UNIVERSITY OF NAPLES “FEDERICO II”

DEPARTMENT OF PHARMACY

Ph.D. IN PHARMACEUTICAL SCIENCE: XXVII CYCLE

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Ph.D. Thesis

Sex-dependent production of eicosanoids in inflammation

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1. INTRODUCTION

1.1 Inflammation

Inflammation is a protective process triggered by the immune response to traumatic, infectious, post-ischaemic, toxic or autoimmune injury in order to eradicate the offending agent and start the healing of the organism *(Nathan C., 2002)*. It has been typified by "*rubor, calor, dolor, tumor and functio lesa"* (redness, heat, pain, swelling lost of function, Celsus AD40s) due to a complex set of mechanisms or reactions that involve the interaction between soluble factors, cells and vascular endothelium. In particular, resident macrophages, mast cells, vascular endothelial cells as well as dendritic cells represent the first line in the recognition of tissue damage or microbial infection. Upon stimulation they produce chemokines, cytokines and other inflammatory mediators such as lipid mediators (LM) or complement factors in order to amplify the inflammatory response. These mediators cause vasodilatation, increased vascular permeability (the leakage of fluid and proteins from blood vessels to the interstitial space) and recruitment of circulating leukocyte (especially neutrophils) to the inflamed *situ (Dimasi et al.,2013)*. After the migration of the neutrophils towards the site of inflammation, they follow the chemo attractant flux and start their phagocytic activity. Then, neutrophils interact with epithelial cells, macrophages and endothelial cells and start to produce LM, which may exhibit "pro-inflammatory", or "pro-resolving" activity *(Wang et al., 2014)*. In fact, the production of anti inflammatory LM as well as of factors that promote the repairing of the damaged tissue render "acute inflammation" a self-resolving process.

On the contrary, the inflammatory reaction can also become excessive and lead to harmful outcomes such as tissue injury, fibrosis and angiogenesis (*Medzhitov R., 2008*).

1.2 Eicosanoids

1.2.1 Arachidonic acid cascade

Cytosolic Phospholipase A ($cPLA_2$) (EC: 3.1.1.4) is a lipolytic enzyme responsible for the release of Arachidonic Acid (AA, 5,8,11,14-eicosatetraenoic acid) from phospholipids. The translocation of this enzyme from the cytosol to the membrane is the essential regulatory step needed for its activation. This step can be mediated by the calcium increase as well as the phosphorylation on Ser-505 by MEK/ERK and p38 MAPK pathway (*Gijòn M.A., 2000*). When activated, $cPLA_2$ catalyzes the hydrolysis of the *sn*-2 position of phosphatidylinositol and/or phosphatidilcoline (PC) to liberate AA.

AA is a polyunsaturated fatty acid (PUFA 20:4 n-6) which has the essential role to be the precursor of eicosanoids (*Capra et al., 2014; Funk CD., 2001*).

When AA is hydrolyzed from membrane phospholipid by PLA_2 it can be substrate for three different enzymatic cascades: (I) cyclooxygenase (COXs) enzymes that lead to prostaglandins (PGs) generation; (II) 5-, 12-, and 15-lipoxygenase (LO) for production of (LTs), lipoxins (LXs) and related hydroxylated AA metabolites; (III) CYP450 oxygenase pathway for the formation of epoxyeicosatetraenoic acids (EETs) and diHETEs (*Smith W.L., 1989*). Although most of the LM (such as LTB_4 and PGE_2) act as "pro-inflammatory" factors, the metabolization of AA by 5-, 12-, and 15-LO can lead also to "pro-resolving" mediators like LXs (**Fig.1**).

