Université de Montréal

Effect of hormone replacement therapy on retinal and optic nerve head blood flow and topography in postmenopausal women, and retinal tissue perfusion in ovariectomized rats

Par

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Cette thèse intitulée:

Effect of hormone replacement therapy on retinal and optic nerve head blood flow and topography in postmenopausal women,

and retinal tissue perfusion in ovariectomized rats

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RÉSUMÉ

But: L'hormonothérapie (HT) aux estrogènes, seuls ou combinés aux progestatifs, est prescrite aux femmes pour contrôler les symptômes reliés à la ménopause. Puisque l'HT augmente le débit sanguin dans les organes non reproducteurs et protège contre l'atrophie du tissu cérébral dans certaines régions du cerveau, et que les récepteurs aux estrogènes et à la progestérone sont présents dans la rétine chez l'homme, nous avons étudié si l'HT améliore le débit sanguin de la rétine et de la tête du nerf optique (TNO), et protège la topographie du TNO et la fonction des cellules ganglionnaires (CGs) de la rétine chez les femmes ménopausées. De plus, les récepteurs aux estrogènes étant présents dans la rétine du rat, nous avons développé un modèle de rate ménopausée afin d'évaluer si l'estradiol altère le débit sanguin de la rétine.

Méthodologie: Soixante-cinq femmes ménopausées d'âges similaires ont été évaluées, vingt-neuf n'ont jamais utilisé l'HT (∅HT) et trente-six ont utilisé l'HT (+HT) depuis le début de leur ménopause. Le débit sanguin de l'artère inféro-temporale de la rétine (AITR), et des régions nasales et temporales périripapillaires a été mesuré dans un œil à l'aide du "Canon Laser Blood Flowmeter" et du "Heidelberg Retina Flowmeter". Les douze paramètres stéréométriques de la TNO et l'électrorétinogramme évoqué par inversion d'un damier (PERG) ont été mesurés à l'aide du "Heidelberg Retina Tomograph" et du système VERIS^{TM}. Pour le modèle de rate ménopausée, des rates matures (*Rattus norvegicus)* ont subi une ovariectomie et l'implantation sous-cutanée de capsules silastic contenant de l'estradiol (E_2) ou placébo. Six semaines plus tard, l'indice de capture rétinien (ICR), un indicateur de la perfusion rétinienne, a été mesuré

après l'injection d'un traceur radioactif diffusible, le N-Isopropyl-p-[^{14}C]-iodoamphétamine ([^{14}C]-IMP). L'ICR a été mesuré dans quatre isoptères concentriques à chaque quadrant, à 1, 2, 3 et 4 mm de distance du centre de la TNO, de la rétine montée à plat.

Résultats: Le groupe +HT a présenté des valeurs significativement plus élevées pour le débit sanguin de l'AITR (15.4 ± 3.4 vs 12.5 ± 2.9 µL/min, p=0.006) et du volume de l'anneau neurorétinien de la région entière de la TNO (0.438 ± 0.136 vs 0.380 ± 0.143 mm^3, p=0.032), ainsi que pour le volume de l'anneau neurorétinien (0.066 ± 0.041 vs 0.050 ± 0.030 mm^3, p=0.042), de la hauteur de la variation du contour (0.353 ± 0.099 vs 0.289 ± 0.101 mm, p=0.011), de l'épaisseur moyenne (0.194 ± 0.053 vs 0.167 ± 0.058 mm, p=0.033) et de la surface (0.238 ± 0.068 vs 0.198 ± 0.073 mm^2, p=0.020) de la couche des fibres nerveuses rétiniennes de la région inféro-temporale de la TNO comparativement au groupe ∅HT. Chez les rates ménopausées, le traitement à l'E_2 a induit une augmentation significative des ICRs de 40.6% (p=0.03) pour l'isoptère à 2 mm, 44.2% (p=0.04) à 3 mm et 31.4% (p=0.04) à 4 mm comparativement aux rates contrôles.

Conclusion: Ces résultats indiquent que l'utilisation de l'HT chez les femmes postménopausées est associé à une amélioration du débit sanguin de la rétine, et une couche des fibres nerveuses rétiniennes plus épaisse. De plus, le traitement à l'E_2 chez les rates ovariectomisées a augmenté la perfusion rétinienne.

Most clés : femmes, ménopause, rats, ovariectomie, estrogènes, hormonothérapie, débit sanguin, rétine, fibres nerveuses rétiniennes, neuroprotection

ABSTRACT

Aim: Postmenopausal hormone therapy (HT) consisting of estrogens alone or combined with progestagens, is prescribed to women in order to alleviate postmenopausal symptoms. Since HT increases blood flow in non-reproductive organs and prevents cerebral tissue atrophy in some brain regions, and estrogen and progesterone receptors are present in the human retina, we investigated whether HT improves retinal and optic nerve head (ONH) blood flow, and protects ONH topography and the function of retinal ganglion cells (RGCs) in postmenopausal women. Moreover, since estrogen receptors are present in the rat retina, we developed a menopaused rat model to assess the effects of estradiol on the retinal blood flow.

Methods: Sixty-five healthy postmenopausal women of similar age were investigated, twenty-nine of them who had never used HT (∅HT) and thirty-six who were HT users (+HT) since their menopause onset. Blood flow of the infero-temporal retinal artery (ITRA), both nasal and temporal peripapillary retinal areas, and ONH rim were measured in one eye using the Canon Laser Blood Flowmeter and the Heidelberg Retina Flowmeter. The 12 standard stereometric parameters of the ONH and the pattern electroretinogram (PERG) were measured using the Heidelberg Retina Tomograph and the VERIS^{TM} system. For the menopaused rat model, mature female rats (*Rattus norvegicus)* were ovariectomized and silastic capsules containing either estradiol (E_2) or placebo were implanted subcutaneously. Six weeks later, the retinal uptake index (RUI), an indicator of the retinal tissue perfusion, was measured following the injection of the blood flow tracer N-Isopropyl-p-[^{14}C]-iodoamphetamine ([^{14}C]-IMP). The RUI was

measured in four concentric isopters of all quadrants, at 1, 2, 3 and 4 mm away from the centre of the ONH, in the whole-mount retina.

Results: The +HT group presented significantly greater blood flow of the ITRA (15.4 ± 3.4 vs 12.5 ± 2.9 μL/min, p=0.006) and greater rim volume for the entire ONH region (0.438 ± 0.136 vs 0.380 ± 0.143 mm^3, p=0.032), as well as greater rim volume (0.066 ± 0.041 vs 0.050 ± 0.030 mm^3, p=0.042), height variation contour (0.353 ± 0.099 vs 0.289 ± 0.101 mm, p=0.011), mean thickness (0.194 ± 0.053 vs 0.167 ± 0.058 mm, p=0.033) and cross-sectional area (0.238 ± 0.068 vs 0.198 ± 0.073 mm^2, p=0.020) of the retinal nerve fiber layer for the infero-temporal region of the ONH compared to those of the ∅HT group. In the menopaused rat model, E_2 treatment significantly induced an increase in the RUIs by 40.6% (p=0.03) for the 2 mm isopter, 44.2% (p=0.04) for the 3 mm isopter and 31.4% (p=0.04) for the 4 mm isopter compared to those in placebo animals.

Conclusion: These findings indicate that the use of HT in postmenopausal women was associated with improved retinal blood flow and thicker retinal nerve fiber layer. Moreover, the E_2 treatment in ovariectomized rats was associated with enhanced retinal tissue perfusion.

Keywords: women, menopause, rats, ovariectomy, estrogens, hormonotherapy, blood flow, retina, retinal nerve fiber layer, neuroprotection

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LIST OF ACRONYMS

AFFPIA	automatic full-field perfusion image analysis
ACG	angle-closure glaucoma
ARMD	age-related macular degeneration
ARVO	Association for Research in Vision and Ophthalmology
AT	angiotensin
AU	arbitrary unit
BF	blood flow
BMI	body mass index
BP	blood pressure
BP_{mean}	mean blood pressure
CBF	cerebral blood flow
CBG	corticosteroid-binding globulin
CCD	charged-coupled device
cd/m^2	candela per metre squared
CDI	color Doppler imaging
CEE	conjugated equine estrogens
CHD	coronary heart disease
CLBF	Canon Laser Blood flowmeter
CV	cardiovascular disease
D	diameter
E_2	17β-estradiol
EDHF	endothelium-derived hyperpolarizing factor

EDV	end diastolic velocity
ER	estrogen receptor
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ET	endothelin
FA	fluorescein angiogram
FMD	flow mediated vasodilation
FRSQ	Fonds de la Recherche en Santé du Québec
GABA	γ-aminobutyric acid
Hct	hematocrit
HDL	high-density lipoprotein
HERS	Heart and Estrogens/Progestins Replacement Study
HRF	Heidelberg Retina Flowmeter
HT	hormone therapy
∅HT	not hormone replacement therapy user
+HT	hormone replacement therapy user
HRT I	Heidelberg Retina Tomograph I
[^{14}C]-IMP	N-Isopropyl-p-[^{14}C]-iodoamphetamine
ICGA	indocyanine green angiogram
IMS	International Menopause Society
IOP	intraocular pressure
IRB	institutional review board
ITRA	infero-temporal retinal artery

LDF	laser Doppler Flowmetry
LDL	low-density lipoprotein
LDV	laser Doppler velocimetry
MPA	medroxyprogesterone
MI	myocardial infarction
NETA	norethindrone acetate
NO	nitric oxide
NOS	nitric oxide synthase
NSERC	Natural Sciences and Engineering Research Council
NTG	normal tension glaucoma
OCT	optical coherence tomography
OAG	open-angle glaucoma
OC	oral contraceptive
ONH	optic nerve head
OPP	ocular perfusion pressure
OVX	ovariectomy
P	progesterone
PVEP	pattern visual evoked potentials
PERG	pattern electroretinogram
PGI_2	prostacyclin
PI	pulsatility index
POBF	pulsatile ocular blood flow
PR	progesterone receptor
PSV	peak systolic velocity

RBC	red blood cell
RGC	retinal ganglion cell
RI	resistivity index
RPE	retinal pigmentary epithelium
rps	reversal per second
RNFL	retinal nerve fiber layer
ROI	region of interest
RUI	retinal uptake index
RVA	Retinal Vessel Analyzer
RY	reproductive year
SERM	selective estrogen receptor modulator
SHBG	sex hormone-binding globulin
SLDF	Scanning Laser Doppler Flowmetry
SOGC	Society of Obstetricians and Gynaecologists of Canada
SPECT	single photon emission computed tomography
T	time
TXA_2	thromboxane A_2
VA	visual acuity
VEGF	vascular endothelial growth factor
VEP	visual evoked potential
WHI	Women's Health Initiative Study

LIST OF ABBREVIATIONS

Vel	velocity
Vel_{mean}	mean velocity
Vol	volume

DEDICATION

To my husband Gordon

for his constant loving

support.

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PREFACE

Menopause is the period corresponding to the permanent cessation of ovarian follicular activity and subsequent fall in endogenous estrogens and progesterone production in aging women. The average age at menopause is around 50 years.

Women undergoing menopause experience physiological and metabolic changes that may or may not be symptomatic. These changes are translated into vasomotor instability (hot flushes, night sweats), urogenital tissue atrophy, vaginal dryness, dry skin, bone and collagen loss, and psychological symptoms such as insomnia, depression, irritability, lethargy/fatigue and loss of libido. As well, ocular changes occur such as the onset of dry eyes (keratoconjunctivitis sicca), senile cataract, presbyopia, ptosis of the eyelids, glaucoma and age-related macular degeneration (ARMD).

Particularly in glaucoma and ARMD, recent epidemiological studies have reported that reduced exposure time to endogenous estrogens defined as late menarche, early menopause onset and decreasing duration of reproductive year increases the risk in the development and progression of glaucoma and ARMD in postmenopausal women. As, well, the use of postmenopausal hormone therapy (HT) decreases this risk. Impaired ocular blood flow contributes to the etiology and progression of glaucoma and ARMD. The apparent protective role of estrogens and HT against glaucoma and ARMD in postmenopausal women could result from the vasomotor effect of estrogens by providing a better blood flow as well as a protective effect on the optic nerve head

(ONH) topography. However, there is no direct evidence that estrogens and HT influence the retina and ONH head flow and the ONH topography.

The research area of this thesis focuses on investigating whether estrogens and HT influence the blood flow in the retina and ONH, and the ONH topography. An introductory chapter will present the basic knowledge of the eye and histology, anatomy of the arterial ocular supply, control of the ocular circulation and the techniques employed to measure the ocular circulation. This chapter will also discuss glaucoma and ARMD, the estrogen and progesterone hormones and the current knowledge on menopause and postmenopausal hormone therapy and their relation to glaucoma and ARMD. The second and third chapters will present the aim of two studies designed to investigate whether estrogens and HT influence the blood flow in the retina and ONH blood, and the ONH topography. An observational study investigates the long-term effects of estrogen depletion and the use of HT on retinal and ONH blood flow, and ONH topography in a group of healthy postmenopausal women. The second study investigates the long-term effect of estrogen depletion and estrogen treatment on the retinal tissue perfusion using a radioactive blood flow tracer, the N-Isopropyl-p-[^{14}C]-iodoamphetamine in a menopausal rat model. A fourth chapter reports the findings of these two studies followed by a discussion chapter on the interpretation of these findings.

We hope that this postdoctoral work provides a better understanding of how menopause, estrogens and HT play roles in the ocular circulation and optic nerve head topography in an attempt to explain the link between the hormonal status of estrogens and progesterone

throughout a woman's life and the risk of developing glaucoma and ARMD in the aging woman.

1.0 INTRODUCTION

1.1 The eye

The eye is the organ of vision. The retina is the photosensitive layer in the innermost part of the eye responsible for the conversion of different light energy quanta into nervous pulses. Its external surface is in contact with the choroid and its internal surface is in contact with the vitreous body (Fig. 1).

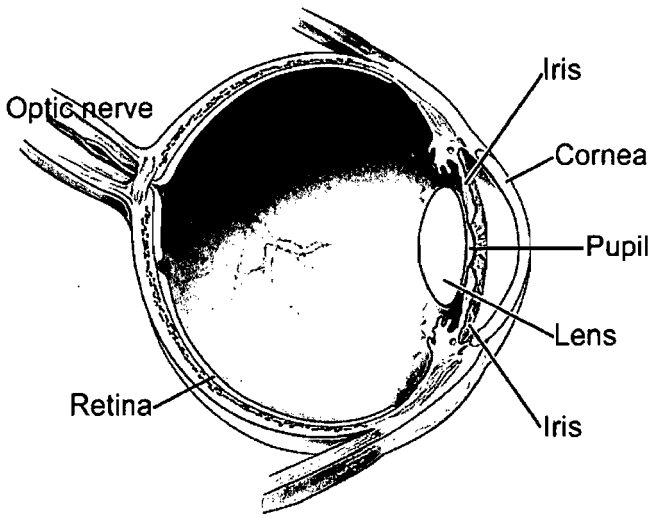

Figure 1: The eye. (From http://www.basiclenses.com/images/uploads/Eye Diagram National Eye Institute Refno NEA08.jpg)

The histology of the retina consists of eleven layers of cells. They are listed as follows along the path of the incident light: (Fig. 2):

1) inner limiting membrane (glial cell fibers separating the retina from the vitreous body

2) nerve fiber layer (astrocytes and bundles of the retinal ganglion cells (RGCs) axons surrounded by neuroglia)
3) retinal ganglion cell layer (cell nuclei)
4) inner plexiform (synapses between the bipolar, horizontal and amacrine cells with RGCs)
5) inner nuclear layer (cell nuclei of the bipolar cells, horizontal and amacrine cells)
6) outer plexiform layer (synapses between the bipolar cells, horizontal and amacrine cells with photoreceptors)
7) outer nuclear layer (cell nuclei of the photoreceptors)
8) outer limiting membrane (sieve-like plate of the rods and cones)
9) layer of the rod and cone outer segments
10) retinal pigmentary epithelium
11) Bruch's membrane (basal membrane separating the retina from the choroids

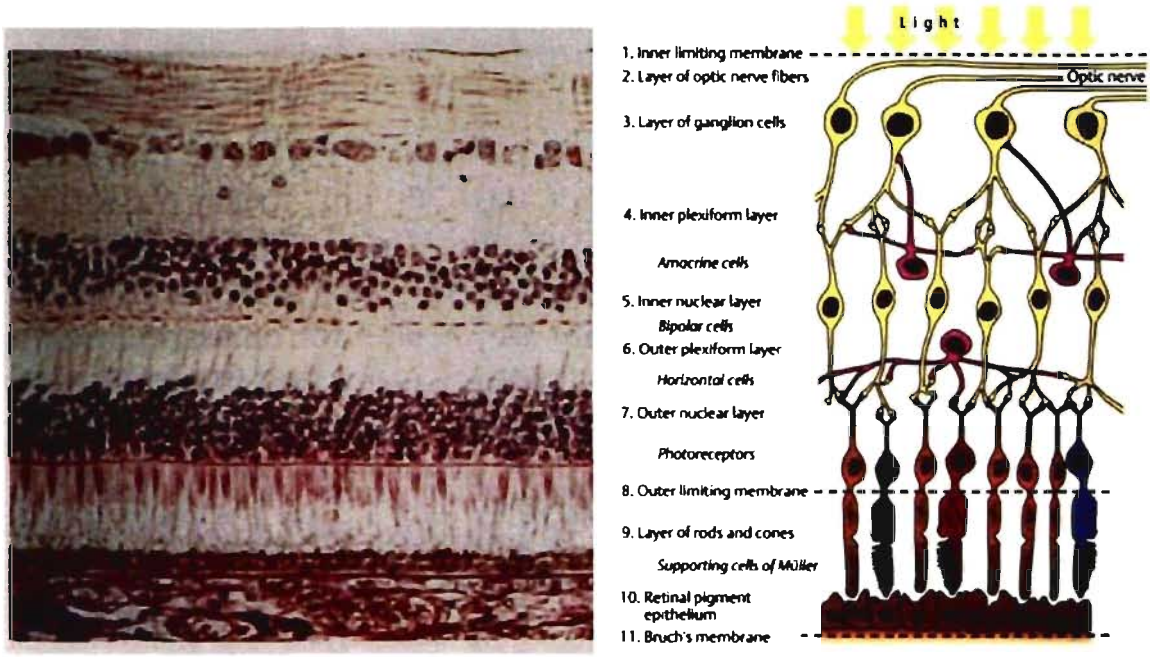

Figure 2: Histology and schematic representation of the retinal layers. (From Lang, GK. Ophthalmology. *A Pocket Textbook Atlas.* Stuttgart, Germany: Georg Thieme Verlag; 2000:301)

All the nerve fiber layer bundles converge to form the neuroretinal rim of the optic nerve head (ONH) (Fig. 3). The cup of the optic disc is a concave region of the optic nerve head. The fibers exit the eye through a sieve-like structure called the lamina cribrosa and form the optic nerve.

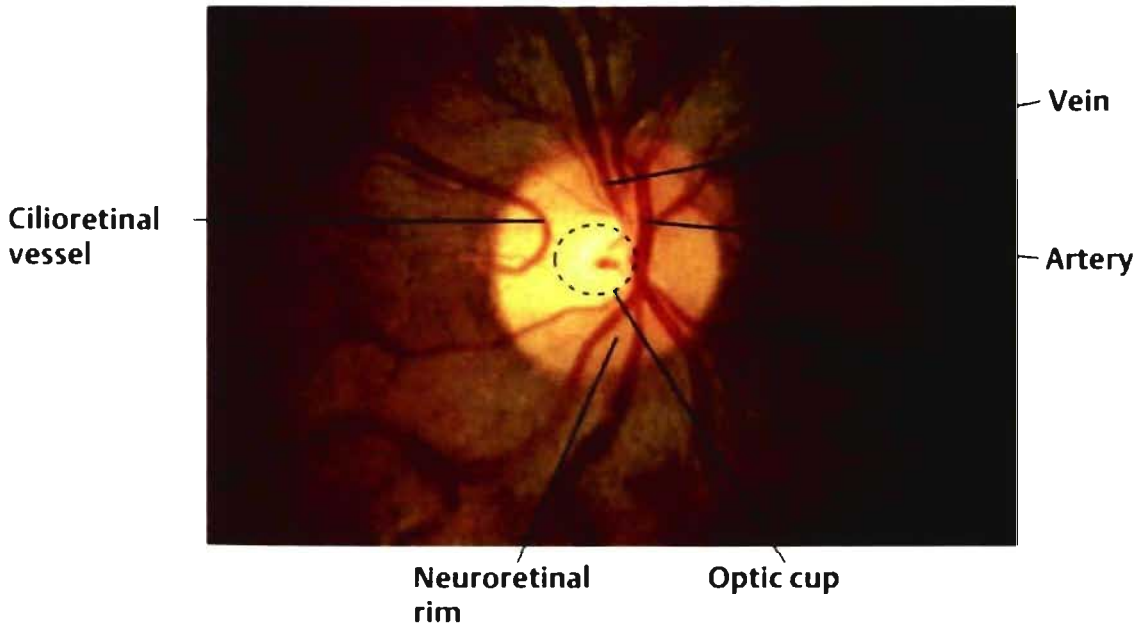

Figure 3: Fundus picture of the optic nerve head and retinal vessels. (From Lang, GK. Ophthalmology. *A Pocket Textbook Atlas.* Stuttgart, Germany: Georg Thieme Verlag; 2000:360)

1.2 Arterial supply to the eye

The ocular circulation is provided by the ophthalmic artery, which is a branch of the internal carotid.[1] The ophthalmic artery enters the orbit of the eye and branches into the central retinal artery, two or three posterior ciliary arteries and several anterior ciliary arteries (Fig. 4). The central retinal artery enters the optic nerve at approximately 10 mm behind the globe, then at the exit of the optic nerve head, is divided into four retinal vessels, each supplying one of the four quadrants of the retina (Fig. 3). The posterior ciliary arteries divide into 10 to 20 short posterior ciliary arteries and, as a rule, two long posterior ciliary arteries.[1] The short and long posterior ciliary arteries pierce the sclera to supply the choroid. The short posterior ciliary arteries pierce the sclera at the

posterior pole, while the long posterior ciliary arteries pierce the sclera toward the periphery. The circle of Zinn-Haller represents the scleral penetration of the short posterior ciliary arteries organized in a ring fashion around the optic nerve.[1] The outer portion of the optic nerve behind the lamina cribrosa is supplied by the pial vessels, whereas the inner portion is supplied by branches of the central retinal artery, while running within the nerve, and anastomose with the superficial pial vessels (Fig. 5).

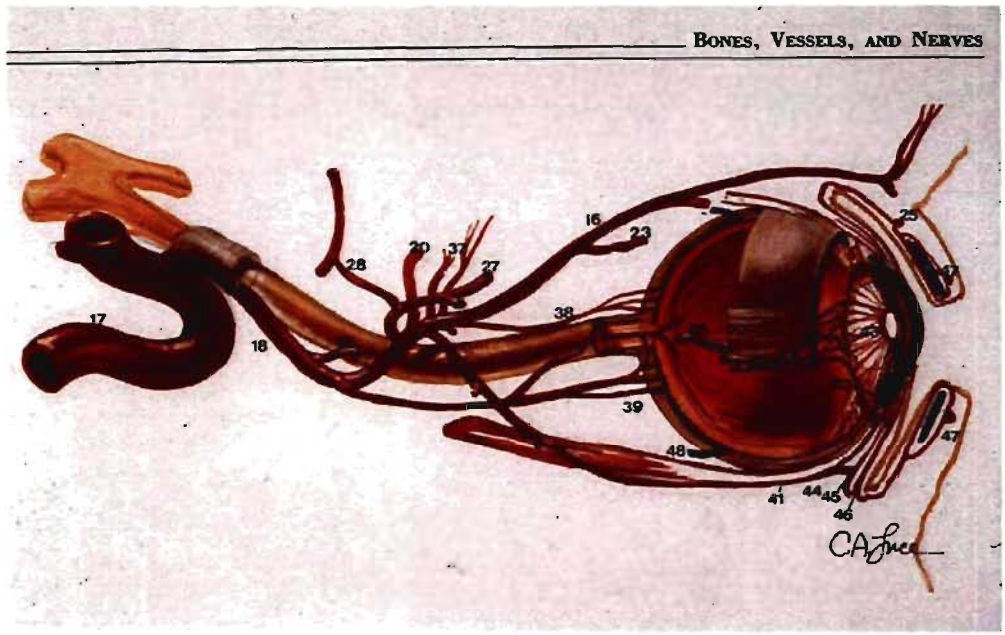

Figure 4: Gross anatomy of the arterial supply to the eye showing the internal carotid (17), ophthalmic artery (18), central retinal artery (35), medial posterior ciliary artery (38) and short ciliary branches of the lateral posterior ciliary artery (39). (From Zide BM, Jelks GW. Bones, vessels and nerves. In Surgical Anatomy of the Orbit. New York, USA: Raven Press, 1985:11)

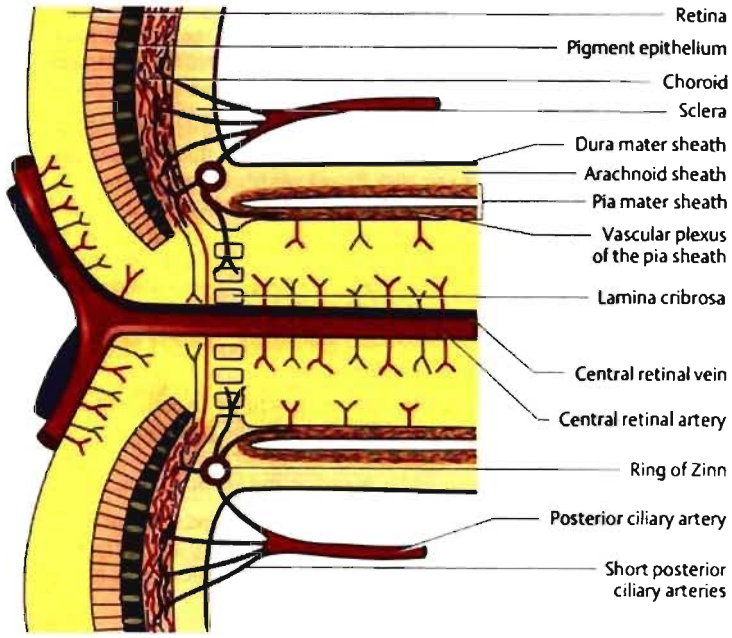

Figure 5: Vasculature of the optic nerve head. (From Lang, GK. Ophthalmology. *A Pocket Textbook Atlas*. Stuttgart, Germany: Georg Thieme Verlag; 2000:362)

1.2.1 Retinal vessels

The retinal vessels are distributed within the inner two-third portion of the retina including the nerve fiber layer, with the exception of a capillary-free zone within the fovea. The fovea is the central region of the retina (diameter 1500 μm) and has the greatest cone density. The foveal avascular zone (FAZ) is within the fovea (500 μm) and comprises the foveola (350 μm), characterized by the absence of inner retinal layers and the presence of cones as the only photoreceptors. The intervening capillaries of the retinal vessels are organized in a multilaminate fashion. In the posterior pole, the retinal capillaries form several layers and are greater in number than in the more peripheral retina.[2] The peripapillary retina has a superficial layer of fine capillaries. If present, a

cilioretinal artery which may be a direct branch of the ciliary or choroidal arteries, supplies a variably sized region of the retina, temporal to the optic nerve.[2]

1.2.2 Choroid

The choroid supplies the outer one third portion of the retina including the photoreceptors, bipolar cells and RPE.[2,3] It extends from the ora serrata anteriorly to the optic nerve head posteriorly. Histologically, the choroid consists of three layers layers: the outermost layer of large vessels (Haller's layer), the medium vessel layer (Sattler's layer) and the inner choriocapillaris layer, which is the capillary layer of the choroid. The suprachoroidal space is a virtual space between the choroid and the sclera. The outer half of Bruch's membrane can be considered as part of the choroid, whereas its inner half can be considered as a part of the retinal pigmentary epithelium. Due to its avascular nature, the central fovea depends entirely on the blood supply provided by the choroid.

1.2.3 Optic Nerve

There are four anatomical regions constituting the optic nerve: 1) the surface layer of the nerve fiber layer, 2) the prelaminar region, 3) the lamina cribrosa and 4) the retrolaminar region.[4] The nerve fiber layer region gets its blood supply from the retinal arterioles. The prelaminar region receives blood supply from the choroid. The lamina cribrosa receives its blood supply from the short posterior ciliary arteries, either directly or from the circle of Zinn-Haller formed by the short posterior ciliary arteries. The retrolaminar region gets a dual source of blood supply, from the peripheral centripetal vascular bed

formed by the pial vessels and from the axial centrifugal vascular bed derived from branches of the central retinal artery (Fig. 5).

1.2.4 Control of the ocular circulation

To maintain the functional integrity of the blood flow in the vascular architecture of the retina, choroid and optic nerve head, several local and systemic factors regulate the ocular circulation. The local factors are the 1) perfusion pressure, 2) endothelium activity, and 3) metabolic demand. The systemic factors are the 1) innervations from the autonomic nervous system and 2) blood gases.[1,5-12]

1.2.4.1 Perfusion pressure

The perfusion pressure is the entering force of the blood through the intraocular blood vessels. It is determined by the difference between the two-third mean arterial pressure (MAP) (the diastolic pressure +1/3 of the systemic blood pressure pulse) in the arteries entering the eye and the pressure in the veins leaving it. The intraocular pressure (IOP) is normally almost identical to the pressure in the veins. Therefore, the ocular perfusion pressure (OPP) is calculated using the following equation:[1]

$$OPP = 2/3\ MAP\text{-}IOP$$

where MAP = Diastolic pressure +1/3 (Systolic pressure- Diastolic pressure)

Consequently, it is expected that a change in IOP or BP could affect the perfusion pressure to the eye. However, several studies have shown that the retinal vessel

circulation in humans has an autoregulatory property allowing constant blood flow regardless of moderate changes in perfusion pressure.[13,14] This is called myogenic autoregulation of the perfusion pressure. Myogenic autoregulation is the maintenance of consistent blood flow in the presence of altered perfusion pressure. The principle is that myocytes sense the transmural pressure, which is the difference in pressure across the wall of vessel, i.e. the internal blood pressure minus the external pressure acting on the vessel.[15] In the event that the internal pressure is raised, the myocytes undergo a mechanical stretch and activate their stretch-sensitive non-selective cation channels and chloride channels. The resulting ionic currents depolarize the myocytes, which increase the open probability of L-type Ca^{2+} channels leading to the contraction of the arterial wall. In ocular circulation, myogenic autoregulation occurs with BP and IOP changes. Myogenic autoregulation occurs in the retinal vessels,[16-20] ON[21,22] and choroid.[23-25] There is clinical evidence that points to impaired autoregulation mechanisms in patients who suffer of diabetes,[26] glaucoma,[27-30] and age-related macular degeneration.[31]

1.2.4.2 Endothelium

The blood vessel wall consists of three different layers: the intima (innermost layer also called the endothelium), media (middle layer also called the smooth muscle), and the adventitia (outer layer) (Fig 6).[15] The intima is a layer of flat endothelial cells and is the main barrier to plasma proteins. It secretes many vasoactive products and is mechanically weak, while the media supplies the mechanical strength and contractile power. The media is made of spindle-shaped smooth muscle cells arranged circularly. The adventitia is a connective tissue sheath with no distinct border. It serves to secure the vessel loosely in place.

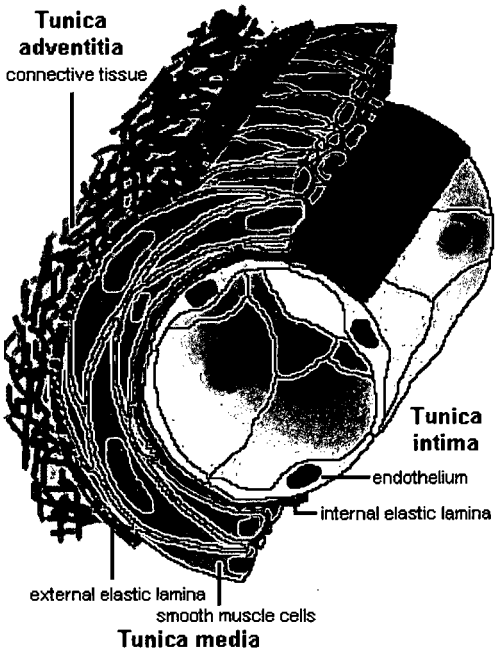

Figure 6: Blood vessel wall.

(From http://www.lab.anhb.uwa.edu.au/mb140/CorePages/Vascular/Vascular.htm)

The endothelium controls the tone of the underlying vascular smooth muscle (VSM) by releasing endothelium-derived relaxing factors (nitric oxide, prostacyclin, and endothelium-derived hyperpolarizing factors) and endothelium-derived contracting factors (endothelin-1, thromboxane A2, angiotensin (AT), superoxide anions, and endoperoxides).[32] The VSM controls the radius of the blood vessels and consequently the blood flow running through it. The VSM contraction is triggered by a rise in Ca^{2+} ion concentration in the cytoplasm leading to the activation or shortening of the actin and myosin filaments.[15]

Nitric oxide (NO), prostacyclin (PGI_2) and endothelin (ET) are strong vasoactive factors (see reviews).[33,34] Nitric oxide is an endothelial vasodilating compound and is believed

to be involved in the basal blood flow in the retina, choroid and optic nerve.[33] Also, nitric oxide acts as a vasodilator mediator for acetylcholine, bradykinin, histamine, substance P and insulin. Prostacyclin is also present in the retina and intravitreal injection of prostacyclin has been shown to increase retinal blood flow.[35,36] Endothelin is a vasoconstrictive peptide released by the endothelium. Its vasoconstrictive effect has been observed in ophthalmic, ciliary and retinal arteries in animal studies.[34] In humans, systemic injection of endothelin induces a reduction of the pulsatile blood flow in the choroid and optic nerve.[37]

1.2.4.3 Metabolic demand

The retina, choroid and optic nerve head induce changes in the ocular circulation in response to dark or light stimulation in order to meet the metabolic demand. Human studies have shown that blood flow at the optic disk surface increases from dark to light condition,[38] and that choroidal blood flow decreases from light to darkness.[39] Moreover, the diameter of retinal arteries and veins, blood velocity in the macular capillary and blood flow at the optic disc surface have been shown to increase during a flicker[40-43] or a pattern stimulation.[44] Therefore, retinal vessels, the choroid and the optic nerve head circulation have an autoregulatory metabolic property in order to meet the physiologic demand in response to the level of ambient light or light stimulation.

1.2.4.4 Nervous control

Sympathetic and parasympathetic innervation are present in the choroid[1,45-48] but not in the retinal vessels.[47,48] Sympathetic innervation of the choroid decreases choroidal

blood flow.[3] The sympathetic adrenergic innervation is present in the central retinal artery but absent in the retinal vessels.[47,48] It has been postulated that the presence of cholinergic innervation in the central retinal artery plays a role in protecting the fragile capillaries of the retina in situations of marked increase of blood pressure. A sudden increase in sympathetic activity, as may occur during stress, causes a marked increase of blood pressure. It is believed that the constriction of the extraocular part of the central retinal artery increases resistance and limits the increase of the intravascular pressure in the fragile capillary bed.[9] The role of parasympathetic innervation of the choroid is less known, other than that parasympathetic innervation seems to increase choroidal blood flow possibly via the vasoactive intestinal peptide (VIP) and nitric oxide (NO).[1,46]

1.2.4.5 Blood gases

Ambient oxygen (O_2) and carbon dioxide (CO_2) are also contributing factors changing the ocular blood flow to the retina, choroid and optic nerve head in humans. In the presence of elevated O_2, blood flow decreases in the retinal vessels and macular capillaries causing a reduction of blood velocity and constriction of the vessels,[49-53] while elevated CO_2 causes an increase of the velocity and dilation of the blood vessels.[50,51,54] Pulsatility, velocity, volume and flow of the neuroretinal rim decrease in the presence of O_2 and increase in the presence of CO_2.[55-57] Finally, O_2 has been shown to have very little effect on choroidal pulsatility, while CO_2 induces an increase on choroidal pulsatility[55,56,58] and flow.[59]

1.3 Techniques employed to assess ocular blood flow

Several techniques have been developed to measure ocular blood flow so as to understand the vascular supply of the retina, choroid and optic nerve as well as the underlying mechanisms of impaired ocular blood flow in eye diseases.

1.3.1 In humans

Techniques to assess ocular blood flow in humans are: fluorescein angiogram (FA), indocyanine angiogram (ICG), pulsatile ocular blood flow (POBF), laser interferometry, blue field entoptic simulation, color Doppler imaging (CDI), laser Doppler velocimetry (LDV), retinal vessel analyzer (RVA), laser Doppler flowmetry (LDF), scanning laser Doppler flowmetry (SLDF) and Canon laser Doppler flowmeter (CLBF).

Fluorescein angiography (FA) is a technique consisting of injecting a bolus of 10 mL of 5% fluorescein sodium into one of the patient's cubital veins.[60-62] The sodium fluorescein has a peak of absorption at 490 nm and emission at 530 nm. Blue and yellow-green filters are positioned along the optical axis of a single-lens reflex camera for viewing and documenting the fundus fluorescence. As the fluorescein dye reaches the retinal and choroidal circulation, it is photographed at several time intervals while the circulation of the fluorescein dye progresses in time. The fluorescein angiogram is a technique which qualitatively assesses blood flow in the retina and optic nerve head and localizes the regions of ischemia and leakage.

The indocyanine green angiography (ICGA) consists of injecting indocyanine dye using the same technique as for the sodium fluorescein dye. It is used for assessing the

choroidal circulation. The indocyanine dye has several advantages over the sodium fluorescein dye. Its fluorescence is near–infrared spectrum. Unlike sodium fluorescein, which absorbs and emits light in the visible portion of the spectrum, indocyanine dye has a peak absorption (805 nm) and fluorescence (835 nm) in the near-infrared range.[62] Light at a wavelength in the near-infrared spectrum penetrates deeper in tissue than light in the visible spectrum.[62] Therefore, light at wavelengths in the near-infrared spectrum penetrates the pigmented layers of the fundus allowing to assess deeper structures of the retina (RPE and Bruch's membrane) and choriocapillaris. Similar to FA, ICG is used for qualitative assessment of the blood flow and localizes the regions of ischemia and leakage.

The pulsatile ocular blood flow (POBF) is the pressure change of the eye in response to the bolus of blood from each heartbeat. Each heart contraction causes a bolus of blood to flow into the ophthalmic artery from the internal carotid artery (ICA) and then spreads rapidly forward through the retinal vessels and choroidal vascular network. The entry of blood into the eye increases the ocular volume with a proportionate increase of the IOP to reach a maximum IOP, which corresponds to the systolic IOP. Following relaxation of the heart and closure of the aortic valve, the IOP falls gradually to a minimal value, which corresponds to the diastolic IOP. The observed changes in pressure reflect the fact that the eye volume must change in response to accommodate changes in the intraocular blood volume induced by the arterial pulse. A relationship between pressure/volume has been established by Silver et al. (1989,1994) from living human eyes.[63,64] From this relationship, the volume of blood entering the eye can be estimated from the corresponding and measured IOP. The total ocular blood flow consists of

pulsatile and non-pulsatile components. In healthy eyes, the pulsatile component is 75% to 85% of the total flow.[65,66] It has been estimated that from the total ocular circulation, 95% is constituted from the choroidal circulation and 5% from the retinal circulation.[65-68] Therefore, POBF is essentially the measurement of blood flow circulating in the choroid with each heartbeat, and is a reliable parameter for evaluating the status of the choroidal circulation, and consequently the overall ocular circulation. However, the POBF technique does not account for the scleral rigidity, which has an impact on how rigid or elastic the eye is and affects its mechanical property to pulsate from each bolus of blood. Scleral rigidity extremes might not reflect the "true" representation of the volume of bolus of blood. As explained before, POBF is used to mainly assess the overall choroidal circulation. Therefore, it does not provide any information on the retinal and optic nerve head circulation and it does not give information on particular regions of the retina, optic nerve or choroid.

