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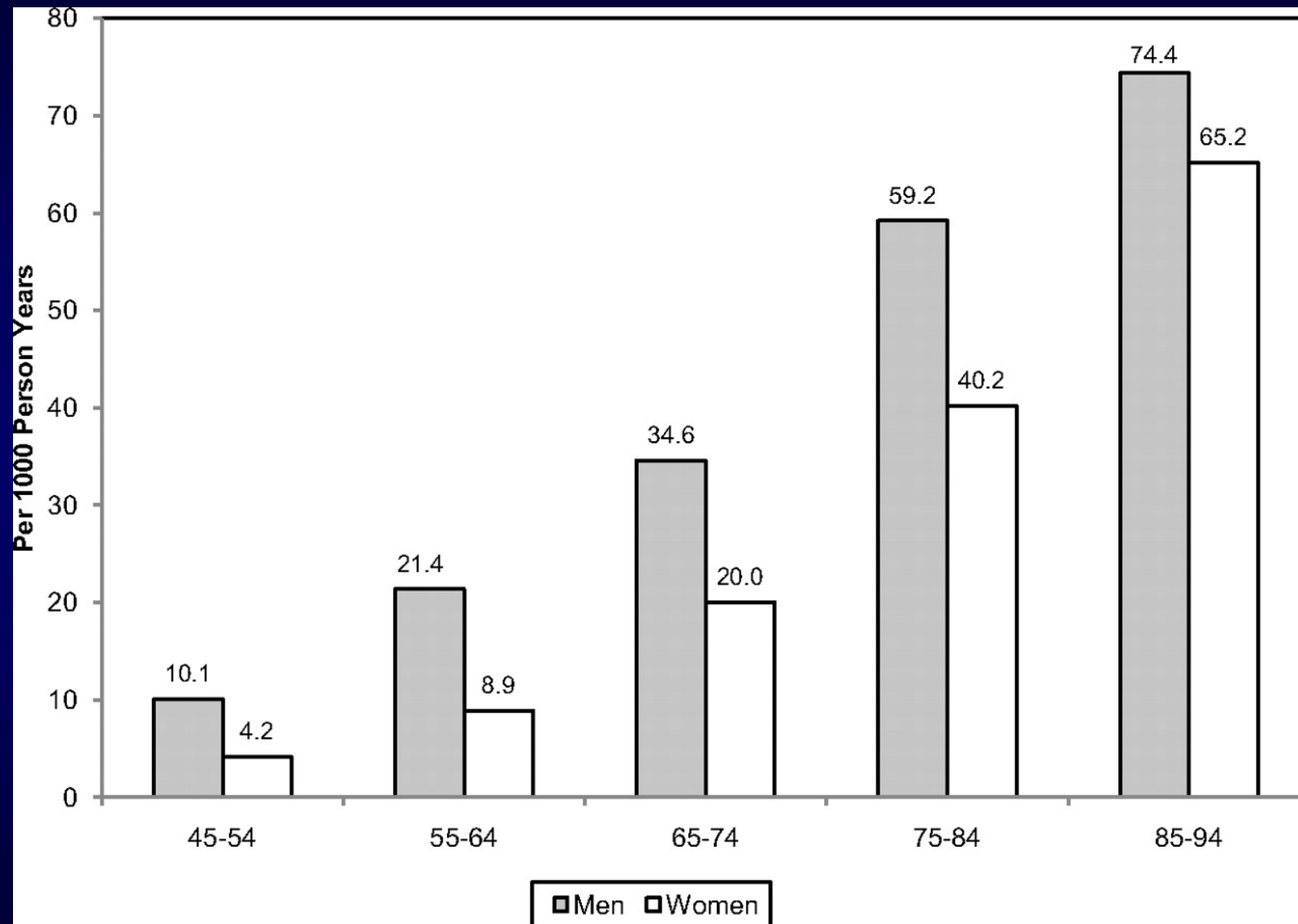
# ***Estrogen signaling and cardiovascular protection: what can we learn after the Women's Health Initiative?***

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**Ana Paula Dantas, PhD**

***Division of Experimental Cardiology  
Institut d'Investigacions Biomèdiques  
August Pi i Sunyer (IDIBAPS)***

**Incidence of cardiovascular disease\* by age and sex (FHS, 1980–2003).**  
**\*Coronary heart disease, heart failure, stroke, or intermittent claudication.**



Roger V L et al. *Circulation* 2011;123:e18-e209

# WHY ARE WOMEN PROTECTED?



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# ***The X chromosome mosaicism theory***

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**According to the heterogametic sex hypothesis, the lack of a second X chromosome in male may lead to lower cardiovascular protection**

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## ***The hormonal theory***

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**Estrogen protects females mammals at a cellular level, so that the incidence and severity of cardiovascular disease in females – whether they are rats, dogs or humans – will generally be lower than in males.**

# The Nurses' Health Study

(Stroke. 1996;27:2020-2025.)

© 1996 American Heart Association, Inc.

## Articles

### Risk Factors for Cerebral Hemorrhage in the Controlled Hypertension

Amanda G. Thrift, PhD; John J. McNeil, PhD; Andrew  
Geoffrey A. Donnan  
Group

the Department of Epidei  
Hospital (A.G.T., J.J.M., A  
Repatriation Hospitals, H

American Family Physician®  
A peer-reviewed journal of the American Academy of Family Physicians

ARTICLE

Coronary

tents

What's Different for

## Journal of Women's Health

### The Nurses' Health Study: 20-Year Contribution to the Understanding of Health Among Women

...nutrition, Vol. 70, No. 3, 412-419, September 1999

Society for Clinical Nutrition

Journal Research Communications

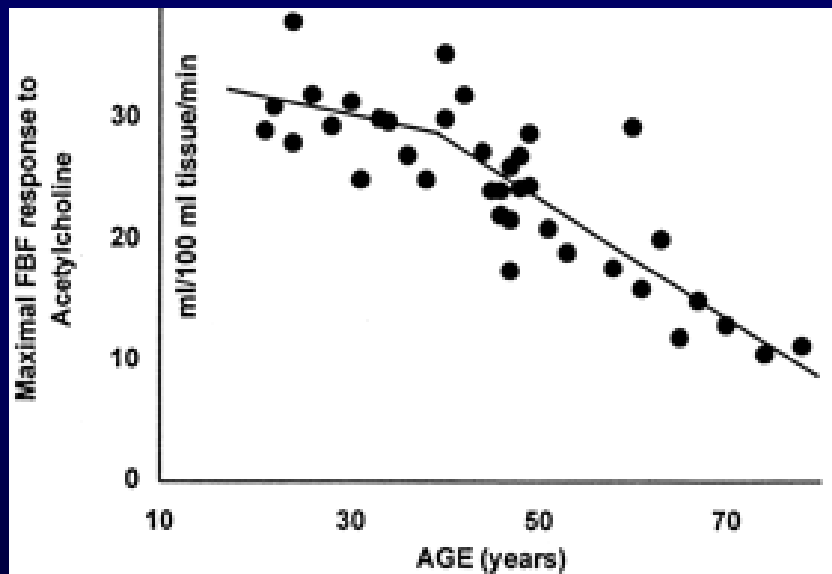
Whole-grain consumption and risk  
of coronary heart disease: results  
from the Nurses' Health Study<sup>1,2,3</sup>

Annals of Intern.

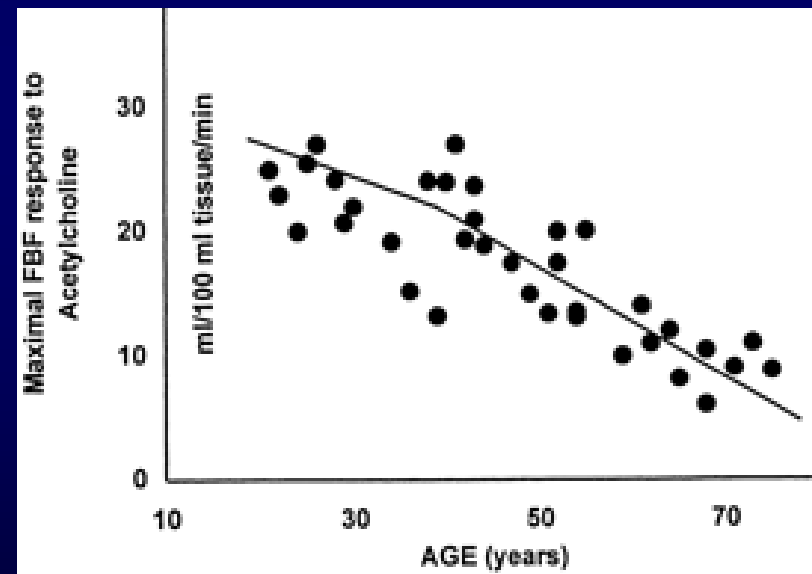
Postmenopausal Hormone Use and  
Events in the Nurses' Health Study  
A Prospective, Observational Study

# Menopause Is Associated With Endothelial Dysfunction in Women

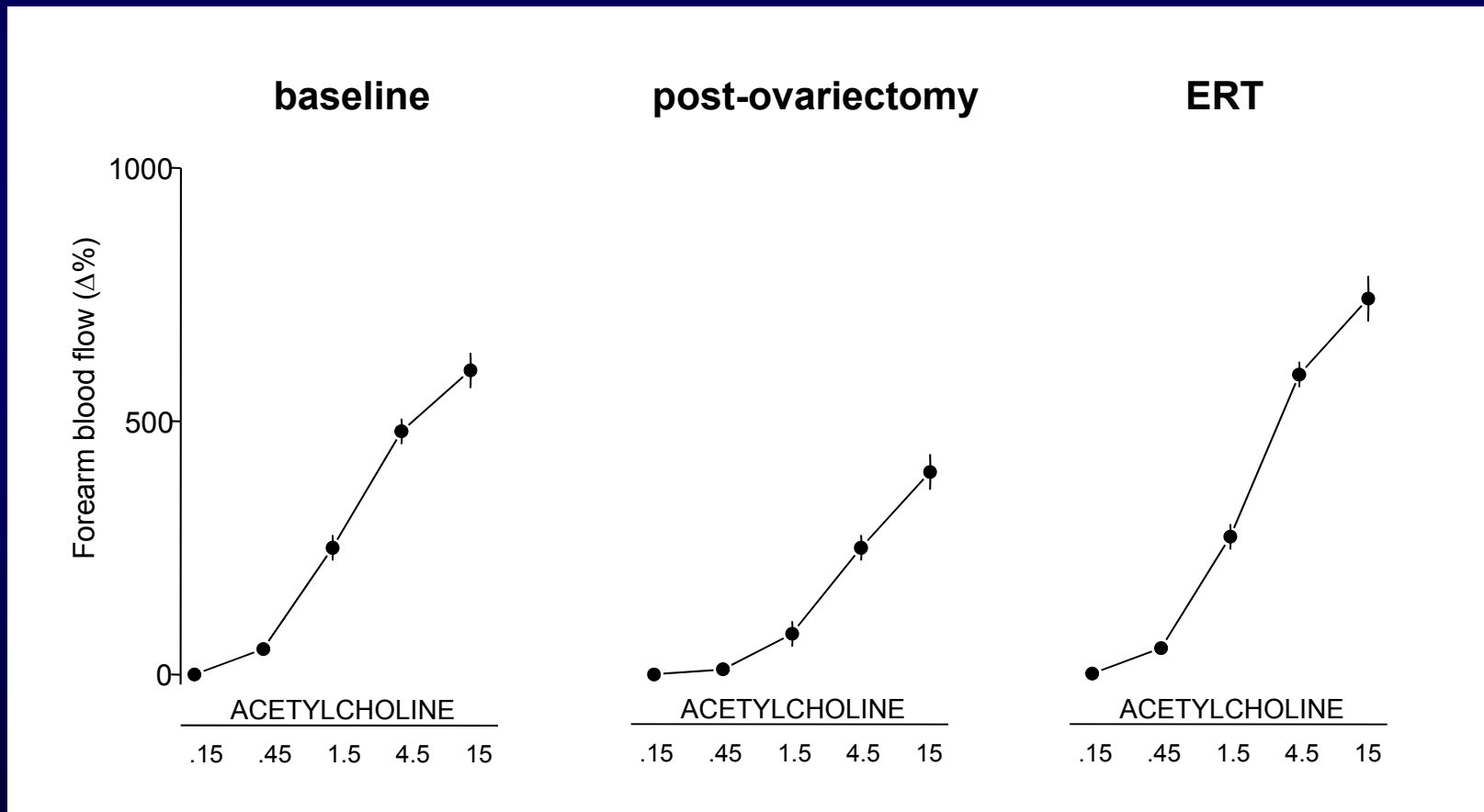
## Hypertensive



## Normotensive

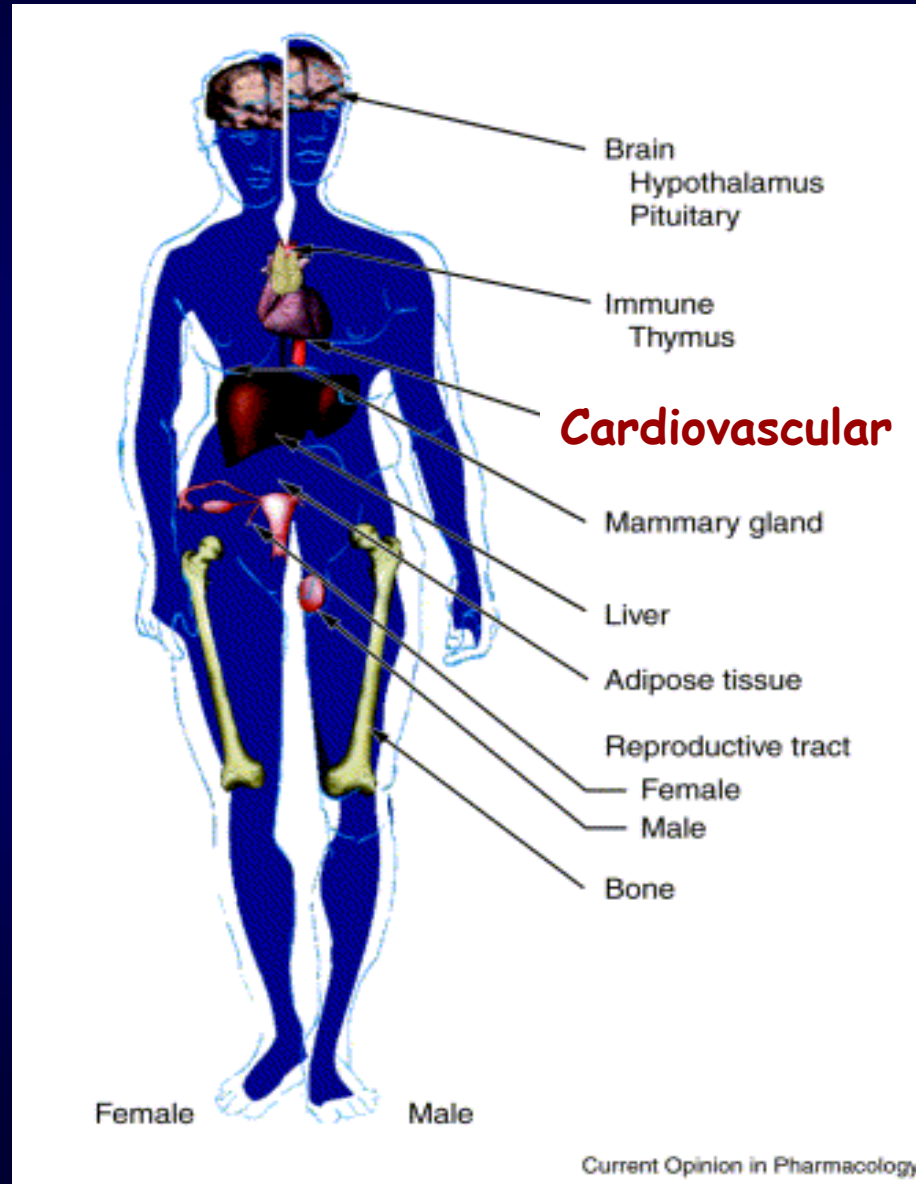


# Ovariectomy also induces hyposensitivity to endothelium-dependent vasodilator ACh in women. Estrogen replacement therapy restored the hyposensitivity to ACh

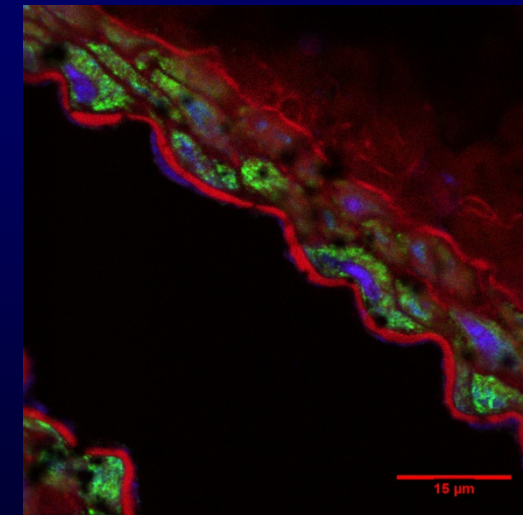
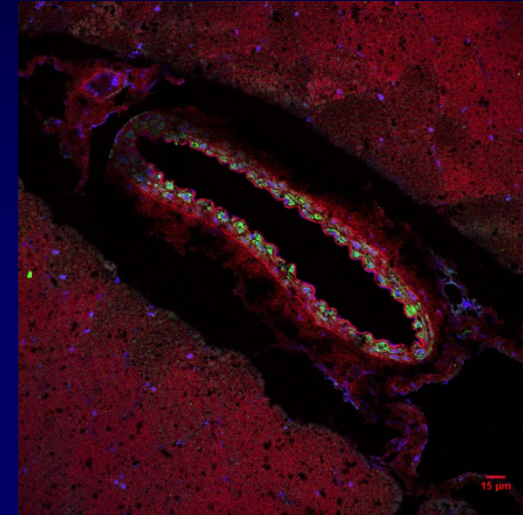




