U.PORTO

FMUP FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

UNRAVELLING THE ROLE OF ESTROGENS AND XENOESTROGENS IN ADIPOSE TISSUE-ORIGINATED INFLAMMATION

DIANA MARINA DA SILVA TEIXEIRA

TESE DE DOUTORAMENTO APRESENTADA
À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO EM
BIOMEDICINA

PORTO · 2015

Aos meus pais

À minha irmã

À Professora Rosário

À Professora Conceição

DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR EM BIOMEDICINA, APRESENTADA À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO

JÚRI DA PROVA DE DOUTORAMENTO

PRESIDENTE:

Doutora Maria Amélia Duarte Ferreira
Diretora da Faculdade de Medicina da Universidade do Porto (por delegação reitoral)

VOGAIS:

Doutora Maria do Rosário Barbosa Monteiro
Professora Auxiliar Convidada da Faculdade de Medicina da Universidade do Porto

Doutor João Francisco Montenegro de Andrade Lima Bernardes
Professor Catedrático da Faculdade de Medicina da Universidade do Porto

Doutora Paula Manuela de Castro Cardoso Pereira
Professora Associada do Instituto Superior de Ciências da Saúde Egas Moniz

Doutor António Pedro Soares Ricardo Graça
Professor Associado Convidado da Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto

Doutora Lélita da Conceição dos Santos
Professora Auxiliar com Agregação da Faculdade de Medicina da Universidade de Coimbra

Doutora Ana Isabel Gonçalves Faria
Professora Auxiliar Convidada da Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto

Artigo 48º, Parágrafo 3º - "A Faculdade não responde pelas doutrinas expendidas na dissertação" (Regulamento da Faculdade de Medicina do Porto – Decreto-Lei nº 19337, 29 de Janeiro de 1931).

CORPO CATEDRÁTICO DA FACULDADE DE MEDICINA DO PORTO

PROFESSORES EFETIVOS – CATEDRÁTICOS

Doutor Manuel Alberto Coimbra Sobrinho Simões

Doutora Maria Amélia Duarte Ferreira

Doutor José Agostinho Marques Lopes

Doutor Patrício Manuel Vieira Araújo Soares-da-Silva

Doutor Daniel Filipe Lima Moura

Doutor Alberto Manuel Barros da Silva

Doutor José Manuel Lopes Teixeira Amarante

Doutor José Henrique Dias Pinto de Barros

Doutora Maria de Fátima Machado Henriques Carneiro

Doutora Isabel Maria Amorim Pereira Ramos

Doutora Deolinda Maria Valente Alves Lima Teixeira

Doutora Maria Dulce Cordeiro Madeira

Doutor Altamiro Manuel Rodrigues da Costa Pereira

Doutor Rui Manuel Almeida Mota Cardoso

Doutor José Carlos Neves da Cunha Areias

Doutor Manuel Jesus Falcão Pestana Vasconcelos

Doutor João Francisco Montenegro Andrade Lima Bernardes

Doutora Maria Leonor Martins Soares David

Doutor Rui Manuel Lopes Nunes

Doutor José Eduardo Torres Eckenroth Guimarães

Doutor Francisco Fernando Rocha Gonçalves

Doutor José Manuel Pereira Dias de Castro Lopes

Doutor António Albino Coelho Marques Abrantes Teixeira

Doutor Joaquim Adelino Correia Ferreira Leite Moreira

Doutora Raquel Ângela Silva Soares Lino

PROFESSORES JUBILADOS OU APOSENTADOS

Doutor Abel Vitorino Trigo Cabral

Doutor Alexandre Alberto Guerra de Sousa Pinto

Doutor Álvaro Jerónimo Leal Machado de Aguiar

Doutor Amândio Gomes Sampaio Tavares

Doutor António Augusto Lopes Vaz

Doutor António Carlos Freitas Ribeiro Saraiva

Doutor António Carvalho Almeida Coimbra

Doutor António Fernandes Oliveira Barbosa Ribeiro Braga

Doutor António José Pacheco Palha

Doutor António Manuel Sampaio de Araújo Teixeira

Doutor Belmiro dos Santos Patrício

Doutor Cândido Alves Hipólito Reis

Doutor Carlos Rodrigo Magalhães Ramalhão

Doutor Cassiano Pena de Abreu e Lima

Doutor Daniel Santos Pinto Serrão

Doutor Eduardo Jorge Cunha Rodrigues Pereira

Doutor Fernando Tavarela Veloso

Doutor Francisco de Sousa Lé

Doutor Henrique José Ferreira Gonçalves Lecour de Menezes

Doutor Jorge Manuel Mergulhão Castro Tavares

Doutor José Carvalho de Oliveira

Doutor José Fernando Barros Castro Correia

Doutor José Luís Medina Vieira

Doutor José Manuel Costa Mesquita Guimarães

Doutor Levi Eugénio Ribeiro Guerra

Doutor Luís Alberto Martins Gomes de Almeida

Doutor Manuel António Caldeira Pais Clemente

Doutor Manuel Augusto Cardoso de Oliveira

Doutor Manuel Machado Rodrigues Gomes

Doutor Manuel Maria Paula Barbosa

Doutora Maria da Conceição Fernandes Marques Magalhães

Doutora Maria Isabel Amorim de Azevedo

Doutor Mário José Cerqueira Gomes Braga

Doutor Serafim Correia Pinto Guimarães

Doutor Valdemar Miguel Botelho dos Santos Cardoso

Doutor Walter Friedrich Alfred Osswald

ORIENTADORA:

Professora Doutora Rosário Monteiro

Professora Auxiliar Convidada - Faculdade de Medicina da Universidade do Porto

CO-ORIENTADORA:

Professora Doutora Conceição Calhau

Professora Auxiliar com Agregação - Faculdade de Medicina da Universidade do Porto

O trabalho experimental foi executado no Departamento de Bioquímica da Faculdade de Medicina da Universidade do Porto e no INSERM 858, Toulouse, França, e subsidiado pela Fundação para a Ciência e Tecnologia (Fundo Social Europeu, Programa Operacional Potencial Humano da EU (POPH); PEst-OE/SAU/UI0038/2011) e Projetos de Investigação na Pré-graduação 2012, Universidade do Porto. A candidata realizou o trabalho experimental com o apoio de uma bolsa de Doutoramento (SFRH/BD/64691/2009) atribuída pela Fundação para a Ciência e a Tecnologia.

CONTRIBUTO PESSOAL NOS TRABALHOS DE INVESTIGAÇÃO APRESENTADOS NESTA DISSERTAÇÃO

A candidata declara que teve uma contribuição determinante em toda a realização do trabalho experimental (programação e execução das experiências), bem como na interpretação e discussão dos resultados apresentados em todos os artigos desta dissertação. A candidata contribuiu ainda ativamente na redação dos trabalhos apresentados.

O caráter interdisciplinar e inovador desta investigação foi possível devido à estreita interligação entre as diferentes áreas relacionadas com o tema, permitindo esta abordagem um olhar integrativo sobre o mesmo. Dessa forma, foi de extrema relevância a cooperação com os centros de referência: REQUIMTE – Instituto Superior de Engenharia do Instituto Politécnico do Porto; Departamento de Cirurgia Geral da Faculdade de Medicina da Universidade do Porto; Serviço de Endocrinologia e Serviço de Imuno-Hemoterapia do Centro Hospitalar de São João – EPE e CINTESIS – Centro de Investigação em Tecnologias e Sistemas de Informação em Saúde.

17β-hydroxysteroid dehydrogenase	**17β-HSD**
alpha serine/threonine-protein kinase	**AKT**
activator protein	**AP**
adipose tissue	**AT**
adipose tissue macrophage	**ATM**
brominated flame retardants	**BFR**
body mass index	**BMI**
bisphenol A	**BPA**
crown-like structures	**CLS**
C-reactive protein	**CPR**
cAMP response element-binding	**CREB**
cardiovascular disease	**CVD**
di-*n*-butyl phthalate	**DBP**
dichlorodiphenyldichloroethylene	**DDE**
dichlorodiphenyltrichloroethane	**DDT**
di-ethyl-2-hexyle phthalate	**DEHP**
dipeptidyl peptidse	**DPP**
17β-estradiol	**E_2**
estrogen receptor	**ER**
extracellular signal-regulated kinases	**ERK**
estrogen-related receptor	**ERR**
G protein-coupled ER	**GPER**
high density lipoprotein	**HDL**
hexachlorobenzene	**HCB**

hexachlorocyclohexane	**HCH**
heat-shock protein	**HSP**
insulin-like growth factor	**IGF**
IκB kinase	**IKK**
interleukin	**IL**
inducible nitric oxide synthase	**iNOS**
c-jun N-terminal kinase	**JNK**
lipoprotein lipase	**LPL**
lipopolysaccharide	**LPS**
classically-activated macrophages	**M1**
alternatively-activated macrophages	**M2**
mitogen-activated protein kinase	**MAPK**
metabolically abnormal obesity	**MAO**
monocyte chemotactic protein	**MCP**
metabolically healthy obese	**MHO**
nuclear factor-κ B	**NF-κB**
plasminogen activator inhibitor-1	**PAI-1**
polychlorinated biphenyls	**PCB**
phosphatidylinositide 3-kinases	**PI3K**
persistent organic pollutant	**POP**
peroxisome proliferator-activated receptor	**PPAR**
regulated on activation, normal T cell expressed and	**RANTES**
subcutaneous adipose tissue	**scAT**
specificity factor	**SP**
type 2 dabetes	**T2D**
transforming growth factor	**TGF**
T-helper 1	**Th1**
T-helper 2	**Th2**
tumor necrosis factor alpha	**TNF-α**

visceral adipose tissue	**vAT**
vascular endotelial growth factor	**VEGF**
world health organization	**WHO**
xenoestrogen	**XE**

CONTENTS

ABSTRACT/ RESUMO

ABSTRACT

Environmental factors used in consumer products or manufacturing (e.g. in plastics, detergents, surfactants, pesticides and industrial chemicals) are thought to have estrogenic activities. These xenoestrogens (XEs) are chemicals derived from a variety of natural and anthropogenic sources that can interfere with endogenous estrogens by either mimicking or blocking their responses via non-genomic and/or genomic signaling mechanisms. There is evidence and an ongoing discussion that XEs may affect human health, increasing the incidence of endocrine disorders and have recently emerged as a new risk factor for obesity and cardiovascular disease (CVD).

In this regard, we evaluated XE (aldrin, *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), hexachlorobenzene (HCB), sum of hexachlorocyclohexane (∑HCH: α-HCH, β-HCH and δ-HCH), and lindane) levels in plasma and in adipose tissue (AT) in a sample of Portuguese obese patients that underwent bariatric surgery. Its putative association with metabolic and inflammatory parameters and 10-year CVD risk (calculated throught the Framingham Risk Score) was assessed, according to menopausal status. We also investigated the multiple actions of XEs (bisphenol A (BPA), di-*n*-butyl phthalate (DBP) and di-ethyl-2-hexyle phthalate (DEHP)) on human monocyte-derived macrophage migration, cytokine release and estrogen-related signaling pathways.

Plasma and AT (from visceral (vAT) and subcutaneous (scAT) depots) samples from obese (BMI ≥ 35 kg/m^2) premenopausal (n=73) and postmenopausal (n=48) women were collected and the levels of XEs were determined by gas chromatography with electron-capture detection. Anthropometric and biochemical data were assessed prior to surgery. Additionally, human peripheral blood monocyte-derived macrophages after classical M1 (lipopolysaccharide, 100 ng/mL) or alternative M2 (IL4 (15 ng/mL)) activation were treated with 17β-estradiol (E_2) or XE alone or in combination with selective estrogen receptor (ER) α or ERβ antagonists. Macrophage cytokine release, migratory capability and estrogen-associated signaling pathways were evaluated.

Our data show that XEs are pervasive in the samples assessed and the distribution and concentration of individual and total XEs differed between plasma, vAT and scAT. Furthermore, the pattern of accumulation was different between pre- and postmenopausal women. In premenopausal women, XEs accumulate preferentially in vAT and although XEs in this location showed a better correlation with metabolic dysfunction, plasma XEs were the best predictors of inflammation and 10-year CVD risk. We also advance that plasma XEs in these women may be released from vAT as consequence of local toxicity and AT dysfunction induced by XEs. Additionally, we observed that postmenopausal women had a worst CVD risk profile.

Aiming at assessing the interference of XEs with inflammatory cells implicated in metabolic inflammation and cardiometabolic risk, we demonstrated that ERs are differently expressed among different stages of macrophage polarization. ERα was expressed in higher levels in macrophages of either subset and most of the effects have been related to this isoform; however, ERβ may also play a role. All tested XEs had a tendency to stimulate M2 migration, an effect that followed the same direction than that of E_2. E_2 significantly induced M1 release of IL10 and IL6 and decreased IL10, IL6, tumor necrosis factor (TNF)-α and IL1β release by M2 macrophages. Moreover, all XEs significantly induced IL10 and decreased IL6 without changing TNF-α release by M1 cells and globally decreased IL10, IL6, TNF-α and IL1β release by M2 macrophages. However, DEHP and DBP significantly increased IL1β release in M1 and M2 macrophages, respectively. We point out that the previous exposure to the lack/decrease of estrogen signaling, since these cells were obtained from postmenopausal women, may render cells to a more pro-inflammatory situation, which may contribute to the manifestation of chronic inflammatory actions. Most effects of E_2 as well as of XEs were shown to be mediated by either ERα or ERβ and/or by orchestrating nuclear factor-κ B, activator protein 1, c-jun N-terminal kinase and extracellular signal-regulated kinases signaling pathways.

This work increased the knowledge on XEs ability to mediate obesity-associated inflammation and related metabolic abnormalities, according to menopausal status. We advance a number of potential effects of XEs, highlighting the fact that their hormone-related effects are likely dependent of endogenous hormonal milieu. From the amount of evidence being presently accumulated, it is becoming clearer that neglecting the novel and emerging knowledge about XEs and metabolic disease will have significant health impacts for the general population, as well as on the generations to come.

RESUMO

Diversos fatores de origem ambiental como os compostos utilizados em produtos de uso diário ou na indústria (por exemplo, em plásticos, detergentes, surfactantes, pesticidas e outros produtos químicos industriais) podem apresentar atividade estrogénica. Os xenoestrogénios (XEs) são compostos, de origem natural ou antropogénica, que podem interferir com a sinalização estrogénica, mimetizando ou bloqueando as respostas às hormonas endógenas através de mecanismos genómicos e/ou não-genómicos. Com base na evidência científica atual, assiste-se a uma discussão sobre o impacto da exposição humana a XEs na saúde, sendo plausível uma associação positiva com a incidência de doenças e complicações endócrinas. Assim sendo, estes compostos emergiram como um novo fator de risco para a obesidade e para as doenças cardiovasculares.

Por conseguinte, foram avaliados os níveis de XEs (aldrina, *p,p'*-diclorodifenildicloroetileno (*p,p'*-DDE), hexaclorobenzeno (HCB), somatório de hexaclorociclohexano (ΣHCH: α-HCH, β-HCH e δ-HCH), e lindano) no plasma e no tecido adiposo de uma amostra de indivíduos com obesidade mórbida, e doença metabólica associada, submetidos a cirurgia bariátrica. Avaliou-se a sua associação com parâmetros metabólicos e inflamatórios e o risco de desenvolver doença cardiovascular a 10 anos (calculado a partir do *Score* de *Framingham*) em mulheres pré- ou pós-menopausa. Por outro lado, investigaram-se os efeitos de diferentes XEs (bisfenol A (BPA), dibutilftalato (DBP) e dietil-2-hexilftalato (DEHP)) na capacidade de migração, na libertação de citocinas e nas vias de sinalização associadas aos estrogénios em macrófagos derivados de monócitos humanos.

Amostras de plasma e de tecido adiposo (subcutâneo e visceral) de mulheres obesas (IMC ≥ 35 kg/m^2) na pré- (n=73) ou pós-menopausa (n=48) foram recolhidas e os níveis de XEs foram determinados por cromatografia gasosa com deteção eletroquímica. Os dados antropométricos e bioquímicos foram avaliados no momento da cirurgia. Adicionalmente, usando um modelo simples de cultura celular, trataram-se com 17β-estradiol (E_2) ou XEs isoladamente ou em combinação com antagonistas seletivos do recetor de estrogénios (ER) α ou ERβ, macrófagos derivados de monócitos do sangue periférico humano ativados pela via clássica M1 (lipopolissacarídeo, 100 ng/mL) ou alternativa M2 (interleucina (IL) 4, 15 ng/mL). A libertação de citocinas, a capacidade de migração e as vias de sinalização associadas aos estrogénios foram avaliadas.

Os nossos dados mostram que os XEs estão extensamente presentes nas amostras obtidas da população obesa estudada e o perfil individual ou total de distribuição e concentração dos XEs difere entre o plasma, o TAV e o TASC. Além disso, o padrão de acumulação foi diferente entre mulheres pré- e pós-menopausa. Nas mulheres pré-menopausa, os XEs acumulam-se preferencialmente no TAV e, ainda que o somatório de XEs nesta localização se correlacione com disfunção metabólica, os XEs de plasma apresentam-se como os melhores preditores de inflamação e de risco de doenças cardiovasculares a 10 anos. Foi também

proposto que os XEs no plasma destas mulheres podem ter origem no TAV, como consequência da toxicidade local e consequente disfunção induzida pelos XEs. Além disso, observou-se que as mulheres em pós-menopausa apresentavam maior prevalência de risco de cardiovascular a 10 anos.

Com o objectivo de determinar a interferência dos XEs com as células inflamatórias implicadas na disfunção metabólica e no risco cardiometabólico, demonstrou-se que a transcrição do ERα e do ERβ é diferente de acordo com o estado de ativação dos macrófagos. O ERα foi o mais expresso nos dois subtipos de macrófagos, mediando a maioria dos efeitos; no entanto, o ERβ pode também desempenhar um papel relevante. Todos os XEs testados apresentavam uma tendência para estimular a migração dos macrófagos M2, mimetizando o efeito do E_2. O E_2 induziu significativamente a liberação de IL10 e IL6 nos macrófagos M1 e diminuiu a libertação de IL10, IL6, fator de necrose tumoral (TNF)-α e IL1β pelos macrófagos M2. Além disso, todos os XEs induziram significativamente a libertação de IL10 e diminuíram a de IL6 sem alterar o TNF-α nos macrófagos M1 e, globalmente, diminuíram a libertação de IL10, de IL6, de TNF-α e de IL1β nos macrófagos M2. No entanto, o DEHP e o DBP aumentaram significativamente a libertação IL1β em macrófagos M1 e M2, respetivamente. Destacamos ainda que a exposição prévia a um ambiente com ausência/diminuição da sinalização mediada pelos estrogénios, visto que as células foram obtidas de mulheres pós-menopausa, pode predispor as células a um estado mais pró-inflamatório, o que pode contribuir para a activação inflamatória crónica. Mostramos ainda que muitos dos efeitos atribuídos ao E_2, bem como os efeitos dos XEs, foram mediados quer pelo ERα ou ERβ e/ou orquestrados pelas vias de sinalização do fator nuclear-κ B, da proteína de ativação 1, da cínase c-jun N-terminal e das cínases reguladas por sinais extracelulares.

Este trabalho permitiu ampliar o conhecimento sobre a capacidade dos XEs mediarem a inflamação associada à obesidade e as complicações metabólicas com ela relacionadas, de acordo com estado de menopausa. São revelados diferentes efeitos possíveis dos XEs, destacando-se que os seus efeitos alteradores endócrinos são provavelmente dependente do ambiente hormonal endógeno. Em última análise, estes resultados tornam evidente que negligenciar o mais recente conhecimento sobre a relação dos XEs com a doença metabólica poderá ter um impacto negativo na saúde da população em geral, bem como nas gerações futuras.

CHAPTER I

INTRODUCTION

AIMS

Obesity and the adipose tissue

Obesity is most definitely a multifactorial disease which is caused by a complex interaction between genetic, behavioral, and environmental factors. The most common causes are thought to be overeating high caloric diets combined with a sedentary lifestyle which is imposed on a background of genetic predisposition for the disease. However, these factors alone fail to solely explain the alarming rate and magnitude of the obesity epidemic [1]. Prevalence of obesity has increased worldwide being a source of concern since the negative consequences of obesity start as early as in childhood [2]. According to the World Health Organization (WHO), 35% of adults worldwide aged more than 20 years were overweight (34% men and 35% women) in 2008, including 10% of men and 14% of women being considered obese [3]. In Portugal, 59% of adults aged more than 20 years were overweight (64% men and 55% women) in 2013 including 21% of obese men and 23% of obese women [4]. New hypotheses are emerging to explain the etiopathogenesis of this condition, including stress, immunological alterations, micronutrient deficits, environmental chemicals and changes in gut microbiota [5, 6].

Increased storage of triacylglycerols leading to an expanded adipose tissue (AT) mass characterizes obesity. Past theories postulated that the main function of AT was the storage of energy under conditions of excess calories and their release in periods of fasting. Later, thermoregulation and mechanical organ protection were added [7]. The subsequent identification and characterization of leptin in 1994 firmly established AT as an endocrine organ [8]. Noteworthy, AT has been acknowledged as the largest endocrine organ secreting several hormones such as leptin and adiponectin, pro- and anti-inflammatory factors, growth factors and complement proteins, which act at both the local (autocrine/paracrine) and systemic (endocrine) level [9, 10]. The important endocrine function of AT is emphasized by the adverse metabolic consequences of both AT excess and deficiency [11].

Far from being a simple organ, AT exhibits a marked heterogeneity both in terms of its anatomical location and in relation to its cellular composition [12]. There are two major anatomic subdivisions of AT, each with unique anatomic, metabolic, endocrine, paracrine, and autocrine properties: visceral (v)AT and subcutaneous (sc)AT. scAT stores more than 80% of total body fat in the body. The most commonly defined and studied subcutaneous sites of accumulation are the abdominal, gluteal and femoral depots. vAT is mostly associated with digestive organs, and include omental, mesenteric, and epiploic AT [13]. The size of each fat compartment results from the integration of the size and number of lipid-laden adipocytes, which represent the main cellular component of AT. However, the adipose organ is composed by various cell types including not only adipocytes but also a stromal vascular fraction which includes preadipocytes, fibroblasts, vascular endothelial cells, pericytes and different immune cells (macrophages,

T-cells, neutrophils, lymphocytes) [9]. These later cellular components can be separated from mature adipocytes by digestion and centrifugation [14]. Differences in preadipocyte populations have been reported, scAT containing a higher number of preadipocytes with higher proliferative capacity, which contributes to higher plasticity and adipogenic potential of this AT depot [15, 16], mainly in women [17]. In general, expansion of AT can occur either by an increase in volume of preexisting adipocytes (hypertrophy) or by hyperplasia in which an increase in AT occurs through recruitment of new preadipocytes. The association between number or size of adipocytes and obesity level is modulated as a function of anatomical localization and sex. Tchoukalova *et al.* [18] have shown that lower AT compartments of obese women contain more adipocytes than those of lean women, whereas obesity is not related to cell number in lower-body compartments in men. These results may suggest that during weight gain, lower-body AT tends to expand through hyperplasia in women, but through hypertrophy in men [19]. With regard to visceral adipocyte size and number, adipocytes are 20%-30% smaller in omental AT when compared to abdominal scAT in women, while they are similar in size in men [20, 21]. Additionally, in lean to moderately obese individuals of both sexes, a strong correlation is observed between abdominal subcutaneous adipocyte size and total AT, suggesting that expansion of abdominal AT may occur by adipocyte hypertrophy in both sexes [18]. Thus, these findings suggest that in women, expansion of the scAT relies more heavily on adipocyte hyperplasia than vAT, which may be predominantly hypertrophic [22]. On the other hand, AT cell hypertrophy or hyperplasia is influenced by vascular supply. In humans, scAT has higher capillary density and angiogenic growth capacity when compared to samples taken from the visceral depot [23]. Further, adipocyte hypertrophy is related with impaired AT function, whereas data linking adipocyte hyperplasia to metabolic alterations are more limited [24, 25]. Another interesting point is that the number of stromal cells (non-adipocytes, mainly macrophages) per gram of AT is greater in omental vAT than abdominal scAT [26, 27]. Thus, central obesity, particularly visceral obesity, but also including accumulation in abdominal scAT (apple-shaped, android obesity), confers increased risk for metabolic complications of obesity, whereas peripheral obesity, mostly accumulation in gluteofemoral region (pear-shaped, gynoid obesity), is associated with lower risk and may be even protective [28]. Moreover, during progression of obesity, there are changes in the number, phenotype, activity and location of immune, vascular and structural cells.

Adipose tissue macrophages and obesity-related inflammation

Metabolic diseases and obesity are accompanied by major alterations of AT functions such as a state of chronic low-grade inflammation. It is well established that during the course of obesity, when the ability of expansion of AT reaches the limit to accommodate the excess of nutrients, functional and morphological changes may occur, regulating the quantity and quality of all its cellular components, together with changes in adipokine secretion profile with unbalanced production of pro- and anti-inflammatory factors

[29-33]. These inflammatory molecules contribute to a marked disturbance of paracrine dialogues, which favor the alteration of AT biology. It has been highlighted that obesity-associated inflammation does not meet the criteria of classical inflammation [31]. In fact, inflammatory activation is often modest and local, being mainly triggered by metabolic surplus with metabolic specialized cells, such as adipocytes activating cellular stress pathways, thus initiating and sustaining the inflammatory program [31, 34, 35]. In fact, the number of AT macrophages (ATM) is increased in obesity which has been set into attention by the work of Weisberg *et al.* [36]. ATMs, like other macrophages, arise from a lineage that includes bone marrow precursors and blood monocytes [37]. Since macrophages were considered to be terminally differentiated cells and postmitotic [38], mechanisms of increased macrophage number in AT during obesity include both enhanced recruitment of blood-borne monocytes [39] by chemoattractant proteins including monocyte chemotactic protein (MCP)-1, chemerin, progranulin, colony stimulating factor 1 and local proliferation [40, 41].

Under normal AT functioning, resident macrophages release anti-inflammatory cytokines and are involved in non-immunological processes, such as remodeling (repair) of AT and lipid metabolism, helping to preserve normal adipocyte function [42].

During the development of obesity, dynamic changes in the number of ATMs and also of their phenotype occur in a way that mirrors the concept of T-cell activation [36, 43]. M1 or “classically activated” macrophages are produced upon exposure to T-helper 1 (Th1) type cytokines or inflammatory mediators such as lipopolysaccharide (LPS), express CD11c in addition to CD11b and F4/80 and are involved in inflammatory processes (such as combating infectious agents) [44]. Thereafter, they release inflammatory cytokines such as interleukin (IL)-6 or tumor necrosis factor (TNF)-α and generate reactive oxygen species. M2 or “alternatively activated” macrophages are produced upon exposure to T-helper 2 (Th2) cytokine such as IL4 or IL13, express phenotypically CD206, CD11b, F4/80 and CD301 markers and factors including IL10, arginase and play a role in immunosuppressive activities (namely as tissue repair) [45-47]. Polarization of macrophages in AT is thought to confer similar properties. As mentioned above, for example M2 macrophages predominantly make up the tissue-resident macrophages and support AT homeostasis, while M1 macrophages in obese AT likely promote inflammation leading to metabolic inflammation [48]. The secretion of inflammatory cytokines and chemokines by ATMs is under the control of two key intracellular inflammatory pathways, c-Jun N-terminal kinase (JNK) - activator protein (AP)-1 and IκB kinase (IKK) β - nuclear factor-κ B (NF-κB), which are initiated by different mediators including oxidative and endoplasmic reticulum stresses, saturated fatty acids, and inflammatory cytokines but converge on the induction of overlapping inflammatory genes [49].

As obesity develops with excess of energy storage, adipocytes will expand by increasing their size and/or increasing their number. Enlarged adipocytes become stressed and subsequently attract immune cells. Several changes in cellular immune processes have been reported. One may be increased infiltration of

macrophages in AT and an increase in the number of generally pro-inflammatory M1 macrophages. It is unclear what initially triggers M1 activation but nutritional signals and/or reduced angiogenesis and local adipose cell hypoxia from adipocyte hypertrophy have been pointed as possible culprits. This increased infiltration of macrophages in inflamed AT, mainly in vAT, contributes to exacerbation of inflammation [50]. In several murine models of diet-induced obesity, a switch in ATM polarization state from M2 to M1 was demonstrated [51]. Newly recruited ATMs display a more pro-inflammatory profile than resident ATMs in lean mice [44] and resident macrophages express genes characteristic of M2 activation state. Nevertheless, ATM subsets show no restrict M1 or M2 polarization [52, 53].

Increases in adipocyte diameter may also contribute to increased macrophage infiltration in the AT since hypertrophic adipocytes are prone to apoptotic and necrotic degeneration where adipocytes will be surrounded by macrophages, forming crown-like structures (CLS). The inflammatory response is amplified, since, for each dead adipocyte, several macrophages are recruited, perpetuating dysfunction in a vicious cycle [54]. Finally, changes in other AT immune cell populations such as an increase in the number of B cells and T CD8+ cells infiltration and a decrease in the number of regulatory T cells may also occur [55, 56].

All changes in AT inflammation including the increase in adipocyte size, increase in the number of infiltrating macrophages and CLS are associated with the increased inflammatory cytokines, chronic low grade-inflammation and comorbidities associated with obesity [57, 58]. Importantly, ATMs may signal AT stress and inflammatory status to other organs and could thereby contribute to the inter-organ cross-talk linking obesity to second organ dysfunction [59]. Evidence presented above suggests that macrophages have paramount importance in the genesis of obesity complications.

Adipocyte dysfunction, inflammation and cardiometabolic risk

It has been suggested that this low-grade inflammation is also a major player in the installation of the metabolic syndrome, a cluster of risk factors including central obesity, impaired glucose tolerance or diabetes, hypertension, and dyslipidemia with high plasmatic concentration of triglycerides and low concentrations of high density lipoprotein (HDL) cholesterol, all which can contribute to the increased risk in developing type 2 diabetes (T2D) and cardiovascular disease (CVD) [54, 60].

Following the onset of obesity, the secretory status of adipocytes can be modified by the changes in the cellular composition of the tissue, including alterations in the number, phenotype and localization of immune, vascular and structural cells. Thus, via the secretion of pro-inflammatory adipokines and emigrating immune cells, including macrophages, “inflamed” AT may signal its functional status to other organs. In fact, adipokines may play specific roles not only in immune response (e.g. ILs,

adipsin/complement factor D, acylation-stimulating protein, serum amyloid A3) and inflammation (e.g. IL1β, IL6, IL8, IL10, C-reactive protein (CRP), MCP-1, osteopontin, progranulin, chemerin) but also in glucose metabolism (e.g. leptin, adiponectin, dipeptidyl peptidase (DPP)-4, fibroblast growth factor, resistin, vaspin), lipid metabolism (e.g. CD36), insulin sensitivity (e.g. leptin, adiponectin, chemerin), adipogenesis and bone morphogenesis (e.g. bone morphogenetic protein-7), regulation of appetite and satiety (e.g. leptin, vaspin), vascular tone (e.g. angiotensinogen), cell adhesion (e.g. plasminogen activator inhibitor (PAI)-1), vascular growth and function (e.g. vascular endothelial growth factor (VEGF)), atherosclerosis development (e.g. cathepsins, apelin) growth (e.g. insulin-like growth factor (IGF)-1, transforming growth factor beta (TGF)-β, fibronectin), among other biological processes. An adipokine pattern with low circulating adiponectin and high concentrations of chemerin, IL6, TNF-α, CRP, DPP-4 and others may reflect AT dysfunction [41, 61]. This diabetogenic, pro-inflammatory and atherogenic adipokine pattern may link adipocyte dysfunction to the metabolic syndrome.

In addition to changes in adipokine secretion, impaired expandability of scAT, ectopic fat deposition and hypoxia may contribute to AT inflammation or dysfunction. The AT expandability hypothesis infers that, for some obese individuals, scAT may reach its maximal storage capacity, the excess of lipids then being reoriented, and thus excess visceral adiposity may be a marker of the relative inability of the scAT to expand through hyperplasia in face of a positive energy balance. Furthermore, as a hypertrophic AT cannot properly expand to store extra dietary lipids, there is, as result, a spillover of its lipid content leading to accumulation at undesired sites (heart, liver, skeletal muscle, pancreas) through a process called ectopic fat deposition [62, 63]. It has been therefore suggested that impaired subcutaneous vascularisation capacity may contribute to lower scAT expandability and metabolic diseases [23]. On the other hand, this enlargement of adipocyte size may result in a shift toward a predominance of pro-inflammatory factors including leptin, IL6, IL8, MCP-1 [64], reduced levels of the insulin-sensitivity-related adipokine adiponectin and IL10 [64, 65], and have increased basal and catecholamine-stimulated lipolysis [66].

Conversely, from 10 to 25% obese individuals are characterized by increased lipid deposition in the scAT depot and conserved AT functions, and these are considered metabolically healthy obese (MHO). In general, healthy obesity describes the absence of any metabolic disorder including T2D, dyslipidemia or hypertension in an obese individual (BMI >30 kg/m^2) [67, 68], thus suggesting that obesity is not equivalent to metabolic dysfunction. Recent studies revealed that independent of body mass index (BMI), age and sex, increased visceral fat accumulation, inflammation in vAT and insulin resistance resulting from AT dysfunction may contribute to unhealthy obesity [69]. However, the causal factors leading to transitions between the MHO to the metabolically abnormal obese (MAO) phenotype are poorly understood. For the reasons described above, the MHO phenotype has received increasing interest in recent years, and indeed, stratification of obesity phenotypes could help improve therapeutic decision-making.

An often underappreciated finding is that AT function and deposition differ with sex. Indeed, the marked sex dimorphism in body fat patterning in humans indirectly suggests that sex hormones play a key role in regional fat accumulation. Women have an overall higher total body fat content when compared to men. Importantly, women differ with respect to distribution of AT. Men tend to accrue more vAT, leading to classic android body shape, which has been highly correlated with CVD risk, whereas premenopausal women have a greater proportion of body fat accumulated in subcutaneous (gluteofemoral) location, a feature associated with protection from negative consequences associated with obesity, although in both genders the subcutaneous is the main site of accumulation [70, 71]. In women, estrogens may protect against increased body adiposity through their effects in appetite suppression (enhancing potential anorexigenic signals, such as cholecystokinin, apolipoprotein A-IV, leptin, brain derived neurotrophic factor, and by decreasing the potency of orexigenic signals such as melanin-concentrating hormone and ghrelin) [72-74] and increase energy expenditure [75, 76]. Interestingly, as a woman reaches menopause, body fat is redistributed, depot differences in adipocyte size tend to be attenuated, since the size of omental cells seem to be specially increased. Women tend to acquire a body fat accumulation pattern similar to men [71, 77], and this redistribution may be linked to the age-related decrease in estrogens levels [78], with the shift to favor the visceral depot being accompanied by a parallel increase in metabolic risk reminiscent to that seen in men. Premenopausal women are less prone to develop cardiovascular and metabolic disease than age-matched men. It is generally accepted that a great part of the increase in cardiometabolic risk after menopause is accounted for by the decline in ovarian production of estrogens [79], illustrating estrogens' protective effects on the cardiovascular system [80, 81], whereas men and postmenopausal women, whose estrogen levels are low, have increased metabolic risk translated into higher likelihood to develop CVD [82].

In parallel, epidemiological studies show that premenopausal women are less likely to develop inflammation compared to age-matched men, suggesting a protective effect of estrogens also against inflammation [83, 84]. The higher propensity for developing abdominal adiposity of postmenopausal women may help explain their increased systemic levels of inflammatory cytokines, also indicating that estrogens can modulate both body adiposity and systemic inflammation [85].

Estrogen metabolism and signaling

At present, it is well recognized that the impact of estrogens in humans is wider than their effect on reproduction, due to their participation in the regulation of a diversity of functions including the differentiation of several tissues, the modulation of inflammation and metabolic processes.

Estrogens are synthesized by aromatase, a cytochrome P450 enzyme located in the endoplasmic reticulum of estrogen producing cells which catalyzes the aromatization of testosterone and androstenedione [86],

to 17β-estradiol (E_2), the most active estrogen, and estrone, respectively [87]. In humans, estrogens are generated in several tissues including ovaries, testis, placenta, fetal but not adult liver, bone chondrocytes and osteoblasts, vascular smooth muscle cells, skin, skeletal muscle, several brain regions, and the AT [86, 88, 89]. The gonads and the adrenals express all the necessary enzymes to synthesize estrogens from cholesterol whereas other tissues, such as the bone or the AT, depend on precursor supply from those organs through the blood [90]. In peripheral tissues, 17β-hydroxysteroid dehydrogenase (17β-HSD) converts the weaker hormones androstenedione and estrone to stronger ones, testosterone and E_2, respectively.

In premenopausal women, most estrogens are produced in the ovaries, released to the circulation in large amounts and exert their effects in many target tissues through endocrine signaling. After menopause, when estrogen production from the ovaries falls, circulating levels of estrogens decrease dramatically [91] and estrogen synthesis is mainly assured by peripheral sources, primarily by the AT [92]. Estrogens are also produced during the entire male life cycle, having an action pattern similar to that observed in postmenopausal women in which endocrine actions lose relevance to paracrine, autocrine, and intracrine actions in the tissues or in the cells that produce them [86]. Thus, regardless of low circulating levels, local estrogen concentrations may be high, reflecting on their biological actions [93].

Estrogen distribution occurs after estrogen sulfotransferase action which constitutes a critical mediator of hormone effects [71]. Although estrogen sulfates are biologically inactive, they have prolonged half-life in the circulation, acting as a reservoir for regeneration of active estrogens by steroid sulfatase-mediated desulfation [94].

Two basic estrogen receptors (ERs) are important in estrogen signaling: the ERα and ERβ. Both ERs are widely distributed throughout the body, displaying distinct but overlapping expression patterns in a variety of tissues [95]. ERs function as ligand-related transcription factors, regulating numerous important physiological process such as the development, reproduction, behavior, metabolism, and regulation of the cardiovascular system [96].

Estrogen signaling mediated through both ERs by classical genomic and non-genomic mechanisms, although largely resulting in activation of distinct targets may also have coinciding activities (Figure 1). Classical genomic signaling through the ERs occurs within hours of ligand binding, activating or repressing target genes. ERs are located as monomers in the cytoplasm in protein complexes involving chaperone heat-shock proteins and estrogen binding promotes their dissociation from this complex and ER dimerization (homodimers of ERα or ERβ or heterodimers of ERα-ERβ). ER dimers bind directly to estrogen response elements of target gene promoters, or indirectly through interaction with other DNA-bound transcription factors like AP-1, NF-κB, and specificity factor (SP)-1 [97, 98].

ERs also regulate gene expression in a ligand independent manner by interacting with other nuclear hormone receptors, such as peroxisome proliferator-activated receptors (PPARs) [99]. After DNA binding, ER dimers regulate gene expression interacting with cofactors (coactivators or cosuppressors) [71, 100, 101]. The nature and concentration of the ligand, the type of dimer formed, the kind of DNA interactions (direct or indirect), and the presence of distinct cofactors according to cell type or condition, all constitute sources of variation of ER-activated transcriptional activity. Furthermore, there appears to be ERα dominance since when both receptors are present this receptor seems to be the driver of the response for either genomic or non-genomic responses, while ERβ when in the presence of ERα tends to antagonize its responses [102]. While most of the reproductive effects of estrogens are mediated through classical ER signaling, metabolic effects seem to be largely mediated through non-nuclear ERs and involves activation of ERs located at the membrane or at extranuclear sites within seconds or minutes [71, 77]. Mechanisms of non-genomic actions are not completely known but second messenger activation after estrogen binding results in changes in Ca^{2+}, K^{+}, cAMP, and nitric oxide levels, action of G protein-mediated events and stimulation of different types of kinases such as extracellular signal-regulated kinases (Ras/Raf/MEK/ERK), phosphoinositide 3-kinases (PI3K), p38 mitogen-activated protein kinase (MAPK), and JNK [103, 104]. MEK/ERK pathway activation also regulates gene expression through activation of transcriptional factors such as cAMP-related element binding (CREB) protein or nuclear factor of activated T-cells [105]. With much relevance to the inflammation modulatory properties of estrogens is their capacity to influence NF-κB, inhibiting directly or indirectly part of its negative effects [101].

G protein-coupled receptor 30, now known as G protein-coupled ER (GPER), located on the plasma membrane [106] or with an intracellular localization [107] is also involved in rapid non-genomic estrogen signaling involving release of intracellular Ca^{2+} and activation of calcium-calmodulin-dependent kinases, PI3K and MAPKs [100]. Estrogen-related receptors (ERRs), a family of orphan receptors closely related to ERs, includes three members, ERRα, ERRβ, and ERRγ, and are expressed in muscle, heart, bone, and AT [108]. These nuclear receptors are not stimulated by estrogens or estrogen-like molecules, being constitutively active in the absence of ligand [109]. However, ERRs can interfere with estrogen signaling, recognizing the same DNA-binding elements as ERs and sharing common target genes [108]. ER and ERR are coexpressed in many tissues and ERRs play important functions in adaptative energy metabolism [109].