Laser interferometry is another technique that has the capability of measuring the pulsatility of the ocular blood flow by measuring fundus pulsation of the eye. It uses an infrared single-mode laser diode (780 nm) integrated to a fundus camera.[69,70] The fundus camera permits the visualization and control of the region of the fundus to be investigated, i.e. the retina, the choroid or the optic nerve head. The principle of this technique is that it measures the distance changes between the fundus and the cornea, called the fundus pulsation. The laser beam is reflected at the front side of the cornea and at the retina. Distance variations between the cornea and the retina lead to a corresponding variation $\Delta N(t)$ of the interference order in the interference pattern

resulting from the two reflected beams.[71] The advantage of this technique compared to the pulsatile ocular blood flow system is that no contact is required with the cornea and no topical anaesthesia is needed. The instrument allows the operator to locally measure the pulsatility at a discrete region of the fundus, either at the retina, the macula, or the neuroretinal rim/cup. However, as for the pulsatile ocular blood flow, it does not account for scleral rigidity, and a relatively clear media is necessary for performing this test.

The blue field simulation technique consists of illuminating the eye fundus with an intense blue light from a narrow optical spectrum centered at a wavelength of 430 nm.[72] Under this condition, bright corpuscles can be seen "flying" in an area of 10 to 15° of arc radius centered at the fovea. These corpuscles correspond to the white blood cells (leukocytes) circulating in the macular capillaries. The leukocytes do not absorb short wavelength as the red blood cells do, therefore they are perceived as shadows on the background of the continuous blood stream. Because the fovea is avascular, the entoptic phenomenon is absent. There are two physical properties drawn from the blue field perception of the leukocytes in the blood stream: one is the velocity and the other is the density. Therefore, based on these two properties, the blue field perception of the leukocytes is a close index of the blood velocity, volume and flow.[73] During the blue field entoptic simulation, one of the subject's eyes is stimulated by the intense blue light at a wavelength of 430 nm, while the subject is asked to observe a CRT screen with the other eye, which is also illuminated with a blue light but at a wavelength of 450 nm to prevent the perception of the entoptic phenomenon. The CRT screen displays computer simulated leukocytes and the subject adjusts the velocity pulsatility, mean velocity and

number of the simulated leukocytes with control buttons in order to match with his own leukocyte motion in the stimulated eye. This is a very subjective technique used to measure retinal blood flow and cannot be used in the presence of opaque media.

The optical property of laser light and the property of ultrasound to shift at a frequency (Doppler shift) corresponding to a specific velocity of a moving particle, for instance the blood cells, have led to a series of new techniques for assessing blood flow in various vascular structures, including the ones from the eye. Color Doppler ultrasound imaging, laser Doppler velocimetry, retinal vessel analyzer, laser Doppler flowmetry, confocal scanning laser Doppler flowmetry techniques and Canon laser Blood Flowmeter have been developed to study the vascular and microvascular (capillary) beds of the retina, choroid and optic nerve head.

The color Doppler ultrasound imaging (CDI) is the only non-invasive system that records the blood circulation from the retrobulbar vessels. It uses ultrasound scanners/transducers (5-10 MHz) which are applied with contact jelly through the closed upper eyelid.[74] Blood flow in the retrobulbar vessels is detected by the production of colour pixels (representing Doppler frequency shifts) on a visual display unit. Pulsed Doppler ultrasound is thereby aimed at the vessel of interest (ophthalmic artery, central retina artery and vein, posterior ciliary artery), and spectral analysis of the resultant Doppler frequency shift is used to obtain a velocity waveform. Peak systolic (PSV) and end diastolic velocities (EDV) are obtained from the signal and are used to calculate two indexes, i.e the resistivity (RI=(PSV-EDV)/PSV) and pulsatility (PI=(PSV-EDV)/Tmax)

indexes. These two indexes provide a measure of peripheral resistance to flow. The resistance index ranging from 0 to 1, where 0 represents no resistance and 1 represents high resistance, is also called the Pourcelot's index. High velocity readings provide better reproducibility than low velocity readings. It has been shown that the coefficient of variation with either the Acuson 128 System or the Siemens Q2000 System varies from 4.0% to 4.8% for the ophthalmic artery, 6.4% to 11.0% for the central retinal artery, and 10.0% to 38.0% for the posterior ciliary artery.[74] Therefore the CDI technique is not a reliable technique for measuring the posterior ciliary arteries. CDI has a limited resolution range for detecting velocity as low as 1-3 cm/s. The angle of incidence between the scanner/transducer and the measured vessel is normally very low as most of the vessels have an antero-posterior orientation. However, in situations where the blood vessels present a tortuosity, the measurement is unreliable.

Laser Doppler velocimetry (LDV) permits the measurement of red blood cell velocity in retinal vessels. A helium-neon laser beam (632 nm) incorporated to a fundus camera is focused on a retinal vessel (50 to 150 um diameter) and illuminates the entire lumen over a short vessel segment.[75] This vessel segment contains many RBCs moving at different velocities all in one direction. The scattered laser light is detected by a detector (optical fiber) located in the retinal image plane of a fundus camera. Two components of this light, one originating from the moving RBCs, the other from the vessel wall, are optically mixed by a photomultiplier tube (PMT). The resultant photocurrent contains a whole spectrum of different frequencies (Δfs) corresponding to the spectrum of different RBC velocities. The power of the photocurrent at a given frequency is proportional to

the number of RBCs moving at the corresponding velocity. A good analogy is that the power spectrum of the photocurrent represents several histograms of RBC velocities. From Poiseuille's mathematical model of the flow, the distribution of the RBC velocities within the vessels is a parabolic function of the radial distance from the vessel axis with an equal number of RBCs flowing at each velocity from V=0 to V=Vmax. Vmax is the maximum (centre line) velocity of the RBCs.[72] In order to increase the accuracy and reproducibility of Vmax at the centre line of the blood vessel and to obtain an absolute measurement of Vmax, a bi-directional laser has been developed.[76] In the bi-directional technique, one incident beam is used to illuminate a site along a retinal vessel. The light scattered by the blood cells at the illuminated site is detected simultaneously in two distinct directions separated by a known fixed angle. Two Doppler shift frequency spectra are measured, each with a particular value of Δf max. The difference of the two values is used to calculate the Vmax in units of speed, i.e. cm/s. By determining the vessel diameter from photograph or laser measurement (CLBF technique described later), the retinal blood flow can be calculated. LDV requires relatively clear media and good fixation from patients.

The retinal vessel analyzer (RVA) is a fundus camera (FF450, Zeiss Jena, Germany). It allows on line measurement of the diameter of a retinal vessel of interest. The diameter measurement is obtained with a charged-coupled device (CCD) camera mounted in the fundus camera. The CCD camera measures diameters of ≥ 90 μm[77] on a vessel segment which can be up to 1 mm maximum length. The CCD camera and analysis software camera determine the diameter using a contrast analysis technique of the column of red

blood cells. Temporal resolution is 40 ms and the diameter resolution is 1 μm. One interesting feature of the RVA system is that it permits the recording of the video signal of the eye fundus (30°) for ≤10 minutes using an SVHS recorder. This feature gives the opportunity to recall data and to analyse different vessels after data acquisition. Since the RVA only gives information on retinal vessel diameter, it does not provide quantitative data of blood flow of the vessel investigated.

Laser Doppler flowmetry (LDF) is a technique that measures the relative velocity, number and flow of red blood cells in the vascular network embedded in tissue. It is used for measuring blood flow in a discrete area of the choroid, the optic nerve superficial layer or retina, where blood vessels are absent. The laser wavelength chosen for the site of measurement determines how deep the measurement is done in the tissue. The longer the laser wavelength is, the deeper is the tissue sampled. The principle of LDF is that the incident laser beam illuminates a small region i.e. the retina, choroid or the optic nerve head free of surface vessels. As the light penetrates the tissue, it is randomly scattered both by nonvascular tissue elements and by blood cells circulating through the capillaries embedded in the tissue. In this situation, each blood cell receives light from a multiplicity of directions. Moreover, the Doppler shifted light scattered by each cell is again randomly scattered by the surrounding nonvascular tissue prior to detection. The power spectrum depends not only on the blood cell speed as for retinal vessels, but also on the light scattering property of the tissue. The Doppler shift power spectrum is recorded via a photodetector placed near the site of illumination of the incident laser. The shape of the power spectrum is logarithmic. The logarithmic fit of

the power spectrum where it meets the baseline is proportional to the velocity of the red blood cells. Bonner and Nossal[78] developed the mathematical model of the broadened Doppler shift spectrum to quantify the total number of blood cells moving in the illuminated tissue volume (X), and to quantify the flux or flow rate of the moving cells (w). The mean blood cell speed is then related to the quotient w/X. In the Occulix system, which is an LDF system, the volume sample corresponds to the area of the laser beam reaching the tissue. The Occulix records in real time, which allows applying stress tests such as the cold provocative test, breathing of different CO_2/O_2 mixtures and light stimulation. However, the output measurements of velocity and flow are in arbitrary units (AU) and therefore it does not allow comparing inter-session measurements. Like the LDV, the Occulix requires relatively clear media and good fixation from patients. The choice of laser wavelength used will determine the depth of the blood flow sampling. The LDF is a single point laser and does not allow the measurment of blood flow in different regions simultaneously.

Scanning laser Doppler flowmetry is the combination of laser Doppler flowmetry (described above) and a scanning laser device. This system has been developed by Heidelberg Engineering.[79] The principle of this system is that the direction of the laser beam (780 nm) entering the eye is periodically changed in two orthogonal directions by means of two oscillating mirrors, so that a two dimensional region is scanned line by line. The scanned field is 10° wide by 2.5° (2.88 mm x 0.72 mm). During the scan along one line, the reflected light intensity at 256 equidistant locations (pixels) is measured and digitized. The pixel distance is 11.2 μm. Each line is scanned 128 times,

then the next line goes through the same process up to a total of 64 lines. The total acquisition time is 2.05 seconds. A discrete fast Fourier over the 128 intensity values for each pixel is performed for generating the power spectrum of the Doppler shift. An automatic full-field perfusion image analysis software (AFFPIA, v3.3),[80,81] is used to determine perfusion analysis of the pixels of the entire image and to obtain the overall volume, velocity and flow. It improves data quality by removing over-exposed pixels and saccades, vessels greater than 30 μm and allows the analysis of large regions of tissue such as the neuroretinal rim. When the acquired image contains the neuroretinal rim area surrounded by the peripapillary retina, inner and outer circles can be drawn by the operator to delimit the neuroretinal rim.

The depth of penetration of the SLDF measurement has been estimated to be 300 to 400 μm.[82] This estimate has been obtained from a monkey model where the central or posterior ciliary arteries were ligatured. Following the occlusion of the central retinal artery, which provides the blood supply to the retinal nerve fiber layer (300 to 400 um thickness), a 51% decrease of the blood flow measured with the SLDF was observed. Following the occlusion of the posterior ciliary arteries, which supply the laminar and retrolaminar region of the ONH, no change in the blood flow was observed.

In normal subjects, SLDF intra-session coefficient of variations for the mean of 5 images of same centration, brightness, and absence of movement, ranges from 11.4% to 16.4%, and the coefficient of reliability ranges from 0.93 to 0.95. Regarding the inter-session, the coefficient of variation ranges from 9.8% to 12.5%, and the coefficient of

reliability ranges from 0.80 to 0.87 respectively.[83] Since it is necessary to obtain at least three good images without blinking and eye drifting, the SLDF system is not a simple test for patients and requires very good cooperation and fixation. Like any other laser Doppler technique, it is necessary to have a relatively clear media.

In order to determine accurately the blood flow from a retinal vessel at a given time, it is necessary to record simultaneously the velocity of the centre line blood column of the vessel and its corresponding width (diameter). The Canon Laser Blood Flowmeter (CLBF) has been developed to achieve this. It uses a bi-directional red diode laser (675 nm, 80 μm x 50 μm oval) that measures the centre line blood column velocity, and a green diode laser (543 nm, 1500 μm x 150 μm) that measures the blood vessel diameter and also serves as a vessel tracking system. These two lasers are mounted in a fundus camera. Refer to the section above describing the principle of the bi-directional laser system. The vessel tracking system corrects for small eye movements. It allows a graph of eye position to be superposed over the velocity time curve to aid in artefact rejection. Two sequent bi-directional readings (i.e. path 1 and path 2) are taken and averaged to give one reading. Multiple diameter readings are simultaneously acquired during the first and final 60 ms of the 2-second velocity measurement window. The CLBF software calculates the blood flow of the vessel of interest once the mean vessel diameter and centre blood velocity are obtained from two paths (2 measurements), using the following formula:

$$\text{Flow } (\mu L/\text{min}) = \text{Velocity}_{\text{mean}}(\pi \text{Diameter}^2/8)$$

where $Velocity_{mean}$ is the time averaged centre line blood velocity (mm/s) during the cardiac cycle and $\pi Diameter^2/8$ is the cross-sectional area of the measurement site (μm^2).

The CLBF software takes into consideration the axial length. The CLBF accuracy relies on the possibility of rejecting or accepting the reading of the path based on visual inspection of the signal in terms of its stability. From our lab experience, we have obtained a coefficient of variation of 3.2 +/- 1.9% for the diameter, 6.7 +/- 3.2% for the velocity, and 7.1 +/- 4.4% for the flow following a standard protocol (paper in preparation). Also, the system keeps in memory the location of the measurement site for follow-up investigations. The CLBF system is user friendly for both technician and patient, but it has minor limitations. It does not allow for measures of an artery if too close to a vein as the laser beams are dragged away from the arterial measurement site by the vein signal. To be performed on patients, CLBF requires a good dilation of at least 7 mm of diameter as the iris blocks the incident laser coming superiorly. Therefore, droopy eyelids or long eyelashes cause problems as the laser signal is blocked. In the presence of lens opacities, the power of the laser can be increased to the next level from 200 to 300 μW for the velocity measurement laser, and from 4 to 6 μW for the tracking and diameter measurement laser.

1.3.2 In animals

All the techniques mentioned in the previous section can be performed on monkeys or other large animals under anaesthesia, which can alter basal hemodynamics and normal physiologic levels. For smaller animals such as rodents, laser Doppler techniques have

been adapted to the confined working area present in the eye, but they still present some limitations.

The laser Doppler flowmetry technique using an intraocular probe can be used to measure retinal blood flow in rodents.[84-86] However, this technique is invasive as it requires one to position the probe near the retinal surface while the IOP has to remain constant. Also, it has to be performed under anaesthesia, which can alter basal hemodynamics and normal physiologic levels. The scanning Laser Doppler Flowmetry (SLDF) (HRF, Heidelberg Engineering, Germany, v1.02) technique can be used in small rodents, but again it must be used in animals under anaesthesia. Moreover, corneal dryness under anaesthesia is a limiting factor. Finally, the SLDF cannot be applied in small rodents for capillary blood flow measurements as it does not have the ability to visualize the capillary layers and it detects blood flow signal from the choroid.[87]

The microsphere technique consists of injecting radioactive microspheres (MS) of a known dosage and size into the left ventricle. MS have been used to measure the retina, optic nerve head and choroidal blood flow.[88,89] As the MS circulate in the blood stream, they become entrapped within the vascular bed in proportion to its regional blood flow. The regional blood flow is determined by calculating the ratio of total MS in the tissue in reference to the arterial contamination obtained from blood samples collected during MS injection. The MS technique presents some limitations, which can over- or under-estimate blood flow.[90-92] A too high or too low dose may lead to over or under MS entrappement. A large size of MS can alter hemodynamics resulting from a blockage of

large vessels. Axial streaming of the MS is also another limiting factor. In the retina, optic nerve and choroid of rats, 8 μm and 10 μm MS are optimal sizes to estimate blood flow in the retina/optic nerve head and choroid respectively.[89] However, since MS are entrapped with the microvascular space and not in the tissue, measurement of the blood flow of the tissue is missed.

Diffusible blood flow tracers such as [^{14}C]-iodoantipyrine,[93] [^{14}C]diazepam,[94] or *n*-[^{14}C]butanol,[95] have been developed to measure regional blood flow in a tissue of interest. The use of a diffusible tracer avoids inaccuracies of the retinal blood flow measurement due to axial streaming, plugging or permeability changes as reported with the classical microsphere technique.[89,92] However, post-mortem intraparenchymal diffusion occurs, which results in weakening the local tracer spatial gradients as observed in the brain,[96] retina, optic nerve and choroid.[92,97-100] The [^{14}C]-iodoamphetamine ([^{14}C]-IMP) is a blood flow tracer, which has been developed and proven to be ideal in measuring blood flow several reasons. First, the [^{14}C]-IMP binds to the amine sites[101,102] and has a very high extraction at the first pass through the microvasculature, which allows a non-restrictive diffusion from blood to tissue despite the blood-retinal barrier. Second, [^{14}C]-IMP has a very low clearance rate and low post-mortem diffusion,[103] due to its high affinity for cerebral tissue. Third, [^{14}C]-IMP is chemically and biologically inert for the duration of the measurement period.[103] These properties have allowed the IMP to be successfully used for measuring cerebral blood flow in small animal research,[96,103,104] and in humans using Single Photon Emission

Computed Tomography. The principle of blood flow evaluation of the [^{14}C]-IMP is identical to that of micrometric microspheres[89] except that molecular microspheres diffuse within the tissue, whereas micrometric microspheres are trapped inside the microvascular space. As yet, there are no published studies reporting the use of IMP in measuring retinal, optic nerve and choroidal blood flow. We have developed a new technique where we used IMP to assess retinal blood flow in a menopause rat model. This is the first time the IMP has been used to measure ocular circulation.[105] Details of the study protocol and findings are presented in the methodology section and paper #2 of the present thesis.

1.4 Glaucoma

Glaucoma affects the retinal ganglion cells and their axons, leading to the excavation of the optic nerve head (ONH) and constriction of visual fields. Recent prevalence estimates conclude that glaucoma is the second leading cause of blindness worldwide.[106] It is also the second leading cause of blindness in the Canadian population aged 50 and over.[107] At least 300 000 Canadians are affected with this disease, and since it is asymptomatic, 50% of patients are unaware that they have it.[108] The three clinical signs of glaucoma are optic nerve head excavation, visual field deterioration and elevated intraocular pressure. There are two causes involved in the aetiology and progression of glaucoma: elevated intraocular pressure (IOP) and/or impaired ocular hemodynamics.[109-111] Elevated intraocular pressure (IOP) results from a poor draining of the aqueous through the angle between the iris and cornea (irido-corneal angle), among other proposed mechanisms, is thought to lead to a mechanical compression of the RGCs and retinal vessels and RGC death. Patients with an open irido-corneal angle and elevated IOP are classified in the open-angle glaucoma (OAG) category, while patients with narrow or closed irido-corneal angle and elevated IOP are classified in an angle-closure glaucoma (ACG) category. Finally, patients with normal IOP, but still present visual field constriction and abnormal excavation of the ONH, fall in the normal tension glaucoma (NTG) category.

There is a large body of evidence indicating impaired ocular blood flow in glaucomatous patients.[110] For example, clinical features indicative of impaired ocular blood flow in glaucomatous patients can be observed through funduscopy. Patients may present pallor of the neuroretinal rim,[112-115] flame haemorrhages of the optic disc[116] and focal

narrowing of the retinal vessels.[117,118] Angiographic studies have demonstrated reduced blood flow,[119-121] filling defects,[120-124] and delayed filling time in glaucoma patients.[125,126] Impaired ocular blood flow demonstrated by quantitative methods has been documented in glaucomatous patients. Color Doppler Imaging technique has demonstrated increased resistivity indexes in the central and short posterior ciliary arteries.[127,128] Pulsatile ocular blood flow findings have indicated reduced flow in primary open-angle glaucoma[65,129] and normal tension glaucoma.[130,131] Scanning laser Doppler flowmetry studies have reported reduced blood flow of the peripapillary and/or neuroretinal rim in primary open-angle glaucoma,[132-137] and normal tension glaucoma.[137,138] A recent study conducted in our laboratory has reported reduced blood flow in the infero-temporal retinal artery measured with the Canon Laser Blood Flowmeter (paper in preparation).[139]

Abnormal peripheral blood circulation underlines the role of impaired ocular blood flow in the aetiology of glaucoma.

Normal systemic blood pressure (BP) and ocular perfusion pressure (OPP = mean BP – IOP), (systolic BP-IOP and diastolic BP-PP) are important hemodynamic factors in providing normal blood perfusion to the retina and optic nerve. Clinical evidence indicates that altered BP and OPP affect the ONH blood flow. First of all, glaucoma[140] and OHT patients[140,141] have reduced OPP and ONH blood flow compared to control subjects as documented by laser Doppler flowmetry and scanning laser Doppler flowmetry studies. Moreover a significant positive correlation between mean blood

pressure (mean BP) and ONH blood flow exists in glaucoma[140] and OHT patients,[140,141] while ONH blood flow remains stable in control subjects despite the BP changes.[141] These findings underline a possible impaired autoregulation to maintain a constant blood flow to the ONH regardless of changes in systemic BP in glaucoma and OHT patients. As well, it indicates that a reduced BP has a stronger negative effect on the ONH blood flow compared to those with a higher BP. This strongly suggests that a higher BP provides a better blood perfusion to the ONH in glaucoma patients. This hypothesis is supported by ONH tomography and visual field studies. Glaucoma patients with normalized diastolic BP resulting from systemic hypertension present a significant decrease of the neuroretinal rim area and an increase in both cup area and cup-to-disc ratio (c/d) of the ONH.[142] In normal tension glaucoma patients, larger circadian OPP and mean BP variations are predicting factors for visual field deterioration.[143,144] Several population-based studies have linked reduced BP and OPP to the glaucoma prevalence and incidence. Low OPP, systolic OPP (systolic OPP = systolic BP-IOP) and/or diastolic OPP values (diastolic OPP= diastolic BP-PP) have been reported to increase significantly the odds ratio of glaucoma prevalence as reported in the Baltimore Eye Survey,[27] Barbados Eye Study,[145] Long Island Glaucoma Case-control Study,[146] Egna-Neumarkt Study,[147] and the Barbados Family Study,[148] and to increase significantly the relative risk ratio of glaucoma incidence as documented in the Barbados Incidence Study of Eye Diseases,[149] Early Manifest Glaucoma Trial,[150] and in the Barbados Eye Study.[151]

Migraines and vasospasticity have been associated with the development and progression of glaucoma.[152] In the Collaborative Normal-Tension Glaucoma Study, it has been reported that glaucomatous female patients who suffered from migraines presented visual field deterioration 2.6 times faster compared to those of females who did not suffer from migraines.[153] In a study involving patients suffering from low-tension glaucoma, reduced finger flow at baseline and following the cold provocation test, which assesses vasospasticity, has been shown.[154]

Finally, and not the least interesting, single photon emission computed tomography (SPECT) studies have been conducted in 31 normal-tension glaucomatous patients.[155] Blood flow measures have reported cerebral blood flow perfusion patterns similar to those of Alzheimer's disease (AD)-like perfusion patterns in seven normal-tension glaucoma patients, while the other NTG patients presented a normal perfusion pattern. Interestingly, the visual field deterioration of the NTG group with an AD-like perfusion pattern had progressed more rapidly than in the group with normal perfusion pattern.

1.5 Age-related macular degeneration (ARMD)

Age-related macular degeneration (ARMD) is thought to be related to the accumulation of metabolite products in the retinal pigmentary epithelium (RPE) of the macular region. The clinical signs are the appearance of drusen (yellow spots) and pigmentation abnormalities (hyper- or hypo-pigmentation), which occur in the early stage, while geographic atrophy and subretinal neovascularization occurs in the later stage. ARMD is the most common cause of vision impairment and blindness worldwide,[106] and in the

Canadian population.[107] The number of ARMD cases is predicted to grow at a rate of 77 000 per year.[108] The accumulation of metabolites may result from impaired choroidal hemodynamics failing to clear the metabolites discharged in the macular region although multiple mechanisms are likely.[156,157]

Angiographic studies have reported a slow[158] and prolonged choroidal filling[159,160] in patients with ARMD. Color Doppler studies have documented higher pulsatility indexes for the ophthalmic,[161] central[162,163] and both nasal and temporal ciliary[162] arteries in ARMD patients. Reduced choroidal blood flow has been documented in ARMD patients with the laser Doppler flowmetry.[156] As well, impaired autoregulation of choroidal blood supply in isometric conditions has been reported.[31] Autoregulation is the ability of a vascular bed to maintain constant blood flow despite changes in perfusion pressure. In normal subjects, subfoveal choroidal blood flow has been shown to remain constant between rest and isometric exercise despite an increase in systemic blood pressure and ocular perfusion pressure, while choroidal blood flow has been shown to increase in ARMD patients with neovascular ARMD.[31] This increase in choroidal blood flow indicates altered autoregulation in response to increased ocular perfusion pressure in ARMD patients.

1.6 Estrogens

Estrogens are a family of hormones which are synthesized in a variety of tissues, such as ovary, placenta, and adipose tissue.[164] Estrogens are responsible for cyclic changes in the vaginal epithelium and the endometrium of the uterus.

There are three kinds of endogenous estrogens; estradiol (E_2), estrone (E_1) and their metabolite product estriol (E_3). Estrogens are formed by the aromatization of testosterone or androstenedione (androgens).[164,165] The aromatization is achieved by the cytochrome P450 enzyme (P450 aromatase) using O_2 and NADPH. In the presence of the testosterone as a substrate, the P450 aromatase leads to the formation of 17β-estradiol, while for the androstenedione it leads to the formation of estrone.

There is a mutual interconversion between estradiol and estrone, and between estrone and estrone sulfate.[166,167] Estrone is then metabolized by two metabolic pathways, the A-ring hydroxylation pathway and the D-ring hydroxylation pathway, each having its own and separate enzyme systems. The two metabolites formed by A-ring hydroxylation are 2-hydroxyestrone and 4-hydroxyestrones, also called catecholestrogens, which can be further metabolized into 2-methoxyestrone and 4-methoxyestrone metabolites. The two metabolites formed by the D-ring hydroxylation are 16α-hydroxyestrone and estriol. Figure 7 illustrates the chemical structures of the parent hormone 17ß-estradiol and its main A- and D-ring metabolites.

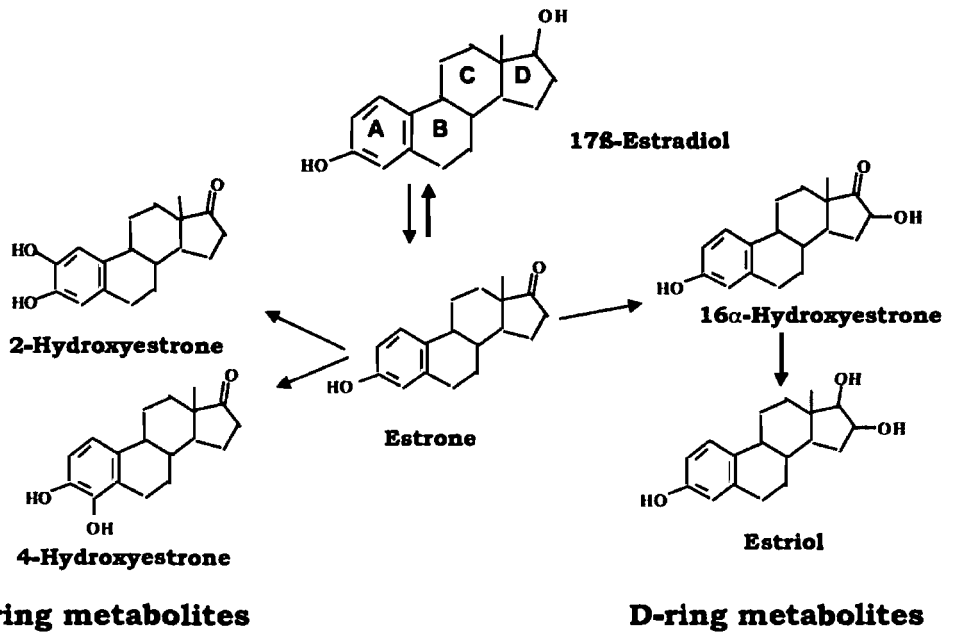

Figure 7: Chemical structures of the parent hormone 17ß-estradiol and its main A- and D-ring metabolites. The cited estrogen metabolites can undergo an additional degradation step by conjugation, either by glucuronidation, sulfation, or methylation. (From Mueck AO, Seeger H, Lippert TH. Estradiol metabolism and malignant disease. *Maturitas*. 2002;43:1-10)

P450 aromatase mRNA and expression are found in several regions of the brain in different species[168] and biosynthesis of 17β–estradiol occurs in the brain.[169,170] Recently, Cascio and al. (2007)[171] also found P450 aromatase mRNA and expression in the adult male rat retina and confirmed that the biosynthesis of 17β-estradiol occurs as well. It is unknown what functional role local estrogen biosynthesis plays in the retina.

In the brain, the proposed role of estrogen biosynthesis is thought to play a role in local trophic and protective effects on neurons.[168] This is discussed in the section 1.6.2.

In the premenopausal period, estradiol and estrone are secreted by the ovaries. Estrone is less biologically active than estradiol and is produced in adipose tissue from androstenedione. Prior to menopause, levels of estradiol are higher than levels of estrone. This is reversed after menopause because estrone continues to be produced in adipose tissue. When released in the blood stream, the estrogens are bound to sex hormone-binding globulin (SHBG).[164] 17β-estradiol, estrone and estriol is 37%, 16% and 1% bound to SHGB respectively, while 61%, 80% and 91% to albumin.[167]

Although the role of estrogens is primarily in reproductive organs, they exert important effects in non-reproductive organs including the bone, the cardiovascular system and neural sites in the brain. The action of estrogens throughout the body is mediated via estrogen receptors (ER) alpha and beta (ERα and ERβ),[172-174] which have been localized throughout the body including the central nervous system, heart and vascular tree, respiratory system, alimentary canal, endocrine system, bone, urinary system and male and female reproductive systems. See reviews for exhaustive list of specific locations of ERs in humans.[167,175,176] In addition to ER, recent studies have reported that estrogens activity is also mediated via the transmembrane G protein-coupled receptors GPR30, which are found in the ovaries, lung, liver, heart breasts, lymphomas and brain.[177]

Estrogen receptors (ERs) have also been localized in the different structures of the eye. This includes the lacrimal gland, meibomian gland, lid, palpebral and bulbar conjunctiva, cornea, lens, iris/ciliary body, lens, uvea, choroid and retinal pigmentary epithelium of rats, rabbits or humans.[178-184] As well, ERs are present in the human,[179,180,185-187] rat,[179,180,188] rabbit[179,180] and bovine retinas.[188] In the human retina, both ERα and ERβ have been identified. ERα are present across the retina, mostly in the inner and outer nuclear layers, in a few cells of the outer plexiform layer and in some nuclei of the ganglion cells layers. A strong presence of ERα has been detected in the axon the RGCs.[186] ERβ are present in the ganglion cell layer and in the choroid.[187] Based on a small sample of eye donors, ERα has been detected in the retina and RPE of two young female eyes still having estrous cycles, but not in eyes from one postmenopausal woman and one elderly man.[186] This indicates a possible age and gender effect on the presence of ERα.

The binding affinity of endogenous estrogens to both ERα and ERβ is greater for the 17β-estradiol, followed by estrone and than estriol.[167]

1.6.1 Vasomotor effect

There is much evidence demonstrating that estrogens have a vasomotor effect by having the ability to influence the vascular tone and blood flow via genomic or non-genomic pathways in reproductive and non-reproductive organs and tissues including the brain

parenchyma. See reviews.[189-191] Experimental studies conducted in female rats showed that 17β-estradiol treatment increased blood flow in some regions of the brain especially the hypothalamus,[192] the frontal cortex, hippocampus, basal ganglia and cerebellum.[193]

The vasomotor effect of estrogens is mediated by a stimulating action on the endothelium-derived relaxing factors, such as nitric oxide (NO),[194-196] prostacyclin (PGI_2),[197-199] pathways and endothelium-derived hyperpolarizing factors (EDHFs), such as potassium (K^+).[191,200,201] The vasomotor effect of estrogens is also mediated by an inhibiting action on the endothelium-derived contracting factors, such as the endothelin (ET),[202-204] angiotensin (AT),[205-207] thromboxane A_2 (TXA_2)[32,191,208] and Ca^{2+} pathways.[191,209] Figure 8 illustrates some of these key players on which estrogens induce vasomotor on the blood vessel wall. Here we are going to review some of those key players.

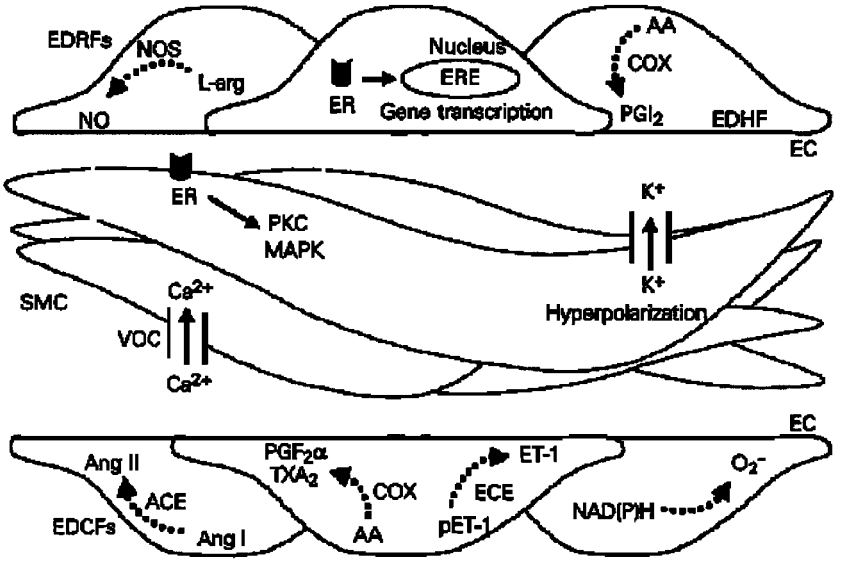

Figure 8: Putative mechanisms for the action of estrogens on the vascular system. (From Tostes RC, Nigro D, Fortes ZB, Carvalho MHC. Effects of estrogen on the vascular system. *Braz J Med Biol Res*. 2003;36:1143-58.

1.6.1.1 Nitric oxide

Nitric oxide (NO) is a gas that is normally produced in the human body. It is endogenously synthesized from the L-arginine via the nitric oxide synthase (NOS). There are three isoforms of NOS, the neuronal NOS (nNOS or NOS-I), endothelial (eNOS or NOS-III) and inducible (iNOS or NOS-II). The e- and nNOS are normally present while the iNOS is present in pathologic conditions.

NO causes vasodilation by inhibiting contraction of the vascular smooth muscle the following way.[15] Once diffused from the endothelial cells to the cytoplasm of the smooth cell layer, NO stimulates the cytoplasmic enzyme guanylate cyclase. This

enzyme catalyzes the production of cyclic guanosine monophosphate (cGMP) from the guanosine triphosphate (GTP). The cGMP activates the protein kinase P, which then reduces the cytosolic Ca^{2+} that is required for the contraction of the smooth muscle cell. In some arteries, NO induces hyperpolarization by directly activating the potassium channels.[15,32] The hyperpolarization reduces the probability of the Ca^{2+}voltage-gated channels to open and consequently reduces the availability of Ca^{2+} necessary for contraction.

Because NO has a short half-life and cannot be stored, its bioavailability is critical. NO production accounts for most of the endothelium-dependent relaxation activity, and there is extensive evidence showing estrogen-induced up regulation of NO production in endothelial cells. The proposed mechanisms are 1) transcriptional regulation of eNOS gene expression; 2) prolongation of the mRNA half-life of eNOS; 3) post-transcriptional modifications and co-factor availability; 4) increase of substrate availability, the L-Arginine; 5) and protection of NO.[210]

Both estrogens receptor α and β are expressed in endothelial and vascular smooth muscle cells.[211] It is believed that ERα are implicated in the increase of nitric oxide synthase III expression and production.[194-196] Little is known of the role of ERβ. The ERβ do not appear to participate in NO production as documented in the mouse aorta.[194] However, it has been reported that ERβ participate in the eNOS activity in endothelial cells[212] and in the vascular smooth muscles.[213]

1.6.1.2 Prostacyclin

Prostacyclin (PGI_2) is produced by arachidonic acid by the cyclo-oxygenase (COX) in the endothelial cells.[15] Arachidonic acid is an unsaturated fatty acid that is generated from the cell membrane phospholipids by the action of another enzyme, phospholipase A_2, which is the rate-limiting step in prostacyclin production.[15,214]

In the vascular smooth muscle, prostacyclin receptors are coupled to adenylate cyclase (AC) leading to an increase of cyclic adenosine 3',5'-monophosphate (cAMP).[34] The cAMP is involved in two vasodilatory mechanisms by: 1)increasing the extrusion of the cytoplasmic Ca^{2+} [32] that is normally necessary for contraction; and 2) stimulating ATP-sensitive K^+ channels causing hyperpolarization of the cell membrane.[32] Consequently this inhibits the contraction of the vascular smooth muscle.

Clinical studies indicate that estrogens have a beneficial effect on prostanoid production. For example, in post-menopausal women, it has been shown that inhibition of COX-1 and COX-2 abolishes skin blood flow after 17β-estradiol administration.[197] In ovariectomized rats treated with 17β-estradiol, the levels of prostacyclin, phospholipase A_2 and COX-1 increase compared to those of non-treated ovariectomized rats.[198] Moreover, in cultured aortic endothelial cells from piglets, rats, and human umbilical vessels, 17β-estradiol stimulates prostacyclin synthesis, and this was also observed in cultured and fresh umbilical vein endothelial cells.[199] To date, there are no published

findings explaining exactly how estrogens are interacting with prostacyclin and whether estrogens receptors are involved.

1.6.1.3 Potassium

Extracellular potassium (K^+) is known to induce hyperpolarization and relaxation in cells. Studies suggest that estrogens interact on the potassium level and channels in vascular smooth muscles. It has been shown that ACh-induced hyperpolarization and relaxation of mesenteric arteries are reduced in ovariectomy (OVx) female and intact male rats compared to intact females and that the differences in the ACh responses in OVx female compared to intact rats were eliminated in the presence of K^+ channel blockers.[200] Moreover, in OVx rats the hyperpolarization and relaxation were restored following 17β-estradiol treatment. In porcine coronary arteries, it has been shown that 17β-estradiol stimulates K^+ channel gating from the smooth muscle leading to K^+ efflux and relaxation.[191] Single-channel and whole cell recording studies performed on isolated human coronary smooth muscle indicate that 17β-estradiol increases the whole-cell current over a range of membrane potentials by likely acting on the calcium- and voltage-activated potassium (BK_{Ca}) channels inducing relaxation.[201]

1.6.1.4 Endothelin

Endothelin (ET) is an important vasoconstrictor. It is a peptide produced and released by the vascular endothelium. It is synthesized from the pro-hormone big endothelin via

the endothelin-converting enzyme (ECE).[34,208,215] There are three isoforms of endothelin (ET-1, ET-2 and ET-3), and the most potent is ET-1. Normally, the plasma level of ET is low (1.5 pg/mL) and has either no direct contractile effect or acts as a vasodilator.[34] During pathological condition, ET production is enhanced by angiotensin II, arginine-vasopressin, thrombin, transforming growth factor, interleukin, epinephrine, calcium ionophore, and phorbol ester, inducing a powerful and sustained vasoconstriction.[15,32,34]

There are two types of endothelin receptors, ET_A and ET_B receptors. The ET-1 binds to ET_A and ET_B receptors of the vascular smooth muscle to induce vasoconstriction[208] by releasing the Ca^{2+} from the sarcoplasmic reticulum leading to an increase of intracellular Ca^{2+}.[34] ET-1 can also interact with ET_B receptors on endothelial cells, triggering the release of nitric oxide (NO) and endothelium-derived hyperpolarizing factors (EDHF).[208]

There is scientific evidence that estrogens interact with the endothelin system. Clinical studies indicate that there is a negative relationship between the levels of estrogens and endothelin levels in women: 1)ET levels are lower in pregnant women than in age-matched non pregnant women;[216] 2)ET-1 levels are higher in postmenopausal women compared to premenopausal women;[217] and 3)ET-1 levels decrease in postmenopausal women after starting estrogens replacement therapy.[217-220] In animal studies it has been reported that 1)antagonist ET_A decreases both mean arterial pressure and ET-1 plasma levels in ovariectomized rats;[221] 2)estradiol metabolites inhibit ET-release in porcine coronary endothelial cells;[202] 3)17β-estradiol attenuates the rabbit coronary arterial

contraction induced by ET-1;[203] and 4)17β-estradiol inhibits both ET-1 production and its gene expression in bovine carotid arterial endothelial cells.[204] Cultured bovine carotid endothelial cells were used to assess whether estrogen receptors interact on the endothelin pathway.[204] Transforming growth factor-*b*1 normally stimulates the ET-1 production in cell and treatment with 17β-estradiol has shown that gene expression and secretion of ET-1 is inhibited. Following treatment with an estrogen receptor antagonist, the ICI 182,780, it was observed that the beneficial effect of 17β-estradiol on ET-1 was blocked. This finding was also confirmed in a more recent study using the same methodological approach and the same ER antagonist on porcine coronary endothelial cells.[202] These studies strongly suggest the participation of estrogen receptors in controlling the endothelin pathway.