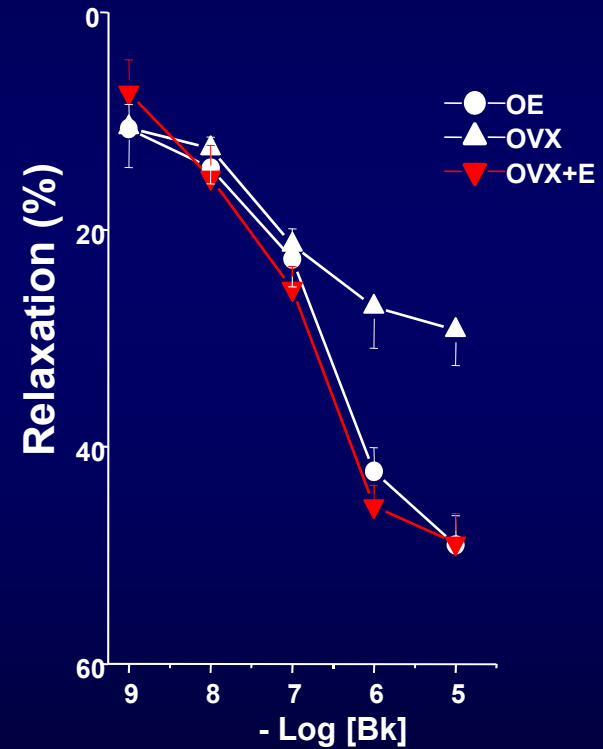
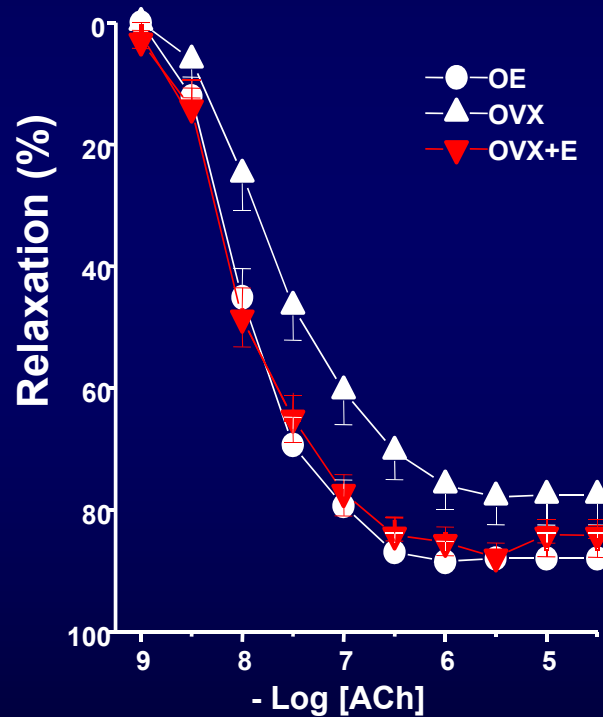
# SITES OF ESTROGEN ACTIONS



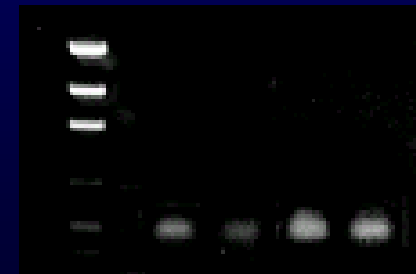
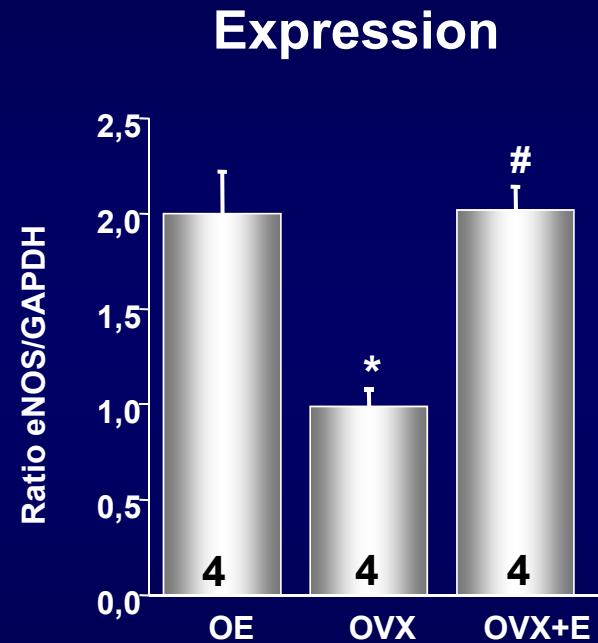
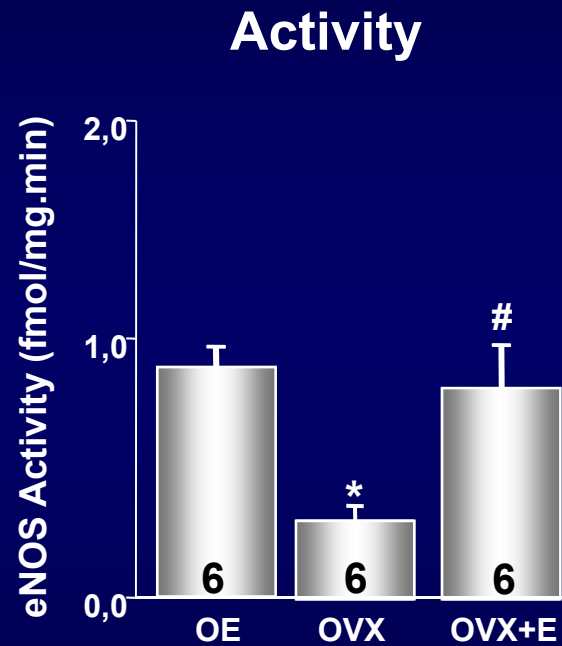
## ER $\alpha$ expression in mice artery



# Estradiol corrects endothelium-dependent relaxation to ACh and Bk in OVX- SHR.



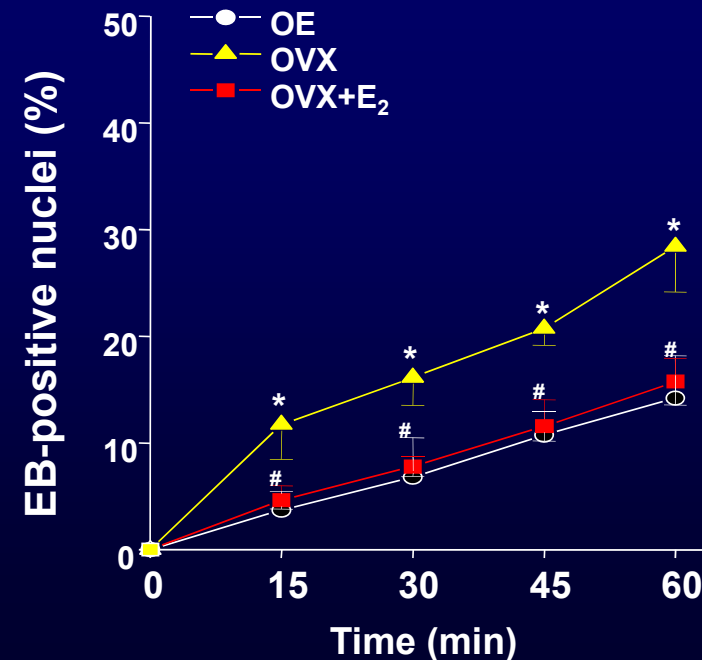
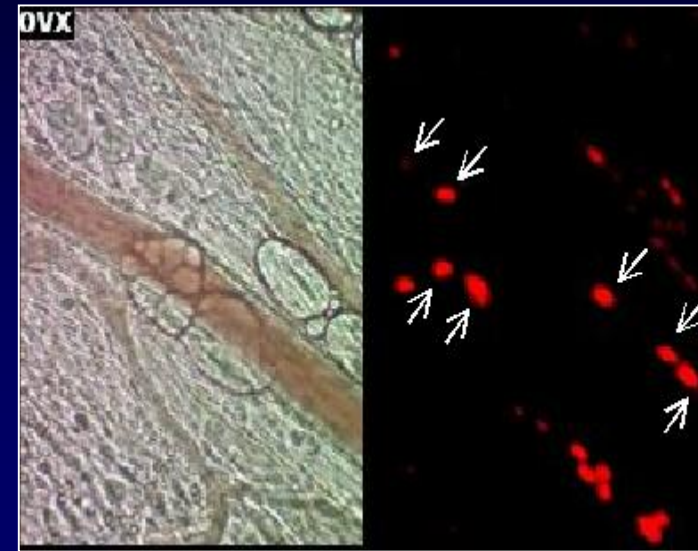
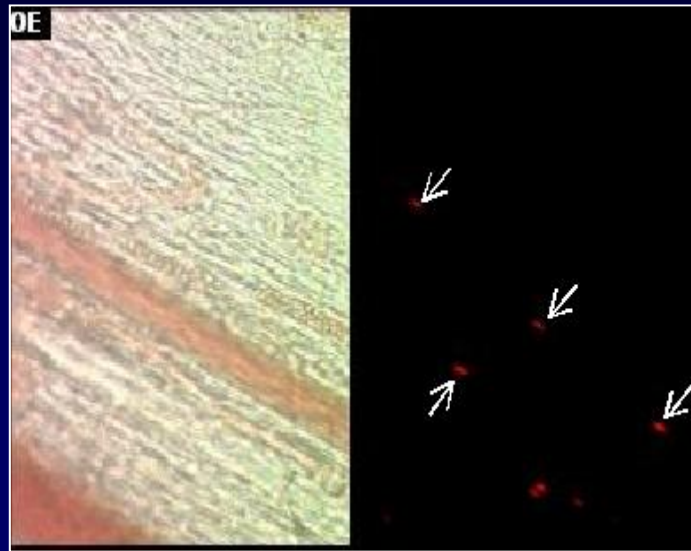
# eNOS activity and gene expression is decreased by ovariectomy in SHR. Estradiol treatment restores eNOS activity/expression

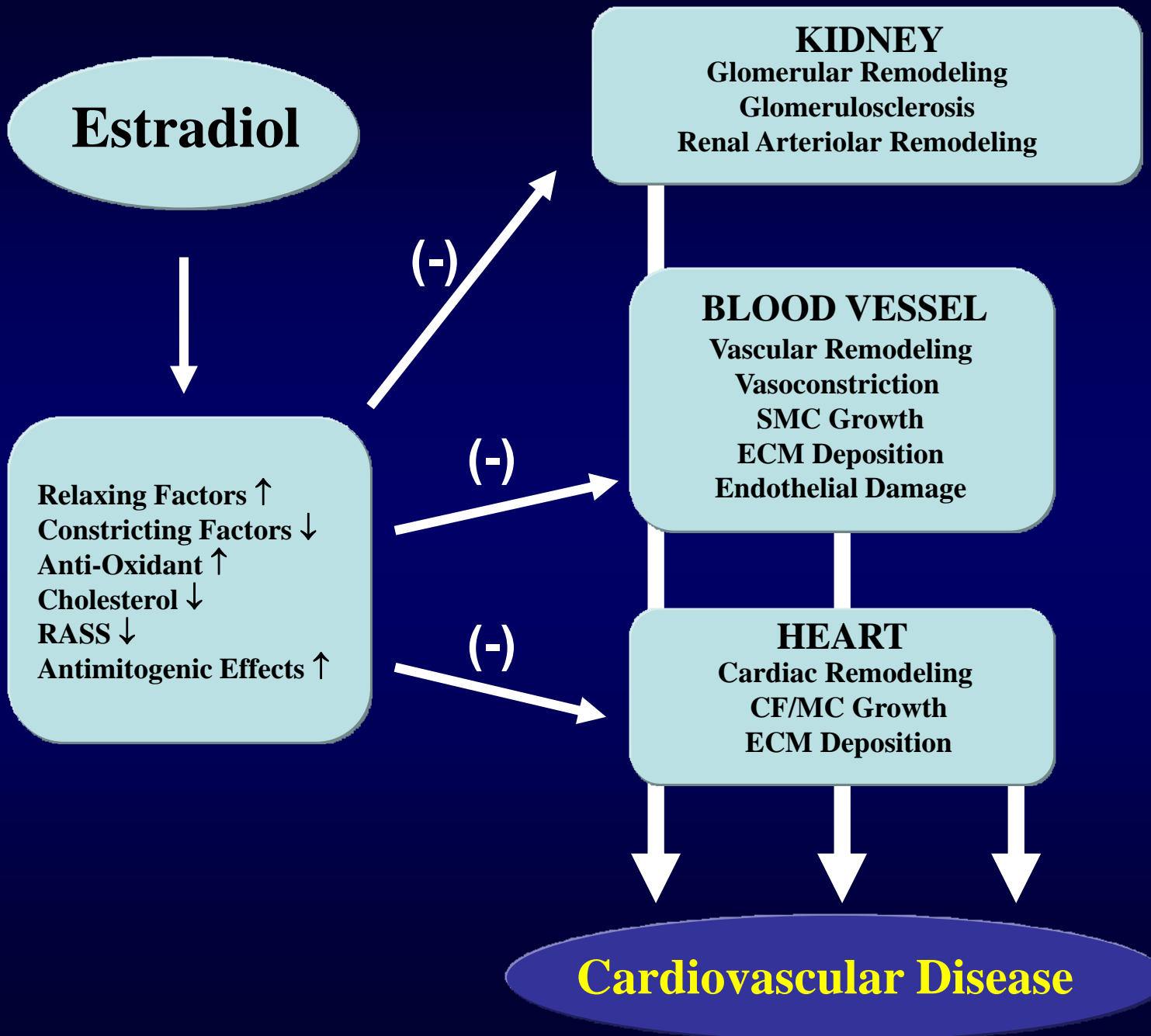


\* p<0.05 vs OE

# p<0.05 vs OVX

# Superoxide generation in SHR arterioles *in vivo in situ*





# Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women

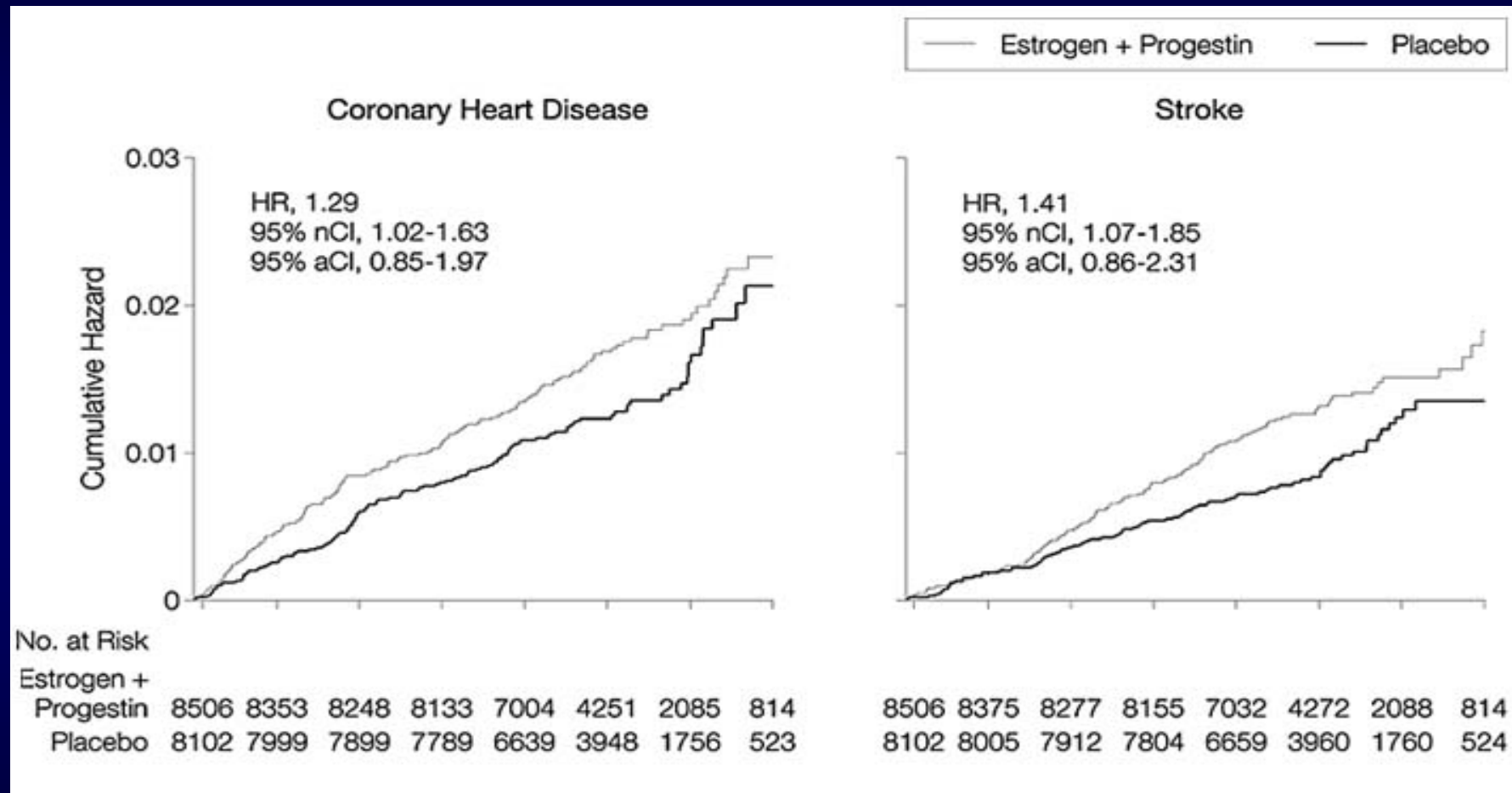
Principal Results From the Women's Health Initiative  
Randomized Controlled Trial