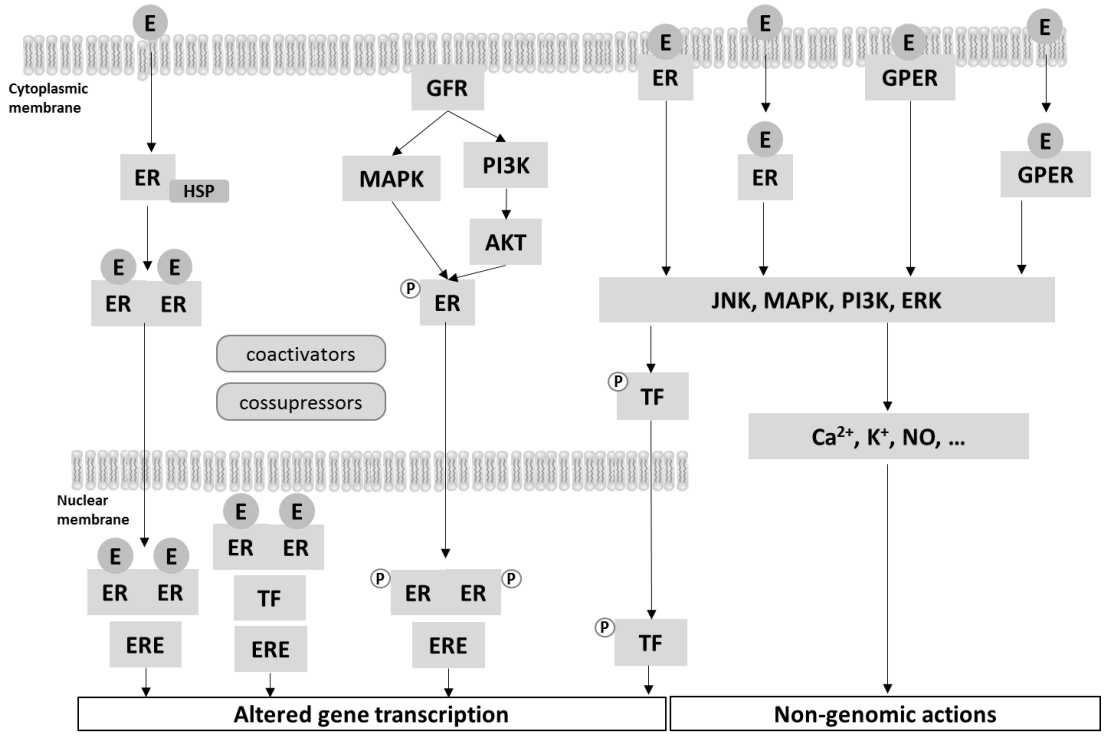


Figure 1. Schematic illustration of how genomic and non-genomic actions of ERs on target gene promoters may converge. Estrogen signaling occurs through both genomic and non-genomic mechanisms. In classical, genomic, estrogen signaling ERs act as ligand-activated transcription factors, activating or repressing target genes within hours of ligand binding. ERs are located as monomers in the cytoplasm in protein complexes involving heat-shock proteins and estrogen binding promotes their dissociation from this complex and ER dimerization. ER dimers bind directly to estrogen response elements of target gene promoters, or indirectly through interaction with other DNA-bound transcription factors. ERs also regulate gene expression in a ligand independent manner being activated downstream to growth factors binding to growth factor receptors, through the action of intracellular kinases or through the formation of heterodimers with different nuclear receptors (not shown). Genomic actions are modulated by cell-specific interaction with cofactors (coactivators or cosuppressors). Metabolic effects of estrogens seem to be largely mediated through non-nuclear ERs, either by interference with gene expression or by exerting non-genomic actions. This involves activation of ERs and G-protein-coupled ER located at the membrane or at extranuclear sites within seconds or minutes resulting in changes in Ca^{2+}, K^{+}, cAMP, and nitric oxide levels, activation of G protein-mediated events, and stimulation of different types of kinases such as extracellular-regulated kinases, phosphoinositide 3-kinases, mitogen-activated protein kinase, and c-Jun N-terminal kinases. E: estrogen; ER: estrogen receptor; ERE: estrogen-response element; ERK: extracellular signal-regulated kinase; GFR: growth factor receptor; GPER: G protein-coupled estrogen receptor; HSP: heat-shock protein; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; NO: nitric oxide; PI3K: phosphoinositide-3 kinase; TF: transcription factor. Adapted from Monteiro *et al.* [110]

Adipose tissue and macrophages as targets of estrogens' actions

The influence of hormones on AT distribution appears to be related both to AT-specific expression of steroid hormone receptors and to local tissue steroid hormone metabolism. AT is an important site for estrogen biosynthesis and storage. Adipocytes from different depots differ in their capacity to produce estrogens, as aromatase expression declines from gluteofemoral, to abdominal scAT, to vAT [111]. Moreover, estrogens are secreted from AT in proportion to total fat mass [112] and are thought to signal the size of body energy stores and influence physiological processes through endocrine actions while also exerting important local effects through paracrine and autocrine actions.

Estrogens can directly inhibit AT deposition by decreasing lipogenesis. This effect results mainly from decreasing activity of AT lipoprotein lipase (LPL), an enzyme that regulates lipid uptake by adipocytes. Ovariectomy increases LPL and lipid deposition in adipocytes and administration of physiological doses of E_2 reverses this increase [113]. On the other hand, E_2 can indirectly affect lipolysis by inducing the lipolytic enzyme hormone-sensitive lipase [114] or by increasing the lipolytic effects of adrenaline [115]. Moreover, fatty acid oxidation can also be increased, which might contribute to the decrease in AT mass induced by E_2. However, contrary to its anti-lipogenic and lipolytic effect, estrogens favor the effect of α_2a-adrenergic receptors in human scAT and decrease lipolysis. This effect could in part account for the increased deposition of scAT in women compared to men [116].

ERα and ERβ are expressed in both scAT and vAT in humans and rodents, denoting that in AT estrogen signaling may occur through either of these ERs [117]. ERα is particularly relevant when considering the metabolic actions of estrogens since diminished ERα activity is associated with obesity in women and men. Furthermore, impaired ERα action is a contributing factor in the pathobiology of the metabolic syndrome [118]. Additionally, human inactivating mutations of ERα recapitulate aspects of the metabolic syndrome [119]. Moreover, global deletion of the ERα gene in mice results in increased adiposity in both males and females, with near doubling of the intra-abdominal AT when compared to age-matched wild type mice [120, 121]. Nevertheless, mice with a deletion in ERβ do not have increased adiposity or metabolic derangements. Several studies have shown associations between ERα polymorphisms and increased AT accumulation particularly in the visceral compartment [122]. Moreover, both ERα and ERβ interfere with AT distribution, inflammation, fibrosis and glucose homeostasis. In 2013, Davis *et al.* [123] in a mouse model with ERα inactivation, especially in adipocytes, showed an increase in AT fibrosis and inflammation which was more marked in males, revealing that the protective effects of estrogens on AT are dependent upon adipocyte ERα-mediated signaling.

Furthermore, dos Santos *et al.* [124] advocates that E_2 can also act through membrane ER in adipocytes, to induce rapid effects through activation of MAPK, AP-1 and CREB protein. ERs are also expressed in preadipocytes [125] whereby estrogens can potentially regulate preadipocyte development and adipocyte differentiation. However, AT is much more than adipocytes. In fact, ERα and ERβ are also expressed in

other cell types found in AT, namely vascular endothelium, vascular smooth muscle and immune cells [121]. Thus, the effect of estrogens on AT must be viewed in light of a concerted action in all its different cell types.

The infiltration of AT by macrophages constitutes an important point of possible regulation of both metabolic and inflammatory processes by estrogens. ERα and ERβ are present in monocytes and macrophages, and estrogens activate these cells [126]. With exception of monocytes, peripheral blood mononuclear cells of postmenopausal women have similar ER expression patterns compared with those of premenopausal women. ERα is expressed in higher amounts in monocytes compared to ERβ from postmenopausal women and men, which may result in altered estrogen responsiveness compared with monocytes of premenopausal women [127].

Macrophage differentiation strongly depends upon the local microenvironment. In activated macrophages, estrogens effects are primarily repressive, by inhibiting the expression of cytokines or modulating other inflammatory mediators by ER-dependent and/or non-genomic pathways in response to inflammatory signals [128, 129]. However, the majority of studies have only addressed the effect of estrogens on M1 cells and less is known about the effect on M2 macrophages. The roles of estrogens in macrophage-related inflammation are clearly complex. In mice, estrogens inhibit LPS-induced mouse homologue of MCP-1 in peritoneal macrophages as well as IL6, IL1 and TNF-α in splenic macrophages. Evidence also points to a role for estrogens in regulating monocyte migration behavior [130, 131].

From the evidence presented above, suppression of inflammatory responses represents a promising strategy to combat obesity and associated disorders. Recent studies establish the mechanistic potential for estrogens to affect the inflammatory process. ERα is the predominant receptor in macrophages and its expression is up-regulated by estrogens (in macrophages but not in monocytes), whereas ERβ is predominantly expressed in monocytes and is unaltered by estrogens in either cell type [132].

Xenoestrogens

Apart from endogenously-produced estrogens, a wide array of xenobiotic substances with potential to interfere with estrogens synthesis, storage/release, metabolism, transport, elimination or receptor binding [133, 134] occur in nature. These compounds are either natural or man-made chemicals and are collectively termed xenoestrogens (XEs) or estrogen disruptors. Molecules like phytoestrogens, but also chemicals produced by industrial processes and released into the environment, namely, the plasticizers phthalates or bisphenol A (BPA), organotins, pesticides, dioxins, polychlorinated biphenyls (PCB), brominated flame retardants (BFR), or alkylphenols belong to this group [135]. These XEs have in common phenolic rings and other hydrophobic components (Figure 2), a characteristic they share with

steroid hormones and related nuclear receptor-activating compounds. They also exhibit potent lipophilic properties and long half-lives [136]. Phytoestrogens are XEs found naturally in certain plants, including foods like whole grains, leafy greens, beans, and garlic, and can mimic the action of estrogen, showing some beneficial effect on bone mineral density, insulin resistance, and CVD risk factors in women after menopause [137, 138]. Another class of XEs belongs to the group of heavy metals (i.e., cadmium, mercury, and arsenic). These metals may be involved in occupational or residential exposure [139, 140].

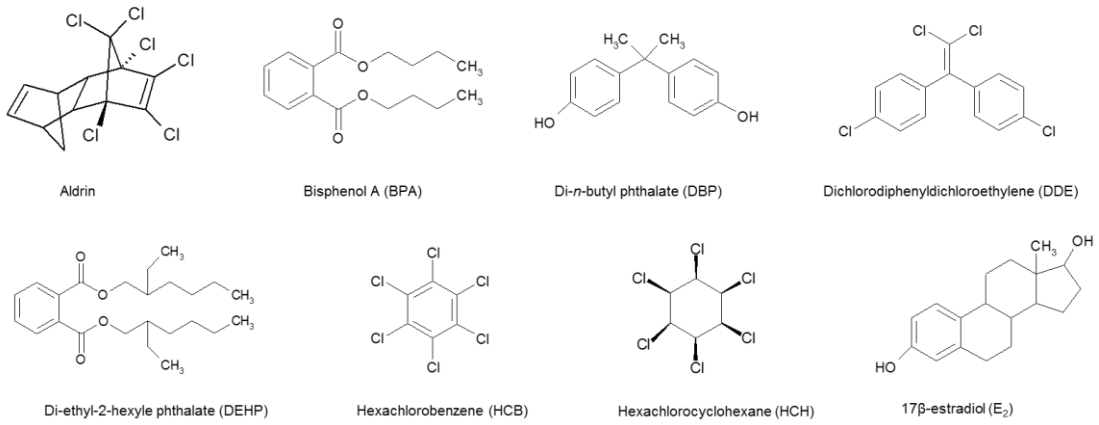


Figure 2. Chemical structures of aldrin, bisphenol A (BPA), di-*n*-butyl phthalate (DBP), dichlorodiphenyldicloroethylene (DDE), di-ethyl-2-hexyle phthalate (DEHP), hexachlorobenzene (HCB), hexachlorocyclohexane (HCH) and 17β-estradiol (E_2).

Some synthetic XEs are persistent in the environment and accumulate in the food chain and in human biological matrices [141]. Epidemiological studies have shown that exposure to XEs is near ubiquitous among humans, and commonly occurs by ingestion of contaminated food or through occupational contact. Environmental estrogens produce non-genomic effects similar, but not identical, to those elicited by E_2, but there is a paucity of data on their ability to mediate non-genomic effects at low concentrations [142-144]. Due to their ubiquity, it is expected that an individual will be contaminated with more than one compound, sometimes from different classes, enabling possible additive or synergistic effects [145].

Such estrogen mimetics were noted for their effect on wildlife in the 1960´s when Rachel Carson drew attention to the endocrine-disrupting effects of some pesticides, notably the organochlorine pesticide dichlorodiphenyltrichloroethane (DDT) [146]. Using cross-sectional data from the 1999–2002 US National Health and Examination Survey, Duk-Hee Lee *et al.* [147] reported a prevalence of a cluster of CVD risk factors that relates to background exposure to a mixture of persistent organic pollutants (POPs), some with xenoestrogenic activity.

Given the lipophilic nature of these contaminants, the AT is a central part of their kinetics on the organism of humans and other animals. Despite the presence of a large number of AT cell types, XE storage in AT is believed to be primarily in the adipocytes [148]; AT storage of a variety of hydrophobic xenobiotic chemicals can be viewed as protective, limiting their systemic toxicity by preventing high blood levels of these XEs and subsequent high exposure of other sensitive organs and tissues, such as the brain, liver and pancreas. However, there are few studies on XE distribution across AT depots and plasma, on the relationship between plasma and tissue levels, or on their different prevalence in different population subgroups.

Furthermore, AT has important endocrine and metabolic functions and could itself be a privileged target of these pollutants (Figure 3) [149-151]. Ample research establishes AT storage of XEs as a central factor in toxicity [149, 152], but significant knowledge gaps remain with regard to mechanisms of action, XE distribution and dynamics in the body, and the molecular pathways disrupted by or involved in XE toxicity.

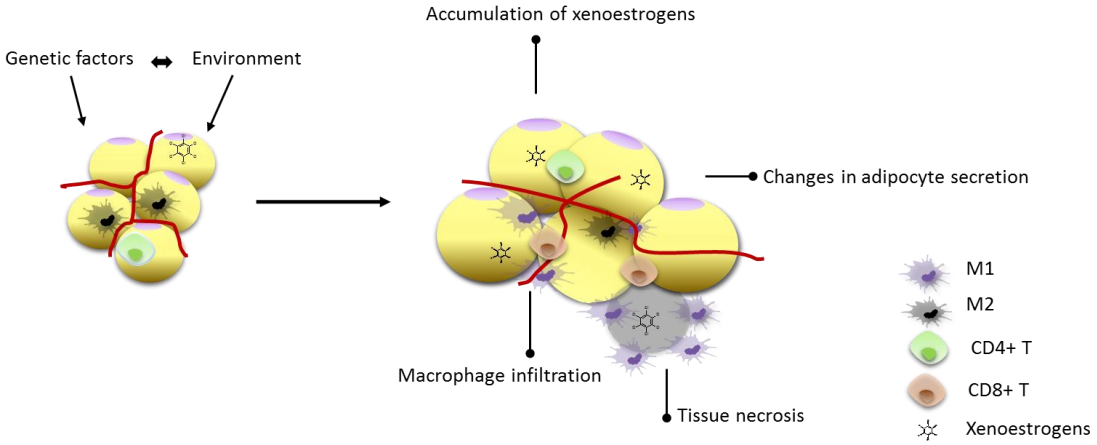


Figure 3. Overview of the changes that occur during adipose tissue growth. Adapted from Myre *et al.* [151]. Adipose tissue (AT) is classically viewed as the main reservoir of energy mobilized from the body. It is composed of a great diversity of cells, such as adipocytes, preadipocytes, vascular cells, neurons, fibroblasts, and immune cells, as resident M2 macrophages. Obesity evolution is characterized by AT growth through adipocyte hypertrophy/hyperplasia, but also by the accumulation of M1 macrophages in AT depots. These macrophages infiltrating the AT are arranged around dead adipocytes, forming characteristic crown-like structures. At the same time, AT store a variety of lipophilic compounds, including xenoestrogens (XEs). The accumulation of XEs within AT is believed to decrease their availability to other cells and tissues, thereby limiting their systemic toxicity. However, XEs released from their storage site in the AT constitute a source of low-level internal exposure, apart from the possibility of exerting local toxicity.

The general aim of this investigation was to assess the effects of endogenous estrogens or xenobiotic molecules with ability to interfere with estrogen signalling systems on the modulation of the low grade systemic inflammatory state that accompanies obesity and the related metabolic abnormalities. The specific aims, related to our different approaches, were the following:

Chapter II

- To determine XEs levels in human samples of blood and AT from subcutaneous and visceral locations of obese women and understanding differences according to menopausal state;
- To search for a putative relationship between plasma and AT XEs with cardiometabolic risk and the possible use of XEs as markers of dysmetabolic obesity;

Chapter III

- To characterize the response of macrophages in different polarization states to estrogens;

Chapter IV

- To determine effect of or XEs in estrogen signaling and behaviour of human macrophages.

CHAPTER II

A- "INFLAMMATORY AND CARDIOMETABOLIC RISK ON OBESITY: ROLE OF ENVIRONMENTAL XENOESTROGENS"

THE JOURNAL OF CLINICAL ENDOCRINOLOGY & METABOLISM, 2015, 100(5):1792-801

DOI: 10.1210/JC.2014-4136

B- "ADIPOSE TISSUE PERSISTENT ORGANIC POLLUTANTS AS POSSIBLE MARKERS OF DYSMETABOLIC OBESITY"

THE JOURNAL OF CLINICAL ENDOCRINOLOGY & METABOLISM, 2014

http://press.endocrine.org/e-letters/10.1210/jc.2013-3935

ORIGINAL ARTICLE

Inflammatory and Cardiometabolic Risk on Obesity: Role of Environmental Xenoestrogens

Diana Teixeira, Diogo Pestana, Cristina Santos, Luísa Correia-Sá, Cláudia Marques, Sónia Norberto, Manuela Meireles, Ana Faria, Ricardo Silva, Gil Faria, Carla Sá, Paula Freitas, António Taveira-Gomes, Valentina Domingues, Cristina Delerue-Matos, Conceição Calhau, and Rosário Monteiro

Departments of Biochemistry (D.T., D.P., S.N., M.M., A.F., R.S., C. Sá, C.C., R.M.), Health Information and Decision Science (C.San.), Endocrinology, Diabetes and Metabolism (P.F.), S. João Hospital General Surgery, Pedro Hispano Hospital (A.T.G.), Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal; Center for Research in Health Technologies and Information Systems (D.P., C.San., G.F., C.C.), 4200-450 Porto, Portugal; Rede de Química e Tecnologia - Instituto Superior de Engenharia (L.C., V.D., C.D.M), Instituto Politécnico do Porto, 4200-072 Porto, Portugal; Faculty of Nutrition and Food Sciences (A.F.), University of Porto, 4200-465 Porto, Portugal; Rede de Química e Tecnologia, Laboratório Associado de Química Verde (A.F.), Faculdade de Ciências, Universidade do Porto, 4169-009 Porto, Portugal; and Department of General Surgery, Oporto Hospital Center (G.F.), 4050-313 Porto, Portugal

Context: Some chemicals used in consumer products or manufacturing (eg, plastics, pesticides) have estrogenic activities; these xenoestrogens (XEs) may affect immune responses and have recently emerged as a new risk factors for obesity and cardiovascular disease. However, the extent and impact on health of chronic exposure of the general population to XEs are still unknown.

Objective: The objective of the study was to investigate the levels of XEs in plasma and adipose tissue (AT) depots in a sample of pre- and postmenopausal obese women undergoing bariatric surgery and their cardiometabolic impact in an obese state.

Design and Participants: We evaluated XE levels in plasma and visceral and subcutaneous AT samples of Portuguese obese (body mass index $\geq$ 35 kg/m^2) women undergoing bariatric surgery. Association with metabolic parameters and 10-year cardiovascular disease risk was assessed, according to menopausal status (73 pre- and 48 postmenopausal). Levels of XEs were determined by gas chromatography with electron-capture detection. Anthropometric and biochemical data were collected prior to surgery. Adipocyte size was determined on tissue sections obtained during surgery.

Results: Our data show that XEs are pervasive in this obese population. Distribution of individual and concentration of total XEs differed between plasma, visceral AT, and subcutaneous AT, and the pattern of accumulation was different between pre- and postmenopausal women. Significant associations between XE levels and metabolic and inflammatory parameters were found. In premenopausal women, XEs in plasma seem to be a predictor of 10-year cardiovascular disease risk.

Conclusions: Our findings point toward a different distribution of XE between plasma and AT in pre- and postmenopausal women, and reveal the association between XEs on the development of metabolic abnormalities in obese premenopausal women. (***J Clin Endocrinol Metab*** **100: 1792–1801, 2015)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Received November 17, 2014. Accepted February 6, 2015.
First Published Online April 8, 2015

Abbreviations: AT, adipose tissue; BMI, body mass index; CVD, cardiovascular disease; HbA1c, glycated hemoglobin; HCB, hexachlorobenzene; HCH, hexachlorocyclohexane; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment value for insulin resistance; HOMA-2B, homeostasis model assessment value for β-cell function; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; MCP1, monocyte chemotatic protein-1; *p,p'*-DDE, dichlorodiphenyldichloroethylene; R_S, Spearman correlation coefficient; scAT, subcutaneous AT; vAT, visceral AT; XE, xenoestrogen; ΣXE, sum of plasma XEs.

doi: 10.1210/jc.2014-4136

Polychlorinated pesticides, widespread in the environment, such as metabolites of dichlorodiphenyltrichloroethane, α-, β- and γ-hexachlorocyclohexane (HCH), as well as hexachlorobenzene (HCB) and aldrin, have estrogenic properties, being called environmental estrogens [xenoestrogens (XEs)] (1).

Environmental estrogens have several unique features that distinguish them from other common chemicals. Due to their long half-life and lipophilicity, they accumulate in adipose tissue (AT) and move within the body bound to lipids (2). Furthermore, humans are exposed to XEs as chemical mixtures due to the coexistence in the environment, food webs, and long-term retention in AT, resulting in virtually everyone in modern society having some XE exposure (3, 4).

Moreover, there is accumulating evidence of potential impacts of XE exposure on human health that might be mediated by a variety of mechanisms, including endocrine disruption locally at the AT and in components of the human immune system (5). Additionally, XE bioaccumulation in AT, a dynamic organ involved in the integrative network that maintains global energy homeostasis, may result in high local concentrations and lead to metabolic disruption in adipocytes (6). On the other hand, it is recognized that chronic low-grade inflammation and an activation of the immune system are involved in the pathogenesis of obesity-related comorbidities and the AT is an important site of inflammation in presence of obesity (7).

Although recent epidemiological evidence has linked environmental chemicals with obesity, insulin resistance, and cardiovascular disease (8–10), few studies link XEs with the complications arising with obesity when it is already installed. In this regard, we have recently reported interesting positive associations between AT concentrations of persistent organic pollutants, some of them with xenoestrogenic activity, and metabolic abnormalities among a sample of obese patients undergoing bariatric surgery. The associations were stronger with chemicals present in visceral AT (vAT). Furthermore, it was highlighted that these anthropogenic chemicals favored dysmetabolism despite the presence of obesity, proposing a shift on the focus to their dysmetabolic, and not only the obesogenic, effect (11).

The reduction of circulatory estrogens is a key factor in the onset of cardiovascular disease (CVD) during the menopausal period. Disturbances of this endocrine signal lead to the development of metabolic syndrome and a higher CVD risk in women, associated with predominant abdominal fat accumulation, even if in premenopausal women (12). However, the relationship between XEs in the presence/absence of these hormones and the cardiometabolic profile of obese women during menopause is not clear.

Moreover, few studies have reported correlations between plasma and AT concentrations of certain XEs and metabolic traits according to menopausal status. Thus, the present study was performed to investigate the levels of XEs in plasma and AT depots in a sample of pre- and postmenopausal obese women undergoing bariatric surgery and their cardiometabolic impact in an obese state.

Materials and Methods

Participants

The study involved 121 obese women (73 premenopausal and 48 postmenopausal as classified after evaluation by clinical endocrinology) with an age range of 19–61 years undergoing bariatric surgery (gastric banding or Roux-en-Y) at the General Surgery Department, S. João Hospital (Porto, Portugal), who were recruited between January 2010 and June 2011. Patients met the criteria for obesity surgery according to the latest criteria of the country's Department of Health. This investigation was conducted according to the Declaration of Helsinki, approved by the hospital's ethics committee. All participants provided written informed consent. Sociodemographic characteristics, anthropometric characteristics, clinical history, lifestyle factors, parity, and occupation were collected from the Medical Support System of S. João Hospital.

Clinical and biological parameters

Anthropometrics, adiposity-related markers, and various clinical variables were measured at baseline. Body mass index (BMI; kilograms per square meter) was calculated from the measured body weight and height. Analysis of blood samples collected after an overnight fast was performed in the Department of Clinical Pathology of S. João Hospital. Routine serum chemistries were measured using conventional methods with an Olympus AU5400 automated clinical chemistry analyzer (Beckman-Coulter, Izasa). Biological parameters evaluated included fasting blood glucose and insulin, lipid profile [total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, and triglycerides] and high-sensitivity C-reactive protein (hsCRP). LDL cholesterol was calculated according to Friedewald's equation (13). The homeostasis model assessment value for insulin resistance (HOMA-IR) and β-cell function (HOMA-2B) were calculated as described previously (14). Lastly, we used the Framingham risk score to estimate the 10-year CVD risk (a person's chance of developing CVD in the next 10 years) of these individuals (15).

Assessment of XE concentrations in plasma and adipose tissue

XEs were quantified in plasma samples stored at −80°C collected prior to surgery. The samples were analyzed by a previously published methodology (16), with some modifications, involving solid-phase extraction and a final determination using a gas chromatograph coupled to an electron capture detector. A total of five XEs were measured: aldrin (purchased from Pestanal; Fluka), *p,p′*-dichlorodiphenyldichloroethylene [dichloro-

diphenyldichloroethylene (*p,p′*-DDE); purchased from Chem Service], HCB (purchased from Pestanal Riedel-de Haën), Σ-hexachlorocyclohexane (sum of α-HCH, β-HCH, and δ-HCH, purchased from Sigma-Aldrich), and lindane (purchased from Pestanal Riedel-de Haën). The recovery percentages of samples spiked with a mixture of XEs were higher than 80%. Agreement in the retention time in the sample and in the reference standard was also required to confirm a positive result. Results were expressed as nanograms per milliliter of plasma and represent a mean of three individual injections of the same sample. XE concentrations were expressed in wet-weight levels because lipid-standardized model produces large biases and appears poorly suited for investigations in regard to cardiometabolic risk (17).

In AT samples [scAT and vAT collected during bariatric surgery], XEs analysis was performed according to the method described by Fernandes et al (18).

Cytokine ELISA

IL-6, IL-10, and monocyte chemotatic protein-1 (MCP1) concentrations in plasma were measured using, respectively, LEGEND MAX human IL-6, IL-10, and MCP1 ELISA kits (BioLegend Inc), according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using SPSS (22.0 version statistical software; IBM Corp). Data were described as frequencies and median (minimum, maximum). A Mann-Whitney test was used to compare clinical and biological characteristics and median of XEs between pre- and postmenopausal women. Friedman tests were used to compare XE levels in plasma, vAT, and scAT, both in pre- and postmenopausal women. The strength of the association between XE concentrations in plasma and AT and various parameters was estimated by Spearman correlation coefficients (R_S). To evaluate contribution of the different variables to plasma concentration of MCP1 (log transformed) or 10-year CVD risk (log transformed), we performed a linear regression analysis. The independent variables were included in the models as a priori knowledge, ie, all the possible covariates with a significant correlation with a dependent variable. A multivariable linear model were adjusted with age, vAT adipocyte area, and sum of plasma XEs (ΣXEs) as independent variables and MCP1 (log transformed) as the dependent variable. Other linear models were adjusted with the number of years of obesity evolution, plasma MCP1 levels, and ΣXEs in plasma as the independent variable and 10-year CVD risk (log transformed) as the dependent variable. All tests were two tailed and $P < .05$ was regarded as significant.

Results

Clinical and biological parameters

The comparison of biological and clinical characteristics between pre- and postmenopausal women is shown in Table 1. Postmenopausal women were obese for more years (37.0 vs 53.0 years, $P < .001$) than premenopausal women. Regarding anthropometric parameters, there was no difference in BMI and waist to hip ratio between the two groups. vAT (4242.7 vs 3834.9 μm^2, $P = .037$) and scAT (6625.4 vs 6134.9 μm^2, $P = .036$) adipocyte area was higher in postmenopausal women. With respect to glucose metabolism, postmenopausal women presented higher fasting glucose (97.0 vs 91.0 mg/dL, $P < .001$) and glycated hemoglobin (HbA1c) (5.8% vs 5.6%, $P < .001$) than premenopausal women. There was no significant difference in HOMA-IR, but HOMA-2B was lower in postmenopausal women (116.5% vs 156.6%, $P = .003$). We found no significant difference in plasma lipid profile or in either systolic or diastolic blood pressure between the two groups. Regarding inflammatory markers, there were no differences in IL-6, IL-10, MCP1, and hsCRP. As expected, the 10-year CVD risk was significantly higher in postmenopausal women (13.3% vs 3.7%, $P < .001$) (Table 1).

XEs in plasma

Among the selected XEs, in premenopausal women, the most frequently detected compound in plasma was ΣHCH (sum of α-HCH, β-HCH, and δ-HCH), present in 79.5% of the samples, followed by HCB, found in 72.6% of the samples. On the contrary, aldrin could be measured only in 6.8% of the samples. In postmenopausal women, the most frequently detected compound found in plasma was HCB, present in 87.5% of the samples, followed by ΣHCH, found in 52.1% of the samples. HCH lindane and *p,p′*-DDE were measured in 18.8% of the samples obtained from postmenopausal women (Table 2). *p,p′*-DDE was not found in the plasma of the premenopausal women and aldrin in postmenopausal women. However, when the Mann-Whitney test was used to compare pre- and postmenopausal women, the median of HCH lindane level (23.61 vs 5.81 ng/mL, $P = .041$) was significantly higher in premenopausal women (Table 2). Median concentrations of the sum of all XE (ΣXEs) present in plasma did not differ significantly between the pre- and postmenopausal women (41.43 vs 35.71 ng/mL, $P = .306$), and for both groups of women, HCB accounted for more than half of the XE plasma burden.

XEs in vAT and scAT

We examined the presence of the five studied XEs both in vAT and scAT samples in pre- and postmenopausal women (Table 2). In premenopausal women, the percentage of samples with detectable concentrations of XEs was 90.4% with median values of 106.72 (0.28, 687.18) ng/g of fat in vAT and 94.5% with median values of 61.62 (1.44, 368.06) ng/g of fat in scAT. In postmenopausal women, the percentage of samples with detectable concentrations of XEs was 97.9% with median values of 141.62 (2.51, 601.32) ng/g of fat in vAT and 97.6% with median values of 161.52 (0.41, 557.17) ng/g of fat in scAT. The most frequently detected XEs, the ΣHCH ap-

doi: 10.1210/jc.2014-4136

Table 1. Clinical and Biological Characteristics of the Patients

Parameters	Premenopausal Women		Postmenopausal Women		*P* Value
	n	Median (Minimum, Maximum)	n	Median (Minimum, Maximum)	
Age, y	73	37.0 (19.0, 59.0)	48	53.0 (36, 62)	<.001[a]
Obesity evolution, y	73	16.0 (5.0, 33.0)	48	24.0 (4.0, 49.0)	.005[a]
Anthropometric and morphometric measurements					
BMI, kg/m^2	73	44.1 (36.8, 56.2)	48	44.6 (36.0, 60.0)	.560
Waist to hip ratio	60	0.85 (0.75, 1.14)	37	0.90 (0.77, 1.08)	.552
vAT adipocyte area, μm^2	70	3834.9 (2001.9, 7220.4)	47	4242.7 (2512.2, 6486.9)	.037[a]
scAT adipocyte area, μm^2	73	6134.9 (2693.3, 10 294.2)	46	6625.4 (3511.6, 11 000.1)	.036[a]
Plasma lipid profile					
Total cholesterol, mg/dL	73	199.0 (114.0, 300.0)	48	210.5 (118.0, 357.0)	.117
Total triglycerides, mg/dL	73	120.0 (53.0, 261.0)	48	114.0 (57.0, 274.0)	.609
HDL cholesterol, mg/dL	73	51.0 (31.0, 81.0)	48	54.0 (32.0, 97.0)	.061
LDL cholesterol, mg/dL	73	131.0 (58.0, 206.0)	48	136.0 (62.0, 290.0)	.324
Glucose homeostasis					
Fasting glycemia, mg/dL	73	91.0 (73.0, 153.0)	48	97.0 (75.0, 297.0)	<.001[a]
HbA1c, %	71	5.6 (4.8, 6.9)	45	5.8 (5.2, 10.3)	<.001[a]
HOMA-IR	70	2.2 (0.1, 7.8)	36	1.8 (0.4, 8.4)	.354
HOMA-2B, %	70	156.6 (13.3, 370.7)	36	116.5 (42.5, 265.9)	.003[a]
Blood pressure					
Systolic blood pressure, mm Hg	58	130.0 (100.0, 171.0)	37	140.0 (109.0, 184.0)	.098
Diastolic blood pressure, mm Hg	58	80.0 (60.0, 120.0)	37	80.0 (63.0, 155.0)	.230
Inflammatory parameters					
IL-6, pg/mL	53	32.7 (4.3, 129.5)	45	30.9 (7.5, 99.0)	.695
IL-10, pg/mL	64	4.8 (0.3, 104.0)	45	3.4 (0.1, 117.4)	.267
IL-6 to IL-10 ratio	52	5.17 (0.47, 317.96)	42	7.60 (0.44, 213.13)	.076
MCP1, pg/mL	67	113.8 (17.0, 792.7)	48	106.1 (20.0, 417.7)	.628
Other parameters					
hsCRP, mg/L	45	3.4 (0.3, 37.0)	29	4.2 (0.3, 45.0)	.373
Estradiol, pg/mL	22	59.4 (15.0, 193.0)	32	26.5 (10.0, 110.0)	.030[a]
Monocytes, %	66	6.0 (0.4, 9.8)	44	6.1 (0.2, 10.2)	.707
10-Year CVD risk, %	51	3.7 (0.3, 27.8)	37	13.3 (1.9, 34.7)	<.001[a]

Ten-year CVD risk was calculated according to D'Agostino et al (15).

[a] Statistical analysis was performed with a Mann-Whitney test: $P < .05$.

peared in 90.4% of vAT and scAT in premenopausal women, also being detected in 95.8% of both vAT and scAT samples in postmenopausal women. Regarding the percentage of XE detection in vAT, HCH lindane was found in 28.8% vs 22.9%, HCB in 5.5% vs 8.3%, aldrin in 15.1% vs 29.2%, and *p,p′*-DDE in 23.3% vs 33.3% of vAT samples from pre- and postmenopausal women, respectively. On the other hand, when comparing the absolute concentration in each AT depot, we verified that the medians of the XEs in vAT were not different between the two groups. Concerning scAT, HCH lindane was found in 23.3% vs 17.0%, HCB was found in 5.5% vs 8.3%, aldrin in 15.1% vs 29.2%, and *p,p′*-DDE in 32.9% vs 43.8% of the samples for pre- and postmenopausal women, respectively. Concentrations of ΣHCH (42.24 vs 98.51 ng/g of fat, $P = .018$), *p,p′*-DDE (1.55 vs 6.21 ng/g of fat, $P = .003$), and ΣXEs (61.62 vs 161.52 ng/g of fat, $P = .020$) were higher in the scAT of postmenopausal women. In this AT depot, ΣHCH was the compound that contributed most in most samples to the total scAT burden of XEs. Finally, in premenopausal women the concentration of XEs in vAT was significantly higher compared with scAT (106.72 vs 61.62 ng/g of fat, $P = .002$). The plasma concentration of XEs was lower than that found in vAT and scAT in pre- and postmenopausal women.

Association between XEs in plasma and AT

Paired-sample comparisons was made among the three evaluated compartments (XEs levels in plasma and the two ATs) both in premenopausal and postmenopausal women (see Supplemental Table 1). In both pre- and postmenopausal women, there was a different distribution of XEs among the three analyzed locations. Although premenopausal women had higher XE accumulation in vAT followed by scAT and plasma, the levels of XEs in vAT and scAT from postmenopausal women were similar, with much lower plasma levels. The levels of XEs in both AT depots were positively correlated in pre- ($R_S = 0.488$, $P < .01$) and postmenopausal women ($R_S = 0.545$, $P < .01$). In premenopausal women, XEs levels in vAT were positively correlated with plasma levels ($R_S = 0.642$, $P < .01$), but plasma levels were not correlated with scAT XEs. In

Table 2. XE Levels in Plasma (Nanograms per Milliliter) and in Both vAT and scAT (Nanograms per Grams of Fat) of the 121 Patients

	%[a]	Premenopausal Women	%[a]	Postmenopausal Women	*P* Value
Plasma					
ΣHCH	79.5	16.20 (2.98, 57.83)	52.1	14.18 (4.41, 26.38)	.336
HCH lindane	16.4	23.61 (2.17, 55.53)	18.8	5.81 (0.46, 33.06)	.041[b]
HCB	72.6	31.52 (21.56, 95.80)	87.5	29.19 (20.75, 58.76)	.195
Aldrin	6.8	0.12 (0.04, 0.38)		<LD	
p,p'-DDE		<LD	18.8	7.10 (4.61, 26.64)	
ΣXEs	93.2	41.43 (2.17, 209.16)	97.9	35.71 (2.80, 101.64)	.306
vAT					
ΣHCH	90.4	103.69 (0.01, 485.67)	95.8	99.57 (1.94, 399.48)	.947
HCH lindane	28.8	15.19 (0.59, 87.44)	22.9	33.96 (5.19, 338.39)	.051
HCB	5.5	87.10 (84.91, 104.00)	8.3	60.67 (0.33, 149.23)	.343
Aldrin	15.1	11.41 (0.47, 48.87)	29.2	6.79 (0.57, 55.72)	.267
p,p'-DDE	23.3	24.83 (1.26, 257.52)	33.3	15.20 (2.02, 289.51)	.873
ΣXEs	90.4	106.72 (0.28, 687.18)	97.9	141.62 (2.51, 601.32)	.442
scAT					
ΣHCH	90.4	42.24 (1.29, 330.30)	95.8	98.51 (0.34, 458.94)	.018[b]
HCH lindane	23.3	30.89 (6.47, 262.81)	17.0	44.77 (24.92, 271.86)	.157
HCB	5.5	104.46 (92.79, 121.77)	8.3	77.85 (14.72, 160.30)	.343
Aldrin	12.3	23.53 (2.17, 112.90)	22.9	9.00 (1.76, 88.85)	.175
p,p'-DDE	32.9	1.55 (0.19, 30.14)	43.8	6.21 (0.30, 308.03)	.003[b]
ΣXEs	94.5	61.62 (1.44, 368.06)	97.6	161.52 (0.41, 557.17)	.020[b]

All data are medians (minimum, maximum).

[a] Percentage of the total number of positive samples.

[b] Values are presented as median (minimum, maximum) ($P < .05$, statistical analysis with Mann-Whitney test).

contrast, in postmenopausal women plasma XEs levels were positively correlated with XEs levels present in either AT compartment (Table 3). The strongest association observed both in pre- and postmenopausal women was that between vAT and plasma XE levels.

XE levels were associated with metabolic dysfunction

The associations between XE levels and the patients' clinical and biochemical parameters were also evaluated. We show correlations between the two most frequently detected XEs in each compartment and biological parameters (see Supplemental Tables 2–4). In premenopausal women, the vAT ΣXEs concentration was significantly and positively correlated with HbA1c and the count of plasma monocytes and inversely correlated with plasma IL-10. However, no significant correlation was found between scAT ΣXEs and the different parameters. Plasma ΣXEs were positively correlated with age, vAT adipocyte area, plasma IL-10, MCP1, and 10-year CVD risk and negatively correlated with HOMA-2B. In postmenopausal women, the only significant correlations found were a positive association between vAT ΣXEs and age and a negative association between plasma ΣXEs and the IL-6 to IL-10 ratio (Table 4).

Plasma XE levels were associated with inflammation and 10-year CVD risk in premenopausal women

In premenopausal women, the concentration of MCP1 was positively correlated with plasma ΣXEs (Table 4). In the linear regression model analysis, only ΣXEs plasma levels ($\beta = 0.006$, $P = .018$) remained statistically associated with plasma MCP1, even when adjusted for age and vAT adipocyte area (Table 5), highlighting the fact that XEs are independent risk factors for inflammation. On the other hand, the 10-year CVD risk correlated positively with ΣXEs plasma levels in premenopausal women (Table

Table 3. Correlation and Comparison Between Levels of XEs Present Simultaneously in Both vAT (Nanograms per Gram of Fat) and scAT (Nanograms per Gram of Fat) and Plasma (Nanograms per Milliliter)

	%[a]	Premenopausal Women	%[a]	Postmenopausal Women
ΣXEs, vAT vs scAT	86.3	0.488[b]	95.8	0.545[b]
ΣXEs , vAT vs plasma	83.6	0.642[b]	95.8	0.625[b]
ΣXEs , scAT vs plasma	87.7	−0.084	95.8	0.416[b]

[a] Percentage of the total number of positive samples in both vAT and scAT.

