UNIVERSITÀ DEGLI STUDI DI MILANO

DIPARTIMENTO DI SCIENZE FARMACOLOGICHE E BIOMOLECOLARI

DOTTORATO DI RICERCA IN SCIENZE FARMACOLOGICHE

XXVIII CICLO

OPTIMIZATION OF THE EXPERIMENTAL SETTINGS FOR THE STUDY OF ESTROGEN ACTION IN INFLAMMATION

BIO/14

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A.A. 2015-2016

ENGLISH ABSTRACT

Estrogen hormones, binding to estrogen receptors (ERs), influence a wide variety of cell types including those of the immune system. When estrogen production ceases, women may suffer of pathologies that are all associated with a strong and disregulated inflammatory response, such as osteoporosis, atherosclerosis and neurodegenerative diseases. Inflammation is mainly driven by macrophages that are able to sense any microenvironment signals and undergo a metabolic and phenotypic adaptations that allow them to remove the insult, repair and resolve tissue damage. Several reports have showed that 17b-estradiol regulates the inflammatory response and restoration the tissue homeostasis through a direct modulation of macrophages. Neverthless, current knowledge on E2 action in macrophages and the identity of estrogen target genes appears limitated since is based on experimental models of inflammatory condition. With the aim to identify the identity of all genes and cellular pathways that are involved in the homeostatic regulation of macrophages in response to estrogen alone, a genome wide-gene expression study of peritoneal macrophages of mice treated *in vivo* with E2 was performed. In particular, we first identified the most faithful macrophage model to perform such study, represented by macrophages isolated from the peritoneum of female mice treated with estrogen *in vivo*. Successively, we performed a gene expression analysis of the response macrophages to estrogen stimulus alone. In order to maintain physiological conditions, we treated mice with physiological concentration of 17b-estradiol (5μg/kg) *in vivo* for 3 or 24 hours and their peritoneal macrophages were then analyzed by gene expression. The experimental groups were chosen according to the endogenous estrogen content. We used: Groups 1-Metaestrous (ME) with lowest levels of estrogen; 2- Estrous (E), that represents the closest phase in proximity to endogenous increase of estrogen that occurs in Proestrous; 3-Metaestrous treated with a SC injection for 3h with 17β-estradiol (ME+3hE_2); and 4- Metaestrous treated with a SC injection for 24h with 17β-estradiol (ME+24hE_2);. Magnetic beads pre-loaded with antibodies against CD11b were used to isolated peritoneal macrophages. Successively, we obtained a list of estrogen target genes, which appear differential regulated among the four hormonal conditions analyzed. In addition, by performing bioinformatic and bibliometric analyses, we obtained indications of functional pathways regulated by estrogen in peritoneal macrophages. Results lead to the identification of possible estrogen target genes in macrophages, that can be grouped in early, late and persistently regulated genes, and can be considered as direct targets of estrogen action in macrophages. More studies on the molecular details of estrogen action in macrophages will shed more light on the biological role of these hormones *in vivo* and the identification of novel therapies and therapeutics targets. The knowledge of new mechanisms by which estrogen regulates the activity of macrophages will be useful to start understanding the physiologic role of this interplay, possibly expanding these information to pathologic inflammatory conditions in which estrogen is also involved, such as in endometriosis, uterine tumors, infertility and reproductive pathologies.

ABSTRACT IN LINGUA ITALIANA

Gli estrogeni, legandosi ai propri recettori estrogenici (ERs), influenzano un'enorme varietà di cellule tra cui quelle del sistema immunitario. Quando la produzione di estrogeni cessa, alcune donne possono manifestare patologie come l'osteoporosi, l'aterosclerosi e le malattie neurodegenerative, tutte caratterizzate da una forte e incontrollata risposta infiammatoria. L'infiammazione è per lo più guidata dai macrofagi, che sono in grado di captare ogni segnale dal microambiente in cui risiedono e di andare in contro a modificazioni fenotipiche e metaboliche che gli permettono di rimuovere gli insulti e di riparare e risolvere il danno tissutale. Esistono diverse evidenze che dimostrano come il 17β-estradiolo sia in grado di regolare la risposta infiammatoria e di ripristinare l'omeostasi tissutale attraverso la modulazione diretta dei macrofagi. Tuttavia, attualmente la conoscenza del meccanismo di azione dell'estrogeno sui macrofagi e la precisa identità dei geni target degli estrogeni appare limitata, dal momento che gli studi a riguardo si basano su modelli sperimentali di condizioni infiammatorie. Con l'obiettivo di identificare tutti i geni e i pathways cellulari coinvolti nella regolazione dell'omeostasi del macrofago in risposta all'estrogeno senza nessun altro stimolo, è stato condotto uno studio dell'espressione genica estesa a tutto il genoma (genome wide-gene expression study) su cellule macrofagiche peritoneali isolate da topi trattati *in vivo* con estrogeno. Inizialmente abbiamo identificato nel macrofago isolato dal peritoneo di topi femmina trattati con estrogeno *in vivo*, il modello cellulare macrofagico più fedele alle condizioni fisiologiche. Successivamente abbiamo condotto l'analisi di espressione genica della risposta macrofagica all'estrogeno. Con l'obiettivo di mantenere il più possibile le condizioni fisiologiche, abbiamo trattato i topi con concentrazioni fisiologiche di 17β-estradiolo (5μg/kg) *in vivo* per 3 o 24 ore e sui loro macrofagi peritoneali è stata condotta l'analisi di espressione genica. I gruppi sperimentali sono stati scelti in relazione al contenuto estrogenico endogeno. In particolare, abbiamo utilizzato: Gruppo 1- femmine in Metaestro (ME), con i più bassi livelli di estrogeni nel sangue; Gruppo 2- femmine in Estro (E), che rappresenta la fase del ciclo estrale temporalmente più prossima all'incremento fisiologico di estrogeni endogeni che si verifica durante la fase di Proestro immediatmente precedente; Gruppo 3- femmine in Metaestro trattate per 3 ore con un'iniezione sottocutanea di 17β-estradiolo (ME+3hE_2); Gruppo 4- femmine in Metaestro trattate per 24 ore con un'iniezione sottocutanea di 17β-estradiolo (ME+24hE_2). Allo scopo di isolare i macrofagi peritoneali, sono state utilizzate biglie magnetiche pre-caricate con anticorpi contro la proteina CD11b. Successivamente, abbiamo ottenuto una lista di geni target dell'estrogeno, i quali appaiono differenzialmente regolati nelle quattro diverse condizioni ormonali analizzate. Attraverso analisi bioinformatiche e bibliometriche, abbiamo ottenuto delle indicazioni sui possibili pathways funzionali regolati dall'estrogeno nei macrofagi peritoneali. I risultati di queste analisi hanno portato all'identificazione di possibili geni target dell'estrogeno nei macrofagi, che possono essere suddivisi in geni precocemente, tardivamente e persistentemente regolati, e che

possono essere considerati come target diretti dell'azione degli estrogeni nei macrofagi. Ulteriori studi sui dettagli molecolari dell'azione degli estrogeni nei macrofagi potranno far luce sul ruolo biologico di questi ormoni *in vivo* e potranno portare all'identificazione di nuove terapie e target terapeutici. La conoscenza di nuovi meccanismi attraverso i quali l'estrogeno regola l'attività dei macrofagi è utile per iniziare a capire il ruolo fisiologico del legame tra estrogeno e macrofagi, possibilmente espandendo queste informazioni alle condizioni patologiche infiammatorie in cui è noto il coinvolgimento dell'estrogeno, come l'endometriosi, i tumori dell'utero, l'infertilità e le patologie del tratto riproduttivo.

Index

1. Introduction

1.1 Macrophage biology

Macrophages are heterogeneous and versatile population of innate myeloid cells. They are highly plastic cells that sense any microenvironment signals and undergo a phenotypic adaptation that allow them to remove the insult, repair and resolve tissue damage. As professional phagocytes, macrophages are able to internalize and dispose cells and microbes, apoptotic cells or senescent erythrocytes as well as extracellular material including lipids and metal ions.

1.1.1 Origins and renewal. Historically, macrophages were considered to derive from hematopoietic stem cells (HSCs) via bone marrow progenitors and circulating blood monocytes intermediates (Fan et al., 2016), (van Furth and Cohn, 1968). However, recent evidences showed that macrophages in adult tissues may have dual origins, either from hematopoietic stem cells in the bone marrow that give rise to progenitors and circulating blood monocytes intermediates, or from embryonic progenitors that migrated from the primitive yolk sac or the fetal liver and that can self-replenish and differentiate during the adult life (Sieweke and Allen, 2013), (Hoeffel et al., 2015). The major part of macrophage populations is established prior to birth (Leid et al., 2016) (Yona et al., 2013), (Schulz et al., 2012), when a first wave of hematopoiesis consists of yolk sac-derived macrophages that migrate and reside within a subset of developing organs including the brain, dermis, and heart. Within the brain, yolk sac-derived macrophages persist and differentiate into microglia throughout life, independent of blood monocyte input. In the embryonic liver, hematopoiesis precursors provide fetal monocytes that differentiate into most of the other tissues, such as alveolar space and liver (Hoeffel et al., 2015; Leid et al., 2016); (Epelman et al., 2014); (Gomez Perdiguero et al., 2013). On the contrary, in dermis and gut tissues macrophages are renewed by adult HSC-derived monocytes (Bain et al., 2014), (Tamoutounour et al., 2013) while in spleen, kidney, and pancreas macrophages with dual origins coexist as essential components of tissue functions (Epelman et al., 2014); (Boiers et al., 2013); (Ginhoux et al., 2010); (Hoeffel et al., 2012); (Schulz et al., 2012); (Yona et al., 2013); (Lavin et al., 2014).

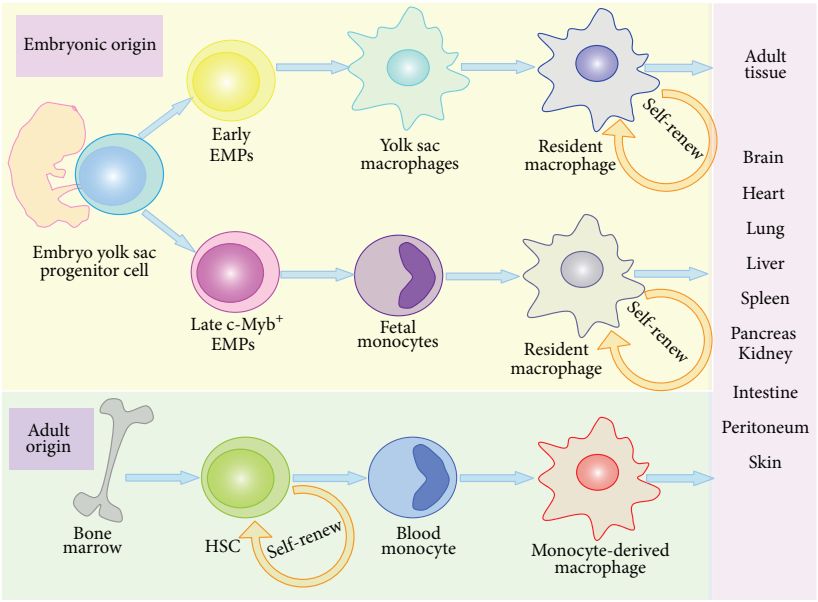

Figure 1A. Origin and self-renewal of macrophage. Recent evidences showed that macrophages in adult tissues may have dual origins, either from hematopoietic stem cells (HSCs) in the bone marrow that give rise to progenitors and circulating blood monocytes intermediates, or from embryonic progenitors that migrated from the primitive yolk sac or the fetal liver and that can self-replenish and differentiate during the adult life (from Fan et al., 2016).

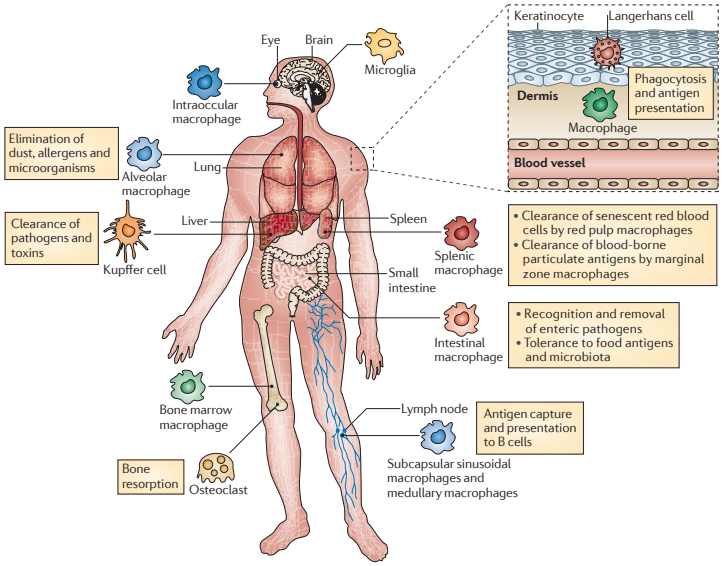

Figure 1B. Tissue macrophages perform important homeostatic functions. Mononuclear phagocytes are generated from committed haematopoietic stem cells located in the bone marrow. Macrophage precursors are released into the circulation as monocytes and quickly migrate into nearly all tissues of the body, where they differentiate into mature macrophages. Various populations of mature tissue macrophages are strategically located throughout the body and perform important immune surveillance activities, including phagocytosis, antigen presentation and immune suppression (from Murray and Winn, 2011).

1.1.2 Role in immunity. Macrophage subpopulations with different functions have been recognized and have initially been classified as two main phenotypes, known as classically activated/inflammatory (M1) and alternatively activated/regenerative (M2) states. These represent the two extremes of a spectrum of intermediate phenotypes that are undertaken by macrophages during activation. In fact, macrophage activation is influenced by a variety of cytokines and microbial products (Toniolo et al., 2015); (Murray and Wynn, 2011).

Macrophage are activated by lipopolysaccharide (LPS) and TH1 cytokines such as IFNγ and TNFα to assume a classically proinfiammatory M1 phenotype which leads to the production of high levels of reactive oxygen and nitrogen species, proinflammatory cytokines (TNF, IL-6 and IL-1), immune mediators such as IL-12 and IL-23. The interaction with bacterial and Th1 cytokines turn macrophages into strong microbicidal killer cells that ensure the inflammatory response (Verreck et al., 2004); (Duffield, 2003; Mosser and Zhang, 2008).

The M1 macrophage action is neutralized by alternatively activated M2 macrophages that become into anti-inflammatory cells in response to TH2 cytokines. M2 macrophages can be further divided into M2a, M2b and M2c major subclasses. IL-4 and IL-13 promote M2a macrophage activation, which are able to release matrix-remodelling cytokines and increase the expression of CD200R and CD86, membrane proteins involved in the recognition of the glycoproteins on the surfaces of myeloid lineage cells such as the NK cells. The M2b subtype is driven by immune complexes in combination with IL-1β or LPS, the function of this phenotype being associated with the immunoregulation of the process. Finally, the induction of the M2c phenotype, resulting from IL-10, TGF-β or glucocorticoids action, leads to the secretion of IL-10 and matrix remodelling factors, such as matrix metalloproteinases (MMPs), and the increased espression of CD163.

As previously mentioned, M1 and M2 classification is a simplified scheme of a much more complex and intricate spectrum of phenotypes (Mosser and Zhang, 2008).

M1 and M2 polarization states are related to the activaty of distinct transcription factors; M1 stimuli activate the signal trasducer and activator of transcription-1, interferon regulatory factor-5, and NF-κB, meanwhile M2 state is regulated by signal transducer and activator of transcription-6, interferon regulatory factor-4, and peroxisome proliferator–activated receptor (PPAR)-γ. These factors act on different categories of the available enhancers.

1.1.3 Activating stimuli: immune and stress signals. Macrophages perform a vast array of homeostatic functions, including phagocytosis, tissue repair, lipid and iron metabolism, processing and presentation of antigens, killing of microbes and cytokines production (Adams and Hamilton, 1984). As professional phagocytes, macrophages express over one hundred distinct surface receptors, such as those for complement components or immunoglobulins, along with major histocompatibility complex class I and class II. Thus, macrophages form effective contacts with pathogens, surrounding cellular components and matrix and other immune cells, forming a crucial bridge between innate and adaptive immunity (Adams and Hamilton, 1984).

The activation of innate immunity is mediated through the recognition of distinct molecules that are present on a broad diversity of microorganisms. These pathogen-associated molecule patterns (PAMPs) are recognized by Toll-like receptors (TLRs) that are expressed by macrophages. Lipopolysaccharide (LPS), a component of the Gram-negative bacterial cell wall, binds to its receptor TLR-4 and activates signaling cascades that result in the production of a vast array of effector molecules (Murphy et al., 2009); (Takeda and Akira, 2005). Indeed, macrophages secrete a wide range of biological products such as lysozymes, proteases, lipases, proteases and phospholipase inhibitors, complement components, coagulation factors, adhesion and binding proteins, reactive oxygen and nitrogen intermediates, superoxide and hydrogen peroxide, nitric oxide and nitrites, prostaglandin E, thromboxane, leukotrienes, and several cytokines such as tumor necrosis factor-α, interleukin-1 and transforming growth factor-β (Adams and Hamilton, 1984).

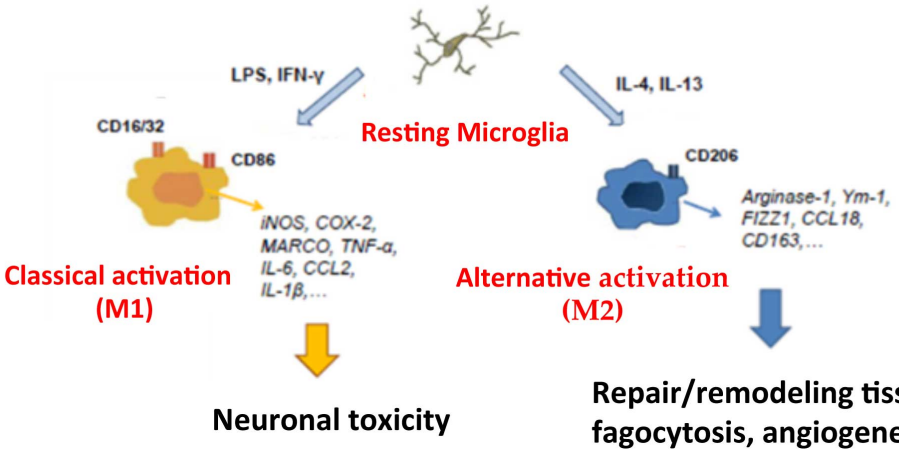

Figure 1C. Macrophage subpopulations. Macrophage subpopulations with different functions have been recognized and have initially been classified as two main phenotypes, known as classically activated/inflammatory (M1) and alternatively activated/regenerative (M2) states. These represent the two extremes of a spectrum of intermediate phenotypes that are undertaken by macrophages during activation.

1.1.4 Macrophages in tissue homeostasis and disease.

Beyond immune protection, macrophages are also involved in physiological reactions that take place in the absence of invading microrganisms or dangerous stimuli. I will briefly summarize some of the major physiopathological roles of macrophages; for some of these an overlapping function of estrogen has also been reported.

Reproductive tissues. Perhaps one of most evident example of tissue remodeling is the one that occurs in reproductive tissues in females, such as the ovarian follicles and uterus. In the ovary, fluctuating numbers of macrophages are present at various stages of the menstrual cycle. During the entire process of oocyte development, macrophages are confined to the theca layer of the growing follicle and their number increases, probably as consequence of CSF-1 action, reaching the highest value just before the ovulation (Thiruchelvam et al., 2013). The action of macrophages in the ovary appears to include the phagocytosis of apoptotic cellular debris and the secretion of cytokines, growth factors and matrix remodeling factors that influence granulosa cells proliferation and follicular growth, and proceeds through ovulation, luteal genesis and regression. In fact, ovulation is comparable to an inflammatory reaction, sharing characteristics of edema, vasodilation, heat and pain. The contribution of macrophages has also been described in the endometrium, where the density of macrophages fluctuates also under the influence of the endocrine milieu, with cell number being lowest at diestrous and increasing at proestrous. Again, it appears that CSF-1 produced by stromal epithelial cells in response to female sex steroid hormones regulates macrophage recruitment and accumulation in uterine stroma. The exact function of uterine macrophages is unknown; it seems to involve phagocytosis, antigen presentation and bactericidal activity which allow pre-implantation embryo growth and implantation as well as clearance of endometrial tissue debris and tissue regeneration during the menstrual cycle, to re-establish tissue integrity and fertility (Thiruchelvam et al., 2013). Although the characterization of the macrophages that populate the walls and fluid of the oviducts has being scarcely investigated, it may be of great clinical to more deeply understand the role of these innate immune cells in this particular tissue considering that the oviductal fluid is a principal factor in tubal functions related with signaling and coating of the fertilized egg and preventing its ectopic tubal implantation or infection by pathogens. Dysregulation of innate immunity at this level may thus create an unsuitable environment that results in infertility. In summary, it appears that macrophages are key players in reproductive tissue homeostasis; however, several aspects of macrophage biology in this system are still indefined, such as the molecular and cellular mechanisms of cell proliferation, the role of immune polarized phenotypes and the consequences of cell activation in response to estrogen on the physiology of reproduction.

***Atherosclerosis*.** Atherosclerosis has been associated with macrophage activity since this pathological condition is both a lipid disorder and an inflammatory disease (Murray and Wynn, 2011); (Woollard and Geissmann, 2010). In particular, macrophages that reside in the intima and subintima of arteries and a particularly form of activated macrophages, called

foam cells, have been shown to participate in the formation of atherosclerotic plaques (Li and Glass, 2002). By contrast, studies have shown that TH2 associated cytokines such as IL10, can block the formation of M1 macrophages in atherosclerotic plaques, demonstrating their protective role. Nevertheless, since cholesterol and other dangerous lipids in the blood are phagocytosed through the scavenging ability of macrophages, these cells may also have a protective role in atherosclerosis (Acton et al., 1996). There is a well-documented evidence for an inverse correlation between plasma estrogen levels and the incidence of cardiovascular disease, which is related to the ability of estrogen to inhibit atherosclerosis (Nofer, 2012). The involvement of macrophages in estrogen action has been reconciled with the favorable regulation in cellular cholesterol discharge, modified LDL uptake and CD36 expression, as further specified in section 1.4.1.

***Tumor*.** Different macrophage immunophenotypes have either protective or pathogenic roles. In fact, M1 macrophages play a protective role in tumorigenesis since they are able to secrete highly toxic molecules that kill tumor cells; further on, they antagonize the negative actions of M2 macrophages and TAMs (tumor associated macrophages) and trigger TH1 responses that help in antitumor response (Murray and Wynn, 2011);(Biswas and Mantovani, 2010).
Conversely, TAMs have an immunosuppressive M2-like phenotype; M2-macrophages foster matrix-remodeling and angiogenetic processes hence favoring cancer progression. The increase in the number of TAMs can contribute to the progression of the tumors and correlates with inauspicious prognosis. Also IL4 and IL13, which promote the differentiation in M2 macrophages, have been shown to have tumor-promoting activities.
Moreover, soluble factors released by cancer cells are able to modulate macrophage differentiation towards an M2c-like phenotype, with increased production of IL-6, IL-8, IL-10 and MMP-9 (Sousa et al., 2015). Duluc and colleagues have shown that INFγ reverses the activities pro-tumoral and immunosuppressive of TAMs, thus its local administration could suppress their cancerogenic activities and induce the protective M1 conversion of macrophages (Murray and Wynn, 2011); (Duluc et al., 2009). Fong and coworkers discovered that the arrest of NFkB pathway is able to promote the switch of TAMs in cells with M1-like phenotype, with antitumor activities (Flynn, 1986; Fong et al., 2008).

Autoimmune diseases. Similarly to other diseases, also in the variety of autoimmune pathologies macrophages have both protective and pathogenic roles. Some cytokines produced by M1 macrophages, such as TNF, IL18, IL12 and IL23 have been shown promote inflammatory and autoimmune diseases, such as Crohn's disease, rheumatoid arthritis and multiple sclerosis (Murray and Wynn, 2011); (Murphy et al., 2003); (Smith et al., 2009); (Platt et al., 2010); (Kamada et al., 2009). Kawane and colleagues showed that TNF, which is secreted by M1 macrophages, leads to the progression of chronic polyarthritis by acting on synovial cells in rheumatoid arthritis (Murray and Wynn, 2011); (Kawane et al., 2006).
However, Smith and colleagues demonstrated that reduced expression of proinflammatory cytokine by macrophages can decrease the ability of macrophages to eliminate potentially

dangerous commensal bacteria from bowel, thus promoting the progression of Crohn's disease (Smith et al., 2009); similarly, Gelderman and colleagues demonstrated that reactive oxygen species produced by macrophages protect mice from arthritis through T cells activation (Gelderman et al., 2007). In multiple sclerosis, a demyelinating diseases of the central nervous system, and in the animal model of this disease (experimental autoimmune encephalomyelitis), M1 macrophages were shown to contribute to axonal loss (Hendriks et al., 2005). Accordingly, Kiefer and colleagues demonstrated that macrophages can promote the apoptosis of T cells and express the anti-inflammatory cytokines TGFβ1 and IL10, helping the resolution of inflammation and suggesting a protective role in multiple sclerosis (Kiefer et al., 2001).

***Allergy*.** Although several studies have indicated the role of M2 macrophages in allergic reaction driven by IL4 and IL13 (Murray and Wynn, 2011); (Prasse et al., 2007), their contribute in allergy and asthma is not still clarified, since some studies underline the role of M2 macrophages in promote the allergic inflammation, whereas other studies show their suppressive role. In support of first hypothesis, a study conducted by Kim and colleagues showed that M2 macrophages are necessary for the progression of airway diseases caused by the infection with a murine parainfluenza virus, called Sendai virus, since M2 macrophages secrete IL13 and their depletion attenuated the inflammation in the lung driven by TH2 (Kim et al., 2008). Nevertheless, van Rijt and colleagues have shown that other types of mononuclear phagocytes, the CD11c+ dendritic cells, instead of macrophages, have a role in the development of airway inflammation and cytokine production in the lungs (van Rijt et al., 2005).
Moreover, Bhatia and colleagues proposed a suppressive role for M2 macrophages in asthma and allergy, since they showed that M2 macrophages are able to uptake and remove fungal conidia, thus inhibiting the manifestation of asthma associated with fungal infections (Bhatia et al., 2011).

Metabolic disorders. Concerning metabolic disorders, M2 macrophages regulate metabolic functions such as maintain adipocyte function, insulin sensitivity and glucose tolerance (Murray and Wynn, 2011). Nevertheless, if obesity progresses, the macrophages associated with adipose tissue can switch from the M2 anti-inflammatory state to a M1 phenotype with dangerous proinflammatory abilities (Odegaard and Chawla, 2011); (Vandanmagsar et al., 2011).

1.1 Estrogen action

Estrogen action is mediated by estrogen receptors (ERs), which are members of the intracellular or nuclear receptor superfamily that includes ligand-dependent transcription factors. ERs establish chromatin interactions and, forming complexes with the genetic regulatory elements, can promote epigenetic changes and transcription of target genes.
More recently has been identified GPR30, an estrogen receptor with a different morphological structure respect to the intracellular dimers, and that mediates some effects of estrogen.

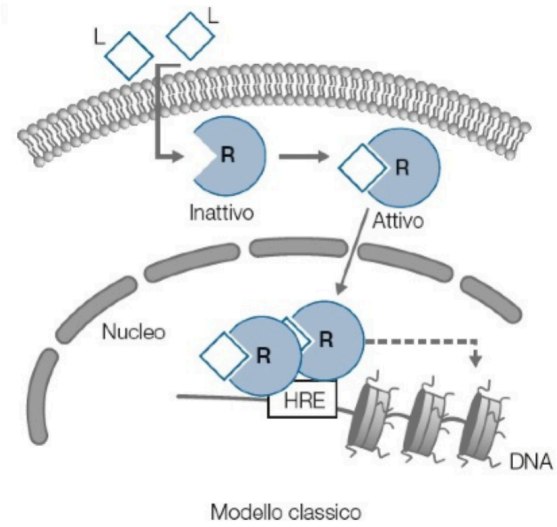

Figura 1D. Ligand-dependent signaling pathway of estrogens. The image taken from "General and molecular farmacology. The molecular mechanism of drugs" Clementi, Fumagalli, "The intracellular receptors" section, Maggi A., Vegeto E., represents the ligand-dipendent signalling pathway of estrogens.

1.2.1 Molecular mechanisms of hormone action (nuclear and membrane receptors)

The structural organization of ERs is highly conserved among species and consists of five homology domains named A/B, C, D, E, and F (from "General and molecular farmacology. The molecular mechanism of drugs" Clementi, Fumagalli, "The intracellular receptors" section, Maggi A., Vegeto E.). The most conserved is the central C domain, which mediates the binding to DNA (DNA binding domain (DBD)) of receptor. Through this domain, the intracellular receptors recognize and bind in a selective manner a short nucleotide sequence, called hormone-responsive element (HRE), which is present in the promoters of target genes. The DBD consists of 66 amino acids containing eight conserved cysteine residues, is a type 2 zinc finger and contains two zinc finger motives. A zinc finger is a DNA binding motif that contains 4 cysteine residues coordinating one zinc ion and that folds into two beta sheets and one alpha-helix structure. When receptor dimerization occours, two DBDs are in close proximity and this allows each monomer to interact with 6 specific nucleotides (half-site) within the HRE DNA sequence. HRE DNA sequence contains two half-sites separated by 1–3 nucleotides. The second zinc finger motif contains a domain, called D-box, which is able to recognize the spacing between the two half-sites of an HRE. The intracellular receptors in fact, can form homodimers or heterodimers. When receptor dimer is formed, it is able to distinguish sequence, spacing and orientation of the half-sites, thereby discriminating between different HRE sequences. The E domain, named ligand-binding domain (LBD), participates in several activities including hormone binding, homo- and/or heterodimerization, interaction with heat shock proteins (HSPs), and transcriptional activation or repression. In this domain, several structural components have been identified: 1) the ligand-binding pocket (LBP), in which the interaction between receptor and lipophilic molecules takes place; 2) the surface of dimerization that allows the association between LBD domains of two monomers thus forming a dimeric receptor; 3) an α-helix motif, called AF-2, that is mainly responsible for the activation of transcription; 4) several sites that mediate the interaction of receptor among HSPs and other inhibitors. Moreover, specific studies have revealed that the E domain contains 12 α-helices (numbered H1–H12). H3, H5, and H6 contribute to delimit LBP hydrophobic cavity by exposing mainly hydrophobic amino acids. The mechanism of activation of the AF-2 functional domain is also very similar within the receptor superfamily and involves the repositioning of H11 and H10 and the opening of H12 because of intramolecular interactions triggered by ligand binding. This affects the receptor affinity for other proteins; the high flexibility of H12 plays a key role in receptor interaction with coactivators and corepressors and thus in receptor activity. The A/B region in N-terminus of the receptor is poorly defined. It is crucial for gene transcription regulation since it contains AF-1, a region that mediates ligand-independent interaction between receptor and transcriptional machinery. In addition, this N-terminal region can interact with cofactors such as coactivators or other transcription factors.