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Writing Group for the  
Women's Health Initiative  
Investigators

JAMA, July 17, 2002 – Vol. 288, No. 3

# Women's Health Initiative (WHI)



# Estrogen Replacement Therapy: before and after the Women's Health Initiative (WHI)

## Before WHI

Estradiol

Relaxing Factors ↑  
Constricting Factors ↓  
Anti-Oxidant ↑  
Cholesterol ↓  
RASS ↓  
Antimitogenic Effects ↑

↓ **Cardiovascular Disease**

## After WHI

Estradiol

Venous thrombo-  
embolism ↑  
Stroke ↑  
Coronary heart  
disease ↑  
Cholesterol ∅

↑ **Cardiovascular Disease**



# WHI?

ORIGINAL CONTRIBUTION

JAMA - EXPRESS

## Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women

Principal Results From the Women's Health Initiative  
Randomized Controlled Trial

Writing Group for the  
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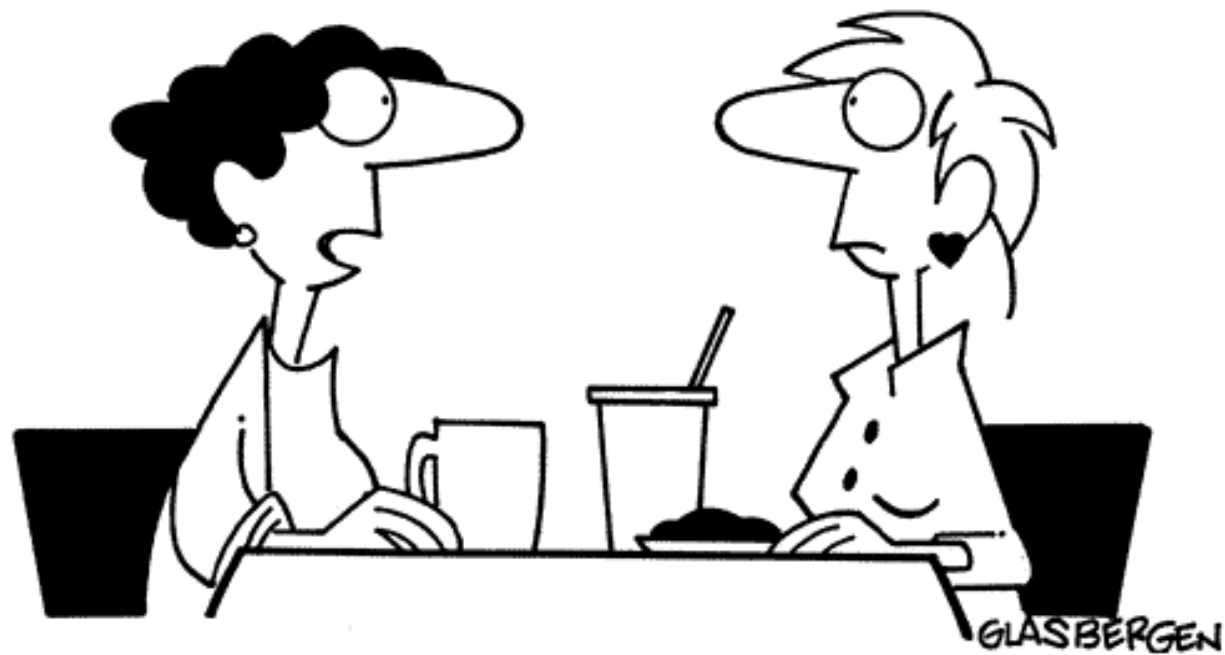
# *WHI?*

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- ✓ **Dose regimen**
- ✓ **Association of estrogen with progestins**
- ✓ **Administration route**
- ✓ **Type of Estrogen**
- ✓ **Average age of women beginning the trial**

# Type of Estrogen

Copyright 2002 by Randy Glasbergen.  
www.glasbergen.com



**“I’m in an experimental program that treats menopause with ostrich hormones. Now I only get hot flashes when I’m laying an egg.”**

# Conjugated Equine Estrogens PREMARIN®

Concentration of estrogen found in Premarin® <http://www.fda.gov/cder/news/celetterjw.htm> and corrected by average of BMI described by the clinical trials HERS, ERA and WHI .

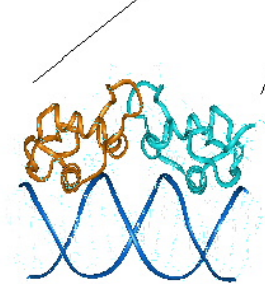
Sodium Estrogen Sulfate	[Estrogen] (mg/tablet of Premarin® 0.625mg)	Estrogen (mg) /BMI (kg/m <sup>2</sup> )
Estrone	0.370	0.013
Equilin	0.168	0.006
17β-Dihydroequilin	0.102	0.004
17α-Estradiol	0.027	9.0 x 10 <sup>-4</sup>
17α-Dihydroequilin	0.011	3.8 x 10 <sup>-4</sup>
Equilenin	0.015	5.2 x 10 <sup>-4</sup>
Δ8,9-dehydroestrone	0.026	9.0 x 10 <sup>-4</sup>

# hypothesis

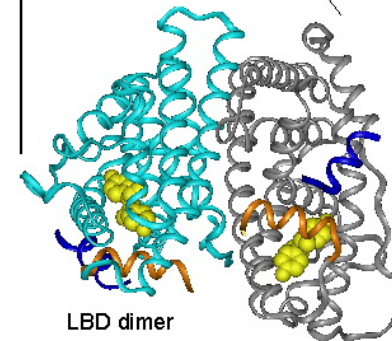
Different estrogenic molecules do not activate transcription of a given gene in the same pharmacological way as  $17\beta$ -Estradiol.

The differential modulation of gene transcription is mostly dependent on how conformational changes of ER allow its binding to DNA and the recruitment of different coregulators molecules rather than ligand binding affinity.

Domains of the hER $\alpha$

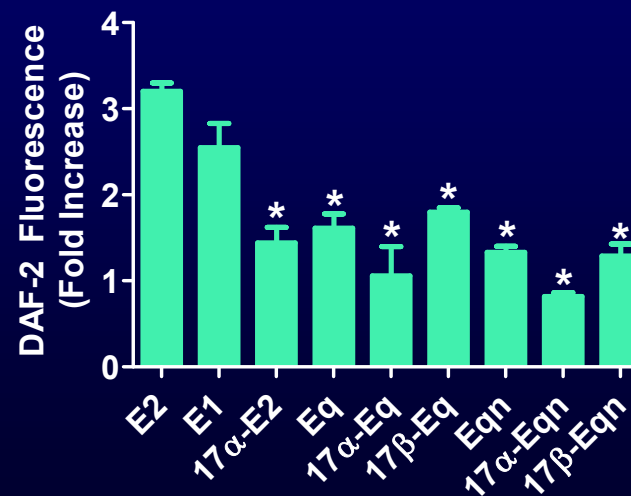
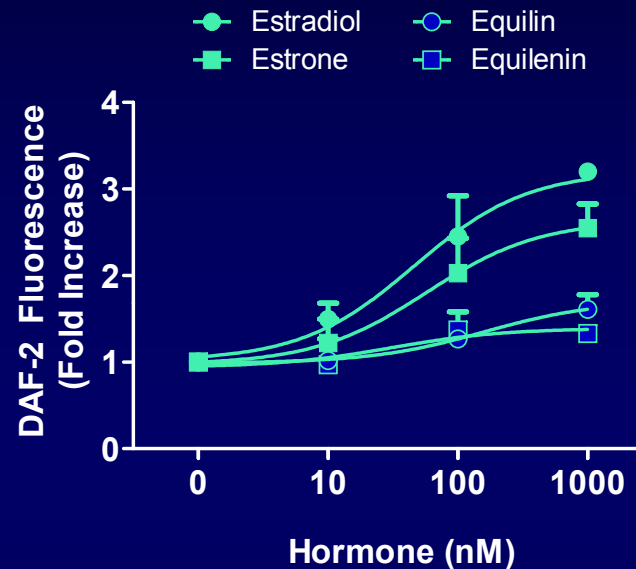
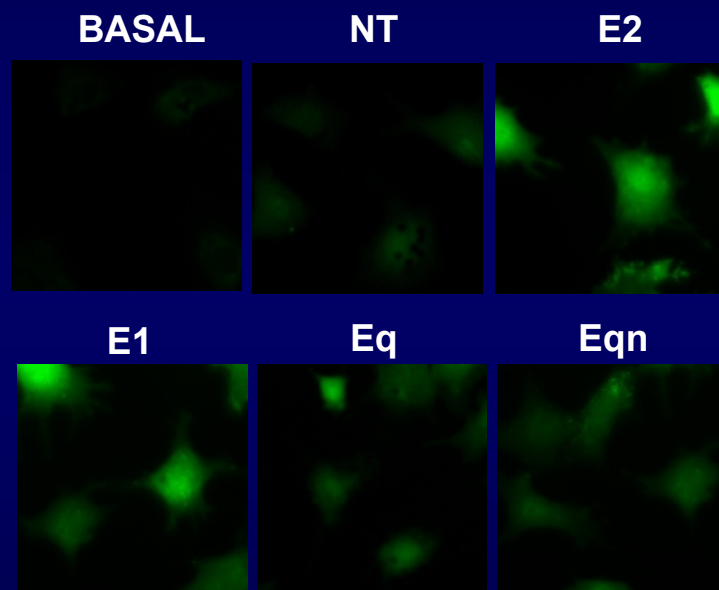


DNA-binding domain dimer  
1HCQ, Schwabe et al

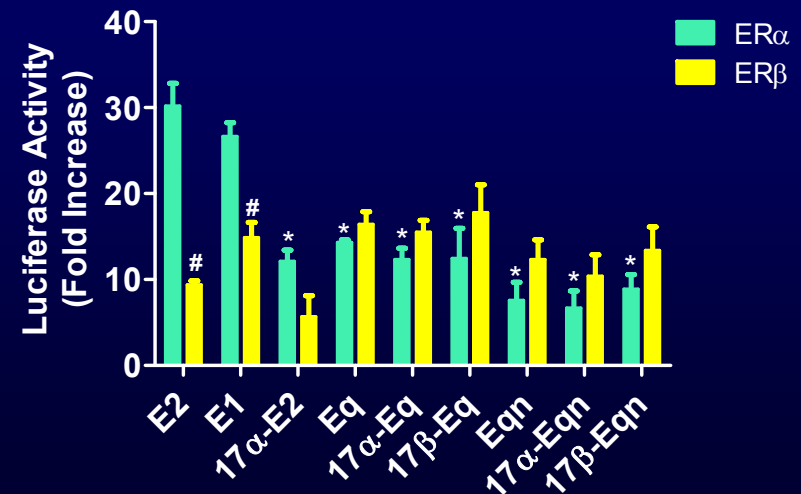
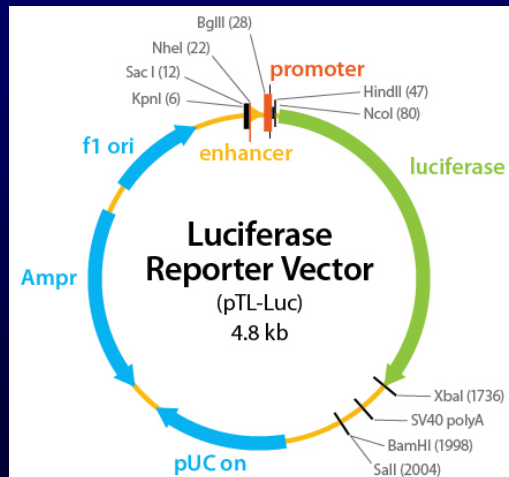
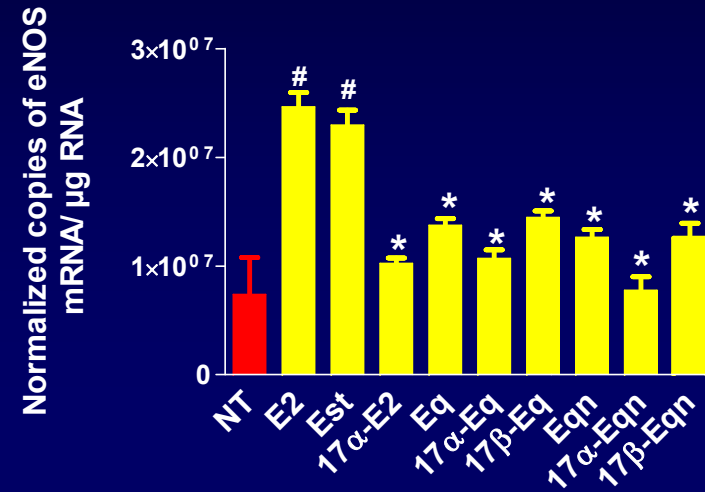
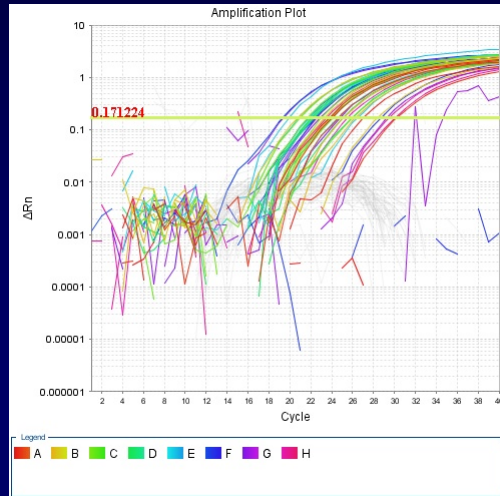