[b] Statistical analysis with Spearman's correlation ($P < .01$).

doi: 10.1210/jc.2014-4136

Table 4. Correlation of Clinical and Biological Characteristics With XE Levels in vAT and scAT (Nanograms per Gram of Fat) and Plasma (Nanograms per Milliliter) of Premenopausal and Postmenopausal Women

	Premenopausal Women			Postmenopausal Women		
Parameters	**vAT XEs**	**scAT XEs**	**Plasma XEs**	**vAT XEs**	**scAT XEs**	**Plasma XEs**
Age, y	0.077	0.102	0.368[a]	0.351[b]	0.074	−0.052
Obesity evolution, y	0.131	−0.097	0.172	−0.012	−0.046	0.219
Anthropometric and morphometric measurements						
BMI, kg/m^2	0.115	0.027	0.001	−0.055	0.106	−0.016
Waist to hip ratio	−0.105	−0.043	0.076	0.026	−0.070	−0.158
vAT adipocyte area, μm^2	−0.014	0.109	0.278[b]	−0.264	−0.208	0.059
scAT adipocyte area, μm^2	0.000	0.201	−0.109	0.047	−0.045	0.138
Plasma lipid profile						
Total cholesterol, mg/dL	0.035	0.091	0.052	−0.024	0.017	0.115
Total triglycerides, mg/dL	0.041	0.004	0.019	0.107	0.206	0.221
HDL cholesterol, mg/dL	−0.035	0.111	−0.174	0.051	0.082	−0.039
LDL cholesterol, mg/dL	0.101	0.148	0.083	−0.060	−0.023	0.156
Glucose homeostasis						
Fasting glycemia, mg/dL	−0.066	0.048	0.074	0.134	0.186	−0.129
HbA1c, %	0.252[b]	0.232	0.085	0.116	0.154	0.037
HOMA-IR	−0.057	0.121	−0.234	−0.01	0.243	0.164
HOMA-2B, %	−0.050	0.096	−0.250[b]	−0.173	0.097	0.162
Blood pressure						
Systolic blood pressure, mm Hg	0.136	0.175	0.165	0.071	0.127	−0.031
Diastolic blood pressure, mm Hg	0.103	0.118	0.255	−0.159	0.166	0.116
Inflammatory parameters						
IL-6, pg/mL	−0.058	−0.152	0.172	−0.138	−0.015	−0.030
IL-10, pg/mL	−0.279[b]	0.017	0.328[b]	0.108	0.131	0.291
IL-6 to IL-10 ratio	0.259	−0.062	−0.131	−0.138	−0.158	−0.365[b]
MCP1, pg/mL	−0.170	−0.004	0.285[b]	−0.053	0.039	0.008
Other parameters						
hsCRP, mg/L	−0.228	−0.158	−0.231	−0.047	−0.019	0.005
Estradiol, pg/mL	0.122	0.022	−0.339	−0.179	−0.311	−0.232
Monocytes, %	0.360[a]	−0.096	−0.054	0.220	0.058	0.014
10-Year CVD risk, %	0.211	0.098	0.363[b]	0.185	0.155	−0.142

Ten-year CVD risk was calculated according to D'Agostino et al (12).

[a] $P < .01$ (statistical analysis with Spearman's correlation).

[b] $P < .05$ (statistical analysis with Spearman's correlation).

4). In the multivariate linear model, ΣXEs plasma levels were also significantly associated with 10-year CVD risk ($\beta = 0.012$, $P = .009$), even when adjusted for the time of obesity and MCP1. A significant interaction ($\beta = -0.001$, $P = 0.006$) between MCP1 and the time of obesity seems to occur, even after adjustment for ΣXEs plasma levels (Table 6) in premenopausal women. Considering postmenopausal women in multivariate models, no independent variables were significantly associated with MCP1 or with 10-year CVD risk (Table 4). Considering the 44 premenopausal woman included in the linear regression

Table 5. Coefficients From Linear Regression Model With Ln (MCP1) as Dependent Variable[a]

	Premenopausal Women		Postmenopausal Women	
	β	***P* Value**	***β***	***P* Value**
ΣXEs in plasma, ng/mL	0.006	**.012**	0.000	.966
Age	−0.017	.108	−0.012	.451
vAT adipocyte	0.000	.845	0.000	.870

β-values from a linear regression model are reported. Significant *P* values are shown in bold.

[a] Adjusted for all variables present in table.

Table 6. Coefficients From Linear Regression Model With Ln (10 Year CVD Risk) as Dependent Variable[a]

	Premenopausal Women		Postmenopausal Women	
	β	***P* Value**	***β***	***P* Value**
ΣXEs in plasma, ng/mL	0.012	**.009**	−0.005	.384
Obesity evolution, y	0.014	**.016**	0.000	.982
MCP1, pg/mL	0.135	**<.001**	0.017	.517
Obesity evolution, y, MCP1, pg/mL[a]	−0.001	**.006**	0.000	.615

β-Values from linear regression model is reported. Significant *P* values are shown in bold.

[a] Adjusted for all variables present in table.

model with Ln 10-year CVD risk as dependent variable, we studied the power of testing the predictor ΣXEs in plasma (nanograms per milliliter), in the presence of two other predictors (obesity evolution, MCP1). The power of the test of a regression coefficient depends on the error SD, the SD of the predictor itself, and the multiple correlation between that predictor and other predictors in the model (related to the variance inflation factor). These parameters were estimated based on the sample enrolled. For an α of .05 and a power of 0.80, a sample size of n = 44 will detect a regression coefficient of 0.013. Considering the 36 postmenopausal woman included in the linear regression model with Ln 10-year CVD risk as a dependent variable, we studied the power of testing the predictor ΣXEs in plasma (nanograms per milliliter), in the presence of two other predictors (obesity evolution and MCP1). With the parameters estimated with the sample enrolled, for an α of .05 and a power of 0.80, a sample size of n = 36 will detect a regression coefficient of 0.015.

Discussion

In the present study, we carried out a novel and comprehensive assessment in pre- and postmenopausal women of the distribution and putative effects of several XEs that are known to be preferentially accumulated in the AT. We show that several environmental estrogens such as ΣHCH, HCH lindane, HCB, aldrin, and *p,p′*-DDE can be currently detected in biological samples obtained from obese patients, although their use has long been banned. Analysis of the association of health-related outcomes with XEs in the different biological samples was considered superior to the analysis of individual compounds because essentially all humans are exposed to many different XEs at the same time (3, 4), although perhaps in different relative concentrations.

A previous work from our group (11) demonstrated that persistent organic pollutants, some of which have xenoestrogenic activity, accumulated preferentially in vAT when women were considered regardless of menopausal status. In the present study, we were able to demonstrate that the distribution of XEs changes with menopausal status. For the compounds analyzed here, we can observe that this is true for premenopausal women but not postmenopausal women whose XE levels in scAT are similar to those in vAT and significantly higher than those found in the scAT of premenopausal women. On the other hand, this observation has important practical implications because it suggests that scAT, despite being an easily accessible compartment for determination of contamination levels, is not representative of the XE distribution in deeper AT depots, in accordance with different studies (19, 20). Plasma levels of XEs also did not reflect total levels accumulated in other compartments, highlighting that using plasma XE levels to estimate exposure may be misleading as well. Apart from being lower than AT levels, the type of compound present in plasma does not mimic the pattern accumulated in AT, possibly due to differences in solubility and toxicokinetics, resulting in different potential toxicities.

In premenopausal women there is a significant positive correlation between XEs in vAT and plasma but not with XEs in scAT. This association makes sense in that vAT adipocytes are metabolically more active and more sensitive to lipolysis than scAT adipocytes (21). On the other hand, plasma XEs correlated both with vAT and scAT XEs in postmenopausal women, suggesting that the increased turnover of the AT after menopause, including higher lipolysis rate due to decreased estrogens (22), may contribute to the concentration of XEs in scAT. Indeed, this pattern change may be a consequence of a changing hormonal milieu because of the menopausal transition, but it may also be modulated by the presence of XEs. Nevertheless, it seems to accompany a dysfunctional AT in this subgroup of women.

We observed that although older, postmenopausal women present higher metabolic dysfunction, as confirmed by our results, we found that XEs are positively associated with markers of impaired metabolism and with CVD risk in younger, premenopausal women. This suggests that the effect of these compounds may be independent of the decline of metabolic function that occurs with age.

Fat accumulated in vAT, in comparison with scAT, is known to positively correlate with metabolic complications, such as CVD, hypertension, and type 2 diabetes (23). Indeed, because XEs in vAT of premenopausal women were found to be more tightly associated with markers of worst metabolic profile compared with XEs in scAT, namely glucose homeostasis and inflammatory parameters, this might suggest that the presence of these compounds in vAT contributes to local toxicity and dysfunction and potentially favors the metabolic complications associated with obesity (6). Therefore, our study highlights the contribution of chronic internal exposure of obese subjects to XEs as possible additional factors leading to AT dysfunction.

In metabolically unhealthy obesity, adipocyte expansion occurs through hypertrophy with overproduction and secretion of signals that recruit immune cells, namely macrophages, leading to increased circulatory proinflammatory (eg, IL-6 and MCP1) and decreased antiinflammatory (IL-10) factors that contribute to local AT inflammation and to systemic low-grade inflammation (24, 25).

doi: 10.1210/jc.2014-4136

Indeed, plasma XEs were correlated with increased vAT adipocyte area, a known marker of adipocyte dysfunction (26, 27).

Additionally, it has been proposed that XEs in AT can be involved in inflammatory activation/perpetuation, a critical condition for metabolically unhealthy AT (28). In accordance, we have found that in premenopausal women the presence of XEs in vAT and plasma may contribute to a more proinflammatory status. Plasma XEs correlated positively with MCP1 levels, which is compatible with higher production by activated cells in the AT. In fact, the mesenteric AT is a major producer of MCP1, which can modulate macrophage trafficking and activation during obesity-related inflammation (29). Furthermore, proinflammatory chemokines such as MCP1 are highly expressed by hypertrophic adipocytes accelerating migration and homing of bone marrow-derived monocytes/macrophages to the AT (30). It is worth highlighting that in the linear regression model, adjustment for age and vAT adipocyte area did not modify the association between plasma XEs and MCP1 levels.

Once again, the increase in vAT XEs is correlated with the increase in the number of monocytes in circulation, a possible intermediate step to their migration to the AT. This reinforces the hypothesis that XE exposure, through its effect on the immune system, may contribute to the high rate of metabolic disorders (31). The negative association between vAT XEs and plasma IL-10 levels adds to this evidence, although an opposite association was observed with plasma XEs.

In addition to inflammatory activation, dysfunctional AT also displays insulin resistance (32). In the same line, we have observed a positive association between vAT XEs and HbA1c. We also observed a negative relationship between plasma XEs and HOMA-2B. Accordingly, several researches provide evidence on possible actions of XEs on β-cell function and insulin resistance (33, 34). An observational study of Greenland Inuits highly exposed to environmental pollutants showed associations of some XEs with HOMA-2B, but not with HOMA-IR (35), indicating that the primary mechanism by which XEs increase the risk of type 2 diabetes is by modulating β-cell function. Furthermore, the association of plasma, but not vAT, XEs with this marker highlights the fact that different compounds are present in plasma and AT and that plasma XEs have many possible targets for toxicity.

Another interesting fact is that XE concentration is not BMI dependent. This is an important result because it demonstrates that body weight per se is not the primary factor explaining plasma XE concentration (36). Plasma XE levels may potentially be affected by a number of factors including the degree and source of exposure, the time since exposure occurred, genetic differences among individuals in rates of metabolism (37), and the number of pregnancies and breast-feeding practices (38).

Our results support the hypothesis that XEs increase systemic inflammation/AT dysfunction in premenopausal women, which may aggravate metabolic status and induce target organ damage. Indeed, plasma XEs helped predict CVD risk, even after adjustment for plasma MCP1 and the time of obesity. In addition, these results indicate that XE exposure or release from AT and inflammation may be independent factors for CVD risk, suggesting that increased plasma XEs may at least partially account for the inflammation and risk elicited by obesity in premenopausal women with the same time of obesity.

As mentioned earlier, the compounds evaluated in this study have been highlighted as endocrine disruptors interfering with estrogens, although also possessing other biological activities (39). However, their capacity to interfere with estrogens' actions is complex, given that these compounds may behave differently, often with opposing actions, if in the presence of absence of the natural hormone and/or depending on its concentration (40). This possibly underlines the fact that most of the associations of XEs with markers of metabolic deterioration observed herein were in the premenopausal women subgroup, leading to the speculation that the effect of XEs is more relevant when in the presence of preserved estrogen endocrine signaling.

Our findings also suggest that XEs and not obesity alone may contribute to increase CVD risk and inflammation, especially in premenopausal women, and thus, these chemicals may have a potential role in the later development of cardiometabolic disease in obese women. Moreover, the associations observed are compatible with the endocrine disruptor character of these compounds by compromising the dynamic protective physiological function of estrogens, leading to the release and redistribution of these pollutants. We also provide new insights into the profile and kinetics of XEs and their putative pathogenic effects according to menopausal status. This is in line with the observation that the relationship between 10-year CVD risk and plasma concentrations of MCP1 tended to be weakened with the time of obesity evolution in premenopausal women.

We acknowledge that the present study is not without limitations: 1) the patients were at the end of the line of obesity treatment, which limits the generalization of our findings to the overall population, and 2) there were multiple comparisons made in this study; however, because the present study is an exploratory study, we believe that it is not needed to include the correction for multiple comparisons, and thus, the significant results found in this

study should be verified in further confirmatory studies (34). Nonetheless, our study has significant assets regarding the large sample size, the characterization of environmental exposures in three tissue compartments (plasma, vAT, and scAT) in obese women undergoing bariatric surgery, and the analysis of the results according to menopausal status, exploring a differential link to the metabolic abnormalities and cardiovascular risk.

Importantly, there is a need to extend the knowledge of the mechanisms of action of XEs, which may alter metabolic function, which will open novel directions for the prevention, and treatment of metabolic disease. The question of whether XEs could serve as biomarkers representing a novel tool to predict cardiometabolic risk remains to be fully answered, but if these findings are reproducible in different populations, it means that as early as possible, any effort to reduce exposure to XEs would be necessary to decrease the social burden of cardiometabolic disease.

Acknowledgments

Address all correspondence and requests for reprints to: Diana Teixeira, BSc, Department of Biochemistry (U38-FCT), Faculty of Medicine, University of Porto, Alameda Professor Hernâni Monteiro, 4200-319 Porto, Portugal. E-mail: dianamst@med.up.pt.

This work was funded by Fundo Social European, Programa Operacional Potencial Humano da European Union; Grants PEst-OE/SAU/UI0038/2011, SFRH/BPD/40110/2007, SFRH/BD/46640/2008, SFRH/BD/64691/2009, SFRH/BPD/75294/2010, and SFRH/BD/93073/2013, and the Projetos de Investigação na Pré-graduação 2011, Universidade do Porto.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Ociepa-Zawal M, Rubis B, Wawrzynczak D, Wachowiak R, Trzeciak WH.** Accumulation of environmental estrogens in adipose tissue of breast cancer patients. *J Environ Sci Health A Tox Hazard Subst Environ Eng*. 2010;45(3):305–312.
2. **Ljunggren SA, Helmfrid I, Salihovic S, et al.** Persistent organic pollutants distribution in lipoprotein fractions in relation to cardiovascular disease and cancer. *Environ Int*. 2014;65:93–99.
3. **Lee DH, Lee IK, Porta M, Steffes M, Jacobs DR Jr.** Relationship between serum concentrations of persistent organic pollutants and the prevalence of metabolic syndrome among non-diabetic adults: results from the national health and nutrition examination survey 1999–2002. *Diabetologia*. 2007;50(9):1841–1851.
4. **Porta M, Pumarega J, Gasull M, Lopez T.** Contamination from endocrine disrupters of the general population at low and high concentrations. In: Litwack G, ed. *Vitamins and Hormones*. Vol 94. Oxford, UK: Elsevier Inc; 2014:168–189.
5. **Kim MJ, Pelloux V, Guyot E, et al.** Inflammatory pathway genes belong to major targets of persistent organic pollutants in adipose cells. *Environ Health Perspect*. 2012;120(4):508–514.
6. **La Merrill M, Emond C, Kim MJ, et al.** Toxicological function of adipose tissue: Focus on persistent organic pollutants. *Environ Health Perspect*. 2013;121(2):162–169.
7. **Cancello R, Henegar C, Viguerie N, et al.** Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes*. 2005;54(8):2277–2286.
8. **Mastin JP.** Environmental cardiovascular disease. *Cardiovasc Toxicol*. 2005;5(2):91–94.
9. **Lind PM, van Bavel B, Salihovic S, Lind L.** Circulating levels of persistent organic pollutants (pops) and carotid atherosclerosis in the elderly. *Environ Health Perspect*. 2012;120(1):38–43.
10. **Lee DH, Steffes MW, Sjodin A, Jones RS, Needham LL, Jacobs DR Jr.** Low dose organochlorine pesticides and polychlorinated biphenyls predict obesity, dyslipidemia, and insulin resistance among people free of diabetes. *PLoS One*. 2011;6(1):e15977.
11. **Pestana D, Faria G, Sa C, et al.** Persistent organic pollutant levels in human visceral and subcutaneous adipose tissue in obese individuals-depot differences and dysmetabolism implications. *Environ Res*. 2014;133C:170–177.
12. **Lizcano F, Guzman G.** Estrogen deficiency and the origin of obesity during menopause. *Biomed Res Int*. 2014;2014:757461.
13. **Friedewald WT, Levy RI, Fredrickson DS.** Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem*. 1972;18(6):499–502.
14. **Wallace TM, Levy JC, Matthews DR.** Use and abuse of homa modeling. *Diabetes Care*. 2004;27(6):1487–1495.
15. **D'Agostino RB Sr, Vasan RS, Pencina MJ, et al.** General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation*. 2008;117(6):743–753.
16. **Covaci A.** Application of solid-phase disk extraction combined with gas chromatographic techniques for determination of organochlorine pesticides in human body fluid. *Pesticide Protocols (Methods in Biotechnology)* 2005:49–59.
17. **Gauthier MS, Rabasa-Lhoret R, Prud'homme D, Karelis AD, Geng D, van Bavel B, Ruzzin J.** The metabolically healthy but obese phenotype is associated with lower plasma levels of persistent organic pollutants as compared to the metabolically abnormal obese phenotype. *J Clin Endocrinol Metab*. 2014:jc20133935.
18. **Fernandes VC, Pestana D, Monteiro R, et al.** Optimization and validation of organochlorine compounds in adipose tissue by spegas chromatography. *Biomed Chromatogr*. 2012;26(12):1494–1501.
19. **Kim MJ, Marchand P, Henegar C, et al.** Fate and complex pathogenic effects of dioxins and polychlorinated biphenyls in obese subjects before and after drastic weight loss. *Environ Health Perspect*. 2011;119(3):377–383.
20. **Arrebola JP, Fernandez MF, Porta M, et al.** Multivariate models to predict human adipose tissue PCB concentrations in southern Spain. *Environ Int*. 2010;36(7):705–713.
21. **White UA, Tchoukalova YD.** Sex dimorphism and depot differences in adipose tissue function. *Biochim Biophys Acta*. 2014;1842(3):377–392.
22. **Berman DM, Nicklas BJ, Rogus EM, Dennis KE, Goldberg AP.** Regional differences in adrenoceptor binding and fat cell lipolysis in obese, postmenopausal women. *Metabolism*. 1998;47(4):467–473.
23. **Wajchenberg BL, Giannella-Neto D, da Silva ME, Santos RF.** Depot-specific hormonal characteristics of subcutaneous and visceral adipose tissue and their relation to the metabolic syndrome. *Horm Metab Res*. 2002;34(11–12):616–621.
24. **Chawla A, Nguyen KD, Goh YP.** Macrophage-mediated inflammation in metabolic disease. *Nat Rev Immunol*. 2011;11(11):738–749.
25. **Sun S, Ji Y, Kersten S, Qi L.** Mechanisms of inflammatory responses in obese adipose tissue. *Annu Rev Nutr*. 2012;32:261–286.
26. **Monteiro R.** Chronic inflammation in the metabolic syndrome: Emphasis on adipose tissue. In: Soares R, Costa C, eds. *Oxidative Stress,*

Inflammation and Angiogenesis in the Metabolic Syndrome. Porto: Springer Netherlands; 2009:65–84.
27. **Monteiro R, de Castro PM, Calhau C, Azevedo I.** Adipocyte size and liability to cell death. *Obes Surg*. 2006;16(6):804–806.
28. **Lee DH, Porta M, Jacobs DR Jr, Vandenberg LN.** Chlorinated persistent organic pollutants, obesity, and type 2 diabetes. *Endocr Rev*. 2014:er20131084.
29. **Yu R, Kim CS, Kwon BS, Kawada T.** Mesenteric adipose tissue-derived monocyte chemoattractant protein-1 plays a crucial role in adipose tissue macrophage migration and activation in obese mice. *Obesity (Silver Spring)*. 2006;14(8):1353–1362.
30. **Kanda H, Tateya S, Tamori Y, et al.** Mcp-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest*. 2006;116(6):1494–1505.
31. **Mathis D.** A gut feeling about arthritis. *Elife*. 2013;2:e01608.
32. **Bays HE, Gonzalez-Campoy JM, Bray GA, et al.** Pathogenic potential of adipose tissue and metabolic consequences of adipocyte hypertrophy and increased visceral adiposity. *Expert Rev Cardiovasc Ther*. 2008;6(3):343–368.
33. **Nadal A, Alonso-Magdalena P, Soriano S, Quesada I, Ropero AB.** The pancreatic β-cell as a target of estrogens and xenoestrogens: implications for blood glucose homeostasis and diabetes. *Mol Cell Endocrinol*. 2009;304(1–2):63–68.
34. **Alonso-Magdalena P, Morimoto S, Ripoll C, Fuentes E, Nadal A.** The estrogenic effect of bisphenol a disrupts pancreatic β-cell function in vivo and induces insulin resistance. *Environ Health Perspect*. 2006;114(1):106–112.
35. **Jorgensen ME, Borch-Johnsen K, Bjerregaard P.** A cross-sectional study of the association between persistent organic pollutants and glucose intolerance among Greenland Inuit. *Diabetologia*. 2008; 51(8):1416–1422.
36. **Zeyda M, Geyeregger R, Poglitsch M, et al.** Impairment of t cell interactions with antigen-presenting cells by immunosuppressive drugs reveals involvement of calcineurin and nf-κb in immunological synapse formation. *J Leukoc Biol*. 2007;81(1):319–327.
37. **Maes J, Belpaire C, Goemans G.** Spatial variations and temporal trends between 1994 and 2005 in polychlorinated biphenyls, organochlorine pesticides and heavy metals in European eel (*Anguilla anguilla* l.) in Flanders, Belgium. *Environ Pollut*. 2008;153(1):223–237.
38. **Bjermo H, Darnerud PO, Lignell S, et al.** Fish intake and breastfeeding time are associated with serum concentrations of organochlorines in a Swedish population. *Environ Int*. 2013;51:88–96.
39. **Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, et al.** Endocrine-disrupting chemicals: an endocrine society scientific statement. *Endocr Rev*. 2009;30(4):293–342.
40. **Welshons WV, Thayer KA, Judy BM, Taylor JA, Curran EM, vom Saal FS.** Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. *Environ Health Perspect*. 2003;111(8):994–1006.

Letter to the Editor

D. Teixeira[1], D. Pestana[1], C. Calhau[1, 2], R. Monteiro[1]

[1] Department of Biochemistry (U38-FCT), Faculty of Medicine, University of Porto, Porto, Portugal

[2] CINTESIS (Center for Research in Health Technologies and Information Systems), Porto, Portugal

Published on October 8, 2014

In their recent paper Gauthier et al. (1) interestingly demonstrate the relationship of persistent organic pollutants (POPs) with the variation in metabolic risk observed among obese individuals showing that the metabolically healthy but obese (MHO) phenotype is associated with lower plasma levels of POPs as compared with metabolically abnormal obese (MAO) subjects.

In fact, many epidemiological studies have used circulating concentrations of POPs as a marker of body burden of the compounds. However, the adipose tissue (AT) is the main reservoir of POPs and, although circulating POP concentrations have been reported to be correlated with those in AT, plasma POP levels are more transient and measurement of AT POP levels are undoubtedly a better way to estimate whole body POP accumulation (2). Usually POP accumulation in AT can be regarded as protective by preventing the systemic effects of these compounds. In addition to its function as POP reservoir, the AT can also be a target of their disruptive effects, with putative local and systemic consequences (3). Recently our group revealed that, POP levels either in subcutaneous and visceral AT (vAT) were higher in subjects with evidence of metabolic abnormalities. This pattern was especially evident for vAT, supported by higher vAT POP levels in patients with increased aggregation of metabolic syndrome components and higher 10-year cardiovascular risk based on the Framingham score (4).

We believe that the higher accumulation of POPs observed in vAT is of paramount importance since this highly metabolically active tissue is held as the main impeller of metabolic dysfunction progression. Moreover, longer exposure times, such as those expected to occur for example in postmenopausal women, may reflect in higher POP concentrations in AT imposing on the likelihood of local toxicity. Further, Bluher et al. (5) advance that inflammation is one of the main factors discriminating 'healthy' from 'unhealthy' obese individuals, and vAT inflammation largely contributes to the inflammatory state associated with obesity. Therefore, higher POP accumulation on vAT of obese individuals may induce or exacerbate local inflammation contributing to metabolic deterioration associated with obesity and constituting a possible factor discriminating the two subpopulations.

Another interesting point of discussion is that vAT is more susceptible to lipolysis probably resulting in increased POP release. As metabolic dysfunction is mainly associated with increased vAT, one can speculate if POPs, which we have demonstrated to be accumulated in higher amounts in this AT depot, are, on the one hand hampering its normal function and, on the other, promoting a consequent POP release into the bloodstream. Our hypothesis is corroborated by the increase of plasma POP levels in MAO and is consistent with the notion that once POPs return to circulation they become free to act in distant organs possibly contributing to cardiometabolic complications.

This implies the broader effect of environmental POPs, in which the impairment of AT normal function seems to play an important role, reinforcing the need for a paradigm shift of the view of POPs as mere obesogen compounds and introducing them as possible markers of dysmetabolic obesity.

References

1. Gauthier MS, Rabasa-Lhoret R, Prud'homme D, Karelis AD, Geng D, van Bavel B, Ruzzin J. The metabolically healthy but obese phenotype is associated with lower plasma levels of persistent organic pollutants as compared to the metabolically abnormal obese phenotype. J Clin Endocrinol Metab. 2014:jc20133935.

2. Porta M, Puigdomenech E, Ballester F, Selva J, Ribas-Fito N, Llop S, Lopez T. Monitoring concentrations of persistent organic pollutants in the general population: the international experience. Environ Int. 2008;34:546-561.

3. La Merrill M, Emond C, Kim MJ, Antignac JP, Le Bizec B, Clement K, Birnbaum LS, Barouki R. Toxicological function of adipose tissue: focus on persistent organic pollutants. Environ Health Perspect. 2013;121:162-169.

4. Pestana D, Faria G, Sa C, Fernandes VC, Teixeira D, Norberto S, Faria A, Meireles M, Marques C, Correia-Sa L, Cunha A, Guimaraes JT, Taveira-Gomes A, Santos AC, Domingues VF, Delerue-Matos C, Monteiro R, Calhau C. 2014. Persistent organic pollutant levels in human visceral and subcutaneous adipose tissue in obese individuals-Depot differences and dysmetabolism implications. Environ Res 133C: 170-7.

5. Bluher M. The distinction of metabolically 'healthy' from 'unhealthy' obese individuals. Curr Opin Lipidol. 2010;21:38-43.

Conflict of Interest: None declared.

CHAPTER III

"ESTROGEN RECEPTOR DEPENDENT AND INDEPENDENT MODULATION OF M1 AND M2 MACROPHAGE FUNCTIONS BY ESTRADIOL"

(SUBMITTED)

Estrogen receptor dependent and independent modulation of M1 and M2 macrophage functions by estradiol

Diana Teixeira[ab], Cláudia Marques[ab], Ana Faria[acd], Diogo Pestana[ab], Sónia Norberto[a], Conceição Calhau[ab], Rosário Monteiro[ae]

[a]Department of Biochemistry, Faculty of Medicine, University of Porto, Centro de Investigação Médica, Porto, Portugal
[b]CINTESIS - Center for Research in Health Technologies and Information Systems, Porto, Portugal
[c]REQUIMTE, Laboratório Associado Em Química Verde, Faculty of Sciences, University of Porto, Porto, Portugal
[d]Faculty of Nutrition and Food Sciences, University of Porto, Porto, Portugal
[e]Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal

ABSTRACT

Purpose: Evidence is being gathered on the effect of estrogens on the immune system. However, little is known about their effects on the differential expression of estrogen receptor subtypes or on the putatively distinct effects on macrophages in different activation states. This study was carried out to evaluate if estrogen treatment of classically (M1)- or alternatively (M2)- differentiated macrophages could affect their behavior.

Methods: Human peripheral blood monocyte-derived macrophages after M1 or M2 activation were treated with 17β-estradiol (E_2) alone or in combination with selective estrogen receptor (ER) α or ERβ antagonists. Macrophage cytokine release, migratory capability and estrogen-associated signaling pathways were evaluated.

Results: ERα and ERβ mRNA were differently expressed among different stages of macrophage activation. E_2 significantly induced M1 release of interleukin (IL) 10 and IL6 and decreased IL10, IL6, TNFα and IL1β release by M2 macrophages. Moreover, E2 also stimulated M2 migration. Most E_2 effects were shown to be mediated by either ERα or ERβ and/or by orchestrating NF-κB, AP1, JNK and ERK signaling pathways.

Conclusions: We provide evidence that both pro- and anti-inflammatory programs can co-exist within the same macrophage and may be programmed by E_2 into either direction depending on the surrounding environment, which may represent an interesting target to modulate inflammation.

Keywords: estrogen, estrogen receptors, M1 macrophages, M2 macrophages, inflammation

INTRODUCTION

Knowledge on the pleiotropic functions regulated by estrogens in different tissues and organs has evolved rapidly during the past decade. Given the widespread role of estrogens in human physiology, it is not surprising that estrogens are also implicated in the development or progression of several common pathological conditions, which include (but are not limited to) asthma, atherosclerosis, osteoporosis, insulin resistance, neurodegenerative diseases, as well as obesity [1-6]. Among other physiological roles, there is growing evidence that estrogens influence the immune system and the processes associated with inflammation [7, 8].

The biological actions of estrogens are largely mediated by two distinct estrogen receptor (ER) isoforms, namely ERα and ERβ, that are distributed in different cell types including monocytes/macrophages [9, 10]. ERs are members of the nuclear receptor family and act as ligand-activated transcription factors to regulate the expression of target genes, which control both specific and overlapping physiological effects of estrogens [11]. ERs mediate not only genomic but also a series of non-genomic actions of estrogens.

Running title: Estrogen on macrophage behaviour

**Corresponding author*: Diana Teixeira. Tel: (+351) 220426680.
E-mail: dianamst@med.up.pt

Moreover, a number of different signaling pathways have been found to be activated by estrogens in different cell types, namely ERα-mediated extracellular signal regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and phosphatidyl-inositol-3-kinase/AKT (PI3K/AKT) pathways, as well as ERβ-mediated p38/MAPK. Furthermore, some transcriptional activity can be regulated by protein-protein interaction of ER with other specific transcription factors, such as activator protein (AP) 1 and nuclear factor-κB (NF-κB) [12, 13].

Newly formed monocytes leave the unique environment of the bone marrow and enter the blood, where they are exposed to a plethora of agents, which are capable of impacting on their functional and phenotypic characteristics. Under stimulation, these cells selectively home to different tissues, undergo differentiation into macrophages, and upon entry, migrate to the inflammatory focus, a central and rate-limiting step in inflammation [14]. Tissue macrophages experience a hierarchy of activation or polarization states that ensure baseline tissue homeostasis and prevent constant inflammation which, in case of occurrence, underlies the pathophysiology of numerous chronic and metabolic diseases [15].

Functional heterogeneity and plasticity are hallmarks of the monocyte/macrophage lineage. In response to various signals, macrophages may undergo classical activation (M1, stimulated by lipopolysaccharide, LPS) or alternative activation (M2, stimulated by interleukin (IL) 4), states that mirror the Th_1-Th_2 polarization of T lymphocytes [16]. In addition, pathological states are frequently associated with dynamic changes in macrophage activation, with classically-activated M1 cells implicated in initiating and sustaining inflammation and M2 in resolving or containing chronic inflammation [17].

Monocyte-derived macrophages (MDM) are commonly accepted as a good surrogate of macrophages infiltrating tissues. Distinct phenotypes are currently obtained upon monocyte exposure to bacterial agents or cytokine/chemokine cocktails allowing M1 or M2 activation [18]. Nevertheless, the potential

influence of estrogens on these divergent cell populations is unclear.

Macrophage function is profoundly affected by the environment to which they are exposed, including not only cytokines and chemokines but also steroid hormones.

Although the prominent role of different types of nuclear receptors in the orchestration of macrophage polarization in several inflammatory diseases is well established, and previous studies have shown that estrogens mediate effects on monocyte and macrophage immune function [19, 20], the exact contribution of estrogen-regulated pathways to macrophage polarization remains unclear. In fact, the connection between macrophage polarization and estrogens is just beginning to be described and seems to be relevant *in vivo* under physiological and pathological conditions [21]. Furthermore, very little is known about whether the expression of the ERs varies with macrophage polarization stage.

The current study was devised in order to further clarify the immunomodulatory potential and underlying mechanisms of estrogen action in human MDM according to activation state. We further hypothesized that estrogen treatment of macrophage after differentiation with LPS or IL4 could modify their profile of cytokine release. Finally, we sought to determine if the effects of estrogen treatment are mediated by estrogen associated signaling pathways.

MATERIALS AND METHODS

Peripheral blood-derived macrophages

Leukocyte-rich buffy coats were obtained from blood donor healthy female volunteers between the ages of 50 and 62 years recruited from the Department of Immunohemotherapy, São João Hospital, Porto, Portugal, following a protocol approved by the Ethical Committee for Health of Hospital São João and after written an informed consent.

Human peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Histopaque®1077 (Sigma Aldrich, Sintra, Portugal), according to the manufacturer's protocol. Briefly, blood was diluted with one volume of phosphate-buffered saline. This mixture (30 mL) was layered over 10 mL of Histopaque® and centrifuged at 800 × g for 30 min at room temperature. After three washes, PBMCs were resuspended in RPMI-1640 phenol-red free medium (Sigma Aldrich, Sintra, Portugal) and counted. PBMCs obtained from six different donors were pooled to reduce variability. Furthermore, all women were postmenopausal as classified after clinical endocrinology evaluation according to American Association of Clinical Endocrinologists Medical Guidelines, having more than one year without menses [22], in order to avoid a possible preconditioning effect of different levels of circulating estrogens from premenopausal women.

Differentiation of monocytes into macrophages occurs spontaneously by adhesion of cells to the plastic culture dishes [9] (21 cm^2; Ø 60 mm; Orange Scientific, Belgium). Cells were maintained in the presence of RPMI-1640 medium with 10% fetal bovine serum (FBS; Gibco, Paisley, UK), 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (Sigma Aldrich, Sintra, Portugal) for 7 days at 37ºC in a humidified atmosphere with 5% CO_2. For macrophage differentiation and alternative activation (M2), freshly-plated monocytes were stimulated with human recombinant IL4 (15 ng/mL; Sigma Aldrich, Sintra, Portugal) added at the beginning of differentiation and for 7 days. The classical activation of macrophages was achieved through the incubation of adherent cells (macrophages) on day 7 in culture medium, with 100 ng/mL LPS (classical activation stimulus, M1; *Escherichia coli* 0111:B4, Sigma Aldrich, Sintra, Portugal) at 37°C for 24 h. Control macrophages (Mϕ) were obtained through a similar procedure (7 days of culture) but received no stimulation. Immunofluorescence analyses of culture cells after differentiation showed that more than 95% were positive for CD68, a marker for differentiated macrophages. Briefly, macrophages were differentiated on 8-well Falcon® slides, washed with PBS, fixed in 4% ρ-formaldehyde and permeabilized with 1% triton X-100. Mϕ cells were stained with rabbit-anti-CD68 (200 µg/mL). M1 cells were stained with rabbit-anti-CD68 (200 µg/mL) and mouse-anti-CD11c (200 µg/mL) and M2 cells were stained with rabbit-anti-CD68 (200 µg/mL) and goat-anti-CD206 (200 µg/mL) followed by incubation with FITC and Texas red secondary antibodies [23, 24]. Cell nuclei were counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Roche, Amadora, Portugal). Fluorescence images were acquired using a fluorescence microscope (Nikon Eclipse 50i®, Melville, USA) at a magnification of x200 and their analysis performed with ImageJ software® (National Institute of Health, Bethesda, USA). Negative controls were carried out by omission of the primary antibody. All antibodies were purchased to Santa Cruz Biotechnology, Heidelberg, Germany. The cells were subsequently washed and cultured in serum-free medium during 24 h and were then treated with 17β-estradiol (E_2, 10^{-11} M; Sigma Aldrich, Sintra, Portugal) in serum-free medium supplemented with 0.05% bovine serum albumin (BSA, Sigma Aldrich, Sintra, Portugal), for 24 h in all experiments. To assess the ER-dependence of the E_2 effect, cells were exposed to the selective ERα antagonist 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP, 10^{-6} M; Sigma Aldrich, Sintra, Portugal) or the selective ERβ antagonist 4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl)phenol (PHTPP, 10^{-6} M; Santa Cruz Biotechnology, Heidelberg, Germany) added at the same time as E_2. After 24 h incubation, supernatants were collected and stored at -80ºC for the determination of cytokine levels.

A stock solution of each compound used for treatments was prepared in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) and kept at -20ºC. Appropriate dilutions of each compound were freshly prepared just prior to every assay. The effect of the vehicle (at a final concentration of 0.1%) was evaluated in all experiments.

RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was isolated from the cells using STAT-60 reagent (AMS Biotechnology, Abingdon, UK) followed by chloroform extraction and isopropanol precipitation. RNA extracts were treated with DNase (RQ1 RNase-free DNase, Promega, Carnaxide, Portugal) to avoid contamination with genomic DNA and its concentration was assessed spectrophotometrically with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA,

USA). For qRT-PCR analysis, total RNA (1 µg) was reversely-transcribed using the NZY First Strand cDNA synthesis kit (NZYTech, Lisbon, Portugal) in accordance to the manufacturer's protocol.

qPCR was carried out using a LightCycler 96 (Roche, Amadora, Portugal). All cDNA samples were analysed in duplicate by qRT-PCRs conducted with SYBR green qPCR mix (FastStart Essential DNA Green Master; Roche, Amadora, Portugal). Cycling conditions were as follows: denaturation (95ºC for 5 min), amplification and quantification [95ºC for 10 s, annealing temperature (AT) for 5 s, and 72ºC for 10 s, with a single fluorescence measurement at the end of the cycle repeated 45 times, a melting curve program [(AT+10)ºC for 15 s and 95ºC with a heating rate of 0.1ºC/s and continuous fluorescence measurement], and a cooling step to 37ºC. Human-specific primers (Table 1), purchased from Sigma Aldrich (Sintra, Portugal) were used for qRT-PCR analysis.

Data were analyzed using LightCycler analysis software (version 1.0). Gene expression was calculated using the $2^{-\Delta CT}$ method [25], where the CT was the threshold concentration, and normalized against the geometric mean expression levels of the endogenous control gene hypoxanthine-guanine-phosphoribosyltransferase (HPRT).

Table 1
Primers used for quantitative real-time PCR.

Gene	Forward primer (5'-3')	Reverse primer (3'-5')	Annealing Temperature
Hprt	TGCTGACCTGCTGGATTACA	TTTATGTCCCCTGTTGACTGG	59
c-Jun	GAGCGCCTGATAATCCAGTCC	GAAGCCCTCCTGCTCATCTGT	62
COX2	CATTCCCTTCCTTCGAAATGC	GAGAAGGCTTCCAGCTTTTG	61
CREB1	ACTGATTCCCAAAAGCGAAGG	CTTCAATCCTTGGCACTCCTG	61
ERα	GAAGAGGGTGCCAGGCTTTGT	CGCCAGACGAGACCAATCATC	64
ERβ	GTTCGACCAAGTGCGGCTCTT	TCCCCTCATCCCTGTCCAGAA	65
ERK2	TGTTGACATTCAACCCACACAA	TCGAACTTGAATGGTGCTTCG	61
Ikβα	TGGGCCAGCTGACACTAGAAA	GGCCTCCAAACACACAGTCAT	62
JNK1	AAGCGGGCCTACAGAGAGCTA	GGCAAAGATTTGCATCCATGA	61
NF-κB	GCCCAGTGAAGACCACCTCTC	AAGCTGAGTTTGCGGAAGGAT	62
RelA	GCTCCTGTGCGTGTCTCCAT	TTCTCCTCAATCCGGTGACG	62

Hprt, hypoxanthine phosphoribosyltransferase; COX2, cyclooxygenase (COX) 2; CREB1, cAMP response element-binding protein 1; ERα, estrogen receptor α; ERβ, estrogen receptor β; ERK2, extracellular signal-regulated kinase 2; IkBα, inhibitor κB α; JNK1, c-Jun N-terminal kinase 1; NF-κB, nuclear factor-κB, p50 subunit; RelA, nuclear factor-κB, p65 subunit

Cytokine ELISA assay

IL1β, IL6, IL10, and TNFα concentrations in cell media were measured using, respectively, LEGEND MAX™ Human IL1β, IL6, IL10, and TNFα ELISA kits (BioLegend Inc., San Diego, CA, USA), according to the manufacturer's instructions.