1.6.1.5 Angiotensin II

Angiotensin II (AT-II) is a powerful vasoconstrictor. It is produced from angiotensinogen cleaved off by the enzyme renin (mainly secreted by the kidneys) to produce angiotensin I (AT-I).[15] Then AT-I is cleaved again by an enzyme situated in the lungs, called angiotensin converting enzyme (ACE) to form angiotensin II. Endothelial cells express receptors to AT-II in some arterial beds.[32] The contraction induced by AT-II in vascular smooth muscle is mediated by AT_1 receptors coupled to the activation of phospholipase C, the mobilization of intracellular Ca^{2+}, and the activation of mitogen-activated protein kinases.

Studies indicate that estrogens interact with the angiotensin by inhibiting its constrictive effect. Women on estrogen replacement therapy have a lower level of renin than women not on estrogen replacement therapy, and premenopausal level women have a lower level of renin than men and postmenopausal women.[191] In another group of postmenopausal women taking HRT, it has been shown that ACE activity is reduced.[222] Moreover, women who are estrogen deficient have a higher level of mRNA for the AT_1 receptor and its density.[191] Animal studies seem to confirm these findings in humans. For example, a study of ovariectomized rats showed that 17β-estradiol reverses the density and expression of AT_1 receptors in smooth muscle cells from the aorta.[205,206] It has also been reported that 17β-estradiol treatment reduces ACE mRNA concentration in kidneys, lungs and the aorta in ovariectomized rats.[207] So far, there is no study looking at whether estrogens receptors are directly interacting with the renin, ACE and angiotensin production.

1.6.1.6 Thromboxane A_2

Thromboxane A_2 (TXA_2) is formed in platelets and is a powerful vasoconstrictor. It is also synthesized via the prostaglandins pathway.[15] Basically, the arachidonic acid transformed by cyclooxygenase produces both prostacyclin (PGI_2) and thromboxane (TX).[223,224] TXA_2 binds to endoperoxide/thromboxane receptors on the smooth muscle to induce contractions.[32,208] Studies indicate that estrogens have an inhibitory effect on the vasoconstrictive property of TXA_2.[191] In ovariectomized rats, it has been shown that estrogen treatment decreases the sensitivity TXA_2 receptor.

1.6.1.7 Calcium

The contraction mechanism requires the presence of intracellular calcium (Ca^{2+}). Functional evidence has suggested that estrogens modulate Ca^{2+} entry or intracellular Ca^{2+} release in the vascular smooth muscle.[191] For example, 17β-estradiol has been shown to inhibit the barium inward current through voltage-dependent L-type Ca^{2+} in cultured rat thoracic smooth muscle. Also, in experiments carried out using Ca^{2+}-free solution in which Ca^{2+} storage was depleted, 17β-estradiol significantly reduced the contraction following the re-addition of Ca^{2+} to the Ca^{2+}-free solution.[225] This suggests that 17β-estradiol restricted the Ca^{2+} entry. In a different experiment, Freay et al. (1997)[209] demonstrated that 17β-estradiol inhibits Ca^{2+} uptake causing relaxation in smooth muscle in rat aorta.

1.6.2 Neuronal effect

As well, there is a growing body of evidence showing that estrogens have a protective effect on neurons. For example, estrogens have been shown to increase neuron survival in several toxicity models of cultured neurons, such as in glutamate, β-amyolid, hydrogen peroxide, and excitatory amino acid toxicity models.[226-229]

Estrogens also have a trophic effect on neurons. Experimental studies have demonstrated that estrogens increase dendritic spines density, which are the post-synaptic sites of excitatory input to a wide variety of neurons in the mammalian brain. This has been observed in the pyramidal cells of the CA1 region of the hippocampus in

rodents.[230-232] As well, estrogens stimulate cell proliferation in the hippocampus.[233,234] A large body of evidence pointed out that the neurotrophic mechanisms of estrogens are mediated by the brain-derived neurotrophic factor (BDNF).[235,236]

1.7 Progesterone

Progesterone is a sexual hormone produced by the corpus luteum and placenta. It is responsible for changes in the endometrium in the second half of the menstrual cycle, preparatory to implantation of the blastocyte, development of the material placenta and development of the mammary glands. Progesterone results from the conversion of pregnolone by the 3β-hydroxysteroid dehydrogenase.[237] Progestagens is a term applied to hormones that produce similar effects to those of progesterone, the only natural progestagen. All other progestagens are synthetic and are often referred to as progestins. Progesterone is 36% bound to corticosteroid-binding globulin (CBG) when released in the blood stream.[167]

Similar to estrogens, progesterone also exerts vascular effects in non-reproductive organs including the vascular system and the brain. The interaction of progesterone throughout the body is mediated via progesterone receptors (PRs). There are two isoforms of progesterone receptors termed A and B (PR-A and PR-B).[238-241] The interaction of progestins is not only with progesterone receptors, but also with other steroid hormones receptors.[242] Some progestins interact with the androgen, estrogen, glucocorticoid or the mineralocorticoid receptors. These interactions may either induce transactivation of a steroid receptor or prevent activation. Therefore, the resulting action

of a progestin may be agonistic or antagonistic based on the ratio of transactivation/inhibition of a steroid receptor.[243]

Progesterone receptors have been localized in the central nervous system,[244-246] skin,[247] heart and vascular tree[248-250] and reproductive system[251] in humans. Progesterone receptors have also been localized in different structures of the eye. These includes the lacrimal gland, meibomian gland, lid, palpebral and bulbar conjunctiva, cornea, lens, iris/ciliary body, lens, uvea, choroid, and retinal pigmentary epithelium of rats, rabbits or humans.[178-181,183,184] As well, progesterone receptors are present in the human,[179,180,185] rat[179,180] and rabbit retinas.[179,180]

1.7.1 Vasomotor effect

Contrary to estrogens, the vasomotor effects of progesterone are divergent. In animal models, P has been shown to either induce vasodilation,[252-255] increase BF[256,257] or have no effect.[258,259] In clinical trials involving postmenopausal women, P administration has been shown to decrease the endothelium-dependent flow mediated vasodilatation (FMD) of the brachial artery,[260] to have no effect[261] or to increase FMD.[262]

To add to the complexity of progesterone vasomotor effects, progesterone also has divergent vasomotor effects when administered with estrogens. In an animal model, progesterone was shown to antagonize the estrogen-induced BF in a combined therapy.[258,259,263] In postmenopausal women, it antagonized,[264] or did not antagonize[262,265] the estrogen-induced FMD compared to estrogen administration alone.

The vasomotor effect of progesterone is mediated by stimulating action on nitric oxide,[266-268] and prostacyclin[267,269] pathways, and inhibiting action on the thromboxane A_2 pathway. [255]

1.7.2 Neuronal effect

With regards to whether progesterone protects neurons, findings are also divergent. P has been shown to increase neuron survival in glutamate toxicity models of neurons from the hippocampus[270] and cerebral cortex,[271] and in a spinal cord genetic model[272] in rodents. However, an increase of neuron survival has not been observed in the axotomized optic nerve model in rodents.[273]

1.8 Menopause

The menopause is the period corresponding to the permanent cessation of ovarian follicular activity and subsequent fall in endogenous estrogens and progesterone production in aging women. Prior to menopause, the ovaries become increasingly resistant to hormonal stimulation. To compensate for this, the hypothalamus and the anterior pituitary gland increase the release of gonadotrophic hormones. Eventually, the remaining ovarian follicles fail to ripen despite continued secretion of follicle stimulating hormone (FSH) and lutenizing hormone (LH), and a subsequent fall in endogenous estrogens and progesterone occurs. Table 1 indicates the concentration ranges of endogenous estradiol, estrone and progesterone during the follicular, luteal and postmenopausal period in women. The average age at menopause is 51 years, although it commonly occurs as early as 45 years or as late as 56 years of age.[274] The perimenopause (or climacteric) is the period immediately before the menopause with endocrinological and clinical features of approaching menopause, and at least the first year after the menopause. The postmenopause is the period following the date of the last menstrual bleed, which cannot be determined until 12 months of amenorrhea.

Symptoms related to the menopause are vasomotor instability (hot flushes, night sweats), urogenital tissue atrophy, vaginal dryness, dry skin, bone density loss and psychological symptoms such as insomnia, depression, irritability, lethargy/fatigue and loss of libido.

Table 1: Concentration ranges of endogenous estradiol, estrone and progesterone during the follicular, luteal and postmenopausal period in women.

	Follicular	Luteal	Postmenopausal
Estradiol (pmol/L)	110.3 – 367.1	257.0 – 1101.3	< 55.1
Estrone (pmol/L)	110.9 – 369.8	332.8 – 591.7	< 147.9
Progesterone (nmol/L)*		11.1 – 119.3	< 0.3

* Luteal progesterone on days ranging from 17 to 23.

(From http://www.esoterix.com/files/expected_values.pdf)

1.9 Postmenopausal hormone therapy (HT)

Postmenopausal hormone therapy (HT) consisting of estrogens alone or combined with progestagens, is frequently prescribed to women in order to alleviate postmenopausal symptoms. The most common routes of administration are oral and transdermal, but it also can be administered vaginally, by injection or via nasal spray. The active ingredients can be bio-identical or synthetic.

1.9.1 Estrogen therapy

The most common forms of estrogens and equivalent doses in HT are listed in Table 2.

Table 2: Common forms of estrogens and equivalent doses in HT *

Estrogens	Dose
17β-estradiol	1.0 – 2.0 mg
Conjugated equine estrogens (CEE)	0.625 – 1.25 mg
Ethinyl estradiol (EE)	5.0 μg
Transdermal 17β-estradiol	50 – 100 μg

* Personal communication from Dr Michèle Moreau, Department of Family Medicine, University of Montreal

When administered orally in its native crystalline state, 17β-estradiol is poorly absorbed by the gastro-intestinal tract because of its extensive first hepatic effect, but in micronized form it is well absorbed.[275] After oral administration of 17β-estradiol 70% is converted to estrone in the liver. Estrone is then further metabolized, resulting in metabolites with decreasing estrogenic activity such as estriol which is the most abundant end product.[274] Transdermal application of 17β-estradiol avoids the entero-hepatic pass.

The term "conjugated equine estrogens" (CEE) refers to a natural mixture of water-soluble sodium salts of estrogen sulfate esters of the ring B saturated and unsaturated estrogens, which are extracted from the urine of pregnant mares.[167,276] Since all

estrogens present in CEE have estrogenic activity, the pharmacological effects of CEE are a result of the sum of these individual activities, which is greater than the 17β-estradiol activity. Ethinyl estradiol is a synthetic estrogen and is more potent than 17β-estradiol because it is not metabolized by the liver, and it is rapidly and almost completely absorbed from the gastro-intestinal tract.[275]

The relative potency of various estrogens is tissue-specific and cannot be generalized.[167] Based on several clinical and metabolic parameters, the relative potency of CEE and EE ranges from 1.1 to 5.0 times and from 120 to 600 times respectively of the 17β-estradiol.

1.9.2 Progestagens therapy

Progestagens are prescribed as a combined HT to menopausal women with an intact uterus so as to regulate the action of estrogens on endometrial cells and prevent endometrial cancer. Progestagens can be prescribed as a cyclical treatment (the first ten or fourteen days of each month) or as a continuous treatment. The types of progestagens prescribed in combination with estrogens in HT are listed in Table 3.

Table 3: Types of progestagens used in combination with estrogens in HT

Progestagens	Cyclical dose	Continuous dose
Progesterone	200- 300 mg	100 mg
Medroxyprogesterone (MPA)	5 -10 mg	2.5 mg
Norethisterone acetate (NETA)	0.7-1.0 mg	0.25 – 0.5 mg
Levonorgestrel	75 μg	250 μg
Desogestrel	150 μg	unknown
Dydrogesterone	10 – 20 mg	2.5 – 2 mg
Cyproterone acetate	1 mg	unknown
Norgestimate	90 μg	90 μg

(From Samsioe G, Lobo RA, Doren M. *Menopause.* London, UK: Elsevier; 2003:36)

The bioavailability of oral progesterone is low and progesterone derivates such as MPA, NETA and levonorgestrel, three commonly used progestagens, have improved bioavailability. Similar to the estrogens, the data on the potency of the various progestagens are tissue-specific. Based on the transformation dose (an indicator of the progestagens hormonal potency on the endometrium),[167] the hormonal potency of the MPA and NETA is 84 times, and for levonorgestrel, it is 840 times of the 17β-estradiol.

1.9.3 Vasomotor effect

The vasomotor effects of estrogens have been observed in postmenopausal women using HT. Clinical studies have demonstrated that the use of HT improved blood flow in the femoral artery[277] and brain parenchyma,[278-281] and pulsatility and/or resistivity indexes (expression of resistance to flow) in the common carotid,[282-284] internal carotid[285-289] and middle cerebral arteries[286,287] in postmenopausal women.

The beneficial vasomotor effects of HT in improving blood flow combined with an improved lipid profile, such as decreased low-density lipoprotein and increased high-density lipoprotein, have characterized HT as being cardioprotective. During the 1990's, HT was increasingly prescribed to postmenopausal women to protect against cardiovascular disease (CVD). Observational studies pointed out that postmenopausal women taking HT presented a reduction in all-cause mortality, which was believed to be primarily due to a reduction in CVD.[290-294] However, since the publication of two large randomized trials, the Heart and Estrogens/Progestins Replacement Study (HERS)[295] and the Women's Health Initiative Study (WHI)[296] indicating that HT does not offer a cardiovascular protection, the use of HT for controlling postmenopausal symptoms has raised controversies.

The HERS[295] study was the first large-scale randomized trial of HT for the prevention of coronary heart disease (CHD). A total of 2 763 women with coronary disease, younger than 80 years old (mean=66.7 years) were randomly assigned to either 0.625 mg of conjugated equine estrogens (CEE)) plus 2.5 mg medroxyprogesterone acetate (MPA) or a placebo treatment. The primary outcome measures were the occurrence of myocardial

infarction (MI) or CHD death. After a 4.1 year follow-up, there were no significant differences between the two groups for MI and CHD death. Women assigned to HT treatment significantly presented an 11% decrease in their low-density lipoprotein (LDL) and a 10% higher high-density lipoprotein (HDL). However they experienced significantly more venous thromboembolic events (odds ratio=2.9, 95% CI: 1.5, 5.6). The design of this study presented some weaknesses. First of all, 18% of the women, who were assigned to the treated group, crossed over to the placebo group during the study[297] and both groups of women used statin, but more in the placebo group.[298] Women enrolled in the study had CHD history and the endothelial lining in these women was likely damaged throughout their vascular system. Finally, the age average of women enrolled in the study was 66.7 years, which is too late an age to initiate HT, for the following reasons; the amount of ERs decreases[299] and ERα gene methylation increases[300] (an indicator of inactivation of gene transcription) in atherosclerotic women, and response to vasodilatation is impaired in postmenopausal women with cardiovascular disease.[301,302] Consequently, the use of HT in older postmenopausal women with a history of CHD would be unlikely to provide a protective effect against cardiovascular disease in the presence of reduced ER expression and activity, and impaired vasodilatation response. The scientific community started to refer to the term *"the window of opportunity"*[303] which means that to be effective against cardiovascular disease and other menopausal symptoms, HT must be prescribed to menopausal women when estrogen receptors are still present and active, and that the cardiovascular system and endothelium are still relatively healthy and responsive to HT, i.e. in the first five years of the menopause.

The WHI study was a large multicentre study that focused on defining the risks and benefits of strategies that potentially could reduce the incidence of heart disease, breast and colorectal cancer, and fractures in postmenopausal women over a period of 8.5 years of treatment.[304] Between 1993-1998, a total of 161 809 women aged between 50 and 79 years old, were enrolled into a set of clinical trials (trial of low fat dietary pattern, calcium and vitamin D supplementation, and two trials of postmenopausal hormone use). The HT trial of 27,500 women (mean age = 63.3 years old) had two arms: 45% of women without uterus were randomized to receive either CEE (0.625 mg/day) or a placebo, while 55% of those with intact uterus received CEE (0.625 mg/day) + MPA (2.5 mg/day). In July 2002, the interim analysis of 5.2 years follow-up of the CEE+P arm study was published.[296] It reported the following odds ratio of 1.29 (95% CI: 1.02, 1.63) for CHD, 1.26 (95% CI: 1.00, 1.59) for breast cancer, 1.41 (95% CI: 1.07, 1.85) for stroke, 2.13 (95% CI: 1.39, 3.25) for pulmonary embolism, 0.63 (95% CI: 0.43, 0.92) for colorectal cancer, 0.83 (95% CI: 0.47, 1.47) for endometrial cancer and 0.66 (95% CI: 0.45, 0.98) for hip fracture. In light of these findings, the data and safety monitoring board recommended discontinuing the CEE+MPA arm as the risks exceeded the benefits and the CEE+MPA did not demonstrate cardiovascular protection. Then, in April 2004, the interim analysis of 6.8 years follow-up of the CEE arm study was published.[305] The following odds ratio were reported; 0.91 (95% CI: 0.75, 1.12) for CHD, 0.77 (95% CI: 0.59, 1.01) for breast cancer, 1.39 (95% CI: 1.010, 1.77) for stroke, 1.34 (95% CI: 0.87, 2.06) pulmonary embolism, 1.08 (95% CI: 0.75, 1.55) for colorectal cancer and 0.61 (95% CI: 0.41, 0.91) for hip fracture for the treatment group. In this second interim analysis, the use of CEE significantly increased stroke risks, but did not affect the

incidence of CHD. Because CEE did not offer any overall benefit, the CEE study ARM was discontinued. However, the outcomes of the WHI study were also controversial. As in the HERS Study, the WHI Study presented similar weaknesses in its design: the average age of the study participants at the initiation of HT was 63.3 years old, which again, is too late an age to initiate HT. As well, some women already had CHD history prior to the study enrolment. Interestingly, when looking at the odds ratio of CHD for different age-groups, the odds ratio were 0.93 (95% CI: 0.65, 1.33) for age group 50 to 59, 0.98 (95% CI: 0.79, 1.21) for 60 to 69 and 1.26 (95% CI: 1.00, 1.59) for 70 to 79 age group.[306] This finding indicated that younger women tended to have reduced CHD risks compared to older women. Moreover, when looking at the odds ratio of CHD risk when HT was initiated at different postmenopausal times, the odds ratio were 0.76 (95% CI: 0.50, 1.16) for less than 10 years of menopause, 1.10 (95% CI: 0.84, 1.45) for 10 to 19 years and 1.28 (95% CI: 1.03, 158) for 20 years or more (p for trend = 0.02, but this trend did not meet the criterion for statistical significance as stated by the investigators of this study). Therefore, women who initiated HT sooner after menopause onset tended to have a reduced CHD risk compared with the increased CHD risk among women who initiated HT later after menopause onset. These two latest observations underline the importance of the "*window of opportunity*" in prescribing HT in postmenopausal women as explained earlier.

In response to both HERS and WHI design weaknesses, another randomized trial, the Kronos Early Estrogen Prevention Study (KEEPS), was designed in 2005, which tested whether the use of HT had cardiovascular benefits.[307] The KEEPS was designed with major improvements of inclusion/exclusion criteria and interventions. Women were

recently menopaused and assigned to oral CEE (0.45 mg/day) + Progesterone (200 mg), transdermal estradiol (50 μg/day) or placebo treatment. The choice of a lower CEE dosage compared to the usual 0.625 mg/day was decided based on the fact that a CEE dose of 0.625 mg may induce a prothrombotic or inflammation effect.[308,309] Also, the choice of adding a transdermal HT treatment group was to eliminate the prothrombotic effect reported with the use of oral CEE.[309-311] Finally, women could not have taken any estrogens- and progestagens-containing medications (contraceptive and HT) within 6 months of randomization and must have presented reduced estradiol levels <91.75 pmol/L as well as FSH ≥1.16 nmol/L. This clinical trial is still in progress and no findings have been published yet.

1.9.4 Neuronal effect

The protective effect of HT on brain tissue has been reported in postmenopausal women using HT. Observational studies using magnetic resonance indicated that women on HT presented larger volumes of some brain regions such as the hippocampus,[312-314] and the lateral prefrontal, fusiform and entorhinal cortex.[314]

1.9.5 Guidelines and recommendations for HT use

In January 2006, the Society of Obstetricians and Gynaecologists of Canada (SOGC) published several general and specific recommendations on HT.[315] The first three general recommendations are listed as follows:

1) Health care providers should discuss and encourage initiation of healthy lifestyle choices in menopausal women
2) The primary indication for hormone therapy (HT) should be for the management of moderate to severe menopausal symptoms
3) HT should not be prescribed for primary or secondary prevention of cardiovascular disease (CVD) or for primary prevention of dementia

These first three general recommendations should guide the reader to understand the SOGC position following the controversial findings of the HERS and WHI clinical trials. In 2007, the International Menopausal Society also published their recommendations on HT.[316] The IMS recommendations reflect those of the SOGC. Both documents listing these recommendations are found in the annex section of the present thesis.

The general message from SOGC and IMS guidelines and recommendations is that HT may still be cardioprotective, but HT should not be prescribed for primary and secondary prevention of cardiovascular disease. .

1.10 Relationship between estrogens and HT with glaucoma and ARMD

Recently, population–based studies indicated a possible link between estrogens and the use of HT with glaucoma and age-related macular degeneration. In the Rotterdam Study (n=3 078),[317] it was reported that women who experienced early menopause onset before the age of 45 (odds ratio=2.6, 95% CI: 1.5, 4.8) had a significantly greater odds ratio of developing open-angle glaucoma. The Blue Mountain Eye Study (n=2 072 women)[318] reported that women who experienced menarche at ≥13 also presented a significantly greater odds ratio (odds ratio=2.1, 95% CI: 1.1, 3.8) of developing glaucoma. Also, in the same Blue Mountain Eye Study,[319] it was reported that an increasing amount of years from menarche to menopause significantly decreased the odds ratio of early ARMD (odds ratio=0.97, 95% CI: 0.95, 0.99). Interestingly, it has been shown that the use of HT in postmenopausal women significantly decreased the odds ratio of developing advanced (odds ratio=0.62, 95% CI: 0.39, 0.96),[320] and neovascular ARMD (odds ratio=0.29, 95% CI: 0.90, 0.92).[321] In the Rotterdam Study, the odds ratio of open-angle glaucoma was lower in women who had used HT than in women who never had used HT, but this was not significant (odds ratio=0.54, 95% CI: 0.17, 1.74).[317] These observations underline that short exposure to endogenous estrogens and depletion of estrogens throughout menopause might be risk factors in the development and progression of glaucoma and ARMD and that the use of HT seems to play a protective role.

This apparent protective role of estrogens and HT against glaucoma and age-related macular degeneration observed in postmenopausal women could result from vasomotor and protective effects of estrogens. Clinical studies reported that postmenopausal

women taking HT presented improved pulsatility and/or resistivity indexes in the retrobulbar vessels, such as the ophthalmic,[288,289,322,323] short posterior[324] and the central retinal arteries,[324-327] as demonstrated by color Doppler Imaging. However, there is no direct evidence that estrogens and HT have a vasomotor effect on the retinal and ONH circulation in postmenopausal women. Additionally, since estrogens have a protective effect on neurons, as HT has been shown to prevent cerebral tissue atrophy in some brain regions and as ERs are present in the ganglion cell layer and axons in human retinas,[186,187] it is unknown whether HT may present a protective effect on optic nerve head (ONH) topography as well as on the function of the retinal ganglion cells (RGCs).

2.0 AIM OF THE STUDY

In an attempt to investigate whether estrogens and HT have a vasomotor effect on the retinal and ONH circulation, as well as a protective effect on the ONH topography and the retinal ganglion cell (RGC) function, we designed two studies. One observational study investigated the retinal and ONH blood flow, ONH topography and the function of RGCs, which were measured in healthy postmenopausal women who were HT users (+HT) since their menopause onset. Results were compared with those obtained from healthy postmenopausal women of similar age and characteristics who had never used HT (∅HT) since their 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 Writing Group for the Women's Health Initiative Randomized Controlled Trial,[296] which is when this study was being initiated. Retinal blood flow measurements were obtained at three different sites: 1) in a major retinal vessel, the infero-temporal retinal artery (ITRA), using the Canon Laser Blood Flow Meter (CLBF, Japan) and in both peripapillary 2) nasal and 3) temporal areas using the Heidelberg Retina Flowmeter (HRF, Germany). For the ONH, the blood flow of the rim was also measured using the HRF. The ONH topography was assessed by measuring the 12 standard stereometric parameters using the Heidelberg Retina Tomograph (HRT I), and the function of RGCs was measured with the pattern electroretinogram (PERG) using the VERIS^{TM} system.

In a second study, we investigated the vasomotor effect of 17β-estradiol (E_2) treatment on the retina in a menopause rat model. *In vivo* retinal tissue perfusion was quantified by autoradiography using the diffusible tracer N-Isopropyl-p-[^{14}C]-iodoamphetamine ([^{14}C]-IMP) in the conscious rat. This methodological approach provided the ability to distinguish the local effects of E_2 on tissue perfusion in different isopters and quadrants of whole-mount retinas. This second study was designed for the purpose of developing a menopause rat model: 1) to confirm our retinal blood flow findings in postmenopausal women on HT, 2) to isolate the estrogen component on retinal blood flow changes by exposing ovariectomized rats to 17β-estradiol treatment only, and 3) to prospectively study the possible vasomotor pathways of estrogens on retinal circulation and protective effect on the ONH and RGCs.

3.0 METHODOLOGY

3.1 Observational study in postmenopausal women

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 enrolment in the study. Subjects were tested in the Ocular Blood Flow Laboratory at the Ophthalmology Research Unit, Maisonneuve-Rosemont Hospital Research Centre.

Eligibility criteria

Healthy postmenopausal women were recruited from both Notre-Dame and Maisonneuve-Rosemont Hospital Menopause Clinics, Montreal, Quebec by personal interview and by posting advertisements in both hospitals and in the local newspaper. To be eligible for the study, all pre-screened healthy menopausal women had to meet the following inclusion criteria: not HT (∅HT) or HT (+HT) users (as prescribed by a physician) since their menopause onset, 45 years of age or older, naturally or surgically menopaused, amenorrhea for at least one year, body mass index equal or less than 30 and non or former smoker. Menopausal women had to be free of systemic and central nervous system diseases and to present normal ocular histories and eye exams. Subjects on vasoactive or anti-inflammatory medications, and allergic to eye drops were excluded. The ∅HT postmenopausal women served as control group.

Study visit

Twelve hours prior to the study visit, subjects were asked to avoid caffeine and alcohol so as not to alter their basal hemodynamic level. As well, they were asked to avoid food with high fat content, which alters the serum estradiol assays. Also, so as to confirm the use of HT and rule out the effects of vasoactive drugs, subjects were asked to bring all their medications, including any herbal medicine with them. The type of HT in terms of their active ingredients was documented.

Medical, gynaecological and ocular histories, and eye exam

At the time of the study visit, demographic, medical, gynaecological and ocular histories were obtained from each subject using a standardized questionnaire administered by one examiner (MCD), who determined the eligibility of the subjects for the study. Medical history included measures of the blood pressure (BP)(mmHg) at rest for 30 minutes, height (m), and weight (kg). These measures were used for calculating the body mass index (BMI=weight/height^2) and mean BP ((BPdiastolic +1/3(BPsystolic – BPdiastolic)). Gynaecological 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 women who had hysterectomies, the age at last menses could not be determined. Therefore, age at last menses, reproductive year and menopause durations were tabulated as missing values. Ocular histories and eye exams were obtained, which included best corrected visual acuity using the Snellen

chart (VA expressed by its numerator divided by its denominator), refraction (expressed in spherical equivalent), and biometry (OcuScan, Alcon® Surgical Inc., USA v3.02). Slit-lamp examination, evaluation of peripheral anterior chamber depth, tonometry (IOP mmHg), and ophthalmoscopy were performed by one ophthalmologist (DD). Ocular perfusion pressure (OPP mmHg) was calculated using the following formula: OPP= 2/3 BP_{mean}-IOP.[1]

Selection of the investigated eye

All retinal and ONH blood flow measurements, as well as ONH topography and PERG measurements were conducted in one eye only, i.e. the eye with the clearest media, better fixation and least lid ptosis. When both eyes were eligible for the study, one eye was randomly chosen. The chosen eye was tested first undilated for the PERG testing, then dilated with tropicamide (Mydriacyl® 1%, Alcon Canada Inc.) for the HRT, SLDF and CLBF testing.

Serum estradiol and progesterone assays

A blood sample was obtained from all subjects and analyzed by the local hospital laboratory service for documenting the total serum estradiol (E_2) and progesterone (P) concentrations by chemiluminescent immunoassay technique (ADVIA Centaur®, Bayer HealthCare Diagnostic, USA). These assays were obtained to confirm the hormonal status of estrogens and progestagens in all postmenopausal women. Some active ingredients present in the HT regimens and used by postmenopausal women were not 17β-estradiol- and/or progesterone-based. Therefore, measures of the serum estradiol

and progesterone concentrations by the hospital laboratory service were not appropriate. Consequently, low serum concentrations of estradiol and progesterone not detected by the assay technique were tabulated as zero. As well, for some postmenopausal women who were not HT users, the endogenous serum 17β-estradiol and progesterone concentrations were below the level of detection by the assay technique. Consequently, their serum concentrations were also tabulated as zero.

Infero-temporal retinal artery

Blood flow (Flow) of the infero-temporal retinal artery (ITRA) was measured using the Canon Laser Blood Flowmeter System (CLBF model 100, Japan). The CLBF is a unique system that quantifies blood flow in retinal vessels by simultaneously measuring both diameter (D) and velocity (Vel) of the blood column in a vessel of interest.[328] To achieve this, the CLBF system is equipped with a dual laser system mounted in an eye fundus camera and an acquisition/analysis software (v2.1.23). An oval (8 μm x 50 μm) red diode laser (675 nm) measures the mean centre line blood column velocity (Vel_{mean}) of the vessel of interest by using a bi-directional laser Doppler velocimetry, the principle of which has been described elsewhere.[329-331] Briefly, the Doppler-shifted light scattered from the flowing blood cells in the targeted vessel is detected simultaneously in two directions by two photomultiplier tube detectors positioned at a fixed angle. The signals from two photomultiplier tube detectors undergo computer-controlled spectrum analysis, and sequential measurements of velocities are performed automatically at every 20 ms (50 Hz sampling rate) over a period of 2 s and time-averaged velocity (Vel_{mean}) is determined. The D is measured by a rectangular (1500 μm x 150 μm) green diode laser

(543 nm) manually positioned perpendicular to the vessel of interest. The laser measures the blood column width by transmittance profile method, the principle of which has been described elsewhere.[332] The D is determined automatically by computer analysis of the signal produced by the image of the vessel on a charge-coupled device (CDD) sensor using the half-height of the transmittance profile to define the blood column edge. It is measured for the first and last 60 ms of the 2 s velocity measurement period, at every 4 ms (250 Hz sampling rate). 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. To compensate for microsaccade of the eye, the green diode laser also serves as a tracking system by scanning repeatedly and rapidly the intensity profile produced by the rectangular green diode laser and by analyzing the scanned intensity profile. The analyzing electronics automatically reposition/stabilize the incident laser to the vessel centre.[333] Since both lasers operate simultaneously, D and Vel_{mean} are obtained in the same time frame.

The CLBF acquisition software has been designed so that one measurement of D and Vel_{mean} obtained by the operator corresponds to one Path. After the acquisition of one Path, the software displays 4 parameter windows allowing the operator to visually inspect the quality of the Path obtained and it is either saved or discarded. The parameter windows are: 1) stability of the relative velocity over the 2 s period, 2) the stability of Q-index over the 2 s period and the overall Q-index (an indicator of the signal quality internally determined by the software), 3) the stability of the vessel tracking over the 2 s period, and 4) the coefficient of variation of the vessel diameter. For accuracy, the CLBF software operates by measuring two Paths (Path1 and Path2),

then automatically calculates the flow following the Poiseuille's law, according to the following equation:

$$\text{Flow } (\mu L/\text{min}) = \text{Velocity}_{mean}(\pi \text{Diameter}^2/8)$$

where Velocity_{mean} is the time averaged centre line blood velocity (mm/s) during the cardiac cycle and $\pi\text{Diameter}^2/8$ is the cross-sectional area of the measurement site (μm^2).

Since study participants underwent several tests during the study visit, retinal blood flow measurements were only obtained from one site, the ITRA. By inference, the blood flow measures from the ITRA can be used as an indicator of the overall retinal arterial circulation. There are two motives for choosing the main retinal artery from the infero-temporal region. First, it is a region that is technically accessible to obtain CLBF measurements, and second, the infero-temporal region is the most common site of early glaucomatous damage. The location of the measurement on the ITRA was done 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 high quality Path1-Path2 pairs and calculated Flow values were obtained. After excluding the incomplete measures (the CLBF automatically rejected flow measurements when one of the two paths was unreplicable) and discarding the highest and lowest flow values, the three closest flow values calculated by the CLBF software were used for averaging D, Vel_{mean}, and Flow. This recording protocol gave us an intra-session coefficient of variation of 3.2+/-1.9% for D, 6.7+/-3.2% for Vel_{mean}, and 7.1 +/-4. % for Flow obtained from the ITRA in 19 normal women (manuscript in preparation). Figure 9 illustrates an example of a CLBF recording from a normal subject.

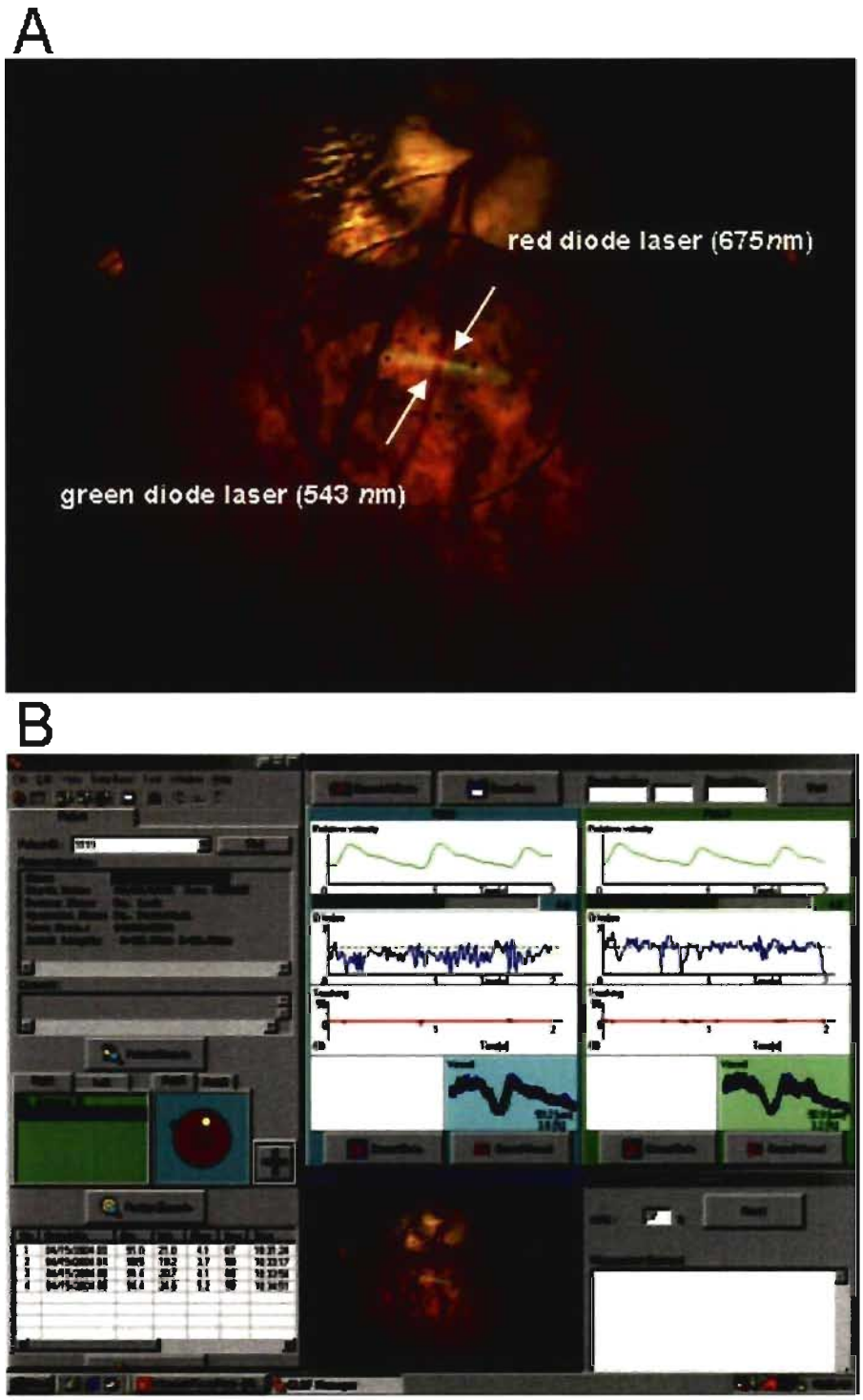

Figure 9: Example of a CLBF measurement obtained from a normal subject. The panel (A) illustrates the position of the red diode laser (675 nm) that measures the mean centre line blood column velocity of the infero-temporal retinal artery (ITRA) and the

orientation of the green diode laser (543 nm) that measures its diameter. The right panel (B) shows both Path measurements, Path 1 and Path 2, with their respective parameter windows. For one Path, the top window displays the relative velocity over a 2 s period, followed by a window, which displays the Q-index over a 2 s period and the overall Q-index (an indicator of the signal quality internally determined by the software). Then a third window displays the stability of the vessel tracking over the 2 s period, and finally the last window displays the coefficient of variation of the vessel diameter. For this particular subject, four Flow measurements (ranging from 3. 7 to 5.2 μL/min) were acquired with their respective D (ranging from 90.5 to 94.4 μm) and Vel (ranging from 19.2 to 24.6 mm/s) as shown on the bottom left of panel B.