LBD dimer  
3ERD, Shiau et al

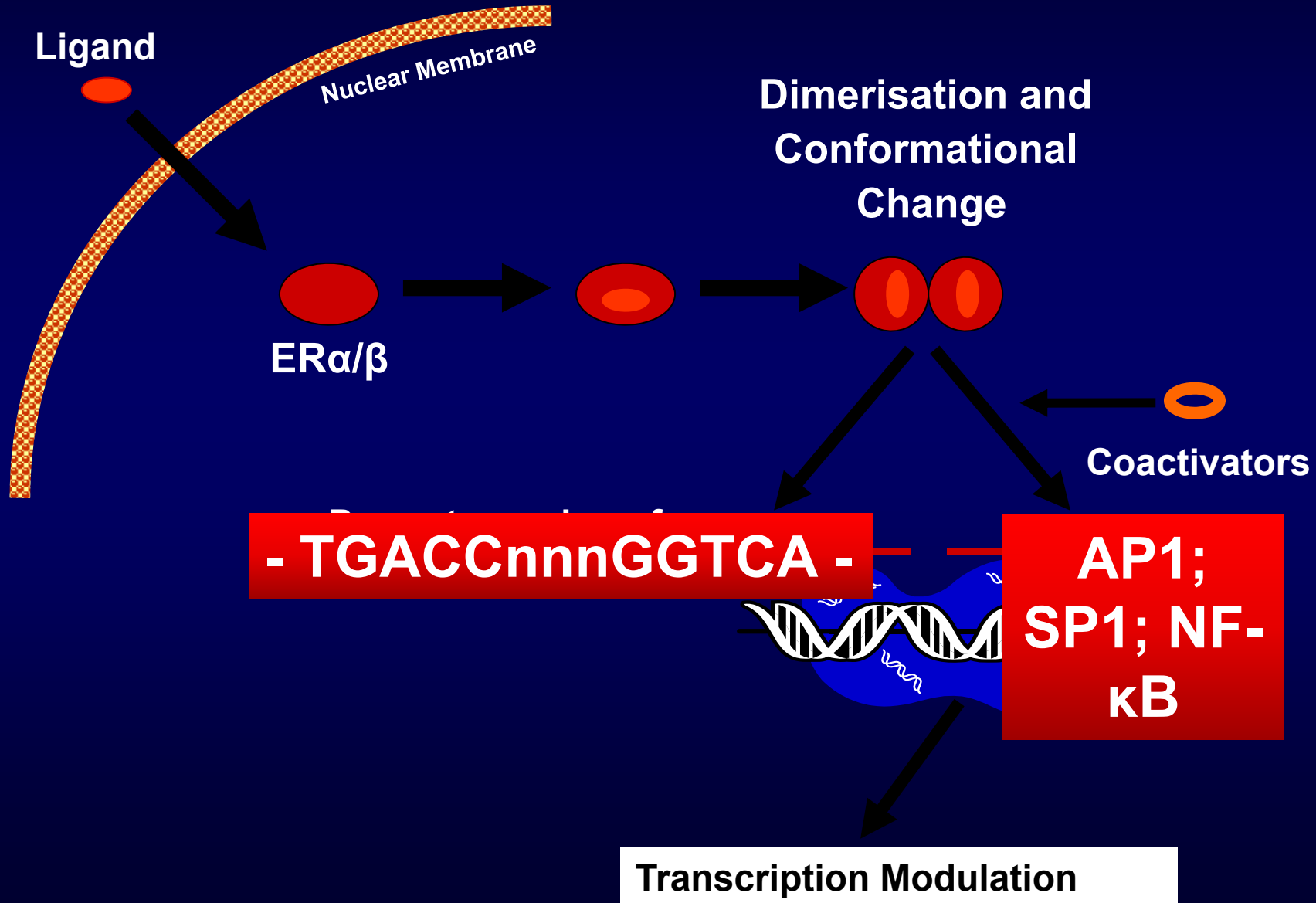
# Estrogen-mediated NO production



# Estrogen effects on eNOS transcription

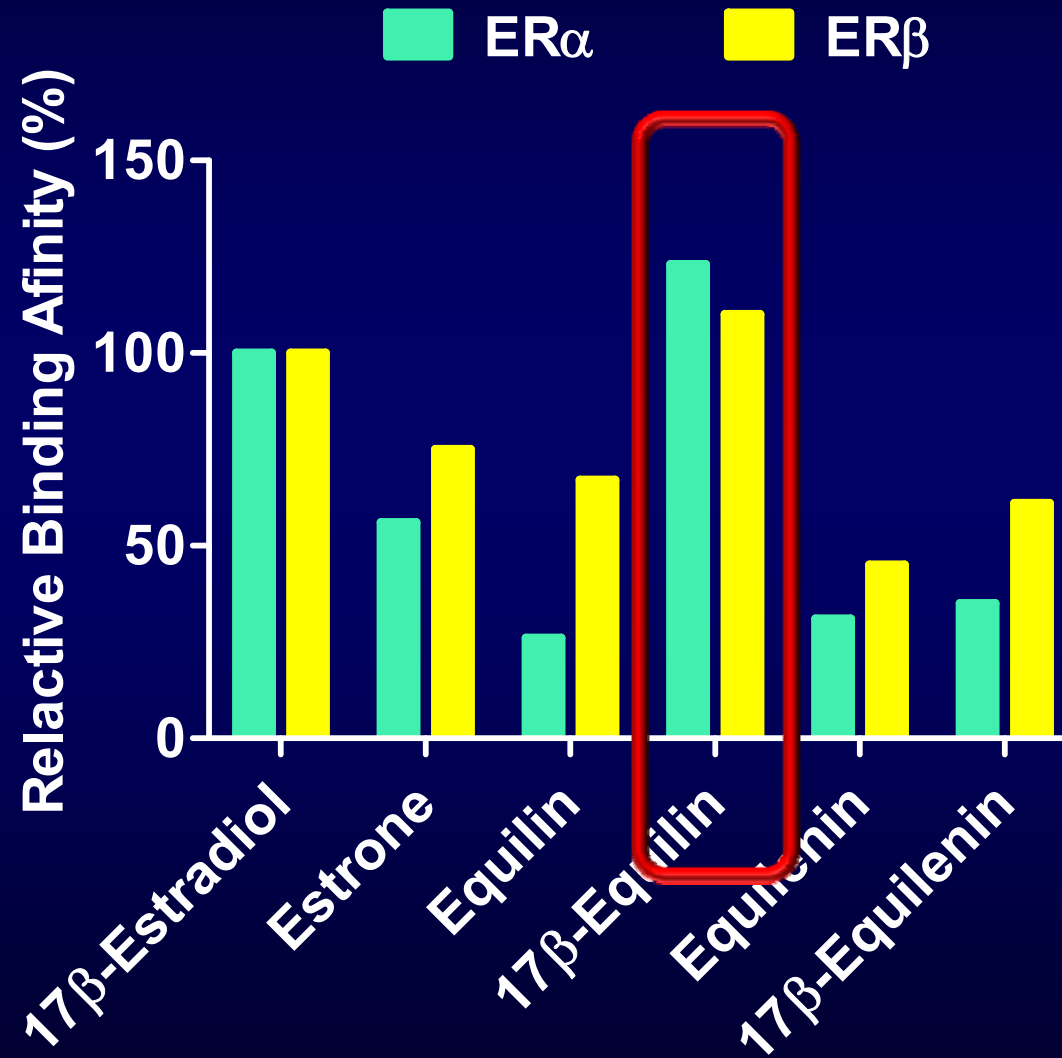


# Estrogen Receptor Signaling

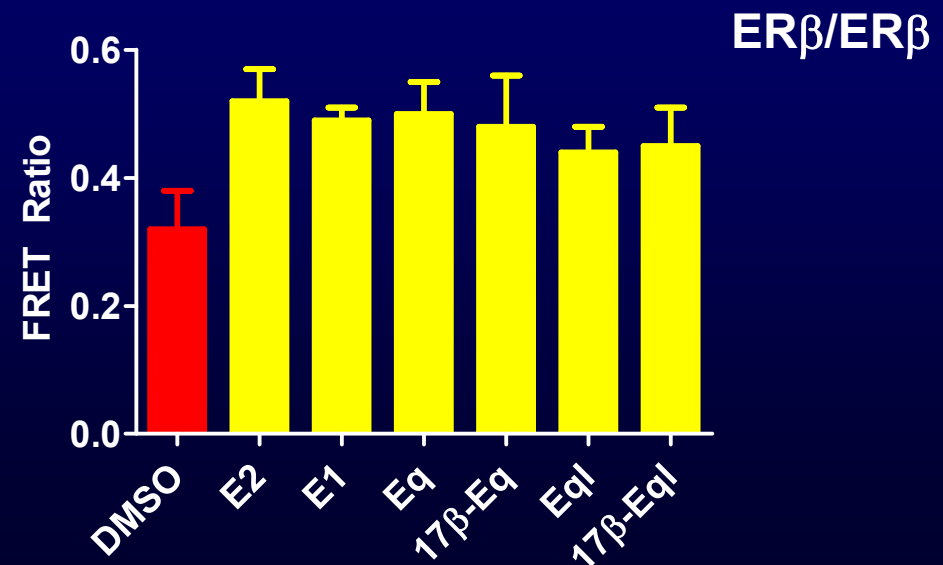
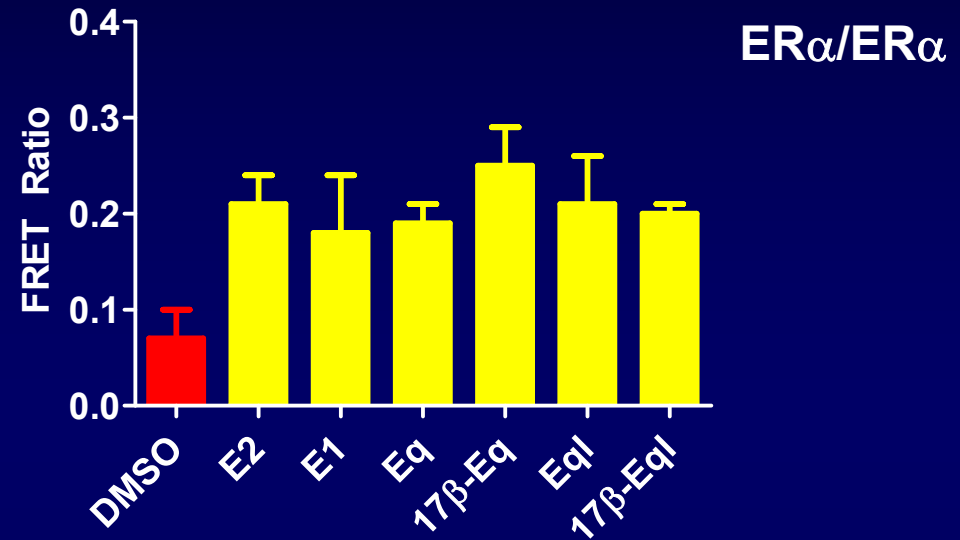
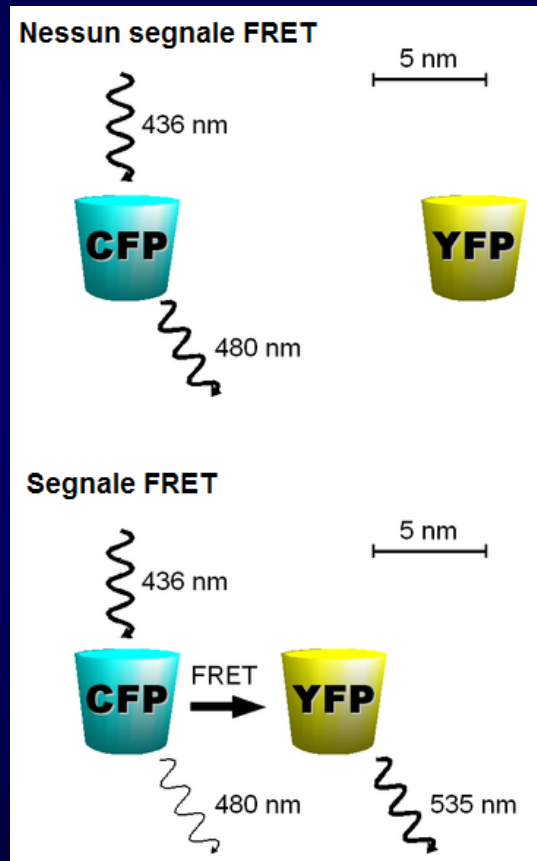




# Relative Binding Affinity



# ER Dimerization – FRET Analysis



## eNOS Promoter

-1600 ATCTGATGCT GCCTGTCACC TTGACCCTGA GGATGCCAGT CACAGCTCCA  
 -1550 TTAAGTGGGA CCTAGGAAAA TGAGTCATCC TTGGTCATGC ACATTTCAA  
 -1500 TGGTGGCTTA ATATGGAAGC CAGACTTGGG TTCTGTTGTC TCCTCCAGCA  
 -1450 TGGTAGAAGA TGCCTGAAAA GTAGGGGCTG GATCCCATCC CCTGCCTCAC  
 -1400 TGGGAAGGCG AGGTGGTGGG GTGGGGTGGG GCCTCAGGCT TGGGGTCATG  
 -1350 GGACAAAGCC CAGGCTGAAT GCCGCCCTTC CATCTCCCTC CTCCTGAGAC  
 -1300 AGGGGCAGCA GGGCACACTA GTGTCCAGGA GCAGCTTATG AGGCCCTTC  
 -1250 ACCCTCCATC CTCCAAACT GGCAGACCCC ACCTTCTTGG TGTGACCCA  
 -1200 GAGCTCTGAG CACAGCCCGT TCCTTCCGCC TGCCGGCCCC CCACCCAGGC  
 -1150 CCACCCCAAC CTTATCCTCC ACTGCTTTTC AGAGGAGTCT GGCCAACACA  
 -1100 AATCCTCTTG TTTGTTTGTG TGTCTGTCTG CTGCTCCTAG TCTCTGCCTC  
 -1050 TCCAGTCTC TCAGTTCCG TTTCTTTCTT AAACCTTCTC TCAGTCTCTG  
 -1000 AGGTCTCGAA ATCACGAGGC TTCGACCCCT GTGGACCAGA TGCCCAGCTA  
 -950 GTGGCCTTTC TCCAGCCCCT CAGATGACAC AGAACTACAA ACCCCAGCAT  
 -900 GCACTCTGGC CTGAAGTGCC TGGAGAGTGC TGGTGTACCC CACCTGCATT  
 -850 CTGGGAAGTCT TAGTTTCCCT AGTCCCCCAT GCTCCCACCA GGGCATCAAG  
 -800 CTCTTCCCTG GCTGGCTGAC CCTGCCTCAG CCCTAGTCTC TCTGCTGACC  
 -750 TCGGGCCCCG GGAAGCGTGC GTCACTGAAT GACAGGGTGG GGGTGGAGGC  
 -700 ACTGGAAGGC AGCTTCCCTG TCTTTTGTGT CCCCCACTTG AGTCATGGGG  
 -650 GTGTGGGGGT TCCAGGAAAT TGGGGCTGGG AGGGGAAGGG ATACCCTAAT  
 -600 GTCAGACTCA AGGACAAAAA GTCACTACAT CTTGCTGGG CCTCTATCCC  
 -550 CAAGAACCCA AAAGGACTCA AGGGTGGGGA TCCAGGAGTT CTTGTATGTA  
 -500 TGGGGGGAGG TGAAGGAGAG AACCTGCATG ACCCTAGAGG TCCCTGTGGT  
 -450 CACTGAGAGT GTGGGCTGCC ATCCCCTGCT ACAGAAACGG TGCTCACCTT  
 -400 CTGCCCAACC CTCCAGGGAA AGGCACACAG GGGTGAGGCC GAAGGCCCTT  
 -350 CCGTCTGGTG CCACATCACA GAAGGACCTT TATGACCCCC TGGTGGCTCT  
 -300 ACCCTGCCAC TCCCAATGC CCCAGCCCCC ATGCTGCAGC CCCAGGGCTC  
 -250 TGCTGGACAC CTGGGCTCCC ACTTATCAGC CTCAGTCTC ACAGCGGAAC  
 -200 CCAGGCGTCC GGCCCCCAC CCTTCAGGCC AGCGGGCGTG GAGCTGAGGC  
 -150 TTTAGAGCCT CCCAGCCGGG CTTGTTCTG TCCATTGTG TATGGGATAG  
 -100 GGGCGGGGCG AGGGCCAGCA CTGGAGAGCC CCCTCCCACT GCCCCCTCCT  
 -50 CTCGGTCCCC TCCCTCTTCC TAAGGAAAAG GCCAGGGCTC TGCTGGAGCA  
 +1 AGCAGAGTGGACGCACAGTA

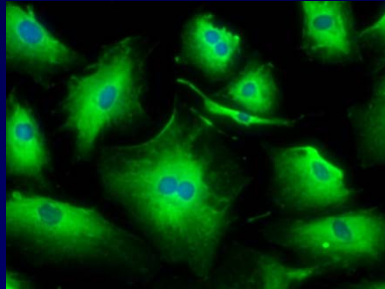
 ERE

 AP1

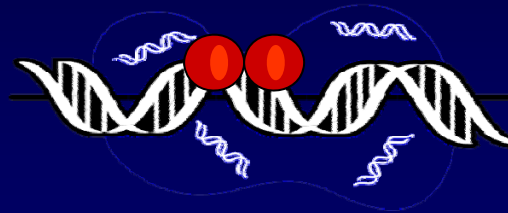
 SP1

# ChIP Assay for E-ER/DNA Biding

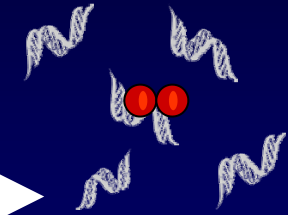
Estrogen (100nM)  
Time (min) →  
5 15 30 45