Migration assay

Human MDM migration was assayed using the Chemicon QCM Migration kit (96-well, 5-µm pore size; Merck Millipore, Madrid, Spain) according to the manufacturer's directions. Freshly isolated human peripheral blood MDM, and after polarization as described previously, were plated at a density of $2x10^4$ cells/well in serum-free culture medium supplemented with 0.05% BSA added to the upper chamber in the presence of E_2, MPP, PHTPP or vehicle. Culture medium with 10% FBS (constituting the chemotactic stimulus of the assay) was added to the lower chamber and cells were incubated at 37ºC and 5% CO_2 for 24 h. After incubation, 75 µL of suspension containing cells that migrated was transferred from the migration feeder tray to the well of a new 96-well plate, while the migrated cells that were still attached to the bottom side of the migration chamber filter were dislodged by incubating in Cell Detachment Solution (Chemicon) for 30 min (37ºC in 5% CO_2). Seventy five µL of dislodged cell suspension were mixed with the cells that were previously collected from the migration feeder tray and incubated for 15 min with 50 mL of Lysis Buffer/Dye Solution (Chemicon) after which fluorescence was read using the 480/520 nm filter set on a fluorometric plate reader. Exposure of macrophages to culture medium with 10% FBS consistently induced migration in control wells of an average of 52% of cells above baseline (cells with serum-free medium).

Statistical analysis

Values are expressed as the arithmetic mean ± standard error of mean (SEM). The statistical significance of the differences between groups was ascertained via one-way ANOVA or two-way ANOVA (treatment and cell type as main factors), followed by Tukey's multiple comparison test. Migration results are expressed as percentage of control (vehicle treatment only). All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). Differences were considered statistically significant when $p<0.05$.

RESULTS

ER expression in human MDM subsets

Estrogens initiate cellular signaling pathways *via* interaction with their receptors expressed primarily as two subtypes, ERα and ERβ, whose distribution varies with different cell types [26]. Recent reports have shown that many of the tissue-specific differences in estrogen action are attributable to selective binding to each ER subtype. Human macrophages have been reported to express both ERα and ERβ [27]; however their

expression in different macrophages subsets had not been evaluated.

In the current study, qRT-PCR was performed to determine estrogen receptor transcription according to macrophage polarization. In agreement with previous reports [28], we identified the transcripts for both α and β forms of ER in primary macrophages (Figure 1). Further, human peripheral blood MDM contained significantly higher levels of ERα mRNA compared to ERβ ($p<0.05$). ERβ transcription is detected at very low levels in these cells. To examine the potential changes in ER transcription as a result of activation and differentiation of human peripheral blood MDM, we compared ER mRNA levels in M1 (LPS-activated) and M2 (IL4-activated) MDM with that of unstimulated cells (Mϕ). ER analysis demonstrated a significant decrease in ERα mRNA transcription in M1 macrophage ($p<0.05$) compared to Mϕ and M2. Activation of human peripheral blood MDM did not significantly alter ERβ in M1 nor ERα in M2 compared to Mϕ. Therefore, our results suggest that ERα and ERβ are expressed differentially depending on macrophage polarization.

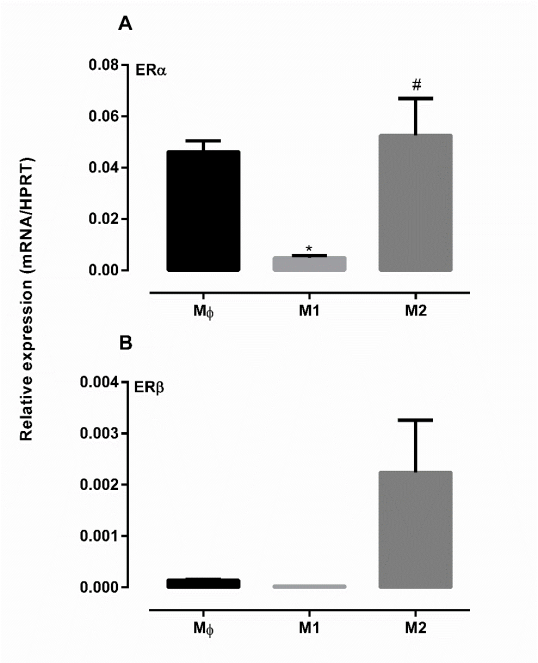


Figure 1. ERα and ERβ mRNA levels in classically (M1, 100 ng/mL LPS) or alternatively (M2, 15 ng/mL IL4) activated human macrophages or unstimulated human macrophages (control, Mϕ). mRNA levels were determined by qRT-PCR and normalized to the housekeeping gene hypoxanthine-guanine-phosphoribosyltransferase (HPRT). Values are represented as mean ± standard error of mean of three independent experiments performed in duplicate (final n=3). Statistical analysis with one-way ANOVA, followed by Tukey's multiple comparison test: *$p<0.05$ vs Mϕ, #$p<0.05$ vs M1.

E_2 modulates human MDM cytokine production

Having determined that peripheral blood MDM express ERs and thus may respond to E_2, we sought to investigate the influence of E_2 on macrophage effector functions, namely the production of IL1β, IL6, IL10 and TNFα, in response to this hormone. Based on this approach, control (Mϕ) peripheral blood MDM and M1 and M2 macrophages were treated with E_2 (10^{-11} M) alone or in combination with the selective ERα antagonist MPP (10^{-6} M), the selective ERβ antagonist PHTPP (10^{-6} M) or with vehicle (DMSO), and cytokine levels were then measured by ELISA, in 24 h culture supernatants.

The basal release of cytokine production in the three cell types is depicted in figure 2 (a, c, e, g). IL10 (13.95 ± 1.04 *vs* 44.38 ± 0.62 pg/mL) and TNFα (1100.00 ± 32.00 vs 2770.00 ± 157.00 pg/mL) release was lower, whereas IL1β (177.78 ± 1.01 *vs* 94.44 ± 11.11 pg/mL) was higher in M1 macrophage when compared to Mϕ. No differences were found between Mϕ and M1 macrophages regarding IL6 release levels. On the other hand, IL6 (122985.05 ± 5970.15 *vs* 36529.85 ± 4738.81 pg/mL) and IL10 (62.90 ± 2.84 *vs* 44.38 ± 0.62 pg/mL) release was more pronounced, and TNFα (3214.00 ± 38.00 *vs* 2770.00 ± 158.00 pg/mL) and IL1β (98.94 ± 0.65 pg/mL *vs* 94.44 ± 11.11 pg/mL) levels were similar in M2 macrophages when compared to Mϕ.

In LPS-activated M1 macrophages, IL10 (51.36 ± 4.26 pg/mL) and IL6 (20223.88 ± 8805.97 pg/mL) release was clearly up-regulated by E_2 treatment (figure 2b, d). This effect of E_2 on IL10 seems to be ERα- and ERβ-independent since it was unaffected by incubation with either MPP or PHTPP, respectively (figure 2b). However, IL6 secretion induced by E_2 was decreased in the presence of MPP and increased in the presence of PHTPP (figure 2d). TNFα and IL1 β release in M1 was unaffected by E_2 treatment (figure 2e, f, g, h).

Furthermore, E_2 strongly down-regulated the IL4-polarized M2 cell release of IL10 (5.79 ± 0.05 vs 62.90 ± 2.84 pg/mL), IL6 (77.69 ± 5.15 vs 122985.05 pg/mL), TNFα (82.00 ± 19.00 vs 219.50 ± 14.50 pg/mL) and IL1β (2.12 ± 1.21 vs 177.78 ±

1.01 pg/mL) (figure 2b, d, f, h). In these cells, cytokine release inhibition by E_2 was blocked by the ERβ antagonist PHTPP in the case of IL6, IL10 and TNFα, and by the ERα antagonist MPP in the case of IL1β, IL10 and TNFα.

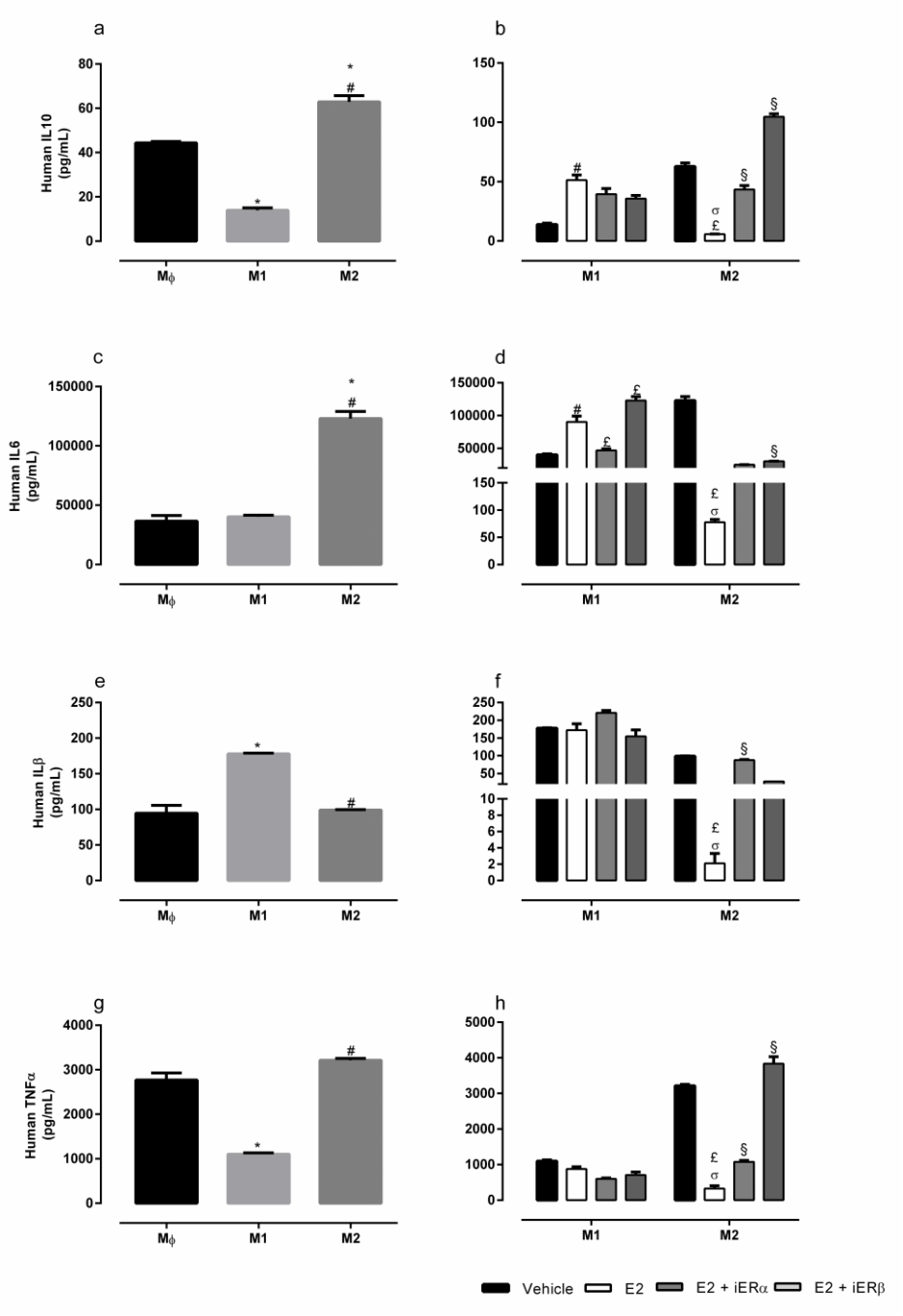


Figure 2. Cytokine release according to macrophage polarization states. Peripheral blood monocyte-derived macrophages either unstimulated (Mϕ) or after polarization with lipopolysaccharide (100 ng/mL, M1) or interleukin (IL) 4 (15 ng/mL, M2) were treated with 17β-estradiol (E_2, 10^{-12} M) alone or in combination with the selective estrogen receptor (ER) α antagonist (iERα, 10^{-6} M) or the selective ERβ antagonist (10^{-6} M, iERβ), and appropriate controls were made by incubation with vehicle, allowing to determine basal cytokine release (a, c, e, g). The levels of IL10 (a, b), IL6 (c, d), tumor necrosis factor (TNF) α (e, f) and IL1β (g, h) were determined in the culture media at the end of the 24 h treatments. Results are presented as mean ± standard error of mean (three individual experiments performed in duplicate). Statistical analysis with one-way (a, c, e, g) or two-way ANOVA, followed by Tukey's multiple comparisons test: *$p<0.05$ vs Mϕ control, $^{\#}p<0.05$ vs M1 control (vehicle), $^{\sigma}p<0.05$ vs M2 control (vehicle), $^{£}p<0.05$ vs M1 treated with E_2, $^{§}p<0.05$ vs M2 treated with E_2.

E_2 alters gene expression profile of human MDM

The presence of ER in human MDM suggests that the modulation of inflammatory responses observed after E_2 treatment may occur at the transcriptional level. Thus, we next investigated the molecular basis for E_2 ability to differentially regulate M1 and M2 targets. Accordingly, M1 and M2 cells were treated with E_2 (10^{-11} M) alone or in combination with the selective ERα and ERβ antagonists as already described above, and the transcription levels of selected inflammation-related genes was analyzed by qRT-PCR analysis.

The MAPK signaling pathway in macrophages is one of the most extensively investigated intracellular signaling cascades involved in pro-inflammatory responses, the extracellular signal-regulated kinases 2 (ERK2) and c-Jun N-terminal kinase (JNK) belonging to this family [29]. Two-way ANOVA (main factors: treatment and cell type) revealed the significant effect of cell type ($F_{1,5} = 6986$, $p<0.05$), treatment ($F_{1,5} = 6896$, $p<0.05$) and an interaction between treatment and cell type ($F_{1,5} = 6757$, $p<0.05$) on JNK1 transcription (figure 3a). The signal intensity of JNK1 gene transcription after 24 h E_2 treatment was significantly higher in M2 cells than that in M1 cells ($p<0.05$, Tukey's), an effect that was found to be ERα- and ERβ-dependent (figure 3b). Concerning ERK2 transcription level, two-way ANOVA disclosed the significant effect of cell type ($F_{1,4} = 23.34$, $p<0.05$) (figure 3c). ERK2 levels were significantly higher ($p<0.05$, Tukey's) in M1 than in M2 E_2-treated macrophages (and the same tendency was observed between M1 and M2 untreated macrophages) and, in accordance, modulation of either ER had no effect on ERK2 transcription (figure 3d).

Members of the NF-κB/Rel family regulate many genes involved in immunity and inflammation [30]. The current analysis showed the significant effects of treatment ($F_{1,5} = 12.65$, $p<0.05$), cell type ($F_{1,5} = 10.34$, $p<0.05$) and an interaction between treatment and cell type ($F_{1,5} = 44.23$, $p<0.05$) on the NF-κB (p50) transcription level (figure 3e). The NF-κB (p50) levels were significantly higher ($p<0.05$, Tukey's) in E_2-treated M2 macrophages than in E_2-treated M1 macrophages or vehicle-treated M2 macrophages. This increase in NF-κB (p50) expression after E_2 treatment in M2 macrophages was found to be ERα- and ERβ-dependent (figure 3f). On the other hand, although the ANOVA revealed no significant effect of treatment and/or cell type on the expression levels of RelA (p65), this NF-κB subunit tends to increase in M1 cells after E_2 treatment (figure 3g, h), an effect that seems to be mediated by ERα and ERβ.

The transcription factor NF-κB is maintained in an inactive form by its inhibitor IκBα, thereby preventing NF-κB-induced transcription of target genes [31]. Two-way ANOVA unveiled the significant effect of treatment ($F_{1,7} = 7.745$, $p<0.05$) on the IκBα transcription level (figure 3i). IκBα levels had a tendency to be higher in E_2-treated M1 macrophages than in vehicle-treated M1 macrophages in an ERβ-dependent manner (figure 3j).

Along with NF-κB, the transcription factor AP1, is also known to be involved in the transcriptional regulation of inflammatory responses [32]. Analysis showed a significant effect of cell type ($F_{1,6} = 10.39$, $p<0.05$), treatment ($F_{1,6} = 6.847$, $p<0.05$) and an interaction between treatment and cell type ($F_{1,6} = 5.994$, $p<0.05$) on c-Jun transcription (figure 3k). E_2 treatment significantly ($p<0.05$, Tukey's) increased c-Jun gene transcription in M2 macrophages in comparison to vehicle-treated M2 macrophages and no effect was observed in M1 cells. This increase in c-Jun expression mediated by E_2 was found to be ERβ-dependent ($p<0.05$, Tukey's) (figure 3l).

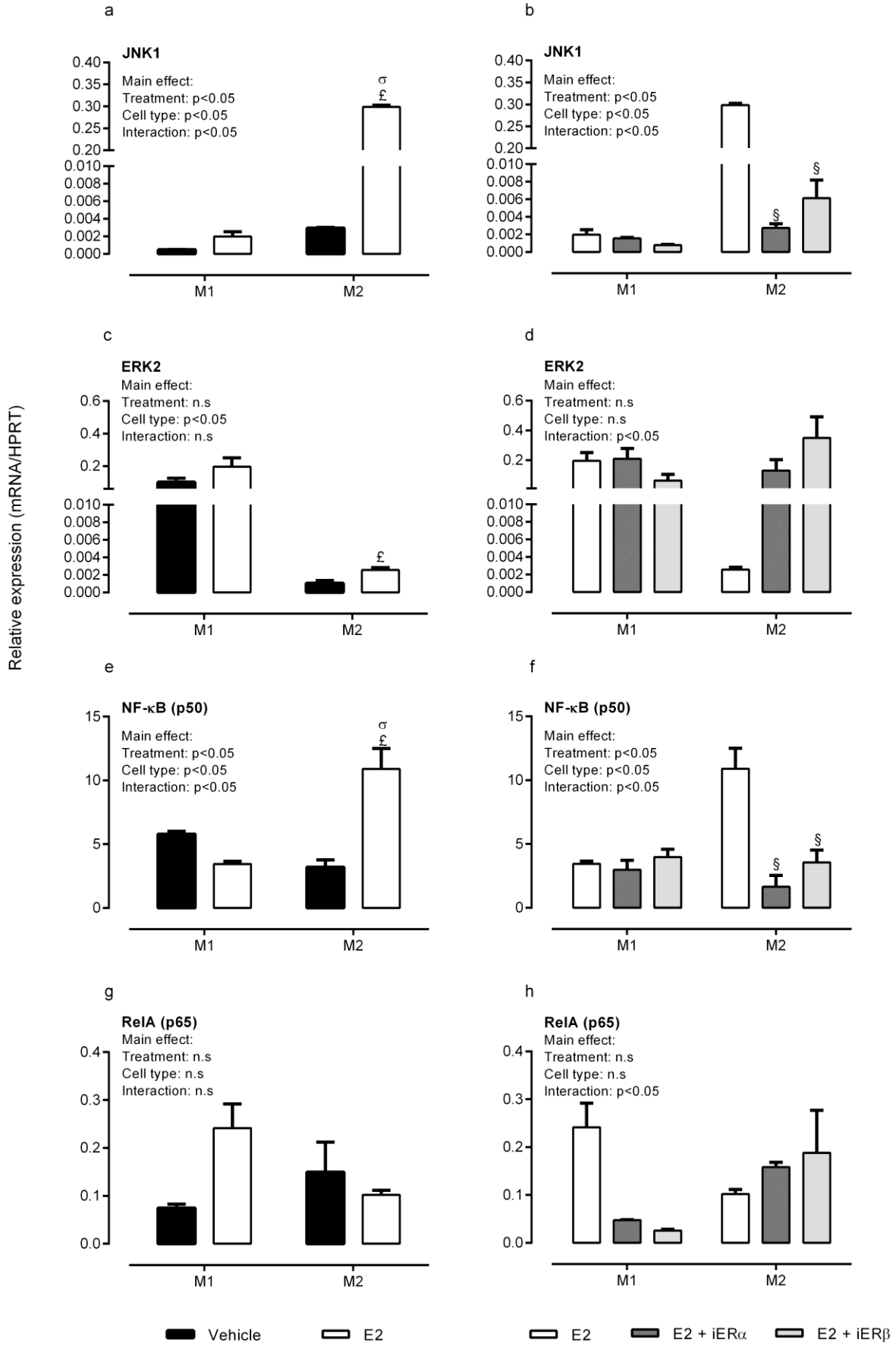
The cAMP response element-binding protein (CREB) 1 is a common target of many signaling pathways and a key transcription factor in processes such as inflammation and cell migration [33, 34]. Two-way ANOVA revealed the significant effect of cell type ($F_{1,6} = 19.92$, $p<0.05$) and an interaction between treatment and cell type ($F_{1,6} = 10.21$, $p<0.05$) on CREB1 transcription (figure 2m). The signal intensity of CREB1 gene transcription after 24 h E_2 treatment was significantly higher ($p<0.05$, Tukey's) in M1 cells than that in M2 cells and no effect was observed after treatment with ER antagonists.

Inflammatory responses are mediated in part by pro-inflammatory cytokines and induction of cyclooxygenase (COX) 2 [35]. Two-way ANOVA revealed a significant effect of cell type ($F_{1,5} = 34.73$, $p<0.05$), treatment ($F_{1,5} = 22.01$; $p<0.05$) and an interaction between treatment and cell type ($F_{1,5} = 32.85$, $p<0.05$) on COX2 transcription (figure 3O). E_2 treatment significantly induced COX2 transcription in M1 macrophages ($p<0.05$, Tukey's) and did not modify COX2 levels in M2 macrophages. This increase in COX2 transcription in M1 macrophages after E_2 treatment was found to be ERβ-dependent ($p<0.05$, Turkey's) (figure 3p).

E_2 regulates MDM cell migration

In order to explore the influence of E_2 on migratory properties of human MDM upon different polarization states, differentiated cells were incubated with E_2 and were allowed to migrate towards a chemotactic stimulus. For this purpose, transmigration chamber assays were performed.

Our results showed that migration of classically- and alternatively-activated untreated macrophages towards RPMI-1640 medium phenol-red free supplemented with 10% FBS was significantly decreased (72.6 ± 4.6% and 66.4 ± 4.5% of the control value, respectively) as compared to non-stimulated Mϕ macrophages ($p<0.05$) (figure 4a). Two-way ANOVA revealed a significant effect of cell type ($F_{1,12} = 6.083$, $p<0.05$), treatment ($F_{1,12} = 4.802$, $p<0.05$) and an interaction between treatment and cell type ($F_{1,12} = 6.083$, $p<0.05$) on polarized MDM migration (figure 4b). The migration capacity of E_2-treated M2 macrophage toward the 10% FBS stimulus was 90% increased as compared to vehicle-treated M2 macrophage, and when comparing to E_2-treated M1 macrophage migrated about 95% more ($p<0.05$, Tukey's). This increase in migration stimulated by E_2 in M2 cells seems to be independent of ERα and ERβ.


a
JNK1
Main effect:
Treatment: p<0.05
Cell type: p<0.05
Interaction: p<0.05
b
JNK1
Main effect:
Treatment: p<0.05
Cell type: p<0.05
Interaction: p<0.05
c
ERK2
Main effect:
Treatment: n.s
Cell type: p<0.05
Interaction: n.s
d
ERK2
Main effect:
Treatment: n.s
Cell type: n.s
Interaction: p<0.05
e
NF-κB (p50)
Main effect:
Treatment: p<0.05
Cell type: p<0.05
Interaction: p<0.05
f
NF-κB (p50)
Main effect:
Treatment: p<0.05
Cell type: p<0.05
Interaction: p<0.05
g
RelA (p65)
Main effect:
Treatment: n.s
Cell type: n.s
Interaction: n.s
h
RelA (p65)
Main effect:
Treatment: n.s
Cell type: n.s
Interaction: p<0.05
Relative expression (mRNA/HPRT)
M1
M2
Vehicle
E2
E2 + iERα
E2 + iERβ

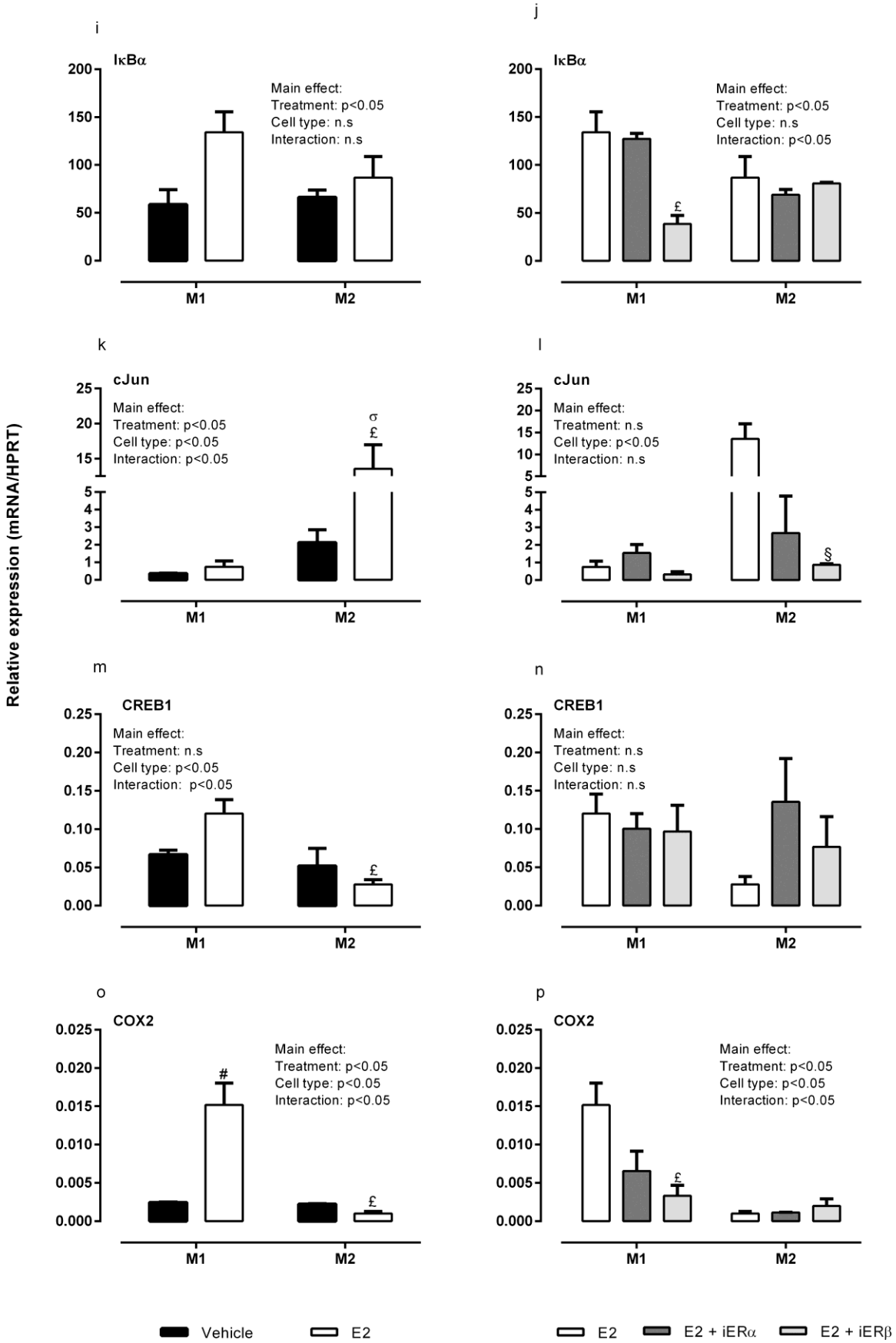

i
IκBα
Main effect:
Treatment: p<0.05
Cell type: n.s
Interaction: n.s
M1
M2
j
IκBα
Main effect:
Treatment: p<0.05
Cell type: n.s
Interaction: p<0.05
£
M1
M2
k
cJun
Main effect:
Treatment: p<0.05
Cell type: p<0.05
Interaction: p<0.05
σ
£
M1
M2
l
cJun
Main effect:
Treatment: n.s
Cell type: p<0.05
Interaction: n.s
§
M1
M2
m
CREB1
Main effect:
Treatment: n.s
Cell type: p<0.05
Interaction: p<0.05
£
M1
M2
n
CREB1
Main effect:
Treatment: n.s
Cell type: n.s
Interaction: n.s
M1
M2
o
COX2
Main effect:
Treatment: p<0.05
Cell type: p<0.05
Interaction: p<0.05
#
£
M1
M2
p
COX2
Main effect:
Treatment: p<0.05
Cell type: p<0.05
Interaction: p<0.05
£
M1
M2
Relative expression (mRNA/HPRT)
Vehicle
E2
E2
E2 + iERα
E2 + iERβ

Figure 3. Effect of 17β-estradiol on the transcription of genes involved in macrophage inflammatory responses. Peripheral blood monocyte-derived macrophages after polarization with lipopolysaccharide (100 ng/mL, M1) or interleukin (IL) 4 (15 ng/mL, M2) were treated with 17β- estradiol (E_2, 10^{-12} M) alone or in combination with the selective estrogen receptor (ER) α antagonist (iERα, 10^{-6} M) or the selective ERβ antagonist (10^{-6} M, iERβ), and appropriate controls were made by incubation with vehicle. mRNA levels were determined by qRT-PCR and normalized to the housekeeping gene (hypoxanthine-guanine-phosphoribosyltransferase, HPRT). Values are represented as mean ± standard error of mean of duplicated samples and are representative of three independent experiments. Statistical analysis with two-way ANOVA, followed by Tukey's multiple comparison test: $^{\#}p<0.05$ vs M1 control (vehicle); $^{\sigma}p<0.05$ vs M2 control (vehicle); $^{£}p<0.05$ vs M1 treated with E_2; $^{\S}p<0.05$ vs M2 treated with E_2. COX2, cyclooxygenase 2; CREB1, cAMP responsive element binding protein 1; ERK2, extracellular signal-regulated kinases 2; IκBα, inhibitor of κBα; JNK, c-Jun N-terminal kinases; NF-κB, nuclear factor-κB.

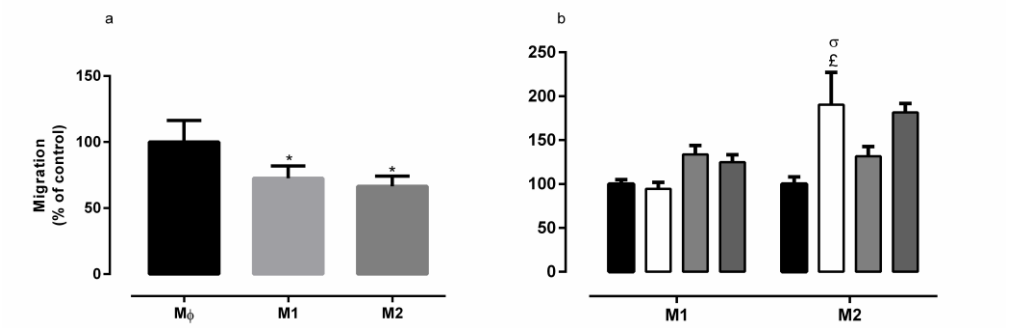


Figure 4. Estrogen regulation of peripheral blood monocyte-derived macrophages migration, either unstimulated (Mϕ) or after polarization with lipopolysaccharide (100 ng/mL, M1) or interleukin (IL) 4 (15 ng/mL, M2). M1 and M2 macrophages were loaded into the upper wells of migration microchambers and were treated with 17β-estradiol (E_2, 10^{-12} M) alone or in combination with the selective estrogen receptor (ER) α antagonist (iERα, 10^{-6} M) or the selective ERβ antagonist (10^{-6} M, iERβ), and appropriate controls were made by incubation with vehicle. The lower wells were filled with 10% FBS RPMI-1640 medium. Cell migration was allowed for 24h. Values are represented as mean of the percentage of control group ± standard error of mean from two independent experiments. The absolute control values (Abs 480/520 nm) were 180.73 ± 16.99, 110.06 ± 9.14 and 132.94 ± 18.47 for Mϕ, M1 and M2 cells, respectively. Statistical analysis with one-way (a) or two-way ANOVA, followed by Tukey's multiple comparisons test: $^{*}p<0.05$ vs Mϕ; $^{\sigma}p<0.05$ vs control M2 (vehicle), $^{£}p<0.05$ vs M1 treated with E_2.

DISCUSSION

Several investigations strongly support that estrogens' actions go far beyond those expected from their designation as a sex hormone; in fact, they act as transcriptional activators regulating important biological functions in almost every tissues of the body [8]. Thereby, the study of estrogens' action on the modulation of human macrophage function is of substantial interest since these versatile cells are implicated in the regulation of immune response and are consequently relevant to pathogenesis of many diseases [36].

During the course of the inflammatory response, phenotypic switching of macrophages from an M1 to an M2 program has been observed, underscoring the dual role of macrophages in initiating and subsequently resolving inflammation. Additionally, Kramer *et al.* [9] have found that absence of estrogen does not have a dramatic effect on the amount of ER protein, suggesting that estrogens do not regulate their own receptors in monocyte-derived macrophages. However, their expression in different macrophage subsets, a feature that may be critical for determining their responsiveness to estrogenic stimuli, had not been fully evaluated.

In the present study, we demonstrated that ERα and ERβ mRNA are differently expressed in different stages of macrophage polarization, in monocyte-derived macrophages (MDM) from postmenopausal women. Using qRT-PCR, we verified that ERα mRNA was expressed at higher levels than ERβ in macrophages that had been differentiated with IL4 for 7 days or with LPS for 24 h at the end of differentiation period. ERβ mRNA is expressed at very low levels in both of these cells. Previous studies have shown a consistence between mRNA transcription of ERs and total protein levels [37, 38]. This finding suggests that macrophage cells may respond to estrogen primarily through ERα rather than ERβ. Corroborating this fact, in different mammalian models, the preponderance of ERα gene over the ERβ gene is accepted to be a mechanism controlling the effects of E_2 on the immune system [8, 39]. In fact, many immune effects attributed to E_2 in macrophages are thought to be mediated through ERα and not ERβ [20, 40, 41]. Phiel *et al.* [42] demonstrated that ERα is increased in monocytes from postmenopausal women which may result in altered estrogen responsiveness compared with monocytes from premenopausal women.

It is widely accepted that ER α/β signaling largely exerts natural anti-inflammatory effects in immune cells of the monocyte lineage [43]. Thus, a decline in circulating E_2 could favor a more pro-inflammatory environment or exacerbate a status of preexisting inflammation. In fact, macrophage responses are not only determined by the polarization state but deeply depend on the specific micro-environmental conditions in which cells were prior to their activation. Regarding the effects of E_2 on macrophage effector functions, conflicting results have been reported from several lines of evidence. While *in vitro* experiments have suggested that E_2 exerts anti-inflammatory properties on monocyte/macrophage cell lines or microglia cells, following activation with LPS [10, 44], opposite results have been independently reported by analyzing the *in vivo* effects of estrogens on the same cell populations [45, 46].

Very little work has been conducted examining the effect of E_2 on cytokine expression in human MDM cells.

Here, we found that upon stimulation with LPS, M1 macrophages secreted higher amounts of IL1β, similar amounts of IL6 and lesser amounts of TNFα and IL10 comparing to unstimulated macrophages. This profile of cytokine production displayed by LPS-polarized macrophages seems to reveal a certain inability to recapitulate typical M1 responses, as one would expect increases in IL6 and TNFα following stimulation. Rachón *et al.* [47] found that among the majority of postmenopausal women, IL6 expression may be present in non-stimulated PBMC isolated directly from venous blood, which suggests the possibility of an endogenous activation of these cells *in vivo.* The absence of circulating estrogens in the postmenopause may play a role, explaining why the production of cytokines may be in the stimulated state, thus not increasing further in response to LPS.

Long-term E_2 treatment at physiological concentrations [48] enhanced the M1 release of IL10 and much more of IL6 (through ERα-dependent mechanisms for the latter) without change in TNFα or IL1β transcription. This is in accordance with previous published data showing that administration of E_2 to ovariectomized female mice markedly increases the expression of numerous inflammatory cytokines by monocytes/macrophages in response to LPS activation *ex vivo* [45]. However, both the dose (postmenopausal/metestrus levels compared with midfollicular phase to pregnancy levels) and the timing (before, during or after macrophage activation) of E_2 treatment may be critical [8, 49, 50]. In this regard, the target of the hormone's action may be the early-phase activation of LPS signaling [48]. This also suggests that once exposed to an estrogen-deprived environment, re-exposure to E_2 at low levels is unable to reverse the acquired pro-inflammatory priming. This may represent that the lack/decrease of estrogen signaling may render subjects to a more pro-inflammatory situation, which might contribute to the manifestation of chronic inflammatory diseases. One important addition to this discussion is the available, although scarce, information about the spontaneous decreases in cytokine activity with natural menopause, suggesting that stimulatory effects of estrogen on cytokine production may perhaps only be elicited in non-physiological conditions, e.g, systemic inflammatory state [51], as supported by the findings of Gregory *et al.* [52]. It is known from the literature that IL4 is the prototypical direct inducer of M2 macrophages which produce high levels of IL10, TGFβ and low levels of IL12 and have the capability to suppress the production of pro-inflammatory mediators by human monocytes activated *in vitro*, at least at the mRNA level [53, 54]. Corroborating this evidence, in the present work we also observed an enhanced production of IL10 and a decrease in IL1β comparing to Mϕ and/or M1 macrophages; however, we also found that these cells released higher amounts of IL6 and TNFα. Again, we can speculate that previous exposure to an estrogen poor environment may change cytokine release from the cells to a more pro-inflammatory profile. This hypothesis is reinforced from observations of Hart *et al.* [55]. This increase in the release of inflammatory cytokines by cells involved in the resolution of inflammation may exacerbate the inflammatory state that accompanies the decline of estrogens namely in menopause.

Nonetheless, the relationship between E_2 and cytokine release is far from being simple. Most often, E_2 effects on macrophages are primarily repressive, by reducing the expression of genes for cytokines or modulating other inflammatory mediators by the ER-dependent and/or non-genomic pathways in response to inflammatory signals [20, 56, 57]. Further, the majority of studies only addressed the effect of E_2 on classically activated M1 cells and less is known about the effect on alternatively-activated macrophages.

There may be several distinct pathways by which E_2 may affect cytokine gene expression, eliciting either net increases or decreases in cytokine production, depending on the activation of these pathways in the individual cellular context. At this time, we focused on the transcription level of several genes with special attention to the signaling factors such as JNK1, ERK2, COX2, nuclear transcription factors like AP1 (c-Jun), NF-κB (p50 and p65) and its inhibitor IκBα and CREB1, to try to understand the molecular basis of E_2 action.

Here, we showed a tendency towards an increase in ERK2 transcription in M1 E_2-treated cells as well as a clear effect of E_2 treatment in favor of JNK1 and p65 NF-κB transcription which may be involved in the observed increase of IL10 production. Indeed, Liu *et al.* [58] demonstrated that p65 NF-κB, ERK1/2 and JNK, were all involved in LPS-induced IL10 expression. These signaling pathways might also regulate IL6 and TNFα. Moreover, in mouse activated macrophages prostaglandin E2 also upregulates the level of IL10 [58]. In line with this, we observed a significant increase in COX2 transcription upon E_2 treatment [59]. Interestingly, many of these effects in gene transcription seem to be mediated by ERβ, highlighting that although ERα is expressed in substantially higher levels and most estrogens effects have been related to this isoform, ERβ also plays a role.

In M2 cells, the inhibitory effect of E_2 in IL6, TNFα and IL1β expression may be a result of E_2-ER mediated binding, and consequently blocking, NF-κB, an effect reported in several *in vitro* studies [60, 61]. In good agreement with this, we observed in M2 E_2-treated cells an increase in p50 NF-κB transcription (and the same tendency was observed in IκBα). As homodimers of the NF-κB p50 can function as transcriptional repressors and IκBα may inhibit the transcriptional activity of p65 NF-κB complexes [62], E_2 treatment in M2 cells may be linked to impaired expression of these NF-κB-dependent genes.

Central to host-defense responses is leukocyte (e.g. monocyte and macrophage) recruitment to the sites of injury. In this study we show that LPS- or IL4-stimulated MDM have lower capacity to cross membranes in the transmigration chamber assays than unstimulated macrophages that readily transmigrate to the bottom of the plate. In line with this finding, in 2008 Bradfield *et al.* [63] have shown that LPS activation leads to an arrest of the monocyte on the lumenal surface whereas non-activated monocytes readily transmigrate across the endothelium. Several other studies that look at the effects of LPS on monocyte migration have been published over the past decade, which appear contradictory [64, 65]. More recently Tajima *et al.* [66] have established that in macrophages, LPS stimulates prostaglandin signaling initiating early migration as well as leading to induction of MCP-1, which contributes to a later phase migration.