Peripapillary retinas and ONH rim areas

The capillary blood flows of the ONH rim and both nasal and temporal peripapillary retinal areas were measured by Scanning Laser Doppler Flowmetry (SLDF) (HRF, Heidelberg Engineering, Germany, v1.02). Briefly, the Heidelberg Retina Flowmeter combines a confocal laser scanning technique and laser Doppler flowmetry. It measures the amount of back scattered light at different locations (pixels) in the region of interest. A parallel infrared laser beam of 3 mm diameter (780 nm, 180 μW) scans line by line a two-dimensional region of the retina. The scan field is 10° wide by 2.5° height, corresponding to a size of 2.88 mm x 0.72 mm. Detailed description of the instrument and measurement technique has been described elsewhere.[334,335] During the scan along one line, the reflected light intensity at 256 equidistant locations (pixels) is measured and digitized (pixel distance 11.2 μm) sequentially. Each line is scanned 128 times at

4000 Hz, which takes 0.032 s. Then, the next line is scanned 128 times until a total of 64 equidistant lines (line distance 11.2 μm) are scanned. Thus, the reflected light intensities of each pixel are obtained as a function of time (fluctuation time curve). The total acquisition time is about 2.5 s. The automatic full-field perfusion image analysis software (AFFPIA, v3.3)[80,81] improved data quality by removing over-exposed pixels, and saccades, and allowed the analysis of large regions of tissue such as the neuroretinal rim. The collected intensity data of each pixel was then analyzed by a discrete Fourier transformation, thus calculating the laser-Doppler frequency shift for each pixel. The data of the laser Doppler frequency shift of the 64 x 256 pixels is the base to generate the perfusion image, and the AFFPIA computes flow (distance travelled by all moving red blood cells per unit of times), volume (number of moving blood cells), and velocity (mean of blood cell speed) for each pixel, all expressed in arbitrary units (AU). For valid capillary blood flow measurement at each pixel, the AFFPIA uses an algorithm to exclude over- or under-exposed pixels, retinal vessel tree and eye saccades (which is identified by the operator). Moreover, the AFFPIA permits an interactive marking of the rim area of the optic nerve head allowing the output of the three hemodynamic parameters, flow (Flow), volume (Vol) and velocity (Vel) for each region of interest, i.e. the nasal peripapillary retina (n), temporal peripapillary retina (t) and neuroretinal rim (r) areas. Figure 10 illustrates an example of a SLDF perfusion image and automatic full-field perfusion image analysis obtained from a normal subject.

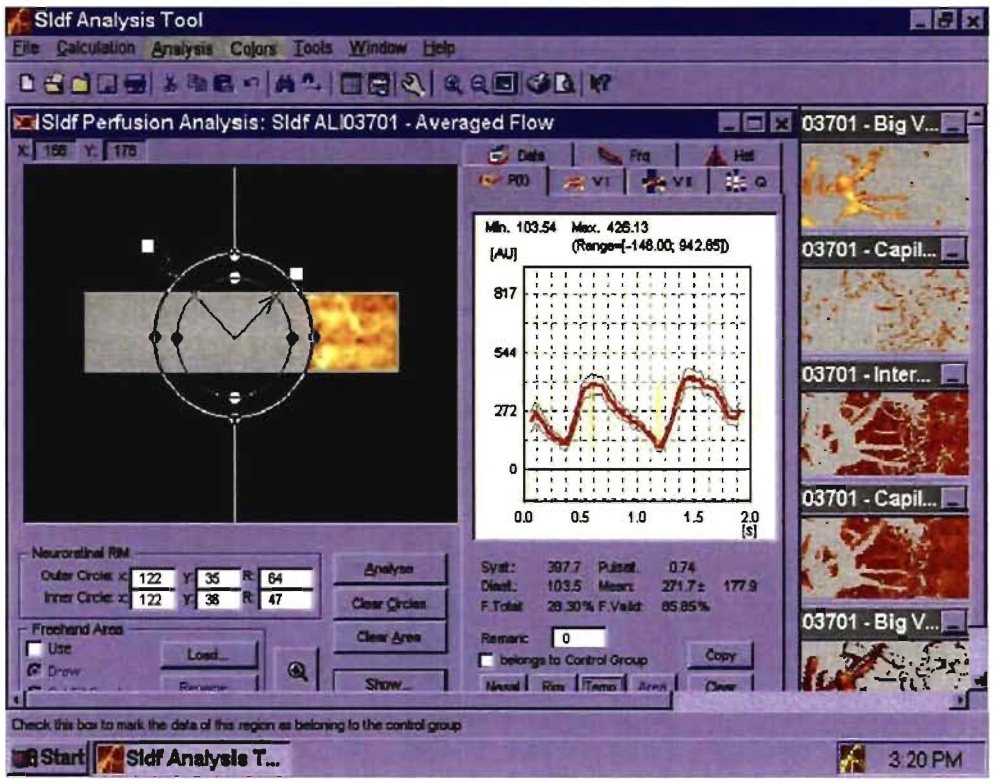

Figure 10: Example of a SLDF perfusion image obtained from a normal subject. The left side shows the marking circle delimitating the rim and both nasal and temporal retina of the optic nerve head region from a normal subject. The right side shows the Flow, Vol and Vel values obtained with the full-field perfusion image analysis (AFFPIA). For this particular subject, the Flow, Vel and Vol values obtained by the AFFPIA for the temporal retina of this SLDF image corresponded to 271.7 AU, 0.9 AU, and 20.7 AU respectively.

Our HRF images were centered on the optic nerve head, 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 focussing, ONH centration, optimum exposure, brightness, and lack of major eye saccades were kept for full-field perfusion image analysis. The obtained Flow, Vel and Vol from these 5 images for the nasal (n), temporal(t) and rim(r) areas were then averaged. This SLDF measurement protocol developed in our lab yields intra-session 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.[83]

ONH topography

The optic nerve head topography was evaluated by measuring the 12 standard stereometric parameters (i.e. disc area, cup area, c/d, 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) in all subjects. This was achieved by using the Heidelberg Retina Tomograph I (HRT I, Heidelberg Engineering, Heidelberg, Germany, v2.01). The HRT I is a confocal laser scanning ophthalmoscope operating three-dimensionally. A diode laser (670 nm) scans the optic nerve head (along the z-axis) in a series of 32 consecutive and equidistant two-dimensional optical section images (x-axis and y-axis of 10° x 10° (256 x 256 pixels). Each pixel is approximately 10 μm. The reflected light at each pixel is measured by the

confocal optical detector system and is used for generating a topography and reflectivity image. The topography image is the location along the *z*-axis where the maximum reflectivity registered is assumed to be the height of the location and the reflectivity image is the sum of the reflectivity measurements along the z-axis. Between five to ten ONH images were obtained and the average of the three closest images in terms of alignment, brightness and clarity was obtained. With the aid of fundus pictures, the external contour of the optic nerve rim was manually drawn and the Heidelberg Retina Tomograph interactive software automatically calculated the twelve standard stereometric parameters. The stereometric parameters were obtained for the entire ONH (0°-360°) and for the infero-temporal region (270°-360°), which is a region most commonly affected by early glaucomatous damage. Figure 11 illustrates the topography and reflectivity images, and an output of the stereometric parameters calculated by the HRT software from a normal subject.

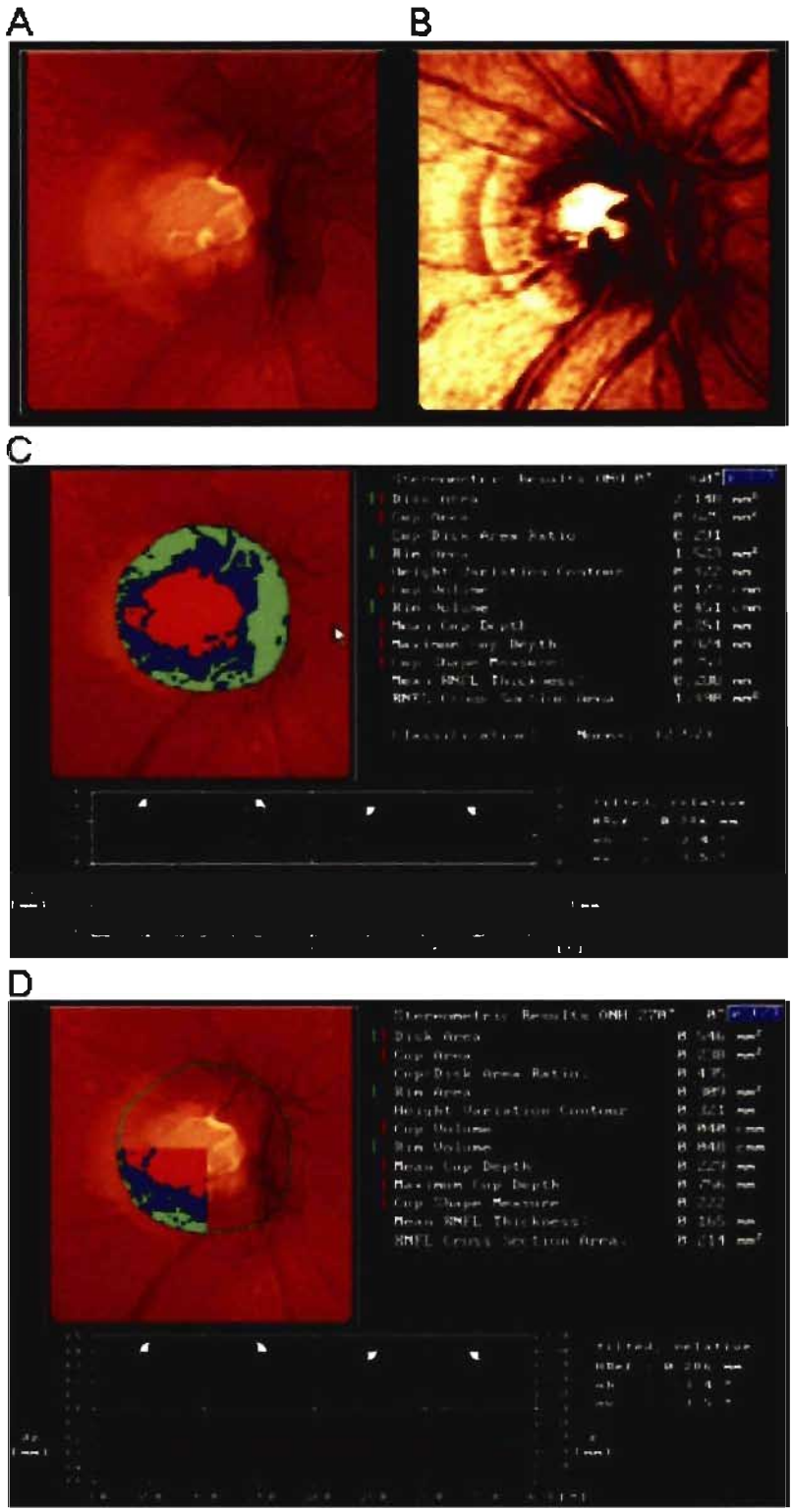

Figure 11: Example of ONH topography measurement obtained from a normal subject.

using the Heidelberg Retina Tomograph I (HRT I). The topography image (panel A) represents the maximum reflectivity registered along the *z*-axis at each pixel, and the reflectivity image (panel B) represents the sum of all the reflectivity measurements along the z-axis. The 12 standard stereometric parameters from the entire ONH (0-360°, panel C) and the infero-temporal region (270-360°, panel D) are automatically calculated by the HRT software after manually marking the external contour of optic nerve head rim.

Functionality of the RGC

The pattern electroretinogram (PERG) represents an objective and direct measure of retinal ganglion cell function.[336-338] It is a retinal biopotential evoked when a high contrast patterned stimulus (alternating stripes or checkerboards) is viewed. The PERG waveform signal begins with a negative deflection called N35, followed by a positive deflection called P50, and then a second negative deflection called N95. Smaller PERG amplitudes and delayed latencies are indicative of RGC loss. Clinical studies where loss of RGCs occur such as in OHT and OAG, have demonstrated that N95 amplitude is reduced and P50 latency is increased,[339] and that N95 amplitude significantly and positively correlates with RNFL thickness while P50 latency negatively correlates.[340,341]

The PERG was recorded using the VERIS™ Multifocal System (Electro-Diagnostic Imaging, Inc., USA, v5.0) following the Guidelines of the International Society for Clinical Electrophysiology of Vision (ISCEV).[342] Transient (4.7 reversal/s (rps)) PERGs (bandwidth 1-100 Hz) were evoked by a black and white reversing checkerboard stimuli of 50° x 38° with checksize of 0.8° generated by a 9-inch monochrome monitor

(75 Hz frame rate). The luminance of the white squares was set at 353 cd/m^2 and for the black squares, it was set at 30 cd/m^2, (of 192 cd/m^2 average luminance, 84% contrast). A sterilized and disposable silver DTL Plus^{TM} (Diagnosys LLC, USA) electrode placed in the lower conjunctival sac served as the active electrode in reference to a gold cup electrode (Grass Technologies, USA) placed at the ipsilateral outer canthus. Subjects were grounded through the forehead with a gold cup electrode. Electrode impedances were checked for 5kΩ or less. The recording session was devided in segments, i.e. 32 segments, each lasting 3.41 s for a total of 109.1 s. This corresponded to an average of 514 responses obtained. During one segment recording, the subject was asked not to blink to minimize artifact since there is no artifact rejection capability built into the system. Two replicable PERG waveforms were obtained. The N35, P50 and N90 waveform components were identified, and latency (N35 lat, P50 lat, N95 lat) and amplitude (P50 amp, N95 amp) were measured. Figure 12 illustrates a transient PERG waveform and measured components obtained from a normal subject.

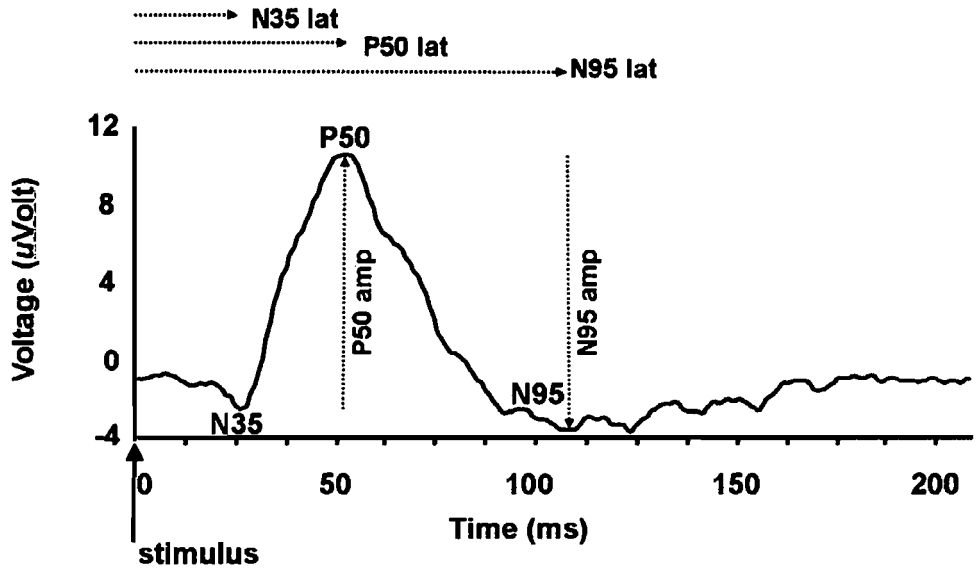

Figure 12: Example of a transient pattern electroretinogram (PERG) obtained from a normal subject. The PERG was evoked by a checkerboard pattern of 50° x 38° with a checksize of 0.8° set at a contrast of 84% (white square luminance of 353 cd/m^2, black square luminance 30 cd/m^2). The PERG was recorded at 4.7 reversal/s. The three main waveform components were identified, N35, P50 and N90, and both latency (N35 lat, P50 lat, N95 lat) and amplitude (P50 amp, N95 amp) were measured as indicated.

Estimation of sample size

The calculation of the sample size was based on a power of 0.80 with an α=0.05 for comparing two means of two normally distributed samples of equal sizes using an unpaired two-sided *t*-test. The following formula to determine the sample size is:

$$n = \frac{(\sigma_1^2+\sigma_2^2)(Z_{1-\alpha/2}+Z_{1-\beta})^2}{\Delta^2}$$

where

σ represents a known standard deviation

Δ represents a known change $\Delta = |\mu_2-\mu_1|$

For our study design we used the following information to determine our sample size: In postmenopausal women who have been on 2 mg of micronized 17β-estradiol for 2 months, the resistivity index in the central retinal artery has been shown to significantly decrease from 0.73 ± 0.08 to 0.66 ± 0.01.[325] Although this central retinal artery resistivity index was not an outcome variable for this observational study, it provided some guidance in calculating the sample size and we predicted we would find approximately the same magnitude of change of blood flow in the retina and optic nerve head. Therefore,

$$n = \frac{(0.08^2 + 0.01^2)(1.96 + 0.84)^2}{(0.73 - 0.66)^2}$$

n = 11 patients in the ∅ HT (control group)

n = 11 patients in the +HT (treated group)

We decided to include 25 subjects in each group hoping to gain power analysis.

Statistical analysis

Unpaired Student *t*-tests were performed for clinical, gynecological and ophthalmological variables between ∅HT and +HT post-menopausal women. Linear regression analysis was performed for the outcome variables for the blood flow, ONH stereometric parameter and PERG between ∅HT and +HT post-menopausal women. As postmenopausal women on HT were either on estrogens alone or combined with progestagens, subsequent linear regression analysis was performed to compare the effect of the type of HT treatment (estrogen therapy vs ∅HT, estrogen + progestagen therapy vs ∅HT and estrogen vs estrogen + progestagen therapies) in an attempt to evaluate the effects of estrogen therapy only. Statistical analysis was done using STATA/IC software (Stata Corp LP, Texas, v10.0). A significance level of $\alpha = 0.05$ was chosen.

3.2 Menopaused rat model

Animals

All experimental methods and animal care procedures were approved by the local institutional animal care committee, "le Comité de Déontologie de l'Expérimentation sur les Animaux" at the University of Montreal, in accordance with the Canadian Council on Animal Care. Twelve mature middle-aged (11-month old) retired female breeder Brown Norway (*Rattus norvegicus,* 225.5 ± 7.9 g) rats from a single colony (Harlan Sprague Dawley, Netherlands), were used in this study. Brown Norway rats have been chosen for the following reasons: 1) rats are widely used in ovarian aging research with well documented pathobiologic, cardiologic and reproductive endpoints, and 2) Brown Norway rats have large eyes compared to other rat species, which have made them ideal to use as glaucoma models. The age of 11 months was chosen as it corresponds approximately to the time when 65% of female rats present irregular estrous cycles,[343] equivalent 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 implantation of capsules

After habituation to their environment, bilateral ovariectomies (OVx) were performed in rats using a posterior surgical approach under isoflurane (3.0%) in order to normalize serum estrogen concentrations to their lowest level. Silastic capsules (I.D. 0.058, O.D. 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, USA) plus 75% cholesterol

(OVx+E_2, n=7, approximately 2 mg E_2 per capsule) or 100% cholesterol (placebo) (Sigma-Aldrich, St. Louis, MO, USA) (OVx, n=5) were implanted in the nape of the neck for time-release. These capsules have been validated as a means of delivering steady and physiological levels of E_2 in the range of 256.9 to 319.3 pmol/mL in mature OVx rats.[344-346] Moreover, the E_2 treatment administered in our OVx rats using silastic capsules is a representative model of HT treatment in menopausal women since the five-fold magnitude increase seen here 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.[285,287] Following the OVx surgery, 5 mL of Ringer's lactate were subcutaneously injected for rehydration, while repetitive subcutaneous doses of 0.1 mL of buprenorphine hydrochloride (Temgesic®, Reckitt Benckiser Health Science Ltd, UK, 0.1 mg/mL) were injected every 12 hours over a two-day period for post-operative pain management. Vaginal smears were performed both to ensure effectiveness of the bilateral OVx surgery and to serve as an indicator of tissue responsiveness to circulating estrogens released from E_2 capsules. Rats were exposed to E_2 or placebo treatment for a period of six weeks. The six-week treatment has been chosen in order to permit a further study investigating the potential vascular protective role of estradiol in a rat glaucoma model.

Surgical procedure for tissue perfusion investigation

Six weeks after OVx surgery and E_2 or placebo treatment, polyurethane catheters (I.D. 0.6 mm, O.D. 0.9 mm, Harvard Apparatus, Holliston, MA, USA) were inserted into the femoral vein and artery under 1.5% isoflurane (induction of anaesthesia with 3% isoflurane for 5 min). During this procedure, body temperature was monitored with a

rectal thermometer and maintained at 37°C by a heating pad (FHC, Bowdoinham, ME). Both blood pressure and heart rate were monitored from the tail using a non-invasive blood pressure cuff system (BP1000, Kent Scientific Corporation, Torrington, CT, USA). At the end of this procedure, 2 mL of Ringer's lactate were given subcutaneously for rehydration. Rats were then installed in a hammock and left under minimal restraint over a 2-hour period to recover from anaesthesia. Body temperature was maintained at 37°C with a heating lamp and both blood pressure and heart rate were monitored until the initiation of measurement experiment for the retinal perfusion. As well, cardiovascular, chemistry and hematological parameters were measured with the i-STAT®, a veterinarian clinical blood gases and electrolytes analyzer (HESKA, Fort Collins, CO), from arterial blood samples collected via the arterial catheter. This was achieved immediately prior to the initiation of measurement experiment for the retinal perfusion to confirm the recovery to normal physiologic level.

Principles of the evaluation of retinal tissue perfusion using IMP

In the aim of evaluating tissue perfusion in retina with a regional resolution, the diffusible blood flow tracer [^{14}C]-IMP was used. The [^{14}C]-IMP binds to the amine sites.[101,102] It was chosen as a blood flow tracer instead of [^{14}C]-iodoantipyrine,[93] [^{14}C]diazepam,[94] or *n*-[^{14}C]butanol,[95] because of three of its essential properties related to cerebral blood flow measurement.[96] These properties avoid the primary limitation of the classical diffusible tracer [^{14}C]-iodoantipyrine autoradiographic technique,[99,100] i.e. its time-dependent post-mortem intraparenchymal diffusion, which results in weakening the local tracer spatial gradients in tissues and regions. First, [^{14}C]-IMP has a very high

extraction at the first pass through the microvasculature, which allows a non-restrictive diffusion from blood to tissue despite the blood-retinal barrier. Second, [^{14}C]-IMP has a very low clearance rate and low post-mortem diffusion,[103] due to its high affinity for cerebral tissue. Third, [^{14}C]-IMP is chemically and biologically inert for the duration of the measurement period.[103] IMP has been successfully used in small animal research,[96,103,104] and in humans using Single Photon Emission Computed Tomography.

Laser Doppler flowmetry technology using an intraocular probe could not have been applied to measure the retinal blood flow in rats.[84-86] This technique is invasive as it requires to position the probe near the retinal surface while the IOP has to remain constant. This technique has to be performed under anaesthesia, which can alter basal hemodynamics and normal physiologic levels. Scanning Laser Doppler Flowmetry (SLDF) (HRF, Heidelberg Engineering, Germany, v1.02) technology could have been used as we did in postmenopausal women, but again it has been used under anaesthesia, and corneal dryness under anaesthesia is another limiting factor. Moreover, the SLDF cannot be apply in small rodents for capillary blood flow measurements as it does not have the ability to visualize the capillary layers and it detects blood flow signal from the choroid.[87]

The principle of blood flow evaluation is identical to that of micrometric microspheres[89] except that this "molecular microsphere" IMP is trapped within the tissue, whereas micrometric microspheres are trapped inside the microvascular space. Since the goal of this experiment is to determine local blood flow gradients in post-mortem tissue, the [^{14}C]-IMP molecular microsphere technique is more appropriate than the classical

microsphere technique. Moreover, the use of a diffusible tracer avoids inaccuracies of the retinal blood flow measurement due to axial streaming, plugging or permeability changes as reported with the classical microsphere technique.[89,92]

Procedure of measurement of the retinal tissue perfusion

The N-Isopropyl-[methyl-1,3-^{14}C]-p-iodoamphetamine (ARC, St Louis, USA) (100 μCi/kg) was dissolved in 600µL of saline (injectable 0.9% NaCl solution) and infused in fully conscious rats over a 30 s period at a constant rate of 1.2 mL/min using an infusion pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA) through the femoral vein. The amount of μCi/kg of injected [^{14}C]-IMP was based on the assumption that all rats were proportional in size. At the end of the infusion, the rats were immediately sacrificed by decapitation and a blood sample was collected for serum E_2 measurements. A small incision on the superior eyelid was made to indicate the orientation of the eyes and the eyes together with the attached superior eyelid were then harvested. They were immediately immersed in a solution of 4% paraformaldehyde for post-fixation. 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 from the eye cup, dissected in four quadrants (superior, inferior, nasal and temporal) and whole-mounted on a glass slide with the ganglion cells layer facing up. A small incision was made on the retina to indicate the superior quadrant. The whole-mount retina was then exposed to an X-ray film (Biomax, Kodak-Eastman Inc, Rochester, New York) for 10 days together with a set of [^{14}C]-standards (GE Healthcare

Ltd, UK). The autoradiograms were analyzed using the computerized image analysis MCID Basic Software (v7.0, Interfocus Imaging, Linton, England).

Calculation of retinal tissue perfusion

Retinal tissue perfusion was evaluated using the principle of indicator-fractionation technique.[96,103] A local retinal uptake index (RUI) was calculated from the following equation,[104]

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

where A is the injected dose (nCi), BW is the body weight (g) and C_{IMP} (T) is the radioactivity measured on the autoradiogram at the time (T) of sacrifice (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 centre of the optic nerve head in all retinal quadrants (Fig. 13). The IMP uptake index in the brain has been shown to correlate well with the local cerebral blood flow (CBF), where the CBF has been determined by positron emission tomography.[104] As the retinal vascular bed is similar to the cerebral vascular bed in terms of permeability (blood-tissue barrier), anatomy (non-fenestrated capillaries) and some local regulatory mechanisms (without autonomic innervation of the vasculature),[94] we considered that the IMP uptake index in the whole-mount retina can provide an approximation of retinal blood flow. However, it cannot be excluded that IMP may also have partly diffused from the choroid prior to retinal dissection.

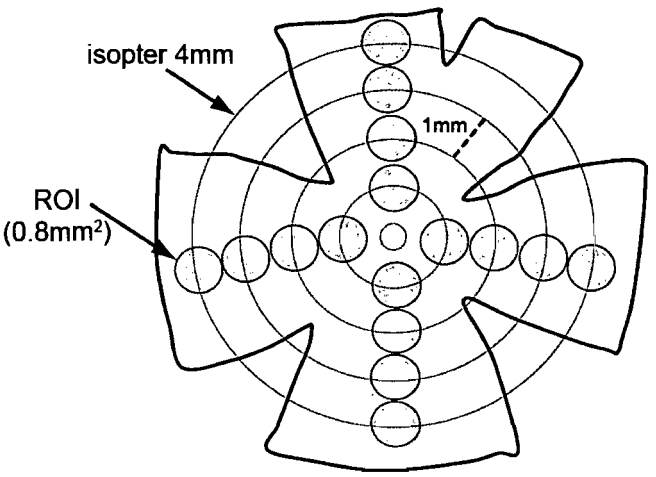

Figure 13: Schematic representation of a whole-mount retina showing the regions of interest (ROI) measured. The retina was divided into four quadrants (the notch indicates the superior quadrant). A circular ROI (filled circles, area 0.8 mm^2) was measured in each quadrant at each isopter (light circular lines at 1, 2, 3 and 4 mm away from the centre of optic nerve head). The four values of $C_{IMP}(T)$ of each quadrant were averaged for each isopter.

It has been demonstrated that the amount of the beta ^{14}C radiation energy reaching a X-ray film decreases with thickness of the tissue being exposed up to $\geq$100 μm where it reaches a plateau level.[347] Therefore, it can be assumed that the measured $C_{IMP}(T)$ from the whole-mount retina autoradiograms originate mainly from the first 100 μm of the inner retina and is mainly representative of the blood supply of the 100 μm inner part of the retina.

Serum assays

Serum E_2 levels were measured using a 3rd Generation Estradiol RIA kit (Diagnostic Systems Laboratories, Webster, TX, USA).[344] Since soy products from animal diets induce vasodilation on coronary arteries *in vitro* in combination with E_2,[348] the concentration of serum equol, a metabolite of daidzein, a phytoestrogen contained in the rat chow used (Ralston Purina Canada Inc), was also measured on a third and distinct group of rats (OVx, n=4; OVx+E_2, n=4) by a time-resolved fluoroimmunoassay kit (Labmaster diagnostic, Turku, Finland) as previously reported.[349]

Statistical analysis

The RUIs measured in each quadrant were averaged for each isopter. Unpaired Student *t*-tests were performed between the OVx group and OVx+E_2 group for (1) the physiological parameters, (2) the E_2 plasma concentrations, and (3) the RUI of each isopter. A significance level of $p \leq 0.05$ was chosen. A Mann-Whitney U-test was used to analyze equol plasma concentrations.

4.0 RESULTS

PAPER #1

This paper will be submitted to the journal

Investigative Ophthalmology and Visual Sciences

Effect of Postmenopausal Hormone Therapy on Retinal and Optic Nerve Head Blood Flow, and Retinal Nerve Fiber Layer in Postmenopausal Women

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The authors have no proprietary interest in the equipment used for testing or in post-menopausal hormone therapy drugs.

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ABSTRACT

PURPOSE: Postmenopausal hormone therapy (HT), consisting of estrogens alone or combined with progestagens, increases blood flow (BF) in both reproductive and non-reproductive organs and prevents atrophy in some brain regions. Since both estrogen and progesterone receptors are present in the human retina, we investigated whether HT improves retinal and optic nerve head (ONH) BF, and protects ONH topography and the function of retinal ganglion cells (RGCs).

METHODS: We investigated 65 healthy postmenopausal women of similar age, 29 who never used HT (∅HT) and 36 who used HT (+HT) continuously since menopause onset. BF of the infero-temporal retinal artery (ITRA), peripapillary retina, and ONH rim were measured in one eye using the Canon Laser Blood Flowmeter and the Heidelberg Retina Flowmeter. ONH stereometric parameters and the pattern electroretinogram (PERG) were measured using the Heidelberg Retina Tomograph and the VERIS^{TM} system.

RESULTS: Compared to the ∅HT group, the +HT group presented significantly greater BF of the ITRA (15.4 ± 3.4 vs 12.5 ± 2.9 μL/min, p=0.006), greater rim volume for the entire ONH region (0.438 ± 0.136 vs 0.380 ± 0.143 mm^3, p=0.032), and greater rim volume (0.066 ± 0.041 vs 0.050 ± 0.030 mm^3, p=0.042), height variation contour (0.353 ± 0.099 vs 0.289 ± 0.101 mm, p=0.011), mean thickness (0.194 ± 0.053 vs 0.167 ± 0.058 mm, p=0.033) and cross-sectional area (0.238 ± 0.068 vs 0.198 ± 0.073 mm^2, p=0.020) of the retinal nerve fiber layer for the infero-temporal region of the ONH. The capillary BF of the temporal peripapillary retina was greater (312.0 ± 75.1 vs 266.0 ± 58.1 AU, p=0.181) and the latency of P50 component of the PERG was shorter (42.2 ±

1.9 vs 43.3 ± 1.8 ms, p=0.063) in the +HT group compared to those of the ∅HT group, but these differences did not reach significance level.

CONCLUSIONS: The use of HT in postmenopausal women was associated with greater retinal arterial BF and thicker RNFL.

The menopause is the permanent cessation of ovarian follicular activity and subsequent fall in endogenous estrogens and progesterone production in aging women. Postmenopausal hormone therapy (HT) consisting of estrogens alone or combined with progestagens, is frequently prescribed to women in order to alleviate postmenopausal symptoms.

As well as their role in function of many cells and organs, estrogens have the ability to influence the vascular tone and blood flow in organs and tissues. In postmenopausal women, HT has been shown to improve blood flow in the femoral artery[1] and brain parenchyma,[2-5] and pulsatility and/or resistivity indexes (an expression of resistance to flow) in the common carotid,[6-8] internal carotid,[9-13] and middle cerebral arteries.[10,11] Moreover, there is a growing body of evidence that HT has a protective effect in some regions of the brain by preventing cerebral tissue atrophy. Observational studies using magnetic resonance indicated that women on HT presented larger volumes of some brain regions such as the hippocampus,[14-16] and the lateral prefrontal, fusiform and entorhinal cortex.[16]

There is a large body of evidence that impaired ocular blood flow is contributing factor in the etiology and progression of glaucoma and age-related macular degeneration (ARMD). Recently, there has been an increasing interest in investigating the possible protective role of HT against glaucoma and ARMD. Epidemiological population–based studies indicated that increased duration of exposure to endogenous estrogens (early menarche, late menopause onset and increasing reproductive year) significantly decreased the odds ratio of developing primary open-angle glaucoma[17,18] and ARMD,[19]

and that the use of HT decreased the odds ratio of advanced[20] and neovascular age-related macular degeneration.[21] This possible protective role of HT may result from improved blood flow to the retina and optic nerve head (ONH). Interestingly, estrogen receptors (ERs) are present in the human retina,[22-26] as are progesterone receptors (PRs),[22-24] and postmenopausal women taking HT have shown to present improved pulsatility and/or resistivity indexes in the retrobulbar vessels, such as the ophthalmic,[12,13,27,28] short posterior[29] and the central retinal arteries.[29-32] However, it is unknown as to whether HT has a vascular effect on the retinal and optic nerve head blood flow in postmenopausal women. Additionally, as HT has a protective effect on some brain regions and ERs are present in the ganglion cell layer and axons in human retinas,[25,26] it is of interest to know whether HT may have a protective effect on the optic nerve head (ONH) topography and on retinal ganglion cell (RGC) function.

In an attempt to answer these two questions, we designed an observational study in which retinal and ONH blood flow, the ONH topography and the function of the RGCs were measured in healthy postmenopausal women who were HT users (+HT) since their menopause onset. Results were compared with those obtained from healthy postmenopausal women of similar age and characteristics who had never used HT (∅HT) since their 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 Writing Group for the Women's Health Initiative Randomized Controlled Trial,[33] which is when this study was being

initiated. Retinal blood flow measurements were obtained at three different sites: 1) in a major retinal vessel, the infero-temporal retinal artery (ITRA), using the Canon Laser Blood Flow Meter (CLBF, Japan) and in both peripapillary 2) nasal and 3) temporal areas using the Heidelberg Retina Flowmeter (HRF, Germany). For the ONH, the blood flow of the rim was also measured using the HRF. The ONH topography was assessed by measuring the 12 standard stereometric parameters using the Heidelberg Retina Tomograph I (HRT I), and the functionality of the RGCs was measured with the pattern electroretinogram (PERG) using the VERIS^{TM} system.

METHODS

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 enrolment in the study. Subjects were tested in the Ocular Blood Flow Laboratory at the Ophthalmology Research Unit, Maisonneuve-Rosemont Hospital Research Centre.

Eligibility criteria

Healthy postmenopausal women were recruited from both Notre-Dame and Maisonneuve-Rosemont Hospital Menopause Clinics, Montreal, Quebec by personal interview and by posting advertisements in both hospitals and in the local newspaper. To be eligible for the study, all pre-screened healthy menopausal women had to meet the following inclusion criteria: not HT (∅HT) or HT (+HT) users (as prescribed by a physician) since their menopause onset, 45 years of age or older, naturally or surgically menopaused by ovariectomy, amenorrhea for at least one year, body mass index equal or less than 30 and non or former smoker. Menopausal women had to be free of systemic, cardiovascular and central nervous system diseases and had to present normal ocular histories and eye exams. 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 prior to the study visit, subjects were asked to avoid caffeine and alcohol so as not to alter their basal hemodynamic level. As well, they were asked to avoid food with high fat content, which alters the serum estradiol level assays. Also, so as to confirm the use of HT and rule out the effects of vasoactive drugs, subjects were asked to bring all their medications, including any herbal medicine with them. The type of HT regimen (estrogens alone or combined with progestagens) and active ingredients were documented for HT users.

Medical, gynecological and ocular histories, and eye exam

At the time of the study visit, demographic, medical, gynecological and ocular histories were obtained from each subject using a standardized questionnaire administered by one examiner (MCD), who determined the eligibility of the subjects for the study. Medical history included measures of the blood pressure (BP)(mmHg) at rest for 30 minutes, height (m), and weight (kg). These measures were used for calculating the body mass index (BMI=weight/height^2) and mean BP ((BPdiastolic +1/3(BPsystolic – BPdiastolic)). Gynecological 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 women who had hysterectomies, the age at last menses could not be determined. Therefore, age at last menses, reproductive year and menopause durations were tabulated as missing values. Ocular histories and eye exams

were obtained, which included best corrected visual acuity using the Snellen chart (VA expressed by its numerator divided by its denominator), refraction (expressed in spherical equivalent), and biometry (OcuScan, Alcon® Surgical Inc., USA v3.02). Slit-lamp examination, evaluation of peripheral anterior chamber depth, tonometry (IOP mmHg), and ophthalmoscopy were performed by one ophthalmologist (DD). Ocular perfusion pressure (OPP mmHg) was calculated using the following formula: OPP= 2/3 BP_{mean}-IOP.[34]

Selection of the investigated eye

All retinal and ONH blood flow measurements, as well as ONH topography and PERG measurements were conducted in one eye only, i.e. the eye with the clearest media, better fixation and least lid ptosis. When both eyes were eligible for the study, one eye was randomly chosen. The chosen eye was tested first undilated for the PERG testing, then dilated with tropicamide (Mydriacyl® 1%, Alcon Canada Inc.) for the HRT, SLDF and CLBF testing.

Serum estradiol and progesterone assays

A blood sample was obtained from all subjects and analyzed by the local hospital laboratory service for documenting the total serum estradiol (E_2) and progesterone (P) concentrations by chemiluminescent immunoassay technique (ADVIA Centaur®, Bayer HealthCare Diagnostic, USA). These assays were obtained to confirm the hormonal status of estrogens and progestagens in all postmenopausal women. Some active

ingredients present in the HT regimens and used by postmenopausal women were not 17β-estradiol- and/or progesterone-based. Therefore, measures of the serum estradiol and progesterone concentrations by the hospital laboratory service were not appropriate. Consequently, low serum concentrations of estradiol and progesterone not detected by the assay technique were tabulated as zero. As well, for some postmenopausal women who were not HT users, the endogenous serum 17β-estradiol and progesterone concentrations were below the level of detection by the assay technique. Consequently, their serum concentrations were also tabulated as zero.

Evaluation of Retinal Blood Flow

Infero-temporal retinal artery. Blood flow (Flow) of the infero-temporal retinal artery (ITRA) was measured using the Canon Laser Blood Flowmeter System (CLBF model 100, Japan). The CLBF is a unique system that quantifies blood flow in retinal vessels by simultaneously measuring both diameter (D) and velocity (Vel) of the blood column in a vessel of interest,[35] and by using eye tracking features. To achieve this, the CLBF system is equipped with a dual laser system mounted in an eye fundus camera and an acquisition/analysis software (v2.1.23). An oval (8 μm x 50 μm) red diode laser (675 nm) measures the mean center line blood column velocity (Vel_{mean}) of the vessel of interest by using a bidirectional laser Doppler velocimetry, the principle of which has been described elsewhere.[36-38] The D is measured by a rectangular (1500 μm x 150 μm) green diode laser (543 nm) manually positioned perpendicular to the vessel of interest. The laser measures the blood column width by transmittance profile method, the principle of which has been described elsewhere.[39] The D is determined automatically

by computer analysis of the signal produced by the image of the vessel on a charge-coupled device (CDD) sensor using the half-height of the transmittance profile to define the blood column edge. It is measured for the first and last 60 ms of the 2 s velocity measurement period, at every 4 ms (250 Hz sampling rate). 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. To compensate for microsaccade of the eye, the green diode laser also serves as a tracking system by scanning repeatedly and rapidly the intensity profile produced by the rectangular green diode laser and by analyzing the scanned intensity profile. Since both lasers operate simultaneously, D and Vel_{mean} are obtained in the same time frame.