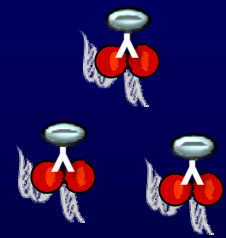
Protein/DNA  
Crosslink



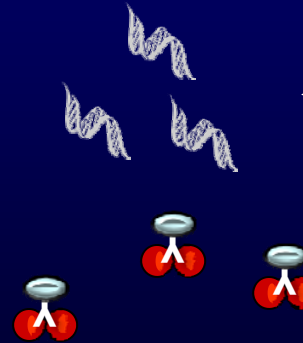
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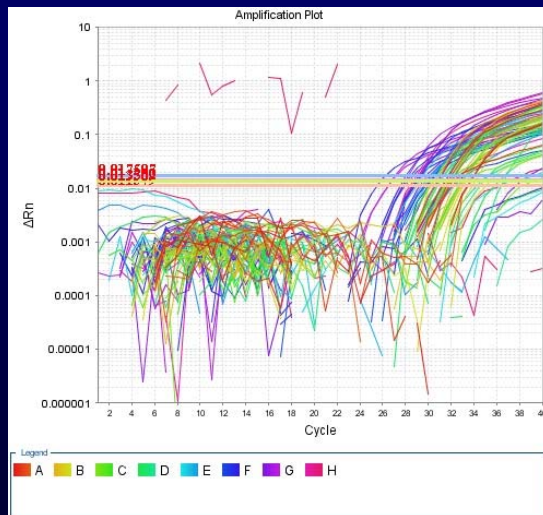
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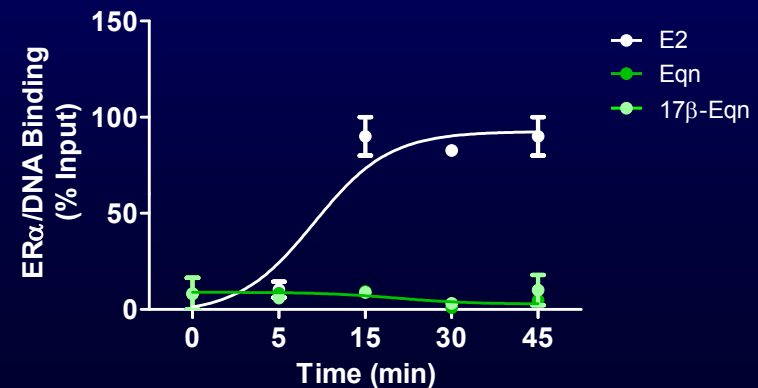
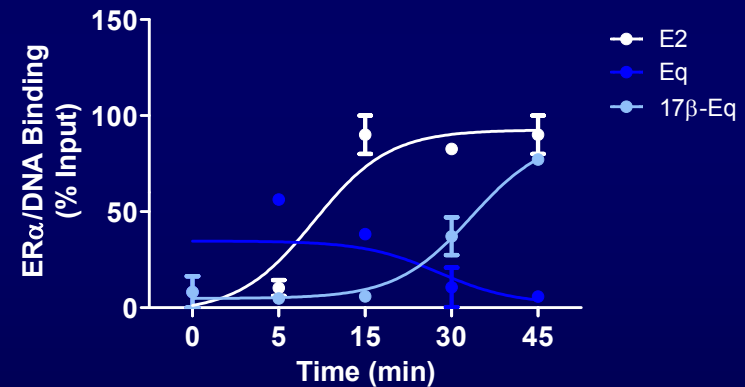
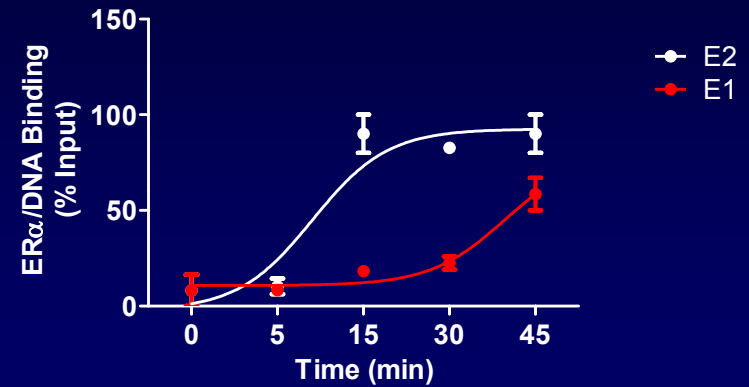
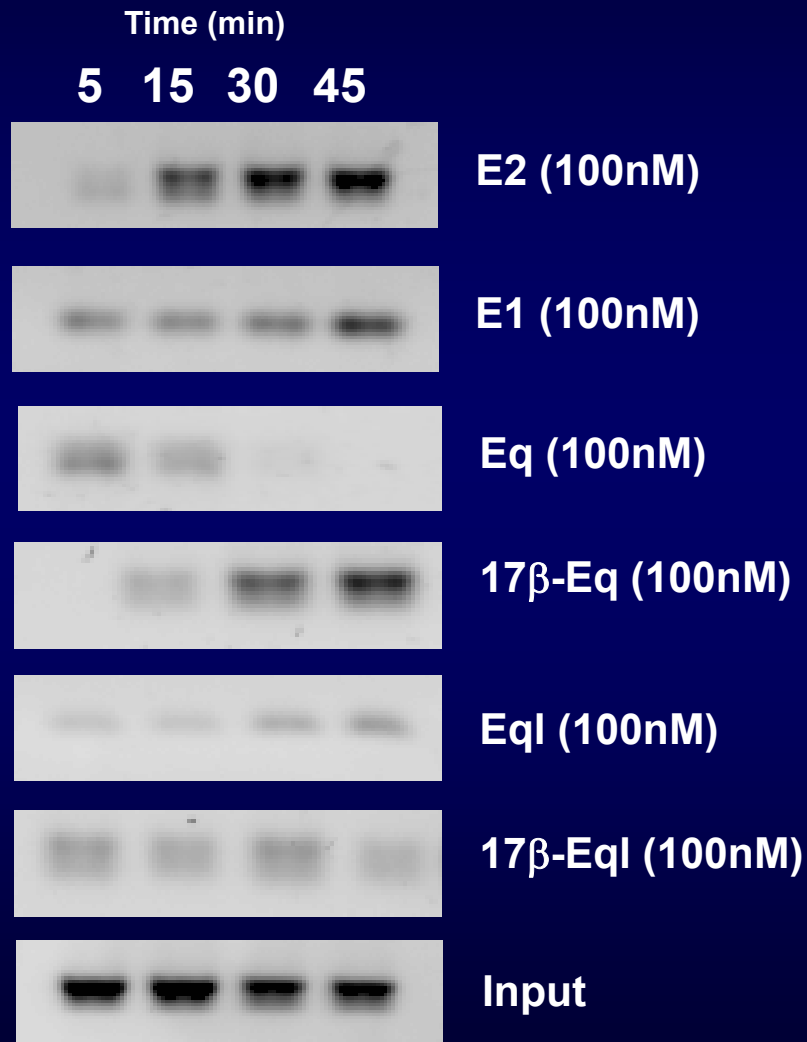
Reverse  
Crosslink



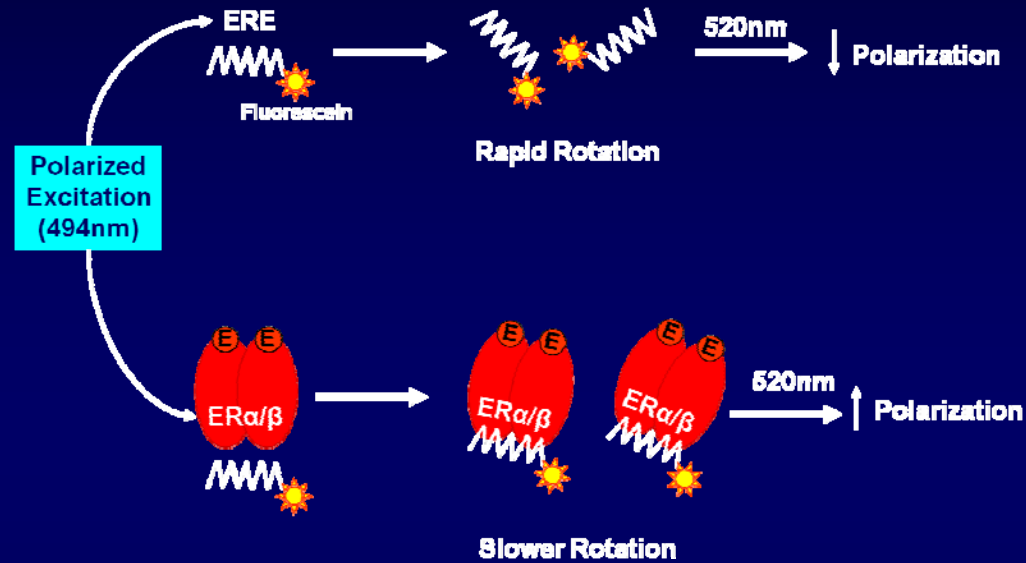
qPCR



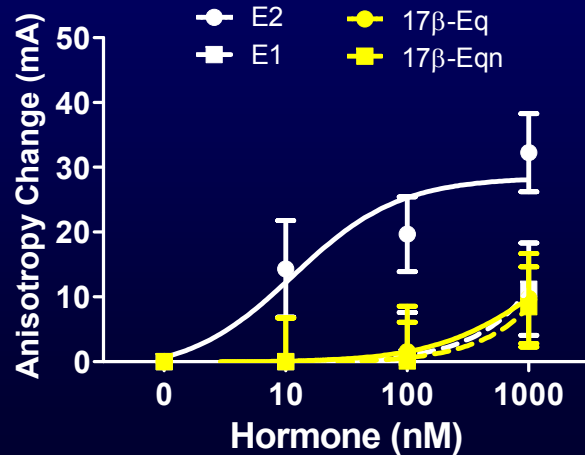
# ChIP Analysis for ER $\alpha$ /DNA Interaction



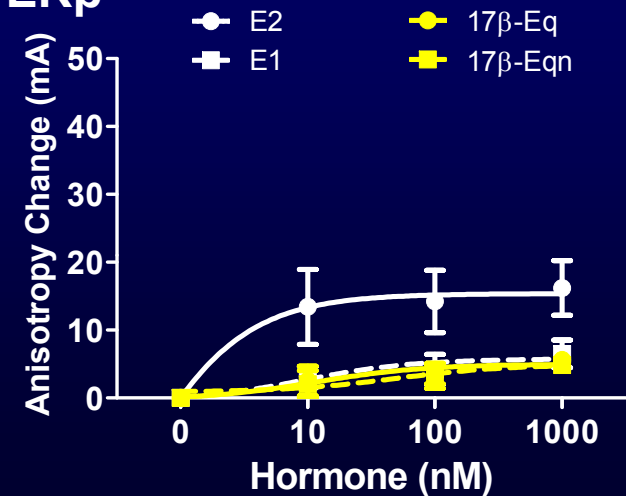
# Fluorescence Anisotropy Analysis of ER $\alpha$ / $\beta$ and SP1 Site Interaction



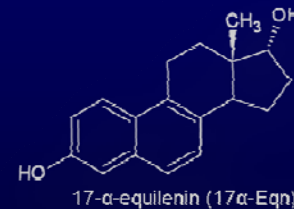
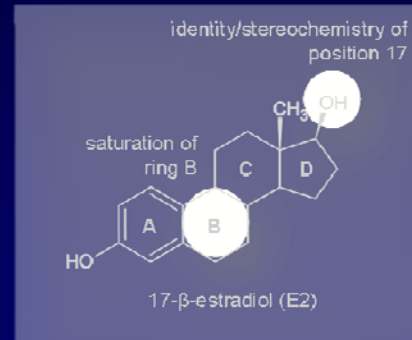
ER $\alpha$



ER $\beta$



# • Molecular Modelling studies

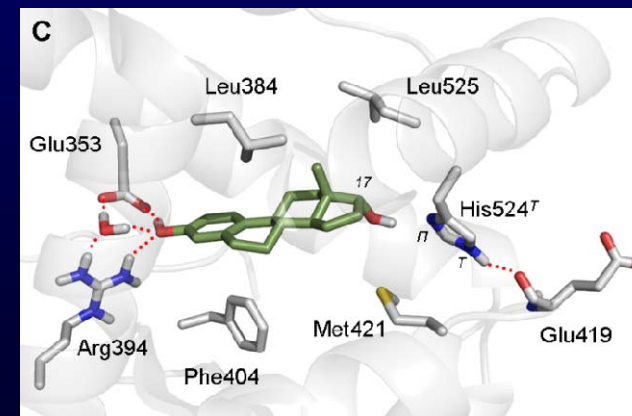
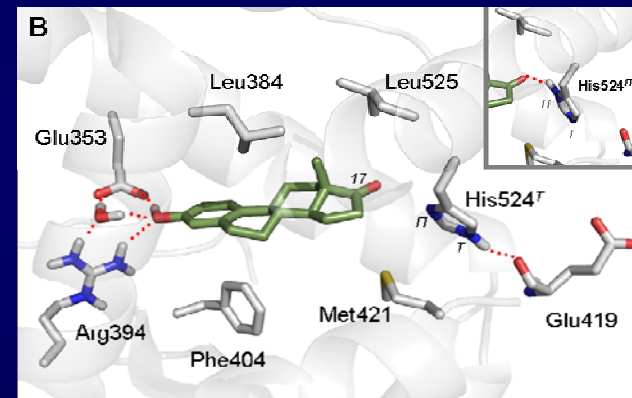
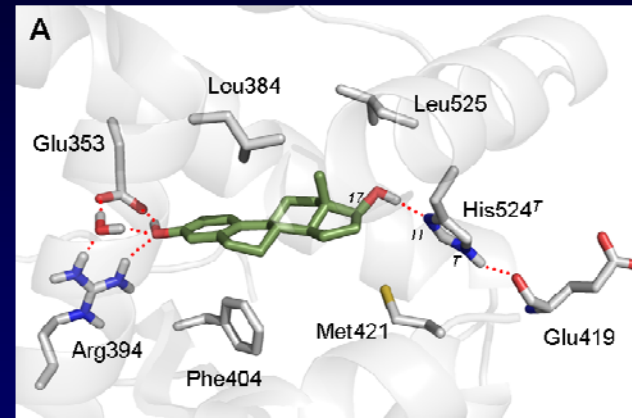
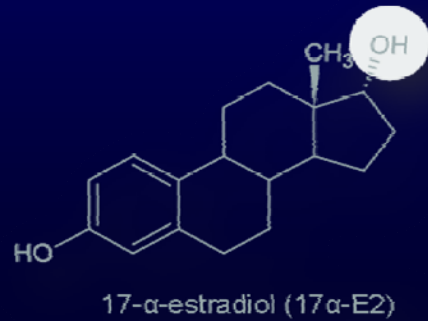
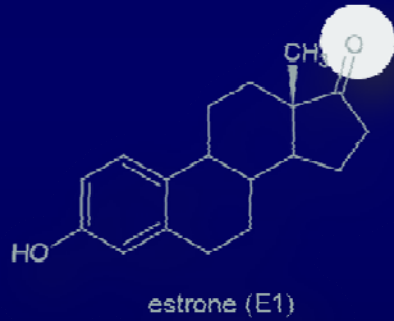
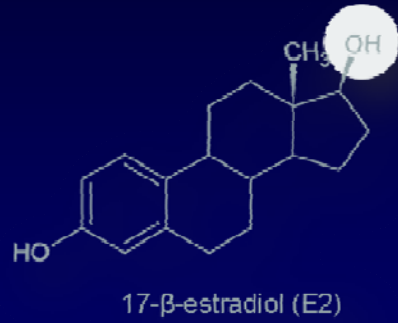


Small structural differences are responsible for significant changes in NO production :

- (i) the chemical moiety and stereochemistry at the position 17
- (ii) the saturation degree of ring B

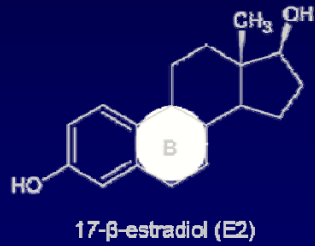
# Chemical Moeity at Position 17

NO Production

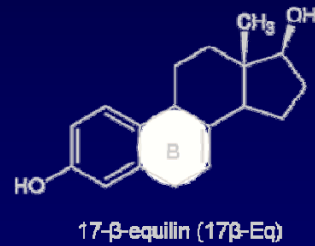




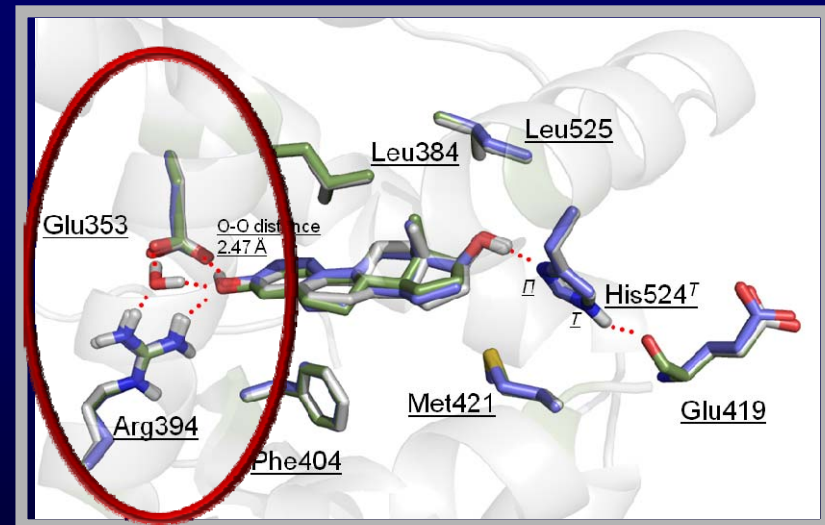
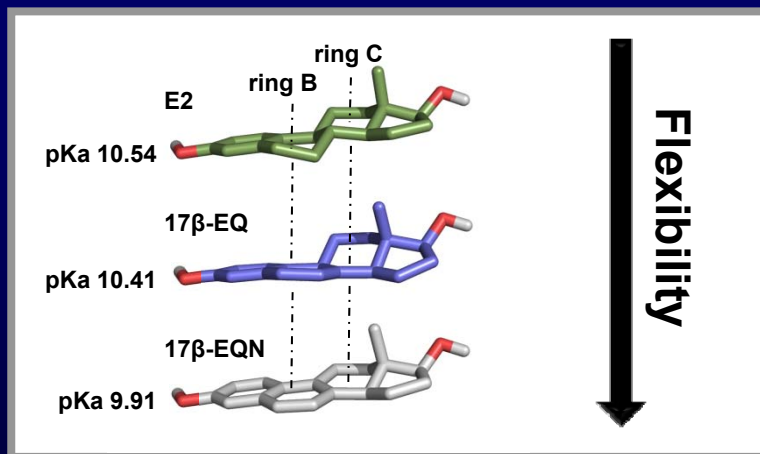
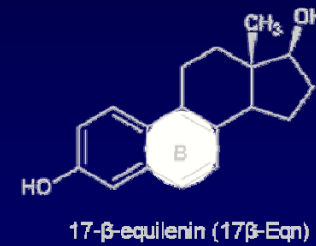
# Saturation of ring B