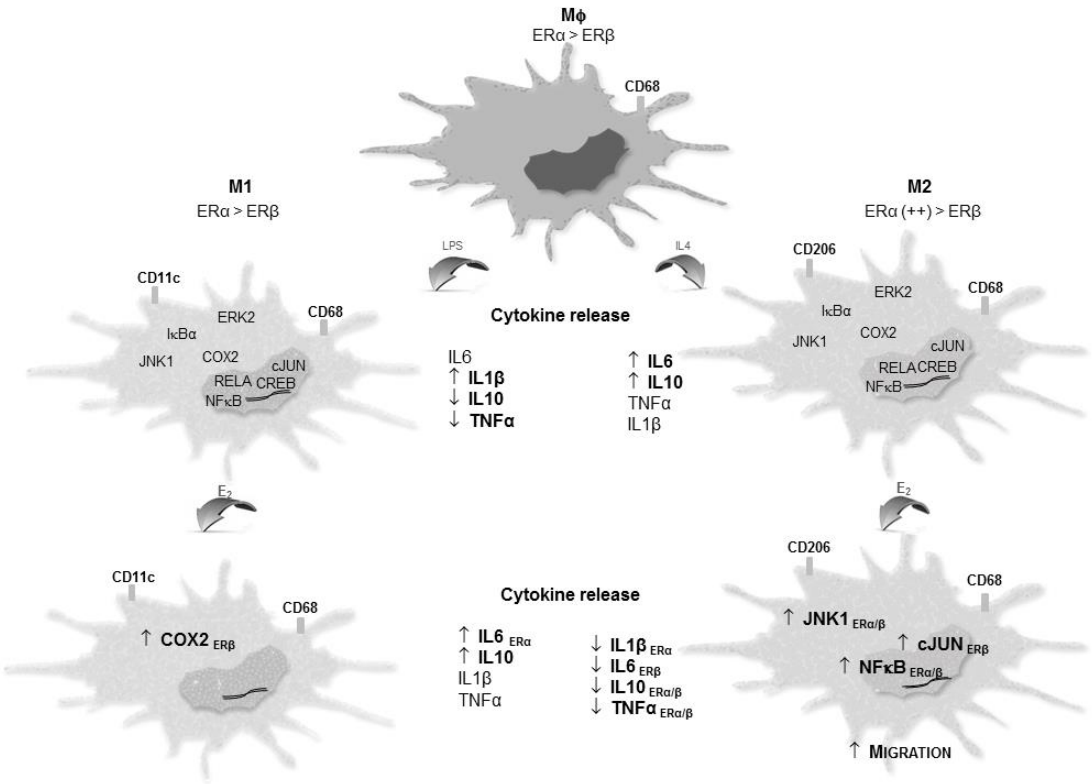


Figure 5. A model illustrating the putative regulation of blood monocyte-derived macrophages (Mϕ) after classically (M1; LPS, 100 ng/mL) or alternatively (M2; IL4, 15 ng/mL) polarization by 17β-estradiol (E2). Estrogen receptor (ER) α was expressed at higher levels than ERβ in Mϕ difference that remained present after polarization. M1 macrophages secreted higher amounts of IL1β, similar of IL6 and lesser amounts of IL10 and TNFα than unstimulated Mϕ macrophages. E2 treatment at physiological concentrations enhanced M1 release of IL10 and much more IL6. M2 macrophages secreted higher IL10 and IL6 amounts than Mϕ, and E2 treatment decreased the release of all cytokines evaluated. E2 increased migration only in M2. The evaluation of the transcription of several genes namely JNK1, ERK2, COX2, nuclear transcription factors like AP1 (c-Jun), NF-κB (p50 and p65) and its inhibitor IκBα and CREB1 and effects mediated by E2 treatment appear highlighted. COX2, cyclooxygenase 2; CREB1, cAMP responsive element binding protein 1; E2, 17β-estradiol; ERK2, extracellular signal-regulated kinases 2; IL, interleukin; IκBα, inhibitor of κBα; JNK1, c-Jun N-terminal kinases 1; LPS, lipopolysaccharides; Mϕ, control macrophages; M1, classically activated macrophages; M2, alternatively activated macrophages; NF-κB, nuclear factor-κB; TNFα, tumor necrosis α; ↑, increase; ↓, decrease. Estrogen receptor α and β dependent effects appear as the subscripts ERα and ERβ, respectively.

However, to date there is a paucity of evidence about the role of E_2 in the migratory capability of clearly differentiated human macrophages. In our study, concerning M1 macrophages, E_2 did not alter the migratory capacity of these cells which may be consistent with their rear guard role against pathogen invasion. Concerning M2 macrophages, as far as we know, this is the first study to document the E_2 stimulation in macrophage migration under static conditions, at a low concentration. As notably reviewed by Straub, E_2 at high levels inhibits migration of innate immune cells to the site of inflammation, although this is mostly reported in conditions of active chronic inflammation. *In vitro* and *in vivo* studies, point toward a marked inhibitory effect of high levels of E_2 on migration of leukocytes into inflamed areas, particularly neutrophils and monocytes, what was accompanied by less severe inflammation [8].

Here, we suggest that low E_2 levels support trafficking of "healing" M2 cells to the inflammation site by serving as a selective stimulus and "phenotype-shaping gate" for macrophages *en route* to the injured site. Recently, several reports have shown that MAPKs, mainly ERK1/2, p38 and JNK1, are involved in chemotaxis induced by serum in leukocytes and smooth muscle cells [67] In the present work, we observed that E_2 significantly increased JNK1 transcription a possible mechanism contributing to increased migration in M2 cells.

The approach used by our study consisted of comparing between E_2 and co-treatment with ER antagonists namely MPP (iERα) and PHTPP (iERβ), revealing the involvement of each receptor subtype in the described effects. As demonstrated for E_2, the effect of treatment with iERs varies according to cell type, thereby granting the possibility to selectively inhibit or

stimulate estrogen-like actions in various tissues. Specifically, our results provide evidence of ERβ functionality on macrophage subtypes. However, it should be noted that a part of estrogens effects are independent of ERs suggesting the involvement of non-classical estrogen signaling, which is supported by the modification of the second messengers and transcription factor here evaluated. Although protein levels and phosphorylation status were not determined for these pathways, the effects observed along with the demonstration of regulation by ER inhibition establishes a role for estrogens in their regulation at least at a transcriptional level. Specifically, one cannot exclude the participation of the nonclassical ER GPR30 to E_2 effects on macrophages. Moreover, it is necessary to point out that dependent on the concentration used, ER antagonists may behave as agonists (which is true for higher concentrations). However, most of our results reflect that they were indeed inhibiting E_2's effect showing their antagonistic action and highlighting that ERs are mediating the effects.

We also provide further evidence that physiological levels of E_2 influence the inflammatory status of macrophages and consequently the capability to orchestrate inflammatory and immune responses (Figure 5). This report supports the view that both pro- and anti-inflammatory programs can co-exist within M1 and M2 macrophages, as recently proposed [68], instead of being mutually antagonistic. This affords a framework to provide homeostasis by "pulling" the cell in opposite directions [69], to which coincident or previous exposure to estrogens may be determining.

In summary, the results presented in this study open a new avenue into the possible molecular mechanism by which E_2 may regulate immune cells, particularly macrophage homeostasis. The interference in MDM functions by E_2 may constitute a valuable target to modulate inflammation pathophysiology.

ACKNOWLEDGEMENTS

The authors kindly thank to Professor Dr. Fernando Araújo and Dr. Cristina Neves from the Department of Immunohemotherapy, São João Hospital, Porto, Portugal. This work was supported by FCT (Fundo Social Europeu, Programa Operacional Potencial Humano da EU (POPH); UID/BIM/04293/2013; SFRH/BD/64691/2009, SFRH/BPD/75294/2010, SFRH/BD/93073/2013), and Projetos de Investigação na Pré-graduação 2011, Universidade do Porto (IJUP).

CONFLICT OF INTEREST

No competing financial interests to declare.

RFERENCES

1. Faienza MF, Ventura A, Marzano F, Cavallo L. Postmenopausal osteoporosis: the role of immune system cells. Clinical & developmental immunology.2013;2013:doi: 10.1155/2013/575936.

2. Bonds RS, Midoro-Horiuti T. Estrogen effects in allergy and asthma. Current opinion in allergy and clinical immunology.2013;13:92-99.

3. Barton M. Cholesterol and atherosclerosis: modulation by oestrogen. Current opinion in lipidology.2013;24:214-220.

4. Matsui S, *et al.* Associations of estrogen and testosterone with insulin resistance in pre- and postmenopausal women with and without hormone therapy. International journal of endocrinology and metabolism.2013;11:65-70.

5. Melcangi RC, Panzica G, Garcia-Segura LM. Neuroactive steroids: focus on human brain. Neuroscience.2011;191:1-5.

6. Mauvais-Jarvis F, Clegg DJ, Hevener AL. The role of estrogens in control of energy balance and glucose homeostasis. Endocrine reviews.2013;34:309-338.

7. Cutolo M, Sulli A, Straub RH. Estrogen metabolism and autoimmunity. Autoimmunity reviews.2012;11:A460-464.

8. Straub RH. The complex role of estrogens in inflammation. Endocrine reviews.2007;28:521-574.

9. Kramer PR, Wray S. 17-Beta-estradiol regulates expression of genes that function in macrophage activation and cholesterol homeostasis. J Steroid Biochem Mol Biol.2002;81:203-216.

10. Vegeto E, *et al.* Estrogen prevents the lipopolysaccharide-induced inflammatory response in microglia. J Neurosci.2001;21:1809-1818.

11. Beato M, Klug J. Steroid hormone receptors: an update. Hum Reprod Update.2000;6:225-236.

12. Webb P, *et al.* The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. Mol Endocrinol.1999;13:1672-1685.

13. Stice JP, Mbai FN, Chen L, Knowlton AA. Rapid activation of nuclear factor kappaB by 17beta-estradiol and selective estrogen receptor modulators: pathways mediating cellular protection. Shock.2012;38:128-136.

14. Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. Journal of leukocyte biology.2004;76:509-513.

15. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nature reviews Immunology.2011;11:723-737.

16. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nature immunology.2010;11:889-896.

17. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. The Journal of clinical investigation.2012;122:787-795.

18. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol.2004;25:677-686.

19. Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. Hum Reprod Update.2005;11:411-423.

20. Suzuki T, *et al.* Tissue compartment-specific role of estrogen receptor subtypes in immune cell cytokine production following trauma-hemorrhage. J Appl Physiol (1985).2007;102:163-168.

21. Bolego C, Cignarella A, Staels B, Chinetti-Gbaguidi G. Macrophage function and polarization in cardiovascular disease: a role of estrogen signaling? Arteriosclerosis, thrombosis, and vascular biology.2013;33:1127-1134.

22. Goodman NF, Cobin RH, Ginzburg SB, Katz IA, Woode DE. American Association of Clinical Endocrinologists Medical Guidelines for Clinical Practice for the diagnosis and treatment of menopause: executive summary of recommendations. Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists.2011;17:949-954.

23. Sindrilaru A, *et al.* An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. The Journal of clinical investigation.2011;121:985-997.

24. Wentworth JM, *et al.* Pro-inflammatory CD11c+CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity. Diabetes.2010;59:1648-1656.

25. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nature protocols.2008;3:1101-1108.

26. Matthews J, Gustafsson JA. Estrogen signaling: a subtle balance between ER alpha and ER beta. Mol Interv.2003;3:281-292.

27. Murphy AJ, Guyre PM, Wira CR, Pioli PA. Estradiol regulates expression of estrogen receptor ERalpha46 in human macrophages. PloS one.2009;4:e5539. doi: 5510.1371/journal.pone.0005539.

28. Mor G, *et al.* Interaction of the estrogen receptors with the Fas ligand promoter in human monocytes. J Immunol.2003;170:114-122.

29. Rao KM. MAP kinase activation in macrophages. Journal of leukocyte biology.2001;69:3-10.

30. Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol.2004;25:280-288.

31. Hoffmann A, Levchenko A, Scott ML, Baltimore D. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science.2002;298:1241-1245.

32. Zhang X, Mosser DM. Macrophage activation by endogenous danger signals. J Pathol.2008;214:161-178.

33. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol.2001;2:599-609.

34. Wen AY, Sakamoto KM, Miller LS. The role of the transcription factor CREB in immune function. J Immunol.2010;185:6413-6419.

35. Kang YJ, Wingerd BA, Arakawa T, Smith WL. Cyclooxygenase-2 gene transcription in a macrophage model of inflammation. J Immunol.2006;177:8111-8122.

36. Gilliver SC. Sex steroids as inflammatory regulators. J Steroid Biochem Mol Biol.2010;120:105-115.

37. Huang H, *et al.* Opposing effects of estradiol and progesterone on the oxidative stress-induced production of chemokine and proinflammatory cytokines in murine peritoneal macrophages. The journal of medical investigation : JMI.2008;55:133-141.

38. Liu L, Zhao Y, Xie K, Sun X, Gao Y, Wang Z. Estrogen-induced nongenomic calcium signaling inhibits lipopolysaccharide-stimulated tumor necrosis factor alpha production in macrophages. PloS one.2013;8:e83072.

39. Campbell L, *et al.* Estrogen Receptor-Alpha Promotes Alternative Macrophage Activation during Cutaneous Repair. The Journal of investigative dermatology.2014;134:2447-2457.

40. Lambert KC, Curran EM, Judy BM, Lubahn DB, Estes DM. Estrogen receptor-alpha deficiency promotes increased TNF-alpha secretion and bacterial killing by murine macrophages in response to microbial stimuli in vitro. Journal of leukocyte biology.2004;75:1166-1172.

41. Lambert KC, Curran EM, Judy BM, Milligan GN, Lubahn DB, Estes DM. Estrogen receptor alpha (ERalpha) deficiency in macrophages results in increased stimulation of CD4+ T cells while 17beta-estradiol acts through ERalpha to increase IL-4 and GATA-3 expression in CD4+ T cells independent of antigen presentation. Journal of immunology.2005;175:5716-5723.

42. Phiel KL, Henderson RA, Adelman SJ, Elloso MM. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. Immunol Lett.2005;97:107-113.

43. Brown CM, Mulcahey TA, Filipek NC, Wise PM. Production of proinflammatory cytokines and chemokines during neuroinflammation: novel roles for estrogen receptors alpha and beta. Endocrinology.2010;151:4916-4925.

44. Vegeto E, Ghisletti S, Meda C, Etteri S, Belcredito S, Maggi A. Regulation of the lipopolysaccharide signal transduction pathway by 17beta-estradiol in macrophage cells. J Steroid Biochem Mol Biol.2004;91:59-66.

45. Calippe B, *et al.* Chronic estradiol administration in vivo promotes the proinflammatory response of macrophages to TLR4 activation: involvement of the phosphatidylinositol 3-kinase pathway. J Immunol.2008;180:7980-7988.

46. Soucy G, Boivin G, Labrie F, Rivest S. Estradiol is required for a proper immune response to bacterial and viral pathogens in the female brain. J Immunol.2005;174:6391-6398.

47. Rachon D, Mysliwska J, Suchecka-Rachon K, Wieckiewicz J, Mysliwski A. Effects of oestrogen deprivation on interleukin-6 production by peripheral blood mononuclear cells of postmenopausal women. J Endocrinol.2002;172:387-395.

48. Ghisletti S, Meda C, Maggi A, Vegeto E. 17beta-estradiol inhibits inflammatory gene expression by controlling NF-kappaB intracellular localization. Molecular and cellular biology.2005;25:2957-2968.

49. Ruh MF, Bi Y, D'Alonzo R, Bellone CJ. Effect of estrogens on IL-1beta promoter activity. J Steroid Biochem Mol Biol.1998;66:203-210.

50. Bernard-Poenaru O, Roux C, Blanque R, Gardner C, de Vemejoul MC, Cohen-Solal ME. Bone-resorbing cytokines from peripheral blood mononuclear cells after hormone replacement therapy: a longitudinal study. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA.2001;12:769-776.

51. Pfeilschifter J, Koditz R, Pfohl M, Schatz H. Changes in proinflammatory cytokine activity after menopause. Endocrine reviews.2002;23:90-119.

52. Gregory MS, Duffner LA, Faunce DE, Kovacs EJ. Estrogen mediates the sex difference in post-burn immunosuppression. J Endocrinol.2000;164:129-138.

53. Woodward EA, Prele CM, Nicholson SE, Kolesnik TB, Hart PH. The anti-inflammatory effects of interleukin-4 are not mediated by suppressor of cytokine signalling-1 (SOCS1). Immunology.2010;131:118-127.

54. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity.2010;32:593-604.

55. Hart PH, Bonder CS, Balogh J, Dickensheets HL, Donnelly RP, Finlay-Jones JJ. Differential responses of human monocytes and macrophages to IL-4 and IL-13. Journal of leukocyte biology.1999;66:575-578.

56. Harkonen PL, Vaananen HK. Monocyte-macrophage system as a target for estrogen and selective estrogen receptor modulators. Annals of the New York Academy of Sciences.2006;1089:218-227.

57. Pioli PA, *et al.* Estradiol attenuates lipopolysaccharide-induced CXC chemokine ligand 8 production by human peripheral blood monocytes. J Immunol.2007;179:6284-6290.

58. Liu YW, Chen CC, Tseng HP, Chang WC. Lipopolysaccharide-induced transcriptional activation of interleukin-10 is mediated by MAPK- and NF-kappaB-induced CCAAT/enhancer-binding protein delta in mouse macrophages. Cellular signalling.2006;18:1492-1500.

59. Kim C, Wilcox-Adelman S, Sano Y, Tang WJ, Collier RJ, Park JM. Antiinflammatory cAMP signaling and cell migration genes co-opted by the anthrax bacillus. Proceedings of the National Academy of Sciences of the United States of America.2008;105:6150-6155.

60. Galien R, Garcia T. Estrogen receptor impairs interleukin-6 expression by preventing protein binding on the NF-kappaB site. Nucleic acids research.1997;25:2424-2429.

61. Kalaitzidis D, Gilmore TD. Transcription factor cross-talk: the estrogen receptor and NF-kappaB. Trends in endocrinology and metabolism: TEM.2005;16:46-52.

62. Porta C, *et al.* Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB. Proceedings of the National Academy of Sciences of the United States of America.2009;106:14978-14983.

63. Bradfield PF, Johnson-Leger CA, Zimmerli C, Imhof BA. LPS differentially regulates adhesion and transendothelial migration of human monocytes under static and flow conditions. International immunology.2008;20:247-257.

64. Galdiero M, Folgore A, Molitierno M, Greco R. Porins and lipopolysaccharide (LPS) from Salmonella typhimurium induce leucocyte transmigration through human endothelial cells in vitro. Clinical and experimental immunology.1999;116:453-461.

65. Shen Y, Sultana C, Arditi M, Kim KS, Kalra VK. Endotoxin-induced migration of monocytes and PECAM-1 phosphorylation are abrogated by PAF receptor antagonists. The American journal of physiology.1998;275:E479-486.

66. Tajima T, *et al.* Lipopolysaccharide induces macrophage migration via prostaglandin D(2) and prostaglandin E(2). The Journal of pharmacology and experimental therapeutics.2008;326:493-501.

67. Ashida N, Arai H, Yamasaki M, Kita T. Distinct signaling pathways for MCP-1-dependent integrin activation and chemotaxis. The Journal of biological chemistry.2001;276:16555-16560.

68. Jenkins SJ, *et al*. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. Science.2011;332:1284-1288.

69. McKay LI, Cidlowski JA. Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. Endocrine reviews.1999;20:435-459.

CHAPTER IV

"EFFECTS OF XENOESTROGENS IN HUMAN M1 AND M2 MACROPHAGE MIGRATION, CYTOKINE RELEASE AND ESTROGEN-RELATED SIGNALING PATHWAYS"

ENVIRONMENTAL TOXICOLOGY. 2015

DOI: 10.1002/TOX.22154

Effects of Xenoestrogens in Human M1 and M2 Macrophage Migration, Cytokine Release, and Estrogen-Related Signaling Pathways

Diana Teixeira,[1] Cláudia Marques,[1,2] Diogo Pestana,[1,2] Ana Faria,[1,3,4] Sónia Norberto,[1] Conceição Calhau,[1,2] Rosário Monteiro[1,5]

[1]Faculty of Medicine, Department of Biochemistry, University of Porto, Centro De Investigação Médica, Porto 4200-319, Portugal

[2]CINTESIS—Center for Research in Health Technologies and Information Systems, Porto 4200-450, Portugal

[3]Faculty of Nutrition and Food Sciences, University of Porto, Porto 4200-465, Portugal

[4]REQUIMTE, Laboratório Associado Em Química Verde, Faculty of Sciences, University of Porto, Porto 4179-007, Portugal

[5]Instituto De Investigação E Inovação Em Saúde, Universidade Do Porto, Porto, Portugal

Received 29 November 2014; revised 27 April 2015; accepted 11 May 2015

ABSTRACT: Bisphenol A (BPA), bis(2-ethylhexyl)phthalate (DEHP) and di(*n*-butyl)phthalate (DBP) are environmental estrogens that have been associated with a wide range of adverse health outcomes for which inflammation has also been hypothesized as a potentially involved mechanism and where macrophages play a central role. This study was carried out to evaluate if xenoestrogen (XE) treatment of classically (M1) or alternatively (M2) activated macrophages could affect their behavior. For this purpose, human peripheral blood monocyte-derived macrophages either unstimulated or activated with lipopolysaccharide (100 ng/mL, M1) or with interleukin (IL) 4 (15 ng/mL, M2) were treated with 17β-estradiol (E_2), BPA, DEHP and DBP alone or in combination with selective ERα or ERβ antagonists. Migratory capability, cytokine release, and estrogen-associated signaling pathways were evaluated to assess macrophage function. All tested XEs had a tendency to stimulate M2 migration, an effect that followed the same direction than E_2. Moreover, all XEs significantly induced IL10 in M1 and decreased IL6 and globally decreased IL10, IL6, TNFα, and IL1β release by M2 macrophages. However, DEHP and DBP significantly increased IL1β release in M1 and M2 macrophages, respectively. Some of the effects described above were shown to be mediated by either ERα or ERβ and were simultaneous to modulation of NF-κB, AP1, JNK, or ERK signaling pathways. We provide new evidence of the effect of XE on

Correspondence to: D. Teixeira, Faculty of Medicine, Department of Biochemistry (U38-FCT), University of Porto, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal. E-mail: dianamst@med.up.pt

Contract grant sponsor: FCT (Fundo Social Europeu, Programa Operacional Potencial Humano da EU (POPH).

Contract grant numbers: UID/BIM/04293/20
40110/2007; SFRH/BD/46640/2008; SF
SFRH/BPD/75294/2010; SFRH/BD/93073/2013.

Contract grant sponsor: Projetos de Investigaçã
2011, Universidade do Porto (IJUP)

Published online 00 Month 2015 in Wil
(wileyonlinelibrary.com). DOI: 10.1002/tox.22154

macrophage behavior and their mechanisms with relevance to the understanding of the action of environmental chemicals on the immune system and inflammation-associated diseases. Environ Toxicol 00: 000–000, 2015.

Keywords: bisphenol A; inflammation; M1 macrophages; M2 macrophages; phthalates; xenoestrogens

INTRODUCTION

Thousands of anthropogenic chemicals are currently being released into the environment and many of them are xenoestrogens (XE), a diverse group of synthetic agents (e.g., pesticides, surfactants, plastic monomers additives) that can mimic and disrupt the action of physiological estrogens (Kerdivel et al., 2013). Two examples are phthalates and bisphenol A (BPA), which are known to disrupt the endocrine system by competing with endogenous hormones for receptor binding or by altering the synthesis and metabolism of these hormones, thereby triggering or preventing hormonal response (Tabb and Blumberg, 2006).

BPA is one of the best-characterized XE members and, at the moment, one of the most produced chemicals worldwide, with over 6 billion pounds produced each year (Vandenberg et al., 2007). BPA is a main ingredient in plastics and resins, and is used in a variety of consumer products including water bottles and the inside lining of cans from which it can migrate into foods (Halden, 2010). In humans, BPA exposure routinely occurs via residues contained in food, beverages, or dental materials. Because of the instability of BPA-based polymers, BPA leaches out of the products and even if not persistent in the environment, virtually all people are continuously exposed to it beginning in intrauterine life (Ikezuki et al., 2002; Vandenberg et al., 2007; vom Saal et al., 2007). A fraction of the absorbed BPA may also be distributed to body storage sites followed by a slow, low-level release of BPA into the bloodstream (Volkel et al., 2002; Fernandez et al., 2007).

Phthalates, which are esters of phthalic acid, are primarily used to enhance plasticity of industrial polymers in many household and personal care products, toys, and medical supplies (Halden, 2010). Human exposure may occur through ingestion, inhalation, and dermal exposure during whole lifetime, including also intrauterine development. Di-(2-ethylhexyl) phthalate (DEHP) is one of the most widespread phthalate plasticizers, used in numerous consumer products, commodities, and building materials and similarly, di-*n*-butyl phthalate (DBP) are two of the most widely used phthalate (Koch and Calafat, 2009). While phthalates are not bio-accumulative, continuous daily exposure leads to a scenario that is similar to persistent and bioaccumulative compounds. Indeed, convincing evidence shows that there is a widespread exposure to BPA and phthalates: 93% of NHANES participants had detectable levels of BPA, and 75% had phthalate metabolites present in their urine (Silva et al., 2004; Calafat et al., 2005). Other studies have also determined the presence of BPA and phthalates in human amniotic fluid, follicular fluid, and semen, placental tissue, umbilical cord blood, and breast milk (Vandenberg et al., 2007; Faniband et al., 2014). More recently some pharmacokinetic experiments demonstrated that XE exposure may be much higher than initially thought (Swan et al., 2010; Taylor et al., 2011; Batista et al., 2012).

Previous studies have demonstrated that BPA and phthalates can bind to both estrogen receptors (ERs). The relative binding affinity of these XEs to ERs is known to be up to 1000 times weaker than that of 17β-estradiol (E_2). Nevertheless, current research has revealed that XEs stimulate cellular responses at very low concentrations through different pathways (Quesada et al., 2002; Zsarnovszky et al., 2005; Welshons et al., 2006; Wetherill et al., 2007; Alonso-Magdalena et al., 2008).

ABBREVIATIONS

ANOVA	analysis of variance;
AP1	activator protein 1;
BPA	bisphenol A;
COX2	cyclooxygenase 2;
CREB	cAMP response element-binding protein;
DBP	di-*n*-butyl phthalate;
DEHP	di-ethyl-2-hexyle phthalate;
E_2	17β-estradiol
ER	estrogen receptor;
ERK	extracellular signal-regulated kinase;
IκBα	inhibitor of κBα;
IL	interleukin;
JNK	c-Jun N-terminal kinase;
LPS	lipopolysaccharide;
M1	classically-activated;
M2	alternatively-activated;
MAPK	mitogen-activated protein kinase;
MDM	monocyte-derived macrophages;
MPP	1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride;
NF-κB	nuclear factor-κB;
PBMCs	peripheral blood mononuclear cells;
PI3K	phosphatidylinositol-3-kinase;
PHTPP	4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl)phenol;
SEM	standard error of mean;
TNF	tumor necrosis factor;
XE	xenoestrogens

Immune function, long known to be sensitive to steroids, has also been identified as a target for XEs. The well-known increase in the incidence of autoimmune diseases, sex differences in immune function, and documented immunomodulatory effects of estrogens have highlighted the potential for these chemicals to affect this dynamic system (Inadera, 2006). Macrophages are key players in the orchestration of inflammation and may exist on a spectrum of diverse phenotypes, from "classically-activated" M1 to "alternatively-activated" M2 macrophages. The environmental conditions, degree and type of the inflammatory process will essentially drive their polarization to either M1 or M2 macrophages to consequently influence inflammation termination and resolution (Gordon, 2003). Despite the existing conflicting data, it has been reported that environmental estrogens may interfere with and disrupt the immune system namely by modulating the function of macrophages resulting in suppression (Pyo et al., 2007; Yoshitake et al., 2008) or enhancement (Ruh et al., 1998) of their activity (Segura et al., 1999).

For all this, there is an urgent need to identify the underlying mechanisms of XE-mediated immune dysfunction, as well as XE immunomodulatory functions and the molecular basis behind their effect.

Herein, we intend to unveil the immunomodulatory effects of three plastic-derived XEs, BPA, DEHP, and DBP on human monocyte-derived macrophages (MDM) upon different activation states, through analysis of their migratory potential, cytokine production, and interference with inflammation relevant estrogen-related signaling pathways. The involvement of ERs in these processes was also investigated using selective ERα and ERβ antagonists.

MATERIAL AND METHODS

Peripheral Blood–Derived Macrophages

Leukocyte-rich buffy coats were obtained from blood donor healthy female volunteers between the ages of 50 and 62 years recruited from the Department of Immunohemotherapy, São João Hospital, Porto, Portugal, following a protocol approved by the Ethical Committee for Health of Hospital São João and after written an informed consent.

Human peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Histopaque®1077 (Sigma Aldrich, Sintra, Portugal), according to the manufacturer's protocol. Briefly, blood was diluted with one volume of phosphate-buffered saline. This mixture (30 mL) was layered over 10 mL of Histopaque® and centrifuged at 800 × *g* for 30 min at room temperature. After three washes, PBMCs were resuspended in RPMI-1640 phenol-red free medium (Sigma Aldrich, Sintra, Portugal) and counted. Although primary macrophages are an excellent model to decipher E_2-induced changes in gene transcription, donor availability as well as donor variability could limit the application of these cells. For this reason, we pooled the PBMCs obtained from six different donors. Furthermore, all women were postmenopausal as classified after clinical endocrinology according to American Association of Clinical Endocrinologists Medical Guidelines having more than one year without menses (Goodman et al., 2011) in order to avoid a possible preconditioning effect of different levels of circulating estrogens.

Differentiation of monocytes into macrophages occurs spontaneously by adhesion of cells to the plastic culture dishes (Kramer and Wray, 2002) (21 cm^2; Ø 60 mm; Orange Scientific, Belgium). Cells were maintained in the presence of RPMI-1640 phenol red-free medium with 10% fetal bovine serum (FBS; Gibco, Paisley, UK) and 2 m*M* L-glutamine, essential for cell growth and function, 100 U/mL penicillin and 100 U/mL streptomycin (Sigma Aldrich, Sintra, Portugal) for 7 days at 37°C in a humidified atmosphere with 5% CO_2. For macrophage differentiation and alternative activation (M2), freshly-plated monocytes were stimulated with human recombinant interleukin (IL) 4 (15 ng/mL; Sigma Aldrich, Sintra, Portugal) added at the beginning of differentiation and for 7 days. On the other hand, at day 7, adherent cells (macrophages) were washed and stimulated in culture medium, with 100 ng/mL lipopolysaccharide (LPS, classical activation stimulus, M1; *Escherichia coli* 0111:B4, Sigma Aldrich, Sintra, Portugal) at 37°C for 24 h. Immunofluorescence analyses of culture cells after differentiation showed that more than 95% were positive for CD68, a marker for differentiated macrophages. Briefly, macrophages were differentiated on 8-well Falcon® slides, washed with PBS, fixed in 4% *ρ*-formaldehyde and permeabilized with 1% triton X-100. Undifferentiated macrophages (Mϕ cells) were stained with rabbit-anti-CD68 (200 µg/mL). M1 cells were stained with rabbit-anti-CD68 (200 µg/mL) and mouse-anti-CD11c (200 µg/mL) and M2 cells were stained with rabbit-anti-CD68 (200 µg/mL) and goat-anti-CD206 (200 µg/mL) followed by incubation with FITC and Texas red secondary antibodies (Wentworth et al., 2010; Sindrilaru et al., 2011). Cell nuclei were counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Roche, Amadora, Portugal). Fluorescence images were acquired using a fluorescence microscope (Nikon Eclipse 50i®, Melville, USA) at a magnification of 200× and their analysis performed with ImageJ software® (National Institute of Health, Bethesda, MD). Negative controls were carried out by omission of the primary antibody. All antibodies were purchased from Santa Cruz Biotechnology, Heidelberg, Germany. The cells were subsequently washed and cultured in serum-free medium during 24 h and were then treated with BPA (10^{-6} M; Sigma Aldrich, Sintra, Portugal), DEHP (10^{-6} M; Sigma Aldrich, Sintra, Portugal) and DBP (10^{-6} M; Sigma Aldrich, Sintra, Portugal) or 17β-estradiol (E_2, 10^{-11} M; Sigma Aldrich, Sintra, Portugal) (Fig. 1) in serum-free medium supplemented with 0.05% bovine serum

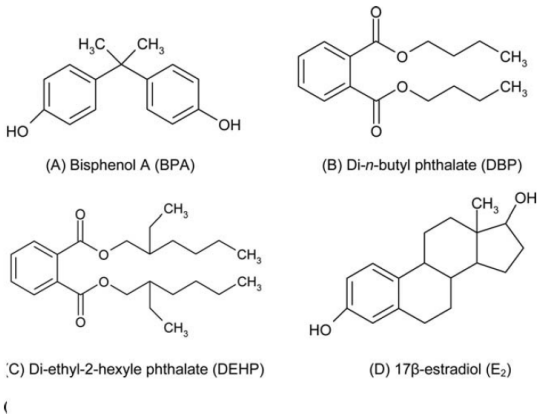


Fig. 1. Chemical structures of (A) bisphenol-A, (B) di-*n*-butyl phthalate, (C) di-ethyl-2-hexyle phthalate, and (D) 17β-estradiol.

albumin (BSA, Sigma Aldrich, Sintra, Portugal), for 24 h in all experiments. To assess ER-dependence of the effects, cells were exposed to the selective ERα antagonist 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)-phenol]-1H-pyrazole dihydrochloride (MPP, 10^{-6} M; Sigma Aldrich, Sintra, Portugal) or the selective ERβ antagonist 4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl)phenol (PHTPP, 10^{-6} M; Santa Cruz Biotechnology, Heidelberg, Germany) added at the same time of the compounds studied. After 24 h incubation, supernatants were collected for the determination of cytokine levels and adherent cells were suspended and lysed in STAT-60 reagent (AMS Biotechnology, Abingdon, UK) for molecular biology and stored at −80°C.

A stock solution of each compound was prepared in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) and kept at −20°C. Appropriate dilutions were freshly prepared just prior to every assay and appropriate controls using the vehicle (at a final concentration of 0.1%) were included in all experiments.

Migration Assay

Human MDM migration was assayed using the two chamber-based Chemicon QCM Migration kit (96-well, 5 μm pore size; Merck Millipore, Madrid, Spain) according to the manufacturer's directions. Freshly isolated human peripheral blood MDM, differentiated as described previously, were plated at a density of 2×10^4 cells/well in serum-free culture medium supplemented with 0.05% BSA added to the upper chamber in the presence of E_2, MPP, PHTPP, or vehicle. Culture medium with 10% FBS (constituting the chemotactic stimulus of the assay) was added to the lower chamber and cells were incubated at 37°C and 5% CO_2 for 24 h. After incubation, 75 μL of suspension containing cells that migrated was transferred from the migration feeder tray to the well of a new 96-well plate, while the migrated cells that were still attached to the bottom side of the migration chamber filter were dislodged by incubating in Cell Detachment Solution (Chemicon) for 30 min (37°C in 5% CO_2). Seventy-five microliter of dislodged cells were mixed with the cells that were previously collected from the migration feeder tray and incubated for 15 min with 50 mL of Lysis Buffer/Dye Solution (Chemicon) after which fluorescence was read using the 480/520 nm filter set on a fluorometric plate reader. Exposure of macrophages to culture medium with 10% FBS consistently induced migration in control wells of an average of 52% of cells above baseline (cells with serum-free medium).

Cytokines ELISA Assay

IL1β, IL6, and IL10 concentrations in cell media were measured using, respectively, LEGEND MAX™ Human IL1β, IL6, and IL10 ELISA kits (BioLegend, San Diego, CA), according to the manufacturer's instructions.

RNA Isolation, cDNA Synthesis, and qRT-PCR

Total RNA was isolated from the cells using STAT-60 reagent followed by chloroform extraction and isopropanol precipitation. RNA extracts were treated with DNase (RQ1 RNase-free DNase, Promega, Carnaxide, Portugal) to avoid contamination with genomic DNA and its concentration was assessed spectrophotometrically with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). For qRT-PCR analysis, total RNA (1 μg) was reversely-transcribed using the NZY First Strand cDNA synthesis kit (NZYTech, Lisbon, Portugal) in accordance to the manufacturer's protocol.

qPCR was carried out using a LightCycler 96 (Roche, Amadora, Portugal). All cDNA samples were analysed in duplicate by qRT-PCRs conducted with SYBR green qPCR mix (FastStart Essential DNA Green Master; Roche, Amadora, Portugal). Cycling conditions were as follows: denaturation (95°C for 5 min), amplification and quantification [95°C for 10 s, annealing temperature (AT) for 5 s, and 72°C for 10 s, with a single fluorescence measurement at the end of the 72°C for each 10 s segment] repeated 45 times, a melting curve program [(AT+10)°C for 15 s and 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement], and a cooling step to 37°C. Human-specific primers (Table I), purchased from Sigma Aldrich (Sintra, Portugal) were used for qRT-PCR analysis.

Data were analyzed using LightCycler analysis software (version 1.0). Gene expression was calculated using the $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008), where the CT was the threshold concentration, and normalized against the geometric mean expression levels of the endogenous control gene hypoxanthine-guanine-phosphoribosyltransferase (HPRT).

TABLE I. Primers used for quantitative real-time PCR

	qPCR primers		
Gene	**Forward Primer (5′–3′)**	**Reverse Primer (3′–5′)**	**Annealing Temperature**
HPRT	TGCTGACCTGCTGGATTACA	TTTATGTCCCCTGTTGACTGG	59
cJun	GAGCGCCTGATAATCCAGTCC	GAAGCCCTCCTGCTCATCTGT	62
COX2	CATTCCCTTCCTTCGAAATGC	GAGAAGGCTTCCAGCTTTTG	61
CREB1	ACTGATTCCCAAAAGCGAAGG	CTTCAATCCTTGGCACTCCTG	61
ERK2	TGTTGACATTCAACCCACACAA	TCGAACTTGAATGGTGCTTCG	61
IκBα	TGGGCCAGCTGACACTAGAAA	GGCCTCCAAACACACAGTCAT	62
JNK1	AAGCGGGCCTACAGAGAGCTA	GGCAAAGATTTGCATCCATGA	61
NF-κB (p50)	GCCCAGTGAAGACCACCTCTC	AAGCTGAGTTTGCGGAAGGAT	62
RelA (p65)	GCTCCTGTGCGTGTCTCCAT	TTCTCCTCAATCCGGTGACG	62

COX2, cyclooxygenase 2; CREB1, cAMP response element-binding protein 1; ERK2, extracellular signal-regulated kinase 2; HPRT, hypoxanthine phosphoribosyltransferase; IκBα, nuclear factor -κB inhibitor alpha; JNK1, c-Jun N-terminal kinase 1; NF-κB, nuclear factor -κB, p50 subunit; RelA, nuclear factor-κB, p65 subunit.

Statistical Analyses

Values are expressed as the arithmetic mean ± standard error of mean (SEM). The statistical significance of the differences between groups was ascertained via one-way analysis of variance (ANOVA) or two-way ANOVA (treatment and cell type as main factors), followed by Tukey's multiple comparison test. Migration results are expressed as a percentage of control (vehicle treatment only). All statistical analyses were performed using GraphPad Prism 6 statistical software (GraphPad Software, San Diego, CA). Differences were considered statistically significant when $p < 0.05$.

RESULTS

Bisphenol A and Phthalates Regulate MDM Cell Migration

Our results showed that the selected XE affected differently cell migration differently capacity, and this seemed dependent of cell subtype. Two-way ANOVA revealed the significant effect of cell type ($F2,\ 37 = 17.86$, $p < 0.05$) on polarized MDM migration [Fig. 2(a)]. Neither E_2 nor the tested XE modified the migration capacity of M1 cells. In M2 cells, the migration capacity of BPA, DEHP, and DBP

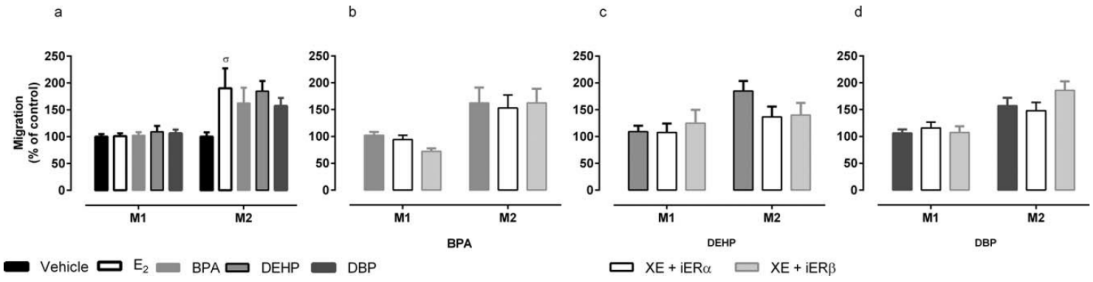


Fig. 2. Xenoestrogen (XE) regulation of peripheral blood monocyte-derived macrophages migration after polarization with lipopolysaccharide (100 mg/mL, M1) or interleukin (IL) 4 (15 ng/mL, M2). M1 and M2 macrophages were loaded into the upper wells of the microchamber and were treated with 17β-estradiol (E_2, 10^{-11} M), bisphenol-A (BPA, 10^{-6} M), di-ethyl-2-hexyle phthalate (DEHP, 10^{-6} M) or di-*n*-butyl phthalate (DBP, 10^{-6} M) alone or in combination with the selective estrogen receptor (ER) α antagonist (iERα, 10^{-6} M, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride) or the selective ERβ antagonist (iERβ, 10^{-6} M, 4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl)phenol), and appropriated controls were made by incubation with vehicle. The lower wells were filled with 10% FBS RPMI-1640 medium. Cell migration was allowed for 24 h. Values are represented as mean of the percentage of control group ± standard error of mean from two independent experiments. The absolute control values (Abs 480/520 nm) were, 110.06 ± 9.14 and 132.94 ± 18.47 for M1 and M2 cells, respectively. Statistical analysis with one-way or two-way ANOVA, followed by Tukeys' multiple comparisons test: $^{\sigma}p < 0.05$ vs. M2 control (vehicle).

treated-cells toward the 10% FBS tended to have a similar effect of that of E2 (approximately, BPA cells migrated 62% more, DEHP 84% more and DBP 57% more than vehicle-treated M2 macrophages); however, without statistical significance. This tendency to increase migration stimulated by plasticizers in M2 cells seems independent of ERα and ERβ [Fig. 2(b–d)], since it was unaffected by simultaneous incubation with ERα and ERβ selective inhibitors.