For accuracy, the CLBF software operates by measuring two Paths (Path1 and Path2 with each its own velocity and diameter), then automatically calculates the flow following the Poiseuille's law, according to the following equation (Eq. 1),

$$\text{Flow } (\mu L/min) = \text{Velocity}_{mean}(\pi \text{Diameter}^2/8) \qquad \text{(Eq. 1)}$$

where Velocity_{mean} is the time averaged center line blood velocity (mm/s) during the cardiac cycle and $\pi\text{Diameter}^2/8$ is the cross-sectional area of the measurement site (μm^2)

Since study participants underwent several tests during the study visit, retinal blood flow measurements were only obtained from one site, the ITRA. By inference, the blood flow measures from the ITRA can be used as an indicator of the overall retinal arterial circulation. There are two motives for choosing the main retinal artery from the infero-

temporal region. First, it is a region that is technically accessible to obtain CLBF measurements, and second, the infero-temporal region is the most common site of early glaucomatous damage. The location of the measurement on the ITRA was done 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 high quality Path1-Path2 pairs and calculated Flow values were obtained. After excluding the incomplete measures (the CLBF automatically rejected flow measurements when one of the two paths was unreplicable) and discarding the highest and lowest flow values, the three closest flow values calculated by the CLBF software were used for averaging D, $Vel_{mean,}$ and Flow. This recording protocol gave us an intra-session 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 (manuscript in preparation). Figure 1 illustrates an example of a CLBF recording from a normal subject.

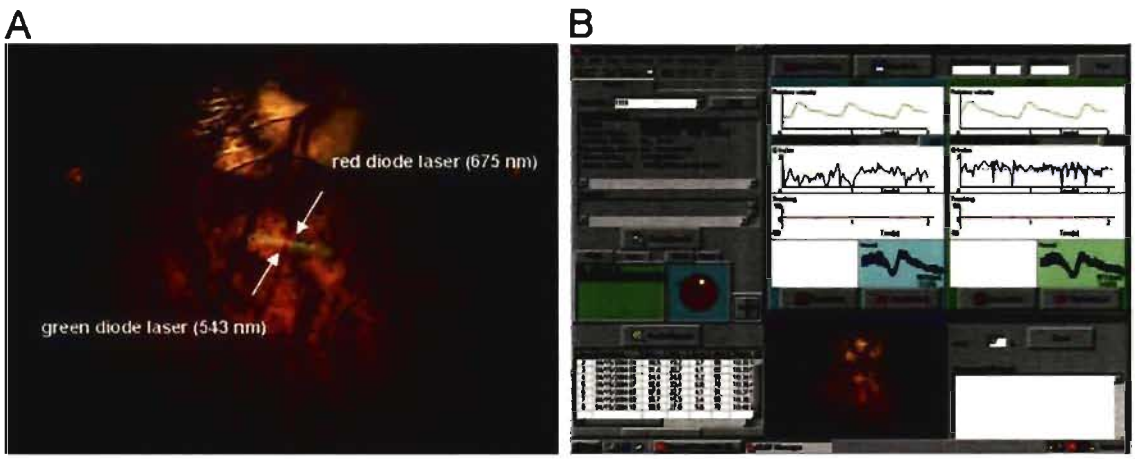

Figure 1: Example of a CLBF measurement obtained from a normal subject. The panel (A) illustrates the position of the red diode laser (675 nm) that measures the mean center line blood column velocity of the infero-temporal retinal artery (ITRA) and the orientation of the green diode laser (543 nm) that measures its diameter. The right panel (B) shows both Path measurements, Path 1 and Path 2, with their respective parameter windows. For one Path, the top window displays the relative velocity over a 2 s period, followed by a window which displays the Q-index over a 2 s period and the overall Q-index (an indicator of the signal quality internally determined by the software). Then a third window displays the stability of the vessel tracking over the 2 s period, and finally the last window displays the coefficient of variation of the vessel diameter. For this particular subject, four Flow measurements (ranging from 3. 7 to 5.2 μL/min) were acquired with their respective D (ranging from 90.5 to 94.4 μm) and Vel (ranging from 19.2 to 24.6 mm/s) as shown on the bottom left of panel B.

Peripapillary retinas and ONH rim areas. The capillary blood flows of the ONH rim and both nasal and temporal peripapillary retinal areas were measured by Scanning Laser Doppler Flowmetry (SLDF) (HRF, Heidelberg Engineering, Germany, v1.02). Briefly, the Heidelberg Retina Flowmeter combines a confocal laser scanning technique and laser Doppler flowmetry. It measures the amount of back scattered light at different locations (pixels) in the region of interest. A parallel infrared laser beam of 3 mm diameter (780 nm, 180 μW) scans line by line a two-dimensional region of the retina. The scan field is 10° wide by 2.5° height, corresponding to a size of 2.88 mm x 0.72 mm. Detailed description of the instrument and measurement technique has been described elsewhere.[40,41] During the scan along one line, the reflected light intensity at 256 equidistant locations (pixels) is measured and digitized (pixel distance 11.2 μm) sequentially. Thus, the reflected light intensities of each pixel are obtained as a function of time (fluctuation time curve). The collected intensity data of each pixel was then analyzed by a discrete Fourier transformation, thus calculating the laser Doppler frequency shift for each pixel. The data of the laser Doppler frequency shift of the 64 x 256 pixels is the base to generate the perfusion image, and the automatic full-field perfusion image analysis software (AFFPIA, v3.3) computes flow (distance travelled by all moving red blood cells per unit of times), volume (number of moving blood cells), and velocity (mean of blood cell speed) for each pixel, all expressed in arbitrary units (AU). For valid capillary blood flow measurement at each pixel, the AFFPIA uses an algorithm to exclude over- or under-exposed pixels, retinal vessel tree and eye saccades (which is identified by the operator). Moreover, the AFFPIA permits an interactive marking of the rim area of the optic nerve head allowing the output of the three

hemodynamic parameters, flow (Flow), volume (Vol) and velocity (Vel) for each region of interest, i.e. the nasal peripapillary retina (n), temporal peripapillary retina (t) and neuroretinal rim (r) areas. Figure 2 illustrates an example of a SLDF perfusion image and automatic full-field perfusion image analysis obtained from a normal subject.

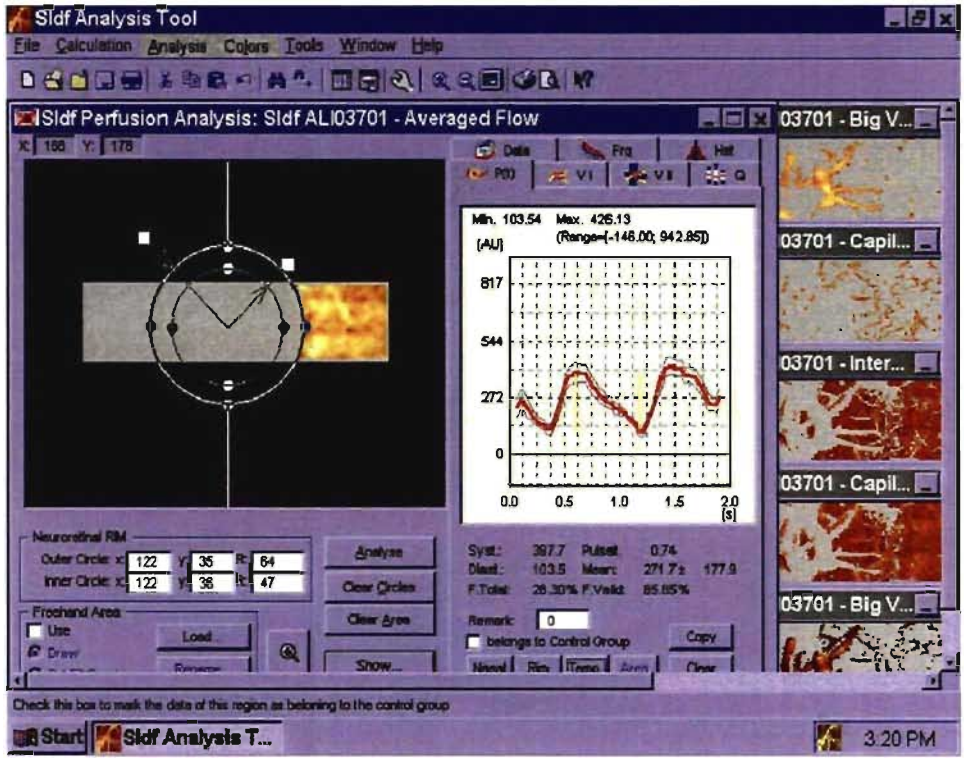

Figure 2: Example of a SLDF perfusion image obtained from a normal subject. The left side shows the marking circle delimitating the rim and both nasal and temporal retina of the optic nerve head region from a normal subject. The right side shows the Flow, Vol and Vel values obtained with the full-field perfusion image analysis (AFFPIA). For this particular subject, the Flow, Vel and Vol values obtained by the AFFPIA for the temporal retina of this SLDF image corresponded to 271.7 AU, 0.9 AU, and 20.7 AU respectively.

Our HRF images were centered on the optic nerve head, optimizing the photodetector sensitivity in order 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 full-field perfusion image analysis. The obtained Flow, Vel and Vol from these 5 images for the nasal (n), temporal(t) and rim(r) areas were then averaged. This SLDF measurement protocol developed in our lab yields intra-session 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.[42]

ONH topography

The optic nerve head topography was evaluated by measuring the 12 standard stereometric parameters (i.e. disc area, cup area, c/d, 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) in all subjects. This was achieved by using the Heidelberg Retina Tomograph I (HRT I, Heidelberg Engineering, Heidelberg, Germany, v2.01). The HRT I is a confocal laser scanning ophthalmoscope operating three-dimensionally. A diode laser (670 nm) scans the optic nerve head (along the *z*-axis) in a series of 32 consecutive and equidistant two-dimensional optical section images (*x*-axis and *y*-axis of 10° x 10° (256 x 256 pixels)). Each pixel is approximately 10 μm. The reflected light at each pixel is measured by the

confocal optical detector system and is used for generating a topography and reflectivity image. The topography image is the location along the *z*-axis where the maximum reflectivity registered is assumed to be the height of the location and the reflectivity image is the sum of the reflectivity measurements along the z-axis. Between five to ten ONH images were obtained and the average of the three closest images in terms of alignment, brightness and clarity was obtained. With the aid of fundus pictures, the external contour of the optic nerve rim was manually drawn and the Heidelberg Retina Tomograph interactive software automatically calculated the twelve standard stereometric parameters. The stereometric parameters were obtained for the entire ONH (0°-360°) and for the infero-temporal region (270°-360°), which is a region most commonly affected by early glaucomatous damage. Figure 3 illustrates the topography and reflectivity images, and an output of the stereometric parameters calculated by the HRT software from a normal subject.

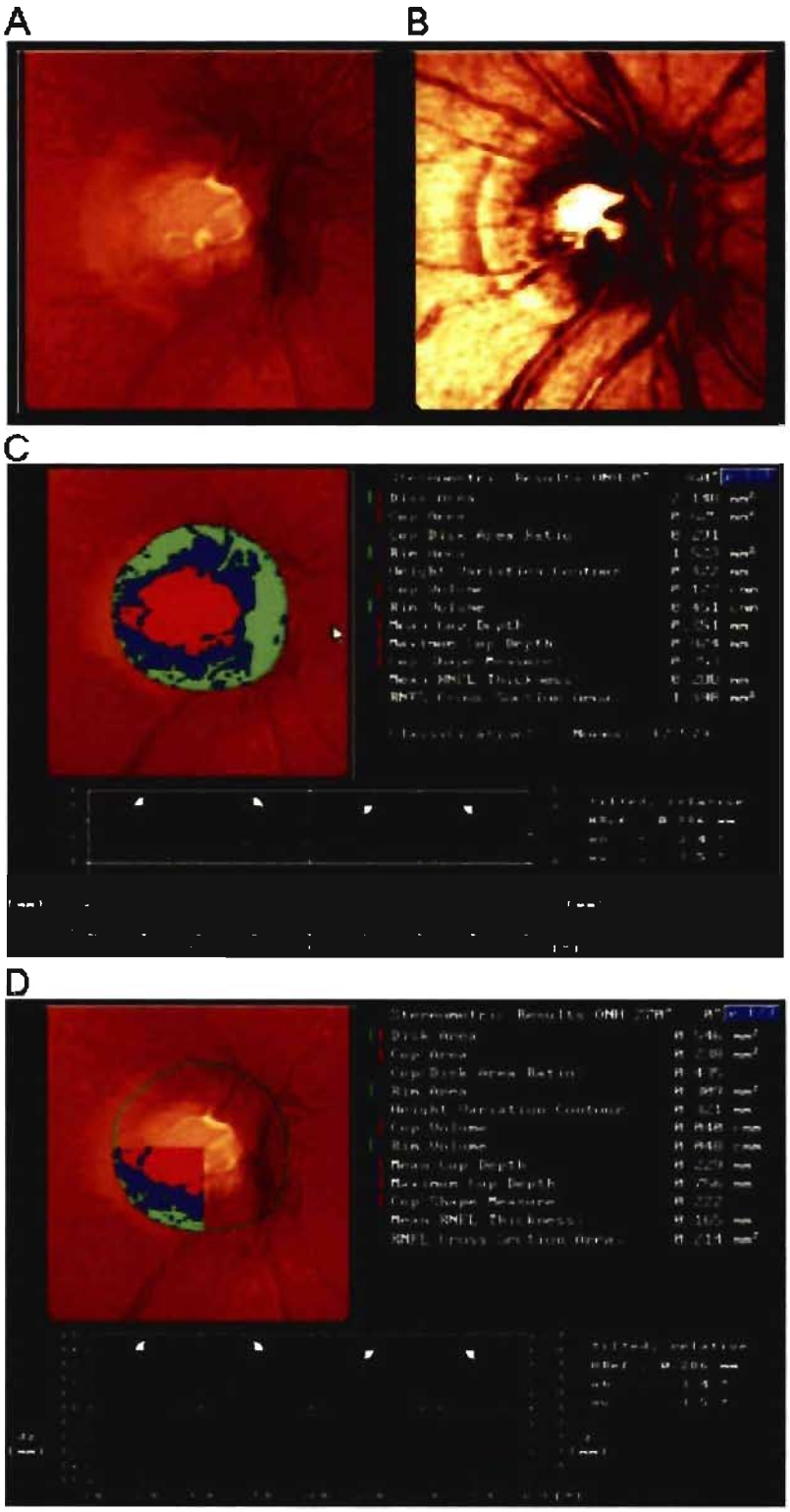

Figure 3: Example of ONH topography measurement obtained from a normal subject

using the Heidelberg Retina Tomograph I (HRT I). The topography image (panel A) represents the maximum reflectivity registered along the *z*-axis at each pixel, and the reflectivity image (panel B) represents the sum of all the reflectivity measurements along the z-axis. The 12 standard stereometric parameters from the entire ONH (0-360°, panel C) and the infero-temporal region (270-360°, panel D) are automatically calculated by the HRT software after manually marking the external contour of the optic nerve head rim.

Functionality of the RGC

The pattern electroretinogram (PERG) represents an objective and direct measure of retinal ganglion cell function.[43-45] It is a retinal biopotential evoked when a high contrast patterned stimulus (alternating stripes or checkerboards) is viewed. The PERG waveform signal begins with a negative deflection called N35, followed by a positive deflection called P50, and then a second negative deflection called N95. The PERG was recorded using the VERIS™ Multifocal System (Electro-Diagnostic Imaging, Inc., USA, v5.0) following 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° x 38° with checksize of 0.8° generated by a 9-inch monochrome monitor (75 Hz frame rate). The luminance of the white squares was set at 353 cd/m^2 and for the black squares it was set at 30 cd/m^2, (of 192 cd/m^2 average luminance, 84% contrast). A sterilized and disposable silver DTL Plus™ (Diagnosys LLC, USA) electrode placed in the lower conjunctival sac served as the active electrode in reference to a gold cup

electrode (Grass Technologies, USA) placed at the ipsilateral outer canthus. Subjects were grounded through the forehead with a gold cup electrode. Electrode impedances were checked for 5kΩ or less. The recording session was devided in segments, i.e. 32 segments, each lasting 3.41 s for a total of 109.1 s. This corresponded to an average of 514 responses obtained. During one segment recording, the subject was asked not to blink to minimize blinking artifacts since there is no artifact rejection capability built into the system. Two replicable PERG waveforms were obtained. The N35, P50 and N90 waveform components were identified, and latency (N35 lat, P50 lat, N95 lat) and amplitude (P50 amp, N95 amp) were measured. Figure 4 illustrates a transient PERG waveform and measured components obtained from a normal subject.

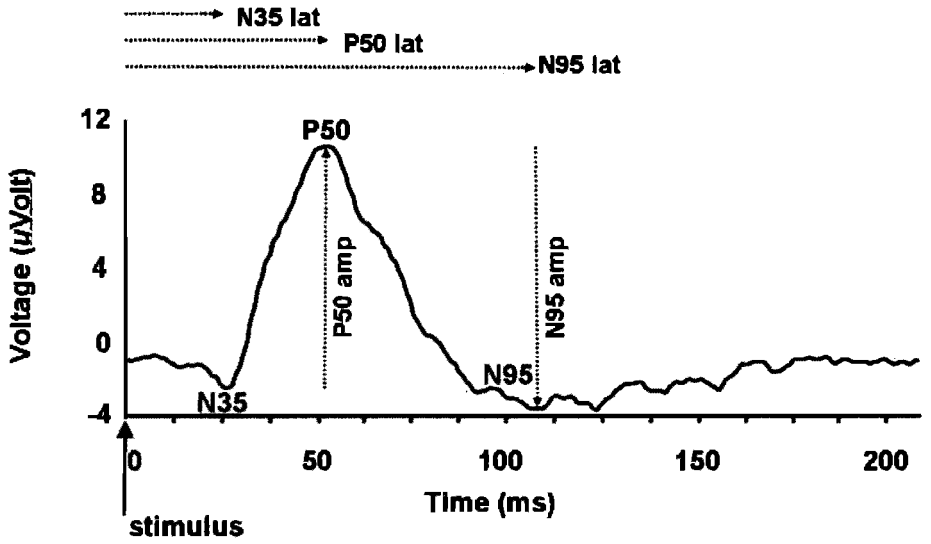

Figure 4: Example of a transient pattern electroretinogram (PERG) obtained of the right eye from a normal subject. The PERG was evoked by a checkerboard pattern of 50° x 38° with a checksize of 0.8° set at a contrast of 84% (white square luminance of 353 cd/m^2, black square luminance 30 cd/m^2). The PERG was recorded at 4.7 reversal/s. The three main waveform components were identified, N35, P50 and N90, and both latency (N35 lat, P50 lat, N95 lat) and amplitude (P50 amp, N95 amp) were measured as indicated.

Estimation of sample size

The calculation of the sample size was based on a power of 0.80 with an α=0.05 for comparing two means of two normally distributed samples of equal sizes using an unpaired two-sided *t*-test. The following formula to determine the sample size is:

$$n = \frac{(\sigma_1^2+\sigma_2^2)(Z_{1-\alpha/2}+Z_{1-\beta})^2}{\Delta^2}$$

where

σ represents a known standard deviation

Δ represents a known change $\Delta = |\mu_2-\mu_1|$

For our study design we used the following information to determine our sample size: In postmenopausal women who have been on 2 mg of micronized 17β-estradiol for 2 months, the resistivity index in the central retinal artery has been shown to significantly decrease from 0.73 ± 0.08 to 0.66 ± 0.01.[30] Although this central retinal artery resistivity index was not an outcome variable for this observational study, it provided some guidance in calculating the sample size and we predicted we would find approximately the same magnitude of change of blood flow in the retina and optic nerve head. Therefore,

$$n = \frac{(0.08^2 + 0.01^2)(1.96 + 0.84)^2}{(0.73 - 0.66)^2}$$

n = 11 patients in the ∅ HT (control group)

n = 11 patients in the +HT (treated group)

We decided to include 25 subjects in each group hoping to gain power analysis.

Statistical analysis

Unpaired Student *t*-tests were performed for clinical, gynecological and ophthalmological variables between ∅HT and +HT post-menopausal women. Linear regression analysis was performed for the outcome variables for the blood flow, ONH stereometric parameter and PERG between ∅HT and +HT post-menopausal women. As postmenopausal women on HT were either on estrogens alone or combined with progestagens, subsequent linear regression analysis was performed to compare the effect of the type of HT treatment (estrogen therapy vs ∅HT, estrogen + progestagen therapy vs ∅HT and estrogen vs estrogen + progestagen therapies) in an attempt to evaluate the effects of estrogen therapy only. Statistical analysis was done using STATA/IC software (Stata Corp LP, Texas, v10.0). A significance level of $\alpha = 0.05$ was chosen.

RESULTS

Study population characteristics

A total of 74 post-menopausal women were enrolled in the study but 9 of them were excluded for the following reasons: four presented significant media opacities that interfered with the testing, one was un-cooperative and presented unreliable test results, one also presented unreliable test results and the pupil was unable to dilate, two did not feel well before starting the study, and one patient stated she was not taking HT but her serum estradiol result indicated an unusually elevated level. Therefore, a total of 65 subjects met the study criteria among whom 29 had never used HT (44.6%) since their menopause onset and 36 used HT (55.4%) currently and continuously since their menopause onset. Among ∅HT and +HT women, one and two of them were surgically menopaused respectively (ovariectomy combined with hysterectomy), while one and ten of them respectively underwent through hysterectomy only.

Clinical, gynecological, and ophthalmological characteristics for the whole study population are given in Table 1. The duration of HT was 8.3 ± 6.0 yr for the +HT group of postmenopausal women. As expected, the only significant differences between the ∅HT and the +HT subjects were the serum E_2 concentration (62.2 ± 60.9 vs 258.4 ± 159.1 pmol/L, $p<0.001$) and serum P concentration (1.9 ± 0.9 vs 11.8 ± 20.1 nmol/L, $p=0.010$). The large standard deviation for both E_2 and P concentration is likely due to a variable endogenous source of these hormones that differs between women and with time. Moreover, since active ingredients present in the HT regimens used by some

postmenopausal women were not 17β-estradiol- and/or progesterone-based, this could also have contributed to the large standard deviation.

Regarding their clinical, ophthalmological, and other gynecological characteristics, the two groups of women were not statistically different, especially in terms of their age, BMI, BP and natural menopause duration. For postmenopausal women who were naturally or surgically menopaused, the sample size used for calculating the averaged age at last menses, natural menopause and reproductive year durations is indicated between parentheses below each value for the whole study population

In the same table, the whole study population is presented in subsets of sub-populations A, B, C, and D that were investigated for the CLBF, SLDF, HRT and PERG testing respectively. Data from some of the tests were either not obtained or discarded for the following reasons: some subjects presented fixation problems (eye movement and/or microsaccade), inability to open the eye wide, and unreliable data measurements for some of the tests conducted. For CLBF testing, data from some subjects was discarded because the main ITRA divided itself into second order arterioles near the outer edge of the ONH. Regarding the ∅HT and +HT groups for all sub-populations, they were also similar in all their characteristics. The exceptions were the serum E_2 and P levels that were statistically different between ∅HT and +HT groups for all the sub-populations. As well, the diastolic blood pressure (79.6 ± 9.0 vs 73.0 ± 8.8 mmHg, p=0.03), the mean BP (95.3 ± 8.9 vs 88.4 ± 10.1 mmHg, p=0.03), and the OPP (49.0 ± 5.9 vs 44.4 ± 6.9 mmHg, p=0.03) were significantly higher in the ∅HT group for the SLDF sub-

population B studied. The sample size used for calculating the averaged age at last menses, menopause and reproductive year duration is also indicated between parentheses below each value for each sub-population for postmenopausal women who were naturally or surgically menopaused.

Table 1: Clinical, Gynecological, and Ophthalmological Characteristics for The Whole Population Study of Women Who Never Used Postmenopausal Hormone Therapy (ØHT) and Who Were HT Users (+HT) and for Sub-Population Study for CLBF (A), SLDF (B), HRT (C) and PERG (D) Assessments

	Whole Population		Sub-Population							
			A		B		C		D	
Characteristics	ØHT (n=29)	+HT (n=36)	ØHT (n=18)	+HT (n=21)	ØHT (n=16)	+HT (n=27)	ØHT (n=27)	+HT (n=33)	ØHT (n=21)	+HT (n=31)
Clinical										
Age (yr)	56.7 ± 5.8	56.8 ± 4.9	57.2 ± 5.9	56.8 ± 4.3	56.6 ± 4.7	56.5 ± 4.9	56.6 ± 5.7	56.7 ± 4.9	56.2 ± 5.4	56.2 ± 4.4
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 ± 3.3	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	73.2 ± 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
Gynecological										
Age at menarche (yr)	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 (yr)	49.3 ± 4.0	47.8 ± 5.7	49.4 ± 4.7	46.9 ± 6.8	49.9 ± 4.5	47.7 ± 5.7	49.7 ± 3.7	48.0 ± 5.6	49.1 ± 3.7	47.1 ± 5.9
(n)	(28)	(26)	(18)	(14)	(16)	(18)	(26)	(24)	(20)	(22)
Duration of RY (yr)	37.0 ± 4.3	34.9 ± 6.3	37.1 ± 4.8	34.2 ± 7.7	37.7 ± 4.8	34.5 ± 6.6	37.5 ± 3.9	35.1 ± 6.1	36.8 ± 3.9	34.3 ± 6.6
(n)	(28)	(26)	(18)	(14)	(16)	(18)	(26)	(24)	(20)	(22)
Duration of menopause (yr)	7.6 ± 6.4	8.9 ± 6.3	7.8 ± 7.1	9.5 ± 7.4	6.8 ± 5.1	8.8 ± 7.2	7.0 ± 5.6	8.6 ± 6.4	7.3 ± 5.4	8.7 ± 6.5
(n)	(28)	(26)	(18)	(14)	(16)	(18)	(26)	(24)	(20)	(22)
HT duration (yr)	0.0 ± 0.0	8.3 ± 6.0*	0.0 ± 0.0	8.5 ± 6.7*	0.0 ± 0.0	7.9 ± 6.7*	0.0 ± 0.0	8.3 ± 6.1*	0.0 ± 0.0	8.2 ± 5.9*
Serum E_1 (pmol/L)	62.2 ± 60.9	258.4 ± 159.1*	70.1 ± 63.6	252.1 ± 173.8*	61.8 ± 65.9	253.4 ± 154.1*	62.7 ± 64.2	243.1 ± 159.8*	57.4 ± 51.5	262.0 ± 160.6*
Serum P (nmol/L)	1.9 ± 0.9	11.8 ± 20.1*	1.8 ± 0.7	11.7 ± 18.1*	1.6 ± 0.8	11.4 ± 19.1*	1.9 ± 1.0	13.9 ± 22.1*	1.9 ± 1.0	10.2 ± 16.9*
Ophthalmological										
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	-0.8 ± 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	-0.7 ± 2.7
IOP (mmHg)	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	14.2 ± 2.3	14.6 ± 1.4	14.0 ± 2.1
OPP (mmHg)	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 means ± standard deviations (SD). Number of subjects is in parenthesis

* Significance level ($P \leq 0.05$) determined by unpaired Student *t*-test between the +HT and ØHT for the whole and sub-populations.

Table 2 shows the list of the active estrogen and progestagen ingredients present in the various HT regimens prescribed and used by postmenopausal women in this observational study. Table 3 indicates the frequency distribution of those active ingredients. In our whole study population of 65 subjects, 36 of them were +HT users (55.4%), 12 (18.5%) were on monotherapy (estrogens alone) and 24 (36.9%) were on a combined therapy (estrogens + progestagens). Of the +HT users 25 (38.5%) of them were taking the active ingredients 17β-estradiol (E_2), which is one of the human bio-identical ingredients.

Table 2: Type of Postmenopausal Hormone Therapy and Their Active Ingredients Prescribed and Used by the +HT Postmenopausal Women (n=36)

HT Trade Name	Manufacturer	Active Ingredients
Estrogens		
Climara®	Bayer HealthCare	17β-estradiol
Estradot®	Norvatis Pharma Canada	17β-estradiol
Estrogel®	Schering Canada	17β-estradiol hemihydrate
Estrace®	Shire Biochem	17β-estradiol micronized
Ogen®	Pfizer Canada	estropipate
Premarin®	Wyeth Canada	conjugated estrogens
Progestagens		
Prometrium®	Schering Canada	progesterone micronized
Provera®	Pfizer Canada	medroxyprogesterone
Norlutate®	Parke Davis Canada	norethindrone acetate
Mirena®	Berlex	levonorgestrel
Estrogens + Progestagens		
Estalis-Sequi®	Norvatis Pharma Canada	17β-estradiol + norethindrone acetate
Premplus™	Wyeth Canada	conjugated estrogens + medroxyprogesterone
Fem HRT™	Warner Chilcott	ethinyl estradiol + norethindrone acetate

Table 3: Frequency Distribution of +HT (n=36) Categorized by Their Active Ingredients.

Therapy Type	Active Ingredients	n (%)
Monotherapy	17β-estradiol	10 (27.7)
	17β-estradiol micronized	1 (2.8)
	conjugated estrogens	1 (2.8)
Combined	17β-estradiol + progesterone micronized	1 (2.8)
Therapy	17β-estradiol + levonorgestrel	2 (5.6)
	17β-estradiol + norethindrone acetate	2 (5.6)
	17β-estradiol micronized + progesterone micronized	1 (2.8)
	17β-estradiol micronized + medroxyprogesterone	1 (2.8)
	17β-estradiol micronized + norethindrone acetate	1 (2.8)
	17β-estradiol hemihydrate + progesterone micronized	5 (13.9)
	17β-estradiol hemihydrate + medroxyprogesterone	1 (2.8)
	estropipate + progesterone micronized	2 (5.6)
	estropipate + medroxyprogesterone	1 (2.8)
	ethinyl estradiol + norethindrone acetate	3 (8.3)
	conjugated estrogens + progesterone micronized	1 (2.8)
	conjugated estrogens + medroxyprogesterone	3 (8.3)
	Total	36 (100.0)

Flow of infero-temporal retinal artery

The D, Vel_{mean} and Flow of the ITRA were obtained from 18 ∅HT and 21 +HT postmenopausal women (Table 1, sub-population A). Table 4 shows D, Vel_{mean} and Flow values and their significance levels obtained from linear regression models both unadjusted and adjusted for age of subject, OPP and age at menarche. In the +HT group, the Flow of the ITRA was significantly greater compared to that of ∅HT women (15.4 ± 3.4 vs 12.5 ± 2.9 μL/min, p=0.006). This significant greater Flow value was mostly due to a significantly larger D of the ITRA measured in the +HT group (133.2 ± 13.9 vs 120.7 ± 11.6 vs μm, p=0.005), while the Vel_{mean} was also greater, but not significantly different between the two groups (37.0 ± 6.2 vs 36.4 ± 7.6 mm/s, p=0.687).

Table 4: Diameter, Velocity and Flow of the Infero-Temporal Retinal Artery Measured in ∅HT and +HT Postmenopausal Women

	∅HT (n=18)	+HT (n=21)	*P* value Unadjusted*	Adjusted†
Diameter (um ± SD)	120.7 ± 11.6	133.2 ± 13.9	0.005*	0.005†
Velocity (mm/s ± SD)	36.4 ± 7.6	37.0 ± 6.2	0.803	0.687
Flow (uL/min ± SD)	12.5 ± 2.9	15.4 ± 3.4	0.008*	0.006†

Values are means ± standard deviations (SD). Number of subjects is in parenthesis.

* Significance level determined by unadjusted linear regression model.

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

Capillary flow of the retinal peripapillary and ONH rim areas

Table 5 represents the capillary blood Vol, Vel and Flow measurements for the rim of the ONH and both temporal and nasal peripapillary retina obtained from 16 ∅HT and 27 +HT postmenopausal women (Table 1, sub-population B) with their significance levels obtained from linear regression models both unadjusted and adjusted for age of subject, OPP and age at menarche. In the +HT group, the capillary Vol (22.3 ± 3.9 vs 19.6 ± 3.4 AU, p=0.120), Vel (1.11 ± 0.26 vs 0.98 ± 0.19 AU, p=0.33) and Flow (312.0 ± 75.1 vs 266.0 ± 58.1 AU, p=0.181) of the temporal peripapillary retina were greater compared to the ∅HT group but did not reach significance level. A post-hoc power calculation indicated that the sample size was inadequately too small and under power for demonstrating a significance level for the Vol and Flow. Calculated power values were 0.66 and 0.61 respectively. A sample size of 34 subjects would have been required in ∅HT and +HT groups in order to observe a significance level. The capillary Vol, Vel and Flow of the rim and nasal peripapillary retina were similar between the ∅HT and +HT groups, although for the rim, the Vol (18.7 ± 3.2 vs 17.8 ± 3.7 AU, p=0.509), Vel (1.06 ± 0.20 vs 0.99 ± 0.23 AU, p=0.391) and Flow (256.8 ± 62.6 vs 251.4 ± 68.3 AU, p=0.912) were slightly greater in the +HT group.

Table 5: 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

	∅HT (n=16)	+HT (n=27)	*P* value Unadjusted*	Adjusted†
Volume (AU ± SD)				
Temporal	19.6 ± 3.4	22.3 ± 3.9	0.030*	0.120
Rim	17.8 ± 3.7	18.7 ± 3.2	0.413	0.509
Nasal	19.1 ± 2.9	19.2 ± 3.1	0.947	0.817
Velocity (AU ± SD)				
Temporal	0.98 ± 0.19	1.11 ± 0.26	0.090	0.333
Rim	0.99 ± 0.23	1.06 ± 0.20	0.324	0.391
Nasal	1.01 ± 0.18	1.01 ± 0.21	0.982	0.780
Flow (AU ± SD)				
Temporal	266.0 ± 58.1	312.0 ± 75.1	0.042*	0.181
Rim	251.4 ± 68.3	256.8 ± 62.6	0.794	0.912
Nasal	274.3 ± 51.7	274.7 ± 58.0	0.984	0.697

Values are means ± standard deviations (SD). Number of subjects is in parenthesis.

* Significance level determined by unadjusted linear regression model.

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

ONH topography

Results of the 12 standard stereometric parameters of ONH topography obtained for both the entire (0-360°) and the infero-temporal (270-360°) regions from 27 ∅HT and 33 + HT subjects (Table 1, sub-population C) are presented in Tables 6 and 7 respectively with their significance levels obtained from linear regression models both unadjusted and adjusted for age of subject, OPP and age at menarche. For the entire ONH region, the rim volume was significantly greater in the +HT group (0.438 ± 0.136 vs 0.380 ± 0.143 mm^3, p=0.032) compared to those of ∅HT group. The mean thickness and cross-sectional area of the RNFL were also greater in the +HT but did not reach a significance level (0.274 ± 0.065 vs 0.256 ± 0.062 mm, p=0.100 and 1.336 ± 0.302 vs 1.235 ± 0.302 mm^2, p=0.066 respectively). When looking at the infero-temporal ONH region, the rim volume (0.066 ± 0.041 vs 0.050 ± 0.030 mm^3, p=0.042), height variation contour (0.353 ± 0.099 vs 0.289 ± 0.101 mm, p=0.011), the mean thickness RNFL (0.194 ± 0.053 vs 0.167 ± 0.058 mm, p=0.033) and RNFL cross-sectional area (0.238 ± 0.068 vs 0.198 ± 0.073 mm^2, p=0.020) were significantly greater in the +HT group compared to those of ∅HT. The remaining stereometric parameters for both the entire (Table 6) and infero-temporal (Table 7) regions of the ONH were similar between both groups of postmenopausal women. Although the rim area was slightly greater in the +HT group for both the entire (1.491 ± 0.276 vs 1.404 ± 0.267 mm^2, p=0.131) and infero-temporal regions (0.324 ± 0.097 vs 0.302 ± 0.092 mm^2, p=0.203) of the ONH, but these differences did not reach a significance level.

Table 6: Standard Stereometric Parameters of the Entire Optic Nerve Head (0-360°) Measured in ∅HT and +HT Postmenopausal Women

	∅HT (n=27)	+HT (n=33)	*P* value Unadjusted*	Adjusted†
Disk are (mm^2 ± SD)	1.855 ± 0.379	1.917 ± 0.315	0.721	0.739
Cup area (mm^2 ± SD)	0.481 ± 0.323	0.426 ± 0.297	0.501	0.318
Cup/disk ratio	0.239 ± 0.142	0.212 ± 0.132	0.448	0.314
Rim area (mm^2 ± SD)	1.404 ± 0.267	1.491 ± 0.276	0.225	0.131
Cup volume (mm^3 ± SD)	0.105 ± 0.107	0.084 ± 0.080	0.389	0.266
Rim volume (mm^3 ± SD)	0.380 ± 0.143	0.438 ± 0.136	0.118	0.032†
Mean cup depth (mm ± SD)	0.209 ± 0.084	0.201 ± 0.091	0.740	0.719
Maximum cup depth (mm ± SD)	0.557 ± 0.211	0.542 ± 0.202	0.778	0.814
Cup shape measure	-0.170 ± 0.060	-0.182 ± 0.064	0.471	0.421
Height variation contour (mm ± SD)	0.298 ± 0.083	0.417 ± 0.093	0.409	0.210
Mean RNFL thickness (mm ± SD)	0.256 ± 0.062	0.274 ± 0.065	0.271	0.100
RNFL cross-sectional area (mm^2 ± SD)	1.235 ± 0.302	1.336 ± 0.302	0.201	0.066

Values are means ± standard deviations (SD). Number of subjects is in parenthesis.

* Significance level determined by unadjusted linear regression model.

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

Table 7: Standard Stereometric Parameters of the Infero-Temporal Region Optic Nerve Head (270-360°) Measured in ∅HT and +HT Postmenopausal Women

	∅HT (n=27)	+HT (n=33)	*P* value Unadjusted*	*P* value Adjusted†
Disk are (mm^2 ± SD)	0.464 ± 0.115	0.493 ± 0.090	0.272	0.309
Cup area (mm^2 ± SD)	0.175 ± 0.112	0.169 ± 0.100	0.822	0.497
Cup/disk ratio	0.348 ± 0.183	0.333 ± 0.182	0.764	0.495
Rim area (mm^2 ± SD)	0.302 ± 0.092	0.324 ± 0.097	0.367	0.203
Cup volume (mm^3 ± SD)	0.037 ± 0.040	0.028 ± 0.024	0.318	0.214
Rim volume (mm^3 ± SD)	0.050 ± 0.030	0.066 ± 0.041	0.096	0.042†
Mean cup depth (mm ± SD)	0.211 ± 0.091	0.210 ± 0.090	0.957	0.867
Maximum cup depth (mm ± SD)	0.510 ± 0.173	0.494 ± 0.183	0.728	0.688
Cup shape measure	-0.108 ± 0.087	-0.110 ± 0.086	0.955	0.812
Height variation contour (mm ± SD)	0.289 ± 0.101	0.353 ± 0.099	0.015*	0.011†
Mean RNFL thickness (mm ± SD)	0.167 ± 0.058	0.194 ± 0.053	0.071	0.033†
RNFL cross-sectional area (mm^2 ± SD)	0.198 ± 0.073	0.238 ± 0.068	0.033*	0.020†

Values are means ± standard deviations (SD). Number of subjects is in parenthesis.

* Significance level determined by unadjusted linear regression model.