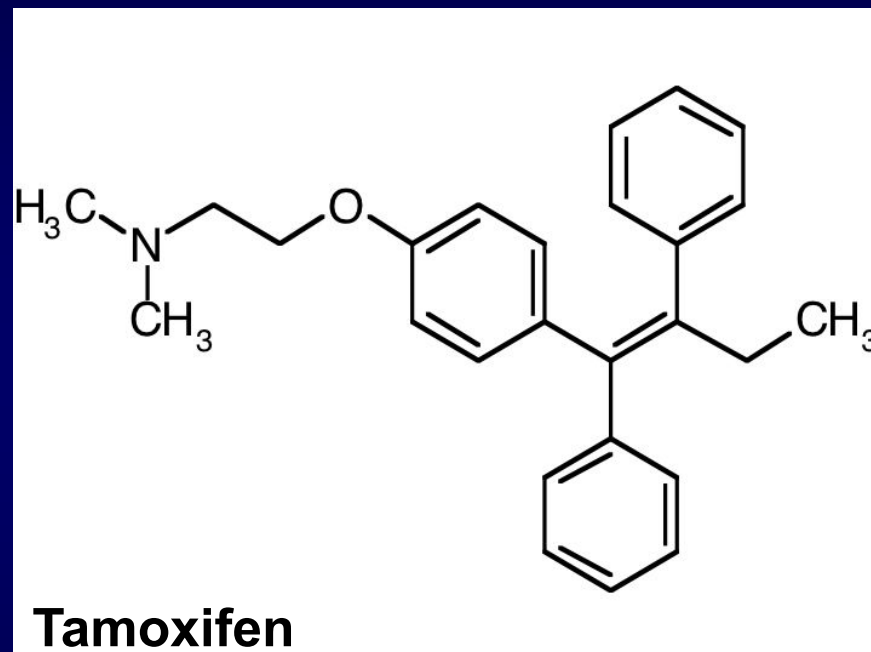
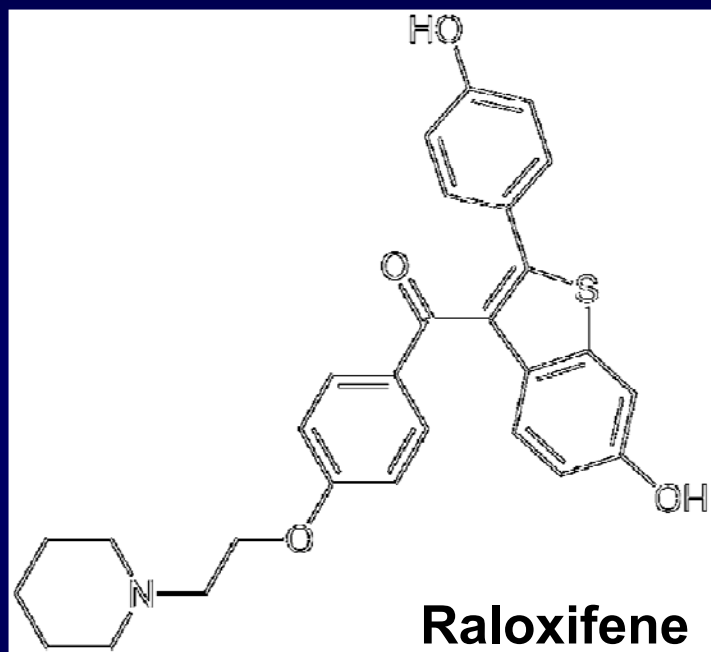
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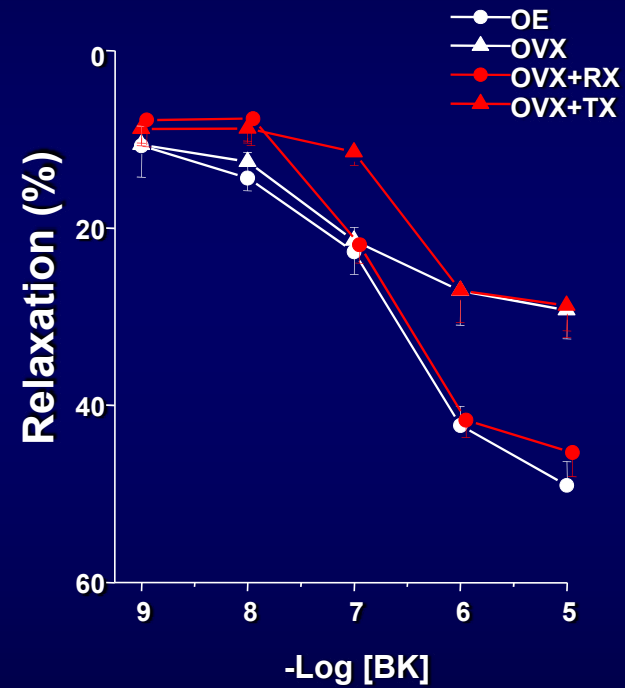
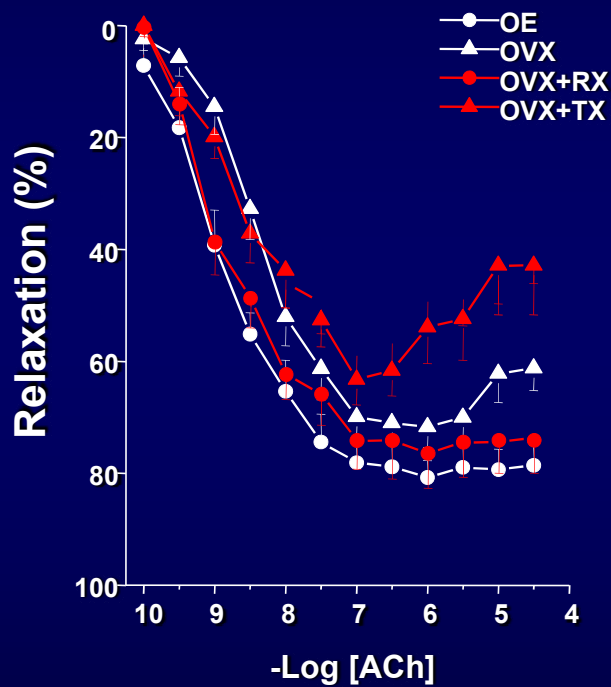


# ***SERMs*** ***(Selective ER Modulators)***

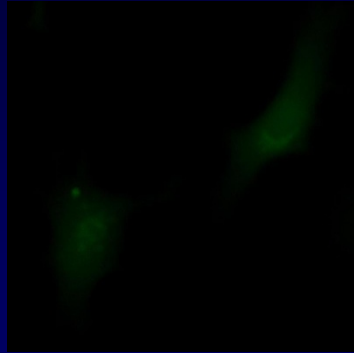


**Molecules designed to express tissue-specific agonist and antagonist activities.**

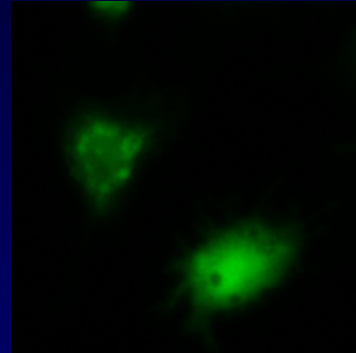
# SERM-induced effects on endothelium-dependent relaxation by ACh and Bk in microvessels of OVX-SHR.



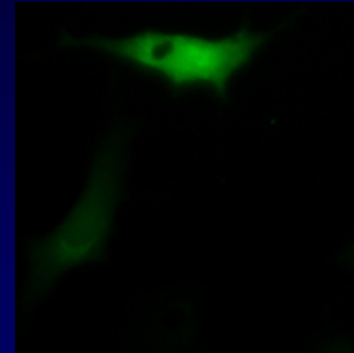
# Nitric Oxide Production by SERMs



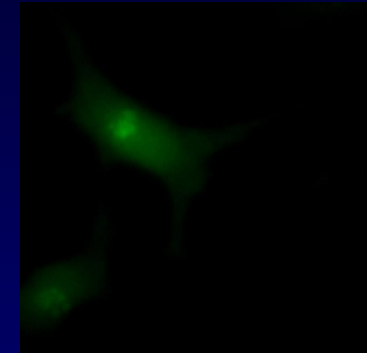
NT



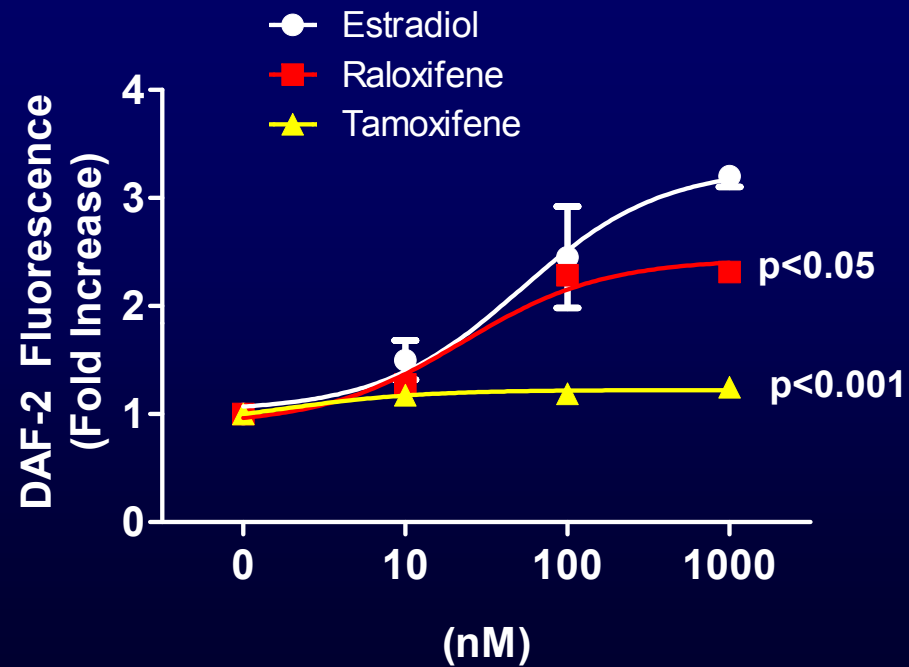
Estradiol



Raloxifene



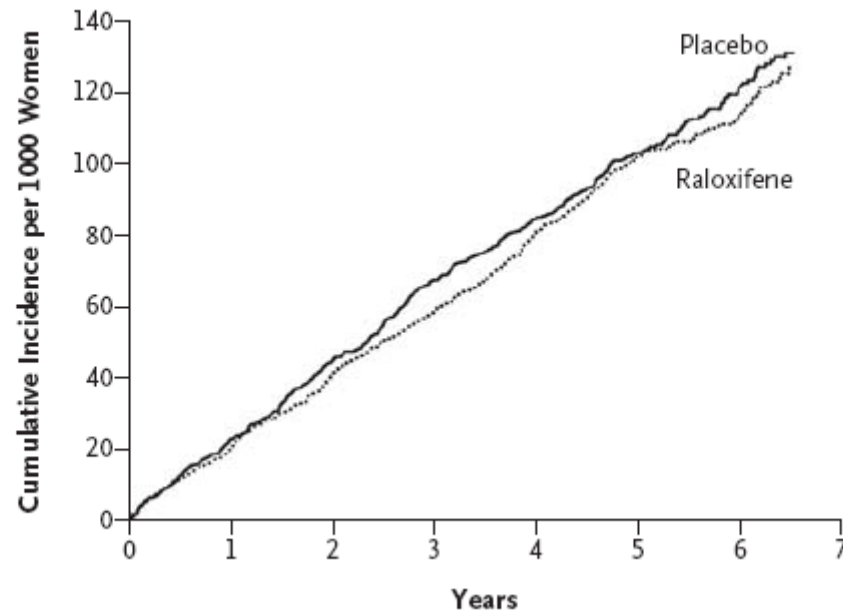
Tamoxifen



# ***RUTH Trial***

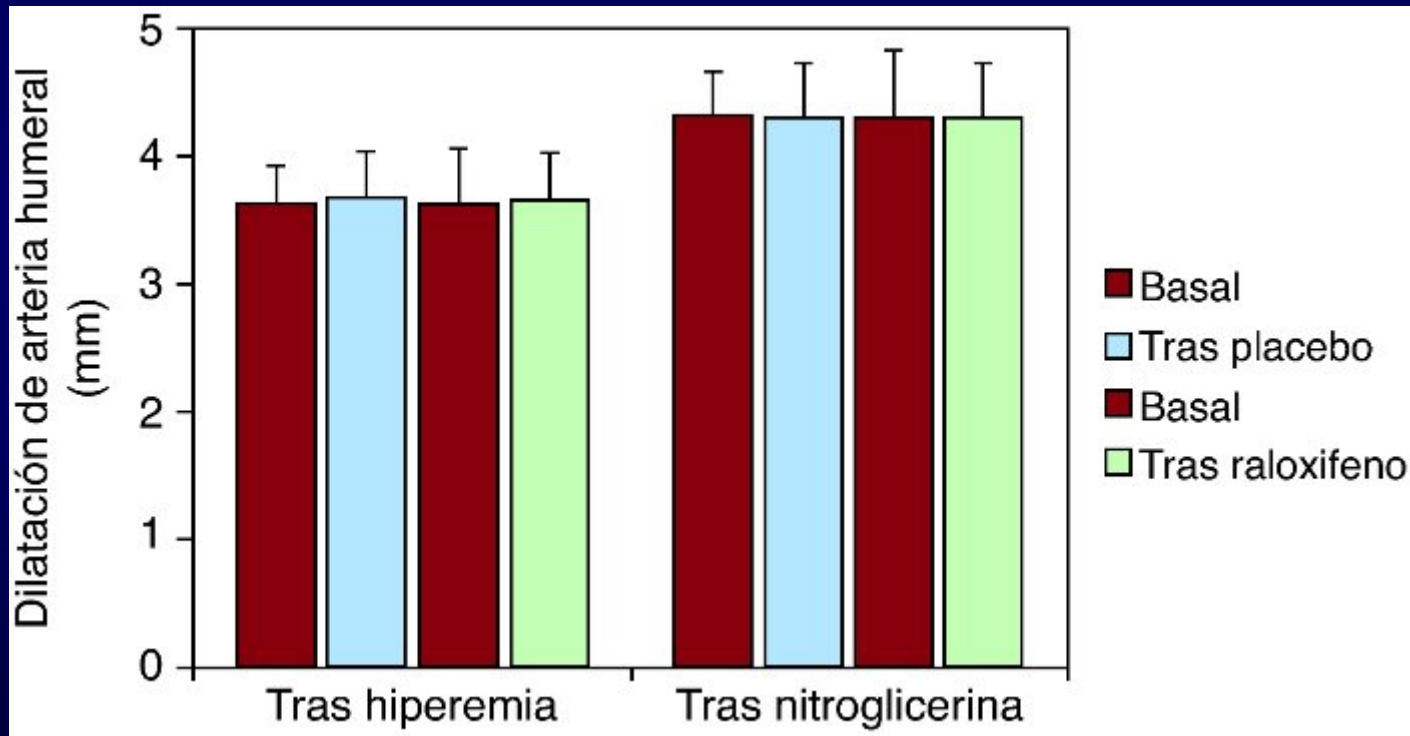
***(Raloxifene Use for the Heart)***

## ***Coronary Events***



# MERGED

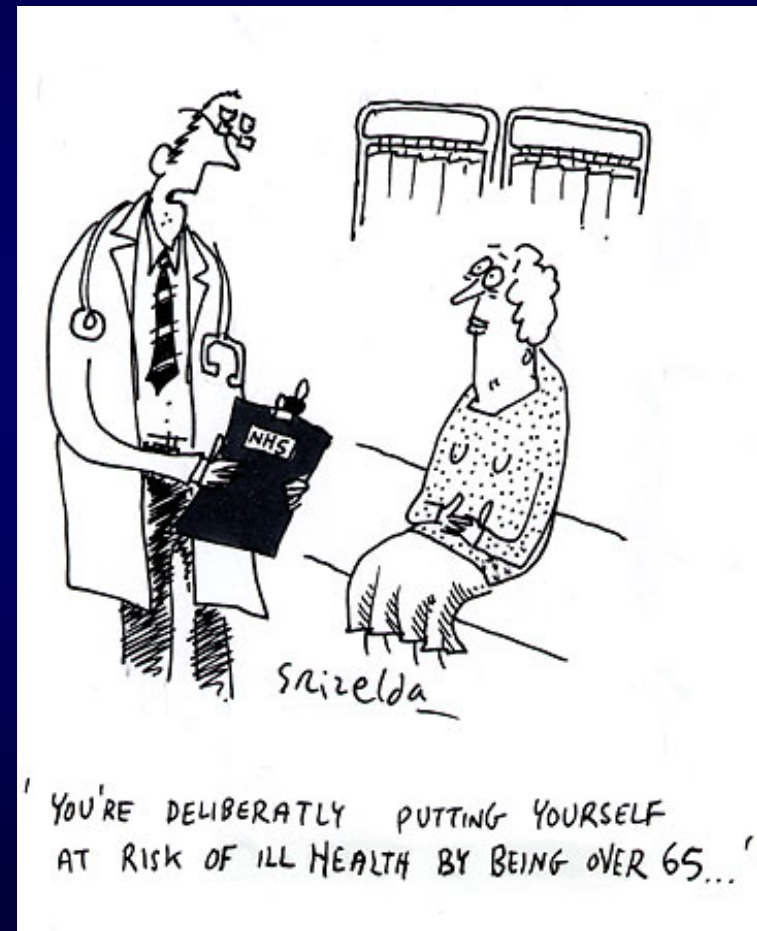
(*ME*nopausia y *R*aloxifeno en la *C*ardiopatía isquémica: *E*fecto en la *D*isfunción endotelial )



WHI?