Two isoforms of ERs, ER α and ER β encoded by the ERS1 and ERS2 genes respectively, exist in mammals, and are members of the nuclear receptor superfamily (Kovats, 2015); (Heldring

et al., 2007). They are mainly, but not exclusively, localized in the cellular nucleus of varius types of cells, including macrophages (Ishihara et al., 2015).
The binding with the cognate ligands starts four classes of mechanisms by which ERs are able to regulate the cellular and physiological process of their target cells. First, the dimerization of two single ER chains α and β, to form $\alpha\alpha$, $\beta\beta$ or $\alpha\beta$ dimers, each of which is functionally distinct, leads to the identification and the binding of ERE (estrogen response element) in the promoter of the target genes. Second, upon binding with E2, ERs can create transcriptional interference without directly bind DNA but through the physical interaction with other transcription factors such as NFKB, SP1, AP-1 and C/EBPβ and as conseguence, can interfere with their trascriptional capacity, since thanks to specific consensus sequences, the transcription factors can bind the regulatory elements of promoters.
Third mechanism observed is the interaction between ERs and cytoplasmatic or nuclear molecules involved in cellular signal transduction, such as PI3K, STATs, Src, MAPK and the modification of their pathways (Maggi et al., 2004; Villa et al., 2016).
In addition, direct interaction beetween ERs and coregulators, for example with histone acetyltransferases p300/CBP and SRC1, has been observed and represents the fourth mechanism of the ERs action. This interaction is foundamental for the modulation of target gene trascription since enables the recruitment of general transcription factors to the TATA box of the target genes and promote the histone modification to facilitate RNA polymerase II transcription (Spencer et al., 1997); (Lonard and O'Malley, 2012). On the contrary, the interaction of ERs with corepressors, such as NCOR, leads to the DNA histone deacetylases and repress the gene transcription.
In particular, Liu and Cheung has reported that ERα isoform is able to bind to regulatory elements or enhancers of those elements that are distant from the promoters of the genes, since ERα is implicated in the establishment of chromatin loops that put in contact distal ERα bound elements to the transcription start sequences (Kovats, 2015); (Liu and Cheung, 2014).

Another estrogen receptor called GPR30 has been described to mediate the estrogen action. In particular, GPR30 is a 7-transmembrane G protein coupled receptor family member that could mediate some effects of estrogen. In 2000, Filardo and colleagues demonstrated the MAP kinase (Erk1/2) mediated activation by estrogen in breast cancer cell lines expressing GPR30 but not in cell lines lacking the receptor (Prossnitz et al., 2008); (Filardo et al., 2000). ER antagonists, ICI 182,780, and 4-hydroxy-tamoxifen at high concentrations, are also capable of mediating Erk activation in GPR30-expressing cells. This response proceeded through the involvement of Gi/o heterotrimeric G proteins. A second phase of GPR30-dependent signaling via adenylyl cyclase has been described, and resulted in the eventual attenuation of Erk activation (Filardo et al., 2002). These and other reports suggest that GPR30 may mediate, in part, the regulation of several cellular functions such as the modulation of the gene expression (Maggiolini et al., 2004), the activation of ion channels (Zhang et al., 2010), the cAMP production (Thomas et al., 2005), the activation of kinases (Revankar et al., 2005); (Filardo et al., 2000) and the Ca++ translocation. All in all, these

reports suggest that GPR30 may mediate, in part, several cellular effects including growth, proliferation and apoptosis.

1.2.2 Estrogen action in innate immunity and inflammation.

It is known that immunity to infection and autoimmunity is sexually dimorphic. This difference has been reconciled with the activity of estrogens. Hormonal mileu in fact for example influences the function of B lymphocytes in terms of immunoglobulin production. Women produce more elevated circulating antibodies than men (Rowley and Mackay, 1969) and consequently, higher levels of autoantibodies when affected by autoimmune diseases (Whitacre et al., 1999). Kanda and colleagues have described that estrogens increase IgG and IgM production by B lymphocytes (Kanda and Tamaki, 1999) directly and through a potentiating effect of IL-10 from monocytes, whereas testosterone inhibits IgM and IgG production, both directly and indirectly, by reducing the production of IL-6 by monocytes (Kanda et al., 1996).
In mice, Latham and colleagues showed that in an arthritis model, estrogen treatment leads to the reduction of immunoglobulins, from a more complement-binding isoform IgG1 subtype to a less complement-binding isoform, increasing the IgG2 subtype (Latham et al., 2003), thus diminishing disease severity.

Since several studies conducted in humans have demonstrated that both hematopoietic progenitors and mature immune cells express, although with differences in subtype and percentage, ERs mRNAs or proteins (Kovats, 2015), it is logic to suppose that estrogens are able to module the cells implicated in the immune system. For example, B lymphocytes express the highest level of ESR1 RNA; CD4+ T lymphocytes, CD8+ T lymphocytes, natural killer cells and plasmacytoid dendritic cells express intermediate levels of ERS1, while monocytes show the lowest levels of ESR1 RNA. B lymphocytes and plasmacytoid dentritic cells express at the highest levels the ESR2 RNA while CD4+ T lymphocytes, CD8+ T lymphocytes, natural killer expressed it at low levels.
In mice, mature immune cells express Esr1 and in some cases Esr2. In particular B lymphocytes, T lymphocytes and natural killer cells express Esr1 and ERα (Grimaldi et al., 2002); (Lambert et al., 2004); (Lelu et al., 2011) while only B lymphocytes and natural killer express ERβ protein (Grimaldi et al., 2002); (Curran et al., 2001).
Bone marrow-derived and peritoneal macrophages express Esr1 and little if any Esr2 (Lambert et al., 2004); (Ribas et al., 2011).

Several authors have described that human monocytes, macrophages, lymphocytes, natural killer cells and monocyte-derived dentritic cells alter their functional responses upon exposure to estrogens (Mor et al., 2003); (Escribese et al., 2008); (Seillet et al., 2012).
Nevertheless, when discussing the data using primary cell cultures or cell lines, it is important to keep in mind that process of primary cell isolation and culture growth conditions may deeply change the abundance, the density, the distribution, and type of ERs and the E2 signalling pathways.
Furthermore, the response to estrogen is also strictly dependent on the concentration and duration of exposure used in the experiments.

Regarding monocytes, a study conducted by Rogers and colleagues demonstrates that estrogen inhibits TNFα secretion by monocytes, but once stimulated with LPS, the effect appears stimulatory, thus the stimulatory effect seems to exceed the inhibitory one, leading to an opposing effect (Rogers and Eastell, 2001). The study carried on by Polan and colleagues shows that the IL1 production by monocytes in response to estrogen is biphasic or not univocal because they observed both a progressive inhibition of IL-1 transcription with increasing concentrations of estrogen (Polan et al., 1989), as well as the induction of IL-1 transcription with high concentrations of estrogen in a monocytic cell line (Ruh et al., 1998). Thus estradiol can have opposing effects on monocytes since low doses enhancing proinflammatory cytokine production including IL-1, IL-6, and TNF-α while high concentrations reduced their production (Bouman et al., 2005).

Concerning to macrophages, it has been shown that estradiol and ERs exert either positive or negative regulatory effects on pro-inflammatory cytokine production.
The studies conducted by Bouman and colleagues and by Rettew and colleagues show that estrogen treatment increases the production of TNFα by stimulated macrophages (Bouman et al., 2001); (Kim et al., 2001); (Rettew et al., 2009), while estrogen appeared to have an inhibitory effect on production of pro-inflammatory cytokines in the study conducted by Kramer and colleagues, where they showed that estrogen modulated CD16 expression on human macrophages and through this pathway inhibits the production of IL-1β, IL-6 and TNF (Kramer et al., 2004). A study carried on murine peritoneal macrophages by Flynn and coworker shows that estrogen increase MHCII expression through the upregulation of IL-1 production (Flynn, 1986).

Studies related to the effects of estrogens on the expression of chemokines and cytokine by TH1 lymphocytes, show that the production of interferon-γ is either increased (Giron-Gonzalez et al., 2000) or not affected (Bouman et al., 2004), while the production of IL2 by TH1 lymphocytes as conseguences of estrogen treatment is decreased (Bouman et al., 2004). The TH2 cytokines IL-4 and IL-10 production instead, are showed by several authors that appear unaffected by estrogens in fertile and postmenopausal women (Kamada et al., 2001); (Cioffi et al., 2002).

As several reports have reported, the activity of natural killer cells is influenced by elevated levels of estrogens, since high dosage elicits a suppressive activity (Ferguson and McDonald, 1985), while low dosage displays no effect. Souza and colleagues shown an increased activity of these cells in post-menopausal women and males compared to fertile females in the luteal phase of the cycle, while during the follicular phase these differences were not evident (Souza et al., 2001). Also other authors described a reduction in the activity of natural killer cells in the periovulatory period compared to healthy male volunteers (Sulke et al., 1985). In addition, several reports indicate that exposure to E2 in vitro enhances natural killer cytotoxicity and production of IFN-γ (Hao et al., 2007; Nakaya et al., 2006; Sorachi et al., 1993) and downregulates the secretion of granzyme B and FasL (Hao et al., 2007).

Concerning the effect of estrogen treatment on human monocyte-derived dendritic cells, Bengtsson and colleagues have shown that the exposition of the immature cells to estrogen increased their IL-6, IL-8 and MCP-1 production, and enhanced their stimulatory capacity on T lymphocytes (Bengtsson et al., 2004). Also the study by Paharkova–Vatchkova and colleagues shows the ability of estrogen in enhancing the differentiation of dendritic cells from bone marrow *in vivo*, and to upregulate the MHC II in the dendritic cells (Paharkova-Vatchkova et al., 2004).

A particular unique physiological condition is represented by pregnancy, since corresponds to an extreme challenge for the immune system (Robinson and Klein, 2012). Concentrations of steroid hormones, including estrogens, are significally higher during pregnancy than during other times in the reproductive cycle of female and increase over the course of pregnancy, with highest levels achieved during the third trimester.
The changes in concentrations of several hormones can contribute to the immunological shifts that occours during pregnancy. In fact, from the perspective of immune cellular component of the pregnant female, the fetus is an allograft that contains foreign antigens from the father. Thus, to permit a successful pregnancy, pregnant female's immune responses shifts away from inflammatory responses that may contribute to fetal rejection, toward an anti-inflammatory immune responses that help in passive transfer of antibodies to permit the correct develop of fetus (Raghupathy, 1997). Sexual hormones contribute essentially to this shift in immune function which occurs over the three trimesters of pregnancy.
In particular, successfully, full-term pregnancies in humans are associated with high IL-4 and IL-10 and reduced IL-2 and IFN-γ production by peripheral blood mononuclear cells, and these differences in cytokine production are more pronunced in the third trimester of pregnancy (Marzi et al., 1996). In fact, inflammatory cytokines like IFN-γ and TNF-α, can damage the placenta and developing fetus either directly or by activating cytotoxic cells, including natural killer cells or T lymphocytes (Raghupathy, 1997). Moreover, during pregnancy has been extensively reported that uterine decidual macrophage that exhibit an anti-inflammatory polarization (M2) phenotype characterized by arginase activity, scavenger receptor expression, and secretion of IL-1 receptor antagonist (Nagamatsu and Schust, 2010) are more numerous than uterine decidual macrophage that exhibit an inflammatory (M1) phenotype, characterized by pronunced secretion of IL-12 and TNF-α, (Nagamatsu and Schust, 2010).

1.3 Molecular mechanisms of estrogen action in macrophages

1.3.1 Genomic effects

The studies reported below provide evidences for a direct effect of estrogen and its receptors on macrophage gene expression; many of the identified estrogen target genes contain estrogen response element (ERE) in their promoters, further supporting a direct transcriptional activity of estrogen in macrophages.

Ribas and colleagues shows that estrogen-activated ERα induces the expression of macrophage transglutaminase 2 (Tgm2); they also reported the presence of an ERE in Tgm2 gene promoter which appears under the control of estrogen through the transient transfections of reporter plasmids containing parts of the Tgm2 promoter (Ribas et al., 2011). In the same manuscript authors showed that other 2 macrophage genes, ApoE and Abca1, which are known to exert atheroprotective effects and contain an ERE in their promoters, were significantly reduced in murine ERα knockout cells (Ribas et al., 2011). This study allowed the authors to link a defective ERα signaling to the metabolic syndrome and the development of atherosclerotic lesions since Tgm2 is an important enzyme implicated in the regulation of the reverse transport of cholesterol, wound healing, phagocytosis of apoptotic cells and the development of atherosclerotic lesions (Boisvert et al., 2006). Also the study of Wang and colleagues on ApoE expression (Wang et al., 2006) suggested a direct effect of ERs and macrophage DNA transcription, as their results indicated that the activation of ERα up-regulated ApoE mRNA and protein expression while the activation of ERβ had an opposite effect.

The evidence reported are in line with a study conducted several years ago by McLaren and colleagues, which showed that the estrogen stimulation of human primary cultures of macrophages, collected from the peritoneal fluid of women suffering from endometriosis, resulted in the increased secretion of VEGF (McLaren et al., 1996). Considering that also the VEGF gene contains ERE elements in its promoter, estrogen action could also be mediated by an increase in VEGF expression

Moreover, other studies reported the existence of estrogen target genes in macrophages. Vegeto and colleagues showed that E2 decreases the MMP-9 RNA levels and prevents reduction of TLR-4 and CD14 induced by the treatment with LPS of RAW 264.7 macrophagic cell line (Vegeto et al., 2004).

Using peritoneal macrophages, Huang and colleagues observed that E2 inhibits the hydrogen peroxide-stimulated cytokine production of TNFα, IL-1β, macrophage inflammatory protein (MIP)-2, and macrophage chemotactic protein (MCP)-1 (Huang et al., 2008). Moreover,

Mor and colleagues demonstrated that estrogen increased FasL expression in monocytes through the binding of ER to the estrogen recognizing elements and AP-1 motifs, both present at the FasL promoter (Mor et al., 2003).

Most of these studies analysed the effect of estrogen in combination with immune or inflammatory mediators, such as LPS. The effect of estrogen alone on macrophages has

never been studied in a thorough and unbiased detail. This lack of knowledge prompted us to undergo the study described in my thesis.

1.3.2 Molecular mechanisms of estrogen cytoplasmic effects

Estrogen action in macrophages may also involve the direct activation of cytoplasmatic effectors. Pioneering studies demonstrated that exposure to estradiol can induce a rapid increase in intracellular calcium mobility in neuronal cells. This event occurs within milliseconds after the stimulus, too short a time interval to be mediated by gene expression and protein neosynthesis. It has been showed that estrogen could rapidly activate protein kinase C even in the presence of protein synthesis inhibitors. This activity can be explained by the fact that estrogen binds to receptor pools outside the nucleus. This pool is represented by both ERs and GPR30.

ERs. Bruce-Keller and colleagues, for example, shown that E2 is able to activate several MAPK pathways, including the p42/44 MAPK that lead to a reduced production of superoxide anion, which production is caused from LPS stimulation (Bruce-Keller et al., 2000). Using MAPK inhibitor PD 98059, they ensure the involvement of MAPK and not of other effectors. Similarly, Wang and colleagues showed that E2 is able to induce M2 polarization of macrophages resident in the endometrium through the increased phosphorylation of $STAT_3$ and P38 (Wang et al., 2015). The use of $STAT_3$ and P38 inhibitors, but not by ERK1/2 and JNK inhibitors, abrogated the differentiation in M2 of macrophages. Furthermore, Calippe and colleagues reported that E2 inhibited Akt phosphorylation induced by LPS (Calippe et al., 2008), through a negative effect on PI3K activity *in vivo*. p38 and p42/44 MAPK activation were not altered while NF-kB activity, assessed by the measurement of nuclear p65 DNA binding, was enhanced.
This work is in line with that published by Ghisletti and colleagues (Ghisletti et al., 2005). Using primary cultures of microglia and RAW 264.7 macrophage cell line stimulated with LPS, these authors demonstrated that E2 inhibits NF-KB activation by preventing its intracellular transport to the nucleus, thereby inhibiting the production of proinflammatory cytokine; this effect was also ascribed to a rapid activation of PI3K and mediated by ERα. More recently, Villa and colleagues shown that estrogen, through the regulation of the $SOCS_3$ and $STAT_3$ signaling pathways, promotes the progression of the inflammatory state toward the IL-10-dependent “acquired deactivation” phenotype, since shortens the proinflammatory phase induced by LPS (Villa et al., 2015).

***GPR30*.** The membrane-localized GPR30, an atypical G-protein-coupled receptor lacking the seven-transmembrane structure, can rapidly stimulate cyclic nucleotide production, calcium flux, and kinase activation in response to estrogen. The study of Rettew and colleagues shows that estrogen elicits a rapid decrease in cell-surface TLR4 expression through GPR30 in macrophages (Rettew et al., 2010). The study by Mori and coworkers focused on the capacity of G1, a specific agonist of GPR30, to induce cell cycle arrest and thus apoptosis in

endometriosis (Mori et al., 2015). In particular, using endometriotic stromal cells (ESCs) from ovarian endometrioma of patients, authors observed that G1 had suppressive effects on the proliferation of ESCs since the agonist was able to arrest the cells at the G2/M phase of cell cycle, without having cytotoxic effects on ESCs. Following this line of evidence, Pelekanou and colleagues demonstrated that GPR30 physically interacts with ERα36-kDa splice variant, acting as coregulator in the process of inhibition of IL6 inflammatory response induced by LPS and resulting in inhibition of NF-kB transcriptional activity (Pelekanou et al., 2016).

1.4 Estrogen action in macrophages

1.4.1 Biological processes as estrogen targets in macrophages

1.4.1.1 Phagocytosis. Macrophage immune activation, along with morphological and functional changes, consists of in the transformation into completly phagocytic cells (Streit and Kreutzberg, 1988).

Professional phagocytes such as monocytes, macrophages, and neutrophils efficiently phagocytose large particles, microorganisms, cell debris and apoptotic cells (Ghigo et al., 2008). This process is initiated by the interaction of cell surface receptors, such as mannose receptors, Fc receptors and lectin receptors, with their ligands, which are present at the particle surfaces, and leads to particle internalization through an actin-dependent mechanism (Aderem and Underhill, 1999). Macrophages moreover contain several phagocytic receptors that interact with apoptotic cells that include complement receptors, Fc receptors, integrins (avb3, avb5), scavenger receptors (SRA, CD36, CD14, and LOX-1), and the presumptive phosphatidylserine receptor (PSR; (Chung et al., 2007); (Stuart and Ezekowitz, 2005). These receptors interact with their ligands on the surface of the apoptotic cells directly or via making a bridge with proteins. Although macrophages use the same receptors to recognize both apoptotic cells and pathogens (Stuart and Ezekowitz, 2005), an inflammatory response take place when macrophages phagocytose pathogens while macrophage recognition of apoptotic cells triggers an anti-inflammatory response, which is mediated by the release of TGF-β, PAF, PGE2 with simultaneous inhibition of TNF-α, IL-12, IL-1β, and IL-8 (Voll et al., 1997). In particular, during the phagocytosis of apoptotic cells, the production of IL-10 was described to prevent inflammation (Chung et al., 2007). In human, some authors showed that human THP-1 macrophages phagocyted apoptotic CTLL-2 cells, leading to the production of human proinflammatory cytokines, such as IL-8 (Kurosaka et al., 1998). Ghigo and colleagues published a work on the mechanism by which Acanthamoeba polyphaga mimivirus was internalized by macrophages by phagocytosis, showing for the first time that a virus is internalized by macrophages via a mechanism normally used by bacteria and parasites (Ghigo et al., 2008). M2 macrophages, involved in resolution of inflammation, exhibit phagocytosis of apoptotic small lymphocytes and neutrophil granulocytes, while M1 macrophages do not show the same capacity (Laria et al., 2016).

Using primary rat microglia and N9 microglia cell line, Bruce-Keller and colleagues in a pioneering work, show that E2 is able to attenuate LPS-induced phagocytosis, measured as cellular uptake of blue latex beads (Bruce-Keller et al., 2000). In particular, these authors trated N9 cells with increasing doses of 17β-estradiol either 24 hours before or during an 18 hours long exposure to LPS. What they observed was a statistically significant reduction of N9 phagocytotic capacity induced by LPS in relation of a pretreatment with E2, while coadministration of 17β-estradiol with LPS doesn't produce the same result.

In 2006, Vegeto and colleagues showed that E2 inhibits Aβ-induced expression of scavenger receptor-A (SR-A) in RAW 264.7 cells (Vegeto et al., 2006). Macrophage scavenger receptor class A (SRA) is one of the main receptors involved in foam cell formation, mediating the

influx of lipids into the macrophages, thus playing a role in lipoprotein uptake. In addition the SR-A has been shown to be important in the inflammatory response in host defense, cellular activation, adhesion, and cell-cell interaction. Moreover, has been described the involment of SR-A in to cause the onset of cell activation induced by Aβ in microglia because SR-A interacts with Aβ and initates membrane-associated signaling pathways (El Khoury et al., 1996). In the work of Vegeto and colleagues (Vegeto et al., 2006), authors associated the reducted expression of SR-A induced by estrogen with the lower activation of microglia that was found in association with amyloid deposition in APP23 mice, an Alzheimer's disease model, under different estrogenic conditions. This mechanism observed in RAW provides a potential mechanism of the anti-inflammatory action of E2 on microglia in APP23 mice.

Zhang and colleagues shown that E2 is able to inhibite the phagocytic activity against titanium dioxide of alveolar macrophages and RAW 264.7 cells (Zhang et al., 2015).

In particular, these authors analyzed murine alveolar macrophages obtained from mice treated in vivo with high doses of E2, in order to mimic the pregnancy hormonal conditions that are associated with reduced pulmonary defects.

Supporting the role of estrogens in the phagocytic process mediated by macrophages, our group showed that estrogen reduces TLR4 and CD14 expression induced by LPS in RAW macrophage cell line, providing another explanation for hormone-induced reduction of phagocytic ability of macrophages induced by pathogen associated signals (Vegeto et al., 2004).

It is still unknown whether estrogen is able to modify the ability of macrophages to phagocytase apoptotic cells.

1.4.1.2 Immune activation phenotype. Several reports have been published on the role of estrogen on the M1 inflammatory phenotype, providing clear evidences of a key role for this hormone in macrophage response; on the other hand, only recently the effect of estrogen on M2 polarization has been approached.

Refering to the effect of estrogen on M1 polarization, pretreatment with E2 before LPS or PMA has been show to decrease superoxide production in a dose-dipendent manner in primary rat microglia and N9 microglia cell line (Bruce-Keller et al., 2000). In particular, superoxide anion, along with other reactive species of oxigen, is involved in the killing of pathogens by phagocytic macrophages, further sustaining previously mentioned evidence on the attenuation of pathogen phagocytosis induced by estrogen.

In RAW 264.7 estrogen pre-treatment has been showed to limit the LPS-induced expression of MMP9, a proteolytic enzyme that acts in tissue remodelling and facilitates the invasion of monocytic cells (Vegeto et al., 2004). To note, MMP9 reduction is mediated by ERα because the use of ER antagonist ICI 182,780 prevented hormone action. Another publication of the same group, showed that E2 is able to block LPS-induced iNOS activity in primary cultures of rat microglia (Vegeto et al., 2000). In particular, the expression of iNOS, the inducible enzyme that catalyzes the production of nitric oxide (NO) from L-arginine, is one of the hall-mark of M1 state, since NO is a free radical and contributes to create an unfavourable environment to infiltrated pathogens.

In agreement with these findings, pretreatment with estrogen 24 h before the stimulation of LPS in N9 microglia cell line resulted in a decrease of LPS-induced superoxide anion production (Bruce-Keller et al., 2000).

Take together several studies have shown that macrophage immune activation can be reduced by estrogens (Vegeto et al., 2000); (Vegeto et al., 2004); (Bruce-Keller et al., 2000), particularly, in their superoxide anion release and cytokine production capacities (Bruce-Keller et al., 2001); (Deshpande et al., 1997); (Hayashi et al., 1998).

However, some studies showed different results and pointed to a pro-inflammatory effect of estrogen. Calippe and colleagues in fact reported that chronic administration of E2 in ovx mice results in the increased expression of proinflammatory cytokines IL1β, IL6, IL12p40 and iNOS by resident tyoglicollate-elicited peritoneal macrophages cultivated *ex vivo* and treated with LPS (Calippe et al., 2008). This effect was also in contrast with the results seen by the same authors, since short-term exposure to estrogen decreases the production of proi-inflammatory cytokines in LPS-activated peritoneal macrophages *in vitro*.
Peritoneal macrophages used for this experimental setting are obtained from the peritoneum of mice elicited with tyoglicollate, which induces a sterile form of peritonitis with a massive infiltration of monocytes from the blood. Activated monocytes are distint

from resident macrophages, this could explain the contrasting data observed by these and other authors in relation with *in vitro* or *ex vivo* and short or long term exposure to estrogen.

More recently, evidences of the capacity of estrogens to promote the resolution of inflammation, preventing the progression of inflammatory condition and, at the same time, encouraging the onset of the restoring phase promoting by M2 macrophages, has been provided by the pioneering datas of Routley and colleagues. In a murine model of ovariectomy and incisional wound repair, they showed that the administration of estrogen is able to re-establish the number of alternatively activated M2 macrophages, represented as Ym1 and Fizz1 positive cells (Routley and Ashcroft, 2009). In fact they showed that ovariectomy results in a diminished presence of Ym1 and Fizz1 positive cells in wounds while and in an increased presence of M1-phenotype macrophages. Ym1 and Fizz1 positive cells are able to promote the resolution of the healing, activating angiogenesis and extra cellular matrix deposition. They showed that macrophages are sensible to reduction of steroid hormones, since when estrogens fall, the phenotype founded in damaged sites is the pro-inflammatory one, while when original estrogen levels are re-established, M2 phenotype can be detected.
Consistently with these observations, the work by Campbell and colleagues that used murine BMDM, murine peritoneal macrophages and murine wound macrophages, demostrated that the effects anti-inflammatory of estrogens on cutaneus wound healing are promoted by ERα, which directly promotes alternative polarization (Campbell et al., 2014).
Using monocytes collected from the blood of healty men, Toniolo and colleagues showed that playing on ERα, pretreatment with E2 is able to prevent the effects of LPS/INFγ stimulation on the reduction of M2 markers (CD163/CD206) observed on hMDM compared with cells not treated with E2 (Toniolo et al., 2015).
Moreover, in an *ex vivo* study conducted on macrophages derived from monocytes collected from blood of postmenopausal women, they demonstrated a impaired capacity of macrophages to express M2 markers as conseguence of IL4/IL13 stimulation respect to their capacity to express M1 markers as conseguence of LPS/INFg stimulation.
Like conseguence of this, in postmenopausal women M1/M2 ratio is moved toward the proinflammatory status and this can suggests that the decreased levels of estrogen that occour during postmenopausal can impair the capacity of macrophages to assume an anti-inflammatory phenotype in response to enviromental cues.

Beyond the capacity of estrogens to promote the anti-inflammatory phenotype in macrophages reported by yet mentionated authors, Villa and colleagues have showed that estrogens display also the capacity to shorten the proinflammatory phase induced by LPS and trigger the resolution of inflammation, thus faciliting the progression of the inflammatory phase toward an acquired deactivation status (Villa et al., 2015). In particular, since IL4 has been described as one of the more important component able to mediate the macrophagical switch toward the resolution state of inflammation, authors want to screen the involvement of estrogens in the response to IL4 of RAW264.7, in terms of expression of

arginase 1, a typical marker of M2 phenotype. Results indicate that when cells are stimulated only with IL4 without estrogens, the cells express arginase 1 more rapidly respect when cells are stimulated with both stimuli. Moreover, if cells are incubated with IL4 and E2 the expression of arginase 1 was reduced respect to the expression obtained if cells are treated only with IL4. The same results were obtained also with Chi3l3 or Ym1, other well-described M2 marker and using another macrophagic cell line, J774A.1. These results matched with the idea that estrogen could interfere on IL4 activity.

An important aspect that the study conducted by Wang and colleagues had highlighted is that M2 state of macrophages, in particular conditions, has not a beneficial role since encourages the onset and the progression of pathologic conditions. In fact, the worsening of endometriotic lesions, a phenomena connected to the development of endometriosis and that will be exhaustively discussed forwards, has been described to be strictly in relation whit the excessive presence of M2 macrophages in peritoneum.
In particular, these authors showed that the treatment with E2 (in addition with TCDD, an environmental contaminanants and a member of the dioxin familiy) is able to induce the M2 polarization state of macrophages resident in endometrium (Wang et al., 2015). They observed that in co-culture of U937, a macrophagic cell line, and endometrial stromal cells, E2 and TCDD used togheter had synergistic effects in moved macrophages activation towards M2 phenotype, expressed as increased expression of CD14 and decreased expression of HLA-DR and CD86.

In summary, the recent evidences on the effect of estrogen on M2 polarization show that hormone is able to promotes alternative polarization in macrophages, since when estrogen levels are low (ovariectomy or postmenopausal period) the number of classicaly activated M1 macrophages are higher than the number of alternatively activated M2 macrophages.
Nevertheless, since in some pathological conditions M2 status plays a detrimental role because permits the disease progression, the effects of estrogen on M2 macrophage polarization appear contradictory.

1.4.1.3 Iron homeostasis. Iron is stored in cells by binding to ferritin; upon request it is released into serum or tissue parenchima by ferroportin-1 (Hamad and Awadallah, 2013).
In serum iron is known to be present in three forms: Fe3+ bound to transferrin, heme linked to hemopexin and hemoglobin bound to haptoglobin (Hubler et al., 2015). Macrophages express receptor for these three forms of iron founded in the serum: transferrin receptor is able to bind Fe3+ -transferrin, CD91 is able to bind heme-hemopexin and CD163 is able to bind haptoglobin-hemoglobin. Iron is essential to heme and hemoglobin biosynthesis in erythrocytes (Soares and Hamza, 2016). The majority of iron necessary for this reaction originates from senescent or damaged red blood cells catabolized by phagolysosomes in macrophages of the spleen, bone marrow and liver (Korolnek and Hamza, 2015).
Erytrophagocytic macrophages are able to phagocytose and process dying red blood cells (Korolnek and Hamza, 2015) thanks to the expression of macrophages lineage-specific genetic program that allows heme-iron recycling starting from hemoglobin and prevents the cytotoxic effects (Haldar et al., 2014); (Kohyama et al., 2009).
Heme is released from hemoglobin and translocated from phagolyososomes into the cytoplasm through a mechanism that involves the heme transporter HRG1 (Korolnek and Hamza, 2015).

Subsequently, heme allows induction of heme oxygenase-1 for the heme-catabolism.
The iron produced through is either exported by ferroportin-1 or accumulated in the cell by ferritin. Upon secreted from erytrophagocytic macrophages, the iron is moved to the plasma through transferrin and is internalized by erythroblasts through the transferrin receptor.
Resident macrophages are implicated in iron recycling in several tissues, such as bone marrow, spleen (Kohyama et al., 2009), liver (Shoden and Sturgeon, 1962) and lung (Corhay et al., 1992). Iron efflux through ferroportin-1 is negatively regulated by hepcidin, a peptide hormone secreted by hapatocytes, adipocytes (Hubler et al., 2015); (Bekri et al., 2006) and macrophages (Wu et al., 2012). Hepcidin induces the endocytosis and degradation of ferroportin-1 (Nemeth et al., 2004); in this way, iron absorption from the intestine, release of iron from hepatocytes and macrophages is regulated by hepcidin.
When increased request of iron occours, for example during hypoxia or iron deficency, there is a downregulation of hepcidin synthesis while excessive levels of iron in the serum upregulate it (Hamad and Awadallah, 2013).

Some effects of estrogen on iron metabolism have been described, yet only marginally understood. Hamad and Awadallah reported that high levels of estrogen regulate the iron homeostasis (Hamad and Awadallah, 2013). In fact studies reported that ovariectomy results in reduced serum iron, decreased iron binding capacity and reduced iron response protein-1 binding activity (Hamad and Awadallah, 2013; Mattace Raso et al., 2009). Moreover, increased levels of serum iron and total iron-binding capacity was found in association with the use of oral contraceptives in women (Campesi et al., 2012) or estrogens in ovariectomized mice where treated with (Ulas and Cay, 2011).

Furthermore, the expression of ferroportin-1 was reported to be high in the presence of elevated levels of estrogen (Stuckey et al., 2006), suggesting an increased uptake of iron by cells and reduced plasma levels.