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

Functionality of the RGC

The PERG findings from 21 ∅HT and 31 +HT (Table 1, sub-population D) postmenopausal women are presented in Table 8 with their significance levels obtained from linear regression models both unadjusted and adjusted for age of subject, OPP and age at menarche. The P50 latency was shorter by 1.1 ms in the +HT group but this was borderline significant (42.2 ± 1.9 vs 43.3 ± 1.8 ms, p=0.063). The remaining PERG components were similar between the ∅HT and +HT groups. The P50 amplitude was smaller by 5% in the +HT group (6.9 ± 1.5 vs 7.3 ± 1.7 μVolt, p=0.741), while the N95 amplitude was greater by 3.2% (9.1 ± 1.9 vs 9.4 ± 1.6 μVolt, p=0.867)

Table 8: Components of the Pattern Electroretinogram Measured in ∅HT and +HT Postmenopausal Women

	∅HT (n=21)	+HT (n=31)	*P* value Unadjusted*	Adjusted†
N35 latency (ms ± SD)	21.6 ± 1.9	21.1 ± 1.8	0.332	0.233
P50 latency (ms ± SD)	43.3 ± 1.8	42.2 ± 1.9	0.044*	0.063
N95 latency (ms ± SD)	80.2 ± 3.3	80.7 ± 4.0	0.649	0.777
P50 amplitude (μVolt ± SD)	7.3 ± 1.7	6.9 ± 1.5	0.400	0.741
N95 amplitude (μVolt ± SD)	9.4 ± 1.6	9.1 ± 1.9	0.598	0.867

Values are means ± standard deviations (SD). Number of subjects is in parenthesis.

* Significance level determined by unadjusted linear regression model.

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

In a subset analysis for all outcome variables, age at menarche, reproduction year and menopause durations were included in the adjusted linear regression model in addition to age, OPP and age at menarche. As age at last menses, reproduction year and menopause durations were unknown for some postmenopausal women, this subset analysis was therefore performed on a smaller sample size for each sub-population. Since age at last menses, reproduction year and menopause durations were observed not to be significant predictors of all the outcome variables, they were removed from the adjusted linear regression model so as to perform the analysis on the entire sample size for each sub-population studied and only adjusting for age, ocular perfusion pressure and age at menarche.

Effects estrogens and estrogens combined with progestestagens therapies.

The Table 9 displays the results of linear regression analysis comparing the effect of the type of HT treatment (estrogen therapy vs ∅HT, estrogen + progestagen therapy vs ∅HT and estrogen vs estrogen + progestagen therapies) for the significant outcome variables. For the infero-temporal retinal artery, postmenopausal women taking either estrogen therapy or estrogen combined with progestagen therapy presented significantly greater Diameter ($p=0.16$ and $p=0.023$) and Flow ($p=0.023$ and $p=0.042$) compared to the control group. The Diameter and Flow values were slightly greater in the estrogen therapy group than in the estrogen combined progestagen therapy group, but these differences were not statistically different.

Regarding the stereometric parameters, postmenopausal women taking either estrogen therapy or estrogen combined with progestagen therapy present greater rim volume for

the entire ONH region, and greater rim volume, height variation contour, mean thickness RNFL and RNFL cross-sectional area for the infero-temporal region for the ONH compared to the control group. However, significance level was reached for both rim volumes of the entire and infero-temporal regions of the ONH in the estrogen user group, and for variation contour, mean thickness RNFL and RNFL cross-sectional area for the estrogen combined with progestagens user group compared to the control group. A post-hoc power calculation indicated that sample sizes were inadequately too small and under power for demonstrating a significance level for the variation contour, mean thickness and cross-sectional area of the RNFL. Calculated power values were 0.24, 0.23 and 0.46 respectively. A sample size of at least 133 subjects in each control and estrogen groups would have been required.

Table 9: Effect of Estrogens Therapy and Estrogens Combined with Progestagens Therapy on Different Outcome Variables

	ØHT	+HT		*P* value Adjusted†		
		Estrogens	Estrogens + Progestagens			
	(n)	(n)	(n)	a	b	c
CLBF	(18)	(19)	(12)			
Diameter (um ± SD)	120.7 ± 11.6	134.6 ± 19.0	132.1 ± 9.3	0.016†	0.023†	0.786
Flow (uL/min ± SD)	12.5 ± 2.9	16.1 ± 4.9	14.9 ± 1.8	0.010†	0.042†	0.485
Stereometric Parameters (0-360°)	(27)	(11)	(22)			
Rim volume (mm^3 ± SD)	0.380 ± 0.143	0.480 ± 0.185	0.416 ± 0.103	0.028†	0.146	0.369
(270-360°)						
Rim volume (mm^3 ± SD)	0.050 ± 0.030	0.082 ± 0.063	0.057 ± 0.022	0.012†	0.287	0.127
Height variation contour (mm ± SD)	0.289 ± 0.100	0.321 ± 0.078	0.370 ± 0.106	0.377	0.003†	0.096
Mean RNFL thickness (mm ± SD)	0.167 ± 0.058	0.184 ± 0.039	0.198 ± 0.0.59	0.391	0.016†	0.242
RNFL cross-sectional area (mm^2 ± SD)	0.198 ± 0.073	0.233 ± 0.059	0.240 ± 0.074	0.170	0.022†	0.552

Values are means ± standard deviations (SD). Number of subjects is in parenthesis.

a Estrogen Therapy vs ØHT

b Estrogen + Progestagen Therapy vs ØHT

c Estrogen Therapy vs Estrogen + Progestagen Therapy

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

DISCUSSION

This observational study in postmenopausal women provides the first clinical evidence that HT (estrogens alone or in combination with progestagens) has a beneficial effect on the arterial retinal circulation and a protective effect on the retinal nerve fiber layer. We observed a greater blood flow in the infero-temporal retinal artery, mostly caused by its greater diameter, and a thicker RNFL. Blood flow was also greater in the temporal peripapillary retina in the +HT group, but this difference did not reach statistical significance in the adjusted regression model, possibly due to our limited sample size.

Effect of HT on Retinal Circulation

These findings of a greater blood flow observed in the infero-temporal retinal artery 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 such as the ophthalmic[13,27,28] and the central retinal[29,31,32] arteries in women taking HT. Moreover, our findings are in agreement with our recent findings observed in a ovariectomized rat model where subcutaneous administration of estradiol (E_2) increased the tissue perfusion in whole-mount retina demonstrated by quantitative autoradiography using the blood flow tracer N-Isopropyl-p-[^{14}C]-iodoamphetamine ([^{14}C]-IMP).[47]

We demonstrated that the significantly greater blood flow measured in the infero-temporal retinal artery in +HT group was mostly due to a significantly larger diameter of

the ITRA. This finding is in contradiction with the findings reported by Leung H et al[48] on the Blue Mountains Eye Study population, and Wong TY et al.[49] on the Beaver Dam Eye Study population. In both studies, a computer-assisted program was used to measure the average arteriolar and venular diameters. In the Blue Mountain Eye Study,[48] no statistically significant difference was found in the averaged arteriolar diameter between never and current HT users for women younger than age 65. There is no mention of the statistical results for the averaged venular diameter for these women. For women aged 65 or older, there were no statistically significant differences of the averaged arteriolar diameter in the current HT users compared to the never HT users, while there was a significant decrease for the averaged venular diameter. In the Beaver Dam Eye study,[49] postmenopausal women who were HT users had significantly narrower averaged arteriolar and venular diameters compared to those who were never HT users. As well, there was a significant trend toward arteriolar and venular diameter narrowing with increasing use of HT when the administration route was oral but not with all combined administration routes together. However, study limitations have been reported by the authors of these two studies such as a confounding by condition bias. A confounding by condition is when a disease condition prompts the use of HT. The underlying disease or risk factor 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 with a history of hypertension or diabetes may have been more likely to use HT for its cardioprotective role and the observed narrower retinal vessel in HT users may simply reflect the effects of elevated blood pressure or diabetes rather than from HT.[48-50] Moreover, women with a history of hypertension and diabetes are likely to have an impaired endothelium and of being on vasodilator treatment or on insulin.

Therefore, a potentially impaired endothelium in patients with vascular diseases may have not been fully responsive to the HT treatment and the endothelium of patients taking vasoactive medications may have not been responsive to additional vasomotor stimulation from HT treatment. As well, the author reported the lack of E_2 assays to confirm the use of HT, interaction of age and elevated BP that were not fully corrected by the adjusted model, 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.

In our study design, we assumed that for women who were HT users that they were taking HT as prescribed by their physicians. Since HT active ingredients taken by +HT group were not all 17β-estradiol and progesterone-based, the assays for quantifying serum 17β-estradiol and progesterone could not have been used to establish patient compliance. It would have been too costly to perform assays for all possible active ingredients that exist in different HT formulations. The digitized technique used in both 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.

Systemic action of HT in postmenopausal women cannot be ruled out due to a number of reports of vasomotor effect of estrogens on various vascular beds.[2,6,9,10,27] A

longitudinal study monitoring the blood pressure changes in HT users and nonusers indicated that systolic blood pressure increased less in HT users than non users over a period of ten years, while the diastolic blood pressure remained stable. 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 compared to HT users. These differences reached a significance level for only the sub-population C. The resulting ocular perfusion pressure was also higher in the women who were not HT users compared to those who were HT users reaching a significance level for only the sub-population C. Higher ocular perfusion pressure has a beneficial vascular effect by providing a better blood flow. However, although higher ocular perfusion pressure was greater in postmenopausal women not on HT, their infero-temporal retinal artery and temporal peripapillary retinal blood flow observed was smaller. Based on these observations, it is most likely that the increased blood flow we observed in the retina results from a local action of HT on retinal circulation and not from a systemic action.

Possible Vasomotor Pathways of Estrogens on Retinal Circulation

The beneficial effect of HT on retinal blood flow is most likely caused by the vasodilating properties of estrogens mediated by the endothelium-derived relaxing factors, primarily nitric oxide (NO) and prostacyclin (PGI_2) pathways, which have been identified in the retinal vascular bed.[51-54] This effect could be mediated through ERα or ERβ, expressed in the retina.[22-26,55] Both ERα and ERβ have been shown to be implicated in the increase of NO synthase (NOS) expression[56,57,57] or NO production.[58] With regards to the prostacyclin pathway, several studies have shown that the stimulating action of estrogens on PGI_2 synthesis,[59,60] can increase retinal blood flow.[53] Finally, studies have indicated that estrogens also antagonize the effects of endothelium-derived contracting factors[61] such as endothelin-1, which has been documented to be a potent vasoconstrictor on the ophthalmic and retinal arteries.[62]

Possible Vasomotor Pathways of Progestagens on Retinal Circulation

Progestagens are prescribed as a combined HT to menopausal women with an intact uterus so as to regulate the action of estrogens on endometrial cells and to prevent endometrial cancer. In our whole study population of sixty-six subjects, 36 subjects (55.4%) were HT users, among which 24 (36.9%) were on estrogens/progestagens combined therapy. The type of progestagen regimen used was heterogeneous in terms of active ingredients, where 11 (18.5%) subjects were using progesterone (P), 6 (9.2%) were using norethindrone acetate (NETA), 5 (7.7%) were using medroxyprogesterone (MPA), and 2 (3.1%) were using levonorgestrel.

However, divergent vasomotor effects of progestagens have been reported. In animal models, P has been shown to either induce vasodilation,[63-66] increase BF[67,68] or have no effect[69,70] when given alone, and to antagonize the estrogen-induced BF in a combined therapy.[69-71] When given alone MPA was shown to have no effect on the hypothalamic blood flow,[72] but to antagonize[73,74] or not antagonize[75] the vasodilating effects of estrogens in a combined therapy. In clinical trials, P administration has been shown to decrease the endothelium-dependent flow mediated vasodilatation (FMD) of the brachial artery,[76] have no effect[77] or increase FMD[78] when given alone. In a combined therapy, P administration was shown to antagonize[79] or not[78,80] the estrogen-induced FMD compared to estrogen administration alone. MPA was shown to antogonize the estrogen-induced FMD.[81] Therefore, possible beneficial or antagonizing vascular interactions of progestagens 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 infero-temporal were slightly greater than in the estrogen combined progestagens therapy, but these differences were not statistically different.

Like estrogens, the vasomotor effects of progestagens can result from an interaction with both NO[82-84] and PGI_2 pathways[85] present in the retinal vascular bed[51,54] mediated through the progesterone receptors,[22-24] which have been identified in the retina.

Regional Effect of HT on ONH Blood Flow

Using the Scanning Laser Doppler Flowmetry technique, we demonstrated greater capillary blood flow in the +HT group the temporal peripapillary retina to a lesser extent in the rim area, but these did not reach statistical significance level. Possible vasomotor effects of HT on the capillary blood flow on the nasal peripapillary retina in postmenopausal +HT cannot be ruled out for the following reasons. The brightness optimization of the laser beam we used in our sampling method may have preferentially generated a stronger signal by the photodetector for the temporal retinal than the rim and nasal peripapillary area signal. Also, the capillary density is higher in the temporal compared to the nasal peripapillary retinas for similar distance from the disc.[86] Therefore, the nasal peripapillary area may be less sensitive to blood flow changes.

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 compared to that of the ∅HT group. For the rim volume, this was significant for both the entire and the infero-temporal region of the ONH, while for the height variation contour, mean thickness and cross-sectional area of the RNFL they reached significance level for the infero-temporal region. The differences observed in these stereometric parameters indicate a thinner retinal nerve fiber layer, in postmenopausal women not on HT compared to those on HT. This finding is similar to the sparing of some brain regions observed in post-menopausal women using HT.[14-16] A sparing of the retinal nerve fiber

layer may result from the vascular properties of estrogens and progestagens, as described earlier, by providing a better blood flow and nutrients to the RGC and the NFL. In addition, sparing of the retinal nerve fiber layer may result from the protective properties of estrogens on neurons independent of blood flow.[87] These protective effects have been documented to increase neuron survival in several toxicity models of cultured neurons, such as glutamate, β-amyolid, hydrogen peroxide, and excitatory amino acid toxicity models.[87] Interestingly, recent studies have reported a protective effect of estrogens on retinal ganglion cells in glutamate toxicity[88] and axotomized optic nerve rodent models.[89]

Similar to its vasomotor effects, progestagens also present divergent effects in terms of protective effects on neurons. When given alone, P has been shown to increase neuron survival in glutamate toxicity models of neurons from the hippocampus,[90] and cerebral cortex,[91] and in a spinal cord genetic model[92] in rodents. However, an increase of neuron survival in presence of progesterone has not been observed in the axotomized optic nerve model in rodents.[89] In combined therapy, P has been shown to increase neuron survival beyond that of E_2 alone in the hippocampal glutamate toxicity model.[90] With regards to MPA, it has shown not to have a neuroprotective effect on neurons when given alone or in combined therapy,[90] or to potentiate neuronal loss[93] in a hippocampal glutamate toxicity model. Therefore, possible beneficial or antagonizing protective effects of progestagens 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 combined progestagen therapy. These differences reached significance level only for the rim volume but not for the height variation contour, mean thickness and cross-sectional area of the RNFL. This likely resulted from under power analysis for those specific variables.

For the height variation contour, mean thickness and cross-sectional area of the RNFL, the ∅HT group of post-menopausal women presented a decrease of 4.6%, 6.7% and 7.6% respectively compared to +HT group for the entire optic nerve region, while for the infero-temporal quadrant, this decrease was 18.1%, 13.9% and 16.8% respectively. Preferential thinning of infero-temporal neural structures is also seen in early glaucoma, and several causes for this have been proposed.[94,95] It is interesting to speculate that the vulnerability of this region may explain some of our findings.

Since our findings on the optic nerve head 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 the optical coherence tomography. However, we were interested in investigating the ONH topography, which was obtained with the HRT I.

Effect of HT on the functionality of RGC

The exact origins of the PERG components have not been identified, but current data suggest that the N95 is a contrast related component of the RGC function and that P50 is partly RGC derived with some contribution from structures distal to RGC.[96] Smaller PERG amplitudes and delayed latencies are indicative of RGC loss. Clinical studies where loss of RGCs occur such as in OHT and OAG, have demonstrated that N95 amplitude is reduced and P50 latency is increased,[97] and that N95 amplitude significantly and positively correlates with RNFL thickness while P50 latency negatively correlates.[98,99]

We observed a borderline significantly shorter P50 latency in the +HT group by only 1.1 ms. 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. Interestingly, our findings are somewhat consistent with the pattern visual evoked potential (PVEP) findings observed in postmenopausal women, where the use of Tibolone over a period of 3 months, a synthetic steroid with prominent estrogen effects, was shown to significantly improve the P100 latencies by 4 ms (5%). As well as a significant increase by 31% in the P100 amplitude.[100] Yilmaz et al. (2000)[100] hypothesized that increasing sensitivity of the central nervous system to catecholamines by changing the opening frequency of voltage-related L-type calcium channels,[101] increasing effects of glutamate[102] and decreasing formation of γ-aminobutyric acid (GABA)[103] induced by estrogens might be the responsible mechanisms in shortening P100 latency and increasing P100 amplitude. So far, it is unknown how estrogens might improve the P50 latency of the PERG. The same mechanisms may apply in shortening

P50 latency in postmenopausal women who were HT users. This remains to be investigated. We also observed a small increase in the N95 amplitude by 3% and a small decrease in P50 amplitude by 6% in the +HT group. However, the magnitude of amplitude changes were not in the 31% magnitude change of the P100 amplitude as reported by Yilmaz al. (2000), and they did not reach statistical significance. 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

As part of our exclusion criteria, postmenopausal women had to be free of systemic and cardiovascular diseases. Potentially impaired endothelium in patients with systemic and cardiovascular diseases may not be fully or partly responsive to the HT treatment. Moreover, these patients may likely be on vasodilator treatments and their endothelium may not be responsive to additional vasomotor stimulation from HT treatment. If we had included postmenopausal women with systemic and cardiovascular diseases, it is possible that the differences we observed in blood flow and RNFL thickness might have been different.

In this heterogeneous population of postmenopausal women we investigated, the type of HT regimens in terms of their active ingredients, routes of administration, dosage and duration of administration was heterogeneous and may have yielded different degrees of vasomotor effects on the retina and protective effects on the retinal nerve fiber layer. In

addition, women were at variable postmenopausal times. As well, the outcome variables measured were collected at a single time point, which does not provide any information on the progress on the outcome variables in both groups of postmenopausal women. Finally, postmenopausal women may not exhibit similar cardiovascular characteristics and risks which could interplay with the efficacy of HT. The ideal study design would be to randomize women to HT at the time of the onset of menopause with documented cardiovascular characteristics and risks, and establish a baseline for blood flow, structure and function before beginning therapy. Then monitor blood flow, structure and function changes at intervals over a five- to ten-year period. Such a protocol could be part of a larger HT study.

CONCLUSION

This is the first observational study that investigated the long-term effects of estrogens depletion compared to the use of postmenopausal hormone therapy on the retina and nerve fiber layer in a population of women. The findings of this study indicated that compared to postmenopausal women who had never used HT, postmenopausal women who have used HT since their menopause onset presented 1) a better retinal blood flow as observed in the infero-temporal retinal artery and capillary blood flow of the temporal retina and 2) a greater rim volume, height variation contour, and mean thickness and cross-sectional area of the RNFL. These findings likely reflect the vasomotor and protective properties of estrogens or estrogens and progestagens combined, and may explain the link between the hormonal status of estrogen and progestagen levels throughout a woman's life and the risk of developing glaucoma[17,18] and age-related macular degeneration.[19-21]

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PAPER #2

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17β-estradiol increases the retinal tissue perfusion in a menopausal rat model

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Abstract

Background: Estrogens, used as postmenopausal hormone therapy, have been shown to improve pulsatility and/or resistivity indexes in the retrobulbar arteries in women. In this study, we investigated whether retinal perfusion could be improved by a chronic treatment of estradiol in a menopausal rat model. **Methods:** Mature female Brown Norway rats were ovariectomized and silastic capsules containing either 17β-estradiol (E_2) or placebo were implanted subcutaneously. Six weeks later, retinal tissue perfusion was measured in four concentric isopters of all quadrants of whole-mount retina by quantitative autoradiography using the diffusible tracer N-Isopropyl-p-[^{14}C]-iodoamphetamine ([^{14}C]-IMP). **Results:** The results showed that the E_2 treatment significantly induced an increase in local retinal uptake index (22% to 45% according the concentric isopter analyzed) compared to placebo animals. **Interpretation:** These findings demonstrate that long term E_2 treatment in ovarian hormone deficient rats enhances retinal tissue perfusion, which may play a protective role in age-related ocular blood flow dysfunction.

Keywords: estrogens, retina, blood flow, iodoamphetamine, ovariectomy

BACKGROUND

The menopause is the permanent cessation of ovarian follicular activity and subsequent fall in endogenous estrogens and progesterone production in women. Postmenopausal hormone therapy (HT) consisting of estrogens alone or combined with progestagens, is frequently prescribed to women in order to alleviate climacteric symptoms. As well as their role in protection of structure and function of many cells and organs, estrogens have the ability to influence the vascular tone and blood flow. In postmenopausal women, HT improves blood flow in the femoral artery[1] and brain parenchyma,[2-5] and pulsatility and/or resistivity indexes in the carotid,[6,7] internal carotid,[8,9] and middle cerebral arteries.[9,10] These blood flow indexes are also decreased in the retrobulbar arteries, such as the ophthalmic,[11-13] the short posterior,[14] and the central retinal arteries.[14-16]

Recently, there has been an increasing interest in investigating the possible vascular protective role of estrogens in age-related retinal blood flow dysfunction, such as glaucoma[17,18] and age-related macular degeneration.[19-21] However, although estrogen receptors (ERs) are present in the retina in the human,[22-24] as well as in the rat,[22,25] it has not been directly determined whether chronic estrogen administration can increase blood tissue perfusion in the retina. In this study, we addressed this question using 17β-estradiol (E_2) treatment in a menopause rat model. *In vivo* retinal tissue perfusion was quantified by autoradiography using the diffusible tracer N-Isopropyl-p-[^{14}C]-iodoamphetamine ([^{14}C]-IMP) in the conscious rat. This methodological approach

provided the ability to distinguish the local effects of E_2 on tissue perfusion in different isopters and quadrants of whole-mount retinas.

METHODS

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 local institutional animal care committee, "le Comité de Déontologie de l'Expérimentation sur les Animaux" at the University of Montreal, in accordance with the Canadian Council on Animal Care. Twelve mature middle-aged (11-month old) retired female breeder Brown Norway (*Rattus norvegicus,* 225.5 ± 7.9 g) rats from a single colony (Harlan Sprague Dawley, Netherlands), were used in this study. This age in rats corresponds approximately to the time when 65% of female rats present irregular estrous cycles, equivalent to the peri-menopausal 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 implantation of capsules

After habituation to their environment, bilateral ovariectomies (OVx) were performed in rats using a posterior surgical approach under isoflurane (3.0%) in order to normalize serum estrogen concentrations to their lowest level. Silastic capsules (I.D. 0.058, O.D. 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, USA) plus 75% cholesterol (OVx+E_2, n=7, approximately 2 mg E_2 per capsule) or 100% cholesterol (placebo)

(Sigma-Aldrich, St. Louis, MO, USA) (OVx, n=5) were implanted in the nape of the neck for time-release. These capsules have been validated as a means of delivering steady and physiological levels of E_2 in mature OVx rats.[26,27] Following the OVx surgery, 5 mL of Ringer's lactate were subcutaneously injected for rehydration, while repetitive subcutaneous doses of 0.1 mL of buprenorphine hydrochloride (Temgesic®, Reckitt Benckiser Health Science Ltd, UK, 0.1 mg/mL) were injected every 12 hours over a two-day period for post-operative pain management. Vaginal smears were performed both to ensure effectiveness of the bilateral OVx surgery and to serve as an indicator of tissue responsiveness to circulating estrogens released from E_2 capsules. Rats were exposed to E_2 or placebo treatment for a period of six weeks. A six-week treatment was chosen in order to permit a further study investigating the potential vascular protective role of estradiol in a rat glaucoma model.

Surgical procedure for tissue perfusion investigation

Six weeks after OVx surgery and E_2 or placebo treatment, polyurethane catheters (I.D. 0.6 mm, O.D. 0.9 mm, Harvard Apparatus, Holliston, MA, USA) were inserted into the femoral vein and artery under 1.5% isoflurane (induction of anaesthesia with 3% isoflurane for 5 min). During this procedure, body temperature was monitored with a rectal thermometer and maintained at 37°C by a heating pad (FHC, Bowdoinham, ME). Both blood pressure and heart rate were monitored from the tail using a non-invasive blood pressure cuff system (BP1000, Kent Scientific Corporation, Torrington, CT, USA). At the end of this procedure, 2 mL of Ringer's lactate were given subcutaneously

for rehydration. Rats were then installed in a hammock and left under minimal restraint over a 2-hour period to recover from anaesthesia. Body temperature was maintained at 37°C with a heating lamp and both blood pressure and heart rate were monitored until the initiation of measurement experiment for the retinal perfusion. As well, cardiovascular, chemistry and haematological parameters were measured with the i-STAT®, a veterinarian clinical blood gases and electrolytes analyzer (HESKA, Fort Collins, CO), from arterial blood samples collected via the arterial catheter. This was achieved immediately prior to the initiation of measurement experiment for the retinal perfusion to confirm the recovery to normal physiologic level.

Principles of the evaluation of retinal tissue perfusion using IMP

In the aim of evaluating tissue perfusion in retina with a regional resolution, the diffusible blood flow tracer [^{14}C]-IMP was used. The [^{14}C]-IMP binds to the amine sites.[28,29] It was chosen as a blood flow tracer instead of [^{14}C]iodoantipyrine,[30] [^{14}C]diazepam,[31] or *n*-[^{14}C]butanol,[32] because of three of its essential properties related to cerebral blood flow measurement.[33] These properties avoid the primary limitation of the classical diffusible tracer [^{14}C]-iodoantipyrine autoradiographic technique,[34] i.e. its time-dependent post-mortem intraparenchymal diffusion, which results in weakening the local tracer spatial gradients in tissues and regions. First, [^{14}C]-IMP has a very high extraction at the first pass through the microvasculature, which allows a non-restrictive diffusion from blood to tissue despite the blood-retinal barrier. Second, [^{14}C]-IMP has a very low clearance rate and low post-mortem diffusion,[35] due to its high affinity for

cerebral tissue. Third, [^{14}C]-IMP is chemically and biologically inert for the duration of the measurement period.[35] IMP has been successfully used in small animal research,[33,35,36] and in humans using Single Photon Emission Computerized Tomography.

The principle of blood flow evaluation is identical to that of micrometric microspheres[37] except that this "molecular microsphere" IMP is trapped within the tissue, whereas micrometric microspheres are trapped inside the microvascular space. Since the goal of this experiment is to determine local blood flow gradients in post-mortem tissue, the [^{14}C]-IMP molecular microsphere technique is more appropriate than the classical microsphere technique. Moreover, the use of a diffusible tracer avoids inaccuracies of the retinal blood flow measurement due to axial streaming, plugging or permeability changes as reported with the classical microsphere technique.[37,38]

Procedure of measurement of the retinal tissue perfusion

N-Isopropyl-[methyl-1,3-^{14}C]-p-iodoamphetamine (ARC, St Louis, USA) (100 μCi/kg) was dissolved in 600μL of saline (injectable 0.9% NaCl solution) and infused in fully conscious rats over a 30 s period at a constant rate of 1.2 mL/min using an infusion pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA) through the femoral vein. At the end of the infusion, the rats were immediately sacrificed by decapitation and a blood sample was collected for serum E_2 measurements. A small incision on the superior eyelid was made to indicate the orientation of the eyes and the eyes together

with the attached superior eyelid were then harvested. They were immediately immersed in a solution of 4% paraformaldehyde for post-fixation. 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 from the eye cup, dissected in four quadrants (superior, inferior, nasal and temporal) and whole-mounted on a glass slide with the ganglion cells layer facing up. A small incision was made on the retina to indicate the superior quadrant. The whole-mount retina was then exposed to an X-ray film (Biomax, Kodak-Eastman Inc, Rochester, New York) for 10 days together with a set of [^{14}C]-standards (GE Healthcare Ltd, UK). The autoradiograms were analyzed using the computerized image analysis MCID Basic Software (v7.0, Interfocus Imaging, Linton, England). It has been demonstrated that the amount of the beta ^{14}C radiation energy reaching a X-ray film decreases with increasing thickness of the tissue being exposed up to ≥100 μm where it reaches a plateau level.[39] Therefore, it can be assumed that the measured C_{IMP} (T) from the whole-mount retina autoradiograms originate mainly from the first 100 μm of the inner retina and is mainly representative of the blood supply of the 100 μm inner part of the retina.

Calculation of retinal tissue perfusion

Retinal tissue perfusion was evaluated using the principle of indicator-fractionation technique.[33,35] A local retinal uptake index (RUI) was calculated from the following equation,[36]

$$RUI = [C_{IMP}(T) / A] \times BW \qquad \text{(Eq. 1)}$$

where A is the injected dose (nCi), BW is the body weight (g) and C_{IMP} (T) is the radioactivity measured on the autoradiogram at the time (T) of sacrifice (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 optic nerve head in all retinal quadrants (Fig. 1). The IMP uptake index in the brain has been shown to correlate well with the local cerebral blood flow (CBF), where the CBF has been determined by positron emission tomography.[36] As the retinal vascular bed is similar to the cerebral vascular bed in terms of permeability (blood-tissue barrier), anatomy (non-fenestrated capillaries) and some local regulatory mechanisms (without autonomic innervation of the vasculature),[31] we considered that the IMP uptake index in the whole-mount retina can provide an approximation of retinal blood flow. However, it cannot be excluded that IMP may also have partly diffused from the choroid prior to retinal dissection.

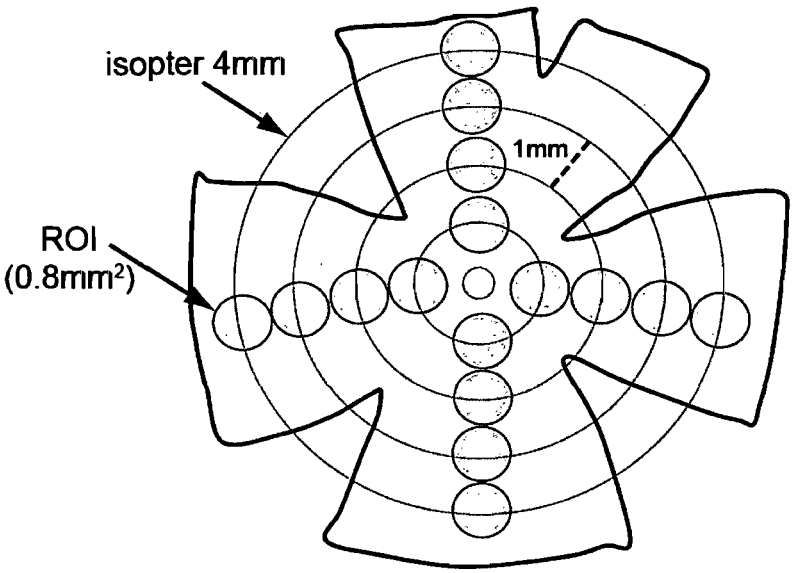

Figure 1: Schematic representation of a whole-mount retina showing the regions of interest (ROI) measured. The retina was divided into four quadrants (the notch indicates the superior quadrant). A circular ROI (filled circles, area 0.8 mm^2) was measured in each quadrant at each isopter (light circular lines at 1, 2, 3 and 4 mm away from the center of optic nerve head). The four values of $C_{IMP}(T)$ of each quadrant were averaged for each isopter.

Serum assays

The total serum E_2 levels were measured using a 3rd Generation Estradiol RIA kit (Diagnostic Systems Laboratories, Webster, TX, USA).[26] Since soy products from animal diets induce vasodilation on coronary arteries *in vitro* in combination with E_2,[40] the concentration of total serum equol, a metabolite of daidzein, a phytoestrogen contained in the rat chow used (Ralston Purina Canada Inc), was also measured on a

third and distinct group of rats (OVx, n=4; OVx+E_2, n=4) by a time-resolved fluoroimmunoassay kit (Labmaster diagnostic, Turku, Finland) as previously reported.[41]

Statistical analysis

The RUIs measured in each quadrant were averaged for each isopter. Unpaired Student *t*-tests were performed between the OVx group and OVx+E_2 group for (1) the physiological parameters, (2) the E_2 plasma concentrations, and (3) the RUI of each isopter. A significance level of $p \leq 0.05$ was chosen. A Mann-Whitney U-test was used to analyze equol plasma concentrations.

RESULTS

Effect of ovariectomy and E_2 treatment on the physiological parameters and serum E_2 and equol levels

Cardiovascular, blood gas and haematological parameters in the OVx and OVx+E_2 groups are summarized in Table 1. The E_2 treatment did not alter most physiological parameters, except body weight and hematocrit level. As expected, OVx rats gained weight ($p = 0.005$) since estrogens play a role in food intake and energy expenditure.[42] The hematocrit level was reduced by 15% in OVx+E_2 group ($p = 0.01$) corresponding to a haemoglobin value of 12.1 ± 0.7 g/dL as compared to 14.2 ± 1.3 g/dL in OVx rats, yet the hematocrit level in OVx+E_2 rats remained within the physiological range (35-57%).[43]

Table 1: Effects of 17β-estradiol (E_2) treatment on the physiological parameters monitored in the conscious OVx rats after two hours of recovery from anaesthesia and prior to the injection of [^{14}C]-IMP.

	OVx (n=5)	OVx+E_2 (n=7)	*P* value
Weight (g ± SD)	247.4 ± 7.5	224.0 ± 13.0	0.005*
Body temperature (°C ± SD)	37.3 ± 0.3	37.7 ± 0.5	0.77
Blood pressure			
systolic (*mm*Hg ± SD)	121.0 ±12.6	130.2 ± 7.9	0.50
diastolic (*mm*Hg ± SD)	95.8 ± 6.2	102.6 ± 9.5	0.50
mean (*mm*Hg ± SD)	104.2 ± 8.3	111.5 ± 8.4	0.27
Arterial pH (±SD)	7.44 ± 0.03	7.44 ± 0.02	0.70
Arterial pO_2 (*mm*Hg ± SD)	80.4 ± 5.6	80.3 ± 5.6	0.97
O_2 saturation (% ± SD)	96.2 ± 0.8	96.1 ± 0.6	0.89
Arterial pCO_2 (*mm*Hg ± SD)	37.1 ± 1.8	37.8 ± 1.2	0.44
Arterial HCO_3 (*mmol*/L ± SD)	25.4 ± 1.1	25.6 ± 1.4	0.83
Hematocrit (*% PVC* ± SD)	41.4 ± 4.6	35.3 ± 2.1	0.01*

pO_2, pCO_2, partial gas pressure of oxygen and carbon dioxide, respectively; HCO_3, bicarbonate. Values are means ± SD. Number of animals in parentheses.

* $p<0.01$, significantly different from OVx placebo (Student *t*-test).

After six weeks of treatment, serum E_2 levels were approximately five-fold higher ($p < 0.01$) in OVx rats treated with E_2 as compared to OVx rats treated with placebo silastic capsules (71.1 ± 7.6 vs 370.4 ± 57.0 pmol/L) (Fig. 2A). Serum equol levels were not statistically different ($p = 0.14$) between OVx and OVx+E_2 rats (511.1 ± 165.2 vs 748.3 ± 217.5 nmol/L) (Fig. 2B).

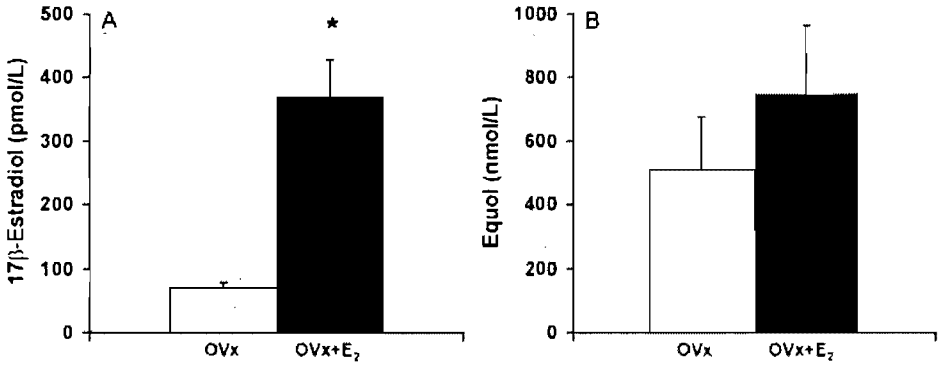

Figure 2: Serum 17β-estradiol (E_2) (A) and equol (B) level in the ovariectomized female rats treated with silastic capsules either containing placebo (OVx, white bars) or E_2 (OVx+E_2, black bars). The E_2 concentrations were five-fold higher in OVx+E_2 rats compared to OVx rats (A), but serum equol levels were similar in both groups (B). * $p<0.05$, significantly different from OVx placebo for the estradiol concentration (Student *t*-test).

Retinal uptake index of [^{14}C]-IMP in regions of whole-mount retina

Pseudocolour autoradiograms of whole-mount retinas generated by the MCID Image analysis system (Fig. 3) display a decreasing gradient of tracer from the centre to the periphery of the retina, and a fairly homogeneous distribution throughout each isopter. Visually, the tracer concentration in the OVx+E_2 group appeared higher than that in the OVx group. Quantified data expressed in RUIs show significantly elevated values in the OVx+E_2 rat in the three furthermost peripheral isopters compared to control rats (Table 2, Fig. 4). This increase in the RUI ranges from 22% to 45%, reaching 45% in the third isopter.

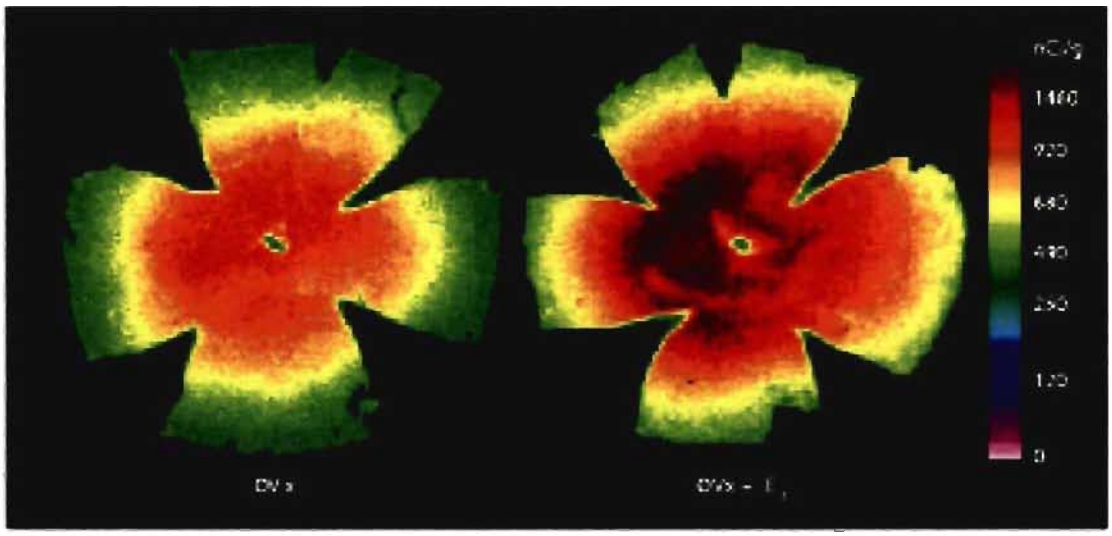

Figure 3: Representative whole-mount retina autoradiograms displayed in pseudocolour from an ovariectomized rat treated with placebo silastic capsules (OVx, left panel) and an OVx+E_2 rat treated with E_2 silastic capsules (right panel). The tissue concentration of [^{14}C]-IMP was higher in the OVx+E_2 rat, indicating that the retinal blood perfusion was greater in E_2 treated animals than in placebo treated animals. The correspondence between pseudocolour scale and C_{IMP} (T) values (nCi/g) is represented on the right side of the figure.