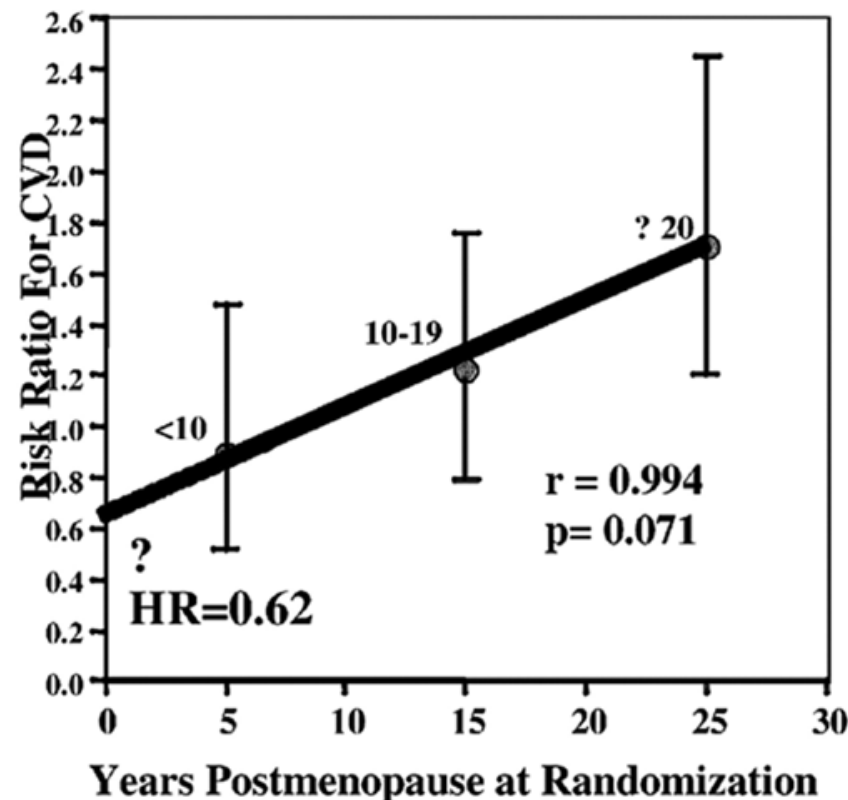
# Average age of women beginning the trial

TRIAL	HRT	AGE
HERS	CEEs	~ 67
WHI	CEEs	~ 64
RUTH	Ralox	~ 67
MERCED	Ralox	~ 60



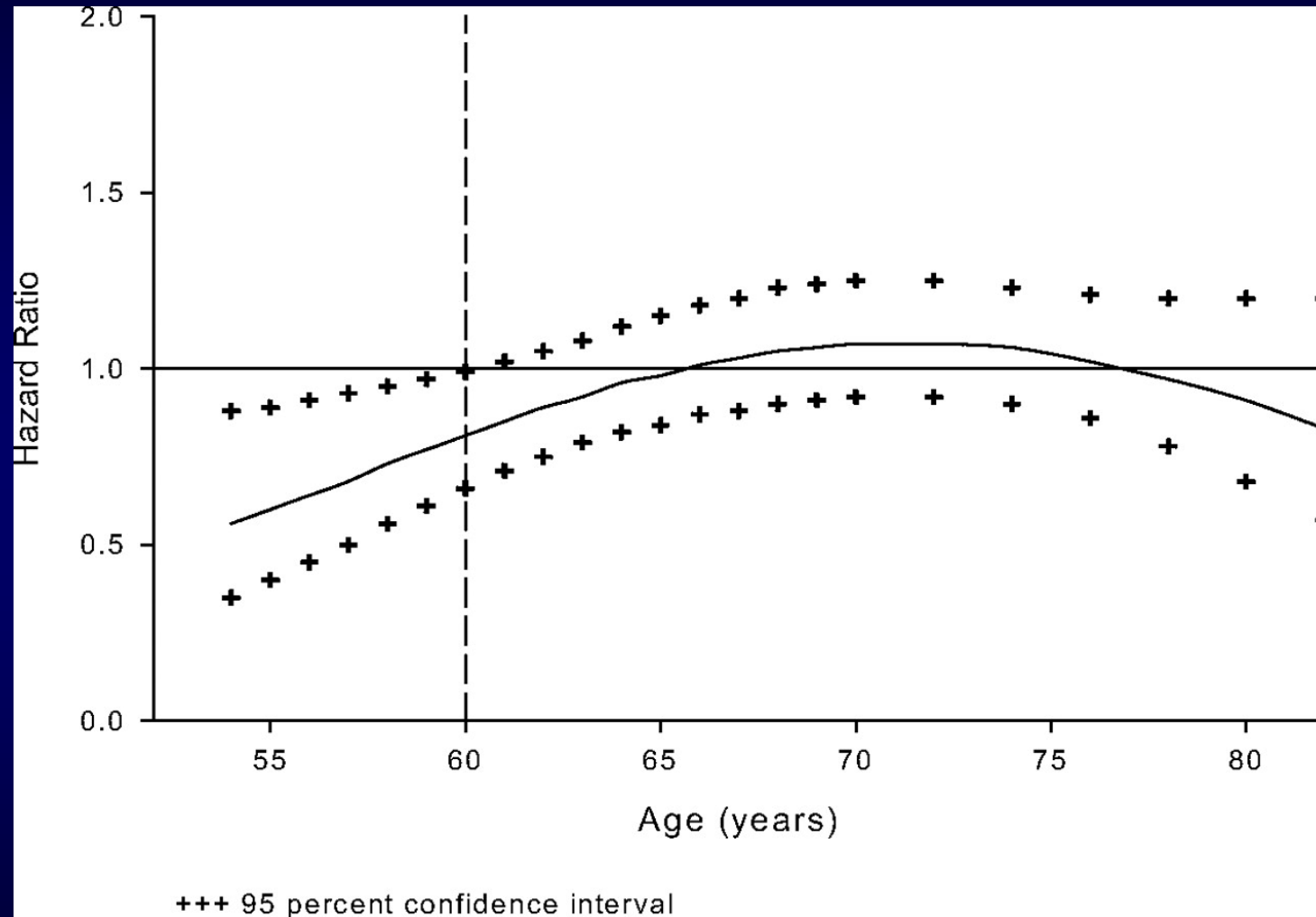
## THE "TIMING" HYPHOTESIS

Estrogen-mediated benefits to prevent cardiovascular disease may occur **only** when treatment is initiated before the detrimental effects of aging or cardiovascular disease are established in the vasculature.



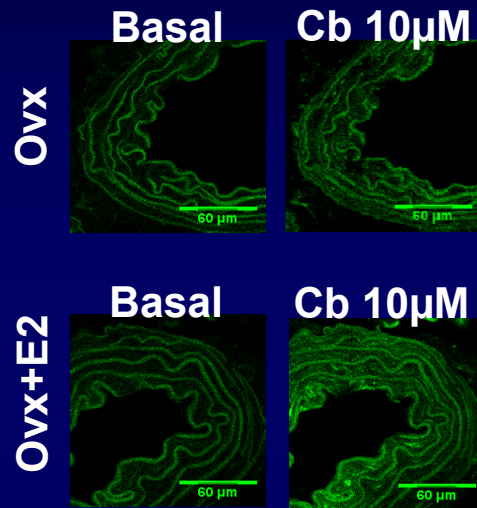


**Effect of raloxifene on the incidence of the primary coronary end point (coronary death, nonfatal MI, or hospitalized ACS other than MI, whichever occurred first) by age.**

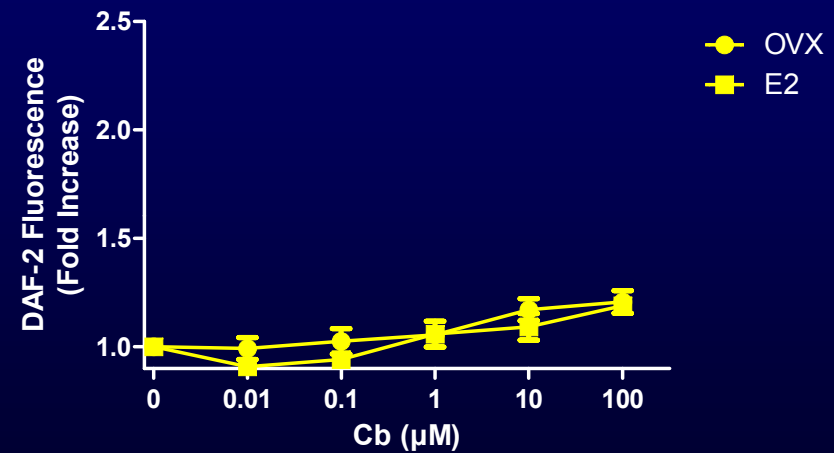
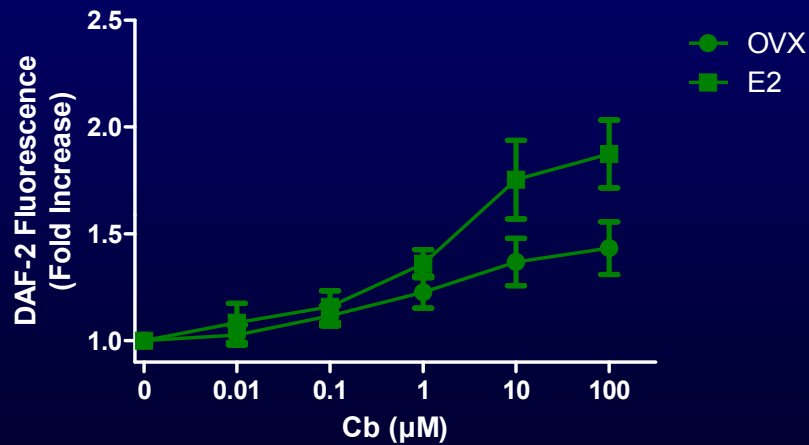
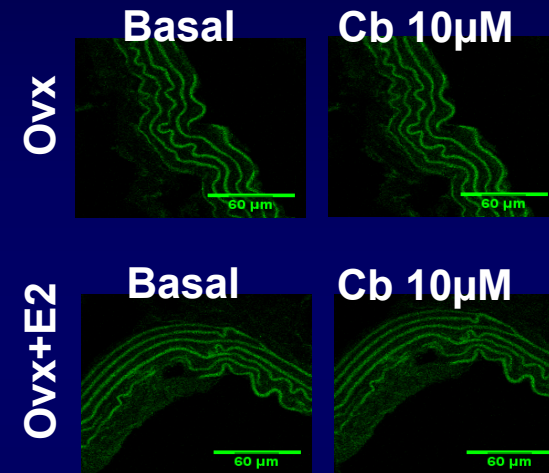


# Aging-associated effects on E2-mediated production of NO

## Young

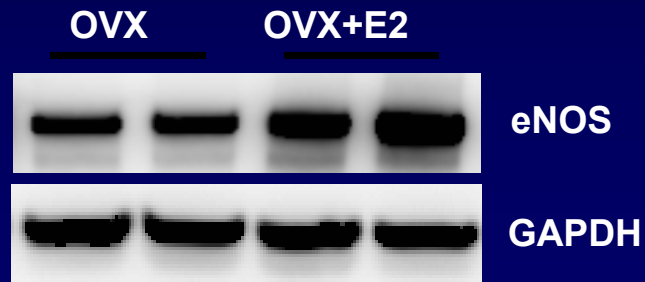


## Aged

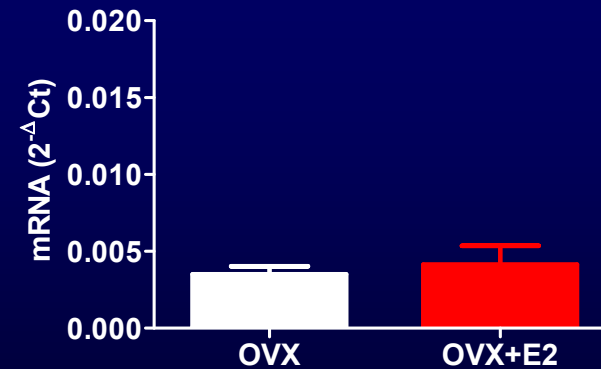
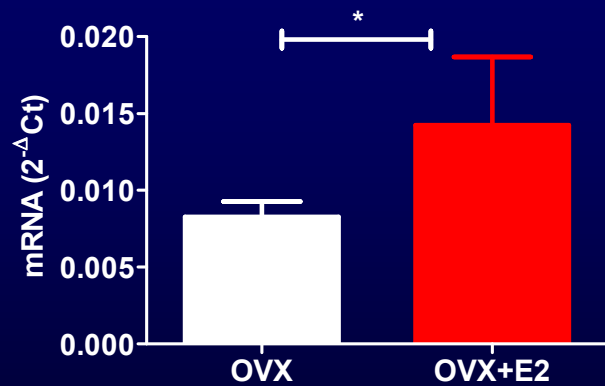
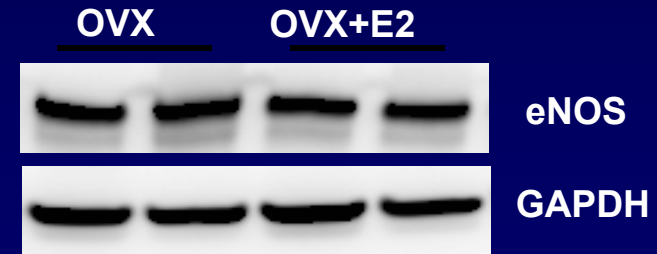


# Aging-associated effects on E2 modulation of eNOS expression

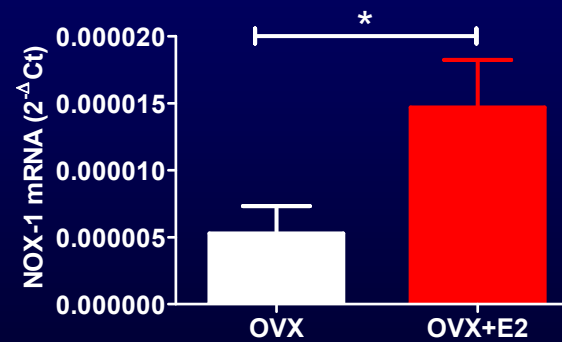
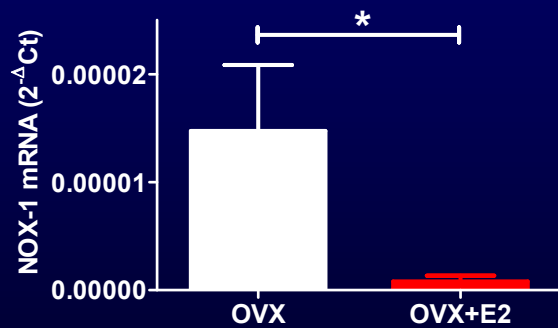
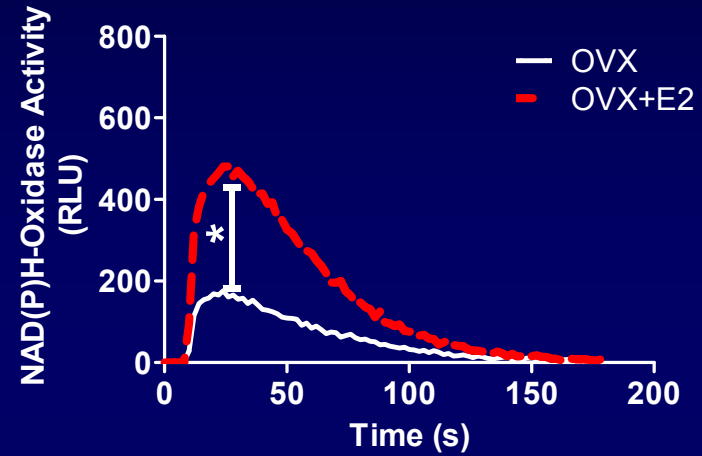
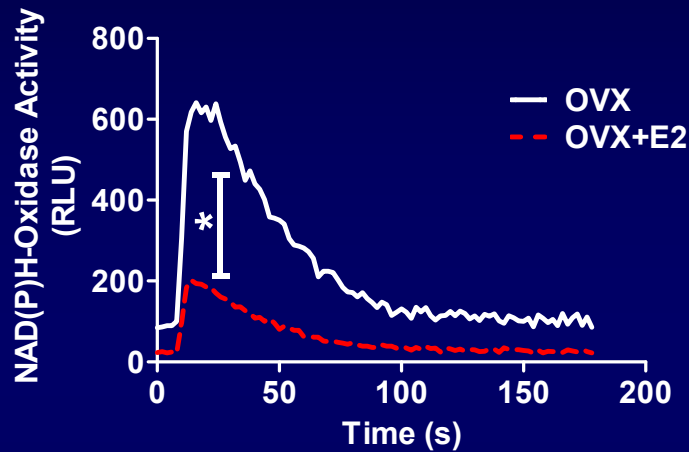
Young