A contradictory study by Qian and colleagues, however shows that the transcription of mRNA and protein of ferroportin was reduced upon E2 treatment in murine bone marrow-derived macrophages (Qian et al., 2015). Importantantly, a functional ERE was identified within the ferroportin promoter leading to hypothesize that the suppressive effect of E2 on ferroportin expression is mediated by ER-ERE binding.

In summary, evidence linking iron homeostasis with estrogen and its effects on macrophages are still scares; however existing evidence point to an increase in iron storage capacity of the organism or macrophages in response to estrogen.

1.4.1.4 Cholesterol metabolism. Macrophages have also a relevant role in cleaning the blood from excess of lipids, particulary cholesterol which leads to the protecting the body from cardiovascular diseases.
In particular, macrophages ingest cholesterol by endocytosis of aggregated and native LDL via the LDL receptor (LDLR), and by uptake of modified lipoproteins through scavenger receptors, such as scavenger receptor class A type 1 (SR-A1) and cluster of differentiation 36 (CD36) (Fernandez-Ruiz et al., 2016); (Steinberg et al., 1989); (Greaves and Gordon, 2009). Cholesteryl esters (CEs) derived from the lipoproteins are hydrolyzed by cholesteryl ester hydrolase or cholesterol esterase (N-CEase), in endosomes/lysosomes to release free cholesterol, which is then distributed to the plasma membrane and peripheral organelles (Liscum and Munn, 1999). Excess free cholesterol is reesterified on the endoplasmic reticulum by acyl CoA:cholesterol acyltransferase (ACAT) (Liscum and Munn, 1999); (Chang et al., 1997) and stored in cytoplasmic lipid droplets. CE in lipid droplets undergo a continual cycle of hydrolysis and reesterification by N-CEase and ACAT, respectively. This lipid-scavenging function of macrophages is initially beneficial, but under conditions of unregulated or increased lipid uptake, it leads to excessive accumulation of CE in macrophages that results in foam cell formation (Chang et al., 1997); (Accad et al., 2000). By reducing excess cellular cholesterol, the cholesterol efflux is critical in preventing foam cell formation. CE hydrolysis to release free cholesterol from the CE stored in lipid droplets is the initial step in cholesterol efflux (Ghosh, 2012). The generated free cholesterol is transported to extracellular acceptors such as ApoA1 or HDL by the ATP-transporters ABCA1 and ABCG1(Kennedy et al., 2005).

It was extensively reported in literature that E2 alters the metabolism of cholesterol in macrophages through two principal mechanisms: 1- reduces the occupancy or the proteins levels of scavenger receptors (SR-A1 and CD36) and subsequently the internalization of cholesterol; 2- increases the efflux of cholesterol by enhancing the cholesteryl ester cycle. Since the reduction in macrophage CE content by E2 could lead to the inhibition of foam cell formation, through the mitigation of foam cell formation, E2 may reduce the progression of coronary heart disease.

Using human peripheral blood monocyte-derived macrophage cells (HMDMs) or the human monocyte/macrophage cell line THP-1, several authors reported that estrogen reduce the cholesteryl ester content. In particular, McCrohon and colleagues showed that the estrogen reduces scavenger receptor occupancy and therefore subsequent internalization of ligand-receptor complexes. This effect was independent of the classic estrogen receptor because was not abrogated by the use of the estrogen receptor antagonist ICI 182780 or reproduced by estrogen receptor agonist diethylstilbestrol cell (McCrohon et al., 1999).
Conversely, the study conducted by Allred and colleagues defines the role that ERα in regulating proteins involved in lipid metabolism in macrophages (Allred et al., 2006), since in the absence of ERα the atherosclerotic lesions size following the HIV protease inhibitor insult

ritonavir are larger. In particular, one possibile mechanism by which E2 modulates the effects of the treatment with ritonavir, could be by preventing the activation of protein kinase C pathway.

Also the work of Corcoran showed the E2-related CE reduction is dependent upon ER activation (Corcoran et al., 2011). Notably, they observed that CE reduction is more evident in those cells that are not exposed to oxLDL, demonstrating that a pro-atherogenic lipoprotein milieu can influence the estrogen modulation of coronary heart disease.

Also the work of Shchelkunova and colleagues support the role of estrogen in protecting from atherosclerosis since they observed that the addition of estrogen at physiological concentration reduces the LDL-induced cholesterol accumulation in HMDMs (Shchelkunova et al., 2013).

Using the human monocyte/macrophage cell line THP-1, Wilson and colleagues showed a reduction mediated by E2 of cholesteryl ester accumulation in response to ritonavir. In particular, E2 provokes a reduction of CD36 proteins levels, while CD36 expression appears unaffected (Wilson et al., 2008).

Napolitano and colleagues observed that E2 reduces the accumulation of cholesterol in macrophages increasing the reverse cholesterol transport by enhancing the cholesteryl ester cycle that generates intracellular unesterified cholesterol, more prone to be excrete from cell (Napolitano et al., 2001).

Congruently, using the J774 A.1 cells, also the work of Tomita and colleagues reported an enhancement of N-CEase activity by estrogen (Tomita et al., 1996).

1.4.1.5 Extracellular matrix and intercellular communication. The capacity that macrophages have to secreting several types of chemical mediators is crucial to biological crosstalk between cells and tissues; this physiological (or in some cases pathological) activity regulates processes such as extracellular matrix formation and remodelling, angiogenesis, wound healing and activation of specific immune response upon antigen presentation. A large body of work have showed that estrogens can regulate the expression and secretion of these different chemical mediators in macrophages.

The extracellular matrix is the non-cellular component that resides within all tissues and organs, and provides not only essential physical scaffolding for the cellular components but also initiates important biochemical and biomechanical processes that are required for tissue homeostasis, morphogenesis and differentiation (Frantz et al., 2010). It is composed of water, proteins and polysaccharides but each tissue has an specific composition and topology of extracellular matrix, which is generated during the development of tissue through crucial dialogue between numerous cellular components (including epithelial, fibroblast, adipocyte, endothelial cells) and the evolving cellular and protein of the microenvironment. Together with the cross talk with other cellular elements, in particular conditions and stimulated with estrogen, macrophages have been described to have a role in the processes that lead to wound healing of the extracellular matrix.

Liu and colleagues, showed that in the environment of wound healing, Fizz1 secreted by alternatively activated macrophages upon estrogen treatment, induces the differentiation of fibroblasts and increases the expression of a-smooth muscle actin and type I collagen and thus contributes directly to the deposition of extracellular matrix (Liu et al., 2004).

Other authors shown that secreted TGFβ1 by M2 macrophages induces fibroblasts to produce extracellular matrix components, thus helping in its formation, which contribute to the resolution of wound healing (Hesse et al., 2001).

A key role of alternatively activated macrophages in deposition of extracellular matrix is reported also by the work of Gratchev and colleagues. They showed that M2 macrophages induced by IL4, overexpress fibronectin and bIG-H3, two components of extracellular matrix, both at mRNA and protein levels (Gratchev et al., 2001).

Concerning to wound healing, Ashcroft and colleagues showed that E2 enhances the deposition of collagen I in the dermis of ovariectomized mice (Ashcroft et al., 2003), while in macrophage-like differentiated THP-1 cells Kanda and Watanabe showed that, through the induction of c-Fos expression via the GPR30/cAMP/protein kinase A signaling pathway thus activating NGF transcription, E2 in vitro enhances the production of NGF, a growth factor for neurons and keratinocytes (Kanda and Watanabe, 2003). In this sense E2 enhances the wound re-innervation and re-epithelialization.

Matrix metalloproteinases (MMPs) is a large family of enzymes that degrade all components of the extracellular matrix (Bellosta et al., 2007); are synthesized as inactive precursors, requires proteolytic activation to express enzymatic activity and once activated, can be inhibited by tissue inhibitors of proteinases (TIMPs).The homestasis and remodelling of extracellular matrix is strongly regulated since uncontrolled proteolysis would contribute to abnormal development and onset of several pathological conditions characterized by either uncontrolled degradation or insufficent degradation of extracellular matrix components (Massova et al., 1998), such as cancer invasion and metastasis, cartilage destruction in arthritis, pulmonary fibrosis, emphysema, neuroinflammation, atherosclerotic plaque rupture and the development of aneurysms (Johansson et al., 2000).

Li and colleagues showed that elevated expression of MMP-26 in macrophages provokes inflammation, that could be a early-onset of emphysema (Li et al., 2004). The presence of an ERE in the MMP-26 promoter can explains the matrix destruction and malignant progression that occour in estrogen-dependent neoplasms.

Vegeto and colleagues showed that the microglia cultured with E2 added 4h before LPS reduces the induction of tissue degradation by MMP-9, indicating that E2 is able to reduce the lytic activity of microglia induced by LPS, decreasing the MMP-9 mRNA content with an ERα-mediating effect (Vegeto et al., 2001).

The study conducted on J774A.1 macrophages by Hwang and coworkers shows that E2 increases MMP-9 activity and Annexin II expression (Hwang et al., 2006); in particular, they observed that the effect of E2 on macrophage MMP activity would be reduced in the presence of statin. Moeover, annexin II plays an crucial role in mediating the action of estrogen and statin on the expression of MMP-9

Fleming and colleagues, in a work on the humanization process of murine mammary gland, showed that E2-stimulated macrophages enhance fibroblast proliferation and invasion through upregulation of the activity of MMP-9 (Fleming et al., 2012).

The study conducted by Bellosta and coworkers shows the involvement of macrophagic ERs in the modulation of expression and activity of MMPs, because raloxifene inhibits gelatinolytic activity of MMP-9 (Bellosta et al., 2007). Moreover, this effect is not due to a physical interaction between raloxifene and MMP-9 but to an inhibition of the trascription of MMP-9 promoter by NF-kB pathway, ER-mediated.

Major histocompatibility complex (MHC) molecules, class I and II, are normally found on the plasmalemma of antigen-presenting cells, including macrophages and are crucial in initiating immune responses.

The study conducted by Dimayuga and colleagues shows that pretreatment with E2 attenuated the surface staining of MHC Class I induced by LPS, without modulating their

gene expression but alterating posttranslational processing and/or degradation of MHC molecules (Dimayuga et al., 2005). E2 reduces the antigen presentation ability of microglia by modifing the expression nd activaty of MHC and coregulatory molecules.

Studies conducted by Kanda and Watanabe shown that E2 enhances VEGF transcription via the GPR30/cAMP/protein kinase A signaling pathway in macrophage-like differentiated THP-1 cells (Kanda and Watanabe, 2002), an effect that is antagonized by androgens and which may be related to the development of granuloma pyogenicum during pregnancy.

1.4.2 Some examples of the involvement of the estrogen-macrophage interplay in tissue homeostasis and disease

In addition to numerous physiological roles carried out by macrophages in different tissues where they reside, many studies have highlighted how their abnormal or incorrect activation is involved in the onset or progression of some important diseases. Below are reported some selected examples of physiopathological conditions in which estrogen has been shown to be a key regulatory element, possibly by modulating macrophage reactivity and function.

1.4.2.1 Wound healing

When cutaneous wound healing occurs, numerous inflammatory cells including macrophages are present at the damaged tissue. Wound macrophages show both M1 and M2 phenotypes; their presence is in strict relation with the local cytokine mileu and a high temporal regulation of these two phenotypes is crucial to the wound repair. The upregulation of Arginase1, Fizz and Ym1, typical of M2 macrophages is a fundamental step for tissue repair.

It has been shown that the protective effects of estrogen on skin and cutaneous healing are mediated, at least in part, by a strong anti-inflammatory activity which involves both ERα and ERβ, (Harkonen and Vaananen, 2006). The initial studies by Ashcroft and colleagues showed that in chronic wounds in the aged women, the exaggerated number of inflammatory cells is due to the decline in sex steroid hormones that occurs in menopause (Ashcroft et al., 1997). Subsequent work provided strong evidence for the molecular mechanism of estrogen and ERs action in reducing inflammation and promoting wound repair (Campbell et al., 2010; Routley and Ashcroft, 2009); (Campbell et al., 2014); the effect of E2 could also be mediated by ERβ, since the ERβ KO model showed an excessive protease activity and reduced matrix deposition, characteristics related to a delayed healing (Campbell et al., 2010).

1.4.2.2 Endometriosis

Endometriosis is a common pathological condition that affects a large number of fertile women; it is characterized by the hormone-dependent persistence and growth of vascularized endometrial tissue at ectopic sites, typically the pelvis, with pain and reduced fertility (Capobianco and Rovere-Querini, 2013); (Lebovic et al., 2001). Lesions that originate from endometrial fragments during menstruations reach the peritoneal cavity via the Fallopian tubes, event called “retrograde menstruation”. Shed endometrium is thought to initially adhere to the peritoneal wall and ovaries, while for the establishment of lesions, the ectopic endometrial cells must invade the underlying basement membrane.

Several authors have underlined that endometriosis is characterized by the increased activation of peritoneal macrophages and elevated levels of inflammatory cytokine (Burns et al., 2012); (Punnonen et al., 1996); (Styer et al., 2008); (Khan et al., 2008), thus this disease is strictly dependent on the ability of ectopic endometrial tissue to attract and activate macrophages. Macrophages are indeed necessary for the growth and spreading of endometrial lesions and their activation, through the production of matrix

metalloproteinases (MMPs) and the increase in pro-angiogenetic factors, further facilitates the myeloid cells recruitment. Macrophages undergo activation as a consequence of signals generated within ectopic lesions or of consequence of the lack of hormone-regulated anti-inflammatory signals in the ectopic endometrium (Zhang et al., 2006);(Herrmann Lavoie et al., 2007); (Lawson et al., 2007); (Minici et al., 2007); (Galleri et al., 2009); (Lousse et al., 2008; Novembri et al., 2011).

The endometrium is the unique adult, physiologic tissue with angiogenesis processes. Estrogen regulates the cyclic distruption/construction of endometrial layers and regulates the growth of endometrial vasculature during the proliferative stage. In particular, the remodeling of endometrial vessels that occurs through the regulation VEGF/VEGF associated receptor and Angiopoietin/Tie-2 system, is modulated by sexual hormones (Girling and Rogers, 2009); (Mints et al., 2010); (Elsheikh et al., 2011); (Lash et al., 2012). Studies have shown that E2 acting on various macrophage signaling pathways, such as MAPK, PI3K/AKT, NF-KB through activity of ERα and ERβ (Couse and Korach, 1999); (Mendelson, 2009); (Wen et al., 1994); (Condon et al., 2006) promotes the vascularization of endometrial lesions.

1.4.2.1 Neurodegenerative diseases

The prolonged or defective inflammatory activation of macrophages has been hypothesized to impair and destroy the adjacent tissues, especially in the central nervous system. In fact, following bacterial invasions, mechanical or chemical injuries, microglia undergoes immune and metabolic modifications that allow the immune response to take place. However, it has been suggested that microglia activation can contribute to the beginning and development of the degenerative steps that take place in neurons and that are directly implicated in the manifestations of neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.

Alzheimer's Disease (AD) is a neurodegenerative disease of the elderly characterized by progressive decline of cognitive functions and memory that lead to dementia, with higher incidence in women than in men. It was one of the first diseases to be associated with toxic effects of activated microglia (Rogers et al., 1988; Yankner et al., 1989). β-amyloid accumulation appears to be a characteristic feature of AD, since has pro-inflammatory capacities and is able to further activate microglia to release neurotoxic factors such as NO, TNFα and superoxide anion that destroy surrounding tissues (Griffin et al., 1998).

Parkinson's Disease (PD) is a neurodegenerative condition with slow and continue progression, that involve functions related to the movement and equilibrium and provokes motor dysfunctions, bradikinesia and cognitive alterations. As in the case of AD, microglia activation is strongly involved in the manifestation of PD causing, through the production of oxidative species, the selective destrucion of neurons of striatum that produce dopamine.

Regarding the effects of estrogens on microglia, research efforts has been direct to understand whether estrogens may delay the onset and reduce the symptomatology of neurodegenerative disease by targeting microglia, that express ERα, ERβ and GPR30 (Almey et al., 2012); (Thompson and Moss, 1994).

Although still limited, the available literature provides evidence that supports the link between estrogen actions in microglia and neuroprotection in PD. Côté and coworkers showed that the activation of GPR30 with E2 and the selective agonist G1 causes neuroprotection of dopaminergicergic neurons situated in myenteric plexus (Cote et al., 2015). Using a MPTP mouse PD model, they evaluate the role of E2 on the recruitment of proinflammatory monocytes/macrophages in gut like a local inflammatory hallmark against MPTP intoxication. In particular, they observed that E2-GPR30 activation on monocytes/macrophages resident in gut causes neuroprotection of dopaminergicergic neurons, since noted increased anti-inflammatory monocytes and diminished proinflammatory population. Authors confirmed these discoveries using also a murine population of mono/macrophages and a human monocytic THP-1 cell line (THP1-XBlue cells).

2. Aim of the work

The aim of the work was to perform a comprehensive analysis of the homeostatic interaction between macrophages and estrogens; this study was performed by a combination of technical achievements, bioinformatics analysys and literature search that allowed to propose novel indications for the future assessment of the physiological role of the estrogen-macrophage interplay in females.

3. Material and methods

3.1 Animal models

Wild type male and female C57BL/6J mice at 4 months of age were supplied by Charles River Laboratories Italia s.r.l (Calco, Italy). All animals were allowed access to food and water *ad libitum* and were kept in temperature-controlled facilities on a 12-hour light and dark cycle. Animals were housed in the animal care facility of the Department of Pharmacological and Biomolecular Sciences at the University of Milan. All of the mouse experiments were carried out according to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institute of Health and in accordance with the European Guidelines for Animal Care and Use of Experimental Animals. In addition, the experiments were approved by the Italian Ministry of Research and University and controlled by a panel of experts at the Department of Pharmacological and Biomolecular Sciences (University of Milan, Milan, Italy).

3.2 Treatments of animals

17β-estradiol (E2; Sigma-Aldrich) was dissolved in corn oil by o/n stirring in the dark at room temperature and administered by a 100 µL s.c. injection at the dosage of 5 µg/kg E2; control animals received corn oil injection alone. Ovariectomy (ovx) or sham surgery was performed under mild anesthesia obtained by s.c. injection of 50 µl solution of ketamine (93.6 mg/kg, Ketavet 100; Intervet) and xylazine (7.2 mg/kg, Rompun; Bayer). At the specified time points, animals were euthanized by intraperitoneal (i.p.) injection of lethal ketamine and xylazine solution (150 and 12 mg/kg, respectively). Animal groups for the RNA sequencing experiment were given an estrogen-free diet (AIN93M; Mucedola) 2 weeks before and throughout the experiment.

3.3 Estral cycle valutation

The phase of the reproductive cycle in female mice was assessed by cytological examination of vaginal smears; vaginal flushing with 20µl sterile physiological solution was performed, vaginal washes were subsequently mounted and air dried on glass microscope slides and stained with May-Grünwald-Giemsa method (MGG Quick Stain Kit; Bio-Optica, Milan, Italy), according to the manufacturer's protocol. Cell and matrix composition was evaluated using an optical microscope.
Murine estral cycle is composed by 4 phases: Metestrous, Diestrous, Proestrous, Estrus. During these phases, occours a fluctuation of the ovaric hormones levels, 17β-estradiol, progesterone, luteinizing gonadotropins (LH) and follicle-stimulating hormone (FSH, Figure 1).

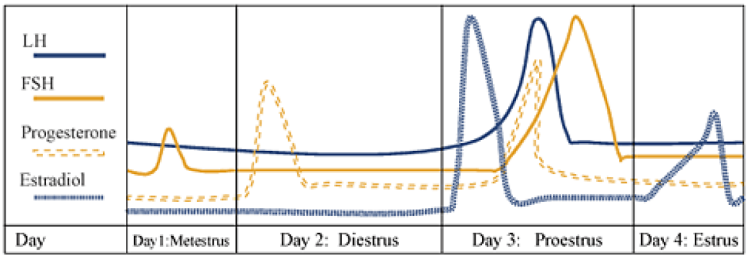

Figure 1. Schematic representation of the hormons's trend (17β-estradiol, progesterone, luteinizing gonadotropins (LH) and follicle-stimulating hormons (FSH)), during the phases of murine estral cycle (Metaestrous, Diestrous, Proestrous, Estrous)

To these fluctuations corresponde a change in cellular type composition into murine vaginal cavity. The type and the number of cellular populations found in the vaginal smears give the indication respect to the estral phase. Usually, cellular populations found in vaginal cavity are: nucleated epithelial cells, cornified epithelial cells and leucocytes

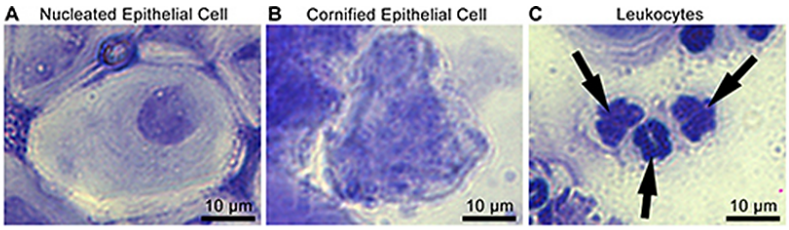

Figure 2 (A, B, C). Cellular populations (nucleated epithelial cells, cornified epithelial cells and leucocytes) observed by optical microscope of a vaginal smears by vaginal mice flushing with the aim to determinate the phase of reproductive cycle (Metestrous, Diestrous, Proestrous, Estrus).

It has been necessary to carry out the cytologic analysis of vaginal smears of female used in the experiment, because the aim of the study was the determination of the effects of estrogen on peritoneal macrophages, isolated in accurate phases of the cycle, such as metaestrous and estrous. In particular, metaestrous represents the phase of estral cycle with lowest levels of estrogen, whereas on the contrary, estrous represents the phase of estral cycle immediately after the proestrous, the phase with highest levels of estrogen.

The proportion of cellular populations found on the glass, permit to establish the estral phase of mouse, in the exact moment of sample collection In vaginal smears collected from female in proestrus, the cells are almost exclusively ovoid nucleated epithelial cells (Figure 3D). In samples collected from female in estrus, cells found are aggregates of cornified epithelial cells with an irregular morphology (Figure 3E). In samples collected from female in metestrus it is possible to find fregmentary cells, cornified epithelial cells and smaller, rounded and dark cells that corresponde to leucocytes (Figure 3F)., those appears in abboundance in the diestrous phase (Figure 3G). In fact in this phase, the number of cornified epithelial cells is small and can be possible to detect the nucleated cells, just before the start of the transition from diestrus and proestrus.

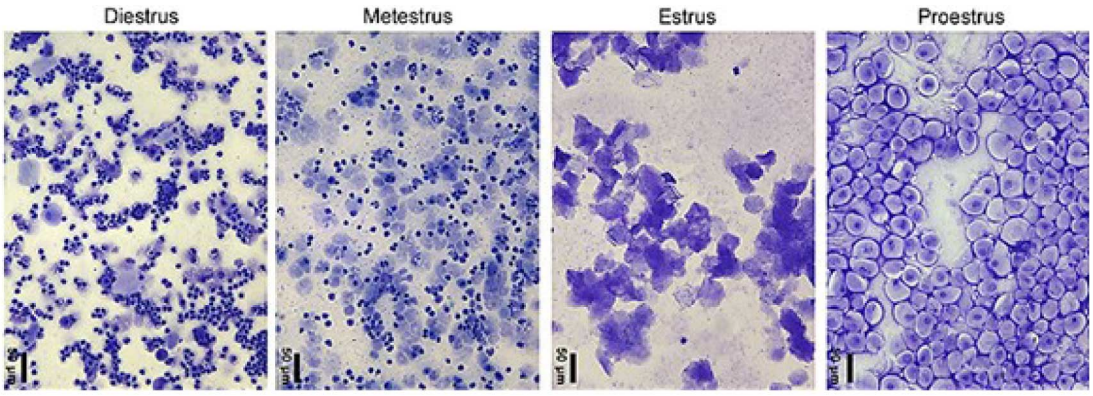

Figure 3. The different proportions of the cellular populations (nucleated epithelial cells, cornified epithelial cells and leucocytes) that it is possible to observe by optical microscope from the vaginal smear, permits to identify the phase of reproductive cycle: Diestrous (numerous rounded and dark cells that are leucocytes, few cornified epithelial cells and nucleated cells); Metestrus (fregmentary cells, cornified epithelial cells and smaller, rounded and dark cells that corresponde to leucocytes); Estrus (aggregates of cornified epithelial cells with an irregular morphology); Proestrous (exclusively ovoid nucleated epithelial cells.

3.4. Isolation and immune magnetic sorting of peritoneal macrophages

The animals were sacrified using CO2 and get on their back on the surgical table. Ventral portion was disinfected with 75% EtOh solution. Peritoneal cells were recovered by peritoneal lavage. 5 ml of pre-chilled 0.9% NaCl were injected into the peritoneal cavity using a 21G needle, cell suspension was collected with a sterile plastic Pasteur pipette from peritoneum. Collected cell suspension was centrifuged and dissolved in PBS + 0.5%BSA. After counting, CD11b MicroBeads (MilitenyiBiotec) were used to isolate resident macrophages following manufacturer's instructions. 10^7 peritoneal cells were suspended in 90 µL PBS + 0.5% BSA, and 10 µL CD11b MicroBeads were added to the cell suspension and incubated for 15 min at 4°C. After washing, cells were resuspended in 500 uL PBS + 0.5% BSA and applied to LS Miltenyi columns (MilitenyiBiotec) for the magnetic separation procedure. After 3 washing steps, CD11b-positive cells were eluted from the columns and counted. Cells were either stored in TRIzol reagent (Invitrogen) for gene expression studies.

3.5 Preparation of bone-marrow-derived macrophages (BMDM)

To prepare BMDMs, bone marrow from the tibia and femur was flushed with RPMI (Life Technology-Invitrogen) using a 21 gauge needle. Cells were centrifuged at 1200 rpm for 5 min at 10°C, seeded in flask cell culture T75 in DMEM+GlutaMAX (Life Technology-Invitrogen) supplemented with 10% endotoxin-free FBS, 1% penicillin/streptomycin and 1% Na pyruvate and incubated over-night. On the next day, the supernatant was collected, seeded at the concentration of 5-8 x 10^6 cells/dish and grown for 6 days in DMEM+GlutaMAX containing 20% endotoxin-free FBS, 30% L929-cell conditioned media, 1% penicillin and streptomycin, and 1% Na pyruvate. After 6 days BMDMs were harvested with Accutase (Merck-Millipore) and plated at the concentration of 5×10^5 cells/ml. On the next day, RPMI medium without phenol red with 10% dextran coated charcoal-FBS was added and cells were treated as specified.

3.6 Gene expression analysis

3.6.1 RNA preparation

RNA was purified using RNeasy minikit protocol (Qiagen) according to the manufacturer's instructions, including a step with the deoxyribonuclease incubation using the Rnase-Free Dnase set (Qiagen).
RNA Quality Control was performed on all RNA samples with an electrophoretic run on a Bioanalyzer instrument using the RNA 6000 Nano Kit (Agilent). RNA Integrity Number was determined for every sample and all the samples were considered suitable for processing based on the RNA integrity (RIN > 8). RNA concentration was estimated through spectrophotometric measurement using a Nanoquant Infinite M200 instrument (Tecan).

3.6.2 cDNA preparation

1 µg RNA has been used for cDNA preparation using 8 U/µl of Moloney murine leukemia virus reverse transcriptase (Promega, dNTPs (AmershamBiosciences) and randon primers (Promega) in a final volume of 25 µl; the reaction was performed at 37°C for 1 h, and the enzyme inactivated at 75°C for 5 min. Control reactions without the addition of the reverse transcription enzyme were also performed.

3.6.3 Semi quantitative RTPCR

A 1:4 cDNA dilution was amplified using GoTaq®qPCR Master Mix technology (Promega) according to the manufacturer's protocol. The PCR was carried out in triplicate on a 96-well plate using 7900HT fast real time PCR system (Applied Biosystems) with the following thermal profile: 2 min at 95°C; 40 cycles, 15 sec at 95°C, 1 min at 60°C. Primer sequences are reported in Table 4. Data were analyzed using the 2-ΔΔCt method.

Table 4. Oligonucleotides used in real time PCR analysis

Gene	Forward sequence	Reverse sequence
Arg1	5'-CAGAAGAATGGAAGAGTCAG-3'	5'-CAGATATGCAGGGAGTCACC-3'
Fizz1	5'-GGAACTTCTTGCCAA TCCAGC-3'	5'-AAGCCACAAGCACACCCAGT-3'
Ym1	5'-GAAGGAGCCACTGAGGTCTG-3'	5'-GAGCCACTGAGCC TTCAAC-3'
36B4	5'-GGCGACCTGGAAGTCCAACT-3	5'-CCATCAGCACCACGGCCTTC-3'
CcnB2	5'-CCGACGGTGTCCAGTGATTT-3'	5'-CTGAGGTTTCTTCGCCACCT-3'
Cdk1	5'-ACACGAGGTAGTGACGCTGT-3'	5'-TCAATCTCTGAGTCGCCGTG-3'
Wee1	5'-TTGGCTGGCTCTGTTGATGA-3'	5'-CAGCTAAACTCCCACCATTACAG-3'
KI67	5'-AGAGCTAACTTGCGCTGACT-3'	5'-TCAATACTCCTTCCAAACAGGCA-3'
Vegfα	5'-AGCAGAAGTCCCATGAAGTGA-3'	5'-ATGTCCACCAGGGTCTCAAT-3'
Cd206	5'-TTCAGCTATTGGACGCGAGG -3'	5'-GAATCTGACACCCAGCGGAA-3'
Tgm2	5'-GGCCACTTCATCCTGCTCTA -3'	5'-TCCAAGGCACACTCTTGATG -3'
ApoE	5'-GGACTTGTTTCGGAAGGAGC-3'	5'-AGGCATCCTGTCAGCAATGT-3'
Angptl4	5'-ATGACTTCAGATGGAGGCTGG -3'	5'-AATTGGCTTCCTCGGTTCCC -3'
E2f1	5'- TTAGCCCTGGGAAGACCTCA-3'	5'- CCGTGGCAATACTGCTTCTTG-3'
CcnD1	5'-TCAAGTGTGACCCGGACTG -3'	5'-ATGTCCACATCTCGCACGTC -3'
ER alfa	5'-GAAGAGTTTGTGTGCCTCAAAT-3'	5'-GTGCCGGATATGGGAAAGGATG-3'
ER beta	5-'CAGTAACAAGGGCATGGAAC-3'	5'-GTACATGTCCCACTTCTGAC-3'
Gper1	5'-CGGCACAGATCAGGACACCC-3'	5'-TGGGTGCATGGCAGAAATGA-3'

3.7 RNA sequencing

Sequencing libraries were prepared using the TruSeqTM RNA Sample Preparation Kit (Illumina) using 1.8 ug of total RNA as input. Polyadenylated transcripts were purified using poly-T oligo-attached magnetic beads. PolyA RNA was fragmented at 94°C for 8 min and retrotranscribed using random hexamers. Multiple indexing adapters were ligated to the ends of the cDNA and the amount of DNA in the library was amplified with 10 PCR cycles. Final libraries were validated and quantified with the DNA1000 kit on the Agilent Bioanalyzer

Instrument. Pooled libraries were sequenced on the Illumina Genome Analyzer IIx producing an average of 13 M reads per library.