Xenoestrogens Modulate Cytokines Production by MDMs

In LPS-activated macrophages, the overall pattern of IL1β release had a tendency to be higher than in M2 cells. Moreover, while E_2, BPA, and DBP did not affect IL1β release, DEHP treatment increased release of this cytokine in M1 cells (258.47 ± 28.63 vs. 157.81 ± 1.04 pg/mL in control cells) [Fig. 3(a)]. IL6 release was down-regulated by all XEs (BPA, 238.38 ± 45.38 pg/mL; DEHP, 369.88 ± 29.13 pg/mL; DBP, 433.50 ± 122.25 pg/mL) when compared to vehicle-treated (40186.56 ± 1380.60 pg/mL) and E_2-treated M1 macrophages (90223.88 ± 8805.97 pg/mL) [Fig. 3(c)]. Concerning IL10, its release was clearly up-regulated by E_2 and XEs (E_2, 51.36 ± 4.26 pg/mL; BPA, 36.36 ± 3.70 pg/mL; DEHP 73.21 ± 9.06 pg/mL; DBP 50.66 ± 2.93 pg/mL vs. 13.95 ± 1.04 pg/mL in control cells) [Fig. 3(e)]. Furthermore, the increase in IL10 release induced by DEHP was more pronounced ($p < 0.05$, Tukey's) than that obtained after incubation with E_2 or DBP treatment. The effects observed upon cytokine release on M1 macrophages seemed to be ERα and ERβ-independent since they were not significantly modified by incubation with MPP or PHTPP (results not shown).

Regarding M2 macrophages, BPA and DEHP did not modify release of IL1β in comparison to vehicle-treated cells, an effect that was similar to that of E_2. However, DBP treatment to M2 cells stimulated IL1β release [Fig. 3(a)], which was dependent upon ERα and ERβ signaling [Fig. 3(b)]. The release of IL6 and IL10 in M2 macrophages was reduced by the three XEs following the same pattern resulting from E_2 treatment. The effect of DBP on IL1β was blocked by the co-treatment with ERα and ERβ antagonists [Fig. 3(b)].

Xenoestrogens Altered the Transcription Profile of Different Genes Involved in Intracellular Signaling Pathways of MDM

Two-way ANOVA (main factors, treatment and cell type) revealed the significant effect of cell type ($F4, 18 = 50.79$, $p < 0.05$), treatment ($F1, 18 = 43.80$, $p < 0.05$) and an interaction between cell type and treatment ($F4, 18 = 47.35$, $p < 0.05$) on CREB1 transcription [Fig. 4(a)]. DEHP behaved as a major stimulator of CREB1 transcription in M2 cells, an effect that was found to be ERα- and ERβ-dependent [Fig. 4(c)]. Neither E_2 nor the other XEs tested changed CREB1 transcription in M1 or M2 cells.

Regarding ERK2 transcription levels, two-way ANOVA revealed the significant effect of cell type ($F1, 14 = 16.53$, $p < 0.05$), treatment ($F4, 14 = 6.36$, $p < 0.05$) and an interaction between treatment and cell type ($F1, 14 = 5.706$, $p < 0.05$) [Fig. 4(e)]. The XEs had a different effect in transcription profile of ERK2 in M1 and M2 cells. BPA and DBP increased ($p < 0.05$, Tukey's) ERK2 transcription in M1 cells (and the same tendency was observed for DEHP). In M2 macrophages, none of the tested compounds significantly affected ERK2 transcription when compared to vehicle-treated. Simultaneous treatment with ERα or ERβ inhibitors did not modify any of the effects described above [Fig. 4(f–h)]. However, DBP treatment led to an increase of ERK2 transcription in M2 cells comparing to E_2 and the other XE. This effect in ERK2 transcription mediated by DBP was abolished by cotreatment with the ERβ antagonist ($p < 0.05$, Tukey's) [Fig. 4(h)].

Concerning JNK1 transcription, two-way ANOVA revealed the significant effect of cell type ($F1,14 = 16.12$, $p < 0.05$), treatment ($F4,14 = 16.12$, $p < 0.05$) and an interaction between treatment and cell type ($F1,14 = 15.92$, $p < 0.05$) [Fig. 4(i)]. JNK1 gene transcription after 24 h XE treatment was not significantly altered in M1 cells. In M2 cells, although XEs did not significantly change JNK1 transcription when compared to vehicle-treated cells, XE-treatment led to a smaller JNK1 transcription comparing to M2 E_2-treated cells ($p < 0.05$, Tukey's) that expressed higher JNK1 mRNA levels that controls. No effect was observed after co-treatment with ER antagonists regarding to BPA and DBP [Fig. 4(j,l)], whereas cotreatment of M2 cells with the DEHP and ERα inhibitor resulted in higher JNK1 transcription ($p < 0.05$, Tukey's) [Fig. 4(k)].

In macrophages, inflammation is regulated primarily at the level of mRNA expression via the involvement of transcriptional factors such as NF-κB. Regarding to NF-κB (p50), the analysis showed the significant effects of cell type ($F\ 1,15 = 11.08$, $p < 0.05$), treatment ($F\ 4,15 = 187.5$, $p < 0.05$) and an interaction between treatment and cell type ($F4,15 = 10.22$, $p < 0.05$) on NF-κB (p50) transcription level [Fig. 5(a)]. The XE DEHP was the one that promoted higher ($p < 0.05$, Tukey's) transcription levels of NF-κB (p50) in M1 and M2 macrophages comparing to correspondent vehicle-treated cells and the other XEs in the both macrophage subtypes. This increase in NF-κB (p50) transcription mediated by DEHP was found to be ER-independent [Fig. 5(c)].

In addition, two-way ANOVA also revealed a significant effect of cell type ($F1,15 = 40.88$, $p < 0.05$), treatment ($F4,15 = 169.0$, $p < 0.05$) and an interaction between both ($F4,15 = 13.72$, $p < 0.05$) on the transcription levels of RelA (p65) [Fig. 5(e)]. Overall, we observed a down-regulation of RELA transcription after 24 h of XE treatment in M1 and M2 macrophages comparing to vehicle-treated control cells

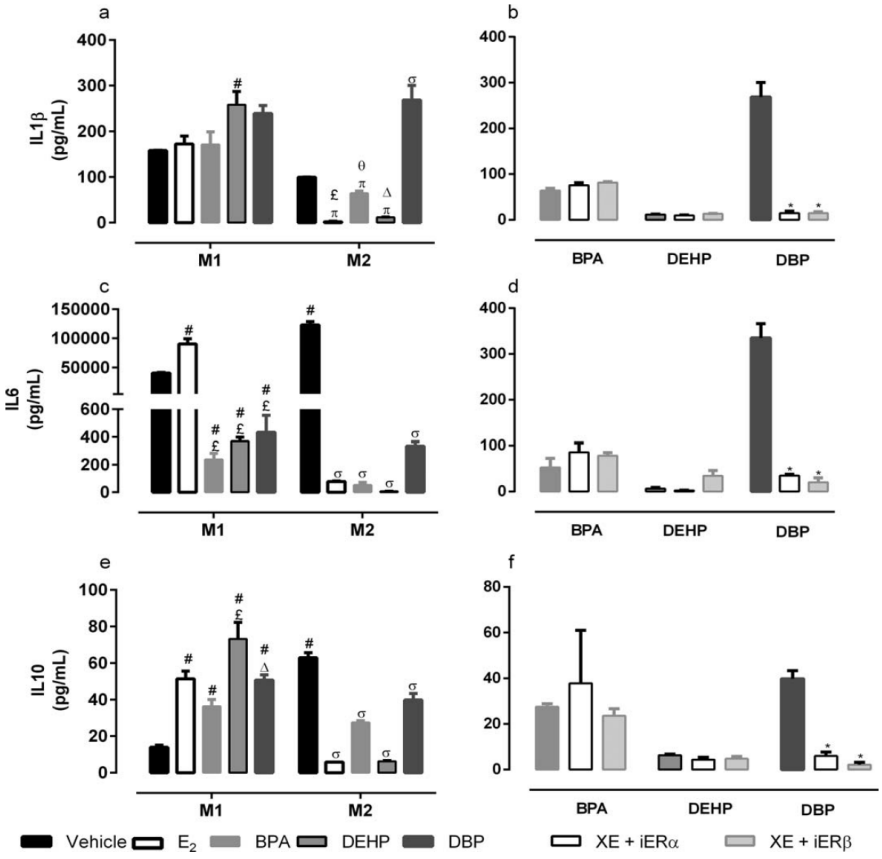


Fig. 3. Effect of xenoestrogens (XE) on macrophage cytokine release. Peripheral blood monocyte-derived macrophages after polarization with interleukin (IL) 4 (15 ng/mL, M2) were treated with 17β-estradiol (E_2, 10^{-11} M), bisphenol-A (BPA, 10^{-6} M), di-ethyl-2-hexyle phthalate (DEHP, 10^{-6} M) or di-*n*-butyl phthalate (DBP, 10^{-6} M) alone or in combination with the selective estrogen receptor (ER) α antagonist (iERα, 10^{-6} M, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride) or the selective ERβ antagonist (iERβ, 10^{-6} M, 4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl)phenol), and appropriate controls were made by incubation with vehicle, allowing to determine basal cytokine release (a, c, e, g). The levels of IL1β (a, b), IL6 (c, d,), IL10 (e, f) were measured in the culture media at the end of the 24 h treatments. Results are presented as mean ± standard error of mean of three independent experiments performed in duplicate (final $n = 3$). Statistical analysis with one-way (b, d, f, h), or two-way ANOVA, followed by Tukeys' multiple comparisons test: $^{\#}p < 0.05$ vs. M1 control (vehicle); $^{£}p < 0.05$ vs. M1 treated with E_2; $^{\Delta}p < 0.05$ vs. M1 treated with BPA; $^{\Delta}p < 0.05$ vs. M1 treated with DEHP; $^{\sigma}p < 0.05$ vs. M2 control (vehicle); $^{\pi}p < 0.05$ vs. M2 treated with DBP, *iER + XE vs. XE alone in macrophages in the same activation state.

($p < 0.05$, Tukey's), although BPA displayed a less exuberant effect. In M1 cells, the effects on RelA (p65) transcription were found to be ER-independent with the exception of DEHP treatment, which effect was found to be ERβ-dependent. In M2 cells, the decrease in RelA (p65) transcription mediated by DEHP and DBP was found to be ERα-dependent [Fig. 5(f–h)].

The transcription factor NF-κB is maintained in an inactive form by its inhibitor IκBα, thereby preventing NF-κB induced transcription of various genes (Hoffmann et al.,

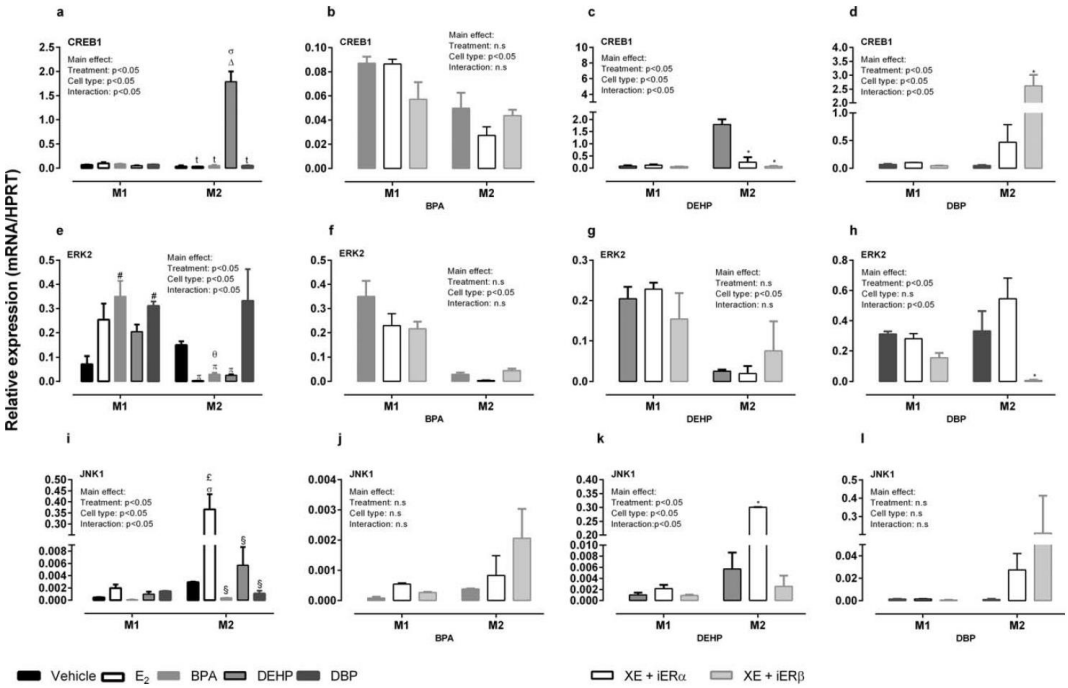


Fig. 4. Effect of xenoestrogens (XE) on the transcription of cAMP responsive element binding protein (CREB) 1, extracellular signal-regulated kinase (ERK) 2 and c-Jun N-terminal kinase (JUNK) 1. Peripheral blood monocyte-derived macrophages after polarization with lipopolysaccharide (100 ng/mL, M1) or interleukin 4 (15 ng/mL, M2) were treated with 17β-estradiol (E_2, 10^{-11} M), bisphenol-A (BPA, 10^{-6} M), di-ethyl-2-hexyle phthalate (DEHP, 10^{-6} M) or di-*n*-butyl phthalate (DBP, 10^{-6} M) alone or in combination with the selective estrogen receptor (ER) α antagonist (iERα, 10^{-6} M, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride) or the selective ERβ antagonist (iERβ, 10^{-6} M, 4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl)phenol), and appropriated controls were made by incubation with vehicle. Gene transcription was calculated using the $2^{-\Delta CT}$ method and normalized to the housekeeping gene (hypoxanthine-guanine-phosphoribosyltransferase, HPRT). Results are presented as mean ± standard error of mean of tree independent experiments performed in duplicate (final $n = 3$). Statistical analysis with two-way ANOVA, followed by Tukey's multiple comparison test: $^{\#}p < 0.05$ vs. M1 control (vehicle); $^{£}p < 0.05$ vs. M1 treated with E_2; $^{\Delta}p < 0.05$ vs. M1 treated with BPA; $^{\Delta}p < 0.05$ vs. M1 treated with DEHP; $^{\sigma}p < 0.05$ vs. M2 control (vehicle); $^{\$}p < 0.05$ vs. M2 treated with E_2; $^{t}p < 0.05$ vs. M2 treated with DEHP; $^{\pi}p < 0.05$ vs. M2 treated with DBP; *iER + XE vs. XE alone in macrophages in the same activation state.

2002). Two-way ANOVA disclosed the significant effect of treatment ($F4,15 = 10.90$, $p < 0.05$) on IκBα transcription level [Fig. 5(i)]. IκBα transcription level was lower in BPA- or DBP-treated M1 macrophages than in E_2-treated M1 macrophages ($p < 0.05$, Tukey's). IκBα transcription decreasing effect of BPA in M1 was found to be ERα-dependent while no modification of DBP's effect was obtained after cotreatment with ER inhibitors in M1 cells. The same two compounds had a lower IκBα transcription level comparing to DEHP treatment, in M2 macrophages ($p < 0.05$, Tukey's). In M2 cells, the decrease in IκBα transcription mediated by DBP was found to be ERα-dependent [Fig. 5(l)].

AP1, another transcription factor involved in the regulation of inflammatory responses, cJun being one member of this family (Zhang and Mosser, 2008). Two-way ANOVA analysis showed a significant effect of cell type ($F1,11 = 10.57$, $p < 0.05$), treatment ($4,11 = 4.941$) and an interaction between treatment and cell type ($F\ 4,11 = 5.070$) on cJun transcription [Fig. 6(a)]. No effect was observed in M1 cells concerning c-Jun gene transcription after 24 h XE

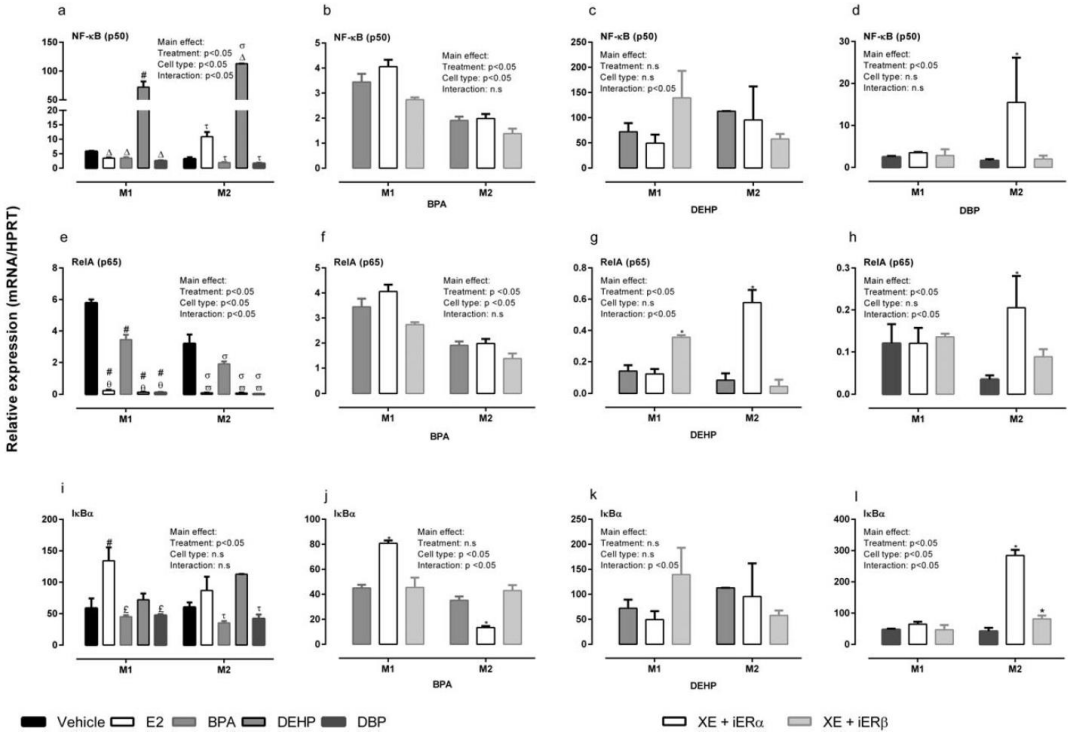


Fig. 5. Effect of xenoestrogens (XE) on the transcription of genes involved in macrophage NF-κB responses. Peripheral blood monocyte-derived macrophages after polarization with lipopolysaccharide (100 ng/mL, M1) or interleukin 4 (15 ng/mL, M2) were treated with 17β-estradiol (E_2, 10^{-11} M), bisphenol-A (BPA, 10^{-6} M), di-ethyl-2-hexyle phthalate (DEHP, 10^{-6} M) or di-*n*-butyl phthalate (DBP, 10^{-6} M) alone or in combination with the selective estrogen receptor (ER) α antagonist (iERα, 10^{-6} M, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride) or the selective ERβ antagonist (iERβ, 10^{-6} M, 4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl)phenol), and appropriated controls were made by incubation with vehicle. Gene transcription was calculated using the $2^{-\Delta CT}$ method and normalised to the housekeeping gene (hypoxanthine-guanine-phosphoribosyltransferase, HPRT). Results are presented as mean ± standard error of mean of tree independent experiments performed in duplicate (final $n = 3$). Statistical analysis with two-way ANOVA, followed by Tukey's multiple comparison test: $^{\#}p < 0.05$ vs. M1 control (vehicle); $^{£}p < 0.05$ vs. M1 treated with E_2; $^{\Delta}p < 0.05$ vs. M1 treated with BPA; $^{\Delta}p < 0.05$ vs. M1 treated with DEHP; $^{\sigma}p < 0.05$ vs. M2 control (vehicle); $^{\varpi}p < 0.05$ vs. M2 treated with BPA; $^{t}p < 0.05$ vs. M2 treated with DEHP; $^{\pi}p < 0.05$ vs. M2 treated with DBP; *iER + XE vs. XE alone in macrophages in the same activation state. IκBα, inhibitor of κB α; NF-κB, factor nuclear κB p50 subunit; RelA, nuclear factor-κB, p65 subunit.

treatment. In M2 macrophages, BPA and DEHP treatments significantly lowered cJun gene transcription comparing to E_2-treated M2 macrophages ($p < 0.05$, Tukey's), which significantly increased its transcription. These effects were ER-independent [Fig. 6(b,c)].

Finally, two-way ANOVA revealed a significant effect of cell type ($F1,12 = 23.82$, $p < 0.05$), treatment ($F4,12 = 15.55$; $p < 0.05$) and an interaction between treatment and cell type ($F1,12 = 23.82$, $p < 0.05$) on COX2 transcription [Fig. 6(e)]. COX2 gene transcription after 24 h XE treatment was significantly higher in M1 macrophages than that in M2 cells ($p < 0.05$, Tukey's). Additionally, in M1 macrophages all XE had lower COX2 gene transcription ($p < 0.05$, Tukey's) comparing to E_2-treament, which increased COX2 transcription. DBP treatment significantly induced COX2 transcription in M1 macrophages comparing to vehicle-treated M1 macrophages and DBP-treated M2 macrophages ($p < 0.05$, Tukey's). In macrophages of both activation states

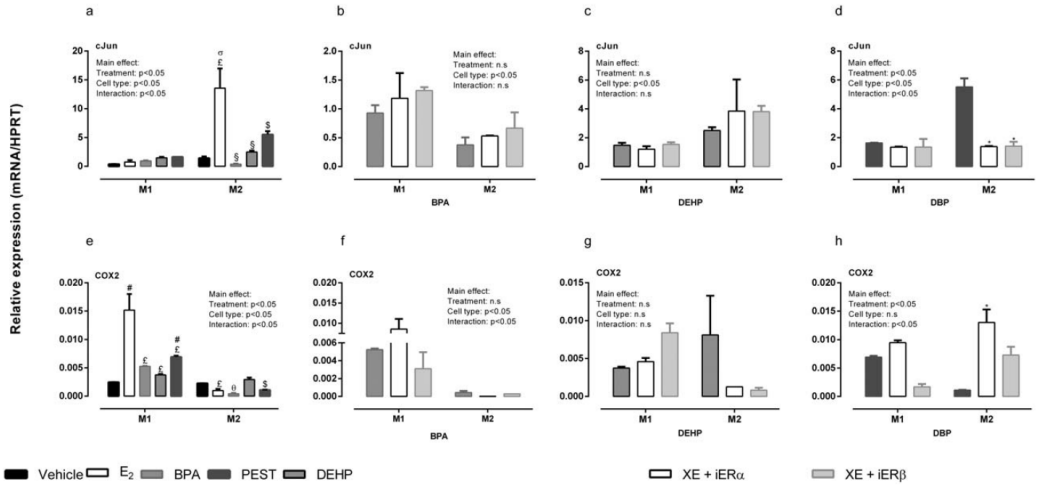


Fig. 6. Effect of xenoestrogens (XE) on the transcription of cyclooxygenase (COX) 2 and cJun. Peripheral blood monocyte-derived macrophages or after polarization with lipopolysaccharide (100 ng/mL, M1) or interleukin 4 (15 ng/mL, M2) were treated with 17β-estradiol (E_2, 10^{-11} M), bisphenol-A (BPA, 10^{-6} M), di-ethyl-2-hexyle phthalate (DEHP, 10^{-6} M) or di-*n*-butyl phthalate (DBP, 10^{-6} M) alone or in combination with the selective estrogen receptor (ER) α antagonist (iERα, 10^{-6} M, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride) or the selective ERβ antagonist (iERβ, 10^{-6} M, 4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl)phenol), and appropriated controls were made by incubation with vehicle. Gene transcription was calculated using the $2^{-\Delta CT}$ method and normalized to the housekeeping gene (hypoxanthine-guanine-phosphoribosyltransferase, HPRT). Results are presented as mean ± standard error of mean of tree independent experiments performed in duplicate (final $n = 3$). Statistical analysis with two-way ANOVA, followed by Tukey's multiple comparison test: $^{\#}p < 0.05$ vs. M1 control (vehicle); $^{£}p < 0.05$ vs. M1 treated with E_2; $^{\Delta}p < 0.05$ vs. M1 treated with BPA; $^{\$}p < 0.05$ vs. M1 treated with DBP; $^{\sigma}p < 0.05$ vs. M2 control (vehicle); $^{\S}p < 0.05$ vs. M2 treated with E_2; $^{\pi}p < 0.05$ vs. M2 treated with DBP; *iER + XE vs. XE alone in macrophages in the same activation state.

effect on COX2 transcription was found to be ER-independent ($p < 0.05$, Tukey's) [Fig. 6(h)].

DISCUSSION

Inflammation is responsible for the onset of numerous chronic diseases including diabetes, atherosclerosis, and obesity (Monteiro and Azevedo, 2010). This notion has instigated many researchers to devote a great deal of effort to identify the triggers for inflammation. Environmental estrogens including phenols and phthalates have been suspected of disturbing immune response in humans (Chalubinski and Kowalski, 2006). Recently, the first Scientific Statement of The Endocrine Society focused on the need to understand the basic mechanisms of action and the physiological consequences of these substances, stating that it is imperative to perform basic *in vitro* molecular studies to identify pathways involved in XE actions (Diamanti-Kandarakis et al., 2009), which are still scarcely known. In line with this recommendation, the present study aimed at elucidating the ability of BPA and phthalates to modify the biology of humanMDM and the mechanisms involved in their actions, using XE concentrations compatible with those obtained in biological samples after long-term exposure (Silva et al., 2004; Vandenberg et al., 2007).

Macrophages are key players in the development, progression and resolution of inflammation. M1 and M2 macrophages are extremes in a continuum of functional states, and dynamic changes in their phenotype have been observed in different models of inflammation (Bystrom et al., 2008; Bolego et al., 2013). Whether (xeno)estrogen/estrogen receptor pathways control the balance between M1/M2 phenotype of macrophages is only partially understood (Liu et al., 2014). Previous results from our group showed that MDM express ERα and ERβ mRNA and levels depend upon state of macrophage activation leading to the speculation that not only these cells are differently modulated by

TABLE II. Summary of the effects of bisphenol-A (BPA), di-(2-ethylhexyl) phthalate (DEHP), di-*n*-butyl phthalate (DBP) ans 17β-estradiol (E_2) on migration, cytokine release, and transcription of factors involved in inflammatory pathways

	M1				M2			
	BPA	DEHP	DBP	E_2	BPA	DEHP	DBP	E_2
Migration	–	–	–	–	↗	↗	↗	↑
Cytokine release								
IL1β	–	↑	–	–	–	–	$\uparrow^{\alpha\beta}$	–
IL6	↓	↓	↓	↑	↓	↓	$\downarrow^{\alpha\beta}$	↓
IL10	↑	↑	↑	↑	↓	↓	$\downarrow^{\alpha\beta}$	↓
Transcription								
CREB1	–	–	–	–	–	$\uparrow^{\alpha\beta}$	–	–
ERK2	↑	↗	↑	↗	–	–	$\nearrow^{\beta}$	–
JNK1	–	–	–	–	–	$\searrow^{\alpha}$	–	↑
NF-Kb (p50)	–	↑	–	–	–	↑	–	–
RelA (p65)	↓	$\downarrow^{\beta}$	↓	↓	↓	$\downarrow^{\alpha}$	$\downarrow^{\alpha}$	↓
$I_\kappa B\alpha$	$\searrow^{\alpha}$	–	↘	↑	↘	↗	$\searrow^{\alpha}$	–
c-Jun	–	–	–	–	–	–	$\nearrow^{\alpha\beta}$	↑
COX2	↓	↓	↑	↑	-	–	–	–

COX2, cyclooxygenase 2; CREB1, cAMP response element-binding protein 1; ERK2, extracellular signal-regulated kinase 2; HPRT, hypoxanthine phosphoribosyltransferase; IκBα, nuclear factor-κB inhibitor alpha; IL, interleukin; JNK1, c-Jun N-terminal kinase 1; NF-κB, nuclear factor-κB, p50 subunit; RelA, nuclear factor-κB, p65 subunit; β, effect mediated by ERβ; α, effect mediated by ERα; ↑, increase; ↓, decrease; ↗, increase tendency; ↘, decrease tendency; –, no effect.

estrogens but that they also may constitute targets of XE actions.

Macrophage tissue infiltration is often a hallmark of several inflammatory disorders (Verollet et al., 2011). However, to date there is little information about the influence of XEs in the migratory capability of differentiated human macrophages. In the present study, with respect to M1 macrophages, XEs did not modify the migration capacity of these cells, mimicking E_2 effect. This is an interesting finding, because although these compounds may cause local tissue inflammation, they did not compromise the role of circulating pro-inflammatory cells on the rear guarding of tissues (Okdah, 2013). As far as the authors know, this is the first study that assessed the effect of XEs regarding M2 macrophage migration, showing that these compounds tended to increase migration, again, mimicking the effect of E_2. This selective effect on macrophages, independent of ERs, may constitute a "phenotype-shaping gate" of M2 macrophages for tissues and sites of inflammation, denoting an immune modulatory character of these compounds.

Environmental estrogens may affect the immune system directly, altering the patterns of cytokine production (Chighizola and Meroni, 2012; Liu et al., 2014). In a previous work, we showed that estrogen signaling in low concentrations (compatible with that observed in menopause) may render macrophages to a more pro-inflammatory profile, which could contribute to the manifestation of chronic inflammatory diseases. The present question is: what would change with the exposure to estrogen-like compounds?

M1 macrophages are efficient producers of cytotoxic effector molecules, such as reactive oxygen intermediates and inflammatory cytokines, including IL1β, and IL6 (Mantovani et al., 2004). Indeed, the increase of IL1β secretion by M1 macrophages after DEHP treatment id supported by other authors (Nishioka et al., 2012). This result may support the role of DEHP in the pathogenesis of diabetes since elevated plasma IL1β may be a predictive of type 2 diabetes (Kim et al., 2013). On the other hand, we observed that XEs enhanced the M1 release of IL10, also through ER-independent mechanisms. Additionally, XEs decreased IL6 secretion in M1 cells. This reflects that XE affect the balance between pro and anti-inflammatory cytokines and while mimicking the effects of estrogens in enhancing secretion of the anti-inflammatory IL10, they oppose to its effects in the increase of pro-inflammatory IL6.

Rapid actions exerted by estrogen or estrogen-like compounds have been explored in recent years, and many intracellular signaling cascades have been found to be involved, also varying with cell type (Madak-Erdogan et al., 2008).

In M1 macrophages we showed an increase in ERK2 transcription in BPA- and DBP-treated cells, DEHP and E_2 having the same tendency. Previous reports have documented that MAPK family, not only p38 but also ERK1/2 and JNK are all involved in IL10 production in LPS-stimulated macrophages (Liu et al., 2006; Chanteux et al., 2007), possibly explaining the observed increase in IL10 release. It is notable that we found a lower transcription of and RelA (p65) subunits and no effect in cJun or JUNK1 transcription on M1 cells. The decrease of IL6 production after XE treatment is

consistent with the decrease in RelA (p65) in these cells (Lambert et al., 2005). In addition, all XE resulted in lower transcription of COX2 when compared to E_2 in M1 cells (although DBP still increased COX2 when compared to vehicle treated M1 cells), which is compatible with RelA down-regulation. Also possibly contributing to the decrease in IL6 and COX2 and increase in IL10 is the opposite regulation of IκBα by XEs and E_2. It is worth noting, however that although ERK2 is also upregulated in XE-treated M1 cells, IL1β secretion was only stimulated after DEHP treatment.

In general, M2 macrophages are characterized by low production of pro-inflammatory cytokines including IL1 and IL6 and high production of IL10. In accordance, we observed a higher basal IL10 and lower IL1β production in M2 as compared to M1 cells. The majority of studies have only addressed the effect of BPA and phthalates in unstimulated macrophages or scarcely in LPS-stimulated macrophages, while less is known about the effect on alternatively-activated macrophages. Herein, we provide evidence that most XEs used suppressed—cytokine production in M2 cells, similar to E_2, with exception of DBP that increased IL1β.

The inhibitory effect of XEs that was found in IL6 and IL10 secretion may be a result of NF-κB blockade, which has been already reported for BPA (Kim and Jeong, 2003). In fact, we observed in XE-treated M2 cells an overall decrease in RelA subunit transcription. In the case of DEHP, the increase in NF-κB (p50) transcription and the tendency to increase IκBα transcription may also contribute for the decrease in cytokine production. It has been postulated that homodimers of the p50 subunit, which lack transactivation domains, are involved in the repression of expression of NF-κB (50) target genes and IκBα may inhibit the transcriptional activity of p65 RelA complexes (Porta et al., 2009). Therefore, XE-treatment in M2 cells may impair the expression of these NF-κB-related genes. Finally, ERK and cJun have been implicated on increased production of IL1β in macrophages (Chanteux et al., 2007; Dalmas et al., 2014). In good agreement with this, we showed in DBP-treated M2 cells an increase in ERK2 and cJun transcription.

The comparisons between BPA, DEHP, and DBP with E_2 support the notion that BPA and phthalates should not be merely considered "estrogenic" since only a relatively small subset of the studied genes is affected in common by BPA or phthalates and E_2. These findings suggested that while BPA, DEHP, and DBP can act as estrogen or antiestrogens depending on certain conditions and cell type. Through it all, the present study demonstrated that XEs exert differential effects on the production of cytokines potentially altering the balance between pro- and anti-inflammatory cytokines. Interestingly, XEs seemed to expose to pro-inflammatory effects of low E2 levels on M1 macrophages and act as E_2 mimetic on M2 (anti-inflammatory) macrophages. This may be of particular importance during the inflammatory state that accompanies the decline of estrogens, for example in menopause. Whether or not these effects remain the same with simultaneous E_2 still remains to be established.

In the present study, comparing the treatment of XEs alone or cotreatment with ER antagonists, namely MPP (iERα) and PHTPP (iERβ), allowed us to disclose the involvement of each receptor subtype in the upper-described effects. As we have previously reported, because of the higher expression levels of ERα than ERβ in these cells, it is possible that macrophages may respond primarely to estrogenic compounds through ERα rather than ERβ. Despite this could occur in most of the cases, one should not disregard the role of ERβ, that even with a low transcription level in these cells seems to have an important role in some of the results presented above. The involvement of ER is most evident for the effects of phthalates in M2 cells, thereby granting the possibility to selectively modulate estrogen-like actions in different tissues. Interestingly, the experiments involving iERs cotreatment indicated that mostly phthalates exerted some of their effects through ER including ERβ, especially in M2 cells, those with greater transcription of both ERs. Still many other do also occur through ER-independent pathways. The effects of tested compounds are summarized in Table II.

The authors kindly thank to Professor Dr. Fernando Araújo and Dr. Cristina Neves from the Department of Immunohemotherapy, São João Hospital, Porto, Portugal.

REFERENCES

Alonso-Magdalena P, Ropero AB, Carrera MP, Cederroth CR, Baquie M, Gauthier BR, Nef S, Stefani E, Nadal A. 2008. Pancreatic insulin content regulation by the estrogen receptor er alpha. PloS One 3:e2069.

Batista TM, Alonso-Magdalena P, Vieira E, Amaral ME, Cederroth CR, Nef S, Quesada I, Carneiro EM, Nadal A. 2012. Short-term treatment with bisphenol-A leads to metabolic abnormalities in adult male mice. PloS One 7:e33814.

Bolego C, Cignarella A, Staels B, Chinetti-Gbaguidi G. 2013. Macrophage function and polarization in cardiovascular disease: a role of estrogen signaling?. Arterioscler Thromb Vasc Biol 33:1127–1134.

Bystrom J, Evans I, Newson J, Stables M, Toor I, van Rooijen N, Crawford M, Colville-Nash P, Farrow S, Gilroy DW. 2008. Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by camp. Blood 112:4117–4127.

Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Ekong J, Needham LL. 2005. Urinary concentrations of bisphenol-A and 4-nonylphenol in a human reference population. Environ Health Perspect 113:391–395.

Chalubinski M, Kowalski ML. 2006. Endocrine disrupters–potential modulators of the immune system and allergic response. Allergy 61:1326–1335.

Chanteux H, Guisset AC, Pilette C, Sibille Y. 2007. Lps induces il-10 production by human alveolar macrophages via mapkinases- and sp1-dependent mechanisms. Resp Res 8:71.

Chighizola C, Meroni PL. 2012. The role of environmental estrogens and autoimmunity. Autoimmun Rev 11:A493–A501.

Dalmas E, Venteclef N, Caer C, Poitou C, Cremer I, Aron-Winewsky J, Lacroix-Desmazes S, Bayry J, Kaveri SV, Clement K, Andre S, Guerre-Millo M. 2014. T cell-derived il-22 amplifies il-1beta-driven inflammation in human adipose tissue: Relevance to obesity and type 2 diabetes. Diabetes 63(6): 1966–77.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. 2009. Endocrine-disrupting chemicals: an endocrine society scientific statement. Endocrine Rev 30:293–342.

Faniband M, Lindh CH, Jonsson BA. 2014. Human biological monitoring of suspected endocrine-disrupting compounds. Asian J Androl 16:5–16.

Fernandez MF, Arrebola JP, Taoufiki J, Navalon A, Ballesteros O, Pulgar R, Vilchez JL, Olea N. 2007. Bisphenol-A and chlorinated derivatives in adipose tissue of women. Reprod Toxicol 24:259–264.

Goodman NF, Cobin RH, Ginzburg SB, Katz IA, Woode DE. 2011. American association of clinical endocrinologists medical guidelines for clinical practice for the diagnosis and treatment of menopause: executive summary of recommendations. Endocr Pract 17:949–954.

Gordon S. 2003. Alternative activation of macrophages. Nat Rev Immunol 3:23–35.

Halden RU. 2010. Plastics and health risks. Annu Rev Pub Health 31:179–194.

Hoffmann A, Levchenko A, Scott ML, Baltimore D. 2002. The ikappab-nf-kappab signaling module: temporal control and selective gene activation. Science 298:1241–1245.

Ikezuki Y, Tsutsumi O, Takai Y, Kamei Y, Taketani Y. 2002. Determination of bisphenol-A concentrations in human biological fluids reveals significant early prenatal exposure. Hum Reprod 17:2839–2841.

Inadera H. 2006. The immune system as a target for environmental chemicals: xenoestrogens and other compounds. Toxicol Lett 164:191–206.

Kerdivel G, Habauzit D, Pakdel F. 2013. Assessment and molecular actions of endocrine-disrupting chemicals that interfere with estrogen receptor pathways. Int J Endocrinol 2013:501851.

Kim JY, Jeong HG. 2003. Down-regulation of inducible nitric oxide synthase and tumor necrosis factor-alpha expression by bisphenol-A via nuclear factor-kappab inactivation in macrophages. Cancer Lett 196:69–76.

Kim JH, Park HY, Bae S, Lim YH, Hong YC. 2013. Diethylhexyl phthalates is associated with insulin resistance via oxidative stress in the elderly: a panel study. PloS One 8:e71392.

Koch HM, Calafat AM. 2009. Human body burdens of chemicals used in plastic manufacture. Philos Trans R Soc London Ser B: Biol Sci 364:2063–2078.

Kramer PR, Wray S. 2002. 17-beta-estradiol regulates expression of genes that function in macrophage activation and cholesterol homeostasis. J Steroid Biochem Mol Biol 81:203–216.

Lambert KC, Curran EM, Judy BM, Milligan GN, Lubahn DB, Estes DM. 2005. Estrogen receptor alpha (eralpha) deficiency in macrophages results in increased stimulation of CD4+ t cells while 17beta-estradiol acts through eralpha to increase il-4 and gata-3 expression in CD4+ t cells independent of antigen presentation. J Immunol 175:5716–5723.

Liu Y, Mei C, Liu H, Wang H, Zeng G, Lin J, Xu M. 2014. Modulation of cytokine expression in human macrophages by endocrine-disrupting chemical bisphenol-A. Biochem Biophys Res Commun 451:592–598.