Aged



# A swap from anti-oxidant to pro-oxidant effect by E2 is observed in aged females



# Anti-inflammatory effects of Estrogen: a matter of timing

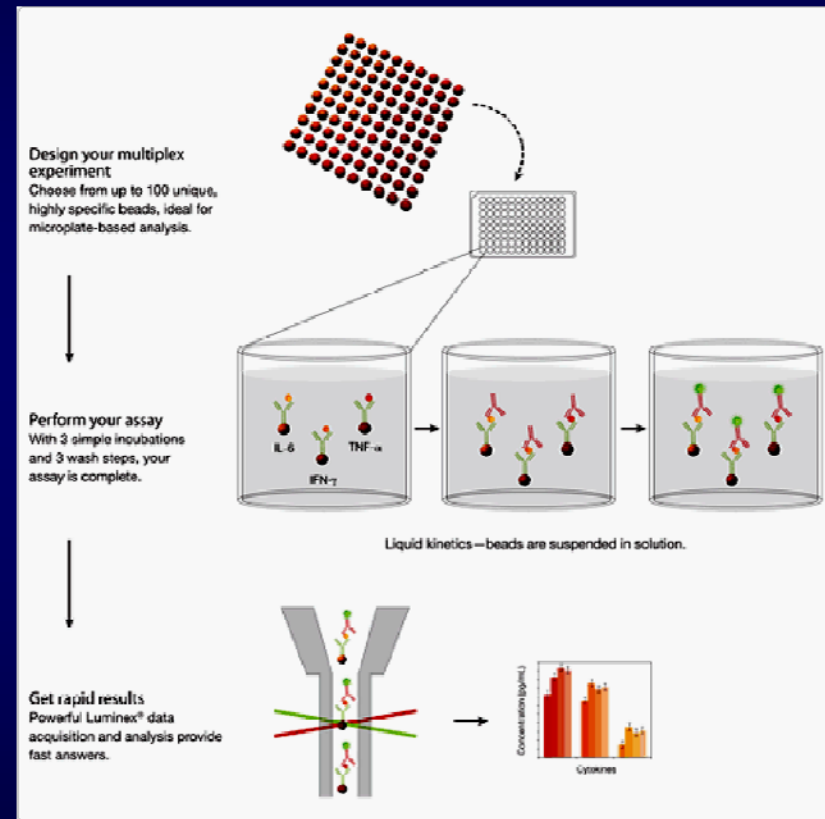
→ Uterine arteries obtained from 68 women (age 41-86) at the moment of hysterectomy were cleaned, divided into three segments and cultured for 24h in tissue culture media containing

17beta-estradiol (100nM),

Raloxifen (100nM) or vehicle.

→ Exclusion criterion: use of hormone replacement therapy, SERMs (Raloxifen, Tamoxifen...), chronic anti-inflammatory therapy, statins, RAS inhibitors, diabetes.

→ Multiplex, immunobead-based assay, was performed to measured 13 cardiovascular-related inflammatory biomarkers.



CVD1: MMP-9; sE-Selectin; s-ECAM; s-VCAM; t-PAI

CVD3: IFN $\gamma$ ; IL-10; IL-1b; IL-6; IL-8; MCP-1; TNF $\alpha$ ; VEGF

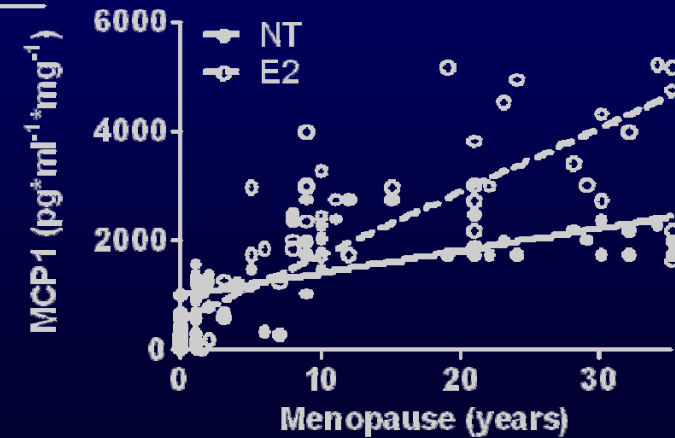
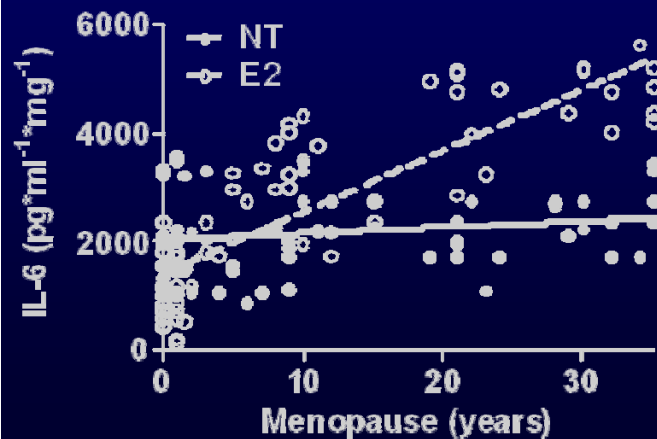
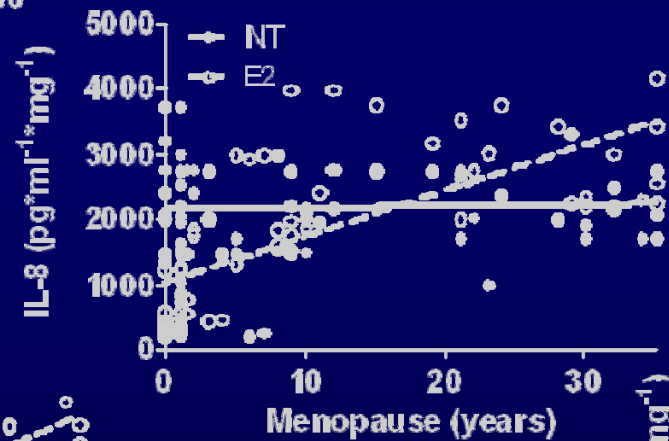
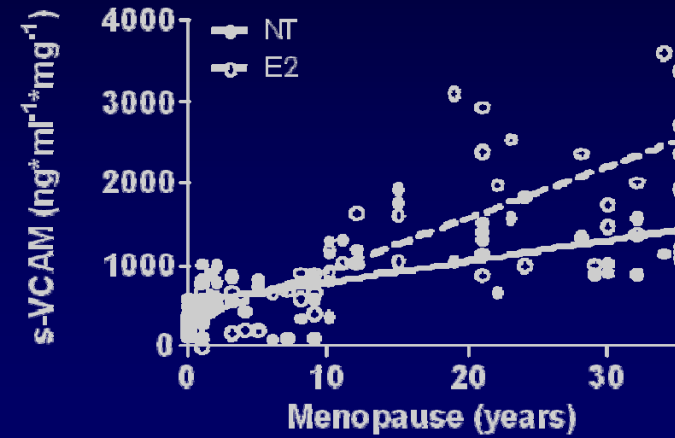
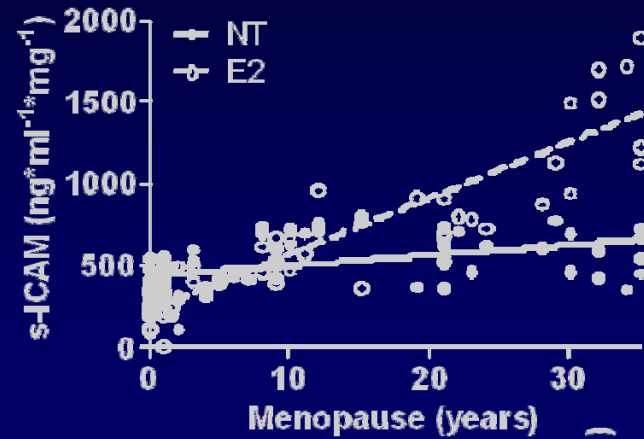
# Anti-inflammatory effects of Estrogen: a matter of timing

## Pearson's Correlation Coefficients

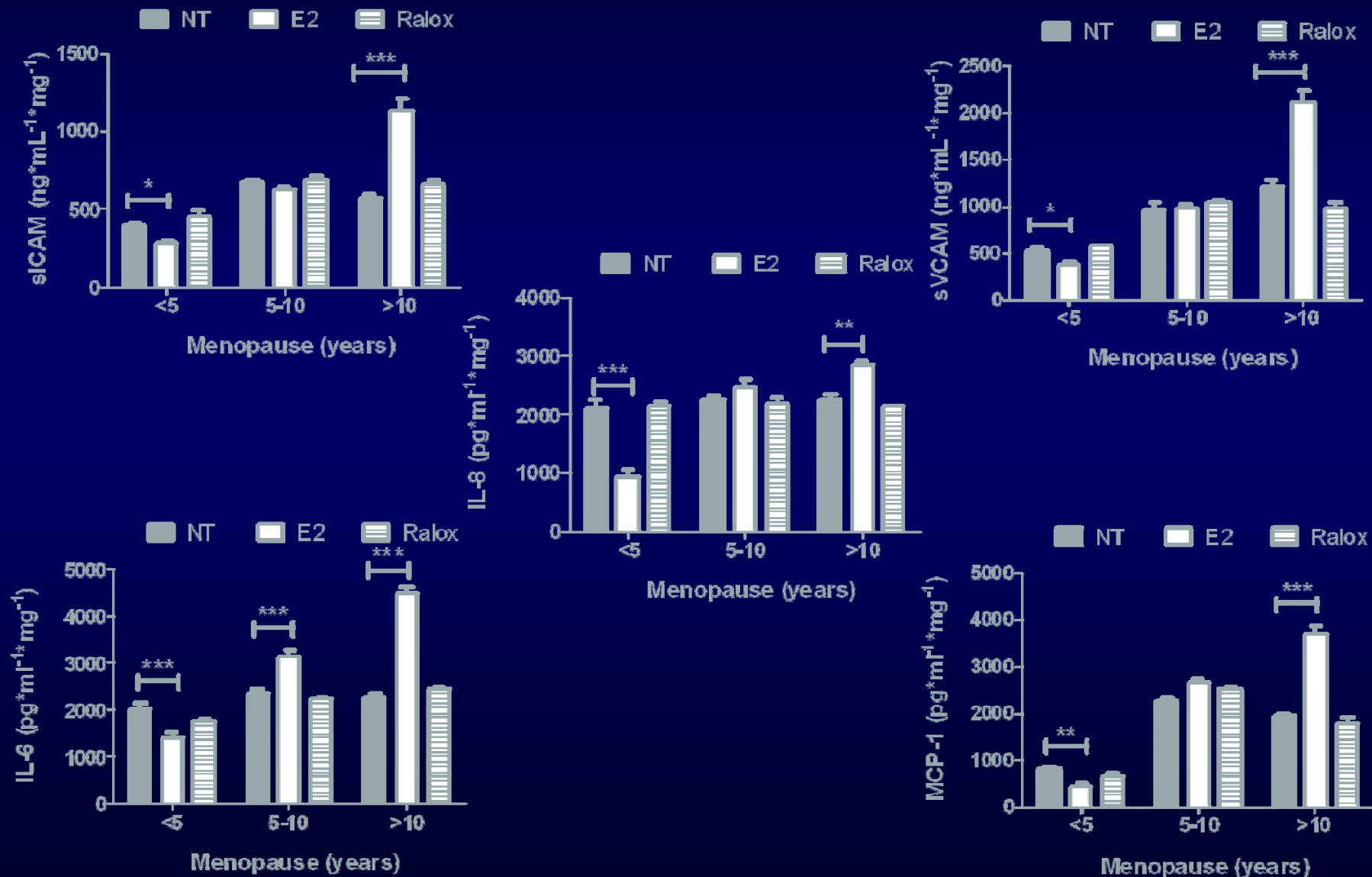
	Untreated				Estrogen				Raloxifene			
	Pierson r	r <sup>2</sup>	P value	Summary	Pierson r	r <sup>2</sup>	P value	Summary	Pierson r	r <sup>2</sup>	P value	Summary
MMP-9	0.1522	0.023	0.5737	ns	0.1459	0.021	0.0987	ns	0.0592	0.004	0.8275	ns
E-Selectin	0.7205	0.519	<0.0001	***	0.7274	0.529	0.0003	***	0.6893	0.475	<0.0001	***
s-ICAM	0.4549	0.207	<0.0001	***	0.9057	0.820	<0.0001	*** (a)	0.5463	0.298	<0.0001	***
s-VCAM	0.6602	0.436	<0.0001	***	0.8463	0.716	<0.0001	*** (a)	0.5723	0.328	<0.0001	***
tPAI	0.6778	0.459	<0.0001	***	0.6221	0.387	<0.0001	***	0.5861	0.344	<0.0001	***
IFN <sub>γ</sub>	0.2048	0.042	0.4467	ns	0.2390	0.057	0.4105	ns	0.1428	0.020	0.8796	ns
IL-1 <sub>β</sub>	0.7161	0.513	<0.0001	***	0.5302	0.281	<0.0001	*** (a)	0.6740	0.454	<0.0001	***
IL-6	0.1715	0.029	0.1587	ns	0.8357	0.699	<0.0001	*** (a)	0.2589	0.067	0.1265	ns
IL-8	0.1319	0.017	0.7479	ns	0.7193	0.518	<0.0001	*** (a)	0.4127	0.170	0.1121	ns
IL-10	0.4440	0.197	0.0849	ns	0.4192	0.176	0.1357	ns	0.5350	0.286	0.0611	ns
TNF <sub>α</sub>	0.6308	0.407	<0.0001	***	0.5161	0.266	<0.0001	*** (a)	0.7104	0.504	<0.0001	***
MCP1	0.6202	0.385	<0.0001	***	0.8311	0.691	<0.0001	*** (a)	0.6729	0.453	<0.0001	***
VEGF	0.6610	0.437	<0.0001	***	0.3362	0.113	0.0047	** (a)	0.5896	0.348	<0.0001	***

(a): Analysis of Covariance (ANCOVA) reveals significant difference ( $p < 0.05$ ) in comparison to untreated group.

**Aging can be associated to a switch from a beneficial anti-inflammatory action by estrogen, at earlier stages of menopause, to a pro-inflammatory profile after 5 year past its onset.**



**Aging can be associated to a switch from a beneficial anti-inflammatory action by estrogen, at earlier stages of menopause, to a pro-inflammatory profile after 5 year past its onset.**





# Conclusions



✓ The complex regulation of cellular responses to estrogen ligands are specific to the ligand and dependent not only on the relative levels of ER in a given tissue, but also on how ligand-induced conformational changes in ERs leading to differential modulation of transcription depending upon the cell type.

✓ Furthermore, cardiovascular responses to estrogens can vary based on different physiological and pathophysiological situations, such as during aging, in part, due to alterations in the tissue methylation status of key regulators of cardiovascular function.

# Conclusions



Gaining a detailed understanding of the cell- and tissue-specific signaling pathways induced by various ER ligands and the subsequent effects on gene regulation under physiological and pathophysiological circumstances, may ultimately lead to the development of new therapeutics for the treatment of cardiovascular disease in both men and women.

# **KEEPS**

## ***(Kronos Early Estrogen Prevention Study )***

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- ✓ **Rationale: “earlier intervention than that performed in the WHI and HERS trials will provide cardiovascular benefit to women”**
- ✓ **Multicenter, randomized, double-blind, placebo-controlled 5-year clinical trial.**
- ✓ **Will evaluate the effectiveness of conjugated equine estrogens or transdermal 17 $\beta$ - estradiol, and placebo in preventing progression of cardiovascular disease in women aged 42-58 years who are within 36 months of their final menstrual period.**

# Acknowledgment



Magda Heras, MD PhD

Mercè Roquè, MD PhD

Laura Novensa, PhD

Nadia Castillo



Jana Selent, PhD

Manuel Pastor, PhD



*Pharmacology*

Maria Helena Carvalho, PhD

Rita CA Tostes, PhD

Zuleica B Fortes, PhD

Graziela Ceravolo, PhD



*Georgetown University  
Medical Center*

Kathryn Sandberg, PhD



Carlos Hermenegildo, MD PhD

Susana Novella, PhD

Pascual Medina, PhD

Gloria Segarra, PhD



*University of  
Wisconsin*

Erin Séanle, PhD

Wei Xu, MD



*Pharmacology*

M. Antonia Busquets, PhD

Mercè Pallas, PhD

## Financial Support