3.8 Bioinformatics analysis

BaseCall files were converted to FastQ files using Casava 1.8.2. Sequencing reads were aligned to the mouse genome (mm10) using TopHat v.2.0.9. Transcripts were reconstructed and quantified using Cufflinks v2.1.1 and differential expression analysis was performed using CuffDiff (Trapnell et al., 2012). CuffDiff uses the test statistics T = E[log(y)]/Var[log(y)], where y is the ratio of the normalized counts between two conditions. A t-test is used to calculate the P value for Differential Expression (Rapaport et al., 2013). A threshold of 0.05 was applied to False Discovery Rate (FDR) adjusted p values in order to select the differentially expressed genes (DEGs) to use in downstream analysis; we also included genes with a log2 fold-change (lgFC) > 1 either showing one anomalous triplicate FPKM value and an FPKM average value between 1 and 2. Heat map of DEGs was made with Genesis software using triplicates mean, after normalization and log2 transformation. Cluster analysis was performed with the Genesis software tool using k-means clustering function (k=8) in order to identify group of genes with a similar regulation at 3 and 24 h of treatment (Sturn et al., 2002); genes with lgFC > +/-0.40 were selected. In each cluster of genes, the regulatory sequences in 20 Kb around the transcription start site were analyzed using iRegulon Cytoscape App and candidate transcription factors were predicted (Janky et al., 2014). Overrepresentation analysis (ORA) on DEGs lists was performed using the Functional Annotation Tool in DAVID website (Huang da et al., 2009). The lists of DEGs at 3 and 24 h of estradiol treatment were used as input gene list and the mouse genome was used as background list. Biological processes, molecular functions and KEGG pathways were investigated focusing on enriched terms with a Benjamini adjusted p-value less than 0.05. A Protein-Protein Interaction Network of the differentially expressed genes has been created using STRING (von Mering et al., 2005).

3.9 Statistical analysis

Unless otherwise stated, statistical significance was carried out with the GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA) by 1-way or 2-way ANOVA followed by Bonferroni *post hoc* test or unpaired *t* test. A value of $p < 0.05$ has been considered statistically significative

4. Results

4.1 Optimizing the growth conditions for the study of diverse macrophage populations

In order to have a deeper understanding of estrogen action in macrophages, we first assayed different sources of this cell type, namely:

1. peritoneal macrophages *ex vivo* (pM *ex vivo*)
2. primary cultures of peritoneal macrophages (pM *in vitro*)
3. bone marrow-derived macrophages (BMDM)

Peritoneal macrophages (pM) are typically used as model systems to study macrophage biology. To recover a high number of pM, animals are usually injected *i.p.* with tyoglicollate (TG) which stimulates the recruitment of immune cells including monocytes/macrophages 2-4 days post injection. However, in our preliminary experiments we noticed that TG-elicited peritoneal macrophages showed an activated morphology, with large and round cell bodies deprived of cellular extrusions. For the study of hormone action on homeostatic pM, both following *in vivo* treatment with estrogens or in primary *in vitro* cultures, we thus decided to isolate peritoneal cells without prior administration of any attractant stimuli so that, although reducing the efficiency of cell recovery from each animal, we could assess resident quiescent macrophages instead of infiltrated or activated cells. The notion that pMs are a resident population that migrates in this cavity during embryogenesis and maintains the ability to proliferate was still unknown at the time my PhD itinerary began. We thus set up the technical and experimental conditions for purifying pM and analyzing gene expression or for culturing pM *in vitro* and challenging with hormone.
On the other hand, I set up also the conditions to obtain BMDMs *in vitro*, which is a consolidated methodology widely present in the literature. BMDM are a reference macrophage population widely used by the scientific community, although it is not a faithful model for the resident macrophages.
Table 1 summarizes the average cell and RNA recoveries from pilot experiments performed to set up these experimental conditions for pM and immediately analyzed (pMP *ex vivo*), further cultivated on plates (pMP *in vitro)* or from BMDMs. Supplementary table 1 in the Supplemental data section summarizes all data related to the number of cells isolated and the RNA obtained from cells, collected throughout my PhD program period.

Table 1. Summary of cell and RNA from macrophage cell populations

Cellular type	N°cells/ animal	RNA recovery (μg/10^6 cell)
Peritoneal Mφ ex vivo	$3,6 \times 10^6$	1,88
Peritoneal Mφ in vitro	$9,01 \times 10^6$	0,87
BMDM	$14,55 \times 10^6$	6,07

4.2. Validation of the estrogen signaling pathway in different macrophage cell populations

With the intent to determine if the above mentioned cell systems are faithful models to study the estrogen signaling and to chose the best model for a thorough analysis of the macrophage response to estrogen, pM *ex vivo*, pM *in vitro* and BMDMs were analyzed for: a) estrogen receptor expression, by measuring the mRNAs levels for ER α, ER β and GPR30 by realtime PCR; b) estrogen transcriptional activity, by assessing ApoE and Tgm2 gene expression, as these two genes were shown to be induced by estrogen in macrophages.

a) Expression of estrogen receptors mRNAs

Although in previous studies we observed that *in vitro* culturing procedures lead to a reduction in the expression of estrogen receptors mRNAs, our results indicate that both primary cultures (pM *ex vivo)* and in *in vitro* cultures (pM *in vitro* and BMDM) express the estrogen receptors, thus are able to respond with a direct mechanism to estrogen stimuli. In fact, Figure 2 shows the expression of ER α, ER β and GPR30 in the different macrophage cell populations, together with positive control tissues. In particular, data show that ER α and GPR30 are expressed in macrophages, although to a much lower extent as compared with receptor-positive tissues, such as the uterus or liver. In particular *in vitro* culturing of pM leads to a 10-fold reduction of receptor expression. Although the possibility to grow pM *in vitro* could be advantageous for reducing the number of animals to be used in the experiments, a reduction in receptor expression could provide different results from those obtained in pM from animals treated *in vivo* with estrogen. Interestingly, the expression of ER β is undetectable in macrophages, while ER β -positive control tissues show a positive result (Supplementary table 2 shows the Ct values of these realtime PCR experiments).

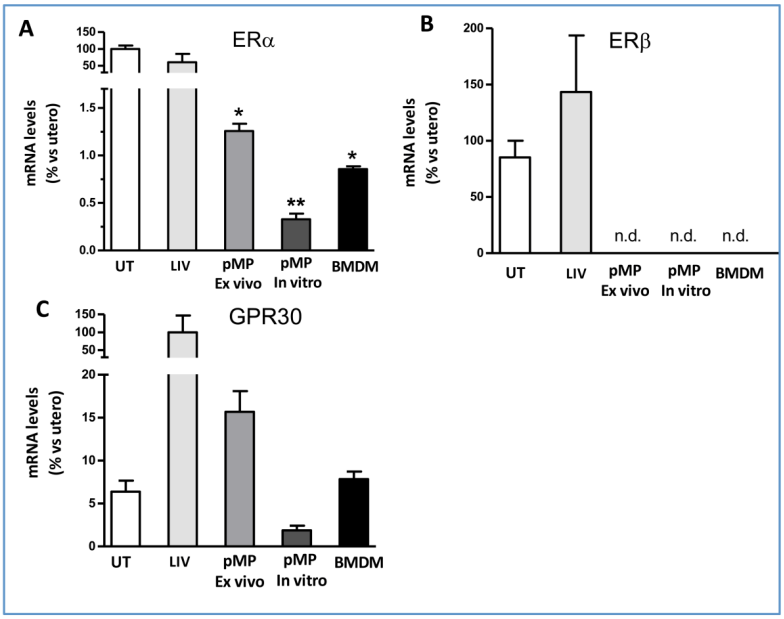

Figure 2. Expression of estrogen receptors isoforms in macrophages. ER α (A), ER β (B) and GPR30 (C) mRNA levels were analyzed in different macrophage cell populations and positive control tissues by realtime PCR. Data were calculated using the 2-ddCt method and represented as % of the levels obtained in the uterus. UT: uterus; LIV: liver.
Bars represent mean values +/-SEM (n=3). Student's unpaired *t*-test, *p<0.05; **p<0.01; ***p<0.001 *versus* UT.

b) Estrogen target genes transcription in different macrophage cell populations

Since the isolation and culturing conditions of macrophage populations did not lead to the loss of estrogen receptors (ER α and GPR30), we evaluated if estrogen could modulate the transcription of target genes similarly to its *in vivo* effects on pM. We thus analysed the mRNA levels of two genes, ApoE and Tgm2, which have been shown to be regulated by estrogen in macrophages. As shown in Figure 3, the i*n vivo* treatment of physiological doses of E2 resulted in the reduction of ApoE mRNA levels in peritoneal macrophages, while in *in vitro* pM and BMDM E2 did not show any effect. These results were apparently in contrast with data reported in the literature and showing a positive regulation of ApoE mRNA by estrogen in brain microglia. This discrepancy could be ascribed either to the cell types used in the present study, which could respond differently from microglia, or to the preparation/culturing conditions used. However, the results of Tgm2 expression show that the estrogen signaling pathway is active in the macrophage cells obtained in my experiments; in fact, Figure 2B shows that the mRNA levels of Tgm2 are increased by estrogen in all macrophages population analyzed. Supplementary table 3 reports the RTPCR values obtained from the analyses of the expression of ApoE and Tgm2 and used to generate these histograms. Thus, *in vitro* cultures of pM lead to a reduction in estrogen transcriptional efficiency, at least in relation with Tgm2 mRNA levels. BMDM are also less responsive to estrogen, further pointing to pM extracted following the *in vivo* treatment as the best choice for reaching the aim of our next genomic studies.

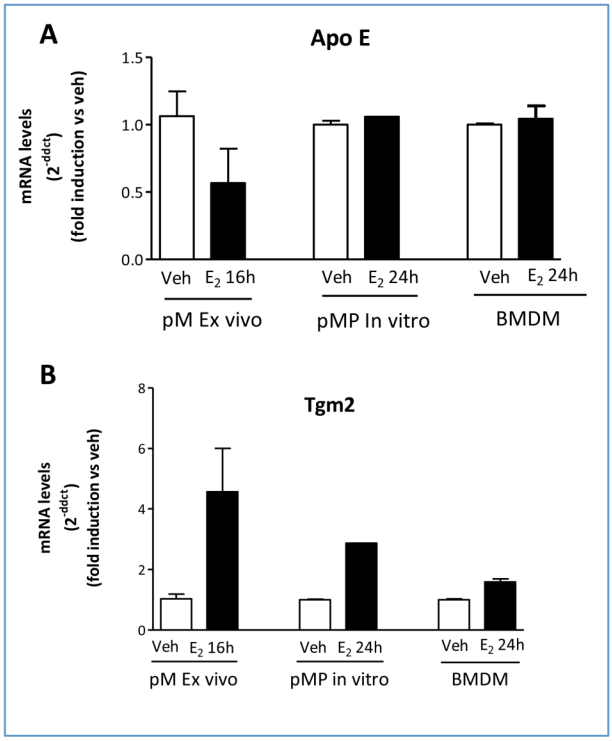

Figure 3. Expression of estrogen target genes in macrophage cell populations. Peritoneal macrophages *ex vivo* cultures (pMp *ex vivo*) were isolated from mice 16 hours after a s.c. injection of vehicle (corn oil) or 5µg/kg 17β Estradiol (E_2). Peritoneal macrophages cultures from nahive female mice (pMP *in vitro*) or BMDMs were treated for 3 hours with vehicle (EtOh 0,01%) or 10^{-7} M E_2. Data were calculated using the 2-ddCt method with respect to the vehicle in pM *ex vivo*. Bars represent mean values +/- SEM (n=3). Student's unpaired *t*-test, *p<0.05; **p<0.01; ***p<0.001 *versus* veh pM ex vivo.

In conclusion, these analyses showed that the procedure of *in vitro* expansion of pM leads to a 10-fold reduction in ERα and GPR30 expression compared to the levels observed in pM freshly isolated from animals; similarly, BMDM express lower levels of ERs mRNAs. Both these last two macrophage populations also show a lower transcriptional activity of estrogen, at least on Tgm2 target gene. We thus decided to use peritoneal macrophages *ex vivo* to perform a genome-wide gene expression analysis of the response of macrophages to estrogen.

4.3 Genome wide gene expression analysis of the macrophage response to estrogens

In order to obtain deep information on the response of macrophages to estrogen, we decided to analyze the changes in the mRNA levels that are induced by estrogen in macrophages *in vivo*. Estrogen action was evaluated in female mice in two ways: either following the exogenous administration of E_2, or at selected phases of the estrous cycle, namely the estrous (E) and metaestrous (ME) phases (group 1). For future experiments, in this phase of the study, we also used ovariectomized/sham operated animals (group 2) and male mice (group 3). Data from these last 2 groups are not the object of my thesis.

4.3.1 Animal selection.

Animals used for the experiment were divided in three groups:

Group 1)

a. intact female in metaestrous (ME)

b. intact female in metaestrous + 3hr E_2 (ME+3hE_2)

c. intact female in metaestrous + 24hr E_2 (ME+24hE_2)

d. intact female in estrous (E)

Group 2)

e. ovarectomized female

f. ovarectomized female + 3hE_2

g. ovarectomized female + 24hE_2

h. sham-operated female

Group 3)

i. male

Group 1). Metaestrous (a) and estrous (d) subgroups refer to animals with the lowest levels of estrogen. Estrous is the phase that immediately follows proestrous, which instead shows the highest levels of circulating estrogen; we reasoned that cells obtained from the E phase represent mid to long term estrogen-responder cells. In fact, proestrous generally lasts for 24h but is generally more difficult to observe; moreover, since our analyses were limited to morning evaluations of the estrous cycle, animals chosen in the proestrous phase would be more heterogeneous in their responses. Thus, females in metaestrous were chosen as "control" samples, with the lower effect of estrogen as more distant from the proestrous phase; a group of animals in ME were also treated for 3h (c) or for 24h with 5μg/kg of 17β-estradiol (E_2) (d). Two time points following E_2 administration were used to obtain information on short and long-term responses of macrophages to estrogen. Females in E (subgroup d) represent mid to long-term responders to the endogenous surge of estrogen hormones.

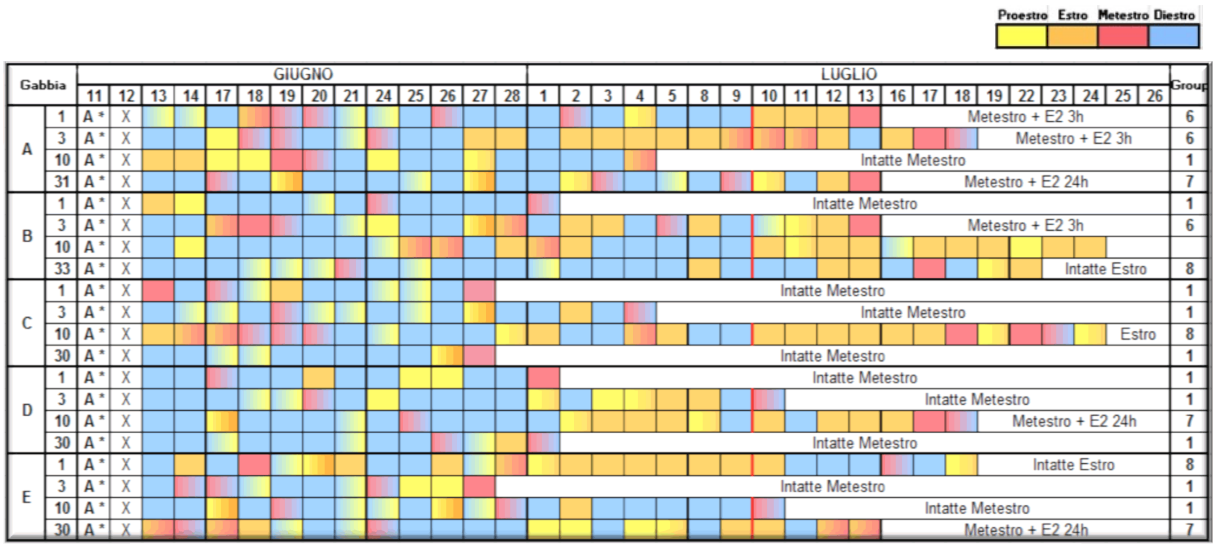

Figure 4. Color-coded assessment of the estrous cycle in animal group 1. A vaginal smear test was used to determine the phase of estrous cycle based on the cellular composition of the vaginal fluid. Twenty animals (named 1, 3, 10 and 30) divided in 5 cases (gabbia, A to E) were analysed in consecutive days during June and July 2014. Colours represent a phase of the estrous cycle, as described in the legend. Where the box appears as mixed colours cells showed characteristics of mixed phases detected in that animal. Animals were sacrificed and put in specific groups (Intatte metestro, (subgroup a); Metestro + 3h E2 (subgroup b); Metestro + 24h E2 (subgroup c); Estro (subgroup d).

4.3.2 RNA sequencing and bioinformatics analyses

Resident peritoneal macrophages were purified from group 1 animals by magnetic immunosorting, the RNA extracted and analyzed by RNA sequencing through Illumina Truseq high throughput sequencer, as reported in Material and Methods section.

The results were aligned with TopHat2 + Bowtie2 softwares and abundances (FPKM values) have been calculated and normalized using Cufflinks software.

Next, bioinformatic analysis calculated the differential expression of the genes, based on FPKM values, by first comparing the experimental subgroups ME, ME+3hE_2 and ME+24hE_2; then, data from the estrous subgroup were analysed by first comparing gene expression with ME, and then with ME+3hE_2 and ME+24hE_2.

4.3.3 List of differential expressed genes (DEGs) identified in the ME, ME+3hE_2 and ME+24hE_2 groups

The comparison of FPKM among ME, ME+3hE_2 and ME+24hE_2 shows that short and long-term hormonal treatments affected expression levels of 565 transcripts (Supplemetary Table 4). Figure 5 shows the graphic representation of the number of genes differentially regulated among the ME/ME+3hE_2 and ME/ME+24hE_2 comparisons. Some genes are in common between the 3h and 24h E_2 treatments (110 genes), while other genes are regulated only at 3h E_2 (128 genes) and others only by 24h E_2 (331 genes). These data show that macrophages are responsive to exogenous E_2 after both short and long-term hormone actions; in our experimental settings 20% of E_2-regulated genes (110 of 569) are both immediately and persistently regulated, being present both in the 3h E_2 and 24h E_2 lists of DEGs. The short exposure to E_2 provides a smaller effect (128 genes, 20% of all DEGs) as compared to the longer 24 h exposure which induces a stronger effect (331 genes, 60% of all DEGs).

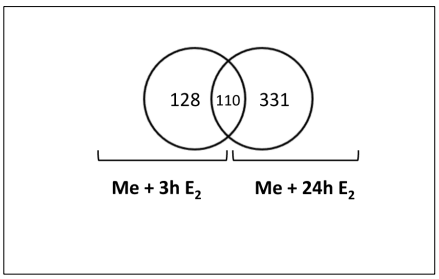

Figure 5. Venn diagram of the number of genes differentially regulated among the ME/ME+3hE_2 and ME/ME+24hE_2 groups. The lists of DEGs obtained from the ME/ME+3hE_2 and the ME/ME+24hE_2 analyses were evaluated for the presence of commonly regulated genes.

4.3.4 List of differential expressed genes (DEGs) in the Metaestrous, and Estrous groups and comparison with exogenous estrogen effects

The analysis conducted on by comparing genes expressed in the Metaestrous versus the Estrous groups shows that 170 transcripts are differentially regulated in these two conditions (Supplemetary Table 5); data are grouped in up- regulated or down-regulated DEGs, according to their FPKM values.

We were than interested in understanding whether the exogenous administration of E_2 provided a similar response in macrophages as that observed following the endogenous fluctuations of estrogen during the estrous cycle. Figure 6 shows the Venn diagram representation of the number of genes regulated among the ME/ME+3hE_2, ME/ME+24hE_2 and ME/E groups. Twenty-seven genes are in common among all the three hormonal conditions (E, 3hE_2 and 24hE_2), probably representing physiological short and long-term estrogen responsive macrophage genes. The E condition has 27 genes in common with 3hE_2, representing physiological early estrogen responsive genes, and 25 in common with 24hE_2 which we could consider as macrophage genes with late estrogen responsiveness. On the contrary, 91 are only regulated by the endogenous estrogen surge, either representing indirect E_2-target genes or genes that are responsive to E in a time frame that is not considered by our experimental settings. In addition, 485 (namely 59+121+305) are only regulated by the exogenous administration of E_2; also for this set of genes we could hypothesize that their responsiveness to E_2 either occurs in a time frame that is not present in the animals chosen at the E phase or that it is masked by physiological signals.

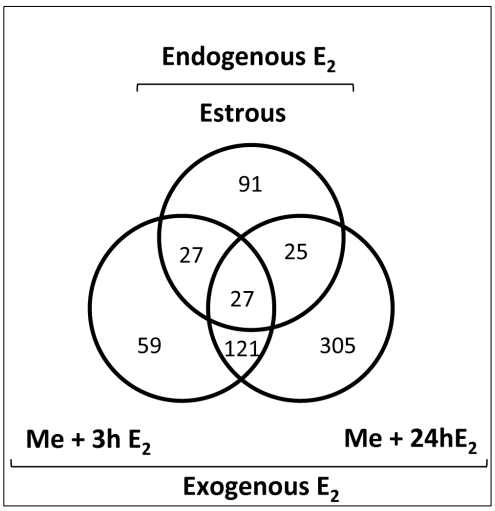

Figure 6. Gene regulation by endogenous or exogenous estrogen. A Venn diagramm is shown to represent the number of genes commonly or differentially regulated in the ME/E, ME+3hE_2, ME+24hE_2 groups.

The overlapping or specificity of exogenous or endogenous estrogen action is also represented as percentage of the total number of DEGs present in the ME/E comparison in Figure 7, or with respect to the total DEGs in the ME+3hE_2 and ME+24hE_2 comparisons in Figure 8.

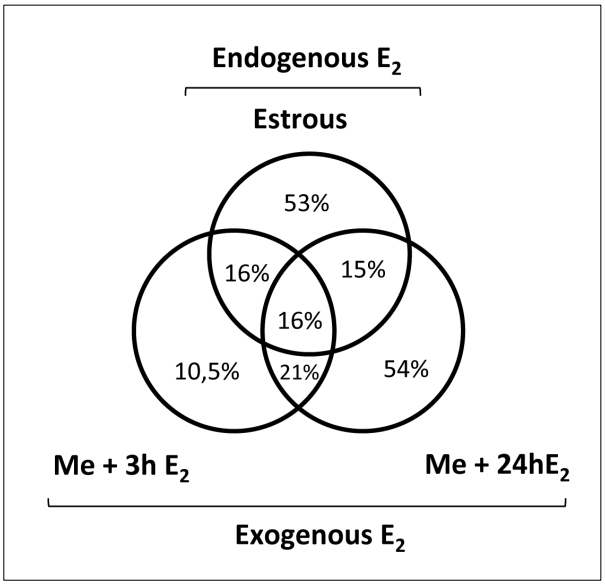

Figure 7. Gene regulation by endogenous or exogenous estrogen. A Venn diagramm is shown to represent the percentage of genes commonly or differentially regulated with respect to the total number of DEGs present in the ME/E comparison.

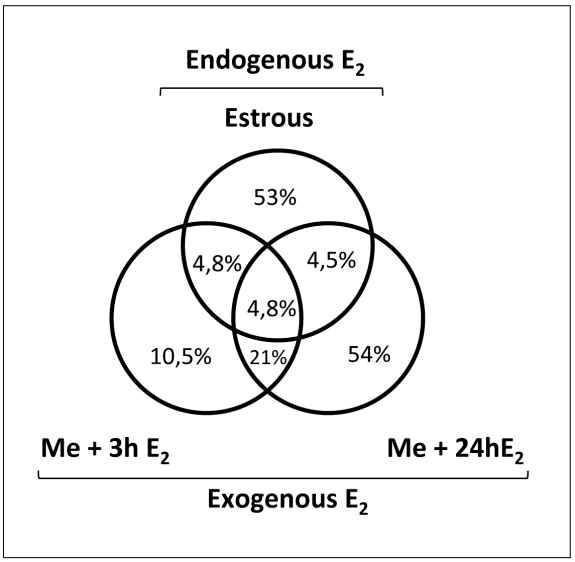

Figure 8. Gene regulation by endogenous or exogenous estrogen. A Venn diagramm is shown to represent the percentage of genes commonly or differentially regulated with respect to the total number of DEGs present in the ME+3hE_2 and ME+24hE_2 comparisons.

4.4. Bioinformatic analysis relative to ME, ME+3hE_2, ME+24hE_2

4.4.1 Gene Ontology analysis

Differentially expressed genes (DEGs) emerged from the comparison among the 3 experimental groups ME, ME+3hE_2 and ME+24hE_2, have been submitted to an ontologic analysis using Gene Ontology (GO) database, with the aim to sort regulated genes on the basis of their biological activity. In addition, DAVID database was used with the aim to obtain functional annotation for DEGs up (Supplementary Table 6) or down (Supplementary Table 7) regulated related within the ME/ME+3hE_2 and ME/ME+24hE_2 comparisons.

Table 2 shows the principal pathways modulated by estrogen: cell cycle (CC); immune response (IR); wound healing (WH); lipid metabolism (LM); regulation of transcription (TX); apoptosis (AP); protein folding (PF); stress response (RS). The identity of DEGs belonging to these pathways is shown together with indications on the up or down regulation effect.

Table 2. Genes regulated by the exogenous estrogen administration are grouped in biologic GO ontologies and further assembled in grouped categories according to overlapping functions.

Ontologies		DEGs at 3h	DEGs at 24h	Grouped categories	Significance of regulation
ID	Name				
GO:0007049	Cell cycle	**CHAF1A, NUF2, CDK1, CCNB2, BUB1B, BIRC5, PRC1, SPC25, SPC24, CENPH, NUSAP1, CHAF1B, CDCA5, UBE2C, GMNN, MKI67, STMN1, UHRF1, TIPIN, LIG1, TACC3, INCENP, MAPK6, CDKN1A, E2F3, CDKN2D, STEAP3**, WEE1, SIK1, BANP, JMY	**NUF2, BIRC5, CCNB2, KIFC1, SPC24, CKAP2, CCNF, CCNB1, BUB1B, CDCA3, CHAF1A, KIF11, MKI67, STMN1, PRC1, NUSAP1, TIMELESS, TPX2, UBE2C, CDC6, SYCE2, AURKB, UHRF1, SLFN1, LIG1, INCENP, STEAP3, MCM2, MAPK6**, DUSP1, KRT7, TACC2, WEE1, CABLES1, BANP, RGS2, E4F1, SIK1	Cell cycle (CC)	17,70%
GO:0006955	Immune response	**VEGFA, H2-M3, CX3CR1, IL1B, NLRP3, BCL3, RAB27A, CCR5, CCR2**, CCL5, IL7R, LAT, OAS1G, OAS2, IL1RL1, CCR7, LTB	**CCL12, CCL22, FCGR1, CX3CR1, RSAD2, CCR5, IRF7, CPLX2, CD300LF, H2-EB2, OAS3, DHX58, LBP, CD300LD, C1RA, TLR1, ICAM1**, CCL3, IL1RL1, CXCL2, IL16, TNFSF8, CD300A, CD14	Immune response (IR)	10,80%
GO:0006935	Chemotaxis		**CCL12, CCL22, FPR2, LBP**, CCL3, CXCL2, TGFB2, CX3CR1, CSF3R		
GO:0006897	Endocytosis		**FCGR1, STXBP1, DAB2, MSR1, LBP, MRC1, LDLR, NME1**, ULK1, CAV1, STON2		
GO:0009611	Response to wounding	**IL1B, ARG1, NLRP3, RAB27A, CCR5, CCR2**, CCL5, LAT, SGMS1, THBD	**CCL12, ARG1, CHI3L3, CCL22, FCGR1, , PAPSS2, MEFV, GATM, CCR5, NGFR, CD163, LBP, C1RA,TLR1, F13A1**, CCL3, THBD, IL6, CXCL2, ORM2, ORM3, SGMS1, NFKBIZ, CD14, TGFB2	Wound healing (WH)	11,34%
GO:0048534	Hemopoietic/ lymphoid organ development	**VEGFA, CCNB2, TGFBR3, TACC3, ID2, BCL3, PLSCR1, CCR2**, IL7R, ZBTB16, KLF11, MLL1, LTB, EPAS1, PTPN22			
GO:0001568	Blood vessel development		**TGM2, NRP1, MYO1E**, CXCR4, EPHA2, FGFR1, NOTCH1, CAV1, EPAS1, ZFP36L1, ZMIZ1, TGFB2, WNT2, JUNB		
GO:0045834	Positive regulation of lipid metabolic process	**IL1B, ABCG1**, ANGPTL4		Lipid metabolism (LM)	3,01%
GO:0008203	Cholesterol metabolic process		**FDPS, SOAT2, IDI1, LDLR**, APOC1, DHCR24		
GO:0010876	Lipid localization		**MSR1, LBP, LDLR**, APOC1, PLIN2, SLC27A1, CAV1, NRIP1		
GO:0006355	Regulation of transcription	**VEGFA, LBX2, CHAF1A, ZFP580, MXD3, CENPK, TOP2A, EGR3, CHAF1B, TGFBR3, CDCA7, SOX7, ASF1B, MCM5, NLRP3, UHRF1, NR4A1, HES1, ID2, KLF4, E2F3, BCL3, ABCG1, PADI4**, KLF15, PER1, ZBTB16, TCF7, MBD1, JDP2, KLF9, ZFP811, PER2, KLF11, MLL1, PER3, NR1D2, TEF, JMY, TFB2M, TSC22D3, ATXN1, RFX2, EPAS1, MLXIP, POU2F1, IL16		Regulation of transcription (TX)	8,33%
GO:0042981	Regulation of apoptosis	**VEGFA, SPP1, BIRC5, CX3CR1, PLEKHF1, NLRP3, NR4A1,CDKN1A, BCL3, RIPK3, RAB27A**, ANGPTL4, IL2RB, ZBTB16, TCF7, SGMS1, JMY, TSC22D3, LTB		Apoptosis (AP)	3,36%
GO:0006457	Protein folding	FKBP5, HSPH1, DNAJB13, HSP90AA1, DNAJA1, DNAJB1, FKBP4, AHSA1		Protein folding (PF)	1,41%
GO:0033554	Cellular response to stress	**CHAF1A, CDK1, RAD51AP1, KIF22, CHAF1B, POLE, UHRF1, TIPIN, LIG1, CDKN1A, BCL3**, ANGPTL4, JMY, EPAS1		Stress response (SR)	2,50%

Genes in bold: up-regulated genes; genes in normal typing: down-regulated genes. Biologic categories: cell cycle (CC); immune response (IR); wound healing (WH); lipid metabolism (LM); regulation of transcription (TX); apoptosis (AP); protein folding (PF); stress response (RS). The significance of their regulation is calculated as the percentage of number of DEGs belonging to each pathway over the total number of DEGs derived from Me/ Me+3h E2 and Me/Me24h E2 comparisons.

4.4.2. Data matching between DEGs and literature evidences

In order to have a complete view of the pathways that are regulated by estrogen in macrophages, I carried out a literature search using the PubMed database and looked for publications focused on the estrogens-macrophages interplay; to this aim I used the key words “estrogens AND macrophages”, “estrogen AND macrophage polarization or activation” encompassing all studies on human or other species. No limits were set for publication dates and type of journals. This study allowed to identifying four pathways, in addition to those emerged from our next generation sequencing experiment, which were reported to be regulated by estrogens in macrophages:

1. iron homeostasis
2. cholesterol homeostasis
3. extracellular matrix components or enzymes
4. phagocytosis

Table 3 indicates the most significant publications in the literature used to identify these additional pathways. These pathways didn’t show up with Gene Ontology analysis due to low statistical significance.

Table 3. Biological pathways reported to be regulated by estrogen in PubMed, as emerged from a personal search.