Liu YW, Chen CC, Tseng HP, Chang WC. 2006. Lipopolysaccharide-induced transcriptional activation of interleukin-10 is mediated by mapk- and nf-kappab-induced ccaat/enhancer-binding protein delta in mouse macrophages. Cell Signal 18: 1492–1500.

Madak-Erdogan Z, Kieser KJ, Kim SH, Komm B, Katzenellenbogen JA, Katzenellenbogen BS. 2008. Nuclear and extranuclear pathway inputs in the regulation of global gene expression by estrogen receptors. Mol Endocrinol 22: 2116–2127.

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25:677–686.

Monteiro R, Azevedo I. 2010. Chronic inflammation in obesity and the metabolic syndrome. Mediat Inflamm 2010. p. 289645. doi: 10.1155/2010/289645.

Nishioka J, Iwahara C, Kawasaki M, Yoshizaki F, Nakayama H, Takamori K, Ogawa H, Iwabuchi K. 2012. Di-(2-ethylhexyl) phthalate induces production of inflammatory molecules in human macrophages. Inflamm Res 61:69–78.

Okdah YA. 2013. Effect of diethylhexyl phthalate on proliferation of ed1-testicular macrophages in rats. J Basic Appl Zool 66: 223–227.

Porta C, Rimoldi M, Raes G, Brys L, Ghezzi P, Di Liberto D, Dieli F, Ghisletti S, Natoli G, De Baetselier P, Mantovani A, Sica A. 2009. Tolerance and m2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappab. Proc Natl Acad Sci U S A 106:14978–14983.

Pyo MY, Kim HJ, Back SK, Yang M. 2007. Downregulation of peritoneal macrophage activity in mice exposed to bisphenol-A during pregnancy and lactation. Arch Pharm Res 30:1476–1481.

Quesada I, Fuentes E, Viso-Leon MC, Soria B, Ripoll C, Nadal A. 2002. Low doses of the endocrine disruptor bisphenol-A and the native hormone 17beta-estradiol rapidly activate transcription factor creb. FASEB J 16:1671–1673.

Ruh MF, Bi Y, Cox L, Berk D, Howlett AC, Bellone CJ. 1998. Effect of environmental estrogens on il-1beta promoter activity in a macrophage cell line. Endocrine 9:207–211.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time pcr data by the comparative c(t) method. Nat Protoc 3:1101–1108.

Segura JJ, Jimenez-Rubio A, Pulgar R, Olea N, Guerrero JM, Calvo JR. 1999. In vitro effect of the resin component bisphenol-A on substrate adherence capacity of macrophages. JEndodont 25:341–344.

Silva MJ, Barr DB, Reidy JA, Malek NA, Hodge CC, Caudill SP, Brock JW, Needham LL, Calafat AM. 2004. Urinary levels of

seven phthalate metabolites in the US Population from the national health and nutrition examination survey (nhanes) 1999–2000. Environ Health Persp 112:331–338.

Sindrilaru A, Peters T, Wieschalka S, Baican C, Baican A, Peter H, Hainzl A, Schatz S, Qi Y, Schlecht A, Weiss JM, Wlaschek M, Sunderkotter C, Scharffetter-Kochanek K. 2011. An unrestrained proinflammatory m1 macrophage population induced by iron impairs wound healing in humans and mice. J Clin Invest 121:985–997.

Swan SH, Liu F, Hines M, Kruse RL, Wang C, Redmon JB, Sparks A, Weiss B. 2010. Prenatal phthalate exposure and reduced masculine play in boys. Int J Androl 33:259–269.

Tabb MM, Blumberg B. 2006. New modes of action for endocrine-disrupting chemicals. Mol Endocrinol 20: 475–482.

Taylor JA, Vom Saal FS, Welshons WV, Drury B, Rottinghaus G, Hunt PA, Toutain PL, Laffont CM, VandeVoort CA. 2011. Similarity of bisphenol-A pharmacokinetics in rhesus monkeys and mice: relevance for human exposure. Environ Health Perspect 119:422–430.

Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. 2007. Human exposure to bisphenol-A (bpa). Reprod Toxicol 24:139–177.

Verollet C, Charriere GM, Labrousse A, Cougoule C, Le Cabec V, Maridonneau-Parini I. 2011. Extracellular proteolysis in macrophage migration: losing grip for a breakthrough. Eur J Immunol 41:2805–2813.

Volkel W, Colnot T, Csanady GA, Filser JG, Dekant W. 2002. Metabolism and kinetics of bisphenol-A in humans at low doses following oral administration. Chem Res Toxicol 15:1281–1287.

vom Saal FS, Akingbemi BT, Belcher SM, Birnbaum LS, Crain DA, Eriksen M, Farabollini F, Guillette LJ, Jr., Hauser R, Heindel JJ, Ho SM, Hunt PA, Iguchi T, Jobling S, Kanno J, Keri RA, Knudsen KE, Laufer H, LeBlanc GA, Marcus M, McLachlan JA, Myers JP, Nadal A, Newbold RR, Olea N, Prins GS, Richter CA, Rubin BS, Sonnenschein C, Soto AM, Talsness CE, Vandenbergh JG, Vandenberg LN, Walser-Kuntz DR, Watson CS, Welshons WV, Wetherill Y, Zoeller RT. 2007. Chapel hill bisphenol-A expert panel consensus statement: integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure. Reprod Toxicol 24:131–138.

Welshons WV, Nagel SC, vom Saal FS. 2006. Large effects from small exposures. Iii. Endocrine mechanisms mediating effects of bisphenol-A at levels of human exposure. Endocrinology 147:S56–S69.

Wentworth JM, Naselli G, Brown WA, Doyle L, Phipson B, Smyth GK, Wabitsch M, O'Brien PE, Harrison LC. 2010. Pro-inflammatory CD11c+CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity. Diabetes 59:1648–1656.

Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, Watson CS, Zoeller RT, Belcher SM. 2007. In vitro molecular mechanisms of bisphenol-A action. Reprod Toxicol 24:178–198.

Yoshitake J, Kato K, Yoshioka D, Sueishi Y, Sawa T, Akaike T, Yoshimura T. 2008. Suppression of no production and 8-nitroguanosine formation by phenol-containing endocrine-disrupting chemicals in lps-stimulated macrophages: involvement of estrogen receptor-dependent or -independent pathways. Nitric Oxide: Biol Chem18:223–228.

Zhang X, Mosser DM. 2008. Macrophage activation by endogenous danger signals. J Pathol 214:161–178.

Zsarnovszky A, Le HH, Wang HS, Belcher SM. 2005. Ontogeny of rapid estrogen-mediated extracellular signal-regulated kinase signaling in the rat cerebellar cortex: potent nongenomic agonist and endocrine disrupting activity of the xenoestrogen bisphenol-A. Endocrinology 146:5388–5396.

CHAPTER V

SUMMARIZING DISCUSSION

CONCLUDING REMARKS

The global prevalence of metabolic diseases like T2D and their colossal economic and social costs represent a major public health concern for present societies, despite medical and political efforts to control their increased prevalence. This growing trend has been attributed to a conjunction of genetic and environmental factors [153]. Likewise, shifts in population genetics are unlikely to occur on a time scale that explains the recent deterioration in human metabolic health. The WHO estimates that "as much as 24% of global disease is caused by environmental exposures that can be adverted" [154]. As such, increasing attention has been turned to the identification of additional factors that may impact on energy metabolism, including exposure to chemical pollutants.

In fact, in the modern world, the environment has been largely affected by an ever increasing number of synthetic pollutants, such as pesticides, organophosphates, polychlorinated bisphenyls, phthalates, and solvents, among others, some of them with estrogenic activity [155]. These may permeate the diet, the air and the ground. Indeed, the exponential rise of obesity-related pathologies worldwide may be associated with the marked increase of toxic chemicals in the environment [156, 157]. This issue is immensely challenging as the list of chemicals to which humans are exposed continues to grow and is now somewhere near 100,000 distinct compounds.

First articulated in 1992, the theory of endocrine disruption postulates that exogenous chemicals can modulate homeostasis by interfering with action of endogenous hormonal axis. Some endocrine disruptors were designed to have long half-lives and therefore are persistent contaminants, do not decay easily, may not be metabolized, or may be broken down into more toxic compounds. Others such as BPA and phthalates, although not very persistent in the environment are so widespread in their use that there is a prolonged human exposure [158]. These man-made chemicals have been used extensively in agricultural, industrial and manufacturing processes. Environmental and human exposure to XEs has been the subject of scientific investigation and political regulation for almost 40 years. The Stockholm Convention on POPs, signed in 2001 and effective from May 2004, is a global treaty to protect human health and the environment from chemicals that remain in the environment for long periods, become widely distributed geographically, accumulate in the fatty tissue of humans and wildlife, and have adverse effects on human health or in the environment. However, they are still ubiquitous in our environment, especially in the food chain [159]. Some highly prevalent endocrine disruptors have immunosuppressive, oxidative, pro-inflammatory, neuroendocrine, metabolic and epigenetic effects [160, 161].

The interest in molecules that can interfere with estrogen signaling, or XEs, has been growing, either to understand why exposure to certain chemicals can influence the incidence of estrogen-dependent diseases or to use them in order to change estrogen signaling in a convenient way. The understanding of

the mechanisms of action and clinical effects of XEs, as endocrine and metabolic disruptors, has increased significantly over a decade: yet, it is far from being complete [161]. Another relevant question is whether current approaches to risk assessment and regulatory control of exposure to XEs are adequate to protect the population against diseases.

In the case of dietary intake, people with increased food intake, such as obese individuals, may need special attention. Moreover, it is well accepted that humans bioaccumulate these lipophilic pollutants in the AT for many years and because of its highly resistance to metabolic degradation levels become increaselly elevated [162]. At the same time, AT may be an endogenous source of these compounds. For these reasons and because the different AT depots may have somewhat different endocrine functions, it is important to know whether or not XEs distribute equally throughout all AT and ultimately to determine how levels of these compounds influence the local AT dynamics in an obese state.

Our initial approach aimed at studying the patterns of accumulation and variation in selected XEs (namely DDT and its metabolites, α-, β- and γ- hexachlorocyclohexane (HCH), hexachlorobenzene (HCB) and aldrin) in plasma and AT samples in obese pre- and postmenopausal women. XEs are pervasive in this obese population (**Chapter IIA**), confirming their bioaccumulation with a higher detection percentage in both AT depots than plasma in pre- and postmenopausal women. Although some recent studies have suggested that some heterogeneity exist with respect to the distribution of XEs across AT depots [163, 164], investigators always denoted the need of more studies to confirm that evidence because of the limited number of subjects that were studied. Our data strongly reinforces the heterogeneity both in the profile of distribution and concentration of XEs in AT depots, with a robust number of subjects studied. Until recently, it has been proposed that the variability of the stored amount of particular XEs in the AT, which can range over several orders of magnitude, depends on dietary exposure, on individual predisposition to store these substances and on the differential lipophilicity of various XEs [163-166]. We additionally suggest that hormonal status is an important determinant for the differences in XEs accumulation between AT depots and in each depot, since premenopausal women accumulate XEs preferentially in vAT. Following menopause and the decline in circulating E_2, women accumulate intra-abdominal AT and tend to develop male-pattern adiposity [167]. In the present study, this AT redistribution is accompanied by changes of XEs pattern of accumulation, XE levels being similar in both scAT and vAT, and significantly higher than those found in premenopausal women scAT, probably reflecting a concentration of XEs in scAT. Firstly, although this may seem paradoxical, it is not surprising because the total amount of scAT mass decreases considerably [149], thereby leading to an increased concentration of XEs in this location. Secondly, this is an important finding with practical implications because it suggests that scAT, despite being an easily accessible compartment for determination of contamination levels, is not representative of the XE distribution in deeper AT depots across the general population, as suggested by different groups [168, 169]. On the other hand, we believe that the higher accumulation of XEs observed in premenopausal vAT is of paramount importance since this highly metabolically active tissue is held as the main impeller of

metabolic dysfunction progression. As known, it has been demonstrated that accumulation of vAT is correlated with increased risk and mortality from diabetes and CDV [170]. Our data supports that accumulation of pollutants with endocrine disrupting capacity might be implicated in metabolic dysfunction associated with increased vAT. Herein, XEs in vAT of premenopausal women were found to be more tightly associated with markers of worst metabolic profile compared to XEs in scAT, namely glucose homeostasis and inflammatory parameters, suggesting that the presence of these compounds in vAT contributes to local toxicity and dysfunction and potentially favors the metabolic complications associated with obesity [149]. Furthermore, this finding strengthens our theory that the biological effects of XEs might differ between AT compartments.

Although XEs accumulate mainly in AT, collecting AT is difficult in practice. Therefore most epidemiological studies of XEs have used their circulating concentrations as a marker of body burden, as circulating XE concentrations are reportedly strongly correlated to those in AT [168, 169]. Contrary to this notion, we noted that plasma levels of XEs did not reflect total levels nor the pattern of compoundsaccumulation in AT compartments, highlighting that using plasma XE levels to estimate exposure may be misleading. On the other hand, several observations both in humans and animals suggest that the release of pollutants from AT is an important source of blood XEs. In humans, most of the evidence has been gathered from studies on drastic weight loss in obese individuals. Several independent studies demonstrated a positive correlation between long-term weight changes and XE plasma concentrations [168, 171, 172]. In our point of view, plasma XEs may reflect AT dysfunction. Recently, Gauthier *et al.* [173] demonstrated the relationship of POPs, some with estrogenic activity, with the variation in metabolic risk observed among obese individuals, showing that the MHO phenotype is associated with lower plasma levels of POPs as compared with MAO subjects. In that report, the authors drew attention to the need to assess the levels of POPs in different AT compartments (i.e., scAT and vAT), to provide better understanding on the reason for the lower plasma POP concentrations observed in MHO compared to MAO women. In a discussion of the publication of Gauthier *et al.* [173] (**Chapter IIB),** we propose that the higher plasma levels of POPs in MAO may arise from dysfunctional vAT, probably after imposing local toxicity. We provide further evidence that this seems to be true especially for the vAT of premenopausal women, in which the sum of XEs (∑XEs) in plasma is positively correlated with ∑XEs in vAT and with vAT adipocyte area, a known marker of adipocyte dysfunction. Another critical issue is whether the release of XEs from the AT could be implicated in metabolic deterioration, eventually through maintaining their availability to exert toxicity in other organs and tissues, because once XEs return to circulation they become free to act in distant targets [149].

Initially known for their ability to promote cancer and affect reproductive functions [174], within the last few years, questions and concerns have also focused on the involvement of XEs in the dramatic rise of metabolic disorders incidence, such as obesity and T2D. More recently XEs have been associated with different cardiometabolic risk factors, including insulin resistance, low HDL-cholesterol, elevated plasma

triglycerides and high blood pressure [5, 175-177]. However, most studies have focused on the effect modification of a single risk factor, and few link XEs with the complications arising with obesity, when it is already installed.

The Framingham Risk Score is the most commonly used scoring system aiming at predicting the risk of CVD, that uses age, gender, smoking history, blood pressure, HDL-cholesterol, low density lipoprotein (LDL)-cholesterol, and blood glucose levels to estimate coronary event risk over the course of ten years among individuals without previously diagnosed coronary heart disease [178]. Here, we demonstrate using the Framingham Risk Score that in premenopausal women, plasma concentrations of XEs may partialy explain variability in CVD risk. Our regression model indicated a statistically significant association for plasma XEs, along with known risk factors such as inflammation and number of years of obesity, on the premenopausal CVD risk. This finding also suggests that environmental XEs might be able to increase the disease burden of CVD at a population level. More importantly, the absence of association between XEs and inflammation and/or CVD risk among postmenopausal women could indicate, on one hand, that the presence of conserved signaling by estrogens is a necessary factor for XEs to increase the risk of inflammation and CVD risk. On the other hand, the worsening of the metabolic degradation that XEs can provide in premenopausal may have a potential role in the later development of cardiometabolic disease in obese women. Then, it is possible that even though XEs might contribute to establishment of increased CVD risk in postmenopausal women, we no longer have an association window that allows this demonstration.

Our results imply that the endocrine disruptor character of these compounds may compromise the well documented cardioprotective action of estrogens in women. Indeed, epidemiological and experimental animal and cellular models have provided intriguing preliminary evidence to suggest a potential contribution of XEs to the pathogenesis of CVD [179-182]. However, precise causal relationships or specific mechanisms are yet unknown.

Additionally, the natural increase in cardiometabolic risk after menopause is paralleled by the redistribution of body fat, contributing from our point of view to the concentration of XEs on scAT, and perhaps extend metabolic dysfunction to this AT localization. It is the dysfunction, rather than the amount of AT that appears to be responsible for generating a state of low-grade inflammation and metabolic abnormalities [183]. Accordingly, perpetuating the state of positive energy balance and under the chronic pressure to store calories, the adipogenic capacity within scAT may falter, leading to overly hypertrophied adipocytes, which inappropriately release free fatty acids and overproduce pro-inflammatory adipokines [184]. Despite this attempt to accomplashish the storage of excess energy, scAT storage capacity is compromised and free fatty acids are relayed to the vAT depot, which is favored by the decrease in E_2 observed in menopause. Thus, an increase of vAT may be a marker for the compromised ability of scAT to store excess energy. This observation is rather worring as the release of XEs from scAT may also occur, and

may contribute to a further exacerbation of the metabolic dysfunction. Alltogether, these results point towards the occurrence of changes in XE toxicokinetics with menopausal status and implicate plasma XEs as possible biomarkers of AT functionality.

An increase in the volume of AT leads to its infiltration by monocytes and macrophages [36]. On the other hand, it is well accepted that the inflammatory response, mainly related to macrophages, is involved in the development of metabolic disease [185]. Particularly in obesity, increased accumulation of macrophages is a hallmark of a pro-inflammatory state that links obesity with systemic inflammation [36]. Thus, AT participation in inflammation is attributed largely to the inflammatory actions of bone marrow monocyte-derived macrophages populations [36]. Moreover, adipocyte-macrophage interaction augments inflammatory changes in AT leading to expression of a variety of chemokines in the AT. Among these, MCP-1 plays a pivotal role in obesity-related macrophage infiltration into AT [186, 187]. Additionally, it has been proposed that XEs in AT can be involved in inflammatory activation/perpetuation [149-151, 188]. Herein (**Chapter II**), we observed that in premenopausal women, the increase in plasma XEs was positively correlated with MCP-1 and, on the other hand, the concentration of XEs in AT, mainly in vAT location, was associated with increased number of monocytes in circulation, a possible intermediate step to their migration to the AT. This reinforces the hypothesis that the contribution of XE exposure to the increasing rate of metabolic disorders may be mediated by their effects on the immune system.

Another key point of our work was to try to understand the mechanisms involved in the possible link between XE actions on the immune system and the development of the state of chronic low-grade inflammation and metabolic dysfunction associated with obesity. Indeed, AT macrophages have received special attention regarding the immune cells involved. To orchestrate the inflammatory process in the AT, macrophages may exist on a spectrum of diverse phenotypes, from M1 to M2. It is well accepted that the environmental conditions, degree and type of the inflammatory process will essentially drive their polarization to either M1 or M2 macrophages and consequently influence inflammation termination or resolution. Estrogens and environmental estrogens have been reported to interfere with and disrupt the immune system, namely modulating the function of macrophages. An incomplete understanding of the tissue-specific effects of hormone action, and ER distribution and function, contribute to our confusion and failure to advance therapeutic strategies to combat estrogen-influenced pathologies [189].

The models used to study human macrophage polarization are limited and gold standard protocols for generating M1/M2 phenotypes *in vitro* are laking. Accordingly, we used human monocyte-derived macrophages that have been spontaneously differentiated *in vitro* when activated with M1-associated (LPS) or M2-associated (IL4) stimuli. Since tissue macrophages are difficult to obtain and handle, this experimental model may be used as a ‘‘surrogate’’ to study them. On the other hand, an advantage of using these cells is that they generate a heterogeneous starting population that reflects the features of plasticity of tissue macrophages. Curiously, such heterogeneity was observed in our study, as reported in

Chapter III and **Chapter IV**, and is consistent with growing evidence that macrophage populations *in vivo* tend to contain concomitantly the characteristics of different differentially activated cells [190]. Moreover, the peripheral blood mononuclear cells from healthy postmenopausal women donors were used to limit the influence of endogenous estrogens levels on study endpoints.

With regard to cells of the myeloid linage, there is evidence that estrogens modulate main activities, such as maturation, differentiation and migration and it has been demonstrated that monocytes express more ERβ while macrophages expresses more ERα [128, 191]. While the contribution of other nuclear receptors to macrophage polarization in different settings is well stablished [192, 193], until now, ER expression in human macrophages in relation to their activation status, a feature that may be critical to determine their responsiveness to estrogen stimuli, had not been evaluated.

In **Chapter III,** we found the ER transcription profile to be significantly affected by different stimuli. In particular, M1 and M2 macrophages expressed higher ERα levels than ERβ, the latter being expressed at very low levels in both cell subtypes. Hence, ER-mediated responsiveness appears to be dependent on the macrophage activation status. On the other hand, this finding may suggest that macrophages may respond to estrogens primarily through ERα rather than ERβ. Corroborating this idea is the observation of Ribas *et al.* [194] who observed that hematopoietic/myeloid-specific deletion of ERα induces defects in macrophage function along with abnormal tissue inflammation and insulin resistance, further suggesting that macrophage ERα is involved in the control of the inflammatory response.

Thus, it becomes evident that these cells may also constitute targets of XE actions. Therefore, through the present work we also hypothesize that the immunomodultory effects of XEs may be crtitical for cardiometabolic dysfunction. Among environmental estrogens, plastic-derived XEs, because of their widespread use in daily life making them measurable in the circulation of almost all individuals in the industrialized world [195, 196], may accordingly have a plausible effect on macrophage biological dynamics, providing a possible mechanism linking inflammation arising in the AT and the exposure to these compounds.

This impelled us to test the immunomodulatory effect of XEs - BPA, DBP and DEHP - in concentrations similar to those in which they are found in the environment [197, 198], in human M1 or M2 macrophages. Moreover, to understand how XEs interfere with the immune system, it is necessary to understand how natural hormones affect these cells. Therefore, comparisons with E_2 were made whenever necessary.

Firstly, in **Chapter III** we verified that M1 and M2 macrophages have lower migratory capacity than unstimulated macrophages. To date there is a paucity of evidence about the influence of E_2 and XEs in the migratory capability of differentiated human macrophages. With respect to M1 macrophages, XEs did not modify the migration capacity of these cells, mimicking E_2 effect. Concerning M2 macrophages, E_2 increased migratory capacity and BPA and the studied phthalates (**Chapter IV**) mimicked the E_2 effect. This

is an interesting finding, because although these compounds may cause local tissue inflammation, they did not compromise the movement of circulating pro-inflammatory cells, and so did not interfere with their role in innate immunity [199].

Regarding M2 macrophages, as far as we know, this is the first time that stimulation of M2 macrophage migration by E_2 has been documented. XEs tended to increase migration, again, mimicking the effect of E_2. This selective effect, independent of ERs, may favor the arrival of M2 macrophages on tissues and sites of inflammation, denoting an immune modulatory character of these compounds. Another plausible hypothesis is that M1 macrophages are actively retained within the AT in response to XEs. Evidence of the detrimental role of M1 macrophages in promoting AT dysfunction has been reported in several studies [42, 200, 201].

A portfolio of cytokines is central to the role of macrophages as sentries of the innate immune system. Cytokines are small soluble proteins that confer instructions and mediate communication among immune and non-immune cells and deregulated cytokine secretion is implicated in several disease states [202]. Very little work has been conducted examining the effect of E_2 and XEs on cytokine expression in human macrophages. Thus, firstly we characterized the chemokine/cytokine release profile of unstimulated (M^{ϕ}), M1 and M2 macrophages. We verified a certain inability of M1 cells to recapitulate typical M1 responses because, although they secreted higher amounts of IL1β, they also secreted similar amounts of IL6 and lesser of TNF-α and IL10 comparing to M^{ϕ} (**Chapter III**). Accordingly, we suggest the possibility of an endogenous activation of these cells *in vivo*, due to the absence of circulating estrogens which allowed us to conclude that macrophage responses are deeply dependent on the specific micro-environmental conditions in which cells were prior to their activation.

Concerning M2 macrophages despite the presence of a characteristic increase in IL10 production and decrease in IL1β, we also found that these cells release higher amounts of IL6 and TNF-α than M^{ϕ} and/or M1 macrophages. Again, we can speculate that previous exposure to an estrogen poor environment may change cytokine release from cells to a more pro-inflammatory profile. This is not surprisingly since macrophage can bear both M1 and M2 characteristics simultaneously, i.e., simultaneously express markers of both classical and alternative activation [203, 204] and thus M2 macrophages are able to secrete pro-inflammatory mediators in specific conditions [205, 206]. This suggests that changes of estrogen signalling and/or obesity may favor the polarization of M2 macrophages, not M1 macrophages. It is also possible that the M1/M2 system is just too simplified to adequately represent the heterogeneity of ATMs in obesity [207]. Moreover, this increase in the release of inflammatory cytokines by cells involved in inflammation resolution may exacerbate the inflammatory state that accompanies the decline of estrogens, namely in menopause.

E_2 significantly induced M1 release of IL10 and IL6 and decreased IL10, IL6, TNF-α and IL1β release by M2 macrophages (**Chapter III**). As we can see, cytokine profiles do not follow the anti-inflammatory *vs* pro-

inflammatory paradigm as expected for E_2. If the endogenous activation of these cells *in vivo* is true, these findings may indicate the way that activated cells respond to coordinately regulate the development of inflammation after menopause. Besides, this finding could help explain why the time of the initiation is so important to the benefits of hormonal replacement therapy, as reported by some groups [208, 209]. Once exposed to an estrogen-deprived environment, re-exposure to E_2 is unable to reverse the acquired pro-inflammatory priming [210, 211].

Moreover, all XEs (**Chapter IV**) significantly altered the pattern of cytokine release by M1 and M2 macrophages, with a globally decreased of IL10 release by M2 macrophages. This may explain in part, why accumulation of XEs in vAT was negatively associated with plasma IL10, as we observed in our epidemiological study **(Chapter II)**. Still, DEHP and DBP significantly increased IL1β release in M1 and M2 macrophages, respectively. Active IL1β is produced by the cleavage of its inactive precursors by the protease caspase-1, which is activated by large multiprotein complexes named inflammasomes. Inflammasomes are central components of the innate immune system. An increase in AT IL1β of obese human, mainly in macrophages, has been reported, and it seems to be directly correlated with insulin resistance, features of metabolic syndrome and severity of T2D. Further, compared to MHO phenotype, the vAT of MAO phenotype is characterized by higher inflammasome activation in macrophages infiltrating this tissue [212]. Therefore, we suggest that XEs may be a trigger for inflammasome activation and an additional factor for global metabolic dysfunction.

Another unresolved issue concerns the exact mechanisms responsible for the observed results either for E_2 or XEs. Until recently, most studies have focused on the classical, or genomic phase of steroid responses, leading to activation or repression of target gene expression [213]. These slow responses (hours to days) occur via a mechanism involving multiple syntheses and modifications of macromolecules, finally generating mature nucleic acid messengers and then proteins. In addition to classical signalling, E_2-ER can act within minutes or seconds via extranuclear and membrane-associated forms of the receptor. In a variety of cell types, membrane and extranuclear pools of ER, activate protein kinases that phosphorylate transcription factors to promote transcriptional action and usually initiate second messenger-triggered signal cascades which emanate from the plasma membrane [214, 215]. A central question pertaining to this field is the cell-specificity of ER action and the molecular mechanisms by which the receptor selectively activates or represses target genes persists.

In order to address this question, we performed qPCR analysis in human macrophages which indeed revealed that some of the effects described above were mediated by either ERα or ERβ and/or by orchestrating NF-κB, AP1, JNK or ERK signaling pathways. However, the detailed understanding of their mechanisms is incomplete and should therefore be the aim of future research. Most recently, it has been reported that an orphan G-protein-coupled receptor, GPER, serves as an ER and mediates E_2 signaling from the membrane [104] or the endoplasmic reticulum [107]. There are examples of such initial rapid

responses for almost every class of steroid and related compounds [216]. We are now beginning to understand that steroid mimetics and steroid-targeted endocrine disruptors can also work via these rapid response pathways [143, 216].

Although the activities of most environmental estrogens have been called "weak" because of their inability to initiate nuclear retention and transcriptional effects [143, 216], we demonstrated that they are quite potent initiators of signal cascades emanating from the membrane. Moreover, the approach used by our study consisted of comparing E_2/XEs and co-treatment with ER antagonists namely MPP (ERα inhibitor) and PHTPP (ERβ inhibitor), revealing the involvement of each receptor subtype in the described effects. Thus, as verified, the effect of treatment with ERs inhibitors varies according to cell type, thereby granting the possibility to selectively inhibit or stimulate estrogen-like actions in various tissues. Curiousely, our results provide proofs of ERβ functionality on macrophage subtypes. Concerning the issue of genomics or non-genomic nature of the results obtained here, knowing that the modulators we used are inhibitors of nuclear ERs and that the membrane ER is the classical nuclear receptor protein localized to cellular domain [217], we belive that in most cases, collaboration between extranuclear and nuclear receptor pools is necessary for final result obtained.

Our study has limitations. Firstly, observational studies (**Chapter II**) do not prove causality, even when exposure is estimated on an individual level and does not allow for inferences regarding the temporality of events. One reason is that the concentration of XEs in plasma/AT at the time of clinical testing does not necessarily reflect the metabolic interference that may have developed some time in the past. In addition, the possibility exists that the observed associations are confounded by dietary factors, physical activity, or other covariables, which could not be addressed in this study. Secondly, our study was performed deliberately in a presumably more susceptible fraction of the population, i.e., obese subjects, and this means that our conclusions do not necessarily apply to healthy subjects. Finally, the associations for individual XEs were not corrected for multiple testingand therefore should be treated with caution. Regarding the study on human macrophages, technical reasons did not allow treating the macrophages with the same compounds evaluated in the epidemiological study. On the other hand, the current paradigm of macrophage activation has been extended from M1 vs M2 polarization to a spectrum model. Therefore, our phenotypes (**Chapter III and IV**) do not enterily reflet the complex biology of macrophage activation in the context of CVD. Also, we can not exclude that the differences observed in M1 and M2 phenotypes in postmenopausal women may be due to confounding factors, other than estrogen loss, including aging, as well as we recognize that the previous exposure to a xenoestrogenic ambient may change cell responsiveness to these compounds *ex vivo*.

Therefore, the results obtained in the present study provide new evidence of the effect of XEs on macrophage behavior and their mechanisms with relevance to the understanding of the action of environmental chemicals on the immune system and inflammation-associated diseases.

Because humans are simultaneously exposed to mixtures of different XEs that have the capacity to function as antagonists or agonists to a variety of hormones including estrogens, it is difficult to speculate about what kind of synergistic, additive or antagonistic actions might occur with XEs mixtures [218]. Importantly, biological effects of one XE can differ widely depending on the presence of another XE. Moreover, the statement of toxicology that "the dose makes poison" has recently been challenged by evidence that many XEs exhibit non-monotonic dose-response relationships in which biological effects are not directly proportional to concentration [219]. Humans are exposed daily to a variety of compounds, and it is thus likely that even if none reach an effective level, the combination or mixture of chemicals may become dangerous. Still, the fact that hormone-related effects of XEs are likely dependent on the endogenous hormonal milieu makes the issue even more complicated. Additionally, the combination of responses could add up to a continuous and sustained response, instead of an oscillating response typical of estrogen hormones, and lead to overstimulation resulting in disruption [6, 220]. This may explain differing potential hazards of exposure during times when endogenous estrogen levels are particularly high or low in either females or males in infancy, pubertal, reproductive and/or post-reproductive stages.

There has been great progress in terms of our understanding of how obesity-induced inflammation is regulated and how modulation of this process can regulate the development of obesity-induced metabolic abnormalities.

A comprehensive consideration of the results presented in this dissertation allows us to provide novel insights regarding the ability of XEs to mediate obesity-associated inflammation and related metabolic abnormalities in a biomedicine dimension (Figure 4). We point out the need to identify biomarkers of XE exposure and discuss the suitability of plasma versus AT XEs measurement to assess whole body burdens of XEs. Indeed, the distribution and concentration of individual and total XEs differed between plasma, vAT and scAT, and the pattern of accumulation was different between pre- and postmenopausal women. In premenopausal women, although vAT XEs were more correlated with metabolic dysfunction, plasma XEs were the best predictors of inflammation and CVD risk. We also advanced that plasma XEs in these women may be released from vAT, as consequence of induction of local toxicity and AT dysfunction by XEs. In postmenopausal women, this AT dysfunction extends to scAT. More importantly, we advance that, other than associating with obesity grade or levels of AT accumulation, XE levels may help predict cardiometabolic risk. In this regard, evidence we have gathered allows us to state that, in our population, XEs may be good indicators of dysmetabolic obesity when premenopausal women are considered. However, it remains to be established if these findings can apply to other populations. Additionally, whether all these observations reflect causal pathways should be determined in longitudinal studies that may link exposure to specific chemicals with early disease development

Although the CVD risk profile worsens in postmenopausal women, the causative mechanisms are incompletely understood. Herein, we advance a number of potential effects of XEs on obesity-derived complications including modulation of the inflammatory response and immune cell function.

We present results showing that macrophage ERs are modulated by XEs, and may therefore be an important target to combat metabolic dysfunction. We further demonstrate that ERs are differently expressed among different stages of macrophage polarization, a feature that may be critical for determining their responsiveness to estrogenic stimuli. We also highlight that although ERα is expressed in substantially higher levels and most estrogen effects have been related to this isoform, ERβ may also play a role. Moreover, the co-treatment with either of the selective ERα and ERβ antagonists MPP and PHTPP, respectively, helped us understand the involvement of each receptor subtype in the described effects. The relationship between estrogens/XEs and inflammation on macrophages was exploited through determinations of intracellular transduction pathways, focusing on the interactions between inflammation-relevant effector kinases and transcription factors, and we point out that the previous

exposure to lack/decrease of estrogen signaling renders cells to a more pro-inflammatory situation, which might contribute to the manifestation of chronic inflammatory diseases.

Evidently, XEs interact with E_2 and estrogen-related signaling pathways leading to differential molecular effects, thus ultimately impacting in the physiological response of sensitive cells. Inflammation was at the center of our research interest, and we were able to demostrate that E_2 and XEs impact on inflammation and immunological processes, which may bridge their effect on metabolism. However, it becomes evident from our research that XEs are more than estrogen mimetics, and that, not only their effects depend on cell type and the nature of the chemicals, but also on the presence of endogenous hormones, highlighting the variation of their effects throughout the life cycle. The implications of these observations relate to the fact that increasing the knowledge on how specific ER modulation may change macrophage behavior can constitute the basis for the development of therapeutic approaches with the aim of altering inflammatory status profile. Additionally, it would be interesting to study the effect of E_2 and XEs in inflammatory and functional markers of AT as we understand they act as mediators that may help to explain the metabolic dysfunction associated with obesity. As our experimental results using inflammatory cells were based on short-term exposure to XEs, the investigation of the effects of long-term exposure to XEs and the underlying molecular mechanisms would also be worth exploring.

Thus, basic and clinical researchers should be aware of the potential health impacts of these ubiquitous environmental chemicals, an understanding that depends both on the knowledge of the extent of the contamination of humans and other biological samples, but also on their effects and the mechanisms endowed. Even though the individuals we studied were assessed in adulthood, likely early life exposures to XEs do also occur, so the hypothesis on developmental determination of health and disease remains relevant. In this point of view, epidemiological studies integrating XEs into that hypothesis would be scientifically important, starting from *in utero* exposure (or even in prenatally in the subject's parents), continuing to early fetal, childhood, adolescence, and adulthood periods, to evaluate how changes in exposure patterns during lifetime affect inflammation and cardiometabolic dysfunction.

Another key point is introduced quoting the latest WHO report on endocrine disrupting chemicals ,"…there is emerging evidence that many chemicals may act additively and, each at levels without individual effect, could act together to cause health problems" [221]. Rather than the old toxicological method of a single-exposure, dose-response approach using pure compounds, it is vital that new risk assessment procedures simulate more closely what occurs in nature. It is crucial to explore the effects of combinations of compounds or mixtures.

Investigation on the action of XEs and other chemicals that alter metabolic function will open novel directions for the prevention and treatment of metabolic diseases, but will also reinforce the importance of international agreements to limit the release of XEs to minimize public health risks. These findings are both of public health and clinical significance in terms of screening and stratification based on XE

contaminations identifying those at greatest cardiometabolic risk for whom appropriate therapeutic or intervention strategies should be developed.

From the amount of evidence being presenly accumulated it is becoming clearer that, negleting the novel and emerging knowledge about XEs and metabolic disease will have significant health impacts for the general population, as well as on the generations to come.

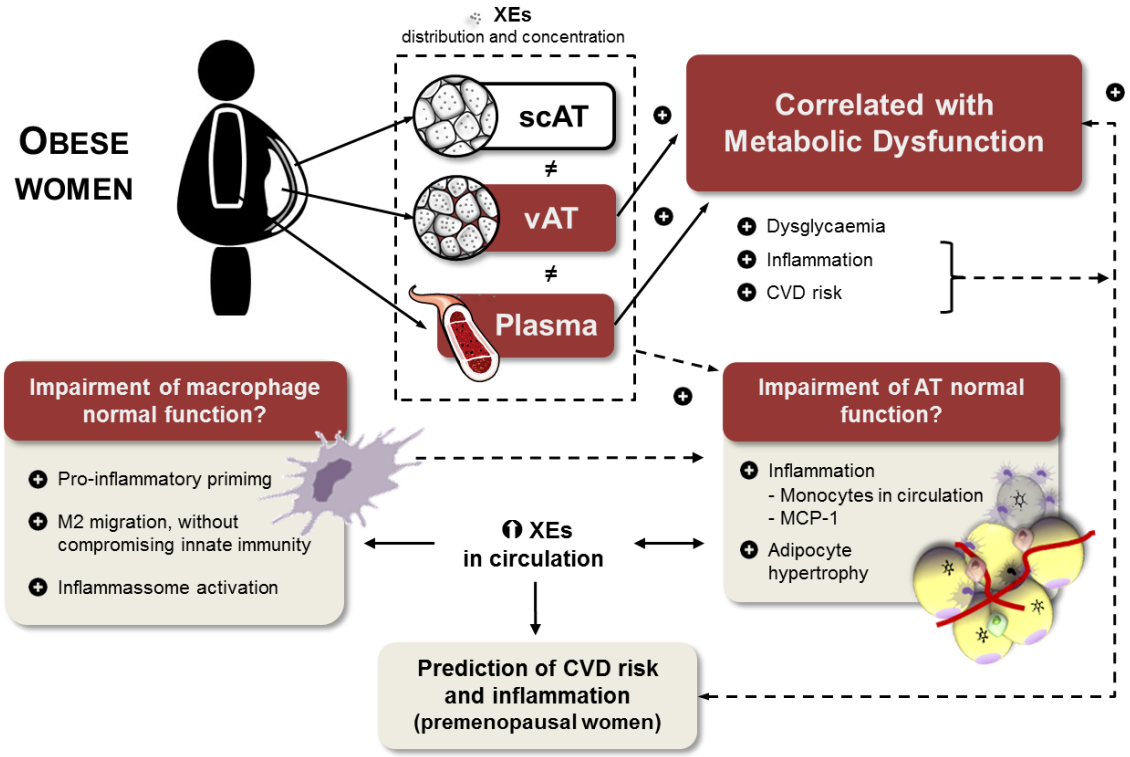


Figure 4. Schematic representation of the proposed mechanisms through which XEs exposure may contribute to cardiometabolick dysfunction. CVD, cardiovascular disease; M2, alternatively-activated macrophages; MCP-1, monocyte chemotactic protein 1; scAT, subcutaneous adipose tissue; vAT, visceral adipose tissue; XEs, xenoestrogens.