Table 2: Effects of E_2 treatment on local retinal uptake index of [^{14}C]-IMP in OVx conscious rats at different isopter distances from the center of the optic nerve head.

	[^{14}C]-IMP local retinal uptake index		
Isopter	OVx (n=5)	OVx+E_2 (n=7)	*P* value
1 mm	8.0 ± 0.7	9.8 ± 0.6	0.11
2 mm	6.9 ± 0.8	9.7 ± 0.8	0.03*
3 mm	5.2 ± 0.6	7.5 ± 0.6	0.04*
4 mm	3.5 ± 0.4	4.6 ± 0.3	0.04*

RUI values are mean ± SD. Number of animals in parentheses.

* $p<0.01$, significantly different from OVx placebo (Student *t*-test).

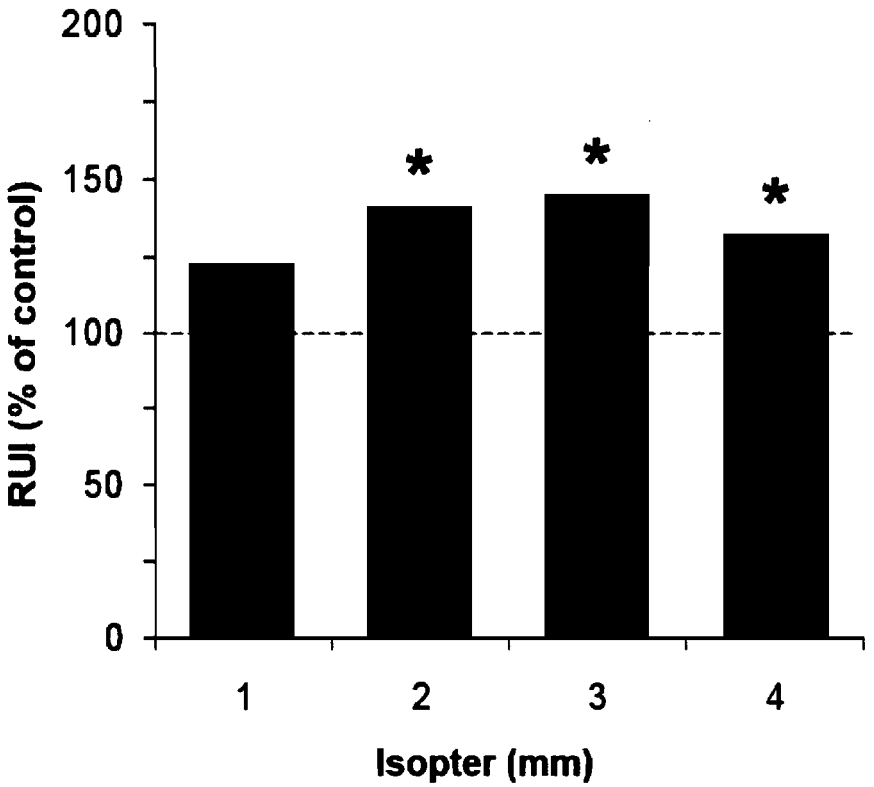

Figure 4: Percent of changes (OVx+E_2 versus OVx) of the local retinal uptake index (RUI) of [^{14}C]-IMP for each isopter. The RUI was increased in OVx+E_2 versus OVx group at each isopter, although only the three farmost peripheral isopters showed statistical significance. * $p<0.05$, significantly different from OVx placebo (Student *t*-test).

INTERPRETATION

These results provide the first evidence that a chronic treatment with E_2, mimicking HT treatment under menopausal physiological conditions, increases retinal tissue perfusion. Our quantitative autoradiographic investigation using [^{14}C]-IMP showed that both the central and peripheral areas of the retina presented a significant blood flow improvement.

Estradiol treatment paradigm

At the time of sacrifice, serum E_2 levels were nearly five-fold higher in OVx rats receiving the E_2 capsules as compared to rats receiving the placebo capsules. The serum E_2 levels for both groups were consistent with values reported in other studies.[27,44] The E_2 treatment administered in our OVx rats using silastic capsules is a representative model of HT treatment in menopausal women since the five-fold magnitude increase seen here 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.[8,10] Also the subcutaneous release of E_2 from silastic capsules is similar to transdermal application of E_2 as both approaches bypass the hepatic degradation of estradiol.

Effect of E_2 on local retinal tissue perfusion

This study provides the first evidence demonstrating that E_2 treatment increased the blood perfusion in all quadrants of rat retina with higher RUI increases in the peripheral retinal. As seen in other species,[45] blood tissue perfusion was higher in the central as compared to peripheral retina, yet E_2 treatment elevated blood flow in the peripheral 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. One explanation for a higher response in the peripheral regions is that the capillary network in the peripheral retina is denser than in the central retina, as shown in corrosion casts in rats,[46] thereby allowing a better tissue perfusion. A second explanation could relate to the regional distribution of estrogen receptors or downstream target enzymes in the retina, which remains to be investigated. Increases in the uptake of [^{14}C]-IMP after the administration of E_2 most likely indicate an increase in retinal blood flow, in accordance with our clinical laser Doppler flowmetry findings showing that menopausal women on HT presented a significantly higher blood flow from the infero-temporal retinal artery compared to age-matched non-users women (paper in preparation). Our results are also in agreement with those reporting improved pulsatility and/or resistivity indexes observed in various retrobulbar vessels, such as the ophthalmic[11-13] or central retinal arteries,[14-16] in women taking HT. However, due to the dual vascular supply of the retina, a fraction [^{14}C]-IMP entrapped in the retina could have diffused through the retinal epithelium from the choroidal circulation.

This regional change of blood tissue perfusion supports a local action of estrogens, although the possibility of a systemic action could not be discarded due to a number of reports of the vasomotor effect of estradiol on various vascular beds.[2,8,9,11,47] In addition, the significance of reduced hematocrit in the OVx+E_2 group on systemic blood flow, and consequently the retinal blood flow, should be taken into consideration. Similar lower hematocrit has been observed in rats[48] and women[49,50] following the administration of ethinyl estradiol. This reduced hematocrit (1) may decrease blood viscosity as seen in women after HT treatment[13,51] and may favour increases in blood flow, or (2) may decrease oxygen delivery and favour local metabolic autoregulation. However, a 6.1 decrease in hematocrit in the OVx+E_2 rats, reflecting approximately 2 g/dL change in haemoglobin, is likely insufficient to induce a 22 to 45% increase in the retinal tissue perfusion.

The effect of E_2 on the retinal blood flow is most likely related to the vasodilator properties of E_2, especially in relation to the endothelium-derived relaxing factors, mostly nitric oxide (NO) and prostacyclin (PGI_2) pathways, which have been identified in the retinal vascular bed.[52-54] This effect could be mediated through ERα or ERβ, since both receptors are expressed in the vascular system of rats,[55] and that ERs are also expressed in their retinas.[22,25] The same mediation may occur in humans.[23,24] Both ERα and ERβ are implicated in the increase of NO synthase (NOS) expression[56,57] or NO production.[58] With regards to the prostacyclin pathway, several studies have shown the stimulating action of estrogens on the PGI_2 synthesis,[59,60] and PGI_2 has been shown to increase retinal blood flow.[54] Finally, studies have indicated that estrogens also

antagonize the effects of endothelium-derived contracting factors.[61] It seems improbable that equol, the metabolite of phytoestrogens from the rat chow, had a direct effect on the retinal tissue perfusion in our rat model since a vasomotor action of equol has been shown only at elevated concentrations (10 μg/mL), but not at lower concentrations (0.1 μg/mL)[62] such as detected in our rats. Moreover, the low binding affinity of equol to both ERs is between 1/250th and 1/1000th of that for E_2.[63]

In conclusion, our findings demonstrate for the first time that estradiol influences the posterior segment ocular blood flow as observed by a significant increase in retinal tissue perfusion ranging from 22% to 45% in this menopausal rat model. The molecular vascular mechanisms of E_2 and its potential vasculoprotective role against age-related retinal blood flow dysfunction remain to be investigated.

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5.0 DISCUSSION

The observational study conducted on postmenopausal women provided the first clinical evidence that the long-term effects of estrogen depletion compared to the use of hormone replacement therapy (estrogens alone or in combination with progestagens) has a negative effect on the retinal arterial circulation and the optic nerve head topography.

5.1 Effects of HT on retinal blood flow

We observed that postmenopausal women on HT presented significantly greater blood flow in the infero-temporal retinal artery, which was mostly due to a significantly larger diameter of the ITRA compared to those of women who never used HT since their menopause onset. These data are in agreement with other clinical studies that used color Doppler imaging and reported improved pulsatility and/or resistivity indexes in retrobulbar vessels such as the ophthalmic[289,322,323] and the central retinal[324,326,327] arteries in women taking HT. Blood flow was also greater in the temporal peripapillary retina in the +HT group, but this difference did not reach statistical significance in the adjusted regression model, possibly due to our limited sample size. Our findings of improved retinal blood flow observed in postmenopausal women on HT were confirmed in a menopause rat model by quantitative autoradiography technique using the blood flow tracer [^{14}C]-IMP. In menopaused rats, chronic treatment with E_2, mimicking HT treatment under menopausal physiological conditions, increased retinal tissue perfusion in whole-mount retina.

We demonstrated that the significantly greater blood flow measured in the infero-temporal retinal artery in +HT group was mostly due to a significantly larger diameter of the ITRA. This finding is in contradiction with the findings reported by Leung H et al.[350] on the Blue Mountains Eye Study population, and Wong TY et al.[351] on the Beaver Dam Eye Study population.

In both studies, a computer-assisted program was used to measure the average arteriolar and venular diameters. In the Blue Mountain Eye Study,[350] for postmenopausal women older than age 65, there was a significant decrease for the averaged venular diameter for HT users compared to those who never used HT. In the Beaver Dam Eye study,[351] postmenopausal women who were HT users had significantly narrower averaged arteriolar and venular diameters compared to those who were never HT users. However, study limitations have been reported by the authors of these two studies such as a confounding by condition bias, lack of E_2 assays to confirm the use of HT, interaction of age and elevated BP that were not fully corrected by the adjusted model, 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 systemic and cardiovascular diseases were excluded, and this may explain why our results differed. Also, the measurements of the infero-temporal retinal artery diameter were obtained with the CLBF system, which accounts for cardiac pulsations, it may have provided more accurate vessel diameter measurements than the computer-assisted program used in both studies.

5.2 Vasomotor mechanisms of estrogens on retinal blood flow

The beneficial effect of E_2 and HT on retinal blood flow in postmenopausal women and ovariectomized rats is most likely caused by the vasodilating properties of estrogens mediated by the endothelium-derived relaxing factors, primarily nitric oxide (NO) and prostacyclin (PGI_2) pathways, which have been identified in the retinal vascular bed.[35,352-354] This effect could be mediated through ERα or ERβ, expressed in the retina.[179,180,185-188] Both ERα and ERβ have been shown to be implicated in the increase of NO synthase (NOS) expression[195,212] or NO production.[194] With regards to the prostacyclin pathway, several studies have shown that the stimulating action of estrogens on PGI_2 synthesis,[355,356] and intravitreal injection of prostacyclin have shown increased retinal blood flow in an animal model.[35] Finally, studies have indicated that estrogens also antagonize the effects of endothelium-derived contracting factors such as endothelin,[191] which has been documented as potent vasoconstrictor on the ophthalmic and retinal arteries.[34]

5.3 Vasomotor mechanisms of progestagens on retinal blood flow

Progestagens are prescribed as a combined HT to menopausal women with an intact uterus so as to regulate the action of estrogens on endometrial cells and to prevent endometrial cancer. In our whole study population of sixty-five subjects, 36 of them (55.4%) were HT users, of which 24 (36.9%) were on estrogens/progestagens combined therapy. The type of progestagen regimen used was heterogeneous in terms of active ingredients, where 11 (16.9%) subjects were using progesterone (P), 6 (9.2%) were

using norethindrone acetate (NETA), 5 (7.7%) were using medroxyprogesterone (MPA) and 2 (3.1%) were using levonorgestrel. However, divergent vasomotor effects of progestagens have been reported. In animal models, P has been shown to either induce vasodilation,[252-255] increase BF[256,257] or have no effect[258,259] when given alone, and to antagonize the estrogen-induced BF in a combined therapy.[258,259,263] When given alone MPA was shown to have no effect on the hypothalamic blood flow,[192] but to antagonize[357,358] or not to antagonize[359] the vasodilating effects of estrogens in a combined therapy. In clinical trials, P administration has been shown to decrease the endothelium-dependent flow mediated vasodilation (FMD) of the brachial artery,[260] to have no effect[261] or to increase FMD[262] when given alone. In a combined therapy, P administration was shown to antagonize[264] or not antagonize[262,265] the estrogen-induced FMD compared to estrogen administration alone. MPA was shown to antagonize the estrogen-induced FMD.[360] Therefore, possible beneficial or antagonizing vascular interactions of progestagens on estrogens in our study population cannot be ignored in our findings. Similar to estrogens, the vasomotor effects of progestagens can result from an interaction on both NO[266-268] and PGI_2 pathways[267,269] present in the retinal vascular bed.[35,352-354] This vasomotor effect is likely mediated through the progesterone receptors, which have been identified in the human retina.[179,180,185]

5.4 Possible vasomotor effect of equol on retinal blood flow

Vasomotor effects of equol, a metabolite of daidzein, a phytoestrogen present in soy products also cannot be excluded in our study results. The conversion of daidzein into equol occurs in the intestinal flora. Equol has been shown to induce vasodilation on

coronary arteries *in vitro* in combination with E_2.[348] In both studies, postmenopausal women and ovariectomized rats likely ingested soy products. However, effects of equol resulting from the ingestion of soy products is likely negligible for the following reasons. Only 27%[361] of women excrete equol and we can assume that both +HT and ØHT postmenopausal women groups were equally exposed to the effect of equol. Moreover, the vasomotor effect of equol has been shown only at elevated concentrations (10 μg/mL), but not at lower concentrations (0.1 μg/mL)[362] such as those levels detected in our rats. Finally, equol has a low binding affinity to both ERs, between 1/250th and 1/1000th of that for E_2.[363]

5.5 Effect of 17β-estradiol on weight, hematocrit and blood pressure

The changes in weight, Hct, blood pressure on systemic blood flow and consequently on retinal tissue perfusion should be taken into consideration.

First of all, the OVx rats gained weight. The amount of [^{14}C]-IMP injected was based on the rat weight to account for smaller and bigger proportional rats. The weight gain likely resulted from higher amounts of adipose tissue, in response to higher food intake and less energy expenditure[364] while the retinal volume does not change with weight. This could have biased the amount of injected [^{14}C]-IMP, which may not have been fully corrected by the formula to determine the RUI index, leading to possible higher RUI values in the OVx rats. A negative relationship exists between weight and SHBG.[365-369] It is possible the SHBG level may have decreased in the OVX rats and this may have led

to a reduced amount of estradiol bounded to SHBG. Consequently, more available free-estradiol could have induced some vasodilating effect in the OVx rats. As well, estrone is produced by adipose tissue from androstenedione. In the OVx rats that gained weight, increased estrone production could have also induced some vasodilating effect, although estrone is less biologically active than estradiol. In our observational study, postmenopausal women had to have and BMI $\leq$30 as part of our inclusion criteria and the BMI in the ∅HT group were similar to those of the +HT group. Therefore, the absence of weight gain effect on the SHBG should not have modified the bioavailability of estrogens in the ∅HT postmenopausal women.

Secondly, the OVx+E2 rat presented lower Hct level by 6.1 (14.7%). Similar lower hematocrit levels have been observed in rats (a 14.3% decrease)[370] and in women (<5% decrease)[371,372] following the administration of ethinyl estradiol. This reduced hematocrit level (1) may decrease blood viscosity as seen in women after HT treatment[289,373] and may favour increases in blood flow, or (2) may decrease oxygen delivery and favour local metabolic autoregulation, necessitate to increased flow of blood to meet the O2-delivery demands. However, a decrease of 6.1 in hematocrit level in the OVx+E_2 rats, reflecting approximately 2 g/dL change in haemoglobin, is likely insufficient to induce a 22% to 45% increase in the retinal tissue perfusion. Additionally, IMP is freely exchangeable between red cells and plasma with a differential concentration of approximately a 1.8/1 with the plasma.[374] Reduced amounts of Hct observed in our treated group of rats may have caused greater IMP availability to bind to the amine sites in the retina and induced higher RUIs (retinal

uptake indexes). In the menopause rat model, the clinical significance of reduced hematocrit in the OVx+E_2 group on systemic blood flow, and consequently the retinal blood flow, as well as on the availability of the [14C]-IMP used to measure RUI is unknown. A future study investigating the vasomotor effect following a short exposure time to estradiol, while avoiding changes in the hematocrit level, is required to narrow down the direct effects of estradiol on retinal circulation. In our postmenopausal women observational study, we have not documented the hematocrit level. Hematocrit, blood viscosity and plasma volume should be documented in future studies investigating ocular blood flow in postmenopausal women.

Finally in our postmenopausal study, we observed lower systolic, diastolic and mean blood pressure in women who were +HT users compared to ∅HT users. The differences for the diastolic and mean blood pressure reached a significance level for only the sub-population C. Our findings are somewhat in agreement with the findings of a longitudinal study monitoring the blood pressure changes in normotensive postmenopausal women.[375] Seventy-seven women used both estrogen and progestagen therapy and 149 women used neither. Over time, it was observed that the systolic blood pressure increased less in HT users than in non HT users over a period of ten years, while the diastolic blood pressure remained stable. Systemic blood pressure (BP) and ocular perfusion pressure (OPP = mean BP–IOP), (systolic BP-IOP and diastolic BP-PP) are important hemodynamic factors in providing normal blood perfusion to the retina. The resulting calculated ocular perfusion pressure was lower in the women who were +HT users compared to those who were ∅HT users reaching a significance level for

only the sub-population C. A high ocular perfusion pressure has a beneficial vascular effect by providing a better blood flow. However, the +HT postmenopausal women with lower ocular perfusion pressure presented greater infero-temporal retinal artery and temporal peripapillary retinal blood flow. This indicates that the increased retinal blood flow we observed was not affected by the systemic blood pressure and that it most likely results from a local action of HT on retinal circulation. In our menopaused rat model, systolic, diastolic and mean blood pressures were slightly higher in the OVx+E_2 rats, although they were not statistically significant. We did not measure the intraocular pressure in rats to determine the OPP. A small increase in systemic blood pressure may have provided a better retinal tissue perfusion in the OVx+E_2 rats. Intraocular pressures should be measured in further studies in rats for determining the OPP.

5.6 Possible effect of 17β-estradiol on vascular density

In addition to the vasomotor effects of estrogens on improving the retinal circulation, angiogenesis may also contribute to increased capillary blood flow in the retina. Estradiol is being recognized as having the ability to induce angiogenesis by stimulating endothelial cell proliferation via the vascular endothelial growth factor (VEGF), as seen in microvascular development in uterus and vascular tissue.[376] In a female rat model, a significant reduction in the capillary density (30%) in the frontal cortex accompanied by a decrease in VEGF level (49%) has been observed following ovariectomy.[377] Administration of 17β-estradiol in the OVx rats has reversed VEGF levels back to similar levels in intact female rats.[377] A possible decreased capillary density in the retina

may have occurred in postmenopausal women who were not using HT and in ovariectomized rats and possible increased capillary retinal blood flow in both studies following estrogen treatment may reflect the retinal capillary density levels being restored. This remains to be investigated.

5.7 Effects of HT 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 compared to that of the ∅HT group. For the rim volume, this was significant for both the entire and the infero-temporal regions of the ONH, while for the height variation contour, mean thickness and cross-sectional area of the RNFL, they reached significance level for the infero-temporal region. The differences observed in these stereometric parameters indicate a thinner retinal nerve fiber layer, in postmenopausal women not on HT compared to those on HT. This finding is similar to the sparing of some brain regions observed in post-menopausal women using HT.[312-314] A sparing of the retinal nerve fiber layer may result from the vascular properties of estrogens and progestagens, as described earlier, by providing a better blood flow and nutrients to the RGC and the NFL. In addition, sparing of the retinal nerve fiber layer may result from the protective properties of estrogens on neurons independent of blood flow.[226] These protective effects have been documented as increasing neuron survival in several toxicity models of cultured neurons, such as glutamate, β-amyolid, hydrogen peroxide, and excitatory amino acid toxicity models.[226] Interestingly, recent studies have reported a protective

effect of estrogens on retinal ganglion cells in glutamate toxicity[378] and axotomized optic nerve rodent models.[273]

Similar to its vasomotor effects, progestagens also present divergent effects in terms of protective effects on neurons. When given alone, P has been shown to increase neuron survival in glutamate toxicity models of neurons from the hippocampus,[270] and cerebral cortex,[271] and in a spinal cord genetic model[272] in rodents. However, an increase of neuron survival in the presence of progesterone has not been observed in the axotomized optic nerve model in rodents.[273] In combined therapy, P has been shown to increase neuron survival beyond that of E_2 alone in the hippocampal glutamate toxicity model.[270] With regards to MPA, it has shown to have no protective effect on neurons when given alone or in combined therapy,[270] or to potentiate neuronal loss[379] in a hippocampal glutamate toxicity model. Therefore, possible beneficial or antagonizing protective effects of progestagens 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 with the estrogen combined progestagen therapy. These differences reached significance level only for the rim volume but not for the height variation contour, mean thickness and cross-sectional area of the RNFL. This likely resulted from under power analysis for those specific variables.

For the height variation contour, mean thickness and cross-sectional area of the RNFL, the ØHT group of post-menopausal women presented a decrease of 4.6%, 6.7% and

7.6% respectively compared to +HT group for the entire optic nerve region, while for the infero-temporal quadrant, this decrease was 18.1%, 13.9% and 16.8% respectively. Preferential thinning of infero-temporal neural structures is also seen in early glaucoma, and several causes for this have been proposed.[380,381] It is interesting to speculate that the vulnerability of this region may explain some of our findings.

Since our findings on the optic nerve head 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 the optical coherence tomography. However, we were interested in investigating the ONH topography, which was obtained with the HRT I. As well, time-domain OCT only became available in our centre after this project was well underway.

5.8 Effect of HT on the functionality of RGC

The exact origins of the PERG components have not been identified, but current data suggest that the N95 is a contrast related component of the RGC function and that P50 is partly RGC derived with some contribution from structures distal to RGC.[382] Smaller PERG amplitudes and delayed latencies are indicative of RGC loss. Clinical studies where loss of RGCs occur such as in OHT and OAG, have demonstrated that N95 amplitude is reduced and P50 latency is increased,[339] and that N95 amplitude significantly and positively correlates with RNFL thickness while P50 latency negatively correlates.[340,341]

We observed a borderline significantly shorter P50 latency in the +HT group by only 1.1 ms. 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. Interestingly, our findings are somewhat consistent with the pattern visual evoked potential (PVEP) findings observed in postmenopausal women, where the use of Tibolone over a period of 3 months, a synthetic steroid with prominent estrogen effects, was shown to significantly improve the P100 latencies by 4 ms (5%), as well as a significant increase by 31% in the P100 amplitude.[383] Yilmaz et al. (2000)[383] hypothesized that increasing sensitivity of the central nervous system to catecholamines by changing the opening frequency of voltage-related L-type calcium channels,[384] increasing effects of glutamate[385] and decreasing formation of γ-aminobutyric acid (GABA)[386] induced by estrogens might be the responsible mechanisms in shortening P100 latency and increasing P100 amplitude. So far, it is unknown how estrogens might improve the P50 latency of the PERG. The same mechanisms may apply in shortening P50 latency in postmenopausal women who were HT users. This remains to be investigated. We also observed a small increase in the N95 amplitude by 3% and a small decrease in P50 amplitude by 6% in the +HT group. However, the magnitude of amplitude changes were not in the 31% magnitude change of the P100 amplitude as reported by Yilmaz al. (2000), and they did not reach statistical significance. 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.

5.9 Possible effect HT on the functionality of other retinal neurons

While the VEP assesses the functionality of the ON and the visual pathways, and the PERG assesses the RGCs, the electroretinogram (ERG) assesses the functionality of the entire retina evoked by a standardized flash of light. The b-wave is the main component of the ERG and represents the Müller cell response to an increase in extracellular potassium generated by the retinal neuron activity. The OPs are a series of successive wavelets of the ERG with a frequency range of 200 Hz. The exact cellular origin of the OPs is still unidentified but they are believed to originate within the inner nuclear and outer plexiform layers.[387]

Since estrogen receptors are present throughout the different retinal layers in human retina,[186,187] estrogens are likely modulating the activity of all retinal neurons and consequently the entire retinal functionality. A study published by Verit et al. (2007)[388] indicated that the use of Tibolone over a 6-month period in postmenopausal women did not significantly affect the P100 amplitude and latencies, but the oscillatory potentials (OPs) of the ERG were overall significantly smaller in the Tibolone user group. In cyclical women, hormonal the variation of estrogens and progesterone have shown to modulate OPs and b-wave amplitudes.[389]

GABA and glutamate are two important neurotransmitter in the retina and particpiate in the generation of the OPs and b-wave amplitude. Increasing effects of glutamate[385] and a decreasing formation of γ-aminobutyric acid (GABA)[386] in the presence of estrogens may have a mixed effect on the b-wave and OP amplitudes. Therefore, the use of HT in

postmenopausal women may have affected the functionality of the retina. This remains to be investigated.

5.10 Possible effect HT on visual function

Although we have shown improved retinal blood flow and greater RNFL thickness in our postmenopausal women on HT, it is unknown how these beneficial effects translate to potentially improved performance on the visual function in these women. Very few studies have reported the use of postmenopausal HT on the visual function. Postmenopausal women using equine conjugated estrogens and dydrogesterone for 1 year or Tibolone for 6 months have demonstrated better performance on the contrast sensitivity test[390] and blue-on-yellow Humphrey visual field tests.[391] This improved performance in postmenopausal women on HT can result from several combined factors such as better blood flow to the visual pathways and protective effect on neurons, as well as an increase in the tear film production[392] and break-up time[393] and better neuronal response along the visual pathways as documented by enhanced pattern VEP signal.[383] Longitudinal effects of HT on visual function assessed by contrast sensitivity and visual field testing in postmenopausal women is warranted.

5.11 Possible neurotrophic effect of HT

Experimental studies have demonstrated that estrogens have a neurotrophic property by increasing dendritic spines density,[230-232] which are the post-synaptic sites of excitatory input to a wide variety of neurons in the mammalian brain and stimulating cell

proliferation.[233,234] These neurotrophic effects are thought to be mediated by the brain-derived neurotrophic factor (BDNF).[235,236] Such neurotrophic activity could also occur in our observational study and menopausal rat model.

5.12 Study Limitations

As part of our exclusion criteria, postmenopausal women had to be free of systemic and cardiovascular diseases. Clinical studies have reported that the use of HT in patients who suffer from diabetes or coronary artery disease did not improve the FMD compared to baseline values.[394,395] In hypertensive patients, the pulsatility indexes of the uterine, carotid and ophthalmic arteries were shown to be higher compared to those of normotensive patients after starting HT.[396] As previously mentioned, potentially impaired endothelium in patients with systemic and cardiovascular diseases may not be fully or partly responsive to the HT treatment. Moreover, these patients may likely be on vasodilator treatment and their endothelium may not be responsive to additional vasomotor stimulation from HT treatment. If we had included postmenopausal women with systemic and cardiovascular diseases, it is possible that the differences we observed in blood flow and RNFL thickness might have been different.

In this heterogeneous population of postmenopausal women we investigated, the type of HT regimens in terms of their active ingredients, routes of administration, dosage and duration of administration was heterogeneous and may have yielded different degrees of vasomotor effects on the retina and protective effects on the retinal nerve fiber layer. In addition, women were at variable postmenopausal times. As well, postmenopausal

women may not exhibit similar cardiovascular characteristics and risks, which could interplay with the efficacy of HT. Finally, the outcome variables measured were collected at a single time point, which does not provide any information on the progress on the outcome variables in both groups of postmenopausal women. Since HT offers cardioprotective effects, potmenopausal women who are not HT users are at greater risk of developing cardiovascular diseases over time. Consequently, a longitudinal study would bring additional information of the retinal blood flow and ONH changes and functionality over time in postmenopausal women. The ideal study design would be to randomize women to HT at the time of the onset of menopause with documented cardiovascular characteristics and risks, and establish a baseline for blood flow, structure and function before beginning therapy. Then monitor blood flow, structure and function changes at intervals over a five- to ten-year period. Such a protocol could be part of a larger HT study.

We successfully developed a menopaused rat model in which retinal tissue perfusion was assessed by quantitative autoradiography technique. This menopaused rat model is a valuable tool in providing a better understanding of our findings of improved retinal blood flow and protective effects on the RNFL. First, the type of estrogens, dose and route of administration as well as the type of progestagen used in combined therapies can be investigated. The longitudinal effect of estrogen depletion and HT can be monitored in different groups of menopaused rats. Finally, possible vasomotor pathways such as nitric oxide, prostacyclin and endothelin, and effect on RGCs and axons can be extensively studied.

We conducted our studies in postmenopausal women and ovariectomized rats, in whom the gonadal source of endogenous estrogens production was either minimalized or eliminated respectively. However, a possible source of endogenous estrogens in the retina could not be excluded. Recently, Cascio and *al.* (2007)[171] found P450 aromatase mRNA and expression in the adult male rat retina and confirmed that the biosynthesis of 17β-estradiol occurs. Similarly, P450 aromatase mRNA and expression are found in several regions of the brain in different species[168] and biosynthesis of 17β–estradiol also occurs in the brain.[169,170] It is unknown what role local estrogen biosynthesis plays in the retina. In the brain, the proposed role is believed to be involved in the trophic and protective effects on neurons.[168] The possible impact of retinal estrogen biosynthesis in the retina relating to our findings in postmenopausal women and menopaused rat model is unknown. However, we assumed that both postmenopausal women and ovariectomized rat groups were equally exposed to retinal estrogen biosynthesis. Intravitreal injection of estrogen receptor antagonists in a rat model may help to narrow down the role of retinal estrogen biosynthesis on retinal circulation and neurons.

6.0 CONCLUSION

This is the first observational study that has investigated the long-term effects of estrogen depletion compared to the use of hormone replacement therapy on the retina and nerve fiber layer in a population of postmenopausal women. The findings of this study indicated that compared to postmenopausal women who never had used HT, postmenopausal women who have used HT since their menopause onset presented 1) a significantly better retinal blood flow as observed in the infero-temporal retinal artery and 2) greater rim volume, height variation contour, mean and cross-sectional area of the RNFL of their optic nerve head. Blood flow was also greater in the temporal peripapillary retina in the +HT group and the latency of the P50 component of the PERG were shorter by 1 ms, but these difference did not reach statistical significance in the adjusted regression model, possibly due to our small sample size. Our results of improved retinal blood flow and protective effect on the ONH topography provided direct evidence that estrogens alone or in combination with progestagens have a vasomotor effect on the retinal circulation and a protective effect on the RNFL.

So as to investigate the vasomotor mechanisms on the retinal circulation and protective effect on retinal nerve fiber layer and function of the RGCs in the near future, a successful menopaused rat model was developed. In this model we demonstrated that chronic subcutaneous treatment of 17β-estradiol significantly improved retinal blood flow as observed by an increase of the retinal uptake index, following the injection of a blood flow tracer, the N-Isopropyl-[methyl-1,3-^{14}C]-p-iodoamphetamine, in whole-mount rat retina.

As mentioned in the introductory section, recent epidemiological population-based studies have reported that early menarche, late menopause onset and increased reproductive year duration, significantly decreased the odds ratio of developing open-angle glaucoma and ARMD and that the use of HT protects against glaucoma and ARMD. These observations strongly suggest that an hormonal component is involved in the aetiology of glaucoma and ARMD possibly linked to the depletion of estrogen level in postmenopausal women.

Glaucoma and ARMD are multifactorial eye diseases and clinical evidence indicates that impaired ocular blood flow is thought to be involved in the aetiology and progression of these two eye diseases. Improved retinal blood flow and protective effects on the RNFL may explain the link between the hormonal status of estrogen and progestagen levels throughout a woman's life and the risk of developing glaucoma[317,318] and age-related macular degeneration.[319-321] by providing a protective effect via improved ocular blood flow and neuronal protection. Rats are often used as glaucoma and ARMD model. Therefore, the application of a menopaused rat model in which glaucoma or ARMD is induced, could bring answers on the possible protective mechanisms of estrogens and HT against glaucoma and ARMD in the postmenopausal women.

These findings of our observational study are a stepping stone for designing studies to further understand the role of estrogens and the consequences of estrogen withdrawal on the retina and optic nerve in postmenopausal women. A clinical trial investigating retinal and optic nerve head blood flow, tomography and functionality (visual field and

visual evoked potentials) in postmenopausal women over a period of time is warranted, where HT will be prescribed to postmenopausal women following the recommendations of the Society of Obstetricians and Gynaecologists of Canada and International Menopause Society.

In the interim, while the science progresses on this, particular attention should be given to postmenopausal women's eyesight. As mentioned earlier, our findings in menopausal women may explain the link between the hormonal status of estrogen and progestagen levels throughout a woman's life and the risk of developing glaucoma[317,318] and age-related macular degeneration.[319-321] Women with early menopause onset or premature ovarian failure caused by chemo/radiotherapy, genetic disorders, autoimmune disease and hypopituitarism[397] should be closely monitored by their eye specialists.

7.0 FUTURE DIRECTIONS

The findings of our observational study in postmenopausal women and ovariectomized rats only reveal a small piece of the puzzle on the role of estrogens in the eye. The possibilities for further research are countless. Here are a few suggestions of what can be undertaken.

Intravitreal injection of agonists and antagonists of estrogens in an ovariectomized animal model, which will avoid possible systemic effects from oral or transdermal treatment, will provide answers to the local role of estrogens and progesterone on retinal and optic nerve head blood flow, tomography and functionality.

The laminar beams composing the lamina cribrosa, are made up of long fibers of elastin and collagen.[398] It is believed that elastin is involved in the optic nerve head compliance to changes in the intraocular pressure. The depletion of estrogens during menopause could likely impair the collagen and elastin in the lamina cribrosa as seen on the skin during menopause.[399] Consequently, the optic nerve head might be less compliant to intraocular pressure changes. This remains to be investigated.

The trabecular meshwork in the eye is responsible for normal flow of aqueous of the eye and prevents increases in the intraocular pressure. Elastin and collagen are also present in the trabecular meshwork.[400] Again, the depletion of estrogens during menopause could likely impair the collagen and elastin in the trabecular meshwork and compromise the aqueous flow. This remains to be investigated.

Selective estrogen receptor modulators (SERMs) for treating menopausal symptoms are being developed. SERMs avoid some side effects from conventional HT use, such as breast cancer. SERMs are synthetic compounds that behave like estrogens in some tissues but block the action of estrogen in others. For example, the SERM raloxifene has been developed to prevent osteoporosis in postmenopausal women. In the Multiple Outcomes of Raloxifene Evaluation (MORE) randomized trial, raloxifene was reported to not significantly affect the risk of cardiovascular events after four years of treatment in postmenopausal women.[401] It also contributed to a 40% reduction of newly diagnosed invasive breast cancer.[402] However, adverse events such as hot flashes and leg cramps and rare venous thromboembolic events have been reported.[403] The development of SERMs is still ongoing to find better molecules with the least side and adverse effects. Since the eye is a window of the body, retinal and optic nerve head blood flow, tomography and functionality assessment in postmenopausal women and ovariectomized animal models can monitor the activity of SERMs and help in designing better molecules.

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9.0 ANNEXES

No 171, February 2006

Canadian Consensus Conference on Menopause, 2006 Update

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ABSTRACT

Objective: To provide guidelines for health care providers on the management of menopause in asymptomatic healthy women as well as in women presenting with vasomotor symptoms, urogenital, sexual, and mood and memory concerns and on specific medical considerations, and cardiovascular and cancer issues.

Outcomes: Prescription medications, complementary and alternative medicine (CAM), and lifestyle interventions are presented according to their efficacy in treating menopausal symptoms.

Evidence: MEDLINE and the Cochrane database were searched for articles from March 2001 to April 2005 in English on subjects related to menopause, menopausal symptoms, urogenital and sexual health, mood and memory, hormone therapy, CAM, and on specific medical considerations that affect the decision of which intervention to choose.

Values: The quality of evidence is rated using the criteria described in the report of the Canadian Task Force on the Periodic Health Examination. Recommendations for practice are ranked according to the method described in this report (see Table 1).

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Key words:

Menopause, estrogen, progestin, androgen, complementary therapies, vasomotor symptoms, urogenital symptoms, mood, memory, estrogen replacement therapy, hormone replacement therapy, cardiovascular diseases, cancer

RECOMMENDATIONS

I. General Recommendations

1. Health care providers should discuss and encourage initiation of healthy lifestyle choices in menopausal women. (II-2A)
2. The primary indication for hormone therapy (HT) should be for the management of moderate to severe menopausal symptoms. (IA)
3. HT should not be prescribed for primary or secondary prevention of cardiovascular disease (CVD) or for primary prevention of dementia. (IA)
4. Local estrogen therapy (ET) is recommended if HT is prescribed for vulvovaginal symptoms alone. (IA)
5. HT should be prescribed for the appropriate duration to achieve treatment goals while taking into consideration risks and benefits and the woman's quality of life. (IIIB)
6. HT should be prescribed at the lowest effective dose, although the long-term risk/benefit ratio of lower dose HT has not been demonstrated. (IIIC)
7. The primary indication for progestin use should be endometrial protection in women using systemic estrogen therapy who have an intact uterus. (IA)
8. HT may be prescribed for an extended period, following proper counselling, if the woman decides that for her the benefits outweigh the risks (II-2A). Periodic re-evaluation is strongly recommended (IIIC).

Criteria for quality of evidence assessment and classification of recommendations

Level of evidence*	Classification of recommendations*
I: Evidence obtained from at least one properly designed randomized controlled trial.	A. There is good evidence to support the recommendation that the condition be specifically considered in a periodic health examination.
II-1: Evidence from well-designed controlled trials without randomization.	B. There is fair evidence to support the recommendation that the condition be specifically considered in a periodic health examination
II-2: Evidence from well-designed cohort (prospective or retrospective) or case-control studies, preferably from more than one centre or research group.	C. There is poor evidence regarding the inclusion or exclusion of the condition in a periodic health examination.
II-3: Evidence from comparisons between times or places with or without the intervention. Dramatic results from uncontrolled experiments (such as the results of treatment with penicillin in the 1940s) could also be included in this category.	D. There is fair evidence to support the recommendation that the condition not be considered in a periodic health examination.
III: Opinions of respected authorities, based on clinical experience, descriptive studies, or reports of expert committees.	E. There is good evidence to support the recommendation that the condition be excluded from consideration in a periodic health examination.

*Woolf SH, Battista RN, Angerson GM, Logan AG, Eel W. Canadian Task Force on the Periodic Health Exam. Ottawa: Canadian Communication Group; 1994. p. xxxvii.

*The quality of evidence reported in these guidelines has been adapted from the Evaluation of Evidence criteria described in the Canadian Task Force on the Periodic Health Exam.

†Recommendations included in these guidelines have been adapted from the Classification of Recommendations criteria described in the Canadian Task Force on the Periodic Health Exam.