Pathway	Authors	Model used
Iron homeostasis	Hamad and Awadallah, 2013	murine serum iron levels
	Qian et al., 2015	murine bone marrow-derived macrophages
Cholesterol homeostasis	McCrohon et al., 1999	female human peripheral blood monocyte-derived macrophage cells
	Napolitano et al., 2001	male human peripheral blood monocyte-derived macrophage cells
	Tomita et al., 1996	J774 A.1 cells
	Wilson et al., 2008	human monocyte/macrophage cell line THP-1
	Allred et al., 2006	peritoneal macrophage from low density lipoprotein receptor null mice
Extracellular matrix components or enzymes	Liu et al., 2004	murine wounds and alternatively activated macrophages
	Gratchev et al., 2001	alternatively activated M2 macrophages
	Li et al., 2004	human macrophages and polymorphonuclear leukocytes, estrogen-dependent neoplasms
	Vegeto et al., 2001	rat microglia
	Johnson and Sohrabji, 2005	rat microglia and cells recruited from blood to the brain
	Hwang et al., 2006	J774A.1 macrophages
	Dimayuga et al., 2005	N9 murine microglial cell line
Phagocytosis	Bruce-Keller et al., 2000	primary rat microglia and N9 microglia cell line
	Vegeto et al., 2006	murine microglia, RAW macrophage cell line
	Zhang et al., 2015	murine primary alveolar macrophages and RAW macrophage cell line
	Vegeto et al., 2004	RAW macrophage cell line

In order to evaluate whether genes related with these novel pathways were also regulated by estrogen in pM, we analyzed our DEGs list for the presence of genes known to be involved in these functions. Table 4 shows the identity of the genes present in our dataset that could be assigned to homeostasis of iron, homeostasis of cholesterol, extracellular matrix components or enzymes and phagocytosis on the basis of functional annotations I found to be reported in the literature. The significance of the regulatory effect of these ontologies has similar values as those found by Gene Ontology database.

Table 4. List of DEGs present in the ME/ME+3hE_2 and ME/ME+24hE_2 comparisons that are known to be involved in the four novel pathways.

DEGs at 3h	DEGs at 24h	Grouped categories	Significance of regulation
STEAP3	**FXN, SFXN5**, SLC22A17, **STEAP3,**	Iron homeostasis	0,88%
ABCG1, CYP11A1, **LDLR, LPL,** SOAT2	**ABCG1,** FABP4, **LDLR, SOAT2**	Cholesterol homeostasis	1,60%
BCL3, CD93, HSPB1,**ITGA7**, **ITGB3**, **LOXL2**, **LRRK2**, **PLOD3, PLSCR1**, SERPINE1, **SLPI**, **SPP1, TGFBR3**	ADAMTSL4, **BCL3, CASK,** CD93, **CKAP4, CLDN20, EDIL3,FAM20C,**HSPB1, **IL1RL1, ITGA9,** ITGB3, LAMA3, **LGALS1, MYO1E, PKVCANM, PLSCR1, RARRES2, SERPINE2,SPP1, TGM2, VCAN,** WNT2	Extracellular matrix	6,38%
CCR2, CCR7, **IL1B,** LAT, **MARCO**, PIK3R5, **RAB27A**	**AIF1,**CD300A, **FCGR, FGR, GAS6, LBP, MARCKSL1, MARCO,** PIK3R, **PLA2G4A, TGM2, TREM2**	Phagocytosis	1,32%

Genes in bold: up-regulated genes; genes in normal typing: down-regulated genes. Biologic categories related to the just individuated four major additional pathways regulated by estrogen in macrophages: iron homeostasis, cholesterol homeostasis, extracellular matrix components or enzymes and phagocytosis with their significance of regulation calculated as the percentage of number of DEGs belonging to each pathway over the total number of DEGs related to the comparison between ME, ME+3hE_2 and ME+24hE_2.

4.5 Bioinformatic analysis relative to the ME/E group

4.5.1 Gene Ontology analysis.

Similarly to the previous analysis, DEGs emerged from the ME/E comparison were submitted to an ontologic analysis using Gene Ontology (GO) database, with the aim to sort genes on the basis of their biological activities (Supplementary Table 5). DAVID database has been also used with the aim to obtain functional annotation for DEGs up or down regulated related to the comparison between ME and Estrous.

This analysis allowed to identify of the following pathways, reported in Table 5: cell cycle (CC); immune response (IR); wound healing (WH); lipid metabolism (LM); regulation of transcription (TX); apoptosis (AP); protein folding (PF); stress response (RS).

Table 5. List of genes regulated by endogenous estrogen fluctuation and categorized by DAVID database.

Ontologies		DEGs	Grouped categories	Significance of regulation
GO ID	Name			
GO:0008283	Cell proliferation	PTGES3, **PDK1, CFB**, CREBBP, **IGF1**, BCL2L1, MYC, MIF, DDIT4	Cell cycle (CC)	8,80%
GO:0051726	Regulation of cell cycle	**SRSF5**, CCND2, PER2, SFN, **ARNTL**, HSPA8		
GO:0002376	Immune system process	**MARCO, C1RA, OLR1, C3, CFB, HP, CD40, PADI4**, MIF	Immune response (IR)	16,47%
GO:0006954	Inflammatory response	**BMP2,** OLR1, **TNFRSF26**, C3, TNFRSF8, CD40, EPHA2, MIF		
GO:0050729	Positive regulation of inflammatory response	**PTGER4, TGM2**, HSPD1		
GO:0030335	Positive regulation of cell migration	COL18A1, **DAB2, BMP2, PLD1**, CXCR4, **IGF1, ITGB3, CXCL12**		
GO:0071560	Cellular response to transforming growth factor beta stimulus	**ARG1, DAB2, CAV1**, ID1, **CX3CR1**	Wound healing (WH)	8,23%
GO:0098609	Cell-cell adhesion	TBC1D10A, **FSCN1**, CREBBP, **HSPA1A**, SFN, DNAJB1, **CXCL12**, AHSA1, **ELMO2**		
GO:0000122	Transcription factor binding	EGR1, TCF7, **CAV1, BMP2, HIST1H1C**, CREBBP, **MLXIPL**, ZBTB16, PLK3, NR1D1, ID1, PER2, PER1, PER3, **NFIL3**, MYC, BCL2L1, **ARNTL**, MYC, **ARHGEF10L,** HSPA8	Regulation of transcription (TX)	12,35%
GO:0043066	Negative regulation of apoptotic process	DAB2, **HPN,** PLK3, **BCL2A1B**, CCND2, ID1, DNAJA1, **SERPINB2**, **IGF1**, BCL2L1, **HSPD1**, **HSPA1B**, MIF	Apoptosis (AP)	7,64%
GO:0006457	Protein folding	HSP90AA1, FKBP5, **CWC27**, FKBP4, DNAJA1, DNAJB1, HSPD1, AHSA1, HSPA8, **HSPA1A**,STIP1	Protein folding (PF)	15,90%
GO:0061077	Chaperone-mediated protein folding	FKBP5, FKBP4, CHORDC1, HSPA8		
GO:0031625	Ubiquitin protein ligase binding	PRKAR2B, **IKBKE,** CXCR4, DNAJA1, PER1, HSPA1A, PER3, **CD40**, HSPD1, MYC, HSPA8, **CKB**		
GO:0001666	Response to hypoxia	EGR1, **BMP2, CAV1, IGF1,** HSPD1, **CXCL12, AGTRAP,** DDIT4,ER2, BCL2L1	Response to hypoxia (RH)	5,90%
GO:0007623	Circadian rhythm	NR1D1, ID1, DBP, PER2, PER1, IGF1, PER3, **ARNTL, NFIL3**,TEF,NR1D2,CREBBP	Circadian rhythm (CR)	10%
mmu04710	Circadian rhythm	NR1D1, PER2, PER1, PER3, **ARNTL**		
GO:0042632	Cholesterol homeostasis	**LPL, HPN, CAV1, ABCA1**, AMPD2, **ABCG1**	Cell metabolism (CM)	8,82%
GO:0046034	ATP metabolic process	**HSPA1A, HSPA1B**, AMPD2, CTNS, HSPA8		
GO:0045821	Positive regulation of glycolytic process	MLXIPL, IGF1, MYC, MIF		

Genes in bold: up-regulated genes; genes in normal typing: down-regulated genes. Biologic categories: cell cycle (CC); immune response (IR); wound healing (WH); lipid metabolism (LM); regulation of transcription (TX); apoptosis (AP); protein folding (PF); stress response (RS) with their significance of regulation calculated as the percentage of number of DEGs belonging to each pathway over the total number of DEGs related to the comparison between ME and Estrous.

4.5.2 Updating pathways with literature evidences

We also evaluated whether the list of DEGs from the ME/E comparison contained genes belonging to the four pathways identified through my personal literature search. Table 6 indeed shows that some DEGs are involved in pathways, such as the homeostasis of cholesterol, extracellular matrix components or enzymes and phagocytosis, with statistical significance.

Table 6. List of DEGs present in the ME/E comparison that are known to be involved in the pathways identified through my personal literature search.

Ontologies Name	DEGs	Grouped categories	Significance of regulation
Cholesterol homesostasis	**ABCA1**, **ABCG1**, AMPD2, ANGPTL4, APOC1, **ARG1**, **CAV1**, CYP11A1, CYP26A1, DGKE, **HGD**, **HPN**, **LPL**, NR1D1, PDK4, PLIN2, **SLC7A8**	Cholesterol homesostasis	10,00%
Extracellular matrix	CLDN20, COL18A1, **FSCN1, HPN**, HSP90AA1,HSPA8, HSPD1, **IGF1**, ITGA7, **ITGB3, LPL**, **LRRK2**, **MYO1E, NTN4,** SERPINE2,**TGM2**	Extracellular matrix	9,41%
Phagocytosis	**ABCA1, C3**, **ELMO2**, HSPA8, **MARCO**, **PLD1, TGM2**	Phagocytosis	4,11%

Genes in bold: up-regulated genes; genes in normal typing: down-regulated genes. Biologic categories related to the just individuated four major additional pathways regulated by estrogen in macrophages: cholesterol homeostasis, extracellular matrix components or enzymes and phagocytosis with their significance of regulation calculated as the percentage of number of DEGs belonging to each pathway over the total number of DEGs related to the comparison between ME and Estrous .

4.6 Comparison between pathways modulated by the endogenous or exogenous estrogen surge

The effects of the endogenous surge of estrogens were compared with those obtained by E_2 injection. Results indicate that, with the exception of circadian rhythm, all pathways are in common between the two hormonal conditions, as shown by Figure 9. Indeed, also the DEGs list from the exogenous administration fo estrogen shows 3 genes that appear to be regulated also in this condition, but did not show up in our analyses due to low statistical significance. We thus believe that also this pathway is modulated by the exogenous administration of estrogen.

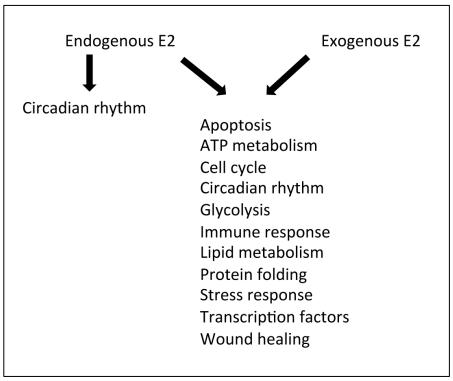

Figure 9. Biological pathways regulated by endogenous and exogenous estrogen. Data derive from the comparisons of the ME/ME+3hE_2, ME/ME+24hE_2 and ME/E groups.

Data reported in Figure 10 shows the comparisons of the two hormonal conditions represented as the percentages of regulated genes in each pathway with respect to the total number of DEGS in the respective comparison. Data in Figure 11 shows a similar analysis conducted on the pathways identified by literature search.

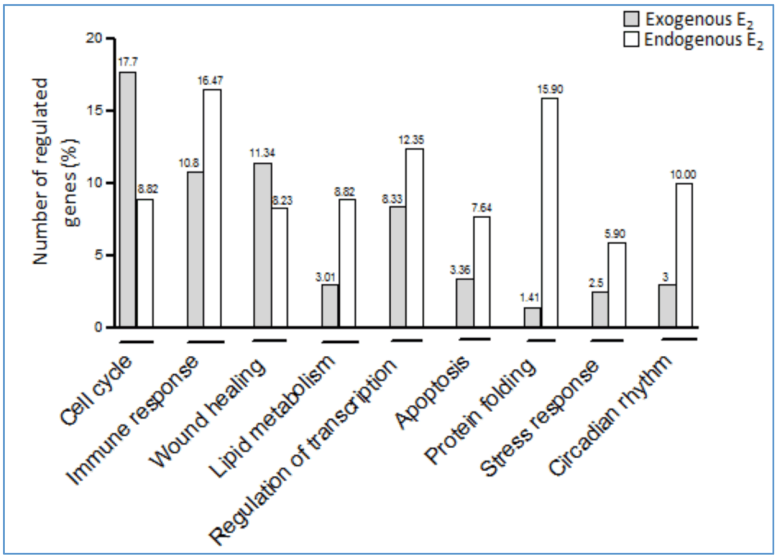

Figure 10. The histogram shows a quantitative representation of the regulation of biological pathways emerged by GO analyses of the comparisons among ME/ME+3hE_2, ME/ME+24hE_2 (exogenous E_2, grey columns) and ME/E (endogenous E_2, white columns).

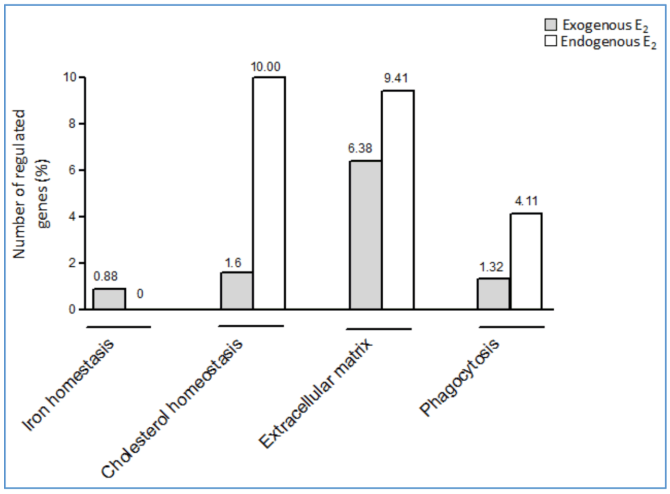

Figure 11. The histogram shows a quantitative representation of the regulation of biological pathways emerged by litearature search in the comparisons among ME/ME+3hE_2, ME/ME+24hE_2 (exogenous E_2, grey columns) and ME/E (endogenous E_2, white colums).

4.7 Validation of estrogen target genes obtained by RNA sequencing

With the aim to confirm the ability of estrogen to modulate gene transcription in macrophages as suggested by our RNA sequencing experiment, some genes representative of the pathways more potently regulated by estrogen were analysed in a novel set of animals, treated as subgroups a-d, group 1) of the ngs experiment. Immunosorted pM were extracted from female in ME, ME+3hE_2, ME+24hE_2 and in E phase, RNA purified and analysed for the expression of typical M2 polarization, cell cycle progression and lipid metabolism. Panels A and B of Figure 12 show that the exogenous administration or the endogenous surge of estrogen induce an increase in the mRNA coding for Tgm2 and CD206; these genes encode for proteins involved in M2 polarization of macrophages. Similarly, Figure 12 C shows that the mRNA levels of lipoprotein lipase (Lpl), an enzyme involved in lipid metabolism, are increased by the short term administration of E_2 and in the Estrous phase, confirming the data obtained by ngs. Finally, cell cycle gene expression, such as those encoding for Ki67, Ube2c and E2f1 are also induced by the hormone in a time-dependent manner, as shown in Figure 12 D-F. The values of dCT are reported in Supplementary Table 8, 9 and 10.

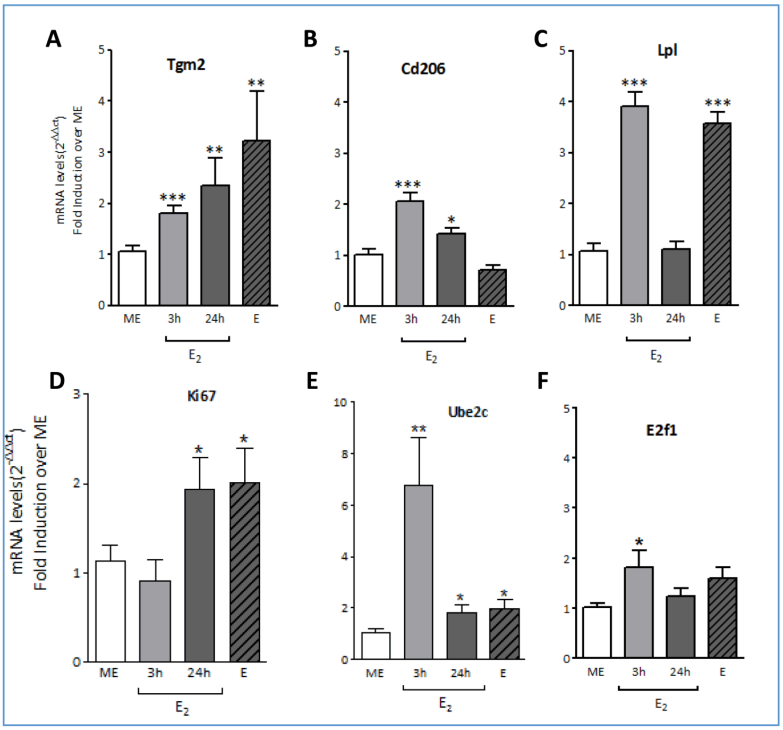

Figure 12. Validation of *in vivo* estrogen target genes in pM. Peritoneal macrophages were obtained from female mice in metaestrous (ME), following 3h or 24h treatment with 5μg/kg E_2 or in Estrous (E). The RNA extracted from these cells was analysed by realtime PCR for the expression of genes identified in ngs experiment. Data were calculated using the 2^{-ddCt} method with respect to the ME group. Bars represent mean values +/-SEM (n=5-10). Student's unpaired *t*-test, *p<0.05; **p<0.01; ***p<0.001 *versus* ME.

5. Discussion

The aim of my work was to have a deeper understanding of estrogen action in macrophages *in vivo.*

Our project was founded by the Cariplo Foundation; we proposed to perform a genome wide analyses of the mRNA present in macrophages under different estrogen levels in order to have thorough description of all possible genomic responses triggered by this hormone.

With my initial work, I performed a series of studies to select the best cellular model to perform such study, either macrophages obtained from female mice or macrophages cells grown in culture. Although avoiding the use of animals, the latter system showed substantial differences in estrogen signaling compared to the *in vivo* macrophages, which led us to opt for an *in vivo* experimental setting. In fact, from first analyses conducted on other types of macrophages, such as peritoneal macrophage cultured and treated *in vitro* and the bone marrow derived macrophage and treated on culture, it is possible to observe that, although these macrophage populations maintain the expression of estrogen receptors and thus are able to respond to the treatment with estrogens, cell culturing leads to a one tenth reduction in receptor mRNA levels and in its transcriptional activity.

We thus analyzed by a next generation sequencing technique peritoneal macrophages isolated from female mice under different hormonal conditions and following treatment with exogenous estrogen. We reasoned that this experimental setting would allow us to understand the physiological responses of macrophages induced by fluctuations in estrogen levels, in the absence of any another stimulus.

Data were submitted to various bioinformatic analysis lead us to group differentially expressed genes in distinct functional biological classes. The comprehensive list of DEGs obtained indicate the specific molecules that are susceptible to regulation by endogenous or exogenous estrogen increases; they represent the molecular mediators of the estrogen-macrophage interplay that, although still indicative of a biological response, provide clues for understanding macrophage responses. In fact, the study performed by other scientists in the lab proved at the biological level the molecular evidence obtained by the experiment. Specifically, Giovanna Pepe investigated the proliferative and polarization effects induced by estrogen in macrophages; the indications obtained by the comparative analyses presented in my thesis I am able to propose additional mechanisms, such as cholesterol and iron homeostasis, circadian rhythm and protein folding, which are worth analyzing in the future. In fact, the genes that belong to these pathways were shown to be under estrogen control both in the ME/E comparison as well as ME/exogenous E_2 analyses, strongly suggesting that these are indeed molecular players of biological reactions under hormonal regulation.

Thus, I believe that my results contributed to provide a relevant insight into our understanding of dialogue between estrogen and macrophages.

6. Conclusions and future plans

The knowledge of new mechanisms by which estrogen regulates the activity of macrophages will be useful to start understanding the physiologic role of this interplay, possibly expanding these information to pathologic inflammatory conditions in which estrogen is also involved, such as in endometriosis, uterine tumors, infertility and reproductive pathologies.
Future studies will:

1. analyze group 2 (ovx replaced with E_2) and group 3 (male mice), a study that will certainly provide interesting informations.
2. extend our observation also to other tissue macrophages.
3. evaluate whether cells that are proliferating are the same that polarize and express ERs.
4. tackle the pathological aspects, by extending the information obtained in this study to human endometriosis or other reproductive pathologies.
5. study the activity of drugs that act on the estrogen receptor, for which it is still unknown whether they behave like agonists or antagonists of estrogen receptor in macrophages; the identity of target genes for different biological processes is available through the present study.

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8 Acknowledgments

I really want to thank my tutor prof. Elisabetta Vegeto for give me the possibility to work in her laboratory and to carry out this hard and fascinating research, hanging around me during all years of phD course and correcting me every time I was wrong, every time with kindness and delicacy.

I want to thank prof. Adriana Maggi for having permit me to learn more than I have imagined before to start this adventure and for let me to work together with a very competent professionists and great colleagues. Giovanna Pepe, Clara Meda, Sara dellaTorre, Roberta Fontana, Valeria Benedusi, Cristina Vantaggiato, Paolo Ciana, Monica Rebecchi, Alessandro Villa, Federica Lolli, Giulia Dell'Omo, Federica Mornata, Elisa Alessandrini, Francesca Moro, Stefania Locatelli, Chiara Sgaragli, Linda Tassetti, Elena Albizzati, Anna Garanzini, Roberta Danza, Marta Clerici have shared with me great and difficult moments during all these years, every day with a smile on face and give me technical assistance when my experiments appeared catastrophics.

I am deeply grateful with Malvina Hoxha, Ashish Dhyani, prof. Elisabetta Gianazza, Stefano Manzini, Marco Busnelli, Giulia Ganzetti, Federica Dellera, prof. Giulia Chiesa, Jerry for their friendship.

Finally, I am immensely grateful to all members of my family: without your love I would not have had the strenght to reach this objective.

This work was supported by grants from the European Union's Seventh Framework Program (FP7/2007-2013; grant no. 278850 INMiND; grant no. 281608 TIMER), the CARIPLO Foundation (grant no. 2011–0591), and Italian Association for Cancer Research (AIRC; grant IG 2013 no. 14685).

4.9 Supplemental data

Supplementary Table 1. Cell and RNA recovery related to murine peritoneal macrophages and BMDM used during the entire period of my PhD program.

Cellular type		name of experiment (and data)	Age of animal (months)	N°	N°cells/ animal	RNA recovery (µg/10^6 cell)	Summary (average)	
							N° cells	RNA (µg/10^6 cell)
Peritoneal Mφ ex vivo	Pilot experiments	Exp #7/8 (4/12/12)	3	1	4,5 ×10^6	0,4	4,5 ×10^6	0,55
				2	4,5 ×10^6	0,7		
		Exp #9/10 (28/1/13)	3	1	3 ×10^6	1,4	2,95×10^6	1,1
				2	2,9 ×10^6	0,8		
		Exp pilota (23/4/13)	4	1	2,7 ×10^6	2,5	4,98 ×10^6	1,57
				2	3,7 ×10^6	2,6		
				3	6,3 ×10^6	1,8		
				4	3,7 ×10^6	2,3		
				5	3,5 ×10^6	0,8		
				6	8,4 ×10^6	0,3		
				7	6,6 ×10^6	0,75		
	RNA sequencing	Exp RNA seq Metaestrous (1/7/13)	4	1	1,04 ×10^6	2,56	1,91×10^6	3,1
				2	3 ×10^6	4,2		
				3	2 ×10^6	4,8		
				4	1,6 ×10^6	6		
		Exp RNA seq Metaestrous+3h (1/7/13)	4	1	3,7 ×10^6	1,6	4,04×10^6	1,24
				2	6,2 ×10^6	2,2		
				3	5,7 ×10^6	2		
				4	1,2 ×10^6	0,3		
				5	3,4 ×10^6	2,1		
		Exp RNA seq Metaestrous+24h (1/7/13)	4	1	2,9 ×10^6	1,5	4,2×10^6	1,9
				2	4,8 ×10^6	1,1		
				3	4,2 ×10^6	2,5		
				4	4,6 ×10^6	2,5		
		Exp RNA seq Estrous (1/7/13)	4	1	2,0 ×10^6	1,52	1,82×10^6	3,7
				2	1,5 ×10^6	5,5		
				3	1,8 ×10^6	6,2		
				4	2 ×10^6	1,76		
Peritoneal Mφ in vitro	Pilot experiments	S Masiero (23/6/12)	6	1	7,4 ×10^6	0,76	4,9×10^6	0,55
				2	2,4 ×10^6	0,4		
			7	1	2,9 ×10^6	0,4	6,9×10^6	0,65
			6	2	10,9 ×10^6	0,9		
		Exp #7 (4/12/12)	3	1	21,3 ×10^6	0,4	21.3×10^6	0,45
				2	21,3 ×10^6	0,4		
				3	21,3 ×10^6	0,5		
		Exp #9 (28/1/13)	3	1	7 ×10^6	2,8	7×10^6	2,7
				2	7 ×10^6	3,2		
				3	7 ×10^6	2,1		
		Exp #10 (14/2/13)	3	1	2 ×10^6	0,8	2×10^6	0,61
				2	2 ×10^6	0,85		
				3	2 ×10^6	0,24		
		Exp #11 (19/2/13)	10	1	12 ×10^6	0,2	12×10^6	0,28
				2	12 ×10^6	0,4		
				3	12 ×10^6	0,24		
BMDM	Pilot experiments	BMDM #1(10/12/15)	4	1	5 ×10^6	4	5×10^6	3,7
				2	5 ×10^6	2,9		
				3	5 ×10^6	4		
				4	5 ×10^6	4		
		BMDM #2 (8/1/16)	4	1	5,2 ×10^6	4,2	5,2×10^6	3,9
				2	5,2 ×10^6	4,2		
				3	5,2 ×10^6	5,3		
				4	5,2 ×10^6	2,3		
		BMDM #3 (27/1/16)	3	1	11,5 ×10^6	10,2	11,5×10^6	9,5
				2	11,5 ×10^6	10,6		
				3	11,5 ×10^6	10,6		
				4	11,5 ×10^6	9,06		
		BMDM S Masiero (23/6/12)	6	1	47 ×10^6	5,9	36,5×10^6	7,2
			6	2	50 ×10^6	8		
			7	3	40 ×10^6	7,2		
			6	4	9 ×10^6	7,5		

Supplementary Table 2. Semiquantitative RTPCR results of the expression of estrogen receptors isoforms (ERα, ERβ and GPR30) mRNAs levels among different macrophage cell populations. pMp *ex vivo*: peritoneal macrophages *ex vivo*, pMP *in vitro*: peritoneal macrophages *in vitro*; BMDM: bone marrow-derived macrophages.

		36B4		ER α						% vs utero	ER β						% vs utero	Gpr 30						% vs utero
		Ct	Media	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}		Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}		Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	
Uterus	1	16,33 16,14	16,23	18,91 18,94	18,92	2,69		0,15	0,90	89,96	27,45 27,17	27,31	11,08		0,00	1,00	100,00	22,86 23,05	22,95	6,72		-0,74	1,67	147,20
	2	17,21 17,10	17,16	19,76 19,35	19,55	2,40		-0,15	1,11 1,01	110,04 100,00	31,55 31,38	31,46	14,31		3,23	0,11	10,66	25,37 25,35	25,36	8,20		0,74	0,60 1,13	52,80 100,00
Liver	1	15,57 16,37	15,97	20,31 19,70	20,01	4,04		1,50	0,35	35,28	25,78 26,41	26,09	10,13		-0,95	1,94	193,68	25,28 25,35	25,31	9,75		2,28	0,21	18,10
	2	15,72 15,57	15,64	18,21 18,59	18,40	2,76		0,22	0,86	85,64	32,38 31,14	31,76	16,12		5,04	0,03	3,04	25,82 26,00	25,91	10,19		2,73	0,15	13,29
pM ex vivo	Me 8	19,22 19,17	19,19	27,91 28,36	28,14	8,94		6,40	0,01	1,18	34,62 36,36	35,49	16,30		5,22	0,03	2,69	30,19 30,16	30,18	10,98		3,52	0,09	7,67
	Me 9	18,35 18,63	18,49	27,15 27,35	27,25	8,76		6,22	0,01	1,33	33,84 32,87	33,36	14,87		3,79	0,07	7,23	29,95 30,17	30,06	11,57		4,11	0,06	5,11
pMP in vitro	1 3h	17,54 17,69	17,61	28,58 28,78	28,68	11,07	2,54	8,53	0,00	0,27	36,28 35,20	35,74	18,13	11,08	7,05	0,01	0,76	31,38 30,80	31,09	13,48	7,46	6,02	0,02	1,36
	4 3h	17,68 17,54	17,61	28,29 28,03	28,16	10,54		8,00	0,00	0,39	33,91 32,86	33,39	15,77		4,69	0,04	3,86	30,24 30,29	30,27	12,65		5,19	0,03	2,41
BMDM	3 3h	17,90 18,06	17,98	27,76 27,11	27,44	9,45		6,91	0,01	0,83	33,63 34,29	33,96	15,98		4,90	0,03	3,35	28,90 29,30	29,10	11,12		3,66	0,08	6,99
	4 3h	18,09 17,90	18,00	27,26 27,44	27,35	9,36		6,81	0,01	0,89	32,70 34,44	33,57	15,57		4,49	0,04	4,44	28,96 28,63	28,80	10,80		3,34	0,10	8,72

Supplementary Table 3. sqRTPCR results of the expression of two estrogen regulated genes apoE and Tgm2 mRNAs levels among different macrophage cell populations. pMp *ex vivo*: peritoneal macrophages *ex vivo*, pMP *in vitro*: peritoneal macrophages *in vitro*; BMDM: bone marrow-derived macrophages.