REFERENCES

1. Newbold, R.R., E. Padilla-Banks, and W.N. Jefferson, Environmental estrogens and obesity. Mol Cell Endocrinol, **2009**. 304(1-2): p. 84-89.
2. Bastien, M.*, et al.*, Overview of epidemiology and contribution of obesity to cardiovascular disease. Prog Cardiovasc Dis, **2014**. 56(4): p. 369-381.
3. WHO, Global Observatory (GBO): Obesity 2008. **2013**: Geneva.
4. Ng, M.*, et al.*, Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet, **2014**. 384(9945): p. 766-781.
5. Thayer, K.A.*, et al.*, Role of environmental chemicals in diabetes and obesity: a National Toxicology Program workshop review. Environ Health Perspect, **2012**. 120(6): p. 779-789.
6. Lubrano, C.*, et al.*, Obesity and metabolic comorbidities: environmental diseases? Oxid Med Cell Longev, **2013**. 2013: p. 640673.
7. Harwood, H.J., The adipocyte as an endocrine organ in the regulation of metabolic homeostasis. Neuropharmacology, **2012**. 63(1): p. 57-75.
8. Zhang, Y.*, et al.*, Positional cloning of the mouse obese gene and its human homologue. Nature, **1994**. 372(6505): p. 425-432.
9. Ahima, R.S. and J.S. Flier, Adipose tissue as an endocrine organ. Trends Endocrinol Metab, **2000**. 11(8): p. 327-332.
10. Galic, S., J.S. Oakhill, and G.R. Steinberg, Adipose tissue as an endocrine organ. Mol Cell Endocrinol, **2010**. 316(2): p. 129-139.
11. Kershaw, E.E. and J.S. Flier, Adipose tissue as an endocrine organ. J Clin Endocrinol Metab, **2004**. 89(6): p. 2548-2556.
12. Frayn, K.N.*, et al.*, Integrative physiology of human adipose tissue. Int J Obes Relat Metab Disord, **2003**. 27(8): p. 875-888.
13. Lee, M.J., Y. Wu, and S.K. Fried, Adipose tissue heterogeneity: implication of depot differences in adipose tissue for obesity complications. Mol Aspects Med, **2013**. 34(1): p. 1-11.
14. Decaunes, P.*, et al.*, Adipose-derived stromal cells: cytokine expression and immune cell contaminants. Methods Mol Biol, **2011**. 702: p. 151-161.
15. Joe, A.W.*, et al.*, Depot-specific differences in adipogenic progenitor abundance and proliferative response to high-fat diet. Stem Cells, **2009**. 27(10): p. 2563-2570.
16. Tchkonia, T.*, et al.*, Fat depot origin affects adipogenesis in primary cultured and cloned human preadipocytes. Am J Physiol Regul Integr Comp Physiol, **2002**. 282(5): p. R1286-1296.
17. Majka, S.M., Y. Barak, and D.J. Klemm, Concise review: adipocyte origins: weighing the possibilities. Stem Cells, **2011**. 29(7): p. 1034-1040.

18. Tchoukalova, Y.D*., et al.*, Subcutaneous adipocyte size and body fat distribution. Am J Clin Nutr, **2008**. 87(1): p. 56-63.
19. Tchoukalova, Y.D*., et al.*, Regional differences in cellular mechanisms of adipose tissue gain with overfeeding. Proc Natl Acad Sci U S A, **2010**. 107(42): p. 18226-18231.
20. Pradhan, A.D., Sex differences in the metabolic syndrome: implications for cardiovascular health in women. Clin Chem, **2014**. 60(1): p. 44-52.
21. Belanger, C*., et al.*, Omental and subcutaneous adipose tissue steroid levels in obese men. Steroids, **2006**. 71(8): p. 674-682.
22. Drolet, R*., et al.*, Hypertrophy and hyperplasia of abdominal adipose tissues in women. Int J Obes (Lond), **2008**. 32(2): p. 283-291.
23. Gealekman, O*., et al.*, Depot-specific differences and insufficient subcutaneous adipose tissue angiogenesis in human obesity. Circulation, **2011**. 123(2): p. 186-194.
24. Michaud, A*., et al.*, Abdominal subcutaneous and omental adipocyte morphology and its relation to gene expression, lipolysis and adipocytokine levels in women. Metabolism, **2014**. 63(3): p. 372-381.
25. Lessard, J*., et al.*, Low abdominal subcutaneous preadipocyte adipogenesis is associated with visceral obesity, visceral adipocyte hypertrophy, and a dysmetabolic state. Adipocyte, **2014**. 3(3): p. 197-205.
26. Harman-Boehm, I*., et al.*, Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity. J Clin Endocrinol Metab, **2007**. 92(6): p. 2240-2247.
27. Cancello, R*., et al.*, Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. Diabetes, **2006**. 55(6): p. 1554-1561.
28. Palmer, B.F. and D.J. Clegg, The sexual dimorphism of obesity. Mol Cell Endocrinol, **2015**. 402: p. 113-119.
29. Itoh, M*., et al.*, Adipose tissue remodeling as homeostatic inflammation. Int J Inflam, **2011**. 2011: p. 720926.
30. Schenk, S., M. Saberi, and J.M. Olefsky, Insulin sensitivity: modulation by nutrients and inflammation. J Clin Invest, **2008**. 118(9): p. 2992-3002.
31. Hotamisligil, G.S., Inflammation and metabolic disorders. Nature, **2006**. 444(7121): p. 860-867.
32. Nishimura, S*., et al.*, Adipogenesis in obesity requires close interplay between differentiating adipocytes, stromal cells, and blood vessels. Diabetes, **2007**. 56(6): p. 1517-1526.
33. Nishimura, S*., et al.*, In vivo imaging in mice reveals local cell dynamics and inflammation in obese adipose tissue. The Journal of clinical investigation, **2008**. 118(2): p. 710-721.
34. Gregor, M.F. and G.S. Hotamisligil, Thematic review series: Adipocyte Biology. Adipocyte stress: the endoplasmic reticulum and metabolic disease. J Lipid Res, **2007**. 48(9): p. 1905-1914.

35. Rudich, A., H. Kanety, and N. Bashan, Adipose stress-sensing kinases: linking obesity to malfunction. Trends Endocrinol Metab, **2007**. 18(8): p. 291-299.
36. Weisberg, S.P.*, et al.*, Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest, **2003**. 112(12): p. 1796-1808.
37. Yona, S.*, et al.*, Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. Immunity, **2013**. 38(1): p. 79-91.
38. Jenkins, S.J.*, et al.*, Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. Science, **2011**. 332(6035): p. 1284-1288.
39. Osborn, O. and J.M. Olefsky, The cellular and signaling networks linking the immune system and metabolism in disease. Nat Med, **2012**. 18(3): p. 363-374.
40. Bluher, M., Adipose tissue dysfunction contributes to obesity related metabolic diseases. Best Pract Res Clin Endocrinol Metab, **2013**. 27(2): p. 163-177.
41. Haase, J.*, et al.*, Local proliferation of macrophages in adipose tissue during obesity-induced inflammation. Diabetologia, **2014**. 57(3): p. 562-571.
42. Lumeng, C.N.*, et al.*, Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. Diabetes, **2008**. 57(12): p. 3239-3246.
43. Xu, H.*, et al.*, Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest, **2003**. 112(12): p. 1821-1830.
44. Lumeng, C.N.*, et al.*, Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. Diabetes, **2007**. 56(1): p. 16-23.
45. Gordon, S., Alternative activation of macrophages. Nat Rev Immunol, **2003**. 3(1): p. 23-35.
46. Gordon, S. and P.R. Taylor, Monocyte and macrophage heterogeneity. Nat Rev Immunol, **2005**. 5(12): p. 953-964.
47. Murray, P.J.*, et al.*, Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity, **2014**. 41(1): p. 14-20.
48. Hill, A.A., W. Reid Bolus, and A.H. Hasty, A decade of progress in adipose tissue macrophage biology. Immunol Rev, **2014**. 262(1): p. 134-152.
49. McNelis, J.C. and J.M. Olefsky, Macrophages, immunity, and metabolic disease. Immunity, **2014**. 41(1): p. 36-48.
50. Balistreri, C.R., C. Caruso, and G. Candore, The role of adipose tissue and adipokines in obesity-related inflammatory diseases. Mediators Inflamm, **2010**. 2010: p. 802078.
51. Murdoch, C., M. Muthana, and C.E. Lewis, Hypoxia regulates macrophage functions in inflammation. J Immunol, **2005**. 175(10): p. 6257-6263.
52. Aron-Wisnewsky, J.*, et al.*, Human adipose tissue macrophages: m1 and m2 cell surface markers in subcutaneous and omental depots and after weight loss. J Clin Endocrinol Metab, **2009**. 94(11): p. 4619-4623.

53. Bourlier, V. and A. Bouloumie, Role of macrophage tissue infiltration in obesity and insulin resistance. Diabetes Metab, **2009**. 35(4): p. 251-260.
54. Monteiro, R., Chronic inflammation in the metabolic syndrome: emphasis on adipose tissue, in Oxidative Stress, Inflammation and Angiogenesis in the Metabolic Syndrome, R. Soares and C. Costa, Editors. **2009**, Springer Netherlands. p. 65-84.
55. Sun, K., C.M. Kusminski, and P.E. Scherer, Adipose tissue remodeling and obesity. J Clin Invest, **2011**. 121(6): p. 2094-2101.
56. Cancello, R.*, et al.*, Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. Diabetes, **2005**. 54(8): p. 2277-2286.
57. Mosser, D.M., The many faces of macrophage activation. J Leukoc Biol, **2003**. 73(2): p. 209-212.
58. Gordon, S., Do macrophage innate immune receptors enhance atherogenesis? Dev Cell, **2003**. 5(5): p. 666-668.
59. Benetti, E.*, et al.*, The NLRP3 Inflammasome as a novel player of the intercellular crosstalk in metabolic disorders. Mediators Inflamm, **2013**. 2013: p. 678627.
60. Alberti, K.G.*, et al.*, Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation, **2009**. 120(16): p. 1640-1645.
61. Bluher, M., Clinical relevance of adipokines. Diabetes Metab J, **2012**. 36(5): p. 317-327.
62. Despres, J.P. and I. Lemieux, Abdominal obesity and metabolic syndrome. Nature, **2006**. 444(7121): p. 881-887.
63. Britton, K.A. and C.S. Fox, Ectopic fat depots and cardiovascular disease. Circulation, **2011**. 124(24): p. e837-841.
64. Skurk, T.*, et al.*, Relationship between Adipocyte Size and Adipokine Expression and Secretion. J Clin Endocrinol Metab, **2007**. 92(3): p. 1023-1033.
65. Bluher, M., Are there still healthy obese patients? Curr Opin Endocrinol Diabetes Obes, **2012**. 19(5): p. 341-346.
66. Laurencikiene, J.*, et al.*, Regulation of lipolysis in small and large fat cells of the same subject. J Clin Endocrinol Metab, **2011**. 96(12): p. E2045-2049.
67. Bluher, M., The distinction of metabolically 'healthy' from 'unhealthy' obese individuals. Curr Opin Lipidol, **2010**. 21(1): p. 38-43.
68. Sims, E.A., Are there persons who are obese, but metabolically healthy? Metabolism, **2001**. 50(12): p. 1499-1504.

69. Badoud, F*., et al.*, Molecular insights into the role of white adipose tissue in metabolically unhealthy normal weight and metabolically healthy obese individuals. FASEB J, **2014**. 19(3): p. 748-758.
70. Lafontan, M., Fat cells: afferent and efferent messages define new approaches to treat obesity. Annu Rev Pharmacol Toxicol, **2005**. 45: p. 119-146.
71. Mauvais-Jarvis, F., D.J. Clegg, and A.L. Hevener, The role of estrogens in control of energy balance and glucose homeostasis. Endocr Rev, **2013**. 34(3): p. 309-338.
72. Zhu, Z*., et al.*, Central expression and anorectic effect of brain-derived neurotrophic factor are regulated by circulating estradiol levels. Horm Behav, **2013**. 63(3): p. 533-542.
73. Shen, L*., et al.*, Estradiol increases the anorectic effect of central apolipoprotein A-IV. Endocrinology, **2010**. 151(7): p. 3163-3168.
74. Clegg, D.J*., et al.*, Gonadal hormones determine sensitivity to central leptin and insulin. Diabetes, **2006**. 55(4): p. 978-987.
75. Xu, Y*., et al.*, Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. Cell Metab, **2011**. 14(4): p. 453-465.
76. Musatov, S*., et al.*, Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome. Proc Natl Acad Sci U S A, **2007**. 104(7): p. 2501-2506.
77. Mauvais-Jarvis, F., Estrogen and androgen receptors: regulators of fuel homeostasis and emerging targets for diabetes and obesity. Trends Endocrinol Metab, **2011**. 22(1): p. 24-33.
78. Hoffstedt, J*., et al.*, Regional impact of adipose tissue morphology on the metabolic profile in morbid obesity. Diabetologia, **2010**. 53(12): p. 2496-2503.
79. Villar, I.C., A.J. Hobbs, and A. Ahluwalia, Sex differences in vascular function: implication of endothelium-derived hyperpolarizing factor. J Endocrinol, **2008**. 197(3): p. 447-462.
80. Ren, J. and R.O. Kelley, Cardiac health in women with metabolic syndrome: clinical aspects and pathophysiology. Obesity (Silver Spring), **2009**. 17(6): p. 1114-1123.
81. Nilsson, B.O., B. Olde, and L.M. Leeb-Lundberg, G protein-coupled oestrogen receptor 1 (GPER1)/GPR30: a new player in cardiovascular and metabolic oestrogenic signalling. Br J Pharmacol, **2011**. 163(6): p. 1131-1139.
82. You, T., A.S. Ryan, and B.J. Nicklas, The metabolic syndrome in obese postmenopausal women: relationship to body composition, visceral fat, and inflammation. J Clin Endocrinol Metab, **2004**. 89(11): p. 5517-5522.
83. Regitz-Zagrosek, V., E. Lehmkuhl, and M.O. Weickert, Gender differences in the metabolic syndrome and their role for cardiovascular disease. Clin Res Cardiol., **2006**. 95(3): p. 136-147.
84. Stork, S*., et al.*, Estrogen, inflammation and cardiovascular risk in women: a critical appraisal. Trends Endocrinol Metab, **2004**. 15(2): p. 66-72.
85. Lobo, R.A., Inflammation, coronary artery disease, and hormones. Menopause, **2008**. 15(6): p. 1036-1038.

86. Simpson, E.R*., et al.*, Aromatase--a brief overview. Annu Rev Physiol, **2002**. 64: p. 93-127.

87. Reed, K.C. and S. Ohno, Kinetic properties of human placental aromatase. Application of an assay measuring 3H2O release from 1beta,2beta-3H-androgens. J Biol Chem, **1976**. 251(6): p. 1625-1631.

88. Chen, S., Aromatase and breast cancer. Front Biosci, **1998**. 3: p. d922-933.

89. Matsumine, H*., et al.*, Aromatization by skeletal muscle. J Clin Endocrinol Metab, **1986**. 63(3): p. 717-720.

90. Labrie, F*., et al.*, DHEA and the intracrine formation of androgens and estrogens in peripheral target tissues: its role during aging. Steroids, **1998**. 63(5-6): p. 322-328.

91. Burger, H.G., The endocrinology of the menopause. J Steroid Biochem Mol Biol, **1999**. 69(1-6): p. 31-35.

92. Simpson, E.R*., et al.*, Aromatase expression in health and disease. Recent Prog Horm Res, **1997**. 52: p. 185-213; discussion 213-184.

93. Simpson, E.R., Sources of estrogen and their importance. J Steroid Biochem Mol Biol, **2003**. 86(3-5): p. 225-230.

94. Reed, M.J*., et al.*, Steroid sulfatase: molecular biology, regulation, and inhibition. Endocr Rev, **2005**. 26(2): p. 171-202.

95. Cui, J., Y. Shen, and R. Li, Estrogen synthesis and signaling pathways during aging: from periphery to brain. Trends Mol Med, **2013**. 19(3): p. 197-209.

96. Heldring, N*., et al.*, Estrogen receptors: how do they signal and what are their targets. Physiol Rev, **2007**. 87(3): p. 905-931.

97. Kushner, P.J*., et al.*, Oestrogen receptor function at classical and alternative response elements. Novartis Found Symp, **2000**. 230: p. 20-26; discussion 27-40.

98. Nilsson, S*., et al.*, Mechanisms of estrogen action. Physiol Rev, **2001**. 81(4): p. 1535-1565.

99. Foryst-Ludwig, A. and U. Kintscher, Metabolic impact of estrogen signalling through ERalpha and ERbeta. J Steroid Biochem Mol Biol, **2010**. 122(1-3): p. 74-81.

100. Faulds, M.H*., et al.*, The diversity of sex steroid action: regulation of metabolism by estrogen signaling. J Endocrinol, **2012**. 212(1): p. 3-12.

101. Stice, J.P. and A.A. Knowlton, Estrogen, NFkappaB, and the heat shock response. Mol Med, **2008**. 14(7-8): p. 517-527.

102. Frasor, J*., et al.*, Response-specific and ligand dose-dependent modulation of estrogen receptor (ER) alpha activity by ERbeta in the uterus. Endocrinology, **2003**. 144(7): p. 3159-3166.

103. Aronica, S.M., W.L. Kraus, and B.S. Katzenellenbogen, Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc Natl Acad Sci U S A, **1994**. 91(18): p. 8517-8521.

104. Filardo, E.J*., et al.*, Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol, **2000**. 14(10): p. 1649-1660.

105. Carlezon, W.A., Jr., R.S. Duman, and E.J. Nestler, The many faces of CREB. Trends Neurosci, **2005**. 28(8): p. 436-445.

106. Filardo, E*., et al.*, Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. Endocrinology, **2007**. 148(7): p. 3236-3245.

107. Revankar, C.M*., et al.*, A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science, **2005**. 307(5715): p. 1625-1630.

108. Giguere, V., Transcriptional control of energy homeostasis by the estrogen-related receptors. Endocr Rev, **2008**. 29(6): p. 677-696.

109. Villena, J.A. and A. Kralli, ERRalpha: a metabolic function for the oldest orphan. Trends Endocrinol Metab, **2008**. 19(8): p. 269-276.

110. Monteiro, R., D. Teixeira, and C. Calhau, Estrogen signaling in metabolic inflammation. Mediators Inflamm, **2014**. 2014: p. 615917.

111. Folkerd, E.J., M.J. Reed, and V.H. James, Oestrogen production in adipose tissue from normal women and women with endometrial cancer in vitro. J Steroid Biochem, **1982**. 16(2): p. 297-302.

112. Bulun, S.E*., et al.*, Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment. Pharmacol Rev, **2005**. 57(3): p. 359-383.

113. Hamosh, M. and P. Hamosh, The effect of estrogen on the lipoprotein lipase activity of rat adipose tissue. J Clin Invest, **1975**. 55(5): p. 1132-1135.

114. Palin, S.L*., et al.*, 17Beta-estradiol and anti-estrogen ICI:compound 182,780 regulate expression of lipoprotein lipase and hormone-sensitive lipase in isolated subcutaneous abdominal adipocytes. Metabolism, **2003**. 52(4): p. 383-388.

115. Ackerman, G.E*., et al.*, Potentiation of epinephrine-induced lipolysis by catechol estrogens and their methoxy derivatives. Endocrinology, **1981**. 109(6): p. 2084-2088.

116. Misso, M.L*., et al.*, Cellular and molecular characterization of the adipose phenotype of the aromatase-deficient mouse. Endocrinology, **2003**. 144(4): p. 1474-1480.

117. Anwar, A*., et al.*, Site-specific regulation of oestrogen receptor-alpha and -beta by oestradiol in human adipose tissue. Diabetes Obes Metab, **2001**. 3(5): p. 338-349.

118. Clegg, D.J., Minireview: the year in review of estrogen regulation of metabolism. Mol Endocrinol, **2012**. 26(12): p. 1957-1960.

119. Smith, E.P*., et al.*, Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med, **1994**. 331(16): p. 1056-1061.

120. Heine, P.A*., et al.*, Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. Proc Natl Acad Sci U S A, **2000**. 97(23): p. 12729-12734.

121. Cooke, P.S.*, et al.*, The role of estrogen and estrogen receptor-alpha in male adipose tissue. Mol Cell Endocrinol, **2001**. 178(1-2): p. 147-154.

122. Okura, T.*, et al.*, Association of polymorphisms in the estrogen receptor alpha gene with body fat distribution. Int J Obes Relat Metab Disord, **2003**. 27(9): p. 1020-1027.

123. Davis, K.E.*, et al.*, The sexually dimorphic role of adipose and adipocyte estrogen receptors in modulating adipose tissue expansion, inflammation, and fibrosis. Mol Metab, **2013**. 2(3): p. 227-242.

124. Dos Santos, E.G.*, et al.*, Rapid nongenomic E2 effects on p42/p44 MAPK, activator protein-1, and cAMP response element binding protein in rat white adipocytes. Endocrinology, **2002**. 143(3): p. 930-940.

125. Dieudonne, M.N.*, et al.*, Evidence for functional estrogen receptors alpha and beta in human adipose cells: regional specificities and regulation by estrogens. Am J Physiol Cell Physiol, **2004**. 286(3): p. C655-661.

126. Murphy, A.J.*, et al.*, Estradiol regulates expression of estrogen receptor ERalpha46 in human macrophages. PLoS One., **2009**. 4(5): p. e5539.

127. Phiel, K.L.*, et al.*, Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. Immunol Lett, **2005**. 97(1): p. 107-113.

128. Harkonen, P.L. and H.K. Vaananen, Monocyte-macrophage system as a target for estrogen and selective estrogen receptor modulators. Ann N Y Acad Sci, **2006**. 1089: p. 218-227.

129. Pioli, P.A.*, et al.*, Estradiol attenuates lipopolysaccharide-induced CXC chemokine ligand 8 production by human peripheral blood monocytes. J Immunol, **2007**. 179(9): p. 6284-6290.

130. Miyagi, M.*, et al.*, Effects of sex hormones on chemotaxis of human peripheral polymorphonuclear leukocytes and monocytes. J Periodontol, **1992**. 63(1): p. 28-32.

131. Okada, M.*, et al.*, Effects of 17 beta-estradiol and progesterone on migration of human monocytic THP-1 cells stimulated by minimally oxidized low-density lipoprotein in vitro. Cardiovasc Res, **1997**. 34(3): p. 529-535.

132. Ghisletti, S.*, et al.*, 17beta-estradiol inhibits inflammatory gene expression by controlling NF-kappaB intracellular localization. Mol Cell Biol, **2005**. 25(8): p. 2957-2968.

133. Gutendorf, B. and J. Westendorf, Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology, **2001**. 166(1-2): p. 79-89.

134. Swedenborg, E.*, et al.*, Endocrine disruptive chemicals: mechanisms of action and involvement in metabolic disorders. J Mol Endocrinol, **2009**. 43(1): p. 1-10.

135. Li, Y.*, et al.*, Endocrine-Disrupting Chemicals (EDCs): In Vitro Mechanism of Estrogenic Activation and Differential Effects on ER Target Genes. Environ Health Perspect, **2013**. 121(4): p. 459-466.

136. Kojima, H.*, et al.*, Comparative study of human and mouse pregnane X receptor agonistic activity in 200 pesticides using in vitro reporter gene assays. Toxicology, **2011**. 280(3): p. 77-87.

137. Marini, H., *et al.*, OPG and sRANKL serum concentrations in osteopenic, postmenopausal women after 2-year genistein administration. J Bone Miner Res, **2008**. 23(5): p. 715-720.

138. Atteritano, M., *et al.*, Effects of the phytoestrogen genistein on some predictors of cardiovascular risk in osteopenic, postmenopausal women: a two-year randomized, double-blind, placebo-controlled study. J Clin Endocrinol Metab, **2007**. 92(8): p. 3068-3075.

139. Tchounwou, P.B., *et al.*, Heavy metal toxicity and the environment. EXS, **2012**. 101: p. 133-164.

140. La Merrill, M. and L.S. Birnbaum, Childhood obesity and environmental chemicals. Mt Sinai J Med, **2011**. 78(1): p. 22-48.

141. Gregoraszczuk, E.L. and R. Kovacevic, The impact of endocrine disruptors on endocrine targets. Int J Endocrinol, **2013**. 2013: p. 340453.

142. Alyea, R.A. and C.S. Watson, Nongenomic mechanisms of physiological estrogen-mediated dopamine efflux. BMC Neurosci, **2009**. 10: p. 59.

143. Bulayeva, N.N. and C.S. Watson, Xenoestrogen-induced ERK-1 and ERK-2 activation via multiple membrane-initiated signaling pathways. Environ Health Perspect, **2004**. 112(15): p. 1481-1487.

144. Wozniak, A.L., N.N. Bulayeva, and C.S. Watson, Xenoestrogens at picomolar to nanomolar concentrations trigger membrane estrogen receptor-alpha-mediated Ca2+ fluxes and prolactin release in GH3/B6 pituitary tumor cells. Environ Health Perspect, **2005**. 113(4): p. 431-439.

145. Diamanti-Kandarakis, E., *et al.*, Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev, **2009**. 30(4): p. 293-342.

146. Carson, R., Silent Spring. **1962**, United States: Houghton Mifflin 365.

147. Lee, H., Relationship between serum concentrations of persistent organic pollutants and the prevalence of metabolic syndrome among non-diabetic adults: results from the National Health and Nutrition Examination Survey 1999–2002. Diabetologia, **2007**. 50: p. 1841–1851.

148. Bourez, S., *et al.*, Accumulation of polychlorinated biphenyls in adipocytes: selective targeting to lipid droplets and role of caveolin-1. PloS One, **2012**. 7(2): p. e31834.

149. La Merrill, M., *et al.*, Toxicological function of adipose tissue: focus on persistent organic pollutants. Environ Health Perspect, **2013**. 121(2): p. 162-169.

150. Lee, D.H., *et al.*, Chlorinated Persistent Organic Pollutants, Obesity, and Type 2 Diabetes. Endocr Rev, **2014**: p. er20131084.

151. Myre, M. and P. Imbeault, Persistent organic pollutants meet adipose tissue hypoxia: does cross-talk contribute to inflammation during obesity? Obes Rev, **2014**. 15(1): p. 19-28.

152. Arrebola, J.P., *et al.*, Adipose tissue concentrations of persistent organic pollutants and total cancer risk in an adult cohort from Southern Spain: preliminary data from year 9 of the follow-up. Sci Total Environ, **2014**. 500-501: p. 243-249.

153. Patel, C.J., J. Bhattacharya, and A.J. Butte, An Environment-Wide Association Study (EWAS) on type 2 diabetes mellitus. PLoS One, **2010**. 5(5): p. e10746.

154. World Health Organization, Almost a quarter of all disease caused by environmental exposure. **2006**, World Health Organization: Geneva, Switzerland

155. Sprague, B.L*., et al.*, Circulating serum xenoestrogens and mammographic breast density. Breast Cancer Res, **2013**. 15(3): p. R45.

156. Baillie-Hamilton, P.F., Chemical toxins: a hypothesis to explain the global obesity epidemic. J Altern Complement Med, **2002**. 8(2): p. 185-192.

157. Neel, B.A. and R.M. Sargis, The paradox of progress: environmental disruption of metabolism and the diabetes epidemic. Diabetes, **2011**. 60(7): p. 1838-1848.

158. Casals-Casas, C., J.N. Feige, and B. Desvergne, Interference of pollutants with PPARs: endocrine disruption meets metabolism. Int J Obes (Lond), **2008**. 32 Suppl 6: p. S53-61.

159. Ruzzin, J*., et al.*, Reconsidering metabolic diseases: the impacts of persistent organic pollutants. Atherosclerosis, **2012**. 224(1): p. 1-3.

160. Alonso-Magdalena, P., I. Quesada, and A. Nadal, Endocrine disruptors in the etiology of type 2 diabetes mellitus. Nat Rev Endocrinol, **2011**. 7(6): p. 346-353.

161. Casals-Casas, C. and B. Desvergne, Endocrine disruptors: from endocrine to metabolic disruption. Annu Rev Physiol, **2011**. 73: p. 135-162.

162. Covaci, A*., et al.*, Determination of polybrominated diphenyl ethers and polychlorinated biphenyls in human adipose tissue by large-volume injection-narrow-bore capillary gas chromatography/electron impact low-resolution mass spectrometry. Anal Chem, **2002**. 74(4): p. 790-798.

163. Ronn, M*., et al.*, Circulating levels of persistent organic pollutants associate in divergent ways to fat mass measured by DXA in humans. Chemosphere, **2011**. 85(3): p. 335-343.

164. Roos, V*., et al.*, Circulating levels of persistent organic pollutants in relation to visceral and subcutaneous adipose tissue by abdominal MRI. Obesity (Silver Spring), **2013**. 21(2): p. 413-418.

165. Smeds, A. and P. Saukko, Identification and quantification of polychlorinated biphenyls and some endocrine disrupting pesticides in human adipose tissue from Finland. Chemosphere, **2001**. 44(6): p. 1463-1471.

166. Yu, G.W., J. Laseter, and C. Mylander, Persistent organic pollutants in serum and several different fat compartments in humans. J Environ Public Health, **2011**. 2011: p. 417980.

167. Shi, H., R.J. Seeley, and D.J. Clegg, Sexual differences in the control of energy homeostasis. Front Neuroendocrinol, **2009**. 30(3): p. 396-404.

168. Kim, M.J*., et al.*, Fate and complex pathogenic effects of dioxins and polychlorinated biphenyls in obese subjects before and after drastic weight loss. Environ Health Perspect, **2011**. 119(3): p. 377-383.

169. Arrebola, J.P*., et al.*, Multivariate models to predict human adipose tissue PCB concentrations in Southern Spain. Environ Int, **2010**. 36(7): p. 705-713.

170. Wajchenberg, B.L., Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocr Rev, **2000**. 21(6): p. 697-738.

171. Hue, O.*, et al.*, Increased plasma levels of toxic pollutants accompanying weight loss induced by hypocaloric diet or by bariatric surgery. Obes Surg, **2006**. 16(9): p. 1145-1154.

172. Lim, J.S.*, et al.*, Inverse associations between long-term weight change and serum concentrations of persistent organic pollutants. Int J Obes (Lond), **2011**. 35(5): p. 744-747.

173. Gauthier, M.S.*, et al.*, The metabolically healthy but obese phenotype is associated with lower plasma levels of persistent organic pollutants as compared to the metabolically abnormal obese phenotype. J Clin Endocrinol Metab, **2014**. 99(6): p. E1061-1066.

174. Ozonoff, D., A. Aschengrau, and P. Coogan, Cancer in the vicinity of a Department of Defense superfund site in Massachusetts. Toxicol Ind Health, **1994**. 10(3): p. 119-141.

175. Lyche, J.L.*, et al.*, Natural mixtures of POPs affected body weight gain and induced transcription of genes involved in weight regulation and insulin signaling. Aquat Toxicol, **2011**. 102(3-4): p. 197-204.

176. Lee, D.H.*, et al.*, Low dose organochlorine pesticides and polychlorinated biphenyls predict obesity, dyslipidemia, and insulin resistance among people free of diabetes. PLoS One, **2011**. 6(1): p. e15977.

177. Airaksinen, R.*, et al.*, Association between type 2 diabetes and exposure to persistent organic pollutants. Diabetes Care, **2011**. 34(9): p. 1972-1979.

178. Wilson, P.W.*, et al.*, Prediction of coronary heart disease using risk factor categories. Circulation, **1998**. 97(18): p. 1837-1847.

179. Ruehlmann, D.O.*, et al.*, Environmental estrogenic pollutants induce acute vascular relaxation by inhibiting L-type Ca2+ channels in smooth muscle cells. FASEB J, **1998**. 12(7): p. 613-619.

180. Jiang, Y.*, et al.*, BPA-induced DNA hypermethylation of the master mitochondrial gene PGC-1alpha contributes to cardiomyopathy in male rats. Toxicology, **2015**. 329: p. 21-31.

181. Felty, Q., Proteomic 2D DIGE profiling of human vascular endothelial cells exposed to environmentally relevant concentration of endocrine disruptor PCB153 and physiological concentration of 17beta-estradiol. Cell Biol Toxicol, **2011**. 27(1): p. 49-68.

182. Marmugi, A.*, et al.*, Adverse effects of long-term exposure to bisphenol A during adulthood leading to hyperglycaemia and hypercholesterolemia in mice. Toxicology, **2014**. 325: p. 133-143.

183. Kloting, N. and M. Bluher, Adipocyte dysfunction, inflammation and metabolic syndrome. Rev Endocr Metab Disord, **2014**. 15(4): p. 277-287.

184. Danforth, E., Jr., Failure of adipocyte differentiation causes type II diabetes mellitus? Nat Genet, **2000**. 26(1): p. 13.

185. Chinetti-Gbaguidi, G. and B. Staels, Macrophage polarization in metabolic disorders: functions and regulation. Curr Opin Lipidol, **2011**. 22(5): p. 365-372.

186. Kanda, H.*, et al.*, MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J Clin Invest, **2006**. 116(6): p. 1494-1505.

187. Kamei, N.*, et al.*, Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. J Biol Chem, **2006**. 281(36): p. 26602-26614.

188. Ibrahim, M.M.*, et al.*, Metabolic impacts of high dietary exposure to persistent organic pollutants in mice. Toxicol Lett, **2012**. 215(1): p. 8-15.

189. Hevener, A.L., D.J. Clegg, and F. Mauvais-Jarvis, Impaired estrogen receptor action in the pathogenesis of the metabolic syndrome. Mol Cell Endocrinol, **2015**.

190. Stoger, J.L.*, et al.*, Distribution of macrophage polarization markers in human atherosclerosis. Atherosclerosis, **2012**. 225(2): p. 461-468.

191. Mor, G. and V.M. Abrahams, Potential role of macrophages as immunoregulators of pregnancy. Reprod Biol Endocrinol, **2003**. 1: p. 119.

192. Bolego, C.*, et al.*, Macrophage function and polarization in cardiovascular disease: a role of estrogen signaling? Arterioscler Thromb Vasc Biol, **2013**. 33(6): p. 1127-1134.

193. Tugal, D., X. Liao, and M.K. Jain, Transcriptional control of macrophage polarization. Arterioscler Thromb Vasc Biol, **2013**. 33(6): p. 1135-1144.

194. Ribas, V.*, et al.*, Myeloid-specific estrogen receptor alpha deficiency impairs metabolic homeostasis and accelerates atherosclerotic lesion development. Proc Natl Acad Sci U S A, **2011**. 108(39): p. 16457-16462.

195. Rubin, B.S., Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects. J Steroid Biochem Mol Biol, **2011**. 127(1-2): p. 27-34.

196. Talsness, C.E.*, et al.*, Components of plastic: experimental studies in animals and relevance for human health. Philos Trans R Soc Lond B Biol Sci, **2009**. 364(1526): p. 2079-2096.

197. Silva, M.J.*, et al.*, Urinary levels of seven phthalate metabolites in the U.S. population from the National Health and Nutrition Examination Survey (NHANES) 1999-2000. Environ Health Perspect, **2004**. 112(3): p. 331-338.

198. Vandenberg, L.N.*, et al.*, Human exposure to bisphenol A (BPA). Reprod Toxicol, **2007**. 24(2): p. 139-177.

199. Okdah, Y.A., Effect of diethylhexyl phthalate on proliferation of ED1-testicular macrophages in rats. The Journal of Basic & Applied Zoology, **2013**. 66(4): p. 223-227.

200. Wentworth, J.M.*, et al.*, Pro-inflammatory CD11c+CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity. Diabetes, **2010**. 59(7): p. 1648-1656.

201. Kraakman, M.J.*, et al.*, Macrophage polarization in obesity and type 2 diabetes: weighing down our understanding of macrophage function? Front Immunol, **2014**. 5: p. 470.

202. Arango Duque, G. and A. Descoteaux, Macrophage cytokines: involvement in immunity and infectious diseases. Front Immunol, **2014**. 5: p. 491.

203. Umemura, N.*, et al.*, Tumor-infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics. J Leukoc Biol, **2008**. 83(5): p. 1136-1144.

204. Shaul, M.E.*, et al.*, Dynamic, M2-like remodeling phenotypes of CD11c+ adipose tissue macrophages during high-fat diet--induced obesity in mice. Diabetes, **2010**. 59(5): p. 1171-1181.

205. Gundra, U.M.*, et al.*, Alternatively activated macrophages derived from monocytes and tissue macrophages are phenotypically and functionally distinct. Blood, **2014**. 123(20): p. e110-122.

206. Zeyda, M.*, et al.*, Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. Int J Obes (Lond), **2007**. 31(9): p. 1420-1428.

207. Lee, B.C. and J. Lee, Cellular and molecular players in adipose tissue inflammation in the development of obesity-induced insulin resistance. Biochim Biophys Acta, **2014**. 1842(3): p. 446-462.

208. Yang, X.P. and J.F. Reckelhoff, Estrogen, hormonal replacement therapy and cardiovascular disease. Curr Opin Nephrol Hypertens, **2011**. 20(2): p. 133-138.

209. Schierbeck, L.L.*, et al.*, Effect of hormone replacement therapy on cardiovascular events in recently postmenopausal women: randomised trial. BMJ, **2012**. 345: p. e6409.

210. Pfeilschifter, J.*, et al.*, Changes in proinflammatory cytokine activity after menopause. Endocr Rev, **2002**. 23(1): p. 90-119.

211. Gregory, M.S.*, et al.*, Estrogen mediates the sex difference in post-burn immunosuppression. J Endocrinol, **2000**. 164(2): p. 129-138.

212. Esser, N.*, et al.*, Obesity phenotype is related to NLRP3 inflammasome activity and immunological profile of visceral adipose tissue. Diabetologia, **2013**. 56(11): p. 2487-2497.

213. Safe, S. and K. Kim, Non-classical genomic estrogen receptor (ER)/specificity protein and ER/activating protein-1 signaling pathways. J Mol Endocrinol, **2008**. 41(5): p. 263-275.

214. Hammes, S.R. and E.R. Levin, Extranuclear steroid receptors: nature and actions. Endocr Rev, **2007**. 28(7): p. 726-741.

215. Mauvais-Jarvis, F., Estrogen sulfotransferase: intracrinology meets metabolic diseases. Diabetes, **2012**. 61(6): p. 1353-1354.

216. Watson, C.S.*, et al.*, Signaling from the membrane via membrane estrogen receptor-alpha: estrogens, xenoestrogens, and phytoestrogens. Steroids, **2005**. 70(5-7): p. 364-371.

217. Levin, E.R., Extranuclear estrogen receptor's roles in physiology: lessons from mouse models. Am J Physiol Endocrinol Metab, **2014**. 307(2): p. E133-140.

218. Kortenkamp, A., Low dose mixture effects of endocrine disrupters: implications for risk assessment and epidemiology. Int J Androl, **2008**. 31(2): p. 233-240.

219. Regnier, S.M. and R.M. Sargis, Adipocytes under assault: environmental disruption of adipose physiology. Biochim Biophys Acta, **2014**. 1842(3): p. 520-533.

220. Vandenberg, L.N., *et al.*, Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr Rev, **2012**. 33(3): p. 378-455.

221. WHO/UNEP, State of the science of Endocrine Disrupting Chemicals 2012, in An assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Programme and World Health Organization. **2013**: Geneva.

ACKNOWLEDGEMENTS/AGRADECIMENTOS

Eis um dos capítulos mais difíceis de escrever. Não tenho referências bibliográficas que sustentem o que sinto, nem resultado estatístico que revele o que pretendo redigir. Na certeza que irei involuntariamente esquecer uma ou outra pessoa, gostaria apenas de explanar aqui o meu profundo e sincero agradecimento.

Aos meus pais, pelo apoio constante. Foi deles que me construi e é também deles este trabalho.

À minha irmã Daniela, pela presença e partilha indubitáveis. Pelo caminho impar e paralelo que sempre trilhamos, em mútuo e constante apoio e companheirismo.

Às Professoras Doutoras Rosário Monteiro e Conceição Calhau, pela amizade e clarificação do caminho a percorrer, pelo rigor científico, pela análise sábia e sabedora, pelos conselhos, pela segurança e tranquilidade que sempre me transmitiram em cada momento desta longa caminhada.

À Professora Doutora Raquel Soares, Diretora do Departamento de Bioquímica, o meu muito obrigada pelo acolhimento e pela possibilidade de concretizar este trabalho.

À Professora Doutora Filipa Carvalho, enquanto Diretora do Programa de Doutoramento em Biomedicina, pela oportunidade de frequentar este programa doutoral, o qual contribuiu para o enriquecimento doa minha formação académica e científica.

À Ana e ao Diogo, pela amizade, pelos sorrisos e palavras de incentivo e por toda a partilha de um inexorável saber, fatores que contribuíram de forma determinante para a concretização deste nosso resultado final.

À Cláudia, à Manuela e à Sónia, pela amizade, pela paciência, pela ajuda e companheirismo.

A todos os meus colegas e amigos do Departamento de Bioquímica da Faculdade de Medicina, pela enriquecedora e profícua aprendizagem e por me proporcionarem um simpático e acolhedor ambiente de trabalho.

À Professora Doutora Cristina Santos, à Professora Doutora Valentina Domingues, à Professora Doutora Paula Freitas, ao Professor Doutor Fernando Araújo, à Drª Cristina Neves, ao Gil Faria e à Luísa Correia-Sá, agradeço pela excelente possibilidade de colaboração, pelo incondicional apoio e disponibilidade nesta jornada interdisciplinar.

To Dr. Anne Bouloumié I would like to thank the possibility of working in her group and the way I have been received there. Working at her lab was really a great experience. I also would like to thank to Professor Pierre Gourdy the opportunity of working in his group. I've really learned a lot with you.

To Professor Max Lafontan I must thank all the good lessons about adipose tissue physiology and science in general.

To all of my friends at the lab in Toulouse - Alexia Zakaroff-Girard, Coralie Sengenès, David Esteve, Elodie Riant, Jean Galitzky, Karrine Lolmède, Marie-Laure Renoud, Nathalie Boulet, Pauline Decaunes, Ludovic Breasson – thank you very much for the sharing of knowledge and for everything.

À Maria João, simplesmente pela sua invulgar amizade. Pelas palavras certas e pelo encorajamento em todos os momentos cruciais desta caminhada.

Agradeço a todos os meus amigos, em especial à Raquel, à Daniela, à Ângela, ao Filipe e ao Ivo por todo o apoio e amizade. O vosso carinho é a maior motivação para tudo o que faço!

Por último, queria também agradecer a todas as pessoas que apesar de não serem referidas, contribuíram de forma determinante para a concretização desta tese, estimulando-me intelectual e emocionalmente.