9. Androgen therapy may be considered for selected women with acquired sexual desire/interest disorders after comprehensive assessment, systemic estrogen therapy and appropriate counselling (II-1B) Androgen therapy is still investigational and long-term safety data are lacking (IIIB).
10. Health care providers may offer identified complementary and alternative medicine with demonstrated efficacy for mild menopausal symptoms. (IB)

II. Specific Recommendations

Chapter 1: Introduction:

No Recommendations

Chapter 2: Menopause and Age-Related Concerns

1. Lifestyle modifications, including reducing core body temperature, regular exercise, weight management, smoking cessation, and controlled breathing may be recommended to reduce mild vasomotor symptoms. (IC)
2. Health care providers should offer HT (ET/estrogen-progestin therapy) as the most effective therapy for the medical management of menopausal symptoms. (IA)
3. Progestins alone or low-dose oral contraceptives can be offered as alternatives for the relief of menopausal symptoms especially during the transition phase. (IA)
4. Non-hormonal prescription therapies, including antidepressant agents, gabapentine, clonidine, and bellergal, can be prescribed as alternatives to HT to reduce vasomotor symptoms. (IB)
5. Complementary and alternative medicine, including black cohosh, red clover (derived isoflavone, and vitamin E) may be recommended for the reduction of mild vasomotor symptoms (IB). Long-term efficacy and safety data are still lacking.
6. Any unexpected bleeding that occurs after 12 months of amenorrhea is considered postmenopausal bleeding and should be investigated. (IA)
7. If prescribing HT to older postmenopausal women, low or ultra-low dose ET is preferred. (IB)

Chapter 3: Urogenital Concerns

1. Conjugated estrogen (CE) cream, an intravaginal sustained-release estradiol ring, or estradiol vaginal tablets are recommended as effective treatment for vulvovaginal atrophy. (IA)
2. Routine progestin co-therapy is not required for endometrial protection in women receiving vaginal estrogen therapy in appropriate dose. (IIIC)
3. Vaginal lubricants may be recommended for subjective symptom improvement of dyspareunia. (IIIC)
4. Health care providers can offer polycarbophil gel (a vaginal moisturizer) as an effective treatment for symptoms of vulvovaginal atrophy including dryness and dyspareunia. (IA)
5. Effective surgical treatment options, including Burch colposuspension and the TVT procedure, are recommended for the treatment of stress urinary incontinence. (IA)
6. Effective non-surgical treatment options, such as weight loss (in obese women), pelvic floor physiotherapy with or without biofeedback, weighted vaginal cones, functional electrical stimulation, and/or intravaginal pessaries, can be recommended for the treatment of stress urinary incontinence. (II-1B)
7. Lifestyle modification, bladder drill (II-1B), and antimuscarinic therapy (IA) are recommended for the treatment of urge urinary incontinence.
8. ET should not be recommended for the treatment of postmenopausal urge or stress urinary incontinence. (IA)
9. Vaginal estrogen therapy can be recommended for the prevention of recurrent urinary tract infections in postmenopausal women. (IA)

Chapter 4: Sexual Concerns

1. A biopsychosexual assessment of preferably both partners (when appropriate), identifying intrapersonal, contextual, interpersonal, and biological factors, is recommended prior to treatment of women's sexual problems. (IIIA)
2. For women with vaginal atrophy, local estrogen should be prescribed to improve vulvovaginal atrophy-associated dyspareunia. (IA)
3. Routine evaluation of sex hormone levels in postmenopausal women with sexual problems is not recommended. Available androgen assays neither reflect total androgen activity, nor correlate with sexual function. (IIIA)
4. Any investigational testosterone therapy included in the management of selected women with acquired sexual desire/interest disorder, typically associated with an arousal disorder, should only be initiated by clinicians experienced in women's sexual dysfunction and with informed consent from the woman. The investigational nature, lack of long-term safety data, need for systemic estrogen therapy, and careful follow-up must be explained. (IC)

Chapter 5: Mood and Memory

1. Estrogen alone may be offered as an effective treatment for depressive disorders in perimenopausal women and may augment clinical response to antidepressant treatment, specifically SSRIs (IB). The use of antidepressant medication, however, is supported with the most research evidence (IA).
2. Estrogen can be prescribed to enhance mood in women with depressive symptoms. The effect appears to be greater for perimenopausal symptomatic women than for postmenopausal women. (IA)
3. Estrogen therapy is not currently recommended for reducing the risk of developing dementia in postmenopausal women or for retarding the progression or deterioration in women with diagnosed Alzheimer's disease. (IB)

Chapter 6: Prescription Drugs

No Recommendations

Chapter 7: Complementary and Alternative Medicine

No Recommendations

Chapter 8: Specific Medical Considerations

1. HT should be offered to women with premature ovarian failure (POF) or early menopause (IA), and its use can be recommended until the age of natural menopause (IIIC).
2. ET can be offered to women who have undergone surgical menopause for the treatment of endometriosis. (IA)
3. Menopausal women undergoing pelvic surgery should be given appropriate thromboembolic prophylaxis. (IA)
4. Health care providers may prescribe HT to diabetic women for the relief of menopausal symptoms. (IA)

Chapter 9: Cardiovascular Disease (CVD)

1. Health care providers should not initiate or continue HT for the sole purpose of preventing CVD (coronary artery disease and stroke). (IA)
2. Health care providers should abstain from prescribing HT in women at high risk for venous thromboembolic disease. (IA)
3. Health care providers should consider other evidence-based therapies and interventions to effectively reduce the risk of CVD events in women with or without vascular disease. (IA)

Chapter 10: Cancer

1. All unscheduled uterine bleeding should be investigated because no estrogen-progestin regimen is completely protective against endometrial carcinoma. (IA)
2. Estrogen-progestin therapy may be offered to women with low-grade adenocarcinoma of the endometrium who have moderate to severe menopausal symptoms. (IB)
3. Health care providers should periodically review the risks and benefits of prescribing HT to a menopausal woman in light of the association between duration of use and breast cancer risk. (IA)
4. Health care providers may prescribe HT for menopausal symptoms in women at increased risk of breast cancer with appropriate counselling and surveillance (IA) (in women in the Women's Health Initiative [WHI] study with high Gael scores were at no greater risk of breast cancer than women with low risk scores).
5. Health care providers should clearly discuss the uncertainty of risks associated with HT after a diagnosis of breast cancer in women seeking treatment for distressing symptoms. (IB)

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IMS Updated Recommendations on postmenopausal hormone therapy

Issued on behalf of the Board of the International Menopause Society by Amos Pines (President), David W. Sturdee (General Secretary), Martin H. Birkhäuser (Treasurer), Hermann P. G. Schneider, Marco Gambacciani and Nick Panay

INTRODUCTION

The past decade has seen marked fluctuations in opinions concerning the merits and risks of postmenopausal hormone therapy. In July 2002, menopause management faced a major turning point when the first data from the Women's Health Initiative (WHI) trial were released. The study was categorized as a primary prevention trial for coronary heart disease, although the fact that mean age at recruitment was 63 years was not given enough importance at that time. WHI investigators concluded that hormone therapy (HT) was not cardioprotective, and, in fact, its risk–benefit ratio did not favor the use of postmenopausal hormones for prevention of chronic diseases. As a result, there was a dramatic change in prescription habits following recommendations to reserve HT for very symptomatic women, and to limit its use to the 'shortest duration needed' and 'to the lowest effective dosage'. This was the atmosphere in which the International Menopause Society (IMS) initiated the IMS Workshop held in Vienna (December 2003) and the IMS Position Paper that was based on the Workshop discussions. Looking at global perspectives, and being independent of local or regional constraints imposed by official health authorities, this IMS Statement called for a more balanced approach in the interpretation of the scientific data on hormone use that were available in 2003. Since then, additional information has been accumulated from both arms of the WHI study, observational trials and from other studies, allowing a more comprehensive review on all issues related to the use of hormones in the postmenopausal period. In view of the above, the IMS Board decided that it is time to update the 2004 Statement and to enlarge its scope to menopause management and adult women's health in general. More than 30 experts from the various fields of menopause medicine reviewed the latest information in a Workshop held in Budapest in February 2007.

The following Recommendations express the views of the IMS on the principles of hormone therapy in the peri- and postmenopausal periods. Throughout the Recommendations, the term HT will be used to cover all therapies including estrogens, progestogens, combined therapies and tibolone.

The previous IMS Statement in 2004 is still valid and serves as a basis for the current Updated Recommendations.

We are aware of the geographical variations related to different priorities of medical care, different prevalence of diseases, and country-specific attitudes of the public, the medical community and the health authorities toward menopause management, which may all impact on hormone therapy. The following recommendations, therefore, give a global and simple overview that serves as a common platform on issues related to the various aspects of hormone treatment. These Recommendations were reviewed and discussed by representatives of more than 60 National and Regional Menopause Societies from all continents. These Recommendations can be easily adapted and modified according to local needs.

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GOVERNING PRINCIPLES

Hormone therapy should be part of an overall strategy including lifestyle recommendations regarding diet, exercise, smoking and alcohol for maintaining the health of postmenopausal women. HT must be individualized and tailored according to symptoms and the need for prevention, as well as personal and family history, results of relevant investigations, the woman's preferences and expectations. The risks and benefits of HT differ for women around the time of menopause compared to those for older women.

HT includes a wide range of hormonal products and routes of administration, with potentially different risks and benefits. Thus, the term 'class effect' is confusing and inappropriate.

Women experiencing a spontaneous or iatrogenic menopause before the age of 45 years and particularly before 40 are at higher risk for cardiovascular disease and osteoporosis. They will benefit from hormone replacement, which should be given at least until the normal age of menopause.

Counselling should convey the benefits and risks of HT in simple terms, e.g. absolute numbers rather than as percentage changes from baseline expressed as a relative risk. This allows a woman and her physician to make a well-informed decision about HT.

HT should not be recommended without a clear indication for its use.

Women taking HT should have at least an annual consultation to include a physical examination, update of medical history, relevant laboratory and imaging investigations and a discussion on lifestyle.

There are no reasons to place mandatory limitations on the length of treatment.

Whether or not to continue therapy should be decided at the discretion of the well-informed hormone user and her health professional, dependent upon the specific goals and an objective estimation of benefits and risks.

Dosage should be titrated to the lowest effective dose. Lower doses of HT than have been used routinely can maintain quality of life in a large proportion of users. Long-term data on lower doses regarding fracture risk and cardiovascular implications are still lacking.

In general, progestogen should be added to systemic estrogen for all women with a uterus to prevent endometrial hyperplasia and cancer. However, natural progesterone and some progestogens have specific beneficial effects that could justify their use besides the expected actions on the endometrium. Low-dose vaginal estrogens administered for the relief of urogenital atrophy do not require progestogen co-medication. Direct delivery of progestogen to the endometrial cavity from the vagina or by an intrauterine system is logical and may minimize systemic effects.

Androgen replacement should be reserved for women with clinical signs and symptoms of androgen insufficiency. In women with bilateral oophorectomy or adrenal failure, androgen replacement has significant beneficial effects, in particular on health-related quality of life and sexual function.

BENEFITS OF HORMONE THERAPY

General

HT remains the most effective therapy for vasomotor and estrogen-deficient urogenital symptoms. Other menopause-related complaints, such as joint and muscle pains, mood swings, sleep disturbances and sexual dysfunction (including reduced libido) may improve during HT. Quality of life and sexuality are key factors to be considered in the management of the aging individual. The administration of individualized HT (including androgenic preparations when appropriate) improves both sexuality and overall quality of life.

Postmenopausal osteoporosis

HT is effective in preventing the bone loss associated with the menopause and decreases the incidence of all osteoporosis-related fractures, including vertebral and hip, even in patients at low risk. Although the magnitude of decline in bone turnover correlates with estrogen dosage, even lower than standard-dose preparations maintain a positive influence on bone indices in most women. Based on updated evidence on effectiveness, cost and safety, HT is an appropriate first-line therapy in postmenopausal women presenting with an increased risk for fracture, particularly under the age of 60 years and for the prevention of bone loss in women with premature menopause. The protective effect of HT on bone mineral density declines after cessation of therapy at an unpredictable rate, although some degree of fracture protection may remain after cessation of HT.

The initiation of standard-dose HT is not recommended for the sole purpose of the prevention of fractures after the age of 60 years. Continuation of HT after the age of 60 years for the sole purpose of the prevention of fractures should take into

account the possible long-term effects of the specific dose and method of administration of HT, compared to other proven therapies.

Cardiovascular disease

Cardiovascular disease is the principal cause of morbidity and mortality in postmenopausal women. Major primary prevention measures (besides smoking cessation and diet control) are weight loss, blood pressure reduction, and diabetes and lipid control. There is evidence that HT may be cardioprotective if started around the time of menopause and continued long-term (often referred to as the 'window of opportunity' concept). HT markedly reduces the risk of diabetes and, through improved insulin resistance, it has positive effects on other risk factors for cardiovascular disease such as the lipid profile and metabolic syndrome.

In women less than 60 years old, recently menopausal and without prevalent cardiovascular disease, the initiation of HT does not cause early harm and in fact reduces cardiovascular morbidity and mortality. Continuation of HT beyond the age of 60 should be decided as a part of the overall risk–benefit analysis.

Other benefits

HT has benefits for connective tissue, skin, joints and intervertebral disks. HT may reduce the risk of colon cancer. HT initiated around the time of menopause or by younger postmenopausal women is associated with a reduced risk of Alzheimer's disease.

POTENTIAL SERIOUS ADVERSE EFFECTS OF HORMONE THERAPY

Studies on the risks of postmenopausal hormone use have mainly focused on breast and endometrial cancer, venous thromboembolism (pulmonary embolism or deep vein thrombosis), stroke and coronary events.

Breast cancer

The incidence of breast cancer varies in different countries. Therefore, currently available data cannot necessarily be generalized. The degree of association between breast cancer and postmenopausal HT remains controversial.

Women should be reassured that the possible risk of breast cancer associated with HT is small (less than 0.1% per annum). For combined HT, observational data from the Million Women Study suggested that breast cancer risk was increased as early as the first year, raising serious reservations on possible methodologic flaws. On the contrary, randomized controlled data from the Women's Health Initiative (WHI) study indicate that no increased risk is observed in women initiating HT, for up to 7 years. It should be noted that the majority of subjects in the WHI study were overweight or obese.

Data from the WHI and Nurses' Health Study suggest that long-term estrogen-only administration for 7 and 15 years, respectively, does not increase the risk of breast cancer in American women. Recent European observational studies suggest that risk may increase after 5 years.

There are insufficient data to evaluate the possible differences in the incidence of breast cancer using different types and routes of estrogen, natural progesterone and progestogens, and androgen administration.

Baseline mammographic density correlates with breast cancer risk. This does not necessarily apply to the increase in mammographic density induced by HT.

The combined estrogen–progestogen therapy-related increase in mammographic density may impede the diagnostic interpretation of mammograms.

Endometrial cancer

Unopposed estrogen administration induces a dose-related stimulation of the endometrium. Women with a uterus should have progestogen supplementation.

Continuous combined estrogen–progestogen regimens are associated with a lower incidence of endometrial hyperplasia and cancer than occurs in the normal population.

Direct intrauterine delivery systems may have advantages. Regimens containing low-/ultra-low-dose estrogen and progestogen cause less endometrial stimulation and less bleeding.

Thromboembolism and cardiovascular events

The HT-related risk for serious venous thromboembolic events increases with age (although minimal until age 60), and is also positively associated with obesity and thrombophilia. By avoiding first-pass hepatic metabolism, transdermal estrogen may avert the risk associated with oral HT. The impact on the risk of a thromboembolic

event may also be affected by progestogen, depending on the type. Late starters of standard-dose HT may have a transient slightly increased risk for coronary events. The risk of stroke is correlated with age. HT may increase the risk of stroke after the age of 60.

Safety data from studies of low-dose and ultra-low-dose regimens of estrogen and progestogen are encouraging.

ALTERNATIVE TREATMENTS

The efficacy and safety of complementary alternative medicines have not been demonstrated and further studies are required.

Selective serotonin reuptake inhibitors, selective noradrenaline reuptake inhibitors and gabapentin are effective in reducing vasomotor symptoms in short-term studies. Their long-term safety needs further evaluation.

There are no medical or scientific reasons to recommend unregistered 'bioidentical hormones'. The measurement of hormone levels in the saliva is not clinically useful. These 'customized' hormonal preparations have not been tested in studies and their purity and risks are unknown.

RESEARCH

There is urgent need for further research especially into the relative merits of lower doses, regimens and routes of administration.

CONCLUSION

The safety of HT largely depends on age. Women younger than 60 years old should not be concerned about the safety profile of HT. New data and re-analyses of older studies by women's age show that, for most women, the potential benefits of hormone therapy given for a clear indication are many and the risks are few when initiated within a few years of menopause. In view of the new data, Regulatory Authorities should review their current recommendations as a priority.

The original IMS Position Statement was published in Climacteric 2004;7:8–11.

APPENDIX: Key messages from the lectures, abstracted by the speakers, on adult women's health presented at the IMS Budapest Workshop, February 2007

HEALTHY LIFESTYLE

Exercise in the menopause – an update

- Any physical activity is better than being sedentary.
- Regular exercise reduces total and cardiovascular mortality.
- Better metabolic profile, balance, muscle strength, cognition and quality of life are observed in physically active persons. Heart events, stroke, fractures and breast cancer are significantly less frequent.
- Benefits far overweigh possible adverse consequences: the more, the better, but too much may cause harm. Injury to the musculo-articulo-skeletal system should be avoided.
- Optimal exercise prescription is at least 30 minutes of moderate-intensity exercise, at least three times weekly. Two additional weekly sessions of resistance exercise may provide further benefit.

Healthy lifestyle

- Obesity (body mass index >30 kg/m^2) affects over 20% of the population and is becoming an increasing problem in the lower socio-economic sectors and also among children.
- Weight loss of only 5–10% is sufficient to improve many of the abnormalities associated with the insulin resistance syndrome.
- The basic components of a healthy diet are: four to five servings/day of fruits and vegetables, whole grain fibers, fish twice a week, and low total fat (but the use of olive oil is recommended). Consumption of salt should be limited and the daily amount of alcohol should not exceed 30 g for men and 20 g for women. Smoking should be prohibited.
- Lifestyle modifications include socializing, and being physically and mentally active.
- The public health approach to lifestyle promotion requires a multidisciplinary approach,

starting from schools through to work places, involving the food and advertising industry, as well as medical insurers and health authorities. A new paradigm in the doctor–patient relations is required, where the doctor becomes more of an advisor and the patient has to take the responsibility for his own health.

UROGYNECOLOGY

- Symptoms such as vaginal dryness, soreness, dyspareunia, urinary frequency and urgency are extremely common in postmenopausal women. Incontinence in women seems to increase with age, from 3–5% at age 20, 8–9% at age 30 and 12–15% at age 50.
- However, there is a huge inter-individual as well as intra-individual sensitivity to these changes, and symptoms and signs of urogenital aging are therefore highly variable within an individual as well as between individuals.
- The loss of lubrication and glandular functions severely impairs sexual desire. Treatment of this condition improves quality of life, not only for the woman but also for her partner.
- Urogenital symptoms respond well to estrogens. Long-term treatment is often required as symptoms can recur on cessation of therapy. Systemic risks have not been identified with local low-potency/low-dose estrogens.
- Use of systemic hormone therapy does not seem to prevent urinary incontinence.
- Antimuscarinic drugs combined with local estrogens constitute first-line treatment in women with urge incontinence and/or overactive bladder.
- Surgery remains the prime option for perimenopausal women with pure stress incontinence in whom hormone therapy may even worsen the situation.

OSTEOPOROSIS

Hormonal therapy (HT)

- HT is effective in preventing the bone loss associated with the menopause.
- HT decreases the incidence of all osteoporosis-related fractures, including vertebral and hip fractures, even in patients at low risk for fractures.
- HT is indicated for the prevention of bone loss in women with premature menopause and secondary amenorrhea.
- HT is indicated in postmenopausal women in the age group 50–60 years presenting with a risk for fracture.
- Potential adverse effects of HT can be limited by using lower than standard doses or by avoiding oral administration, without compromising the beneficial effect of HT on bone.
- The protective effect of HT on bone mineral density is lost after cessation of therapy at an unpredictable rate. Although some degree of fracture protection may remain after cessation of HT, the patient at risk for fracture should receive additional therapy with proven bone-sparing medication.
- The continuation of HT after the age of 60 for the sole purpose of the prevention of fractures should take into account the possible side-effects in the individual of the specific dose and method of administration of HT, compared to other proven therapies.
- The initiation of HT for the sole purpose of the prevention of fractures is not recommended after the age of 60 years.

Non-hormonal therapy

- *Calcium and vitamin D*: Some studies suggest that the combination of calcium and vitamin D is able to reduce the risk of falling, and decreases the hip fracture risk, provided the dose of vitamin D is more than 700 IU per day. Other data suggest that the efficacy of the combined calcium and vitamin D regimen primarily relies on the component calcium.
- *Bisphosphonates*: With bisphosphonates, bone turnover normalizes within weeks and no further suppression is seen during long-term use with up to 10 years of continuous administration. Both vertebral and non-vertebral antifracture efficacies are detectable after 6 months of treatment. The antifracture efficacy seems to last more than 5 years after cessation of treatment.
- *SERMs*: The selective estrogen receptor modulator (SERM) raloxifene reduces the risk of vertebral fracture in postmenopausal women with or without prevalent vertebral fracture. New SERMs and a SERM/HT combination are in final stages of development.
- *Parathyroid hormone* (PTH) produces a significant reduction in the risk of vertebral and non-vertebral fracture. There is no indication that combining PTH with a bone resorption inhibitor has any additional benefit to giving either drug alone.
- *Strontium ranelate* is a new agent for the treatment of osteoporosis, combining mild decreased bone resorption and maintained bone formation. It significantly decreases the

risk of new vertebral fracture and also reduces the relative risk of non-vertebral fractures.
- *New therapeutic possibilities* such as the humanized monoclonal antibody to the receptor activator of nuclear factor-kappa B (RANK) ligand (RANKL) denosumab are under investigation. First results are promising.

Guidelines on osteoporosis

- Optimal skeletal health is dependent on an appropriate lifelong balance of calcium/vitamin D nutrition and exercise.
- Bone mineral density (BMD) assessment by DXA is the basis for the diagnosis of osteoporosis.
- The various bone assessment techniques, including DXA, ultrasound and computed tomography, offer complementary tools for the assessment of fracture risk, but the most important single measure is total hip DXA.
- Site-specific assessments provide the best guide to future fracture risk at that site.
- A large proportion of fractures occur in individuals who do not have BMD-defined osteoporosis.
- BMD is not a cost-effective population screening tool but is best applied on a selective basis, based on age and other risk factors, some of which have an influence on fracture risk that is independent of BMD.
- The goal of management in osteoporosis is the prevention of fracture.
- Ten-year fracture probability is the most useful estimate for therapeutic intervention.
- Individual fracture probability should be based on the combination of bone mineral density, age and other clinical risk factors.
- Hip fracture is responsible for a large proportion of the financial burden of osteoporosis to health-care systems but other osteoporosis-related fractures, particularly vertebral fractures, cause considerable morbidity which can be long-standing.
- Choice of therapy should be based on a balance of effectiveness, risk and cost.

SKIN, CARTILAGE AND OTHER CONNECTIVE TISSUES

Skin, the carotid artery and intervertebral discs

- Menopause inflicts a negative effect on the connective tissue in the dermis of the skin. Such an effect is prevented and in some cases reversed with estrogen therapy.
- Similar changes in connective tissue are observed at the arterial media layer.
- Apart from its positive effect on the bone, it was recently found that estrogen induces favorable changes at the intervertebral discs. The menopause, on the other hand, has a negative effect on discs, just like in bone.
- Estrogen deprivation, on the one hand, and estrogen therapy, on the other, probably leads to changes in the connective tissue matrix at many other sites and organs in addition to the aforementioned tissues.

Articulated joints and the menopause

- The marked predominance of polyarticular osteoarthritis in women and, in particular, the marked increase of osteoarthritis in women after the menopause both point to a likely involvement of female sex steroids in the maintenance of cartilage homeostasis.
- In postmenopausal women treated with levormeloxifene, the urinary excretion of C-telopeptide of type II (CTX-II), a biomarker for cartilage turnover, is decreased by approximately 50%. CTX-II levels are restored to the premenopausal range. Bone resorption is similarly restored to premenopausal levels.
- Timely initiated estrogen/SERM treatment can effectively prevent both bone and cartilage loss accompanying the menopause, involving both direct and indirect mechanisms.

CARDIOVASCULAR PROBLEMS

Gender-specific characteristics of atherosclerosis in menopausal women

- The clinical course of cardiovascular disease has gender-specific characteristics.
- Menopause may be considered a risk factor for coronary artery disease in women due to the potential effects of ovarian senescence on cardiac function, blood pressure and various metabolic parameters (glucose tolerance, lipid profile).
- Arterial hypertension and diabetes are more important cardiovascular risk factors in women than in men.
- Preventative strategies should be focused in women on reducing blood pressure and controlling weight and glucose metabolism.
- Women often have angina with normal coronary arteries but, when they develop an infarct, their prognosis is significantly worse than that of men.

Postmenopausal hormones and coronary artery disease

- The majority of preclinical data and observational studies support the potential HT benefits in reducing risk of coronary artery disease (CAD).
- Randomized controlled trials (RCTs) reported mixed results. RCTs exploring possible association between cardioprotection and hormone use mainly included women with known CAD or potentially having subclinical atherosclerosis. Those RCTs had insufficient power to assess the effects of hormones on coronary risk in younger symptomatic women initiating therapy at the onset of menopause.
- In both the randomized and observational WHI hormonal trials, although the overall data were not significant for benefit or harm, over the course of the studies there was a significant trend for decreasing coronary disease with time.
- Patient selection and timing of initiation may explain these apparently conflicting results. Evidence from the major randomized and observational trials point to the importance of age at initiation of hormone use. A coronary benefit has been shown to be confined to women <10 years from the onset of menopause.
- Initiation of hormone therapy has been related to more coronary events (termed 'early harm') during the first year of use. However, this increased risk appears to be applicable only to elderly women with prevalent coronary disease.
- Emerging data strongly suggest a possible coronary benefit in younger healthy women who also do not experience early harm. These suggestions remain to be confirmed in prospective trials.
- Whether there is a difference in the coronary effects of added progestogen compared to estrogen alone has not been established, but effects may be more favorable with the use of estrogen alone.
- Based on currently available evidence, it is clear that hormonal therapy has no place in the treatment of older women with coronary disease, and this includes recent data on raloxifene.

Menopause and stroke: special features and impact of HT

- Although both CAD and stroke are arterial vascular diseases, the effects of postmenopausal hormones on those very common conditions are not necessarily similar.
- Hormone therapy has been listed as a risk factor for stroke, although the data in menopausal women are not consistent. The existence of hypertension was shown to significantly increase the risk.
- The increased risk for ischemic stroke in the WHI population was in the order of 1 additional case/1000 women-years, which makes it by definition a rare event. However, the risk was not increased in the 50–59-year-old age group, which is in keeping with data from recent observational trials in younger, normotensive cohorts.
- Subanalyses of observational cohorts suggested the risk to be less with lower doses of estrogen, particularly when lower doses are prescribed soon after menopause. In addition, the risk is possibly even lower with non-oral therapy.
- There is a body of evidence from basic science studies which reaffirms the neuronal and stroke-protective effects of estrogen. Thus, the discrepancy between these data and clinical data showing no benefit or increased risk of stroke remains to be explained.
- Data on progestogen use versus unopposed estrogen use have not been consistent.

COAGULATION

Venous thromboembolism safety

- Venous thromboembolism (VTE) is one of the major adverse events during HT. The risk increases with age and body mass index, but is also particularly increased during the first years of therapy.
- Oral estrogens reduce fibrinolysis.
- Non-oral estrogens, by avoiding the hepatic first-pass effect, have minimal effect on coagulation, and may be preferable for those at increased risk of VTE.
- Specific progestogens are capable of reducing the impact of oral estrogens on anticoagulant factors.
- Population screening for thrombophilia is not indicated. Selective screening can be indicated on the basis of personal and familial history.

Arterial disease safety

- HT induces both pro-inflammatory (liver biomarkers) and anti-inflammatory (vascular

biomarkers) effects. Modification of inflammation in either direction can be good or bad for arterial disease depending upon the individual status of inflammation in the vascular wall.
- The liver-derived pro-inflammatory effects of estrogen may be avoided by a non-oral route of administration. Using low doses of oral estrogen potentially decreases these changes, but the dose-response curve is close to the dose-response curve for efficacy.
- Limitation of vascular anti-inflammatory effects is a target not yet achieved. Both oral and non-oral HT exhibit vascular-derived anti-inflammatory actions, although possibly with different magnitude.
- There is limited evidence that different progestogens modulate liver and vascular inflammatory effects.

CENTRAL NERVOUS SYSTEM/ PSYCHIATRY

Cognition and cognitive aging

- For younger women, observational evidence suggests no substantial cognitive sequelae from the natural menopausal transition; limited evidence from clinical trials suggests that HT has no substantial cognitive effect after natural menopause, at least in the short term.
- For younger women, there is limited evidence from clinical trials that estrogen therapy may be of short-term cognitive benefit in the setting of surgical menopause.
- For older women, HT started in the late postmenopause probably does not have a substantial impact on cognitive abilities.
- The long-term cognitive consequences of HT initiated during the menopausal transition or early postmenopause are unknown. The need for further research in this area is urgent.

Alzheimer's disease and other neurological disorders

- During development and adulthood, the human brain is a target for estrogen and other steroid hormones. Estrogen influences neural function and neurological disease directly, through effects on neurons and glia, and indirectly, through effects on the cerebral vasculature and immune system.
- With menopause, the cessation of ovarian estrogen production and the initiation of HT have the potential to influence processes in the central nervous system relevant to a variety of neurological disorders.
- For women with Alzheimer's disease (AD), limited evidence from clinical trials indicates that HT does not improve symptoms or slow disease progression.
- There is limited evidence from clinical trials that HT increases dementia risk when initiated after the age of 64.
- Observational evidence implies that HT used by younger women around the time of menopause is associated with lower risk of AD. However, findings may be biased, and further research is needed to determine whether there might exist an early window during which the effects of HT on AD risk are beneficial rather than harmful.
- Potential effects of HT on the incidence or symptoms of Parkinson's disease are largely unknown.
- Based on evidence from a single clinical trial, combined HT may increase seizure frequency in postmenopausal women with epilepsy.

Estrogen: effects on normal brain function, and neuropsychiatric disorders

- Many women complain of memory and other cognitive/emotional difficulties at times that are associated with changes in estrogen levels.
- However, the biological mechanisms through which estrogen may exert these effects remain poorly understood. Also, the effect of estrogen treatment on cognition and brain function in healthy women and those with Alzheimer's disease is controversial.
- There is evidence that, in healthy women, estrogen affects the dopaminergic, serotoninergic and cholinergic systems, and brain regions crucial to higher cognitive function and mood.
- New results from recent *in vivo* randomized, controlled neuroimaging experiments demonstrate that, in young females and those in midlife:
  - brain function is modulated by normal variation in ovarian function;
  - acute loss of ovarian hormones increases neuronal membrane breakdown;
  - acute suppression of ovarian function is associated with reduced activation of brain regions critical to memory.

ONCOLOGY

Breast cancer prevention

- The lobular breast attains its maximum development during pregnancy and lactation (Lob. 4). After menopause, mammary lobules in both nulliparous and parous women regress to structures designated Lob. 1.
- Undifferentiated lobules (Lob. 1) in the breast of nulliparous women retain a high concentration of epithelial cells (Stem cells 1) that are targets for carcinogens and therefore susceptible to undergo neoplastic transformation.
- Early first full-term pregnancy imprints in the breast epithelial cells a genomic signature (Stem cells 2), making them refractory to transformation.
- The Stem cell 2 contains specific genes controlling transcription, RNA processing, immune response, apoptosis, DNA repair and DNA recombination. Their coded proteins may serve as biomarkers for breast cancer protection.
- Clinical studies are under way to induce in the human breast a genomic signature of Stem cell 2 and thereby confer cancer protection. This concept may pave the way to long-term oncological prevention.

Hormone therapy and breast cancer

- Estrogen associated with breast cancer development is not circulating estrogen but rather that produced locally within the breast.
- Excessive formation of catechol estrogen quinones initiates a series of events leading to breast cancer, by reacting with DNA. Endogenous estrogen is detrimental primarily in those women with genetic susceptibility.
- The WHI study demonstrated that 7.1 years of treatment with estrogen only did not increase the risk of breast cancer in hysterectomized women. The prospective cohort in the Nurses' Health Study also reported that unopposed estrogen did not increase the risk of breast cancer until after 15 years of estrogen exposure.
- Data from the estrogen plus progestogen arm of the WHI showed an increase in breast cancer risk at an average follow-up of 5.6 years. However, women who had not used HT prior to the study were not at a higher risk for breast cancer for up to 7 years after initiation of therapy.
- Micronized progesterone or dydrogesterone used in association with oral or percutaneous estradiol may be associated with no increase in risk or lower risk than use of synthetic progestogens for at least 4 years, and perhaps even 8 years, of treatment.
- The risk of breast cancer decreases rapidly after cessation of HT; by 5 years, the risk may not be greater than that in women without any history of exposure.

Endometrial safety, bleeding, hormone therapy and endometrium

- Progestogen prevents the endometrial proliferation of estrogen.
- Continuous combined regimens are associated with a lower risk of endometrial cancer than in the untreated population.
- New lower-dose regimens cause less endometrial stimulation and less bleeding.
- Intrauterine delivery of progestogen is a suitable and logical route of administration.
- The protective effect of progestogen on the endometrium has to be balanced against the apparent adverse effect on breast cancer risk.
- Data on the effect of tibolone on the endometrium from randomized, controlled trials suggest a similar effect to continuous combined regimens.

NEW ATTITUDES TO SEXUALITY AND QUALITY OF LIFE IN THE MENOPAUSE

Clinical evaluation/diagnosis

- A complex interplay of biological, psychological and socio-relational factors determines women's sexual health. This may negatively affect the entire sexual response cycle, inducing significant changes in desire, arousal, orgasm and satisfaction at menopause and beyond.
- Both age and declining sex hormones have detrimental effects on sexual functioning, with a significant increase in vaginal dryness/dyspareunia and a significant decrease in desire and sexual responsiveness.
- Desire difficulty is the most common sexual complaint experienced by women and the proportion of women with low desire increases with age. However, there are age-related changes in sexually related personal distress, which are especially evident in surgically menopausal women. These women are at increased risk for hypoactive sexual desire disorder.

- Women may not be willing to initiate a conversation on sexual interest, behavior and activity themselves, but they usually appreciate being questioned by doctors.
- Validated tools (self-administered questionnaires/daily diaries and event logs/semi-structured interviews) may be used properly to diagnose sexual symptoms and to gain information on sexual constructs and relationships, while hormonal assays are presently not considered a standard of routine practice.
- An accurate sexual history and a focused clinical evaluation may help clinicians in the management of sexual symptoms that are causing significant distress. Hormonal and non-hormonal treatments, and/or psychosexual strategies should be individualized and tailored according to a woman's history and current needs.

Menopause and aging, quality of life and sexuality

- Healthy status represents a major determinant of quality of life, particularly in elderly people, but sexuality is an important factor at all ages as well.
- Sexuality is less often a problem for women than for men.
- Hormonal changes which are associated with aging or menopause may deeply affect quality of life.
- Therapeutic interventions such as hormonal or non-hormonal treatments, targeting selected diseases or components of the aging process, may improve quality of life and sexuality in both sexes.

NEW HORMONAL THERAPIES AND REGIMENS

Regulatory authorities' statements

- Regulatory Authorities are constituted in the interests of Public Health and not the individual patient.
- The composition of the committees is of paramount importance in determining outcome.
- Publications since 2003 have not impacted much on either Recommendations or information published.
- Further review by an independent organization is to be recommended.

New products and regimens since 2003

- New products are being developed which maintain benefits and minimize risks. However, some useful products have been withdrawn by pharma companies through profitability decisions.
- New ultra-low-dose oral preparations appear to maintain benefits for symptom relief and osteoporosis whilst minimizing side-effects and risks.
- New progestogens can minimize progestogenic side-effects through anti-androgenic and anti-mineralocorticoid effects, e.g. drospirenone.
- Endometrial protection may not be needed with the ultra-low-dose transdermal system (14 μg/day).
- A new female androgen patch will be licensed for treatment of female androgen deficiency causing distress (hypoactive sexual desire disorder).
- A non-hormonal option, selective noradrenaline reuptake inhibitor, for vasomotor symptom management is currently in development in phase III clinical trials.
- A SERM/estrogen therapy combination is in phase III clinical trials and showing encouraging data for efficacy/risks.

Route of administration and timing of initiation

- Non-oral estradiol and progestogens circumvent the first-pass metabolism and therefore have the potential for a lesser stimulation of the liver proteins and a relevant neutral metabolic profile, which might be more favorable in terms of cardiovascular and venous thromboembolism risk.
- The risk of venous thromboembolism has been shown to differ significantly when transdermal estradiol was compared with oral estradiol. However, whether this is related to a differential impact of estradiol on clotting factors synthesized in the liver has not been confirmed.
- It is assumed that lower circulating levels of progestogens will have less negative impact on breast cancer risk, if any.
- First uterine pass of vaginal delivery of progestogens leads to adequate local concentrations and good endometrial protection, but with very low systemic progestogen levels.
- Combination of non-oral administration of estradiol and direct intrauterine delivery

of progestogen may improve compliance and minimize the risks of hormone replacement. However, long-term, good-quality studies to confirm this hypothesis are still needed.
- Further analysis of randomized, controlled and prospective studies indicates that early administration of hormone therapy in younger postmenopausal women can afford protection against cardiovascular disease, while initiation of therapy at an older age, after 10 years without endogenous estrogen, is harmful.

Androgens

- Androgen production is usually preserved in the menopause, and therefore, postmenopausal women usually do not suffer from androgen deficiency and do not require routine androgen replacement.
- The definition of female androgen deficiency is not precise enough and may lead to overdiagnosis.
- Androgen replacement in healthy postmenopausal women has shown no beneficial effect in published trials and currently cannot be recommended.
- Androgen replacement should be reserved for women with severe androgen deficiency due to an established cause and matching clinical signs and symptoms.
- Randomized, controlled trials on androgen replacement in women with bilateral oophorectomy or adrenal failure have shown significant beneficial effects, in particular on health-related quality of life and sexual function, which were impaired at baseline in the participating cohorts.

Non-estrogenic approaches to the management of menopausal symptoms

- Lifestyle and diet modifications may improve both hot flushes and mood. Regular exercise, weight reduction, avoiding too much caffeine and cessation of smoking appear to improve hot flushes. Relaxation techniques, meditation, and pace respiration may help too, although there is relatively very little support for that effect from good clinical trials.
- Plant-derived compounds (i.e. isoflavones, evening primrose, black cohosh and ginseng) are very popular as remedies for vasomotor symptoms, sleep disturbances and bad mood. Although some studies found those products to be helpful, the magnitude of the effect – when present – is small and not much greater than that of placebo.
- The most tested pharmacological alternatives to estrogens are serotonin reuptake inhibitors (SSRI). At their best, SSRIs reduce hot flushes by 50–60% and their effect appears only short-term. The more positive results were seen in breast cancer survivors, whereas the chance for negative results was greater in healthy women. SSRIs improve mood independently of their effect on hot flushes. When used for the treatment of the climacteric syndrome, SSRIs do not adversely affect libido. Withdrawal symptoms may occur after long-term use; thus, SSRIs should not be stopped abruptly.

GENE FINGERPRINTING – ITS ROLE IN HT

- Genetic variability may be important in determining efficacy of therapy as well as susceptibility for adverse events. Clinical implications of genomic medicine have been investigated also in menopause medicine.
- Enzyme activity may depend on single nucleotide polymorphism. This was demonstrated in regard to steroid synthesizing enzymes and estrogen metabolizing enzymes (i.e. strains of the cytochrome P system).
- Factor 5 and factor 2 polymorphisms are recognized as strong risk predictors for thrombosis in hormone users.

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Suggested Reading

A more comprehensive list of references will be found in a special Supplement to *Climacteric*; this will be published in August 2007 and will include full-length articles written by the speakers at the 7th IMS Workshop in Budapest.

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