		36B4		Apo E						Tgm2					
		Ct	Media	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}
pM ex vivo	1	18,90 19,06	18,98	16,20 16,17	16,18	-2,80	-2,66	-0,14	1,10	28,89 28,96	28,92	9,94	10,05	-0,11	1,08
	2	19,39 19,33	19,36	17,60 17,60	17,60	-1,76		0,90	0,54	29,82 29,90	29,86	10,50		0,46	0,73
	8	19,37 19,02	19,19	16,01 16,52	16,27	-2,93		-0,27	1,21	Undetermined Undetermined					
	10	19,75 19,28	19,52	16,31 16,44	16,37	-3,14		-0,49	1,40	28,86 29,56	29,21	9,69		-0,35	1,28
	29	21,15 21,87	21,51	20,63 20,91	20,77	-0,74		1,92	0,27	30,51 30,77	30,64	9,13		-0,92	1,89
	30	20,07 19,82	19,94	17,70 17,94	17,82	-2,12		0,53	0,69	30,64 30,25	30,44	10,50		0,45	0,73
	31	21,27 21,27	21,27	19,43 19,56	19,50	-1,78		0,88	0,54	29,89 29,88	29,88	8,61		-1,43	2,70
	32	19,44 19,37	19,40	19,80 20,06	19,93	0,53		3,18	0,11	25,49 25,41	25,45	6,05		-4,00	16,00
	33	20,44 20,29	20,37	18,11 18,47	18,29	-2,08		0,58	0,67	28,27 28,12	28,20	7,83		-2,22	4,65
	34	20,37 20,63	20,50	17,48 18,02	17,75	-2,76		-0,10	1,07	28,24 28,43	28,34	7,83		-2,21	4,64
	36	21,82 21,97	21,89	21,12 21,45	21,29	-0,61		2,05	0,24	30,84 30,97	30,90	9,01		-1,04	2,05
	37	19,66 19,38	19,52	18,10 18,32	18,21	-1,31		1,35	0,39	26,87 26,64	26,75	7,23		-2,81	7,02
	38	22,11 22,45	22,28	19,77 19,98	19,87	-2,41		0,25	0,84	29,58 29,74	29,66	7,38		-2,67	6,34
	39	19,29 19,29	19,29	17,54 17,69	17,61	-1,67		0,98	0,51	26,92 26,58	26,75	7,46		-2,58	5,99
	40	18,99 19,05	19,02	17,76 17,63	17,70	-1,33		1,33	0,40	27,73 27,85	27,79	8,76		-1,28	2,43
	41	19,69 19,99	19,84	17,98 18,63	18,30	-1,54		1,12	0,46	29,35 29,12	29,24	9,40		-0,65	1,57
pMP in vitro	1 veh 3h	19,90 20,03	19,96	17,63 17,84	17,74	-2,23	-2,18	-0,04	1,03	26,36 26,37	26,37	6,40	6,43	-0,02	1,02
	4 veh 3h	19,65 19,55	19,60	17,42 17,50	17,46	-2,14		0,04	0,97	26,16 25,94	26,05	6,45		0,02	0,98
	3 $E_2$3h	20,17 20,41	20,29	17,95 18,09	18,02	-2,27		-0,08	1,06	25,31 25,07	25,19	4,91		-1,52	2,87
BMDM VII	3 veh 3h	17,46 17,76	17,61	16,74 16,85	16,79	-0,81	-0,76	-0,06	1,04	22,94 23,03	22,99	5,38	5,52	-0,14	1,10
	4 veh 3h	17,60 17,75	17,68	16,99 16,97	16,98	-0,70		0,06	0,96	23,34 23,35	23,34	5,67		0,14	0,91
	5 E_2 3h	17,62 17,56	17,59	16,91 16,92	16,91	-0,68		0,08	0,95	22,18 22,34	22,26	4,67		-0,86	1,81
	6 $E_2$3h	17,50 17,42	17,46	16,60 16,71	16,66	-0,80		-0,04	1,03	22,44 22,35	22,40	4,94		-0,59	1,50
BMDM IX	1 veh 24h	17,51 17,72	17,61	16,97 16,96	16,97	-0,64	-0,63	-0,01	1,01	22,41 22,62	22,52	4,90	4,94	-0,04	1,03
	2 veh 24h	17,85 17,90	17,88	17,23 17,29	17,26	-0,62		0,01	0,99	22,87 22,84	22,86	4,98		0,04	0,97
	5 E_2 24h	17,74 17,97	17,86	17,22 17,38	17,30	-0,56		0,08	0,95	22,20 22,24	22,22	4,36		-0,58	1,49
	6 E_2 24h	18,13 18,14	18,14	17,34 17,30	17,32	-0,82		-0,19	1,14	22,19 22,45	22,32	4,18		-0,76	1,69

Supplementary Table 4. Differentially regulated genes (DEGs) in macrophages following 3 and 24 hours of estrogen administration to Metaestrous female (Group 1) using for the experiment of RNA sequencing, that are listed in according to their logFC. Moreover, the table shows the number of ERE in the promoters of genes and the ontologies where DEGs are included.

GENE	ERE	logFC 3h	logFC 24h	Cluster	Ontology	Correspondence with Me/E matching
Vegfa	1	3,22		I	IR; WH; TX; AP	
H2-M3		1,97		I	IR	
Lbx2		1,80		I	TX	
Rpl39l		1,56		I	n.s.	
Hgd		1,53		I	n.s.	+
Fam187b		1,47		I	n.s.	
Prelid2		1,43		I	n.s.	
Ubxn6		1,41		I	n.s.	
Gsg1		1,40		I	n.s.	
Cdk1		1,39		I	CC; SR	
Ofcc1		1,32		I	n.s.	+
Rad51ap1		1,27		I	SR	
Ckap2l		1,22		I	n.s.	
Kif22		1,18		I	SR	
Oaf		1,17		I	n.s.	
Mxd3		1,16		I	TX	
Itga7		1,15		I	n.s.	
Spc25		1,15		I	CC	
Cd46		1,13		I	n.s.	
Trappc1		1,13		I	n.s.	+
Egr3		1,12		I	TX	
Cenph		1,09		I	CC	
Depdc1b		1,08		I	n.s.	
Il1b		1,08		I	IR; WH; LM	
Plekhf1		1,07		I	AP	
Chaf1b		1,07		I	CC; TX; SR	
Cdca5		1,06		I	CC	
Pole	1	1,04		I	SR	
Lpl	3	1,03		I	n.s.	+
Tgfbr3		1,03		I	WH; TX	
Fam101b		1,01		I	n.s.	
Cdca7		0,99		I	TX	
Sox7	1	0,97		I	TX	
Gmnn		0,95		I	CC	
Plod3		0,95		I	n.s.	
Asf1b		0,90		I	TX	
Cxcr7		0,83		I	n.s.	+
Lonrf3		0,83		I	n.s.	+
Loxl2		0,81		I	n.s.	
Nlrp3		0,79		I	IR; WH; TX; AP	
Tipin		0,76		I	SR; CC	
Nr4a1	1	0,76		I	TX; AP	
Hes1		0,71		I	TX	
Tacc3		0,70		I	CC; WH	
Itgb3		0,69	-0,63	I	n.s.	-
Fabp7	1	0,67		I	n.s.	+
Slpi	1	0,65		I	n.s.	
Cdkn1a	1	0,63		I	CC; AP; SR	
Id2		0,63		I	WH; TX	
Klf4		0,62		I	TX	
Rab27a		0,52		I	IR; WH; AP	
Lrrk2		0,51		I	n.s.	+
Cdc42ep4	1	0,47		I	n.s.	
Ccr2		0,44		I	IR; WH	
Fhit		-2,17		I	n.s.	
Ccl5		-2,14		I	IR; WH	
Ms4a4b		-2,12		I	n.s.	+
Il7r	1	-1,89		I	IR; WH	
Cldn20		-1,59		I	n.s.	
Lat		-1,56		I	IR; WH	
Il2rb	2	-1,45		I	AP	
Slc18a2		-1,43		I	n.s.	
Mylpf	2	-1,42		I	n.s.	
Gm20735		-1,36		I	n.s.	
Serpine1	1	-1,27		I	n.s.	
Dnajb13		-1,24		I	PF	
Oas1g		-1,24		I	IR	
Gm14393		-1,17		I	n.s.	
Dhrs13		-1,16		I	n.s.	
Sec16b		-1,15		I	n.s.	
Bri3		-1,13		I	n.s.	
Per1	1	-1,13		I	TX	+
Ifitm1		-1,10		I	n.s.	
Tcf7	1	-1,09		I	TX; AP	+
Tmem220		-1,02		I	n.s.	
Cyp11a1		-1,01		I	n.s.	
Lyve1		-0,81		I	n.s.	+
Usp18		-0,80		I	n.s.	
Dnajb1		-0,68		I	PF	
Per3		-0,63		I	TX	+
Tbc1d9		-0,61		I	n.s.	
Ccr7		-0,57		I	IR	
Frmd4b		-0,57		I	n.s.	
Ltb	1	-0,51		I	IR; WH; AP	
Pou2f1		-0,49		I	TX	
Manf		-0,47		I	n.s.	
March1		-0,44		I	n.s.	
Chaf1a	1	1,55	1,34	II	CC; TX; SR	
S100a5	1	1,37	1,45	II	n.s.	
Zfp580		1,32	1,03	II	TX	
Bub1b		1,30	1,38	II	CC	
Prc1		1,22	1,23	II	CC	
Cx3cr1	1	1,20	1,13	II	IR; AP	+
Ccr6		1,16	1,29	II	n.s.	
Cenpk		1,14	1,20	II	TX	
Nusap1		1,08	1,19	II	CC	
Serpinb2		1,03	1,00	II	n.s.	+
Ube2c		1,00	1,07	II	CC	
Fam217b		0,95	0,95	II	n.s.	
Slc16a3		0,92	0,59	II	n.s.	
Ldlr		0,82	0,47	II	IR; LM	
Mcm5		0,82	0,78	II	TX	
Uhrf1		0,77	0,81	II	CC; TX; SR	
Lig1		0,76	0,83	II	CC; SR	
Ptgir		0,74	0,50	II	n.s.	
Hk2		0,72	0,60	II	n.s.	
Incenp	2	0,67	0,60	II	CC	
Mapk6		0,64	0,51	II	CC	
Adam8		0,63	0,61	II	n.s.	
E2f3		0,60	<0.4	II	CC; TX	
Bcl3		0,60	<0.4	II	IR; WH; TX; AP; SR	
Fam171a1		0,59	<0.4	II	n.s.	
Slc9a9		0,59	<0.4	II	n.s.	
Cd40		0,58	<0.4	II	n.s.	+
Cdkn2d		0,57	<0.4	II	CC	
Arf2		0,57	0,45	II	n.s.	
Dram1		0,57	<0.4	II	n.s.	+
Ripk3		0,56	0,57	II	AP	
Kremen1		0,54	<0.4	II	n.s.	
Abcg1	1	0,54	<0.4	II	LM; TX	+
Padi4	1	0,53	<0.4	II	TX	+
5430435G22Rik /Rab7b		0,51	<0.4	II	n.s.	
Arhgef10l		0,50	<0.4	II	n.s.	+
Steap3		0,49	0,57	II	CC	
Plscr1	1	0,46	<0.4	II	WH	
B430306N03Rik /Treml6	2	0,45	0,46	II	n.s.	
Dmpk		0,44	<0.4	II	n.s.	
Mcm2		<0.4	0,53	II	CC	
Mrgprb2		-5,04	-2,44	II	n.s.	
Mrgprb1		-3,57	-1,21	II	n.s.	
Angptl4	2	-2,76	-1,51	II	LM; AP; SR	
Slc6a4	1	-2,50	-2,32	II	n.s.	
Pdk4	2	-2,13	-2,46	II	n.s.	
Ly6k		-1,86	-1,74	II	n.s.	
Cxcr4		-1,32	-0,83	II	WH	+
Hsph1		-1,29	-1,28	II	PF	+
Wee1	2	-1,23	-1,13	II	CC	+
Dgke		-1,10	-0,74	II	n.s.	+
Mbd1	1	-1,04	-1,09	II	TX	
Jdp2		-0,96	-0.48 (n.s.)	II	TX	
Klf9		-0,92	-0,65	II	TX	
Fam46c		-0,91	-0.74 (n.s.)	II	n.s.	
Gas5,Mir5117		-0,90	-0,95	II	n.s.	
Per2	2	-0,85	-0,81	II	TX	+
Stip1	1	-0,77	-0,58	II	n.s.	+
Cpt1a		-0,77	-0,56	II	n.s.	

Cep85		-0,77	-0,91	II	n.s.	+
Pik3r5		-0,76	-0,79	II	n.s.	
Hsp90aa1		-0,74	<0.4	II	PF	-
Ddit4		-0,74	-0,62	II	n.s.	+
Pgm2l1		-0,73	-0,55	II	n.s.	
Herpud1	1	-0,72	-0,56	II	n.s.	+
Trim65		-0,70	<0.4	II	n.s.	
Sfn		-0,69	<0.4	II	n.s.	-
Sik1		-0,68	-0,54	II	CC	
Dnaja1	1	-0,68	-0,62	II	PF	+
Cacna1e		-0,68	<0.4	II	n.s.	
Mll1		-0,67	-0,60	II	WH; TX	
Tagap		-0,67	-0,56	II	n.s.	
Oas2		-0,66	<0.4	II	IR	
Trim47		-0,66	-0,73	II	n.s.	
Stxbp3a		-0,66	-0,71	II	n.s.	
Trim7		-0,63	-0.56 (n.s.)	II	n.s.	
Nr1d2		-0,62	<0.4	II	TX	-
Erdr1		-0,60	-0,50	II	n.s.	
Tef		-0,60	<0.4	II	TX	-
Ino80d		-0,60	-0,54	II	n.s.	
Rusc2		-0,59	-0,66	II	n.s.	
Slc37a2		-0,59	-0,54	II	n.s.	+
Jmy		-0,58	<0.4	II	CC; TX; AP; SR	
Rnf169		-0,57	<0.4	II	n.s.	
Fkbp4	1	-0,56	<0.4	II	n.s.	-
Tsc22d3		-0,56	<0.4	II	TX; AP	
Atxn1		-0,56	<0.4	II	TX	
Lmo7		-0,51	<0.4	II	n.s.	-
Tfb2m		-0,51	<0.4	II	TX	
Rabgap1l		-0,51	<0.4	II	n.s.	
Ctns	1	-0,51	-0,53	II	n.s.	+
Phf15		-0,50	<0.4	II	n.s.	
Epas1	1	-0,50	-0,56	II	WH; TX; SR	
Mlxip		-0,50	-0,44	II	TX	
Slc16a10		-0,48	<0.4	II	n.s.	
Ypel2		-0,47	-0,49	II	n.s.	
Ptpn22		-0,46	<0.4	II	WH	
Cacybp	1	-0,45	<0.4	II	n.s.	-
Ubfd1		-0,44	<0.4	II	n.s.	
Glcci1	1	-0,44	-0,50	II	n.s.	
Il16	1	-0,43	<0.4	II	IR; TX	
Rc3h1		-0,43	<0.4	II	n.s.	
Ahsa1		-0,43	<0.4	II	PF	-
Crebbp		<0.4	-0,50	II	n.s.	-
Diras2		<0.4	-0,41	II	n.s.	
Bex6			4,46	III	n.s.	
Spp1		1,51	3,96	III	AP	
Arg1		0,81	2,81	III	WH	+
Trem1	2	1,72	2,09	III	n.s.	
Nuf2		1,41	1,86	III	CC	
Birc5		1,26	1,78	III	CC; AP	
Ccnb2	1	1,38	1,76	III	CC; WH	
Kifc1			1,72	III	CC	
Gins2		1,34	1,71	III	n.s.	
Spc24		1,09	1,70	III	CC	
Kcnmb3			1,57	III	n.s.	
Fignl1	1	1,18	1,47	III	n.s.	
Ccnb1			1,45	III	CC	
Ccnf			1,45	III	CC	
Top2a	1	1,14	1,43	III	TX	
Mcm10		1,07	1,41	III	n.s.	
Camkk1	1		1,40	III	n.s.	
Gm16907			1,39	III	n.s.	
Cdca3			1,36	III	CC	
Mag			1,35	III	n.s.	
Kif11			1,29	III	CC	
Rn45s		0,59	1,26	III	n.s.	+
Slc35g2			1,25	III	n.s.	
Cryzl1	1		1,25	III	n.s.	
Mki67		0,94	1,24	III	CC	

Stmn1		0,89	1,24	III	CC	
Timeless	1		1,19	III	CC	
Tpx2			1,16	III	CC	
Fxn			1,15	III	n.s.	
Slc7a8		0,85	1,13	III	n.s.	+
Smim5			1,09	III	n.s.	
Gm10814			1,08	III	n.s.	
Dtl			1,07	III	n.s.	
Mefv			1,07	III	WH	
Cdc6			1,04	III	CC	
Ccr5		0,50	1,02	III	IR; WH	
Kif23			1,02	III	n.s.	
Syce2			1,01	III	CC	
Echdc3			1,01	III	n.s.	
Lair1		0,54	0,99	III	n.s.	
Dnajb5	1		0,98	III	n.s.	
Rrm2	2		0,95	III	n.s.	
Tgm2	1		0,94	III	WH	+
Slfn9			0,94	III	n.s.	
Marco	1	0,46	0,93	III	n.s.	+
Fcgr4		0,45	0,91	III	n.s.	
Tgm1	1		0,91	III	n.s.	
Aurkb			0,86	III	CC	
Arhgap19			0,86	III	n.s.	
Emp1			0,86	III	n.s.	
Layn			0,83	III	n.s.	+
Idi1			0,82	III	LM	
Guca1a	1		0,79	III	n.s.	
Stxbp1	1		0,78	III	IR	
Cd300lf			0,78	III	IR	
Dab2	1		0,77	III	IR	+
Il21r			0,77	III	n.s.	
Ckb			0,76	III	n.s.	+
Cwc27			0,74	III	n.s.	+
Gm6682			0,74	III	n.s.	
Dusp16			0,73	III	n.s.	
Arl4c			0,70	III	n.s.	
Srxn1			0,67	III	n.s.	
Dhcr24	2		0,65	III	LM	
Sel1l3			0,65	III	n.s.	
Kpna2	1		0,64	III	n.s.	
Kank2		0,51	0,64	III	n.s.	
Msr1			0,62	III	IR; LM	
Fcrl5			0,62	III	n.s.	
Pgk1			0,61	III	n.s.	
Fmnl3			0,60	III	n.s.	
Dpysl3			0,60	III	n.s.	+
Pkm			0,60	III	n.s.	
Lbp			0,60	III	IR; WH; LM	
Cd300ld			0,59	III	IR	
Jam2			0,59	III	n.s.	
Pla2g4a	1		0,58	III	n.s.	
Fus			0,57	III	n.s.	
Fgr			0,57	III	n.s.	
C1ra			0,57	III	IR; WH	+
Cd22	1		0,56	III	n.s.	
Phyh			0,56	III	n.s.	
Pdk1			0,56	III	n.s.	+
Rfx5			0,54	III	n.s.	
Rhoh			0,53	III	n.s.	
Lars			0,53	III	n.s.	
Tlr1	1		0,51	III	IR; WH	
Nin			0,50	III	n.s.	
Itga9	1		0,50	III	n.s.	
Ifi30,Pik3r2			0,50	III	n.s.	
Lrrc25			0,49	III	n.s.	
Myo1e			0,49	III	WH	+
Rhoc			0,47	III	n.s.	
Impdh1			0,46	III	n.s.	
Tpi1			0,46	III	n.s.	
Ap2m1	1		0,46	III	n.s.	
Ldha			0,45	III	n.s.	
Jarid2			0,44	III	n.s.	

Serpine2	1		-2,66	III	n.s.	+
Rasl10a		-1,19	-2,55	III	n.s.	
Gm16701			-2,44	III	n.s.	
Fkbp5	2	-1,35	-2,37	III	PF	+
Zbtb16		-1,09	-2,32	III	WH; TX; AP	+
Hspb1		-1,33	-2,29	III	n.s.	
Slc15a2			-2,19	III	n.s.	
Klf15		-1,34	-2,05	III	TX	
Tcf23			-1,91	III	n.s.	
Zfp811		-0,87	-1,89	III	TX	
Rfx2		-0,55	-1,64	III	TX	+
Tppp		-0,51	-1,61	III	n.s.	
Cyp26a1		-0,96	-1,58	III	n.s.	
Plekha6		-0,56	-1,54	III	n.s.	
Gpr114			-1,53	III	n.s.	
Serf1	1		-1,50	III	n.s.	
Map3k6	1	-0,72	-1,49	III	n.s.	+
Shank3	1		-1,46	III	n.s.	
Ralgds		-0,74	-1,45	III	n.s.	+
Lims2			-1,44	III	n.s.	
Prtg			-1,39	III	n.s.	
Krt7			-1,36	III	CC	
Eps8l1			-1,35	III	n.s.	
Apoc1		-0,54	-1,29	III	LM	
Fam222a			-1,28	III	n.s.	
Thbd		-0,60	-1,26	III	WH	
Ryr2			-1,24	III	n.s.	
Tacc2			-1,18	III	CC	
Klf11		-0,68	-1,18	III	WH; TX	
Tmem150b			-1,12	III	n.s.	
Il6			-1,10	III	WH	
Orm2,Orm3			-1,09	III	WH	
Plin2		-0,78	-1,05	III	LM	
Il1rl1	2	-0,64	-1,05	III	IR	
Ccdc15			-1,04	III	n.s.	
Frat2			-1,01	III	n.s.	
Slc10a6			-0,98	III	n.s.	
Tnfsf8			-0,93	III	IR	
Tnfrsf8			-0,92	III	n.s.	+
Cpm			-0,92	III	n.s.	
Ier3			-0,91	III	n.s.	
Cd93		-0,53	-0,90	III	n.s.	
Ccm2l			-0,89	III	n.s.	
Banp		-0,64	-0,89	III	CC	+
Stxbp3b			-0,88	III	n.s.	
Sgms1		-0,60	-0,84	III	WH; AP	
Rai14			-0,84	III	n.s.	
Glul			-0,80	III	n.s.	
Hilpda			-0,80	III	n.s.	
Sult1a1			-0,78	III	n.s.	
Engase		-0,50	-0,77	III	n.s.	
Hist1h2bc			-0,76	III	n.s.	
Nfkbiz			-0,76	III	WH	
Zfp97			-0,75	III	n.s.	
Elovl5			-0,75	III	n.s.	
Mst1r	2		-0,73	III	n.s.	
Snta1	1		-0,73	III	n.s.	
Nrip1	1		-0,71	III	LM	
Irf2bp2			-0,71	III	n.s.	
Zkscan3			-0,69	III	n.s.	
Mdm1			-0,69	III	n.s.	
Cdan1			-0,69	III	n.s.	
Tns1			-0,68	III	n.s.	
Epha2	1		-0,68	III	WH	+
Igf1r			-0,68	III	n.s.	
Dusp6			-0,68	III	n.s.	+
Slc27a1	1		-0,67	III	LM	
Prkar2b	1	-0,49	-0,66	III	n.s.	+
Snhg11			-0,66	III	n.s.	
Chordc1	1		-0,66	III	n.s.	-
Dapk1			-0,64	III	n.s.	
Dhrs3			-0,63	III	n.s.	
Serpine3	1,1		-0,32	III	n.s.	+

Cd300a			-0,62	III	IR	
Sh3bgrl2			-0,61	III	n.s.	
Ulk1	1		-0,61	III	IR	
Notch1			-0,61	III	WH	
Edil3			-0,60	III	n.s.	
Zbtb44			-0,58	III	n.s.	
Mrvi1			-0,57	III	n.s.	
Zfp36l1	1		-0,57	III	WH	
Cds2			-0,56	III	n.s.	
Klhl24			-0,56	III	n.s.	
Cnst			-0,55	III	n.s.	
Dennd4c			-0,55	III	n.s.	
Efcab4a,Pnpla2			-0,55	III	n.s.	
Dnase1l2,E4f1			-0,55	III	CC	
Net1			-0,54	III	n.s.	
2210018M11Rik/Emsy			-0,53	III	n.s.	
Sorbs3			-0,53	III	n.s.	
Pcmtd2			-0,53	III	n.s.	
Ppl	2		-0,52	III	n.s.	
Hspa12a			-0,51	III	n.s.	
Arrdc3			-0,51	III	n.s.	
Zmiz1			-0,51	III	WH	
Fam20c			-0,51	III	n.s.	
Fam46a			-0,50	III	n.s.	
Mob3b			-0,50	III	n.s.	
Pgap1			-0,49	III	n.s.	
Rfwd2			-0,48	III	n.s.	
Ubn2			-0,48	III	n.s.	
Bach1			-0,48	III	n.s.	
Ube2h			-0,48	III	n.s.	
Jag1	1		-0,47	III	n.s.	
Wnt2			-0,47	III	WH	
Clec10a			-0,47	III	n.s.	
Pnpla7			-0,46	III	n.s.	
Dapp1			-0,46	III	n.s.	
Pan3			-0,46	III	n.s.	
Rassf3			-0,45	III	n.s.	
Heca			-0,44	III	n.s.	
Slc25a37			-0,43	III	n.s.	
Zscan26			-0,43	III	n.s.	
Dusp11			-0,42	III	n.s.	
Ccl12			4,10	IV	IR; WH	
Upp1			3,30	IV	n.s.	
Rab3il1			2,31	IV	n.s.	
Chi3l3	1		2,16	IV	WH	
AA467197/Nmes1			2,05	IV	n.s.	
Vcan			2,00	IV	n.s.	
3110057O12Rik/Abhd18			1,95	IV	n.s.	
Rarres2			1,75	IV	n.s.	
Gas6	1		1,68	IV	n.s.	
Ckap2			1,67	IV	CC	
Vpreb3	2		1,66	IV	n.s.	
Siglec1			1,60	IV	n.s.	
Aif1	2		1,55	IV	n.s.	
Ccl22			1,53	IV	IR; WH	
Gm9920			1,50	IV	n.s.	
Asns			1,42	IV	n.s.	
Ucp1			1,40	IV	n.s.	
Isg15	1		1,37	IV	n.s.	
Chst11			1,37	IV	n.s.	
Lars2			1,36	IV	n.s.	+
Rnf128			1,33	IV	n.s.	
Eme1			1,32	IV	n.s.	
Ifit3			1,31	IV	n.s.	
Nxpe5			1,29	IV	n.s.	+
Il2ra			1,27	IV	n.s.	
Cmss1			1,23	IV	n.s.	+
Fdps	2		1,19	IV	LM	
Fcgr1			1,17	IV	IR; WH	
Sfxn5			1,13	IV	n.s.	
Papss2			1,12	IV	WH	
Trem2			1,09	IV	n.s.	
Ngfr			1,08	IV	WH	

Gatm			1,04	IV	WH	
Rsad2			1,03	IV	IR	
Soat2			0,97	IV	LM	
Mvb12b			0,95	IV	n.s.	
Nme4			0,95	IV	n.s.	
Tmem8	1		0,95	IV	n.s.	
Fam213b			0,94	IV	n.s.	
P2ry6			0,94	IV	n.s.	
Folr2			0,93	IV	n.s.	
Irf7			0,87	IV	IR	
Cbr2			0,86	IV	n.s.	
Ifi27l2a			0,86	IV	n.s.	
Slfn1	1		0,85	IV	CC	
Cplx2			0,85	IV	IR	
Ms4a6c			0,83	IV	n.s.	
Mlkl			0,82	IV	n.s.	
Ppapdc1b			0,82	IV	n.s.	
Adap2			0,80	IV	n.s.	
Galk1			0,77	IV	n.s.	
Glrx			0,76	IV	n.s.	
H2-Eb2			0,76	IV	IR	
Marcksl1			0,76	IV	n.s.	
Zbp1			0,75	IV	n.s.	
Nrp1			0,74	IV	WH	
Fpr2			0,72	IV	IR	
Oas3			0,72	IV	IR	
Cpne2			0,71	IV	n.s.	
Pram1			0,70	IV	n.s.	
Cd163			0,68	IV	WH	
Pam			0,68	IV	n.s.	
Nlrc3			0,67	IV	n.s.	
Ms4a6d			0,66	IV	n.s.	
Dhx58			0,64	IV	IR	
Ddx60			0,64	IV	n.s.	
Fcrl1			0,63	IV	n.s.	
Epb4.1l1			0,62	IV	n.s.	
Tnfrsf11a			0,61	IV	n.s.	
Rbm3			0,61	IV	n.s.	
Dhx29			0,57	IV	n.s.	
Pik3ap1			0,56	IV	n.s.	
Rhobtb1			0,55	IV	n.s.	
Slfn5			0,55	IV	n.s.	
Ctsc			0,54	IV	n.s.	
Ckap4			0,54	IV	n.s.	
Acp2	2		0,53	IV	n.s.	
Lpin2			0,52	IV	n.s.	
Plcb1			0,52	IV	n.s.	
Dck			0,52	IV	n.s.	
Atrip,Trex1			0,52	IV	n.s.	
Tpcn2			0,52	IV	n.s.	
Lgals1	3		0,51	IV	n.s.	
Lman1			0,49	IV	n.s.	
Lrrc59			0,49	IV	n.s.	
F13a1			0,49	IV	WH	-
Mrc1			0,49	IV	IR	
Etv5	1		0,49	IV	n.s.	
Rtp4			0,48	IV	n.s.	
Icam1	1		0,48	IV	IR	
Cd38			0,46	IV	n.s.	
Nme1			0,46	IV	IR	
Ncbp1			0,45	IV	n.s.	
Stat1			0,45	IV	n.s.	
Ostc			0,44	IV	n.s.	
Rgs1			-2,44	IV	n.s.	
Mylk3			-1,88	IV	n.s.	
Abca6	1		-1,84	IV	n.s.	
Spa17			-1,72	IV	n.s.	
Hhipl1			-1,71	IV	n.s.	-
Ccl3			-1,66	IV	IR; WH	
Nupr1l			-1,66	IV	n.s.	
Dusp1			-1,49	IV	CC	
Fos			-1,34	IV	n.s.	
Xlr3b			-1,26	IV	n.s.	

Nr1d1	2		-1,19	IV	n.s.	+
Rab44			-1,15	IV	n.s.	
Gm15471			-1,15	IV	n.s.	
Cxcl2	2		-1,10	IV	IR; WH	
Cables1			-1,07	IV	CC	
Hgf	1		-1,03	IV	n.s.	
Gfod1			-1,01	IV	n.s.	
Art3			-1,01	IV	n.s.	
Zfp72			-1,00	IV	n.s.	
Egr1	1		-1,00	IV	n.s.	+
Tppp3			-0,99	IV	n.s.	
Adamtsl4			-0,95	IV	n.s.	
Fosb	1		-0,93	IV	n.s.	
Nt5e			-0,91	IV	n.s.	
Zfp36	2		-0,87	IV	WH	
Ccrl2			-0,87	IV	n.s.	
Tox2			-0,86	IV	n.s.	
Rgs2			-0,85	IV	n.s.	
Ppap2a	1		-0,84	IV	n.s.	
Cyp2ab1			-0,84	IV	n.s.	
Insr			-0,81	IV	n.s.	
Nedd4			-0,79	IV	n.s.	
Tmem62			-0,76	IV	n.s.	
Aqp9			-0,75	IV	n.s.	
Hr	1		-0,74	IV	n.s.	
Fabp4			-0,72	IV	n.s.	
Jun			-0,69	IV	n.s.	
Garnl3			-0,65	IV	n.s.	
Ocln			-0,65	IV	n.s.	
Abca9			-0,64	IV	n.s.	
Rbpms			-0,62	IV	n.s.	
Cd14	2		-0,60	IV	IR; WH	
Fgfr1			-0,60	IV	WH	
Hist1h1c			-0,60	IV	n.s.	-
Slc22a17			-0,60	IV	n.s.	
Tln2			-0,60	IV	n.s.	
Cav1			-0,58	IV	IR; WH; LM	-
Plcb4			-0,58	IV	n.s.	
Nfia			-0,57	IV	n.s.	
Egln3			-0,57	IV	n.s.	-
Ston2			-0,56	IV	IR	
Tgfb2	2		-0,53	IV	IR; WH	
Calcoco1			-0,52	IV	n.s.	
Myadm			-0,51	IV	n.s.	
Pi16			-0,51	IV	n.s.	
Icam2	1		-0,50	IV	n.s.	
Gata6	1		-0,50	IV	n.s.	
Csf3r			-0,50	IV	IR	
Cask	1		-0,47	IV	n.s.	
Lama3			-0,46	IV	n.s.	
Parvb			-0,46	IV	n.s.	
Fcrls			-0,46	IV	n.s.	
Slc9a3r2			-0,46	IV	n.s.	
Junb			-0,45	IV	WH	
Mgst1			-0,43	IV	n.s.	

Supplementary Table 5. Differentially regulated genes (DEGs) in macrophages of Metaestrous and Estrous female (Group 1) using for the experiment of RNA sequencing, that are listed in according to their logFC. Moreover the table shows the number of ERE in the promoters of genes and the ontologies where DEGs are included.

GENE	fpkm ME	fpkm E	logFC	Ontology	Comparison with Me, Me+3h E_2, Me+24h E_2 matching
Ofcc1	3,86	13,18	1,77		+
Lpl	35,32	107,92	1,61	CM	+
Cx3cr1	0,76	2,28	1,59	IR	+
Rnd2	0,74	2,17	1,56		
1700023L04Rik/Smk	0,77	2,23	1,53		
Lonrf3	1,39	3,97	1,52		+
Rprm	1,56	4,39	1,50		
Hgd	1,08	2,85	1,41	CR	+
Arntl	4,25	10,95	1,37	CR; CC	
Cxcr7	8,35	21,52	1,37		+
Cmss1	96,07	244,63	1,35		+
Trappc1	1,08	2,73	1,34		+
Rn45s	111,50	262,58	1,24		+
Lars2	52,38	123,20	1,23		+
Slc7a8	4,74	11,08	1,22		+
1810024B03Rik	1,17	2,73	1,22		
3300005D01Rik	1,02	2,37	1,21		
Saa3	4790,23	11029,00	1,20		
Snhg1, Snord22	71,74	159,32	1,15		
Olr1	1,35	2,94	1,12	IR	
Ntn4	1,06	2,31	1,12		
Pyroxd2	2,33	4,88	1,07		
Marco	141,79	295,19	1,06	IR	+
Zfp825	1,23	2,48	1,01		
Als2cl	2,45	4,46	0,87		
Stard13	2,46	4,49	0,87		
Lgalsl	1,32	2,41	0,87		
Bcl2a1b	41,86	75,42	0,85	AP	
Rims3	2,56	4,55	0,83		
Layn	3,94	6,83	0,79		+
Nfil3	9,34	16,08	0,78	CR	
Cadps2	3,47	5,96	0,78		
Clec12a	3,26	5,54	0,77		
Serpinb2	159,14	269,00	0,76	AP	+
Zc3h6	1,62	2,74	0,76		
C3	75,88	126,79	0,74	IR	
Tnfrsf23	2,23	3,73	0,74		
Tnfrsf26	3,89	6,44	0,73	IR	
Rassf8	4,36	7,19	0,72		
Tgm2	4,39	7,21	0,72	IR	+
Izumo4	7,63	12,35	0,70		
Epha4	2,88	4,66	0,69		
Cwc27	4,83	7,70	0,67	PF	+
Igf1	9,65	15,37	0,67	CC; AP; CM; RH	
Fscn1	29,71	46,81	0,66		
Armc2	7,76	12,19	0,65		
Nxpe5	17,56	27,42	0,64		+
Rap1gap2	18,22	28,16	0,63	AP	
Hpn	9,11	14,06	0,63	CM	
Tnik	1,65	2,54	0,62		
Hp	88,54	136,12	0,62	IR	
Bmp2	5,91	9,08	0,62	TX; IR; RH	
Cfb	177,83	272,03	0,61	CC; IR	
Cald1	28,32	43,26	0,61		
Mlxipl	31,10	47,25	0,60	TX; CM	
Abca1	24,51	37,24	0,60	CM	
Neat1	276,96	418,97	0,60		
Hist1h1c	17,57	26,44	0,59	TX	-
Hspa1a, Hspa1b	16,29	24,51	0,59	PF; AP; CM	
C1ra	6,41	9,58	0,58	IR	+
Zmym3	4,73	7,07	0,58		
Fbxl4	6,94	10,34	0,58		
Plek	179,61	267,59	0,58		
Pdk1	7,50	11,17	0,57	CC	+
Egln3	11,43	17,00	0,57		-
Cd200r1	13,84	20,54	0,57		
Padi4	298,00	440,58	0,56	IR	+
Fabp7	42,26	62,14	0,56		+
Itgb3	5,01	7,36	0,56		-
Myo1e	25,11	36,69	0,55		+
Dab2	29,48	42,96	0,54	AP; IR	+
Arhgef10l	7,24	10,54	0,54		
H1f0	11,31	16,39	0,54		+
Ckb	52,63	76,13	0,53	PF	
P2rx1	15,40	22,22	0,53		
Elmo2	23,45	33,74	0,52		
Rnf144b	15,89	22,82	0,52		
Enpp5	9,39	13,48	0,52		
1810011O10Rik/AW121743, AW321058	30,84	43,97	0,51		
Ctsf	14,66	20,77	0,50		
Ctsl	61,71	87,39	0,50		
Cav1	13,43	18,97	0,50	CM; IR	-
Ikbke	26,05	36,78	0,50	PF	
Cd40	21,27	29,98	0,50	PF; IR	+
Cxcl12	16,51	23,19	0,49	RH	
Cers6	23,24	32,64	0,49		
Lrrk2	9,19	12,89	0,49		+
Abcc3	7,68	10,77	0,49		
Ptger4	13,41	18,79	0,49	IR	
Arg1	23,11	32,32	0,48	IR	+
Srsf5	172,25	240,63	0,48	CC	
Abcg1	13,78	19,20	0,48	CM	+
Pld1	10,00	13,89	0,47		
Dpysl3	6,89	9,54	0,47		+
Dram1	13,17	18,22	0,47		+
Lpcat2	22,09	30,54	0,47		
Tnfaip2	20,74	28,67	0,47		
Myo9a	3,74	5,16	0,46		
Evi5	41,73	56,22	0,43		
Agtrap	30,54	40,81	0,42	RH	
Col18a1	1,88	1,09	0,00		
Hhipl1	1,91	1,00	0,00		-
Dlgap4	0,61	1,85	0,00		
Ampd2	22,92	16,88	-0,44	CM	
Ptges3	66,43	48,74	-0,45	CC	
Zfp831	5,92	4,34	-0,45		
Phgdh	25,44	18,34	-0,47		
Atp8b2	16,10	11,52	-0,48		
Lmo7	8,88	6,33	-0,49		-
Ctns	13,95	9,81	-0,51		+
Gramd4	30,90	21,68	-0,51		
Ahsa1	79,85	55,96	-0,51	PF	-
Mif	85,50	59,34	-0,53	CC; AP; IR; CM	
Dnajb1	32,41	22,43	-0,53	PF	
Myc	52,68	36,19	-0,54	TX; CC; CM; PF	
Sorl1	11,82	8,12	-0,54		
Hspd1	77,47	53,16	-0,54	PF; AP; IR; RH	
Epha2	32,97	22,63	-0,54	IR	+
Slc37a2	12,39	8,48	-0,55		+
Ccnd2	19,53	13,25	-0,56	CC; AP	
P2ry2	10,60	7,11	-0,58		
Dusp6	57,87	38,63	-0,58		+
Fkbp4	67,58	45,11	-0,58	PF	-
Crebbp	8,83	5,89	-0,58	CR; CC	-
Inpp4b	4,26	2,81	-0,60		
Per1	19,08	12,47	-0,61	CR; PF	+
Tbc1d10a	13,74	8,93	-0,62		
F13a1	135,43	87,64	-0,63		-
Herpud1	101,71	65,30	-0,64		+
Bcl2l1	9,29	5,82	-0,67	TX; CC; AP; RH	
Cacybp	36,05	22,45	-0,68		-
Sfn	14,30	8,91	-0,68	CC	-
Chordc1	38,24	23,76	-0,69		-
Tnfrsf8	10,51	6,49	-0,70	IR	+
Hspa8	1493,78	919,32	-0,70	PF; CC; CM	
Ifrd2	10,51	6,44	-0,71		
Banp	5,32	3,25	-0,71		+
Cep85	95,32	57,56	-0,73		+
Prkar2b	40,78	24,43	-0,74	PF	+
Tppp	27,93	16,43	-0,77		
Egr1	8,59	5,05	-0,77	TX; RH	+
Acot7	8,09	4,73	-0,78		
Per3	8,87	5,17	-0,78	CR	+
Dnaja1	54,59	31,43	-0,80	PF; AP	+
Hsp90aa1	145,33	83,59	-0,80	PF	-
Stip1	71,65	40,45	-0,82	PF	+
Cxcr4	62,30	34,43	-0,86	PF	+
Rfx2	12,01	6,63	-0,86		+
Plk3	5,56	3,05	-0,87	TX; AP	
Ddit4	22,63	12,10	-0,90	CC; RH	+
Map3k6	9,97	5,25	-0,93		+
Dgke	5,72	2,93	-0,97		+
Tef	36,37	18,09	-1,01	CR	-
Cd5	3,03	1,51	-1,01		
Lyve1	32,04	15,23	-1,07		+
Ralgds	6,73	3,09	-1,12		+
Per2	7,77	3,55	-1,13	CR; CC; RH	+
Nr1d2	23,40	10,37	-1,17	CR	-
Id1	3,96	1,76	-1,17	CR; AP; IR	
Ms4a4b	4,51	1,88	-1,26		+
Zbtb16	11,95	4,89	-1,29	TX	+
Hsph1	44,15	17,59	-1,33		+
Slc25a33	2,04	0,78	-1,38		
Wee1	6,17	2,35	-1,39	CC	+
Serpine2	2,23	0,72	-1,63		+
Dbp	14,36	4,62	-1,64	CR	
Gm20594	8,20	2,63	-1,64		
Tcf7	2,53	0,76	-1,74	TX	+
Fkbp5	52,30	12,05	-2,12	PF	+
Nr1d1	19,42	3,89	-2,32	CR	+

Supplementary Table 6. Functional annotation of up-regulated DEGS related to the comparison between ME, ME+3hE_2 and ME+24hE_2, founded using DAVID database with the aim to obtain functional annotation of the genes.

Gene	F.i. 3h	F.i. 24h	ERE	Function	Annotation
Vegfa	3,22		1	secreted protein, vascular endothelial growth factor receptor binding	Growth factor active in angiogenesis, vasculogenesis and endothelial cell growth. Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels. Binds to the FLT1/VEGFR1 and KDR/VEGFR2 receptors, heparan sulfate and heparin.
H2-M3	1,97			Membrane protein. Antigen processing and presentation of peptide antigen via MHC class I, positive regulation of T cell and natural killer cell mediated cytotoxicity	
Lbx2	1,80			Nuclear protein involved in regulation of transcription,DNA-templated	
Trem1	1,72		2	Receptor (secreted and membrane protein) involved in immune response, cell migration, neutrophil chemotaxis and extravasation	Stimulates neutrophil and monocyte-mediated inflammatory responses. Triggers release of pro-inflammatory chemokines and cytokines, as well as increased surface expression of cell activation markers. Amplifier of inflammatory responses that are triggered by bacterial and fungal infections and is a crucial mediator of septic shock.
Rpl39l	1,56			Cytosolic proein involved in translation	This gene encodes a protein sharing high sequence similarity with ribosomal protein L39. It is not currently known whether the encoded protein is a functional ribosomal protein or whether it has evolved a function that is independent of the ribosome.
Chaf1a	1,55		1	Nuclear complex involevd in cell cycle, DNA replication and repair, DNA replication-dependent nucleosome assembly, cellular response to DNA damage stimulus	Core component of the CAF-1 complex, a complex thought to mediate chromatin assembly in DNA replication and DNA repair. Assembles histone octamers onto replicating DNA in vitro. CAF-1 performs the first step of the nucleosome assembly process, bringing newly synthesized histones H3 and H4 to replicating DNA; histones H2A/H2B can bind to this chromatin precursor subsequent to DNA replication to complete the histone octamer. CHAF1A binds to histones H3 and H4. It may play a role in heterochromatin maintenance in proliferating cells by bringing newly synthesized cbx proteins to heterochromatic DNA replication foci
Hgd	1,53			Cytosolic nand extracellular protein involved in: cellular amino acid metabolic process, L-phenylalanine catabolic process, tyrosine catabolic process, oxidation-reduction process	This gene encodes the enzyme homogentisate 1,2 dioxygenase. This enzyme is involved in the catabolism of the amino acids tyrosine and phenylalanine. Mutations in this gene are the cause of the autosomal recessive metabolism disorder alkaptonuria
Spp1	1,51			Secreted protein, involved in neutrophil chemotaxis, ossification, osteoblast differentiation	Secreted phosphoprotein 1 (osteopontin),regulated by CBFA1 and ETS1 in the skeletal tissue. Binds tightly to hydroxyapatite. Appears to form an integral part of the mineralized matrix. Probably important to cell-matrix interaction. Acts as a cytokine involved in enhancing production of interferon-gamma and interleukin-12 and reducing production of interleukin-10 and is essential in the pathway that leads to type I immunity.
Fam187b	1,47			Membrane protein, expressed in testis in mice	
Prelid2	1,43			Mitochondrial protein, involvedi in phospholipid transport	
Ubxn6	1,41			Involved in protein processing in endoplasmic reticulum	Acts in a complex with VCP and cooperates with USP7 in promoting MDM2 deubiquitination and stabilization. MDM2 stabilization leads to MDM2-dependent TP53 degradation.
Nuf2	1,41			Nuclear protein involved in cell cycle, mitotic nuclear division, attachment of spindle microtubules to kinetochore	Acts as a component of the essential kinetochore-associated NDC80 complex, which is required for chromosome segregation and spindle checkpoint activity. Required for kinetochore integrity and the organization of stable microtubule binding sites in the outer plate of the kinetochore. The NDC80 complex synergistically enhances the affinity of the SKA1 complex for microtubules and may allow the NDC80 complex to track depolymerizing microtubules.
Gsg1	1,40			Component of endoplasmic reticulum membrane. Germ cell-specific gene1, expressed in testis in mice	It interacts with Testis-specific poly(A) polymerase (TPAP) that is highly expressed in round spermatids
Cdk1	1,39			Nuclear component, which is a cyclin-dependent protein serine/threonine kinase activity	Plays a key role in the control of the eukaryotic cell cycle by modulating the centrosome cycle as well as mitotic onset; promotes G2-M transition, and regulates G1 progress and G1-S transition via association with multiple interphase cyclins.
Ccnb2	1,38		1	Cytoskeleton and nuclear protein. Involeved in: in utero embryonic development, cell cycle, T cell homeostasis, thymus development	Essential for the control of the cell cycle at the G2/M (mitosis) transition. Cyclin B2 also binds to transforming growth factor beta RII and thus cyclin B2/cdc2 may play a key role in transforming growth factor beta-mediated cell cycle control.
S100a5	1,37		1	S100A5 is a nuclear protein, novel member of the EF-hand superfamily of calcium-binding proteins	S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. This protein is expressed in very restricted regions of the adult brain.
Gins2	1,34			Nuclear protein, involved in DNA replication, double-strand break repair via break-induced replication	The GINS complex plays an essential role in the initiation of DNA replication, and progression of DNA replication forks. GINS complex seems to bind preferentially to single-stranded DNA

Zfp580	1,32			Nuclear protein involved in endothelial cell proliferation and migration, leukocyte chemotaxis	Involved in the regulation of endothelial cell proliferation and migration. Mediates H_2O_2-induced leukocyte chemotaxis by elevating interleukin-8 production and may play a role in inflammation. May be involved in transcriptional regulation. Up-regulated in presence of reactive oxygen species (ROS), like H_2O_2, through the NF-kappaB signaling pathway. Up-regulated by sphingosine-1-phosphate (SP1) through the p38 MAPK signaling pathway (at protein level).
Ofcc1	1,32			Intracellular protein	Diseases associated with OFCC1 include orofacial cleft and chronic tic disorder.
Bub1b	1,30			Cytoskeleton and nuclear protein, involevd in protein phosphorylation, metaphase/anaphase transition of mitotic cell cycle	Essential component of the mitotic checkpoint. Required for normal mitosis progression. Negatively regulates PLK1 activity in interphase cells and suppresses centrosome amplification. Also implicated in triggering apoptosis in polyploid cells that exit aberrantly from mitotic arrest. May play a role for tumor suppression.
Rad51ap1	1,27			Nuclear protein involved in DNA repair and recombination, cellular response to DNA damage stimulus	May participate in a common DNA damage response pathway associated with the activation of homologous recombination and double-strand break repair. Functionally cooperates with PALB2 in promoting of D-loop formation by RAD51. Binds to single and double stranded DNA, and is capable of aggregating DNA. Also binds RNA.
Birc5	1,26			Cytoskeleton,cytosolic and nuclear protein. Member of the inhibitor of apoptosis gene family, encode negative regulatory proteins that prevent apoptotic cell death	Multitasking protein that has dual roles in promoting cell proliferation and preventing apoptosis. Component of a chromosome passage protein complex (CPC) which is essential for chromosome alignment and segregation during mitosis and cytokinesis. Acts as an important regulator of the localization of this complex
Prc1	1,22			Cytosolic and nuclear protein. It's involved in positive regulation of cell proliferation, microtubule cytoskeleton organization, cytokinesis	Protein that is involved in cytokinesis. The protein is present at high levels during the S and G2/M phases of mitosis but its levels drop dramatically when the cell exits mitosis and enters the G1 phase.Essential for controlling the spatiotemporal formation of the midzone and successful cytokinesis.
Ckap2l	1,22			Mitotic spindle protein important to neural stem or progenitor cells	Microtubule-associated protein required for mitotic spindle formation and cell-cycle progression in neural progenitor cells. Expression is cell-cycle dependent. Undetectable in interphase and prophase, strong expression at the spindle pole throughout metaphase to telophase.
Cx3cr1	1,20		1	Membrane protein. Function: microglial cell activation involved in immune response	
Fignl1	1,18		1	Extracellular and nuclear protein. Involved in:regulation of double-strand break repair via homologous recombination, negative regulation of apoptotic process,regulation of cell cycle	Involved in DNA double-strand break (DBS) repair via homologous recombination (HR). Recruited at DSB sites independently of BRCA2, RAD51 and RAD51 paralogs in a H2AX-dependent manner. May regulate osteoblast proliferation and differentiation.
Kif22	1,18			Cytosolic and nuclear protein, involved in DNA repair and microtubule-based movement	Member of the kinesin-like protein family, involved in spindle formation and movements of chromosomes during mitosis and meiosis. Binds to microtubules and to DNA
Oaf	1,17			Extracellular protein	
Mxd3	1,16			Nuclear protein. Involoved in negative regulation of transcription, DNA-templated	Member of the Myc superfamily of basic helix-loop-helix leucine zipper transcriptional regulators, forms a heterodimer with the cofactor MAX which binds specific E-box DNA motifs in the promoters of target genes and regulates their transcription
Ccr6	1,16			Membrane protein. Involved in chemotaxis, immune response	Important for B-lineage maturation and antigen-driven B-cell differentiation, may regulate the migration and recruitment of dentritic and T cells during inflammatory and immunological responses
Itga7	1,15			Membrane protein. Integrin-mediated signaling pathway,regulation of cell shape,cell migration, cell adhesion	Receptor for the basement membrane protein laminin-1 on skeletal myoblasts and adult myofibers. During myogenic differentiation, it may induce changes in the shape and mobility of myoblasts, and facilitate their localization at laminin-rich sites of secondary fiber formation.
Spc25	1,15			Nuclear and cytosolic protein, involved in cell cycle: mitotic spindle organization, chromosome segregation	Component of the essential kinetochore-associated NDC80 complex, which is required for chromosome segregation and spindle checkpoint activity. Required for kinetochore integrity and the organization of stable microtubule binding sites in the outer plate of the kinetochore
Cenpk	1,14			Nuclear and cytosolic protein, which is involved in positive regulation of transcription from RNA polymerase II promoter	Component of a complex recruited to centromeres which is involved in assembly of kinetochore proteins, mitotic progression and chromosome segregation.
Top2a	1,14		1	Nuclear protein. Involved in regulation of apoptotic process, cellular response to DNA damage stimulus, hematopoietic progenitor cell differentiation	DNA topoisomerase II alpha,markely upregulated in proliferating cells,catalyzing the ATP dependent breakage and rejoining of double strand of DNA,relaxing both positively and negatively supercoiled DNA,during DNA transcription and replication.
Cd46	1,13			Membrane and extracellular protein. Involved in positive regulation of interleukin-10 production, positive regulation of memory and regulatory T cell differentiation	Cofactor for complement factor I which protects autologous cells against complement-mediated injury by cleaving C3b and C4b deposited on host tissue. Also acts as a costimulatory factor for T-cells which induces the differentiation of CD4+ into T-regulatory 1 cells. T-regulatory 1 cells suppress immune responses by secreting interleukin-10, and therefore are thought to prevent autoimmunity

Trappc1	1,08	2,36	2,08	1,13			Present in endoplasmic reticulum and Golgi apparatus. Involved in ER to Golgi vesicle-mediated transport	This gene product plays a role in vesicular transport of proteins to the Golgi apparatus from the endoplasmic reticulum. The encoded protein is a component of the multisubunit transport protein particle (TRAPP) complex.
Egr3	0,93	2,02	1,65	1,12			Nuclear protein. Involved in positive regulation of endothelial cell proliferation; positive regulation of T cell differentiation in thymus; negative regulation of apoptotic process; regulation of transcription	Transcriptional regulator that belongs to the EGR family of C2H2-type zinc-finger proteins. It is an immediate-early growth response gene which is induced by mitogenic stimulation. The protein encoded by this gene participates in the transcriptional regulation of genes in controling biological rhythm.
Spc24	0,66	1,42	2,16	1,09			Nuclear and cytosolic protein. Involved in cell cycle, mitotic nuclear division	Component of the essential kinetochore-associated NDC80 complex, required for chromosome segregation and spindle checkpoint activity. It's required for kinetochore integrity and the organization of stable microtubule binding sites in the outer plate of the kinetochore . The NDC80 complex synergistically enhances the affinity of the SKA1 complex for microtubules and may allow the NDC80 complex to track depolymerizing microtubules
Cenph	0,66	1,41	0,95	1,09			Nuclear protein. Involved in kinetochore assembly and organization, mitotic nuclear division, chromosome segregation	Component of the CENPA-NAC (nucleosome-associated) complex, a complex that plays a central role in assembly of kinetochore proteins, mitotic progression and chromosome segregation. Required for chromosome congression and efficiently align the chromosomes on a metaphase plate.
Depdc1b	0,54	1,14	1,06	1,08			Cytosolic protein. Involved in intracellular signal transduction, cell migration	
Nusap1	1,09	2,30	2,49	1,08			Microtubule-associated protein. Involved in cytoskeleton organization, establishment of mitotic spindle localization, mitotic sister chromatid segregation	Microtubule-associated protein with the capacity to bundle and stabilize microtubules . May associate with chromosomes and promote the organization of mitotic spindle microtubules around them
Il1b	1,55	3,27	1,91	1,08			**Secreted** protein. Involved in inflammatory response, immune response, positive regulation of vascular endothelial growth factor production,	This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1. It is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The induction of cyclooxygenase-2 by this cytokine in the central nervous system is found to contribute to inflammatory pain hypersensitivity.
Mcm10	0,50	1,04	1,32	1,07			Nuclear protein. Involved in DNA replication; cellular response to DNA damage stimulus; cell proliferation	Acts as a replication initiation factor that brings together the MCM2-7 helicase and the DNA polymerase alpha/primase complex in order to initiate DNA replication. Additionally, plays a role in preventing DNA damage during replication. Key effector of the RBBP6 and ZBTB38-mediated regulation of DNA-replication and common fragile sites stability
Plekhf1	0,68	1,42	0,83	1,07			Nuclear protein. Its function: positive regulation of intrinsic apoptotic signaling pathway; positive regulation of autophagy	May induce apoptosis through the lysosomal-mitochondrial pathway. Translocates to the lysosome initiating the permeabilization of lysosomal membrane (LMP) and resulting in the release of CTSD and CTSL to the cytoplasm. Triggers the caspase-independent apoptosis by altering mitochondrial membrane permeabilization (MMP) resulting in the release of PDCD8.
Chaf1b	1,00	2,09	1,77	1,07			Nuclear protein. Involved in: DNA replication and repair; DNA replication-dependent nucleosome assembly; regulation of transcription, DNA-templated; cellular response to DNA damage stimulus	Chromatin assembly factor I (CAF-I) is required for the assembly of histone octamers onto newly-replicated DNA. CAF-I is composed of three protein subunits, p50, p60, and p150. The protein encoded by this gene corresponds to the p60 subunit and is required for chromatin assembly after replication. The encoded protein is differentially phosphorylated in a cell cycle-dependent manner and it is normally found in the nucleus except during mitosis, when it is released into the cytoplasm. This protein may also be involved in DNA repair.
Cdca5	0,74	1,55	1,38	1,06			Nuclear and cytosolic protein. Involved in double-strand break repair, mitotic sister chromatid cohesion, G1/S transition of mitotic cell cycle	Regulator of sister chromatid cohesion in mitosis stabilizing cohesin complex association with chromatin. May antagonize the action of WAPAL which stimulates cohesin dissociation from chromatin. Cohesion ensures that chromosome partitioning is accurate in both meiotic and mitotic cells and plays an important role in DNA repair. Required for efficient DNA double-stranded break repair
Pole	0,77	1,58	1,16	1,04		1	Nuclear and membrane protein. Involved in: DNA replication and repair Nucleotide-excision repair, DNA gap filling	This gene encodes the catalytic subunit of DNA polymerase epsilon. The enzyme is involved in DNA repair and chromosomal DNA replication. Mutations in this gene have been associated with colorectal cancer 12 and facial dysmorphism, immunodeficiency, livedo, and short stature
Lpl	35,32	72,24	46,98	1,03		3	Extracellular and plasma membrane protein. Involved in Lipid metabolic process, positive regulation of cholesterol storage; positive regulation of macrophage derived foam cell differentiation	The primary function of this lipase (expressed in heart, muscle, and adipose tissue) is the hydrolysis of triglycerides of circulating chylomicrons and VLDL. Binding to heparin sulfate proteogylcans at the cell surface is vital to the function. The apolipoprotein, APOC2, acts as a coactivator of LPL activity in the presence of lipids on the luminal surface of vascular endothelium
Serpinb2	159,14	325,06	317,78	1,03			**Secreted protein**. Role: negative regulation of peptidase activity; negative regulation of apoptotic process; wound healing	Inhibits urokinase-type plasminogen activator. The monocyte derived PAI-2 is distinct from the endothelial cell-derived PAI-1

Tgfbr3	4,80	9,77	5,04	1,03			Membrane and **secreted** protein. Transforming growth factor beta(TGF beta),receptor type III: could be involved in capturing and retaining TGF-beta for presentation to the signaling receptors	The encoded receptor is a membrane proteoglycan that often functions as a co-receptor with other TGF-beta receptor superfamily members. Ectodomain shedding produces soluble TGFBR3, which may inhibit TGFB signaling. Decreased expression of this receptor has been observed in various cancers.
Fam101b	0,57	1,15	0,78	1,01			Cytosolic protein. Involved in: actin cytoskeleton organization; negative regulation of chondrocyte development; skeletal system morphogenesis	Involved in the regulation of the perinuclear actin network and nuclear shape through interaction with filamins. Plays an essential role in the formation of cartilaginous skeletal elements.
Ube2c	2,84	5,69	5,94	1,00			Nuclear and cytosolic protein. Involved in: ubiquitin-dependent protein catabolic process and cell cycle	The encoded protein is required for the destruction of mitotic cyclins and for cell cycle progression, and may be involved in cancer progression.
Mki67	2,21	4,24	5,23	0,94	1,24		This gene encodes a nuclear protein that is associated with and may be necessary for cellular proliferation	The encoded protein is predominantly localized in the G1 phase in the perinucleolar region, in the later phases it is also detected throughout the nuclear interior, being predominantly localized in the nuclear matrix. In mitosis, it is present on all chromosomes
Stmn1	9,85	18,29	23,28	0,89	1,24		Cytosolic protein, involved in: regulation of cytoskeleton organization, negative regulation of microtubule polymerization, mitotic spindle organization, neuron projection development, intracellular signal transduction	This gene belongs to the stathmin family of genes. It encodes a ubiquitous cytosolic phosphoprotein proposed to function as an intracellular relay integrating regulatory signals of the cellular environment. The encoded protein is involved in the regulation of the microtubule filament system by destabilizing microtubules. It prevents assembly and promotes disassembly of microtubules.
Slc7a8	4,74	8,53	10,40	0,85	1,13		Integral component of plasma membrane, involved L-alpha-amino acid transmembrane transport, organic cation and toxin transport. It is also involved in metal ion homeostasis and leukocyte migration	Sodium-independent, high-affinity transport of small and large neutral amino acids, acting as an amino acid exchanger. It's involved in the uptake of methylmercury when administered as the L-cysteine or D,L-homocysteine complexes, so plays a role in metal ion homeostasis and toxicity. it's involved in the cellular activity of small molecular weight nitrosothiols. It plays an essential role in the reabsorption of neutral amino acids from the epithelial cells to the bloodstream in the kidney.
Arg1	23,11	40,45	161,79	0,81	2,81		Cytosolic protein, involved in urea cycle, positive regulation of endothelial cell proliferation, cellular response to various stimuli such as LPS, IL-4 and TGFb	Arginase catalyzes the hydrolysis of arginine to ornithine and urea. Two isoforms of mammalian arginase exist (types I and II). The type I isoform encoded by this gene, is a cytosolic enzyme and expressed predominantly in the liver as a component of the urea cycle. Inherited deficiency of this enzyme results in argininemia, an autosomal recessive disorder characterized by hyperammonemia.
Rn45s	111,50	167,33	267,82	0,59	1,26		This gene is a representative copy of the 45S pre-rRNA transcript	The sequences coding for ribosomal RNAs are present as rDNA repeating units in the p12 region of chromosomes 13, 14, 15, 21 and 22. A 45S rRNA which serves as the precursor for the 18S, 5.8S and 28S rRNA, is transcribed from each rDNA unit by RNA polymerase I. The number of rDNA repeating units varies between individuals and from chromosome to chromosome
Ccr5	24,09	34,11	49,01	0,50	1,02		Cell membrane protein, it acts as a receptor for a number of inflammatory CC-chemokines and subsequently transduces a signal by increasing the intracellular calcium ion level.	Member of the beta chemokine receptor family, which is predicted to be a seven transmembrane protein similar to G protein-coupled receptors. This protein is expressed by T cells and macrophages, and is known to be an important co-receptor for macrophage-tropic virus, including HIV, to enter host cells. Defective alleles of this gene have been associated with the HIV infection resistance. Expression of this gene was also detected in a promyeloblastic cell line, suggesting that this protein may play a role in granulocyte lineage proliferation and differentiation

Supplementary Table 7. Functional annotation of down-regulated DEGS related to the comparison between ME, Me+3hE_2 and ME+24hE_2, founded using DAVID database with the aim to obtain functional annotation of the genes.

Gene	fpkm Me	fpkm 3h	fpkm 24h	F.i. 3h	F.i. 24h	ERE	Function	Annotation
Mrgprb2	1,02	0,03	0,19	-5,04	-2,44		Plasma membrane protein. G-protein coupled receptor signaling pathway	A range of cationic substances, collectively called basic secretagogues, including inflammatory peptides and drugs associated with allergic-type reactions activate mouse mast cells in vitro and in vivo through a single receptor, Mrgprb2. Secretagogue-induced histamine release, inflammation and airway contraction are abolished in Mrgprb2-null mutant mice.
Mrgprb1	1,26	0,11	0,54	-3,57	-1,21		Plasma membrane protein. G-protein coupled receptor signaling pathway	
Angptl4	7,20	1,06	2,53	-2,76	-1,51	2	**Secreted** protein. Involved in negative regulation of endothelial cell apoptotic process, positive regulation of lipid metabolic process, negative regulation of lipoprotein lipase activity	Protein with hypoxia-induced expression in endothelial cells. May act as a regulator of angiogenesis and modulate tumorigenesis. Inhibits proliferation, migration, and tubule formation of endothelial cells and reduces vascular leakage. It is directly involved in regulating glucose homeostasis, lipid metabolism, and insulin sensitivity.
Slc6a4	1,22	0,22	0,24	-2,50	-2,32	1	Integral membrane protein. Involved in: serotonin transport, synaptic transmission, memory, circadian rhythm,response to toxic substance	It transports the neurotransmitter serotonin from synaptic spaces into presynaptic neurons and recycles it in a sodium-dependent manner. This protein is a target of psychomotor stimulants, such as amphetamines and cocaine, and is a member of the sodium:neurotransmitter symporter family. A repeat length polymorphism in the promoter of this gene has been shown to affect the rate of serotonin uptake and may play a role in sudden infant death syndrome, aggressive behavior in Alzheimer disease patients, and depression-susceptibility in people experiencing emotional trauma.
Fhit	1,58	0,35	1,23	-2,17			Localized in cytoplasm, mitochondrion, nucleus. Intrinsic apoptotic signaling pathway by p53 class mediator, regulation of transcription, DNA-templated, DNA replication	This gene, a member of the histidine triad gene family, encodes a hydrolase involved in purine metabolism.Plays a role in the induction of apoptosis via SRC and AKT1 signaling pathways. Inhibits MDM2-mediated proteasomal degradation of p53/TP53 and thereby plays a role in p53/TP53-mediated apoptosis.
Ccl5	8,55	1,94	4,18	-2,14			**Secreted** protein. Involved in chemotaxis, inflammatory response, immune response	Chemoattractant for blood monocytes, memory T-helper cells and eosinophils. Causes the release of histamine from basophils and activates eosinophils. May activate several chemokine receptors. One of the major HIV-suppressive factors produced by CD8+ T-cells.
Pdk4	9,00	2,06	1,63	-2,13	-2,46	2	Mitochondrial protein kinase: inhibits the pyruvate dehydrogenase complex by phosphorylating one of its subunits. It contribuites to regulate glucose metabolism and fatty acid biosynthetic process	Inhibits pyruvate dehydrogenase activity, so down-regulates aerobic respiration and inhibits the formation of acetyl-coenzyme A from pyruvate. Inhibition of pyruvate dehydrogenase decreases glucose utilization and increases fat metabolism in response to prolonged fasting and starvation. Plays an important role in maintaining normal blood glucose levels under starvation, and is involved in the insulin signaling cascade. In the fed state, mediates cellular responses to glucose levels and to a high-fat diet. Regulates both fatty acid oxidation and de novo fatty acid biosynthesis. Plays a role in the generation of reactive oxygen species. Protects detached epithelial cells against anoikis. Plays a role in cell proliferation via its role in regulating carbohydrate and fatty acid metabolism.
Ms4a4b	4,51	1,04	3,06	-2,12			Integral component of plasma membrane	CD20 homologue in T cells, it's a novel member of the MS4A gene family in mice. MS4a4B negatively regulates mouse T cell proliferation. Cell cycle analysis showed that MS4a4B regulated T cell proliferation by inhibiting entry of the cells into S-G2/M phase (PubMed)
Il7r	1,50	0,40	0,83	-1,89		1	**Secreted** protein. Receptor for interleukine 7 (IL7): positive regulation of T cell differentiation in thymus; negative regulation of T cell mediated cytotoxicity	The function of this receptor requires the interleukin 2 receptor, gamma chain (IL2RG), a common gamma chain shared by the receptors of various cytokines. This protein has been shown to play a critical role in the V(D)J recombination during lymphocyte development. Knockout studies in mice suggested that blocking apoptosis is an essential function of this protein during differentiation and activation of T lymphocytes. The functional defects in this protein
Ly6k	6,65	1,83	2,00	-1,86	-1,74		**Secreted**, cytoplasmatic and cell membrane protein. Involved in binding of sperm to zona pellucida; sperm motility	It has also been discovered as a novel marker for mouse plasma cells. Expression profiling confirmed that mLy-6K is expressed by plasma cells but not B cells or tissues not containing plasma cells.
Cldn20	1,15	0,38	0,28	-1,59			Plasma membrane protein. Member of the claudin family	Claudins are integral membrane proteins and components of tight junction strands. Tight junction strands serve as a physical barrier to prevent solutes and water from passing freely through the paracellular space between epithelial or endothelial cell sheets, and also play critical roles in maintaining cell polarity and signal transductions.Plays a major role in tight junction-specific obliteration of the intercellular space, through calcium-independent cell-adhesion activity
Lat	1,00	0,34	0,64	-1,56			Membrane protein. Involved in adaptive immune response; immune system process: lymphocyte homeostasis, regulation of T cell activation; inflammatory response	Required for TCR (T-cell antigen receptor)- and pre-TCR-mediated signaling, both in mature T-cells and during their development. Involved in FCGR3 (low affinity immunoglobulin gamma Fc region receptor III)-mediated signaling in natural killer cells and FCER1 (high affinity immunoglobulin epsilon receptor)-mediated signaling in mast cells

Il2rb	1,90	0,69	1,41	-1,45		2	Plasma membrane protein. Receptor for interleukin-2. Cytokine-mediated signaling pathway; natural killer cell activation; interleukin-2-mediated signaling pathway;negative regulation of apoptosi	Receptor for interleukin-2 is present in 3 forms with respect to ability to bind interleukin 2. The low affinity form is a monomer of the alpha subunit and is not involved in signal transduction. The intermediate affinity form consists of an alpha/beta subunit heterodimer, while the high affinity form consists of an alpha/beta/gamma subunit heterotrimer. Both the intermediate and high affinity forms of the receptor are involved in receptor-mediated endocytosis and transduction of mitogenic signals from interleukin 2. The protein encoded by this gene represents the beta subunit and is a type I membrane protein. It's involved in receptor mediated endocytosis and in T cell-mediated immune responses. Transduces the mitogenic signals of IL2
Slc18a2	1,41	0,52	0,73	-1,43			Plasma membrane protein. Involved in: aminergic neurotransmitter loading into synaptic vesicle; response to amphetamine	It's a vescicular monoamine transporter,involved in the ATP-dependent vesicular transport of biogenic amine neurotransmitters into synaptic vesicles using the proton gradient maintained across the vesicular membrane. Requisite for vesicular amine storage prior to secretion via exocytosis
Mylpf	1,45	0,54	0,99	-1,42		2	Lysosome and cytosolic protein. Involved in skeletal muscle tissue development	
Gm20735	2,72	1,06	2,76	-1,36				
Fkbp5	52,30	20,47	10,13	-1,35	-2,37	2	Cytoplasmatic and nuclear protein. It's involved in chaperone-mediated protein folding	The protein encoded by this gene is a member of the immunophilin protein family, which play a role in immunoregulation and basic cellular processes involving protein folding and trafficking. It binds to the immunosuppressants FK506 and rapamycin. It is thought to mediate calcineurin inhibition.
Klf15	1,03	0,41	0,25	-1,34	-2,05		Nuclear protein. Involved in: regulation of transcription, DNA-templated; glial cell differentiation	Regulates KCNIP2 circadian expression in the heart . It's a repressor of CTGF expression, involved in the control of cardiac fibrosis. It is also involved in the control of cardiac hypertrophy acting through the inhibition of MEF2A and GATA4. Inhibits NF-kappa-B activation
Hspb1	9,73	3,86	1,99	-1,33	-2,29		Cytoplasmatic and nuclear protein. Heat shock protein 1: involved in stress resistance and actin organization; negative regulation of apoptotic signaling pathway	It translocates from the cytoplasm to the nucleus upon stress induction. iIt's expressed in response to environmental stresses such as heat shock, or estrogen stimulation in MCF-7 cells. Up-regulated in response to enterovirus 71 (EV71) infection
Cxcr4	62,30	24,90	35,15	-1,32	-0,83		Extracellular,lysosome and plasma membrane protein. Involved in: chemotaxis; germ cell development ; nervous system development; organ morphogenesis	It transduces a signal by increasing intracellular calcium ion levels and enhancing MAPK1/MAPK3 activation. Involved in hematopoiesis and in cardiac ventricular septum formation. It has essential role in vascularization of the gastrointestinal tract, probably by regulating vascular branching and/or remodeling processes in endothelial cells. Involved in cerebellar development. Acts as a coreceptor (CD4 being the primary receptor) for human immunodeficiency virus-1/HIV-1 X4 isolates and as a primary receptor for some HIV-2 isolates.
Hsph1	44,15	18,02	18,18	-1,29	-1,28		Extracellular,cytosolic and nuclear protein. Involved in: response to unfolded protein; negative regulation of apoptotic signaling pathway; positive regulation of MHC class I biosynthesis	Prevents the aggregation of denatured proteins in cells under severe stress, on which the ATP levels decrease markedly. Inhibits HSPA8/HSC70 ATPase and chaperone activities
Serpine1	1,56	0,64	0,60	-1,27		1	**Secreted** protein. Roles: positive regulation of leukotriene production involved in inflammatory response; negative regulation of fibrinolysis; negative regulation of extrinsic apoptotic signaling pathway via death domain receptors	Member of the serine proteinase inhibitor (serpin) superfamily. This member is the principal inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), and hence is an inhibitor of fibrinolysis. Defects in this gene are the cause of plasminogen activator inhibitor-1 deficiency (PAI-1 deficiency), and high concentrations of the gene product are associated with thrombophilia
Dnajb13	0,15	0,52	0,58	-1,24			Cytoplasmatic protein. Involved in protein folding	
Oas1g	2,22	0,94	2,25	-1,24			Localized in nucleus and cytosol. Protein family of interferon-induced enzymes characterized by their ability to catalyze the synthesis of 2'-5'-linked oligomers of adenosine from ATP (2-5A). 2-5A bind to the latent Ribonuclease L (RNase L), which subsequently dimerizes into the active form	Several studies indicate that OAS1 is an important inducer of apoptosis in human cancer cells and that it may be regulated by 17beta-estradiol (E(2)).
Wee1	6,17	2,63	2,81	-1,23	-1,13	2	Nuclear protein. Tyrosine kinase involved in cell cycle (mitotic nuclear division)	Acts as a negative regulator of entry into mitosis (G2 to M transition) by protecting the nucleus from cytoplasmically activated cyclin B1-complexed CDK1 before the onset of mitosis by mediating phosphorylation of CDK1 on Tyr-15.

Rasl10a	1,35	0,59	0,23	-1,19	-2,55		Nuclear and plasma membrane protein. Small GTPase mediated signal transduction	Potent inhibitor of cellular proliferation
Gm14393*	4,53	2,02	3,02	-1,17				
Dhrs13	1,03	0,46	0,69	-1,16			**Secreted** protein. Involved in: metabolic process; oxidation-reduction process	DHRS13 (Dehydrogenase/Reductase (SDR Family) Member 13) is a putative oxidoreductase.
Sec16b	4,78	2,15	3,23	-1,15			ER and Golgi membrane protein. Required for secretory cargo traffic from the endoplasmic reticulum to the Golgi apparatus and for normal transitional endoplasmic reticulum organization	Mammalian homolog of S. cerevisiae Sec16 that is required for organization of transitional endoplasmic reticulum (ER) sites and protein export
Bri3	4,48	2,05	4,80	-1,13			Integral component of membrane	Bri3 is a recently identified proline-rich transmembrane polypeptide up-regulated during TNF-mediated inflammation and immunity
Per1	19,08	8,75	14,17	-1,13		1	Nuclear protein. Involved in: circadian regulation of gene expression; regulation of cytokine production involved in inflammatory response; regulation of p38MAPK cascade	Transcriptional repressor which forms a core component of the circadian clock. It is expressed in a circadian pattern in the suprachiasmatic nucleus, the primary circadian pacemaker in the mammalian brain. Genes in this family (Period family) encode components of the circadian rhythms of locomotor activity, metabolism, and behavior.
Ifitm1	1,87	0,87	3,26	-1,10			Plasma membrane protein. Involved in: defense response to virus, negative regulation of viral entry into host cell; response to interferon-gamma, alpha and beta	IFN-induced antiviral protein which inhibits the entry of viruses to the host cell cytoplasm, permitting endocytosis, but preventing subsequent viral fusion and release of viral contents into the cytosol. Active against multiple viruses. Acts as a positive regulator of osteoblast differentiation
Dgke	5,72	2,67	3,43	-1,10	-0,74		Membrane protein. Diacylglycerol kinases: protein kinase C-activating G-protein coupled receptor signaling pathway; intracellular signal transduction	Diacylglycerol kinases are thought to be involved mainly in the regeneration of phosphatidylinositol (PI) from diacylglycerol in the PI-cycle during cell signal transduction. When expressed in mammalian cells, DGK-epsilon shows specificity for arachidonyl-containing diacylglycerol
Zbtb16	11,95	5,60	2,39	-1,09	-2,32		Nuclear protein. Roles: positive regulation of NK T cell differentiation; positive regulation of apoptotic process; positive regulation of fat cell differentiation	Probable transcription factor. May play a role in myeloid maturation and in the development and/or maintenance of other differentiated tissues. Probable substrate-recognition component of an E3 ubiquitin-protein ligase complex which mediates the ubiquitination and subsequent proteasomal degradation of target proteins.
Tcf7	2,53	1,19	2,31	-1,09		1	Nuclear protein. Involved in: immune response; regulation of transcription, DNA-templated	This gene is expressed predominantly in T-cells. It's a transcriptional activator involved in T-cell lymphocyte differentiation. Necessary for the survival of CD4(+) CD8(+) immature thymocytes.
Mbd1	47,30	22,97	22,21	-1,04	-1,09	1	Nuclear protein. Involved in: methylation-dependent chromatin silencing; negative regulation of transcription, DNA-templated;negative regulation of astrocyte differentiation	It is a member of a family of nuclear proteins related by the presence of a methyl-CpG binding domain (MBD). These proteins are capable of binding specifically to methylated DNA, and some members can also repress transcription from methylated gene promoters.
Tmem220*	1,16	0,57	1,42	-1,02			Transmembrane Protein 220	
Cyp11a1	1,05	0,52	0,99	-1,01			Mitochondrial membrane protein. Member of the cytochrome P450 superfamily of enzymes. Involved in synthesis of cholesterol, steroids and other lipids.	This protein localizes to the mitochondrial inner membrane and catalyzes the conversion of cholesterol to pregnenolone, the first and rate-limiting step in the synthesis of the steroid hormones.
Cyp26a1	6,42	3,31	2,14	-0,96	-1,58		Endoplasmic reticulum protein. It's involved in: retinoic acid catabolic process and central nervous system development, by negative regulation of retinoic acid receptor signaling pathway	Member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein acts on retinoids, including all-trans-retinoic acid (RA), with both 4-hydroxylation and 18-hydroxylation activities and regulates the cellular level of retinoic acid which is involved in regulation of gene expression in both embryonic and adult tissues.
* Zfp811	3,87	2,12	1,04	-0,87	-1,89		Zinc finger protein 811	
Plin2	235,20	137,44	113,84	-0,78	-1,05		The protein encoded by this gene belongs to the perilipin family, members of which coat intracellular lipid storage droplets	It's associated with the lipid globule surface membrane material and maybe involved in development and maintenance of adipose tissue. However, it is not restricted to adipocytes, but is found in a wide range of cultured cell lines, for example fibroblasts. It can be found in hepatocytes in alcoholic liver cirrhosis, suggesting that it may be a marker of lipid accumulation in diverse cell types and diseases.
Ralgds	6,73	4,04	2,47	-0,74	-1,45		Cytosolic and nuclear protein. It's involved in Ras protein signal transduction, acting as a positive regulator of GTPase activity	Guanine nucleotide dissociation stimulators (GDSs, or exchange factors), such as RALGDS, are effectors of Ras-related GTPases that participate in signaling for a variety of cellular processes
Map3k6	9,97	6,07	3,56	-0,72	-1,49	1	Intracellular protein. It's involved in activation of MAPKK activity,protein phosphorylation and signal transduction	Serine/threonine protein kinase that forms a component of protein kinase-mediated signal transduction cascades. The encoded kinase participates in the regulation of vascular endothelial growth factor (VEGF) expression

Klf11	10,52	6,56	4,65	-0,68	-1,18		Nuclear protein, involved in: negative regulation of transcription, DNA-templated; negative regulation of cell proliferation; positive regulation of apoptotic process	The protein encoded by this gene is a zinc finger transcription factor that binds to SP1-like sequences in epsilon- and gamma-globin gene promoters. This binding inhibits cell growth and causes apoptosis.
Il1rl1	7,52	4,84	3,65	-0,64	-1,05	2	Cell membrane or **secreted** protein depending on isoforms. It's involved in: negative regulation of T-helper 1 type immune response and NF-kappaB signaling; positive regulation of macrophage activation, inflammatory response and chemokine secretion	Member of the interleukin 1 receptor family. Studies of the similar gene in mouse suggested that this receptor can be induced by proinflammatory stimuli, and may be involved in the function of helper T cells. Receptor for interleukin-33; signaling requires association of the coreceptor IL1RAP. This gene, interleukin 1 receptor, type I (IL1R1), interleukin 1 receptor, type II (IL1R2) and interleukin 1 receptor-like 2 (IL1RL2) form a cytokine receptor gene cluster in a region mapped to chromosome 2q12.
Thbd	25,48	16,78	10,67	-0,60	-1,26		Plasma membrane protein. It plays a role in negative regulation of platelet activation and blood coagulation; it's also involved in response to lipopolysaccharide and leukocyte migration	The encoded protein is an endothelial-specific type I membrane receptor that binds thrombin. This binding results in the activation of protein C, which degrades clotting factors Va and VIIIa and reduces the amount of thrombin generated
Plekha6	13,53	9,15	4,66	-0,56	-1,54		Pleckstrin Homology Domain Containing A6 is a cytosolic and nuclear protein	Pleckstrin homology domain containing 6 protein (PLEKHA6) polymorphisms are associated with psychopathology and response to treatment in schizophrenic patients
Rfx2	12,01	8,19	3,84	-0,55	-1,64		Nuclear protein. Trascription factor, whose role is regulation of transcription from RNA polymerase II promoter. Also involved in cilium assembly and spermatid development	It is a member of the regulatory factor X gene family, which encodes transcription factors that contain a highly-conserved winged helix DNA binding domain. It is a transcriptional activator that can bind DNA as a monomer or as a heterodimer with other RFX family members. It acts as a key regulator of spermatogenesis such as genes required for cilium assembly and function
Apoc1	133,27	91,58	54,66	-0,54	-1,29		**Secreted** protein. It's involved in plasma lipoprotein particle remodeling, lipoprotein, triglyceride and cholesterol metabolic process. It's a negative regulator of receptor-mediated endocytosis	This gene is a member of the apolipoprotein C1 family. It is expressed primarily in the liver, and it is activated when monocytes differentiate into macrophages. The encoded protein plays a central role in high density lipoprotein (HDL) and very low density lipoprotein (VLDL) metabolism. This protein has also been shown to inhibit cholesteryl ester transfer protein in plasma and lipoprotein binding to the LDL receptor and VLDL receptor
Tppp	27,93	19,62	9,14	-0,51	-1,61		TPPP (Tubulin Polymerization Promoting Protein) is a cytoskeleton and nuclear protein. It's involved in promoting microtubule polymerization and microtubule bundle formation	The encoded protein may play a role in the polymerization of tubulin into microtubules, microtubule bundling and the stabilization of existing microtubules, thus maintaining the integrity of the microtubule network. It also may play a role in mitotic spindle assembly and nuclear envelope breakdown.

Supplementary Table 8. RTPCR results of the expression of typical M2 polarization genes CD206, Tgm2 and of lipid metabolism, Lpl, on CD11b peritoneal macrophages extracted from female in Metaestrous (ME), Metaestrous following 3h of treatment with 5μg/kg E_2 (ME+3h E_2), Metaestrous following 24h of treatment with 5μg/kg E_2 (ME+ 24hE_2) and Estrous female.

		36B4 19.2.15		**CD206** 21.7.15						**Tgm2** 2.3.16						**LPL** 26.2.15					
		Ct	media	Ct	media	dCT	Media ddCt	ddCT	2-ddct	Ct	media	dCT	Media ddCt	ddCT	2-ddct	Ct	media	dCT	Media ddCt	ddCT	2-ddct
Metaestrus	1	18,90 19,06	18,98	25,29 25,51	25,40	6,42	6,61	-0,20	1,15	28,89 28,96	28,92	9,94	9,70	0,24	0,85	24,64 24,63	24,64	5,66	5,85	-0,19	1,14
	2	19,39 19,33	19,36	25,69 25,91	25,91	6,55		-0,07	1,05	29,82 29,90	29,86	10,50		0,80	0,57	25,96 25,68	25,82	6,46		0,62	0,65
	3	19,08 18,90	18,99	24,94 24,79	24,86	5,88		-0,74	1,67	28,61 28,40	28,51	9,52		-0,19	1,14	24,96 24,93	24,94	5,95		0,11	0,93
	4	18,82 20,12	19,47	25,46 25,48	25,47	6,00		-0,61	1,53	28,58 28,45	28,52	9,05		-0,66	1,58	24,45 24,51	24,48	5,01		-0,83	1,78
	5	18,67 19,33	19,00	25,54 25,44	25,49	6,49		-0,12	1,09	Undetermined Undetermined				-9,70		23,87 23,96	23,91	4,91		-0,93	1,91
	6	18,67 19,38	19,02	25,38 25,31	25,35	6,33		-0,29	1,22	27,96 28,09	28,02	9,00		-0,70	1,63	25,30 25,44	25,37	6,35		0,50	0,71
	7	19,47 19,48	19,48	25,70 29,60	27,65	8,17		1,55	0,34	29,72 30,18	29,95	10,48		0,77	0,59	25,44 25,47	25,46	5,98		0,13	0,91
	8	19,37 19,02	19,19	26,24 26,21	26,23	7,03		0,42	0,75	Undetermined Undetermined				-9,70		24,62 24,83	24,72	5,53		-0,32	1,24
	9	18,84 19,27	19,05	25,62 25,69	25,65	6,60		-0,02	1,01	28,40 28,62	28,51	9,46		-0,25	1,19	25,27 25,26	25,26	6,21		0,36	0,78
	10	19,75 19,28	19,52	25,97 26,42	26,20	6,68		0,06	0,96	28,86 29,56	29,21	9,69		-0,01	1,01	25,92 25,91	25,91	6,39		0,55	0,68
Met + 3h E2	29	21,15 21,87	21,51	27,28 27,43	27,35	5,84		-0,77	1,71	30,51 30,77	30,64	9,13		-0,58	1,49	25,37 25,64	25,51	3,99		-1,85	3,61
	30	21,29 21,39	21,34	26,89 27,16	27,03	5,69		-0,92	1,89	30,64 30,25	30,44	9,11		-0,60	1,51	25,38 25,63	25,50	4,17		-1,68	3,20
	31	21,27 21,27	21,27	26,48 26,54	26,48	5,20		-1,41	2,65	29,89 29,88	29,88	8,61		-1,09	2,13	25,54 24,99	25,26	3,99		-1,85	3,61
	32	19,44 19,37	19,40	25,04 24,89	24,97	5,56		-1,05	2,07	25,49 25,41	25,45	6,05		-3,66	12,63	23,04 23,10	23,07	3,67		-2,18	4,53
	33	19,63 19,52	19,58	25,14 25,21	25,18	5,60		-1,01	2,01	28,27 28,12	28,20	8,62		-1,08	2,12	23,19 23,25	23,22	3,64		-2,20	4,60
Met + 24h E2	34	19,01 19,12	19,06	25,23 25,32	25,28	6,21		-0,40	1,32	28,24 28,43	28,34	9,27		-0,43	1,35	24,61 24,55	24,58	5,51		-0,33	1,26
	35	36,33 36,06	36,20											-9,70				-36,20			
	36	21,82 21,97	21,89	27,88 28,11	27,88	5,99		-0,62	1,54	30,84 30,97	30,90	9,01		-0,70	1,62	25,52 25,57	25,54	3,65		-2,20	4,58
	37	18,86 19,01	18,93	24,69 24,94	24,82	5,88		-0,73	1,66	26,87 26,64	26,75	7,82		-1,89	3,70	24,92 24,83	24,87	5,94		0,09	0,94
Estro	38	22,11 22,45	22,28	27,47 26,05	26,76	4,48		-2,13	4,39	29,58 29,74	29,66	7,38		-2,32	5,01	23,69 23,89	23,79	1,51		-4,34	20,21
	39	19,29 19,29	19,29	25,55 27,40	26,48	7,19		0,58	0,67	26,92 26,58	26,75	7,46		-2,24	4,73	23,57 23,33	23,45	4,16		-1,68	3,21
	40	18,99 19,05	19,02	26,49 26,38	26,38	7,36		0,75	0,59	27,73 27,85	27,79	8,76		-0,94	1,92	23,13 23,01	23,07	4,05		-1,80	3,48
	41	19,69 19,99	19,84	26,41 26,60	26,50	6,66		0,05	0,97	29,35 29,12	29,24	9,40		-0,31	1,24	23,63 23,73	23,68	3,84		-2,00	4,01

Supplementary Table 9. RTPCR results of the expression of typical cellular proliferation genes Ki67, Ube2c and E2F1 on CD11b peritoneal macrophages extracted from female in Metaestrous (ME), Metaestrous following 3h of treatment with 5μg/kg E_2 (Me+3hE_2), Metaestrous following 24h of treatment with 5μg/kg E_2 (ME+24hE_2) and Estrous female.

		36B4 19.2.15		KI67 8.7.15						UBE2C 22.12.15						E2F1 26.11.15					
		Ct	media	Ct	media	dCT	Media ddCt	ddCT	2-ddct		media	dCT	Media ddCt	ddCT	2-ddct	Ct	media	dCT	Media ddCt	ddCT	2-ddct
Metaestrous	1	18,90	18,98	32,80	32,44	13,46	12,04	1,42	0,37	30,82	30,62	11,64	10,97	0,67	0,63				9,04		
		19,06		32,08						30,42											
	2	19,39	19,36	31,92	32,34	12,98		0,94	0,52	31,37	31,17	11,81		0,84	0,56	28,73	28,82	9,46		0,42	0,75
		19,33		32,77						30,97						28,91					
	3	19,08	18,99	30,24	30,30	11,31		-0,73	1,66	29,29	29,27	10,28		-0,69	1,61	28,32	28,34	9,35		0,31	0,81
		18,90		30,35						29,25						28,36					
	4	18,82	19,47	28,39	28,50	9,04		-3,00	8,01	27,90	27,81	8,35		-2,63	6,18					-9,04	
		20,12		28,62						27,73											
	5	18,67	19,00	31,49	31,35	12,35		0,31	0,80	29,92	30,01	11,01		0,03	0,98					-9,04	
		19,33		31,22						30,09											
	6	18,67	19,02	30,50	30,34	11,32		-0,72	1,65	29,82	29,79	10,77		-0,21	1,16	27,66	27,78	8,76		-0,28	1,22
		19,38		30,18						29,76						27,90					
	7	19,47	19,48	30,90	30,84	11,36		-0,68	1,60	31,47	30,67	11,19		0,22	0,86					-9,04	
		19,48		30,77						30,67											
	8	19,37	19,19	31,52	31,43	12,23		0,19	0,87	30,36	30,17	10,98		0,00	1,00	27,63	27,82	8,63		-0,41	1,33
		19,02		31,33						29,98						28,01					
	9	18,84	19,05	30,97	31,14	12,08		0,05	0,97	29,92	29,69	10,63		-0,34	1,27					-9,04	
		19,27		31,31						29,45											
	10	19,75	19,52	30,59	30,77	11,25		-0,79	1,73	29,94	29,97	10,45		-0,52	1,43	28,32	28,52	9,00		-0,04	1,03
		19,28		30,94						30,01						28,71					
Met + 3h E2	29	21,15	21,51	33,89	34,27	12,75		0,72	0,61	30,64	30,67	9,16		-1,81	3,52	30,11	30,05	8,54		-0,50	1,41
		21,87		34,64						30,71						29,99					
	30	21,29	21,34	32,24	32,90	11,56		-0,48	1,39	29,94	30,03	8,69		-2,28	4,87	30,19	30,27	8,94		-0,10	1,07
		21,39		33,55						30,11						30,36					
	31	21,27	21,27	33,72	33,78	12,50		0,47	0,72	28,61	28,66	7,39		-3,59	12,03	29,95	29,85	8,57		-0,46	1,38
		21,27		33,83						28,70						29,74					
	32	19,44	19,40	27,24	27,26	7,85		-4,18	18,17	27,00	26,99	7,59		-3,39	10,46	27,01	26,96	7,56		-1,48	2,79
		19,37		27,27						26,98						26,92					
	33	19,63	19,58	29,61	29,50	9,92		-2,12	4,33	28,79	28,99	9,42		-1,56	2,95	27,82	27,88	8,31		-0,73	1,66
		19,52		29,39						29,19						27,94					
Met + 24h E2	34	19,01	19,06	30,22	30,41	11,34		-0,69	1,62	29,86	29,52	10,45		-0,52	1,43	27,23	27,30	8,23		-0,81	1,75
		19,12		30,60						29,18						27,36					
	35	36,33	36,20			-36,20		-48,24				-36,20		-47,17						-9,04	
		36,06																			
	36	21,82	21,89	34,31	34,60	12,70		0,66	0,63	29,90	29,92	8,02		-2,95	7,75					-9,04	
		21,97		34,88						29,93											
	37	18,86	18,93	29,73	29,83	10,90		-1,14	2,21	28,92	28,85	9,92		-1,06	2,08	27,41	27,46	8,53		-0,51	1,43
		19,01		29,94						28,78						27,51					
Estro	38	22,11	22,28	33,09	33,30	11,02		-1,02	2,03	29,42	29,24	6,95		-4,02	16,23					-9,04	
		22,45		33,51						29,05											
	39	19,29	19,29	30,33	30,32	11,03		-1,01	2,01	29,35	29,39	10,11		-0,87	1,82	27,15	27,32	8,03		-1,01	2,01
		19,29		30,31						29,44						27,49					
	40	18,99	19,02	29,33	29,50	10,48		-1,56	2,95	28,91	28,96	9,94		-1,04	2,05	27,73	27,73	8,71		-0,33	1,26
		19,05		29,67						29,01						27,73					
	41	19,69	19,84	31,49	31,83	11,99		-0,04	1,03	30,73	30,73	10,89		-0,08	1,06	28,32	28,30	8,46		-0,58	1,50
		19,99		32,18						undetermined						28,27					

Supplementary Table 10. RTPCR results of the expression of typical cholesterol homeostasis gene Ldlr on CD11b peritoneal macrophages extracted from female in Metaestrous (ME), metaestrous following 3h of treatment with 5μg/kg E_2 (ME+3hE_2), metaestrous following 24h of treatment with 5μg/kg E_2 (ME+24hE_2) and estrous female.

		36B4 19.2.15		LDLR					
		Ct	media	Ct	media	dCT	Media	ddCT	2-ddct
Metaestrous	1	18,90 19,06	18,98	26,39 26,54	26,47	7,48	6,24	1,24	0,42
	2	19,39 19,33	19,36	26,90 27,13	27,02	7,66		1,42	0,37
	3	19,08 18,90	18,99	25,00 25,04	25,02	6,03		-0,21	1,16
	4	18,82 20,12	19,47	24,78 24,61	24,70	5,23		-1,01	2,02
	5	18,67 19,33	19,00	23,13 23,34	23,24	4,23		-2,01	4,02
	6	18,67 19,38	19,02	25,33 25,21	25,27	6,24		0,00	1,00
	7	19,47 19,48	19,48						
	8	19,37 19,02	19,19	25,78 25,89	25,83	6,64		0,40	0,76
	9	18,84 19,27	19,05						
	10	19,75 19,28	19,52	25,93 25,93	25,93	6,41		0,17	0,89
Met + 3h E2	29	21,15 21,87	21,51	26,24 26,37	26,31	4,79		-1,45	2,73
	30	21,29 21,39	21,34	24,38 24,29	24,34	3,00		-3,24	9,45
	31	21,27 21,27	21,27	25,65 25,77	25,71	4,44		-1,81	3,49
	32	19,44 19,37	19,40	23,68 23,73	23,71	4,30		-1,94	3,83
	33	19,63 19,52	19,58						
Met + 24h E2	34	19,01 19,12	19,06	24,23 24,42	24,32	5,26		-0,98	1,97
	35	36,33 36,06	36,20						
	36	21,82 21,97	21,89	27,85 27,80	27,82	5,93		-0,31	1,24
	37	18,86 19,01	18,93	23,45 23,55	23,50	4,57		-1,68	3,20
Estrous	38	22,11 22,45	22,28	25,95 25,93	25,94	3,66		-2,58	5,99
	39	19,29 19,29	19,29	24,51 24,55	24,53	5,24		-1,00	2,00
	40	18,99 19,05	19,02	24,47 24,52	24,50	5,48		-0,76	1,70
	41	19,69 19,99	19,84	25,43 25,34	25,39	5,55		-0,69	1,62