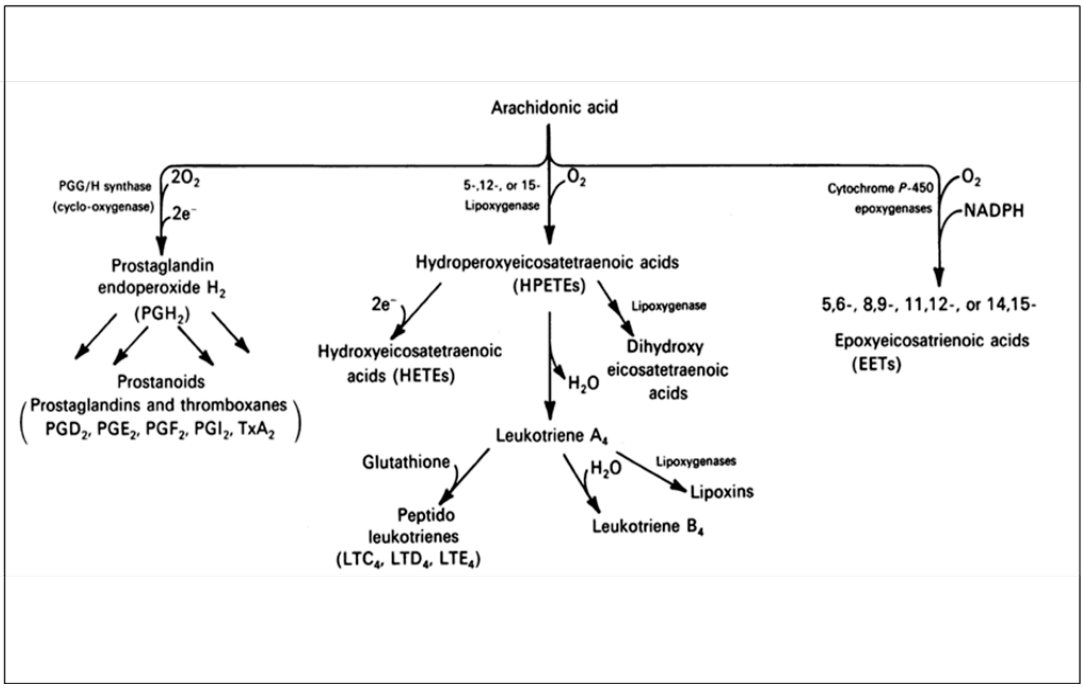


Fig.1 The Arachidonic Acid cascade (*Nathan C. , Nature 2002*)**.**

1.2.1.1 5-Lipoxygenase

5-Lipoxygenase (5-LO) (EC 1.13.11.34) is a dioxygenase with an estimated molecular mass of 72 to 80 kD first discovered in 1976. It is a key enzyme for LT biosynthesis expressed in several leukocytes such as: mast cells, monocyte/macrophages, polymorphonuclear leukocyte, dendritic cells and B lymphocytes. Mammalian 5-LOs are monomeric enzymes with 672 or 673 amino acids and contain two domains: an N-terminal C2-like regulatory domain (β-sandwich) and a C terminal catalytic domain (α-helices). 5-LO contains a non-heme iron in the active site, coordinated by His-367, His-372, His-550 and the C- terminal Ile, that acts as an electron acceptor or donator during catalysis (*Rådmark O., 1995*). The ferrous state (Fe^{2+}) consists in the inactive enzyme, while the ferric (Fe^{3+}) one, in the active enzyme. After activation the ferric 5-LO uses a reductive agent to re-establish the ferrous form (*Chasteen et al., 1993; Hammarberg et al., 2001*).

5-LO has two catalytic functions: an oxygenase activity that corresponds to the addition of oxygen molecules to AA and the subsequent formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and an epoxyde activity that leads to the formation of LTA_4 (unstable epoxyde). 5-HPETE can be also substrate of glutathione peroxidases and reduced to alcohol 5-hydroxyeicosatetraenoic acid (5-HETE). The unstable LTA_4 can be then converted in LTB_4 by stereo specific hydrolysis made by LTA_4-hydrolase or conjugate with gluthatione forming LTC_4, by LTC_4-synthase. The consecutive cleavage of the glutathione tripeptide leads to the other cysteinil LT (cysLT): LTD_4 (through the depletion of γ-glutamyl residue by the γ-glutamyltranferase) and LTE_4 (after the elimination of glycine residue by dipeptidase) (*Rådmark, et al.,2014*) **Fig. 2**. In LT biosynthesis plays an interesting

role also the communication between immune cells. In particular, has been shown by McGee and Fitzpatrick that erythrocytes, cells which are not suppose to synthesize any 5-LO product, when are co-incubated with neutrophils, produce LTA_4 hydrolase that leads to a LTB_4 production (starting from LTA_4 produced in neutrophils) much higher then neutrophils alone. (*McGee and Fitzpatrick, 1986*). Furthermore, LTC_4 formation due to an intercellular transfer of LTA_4 from neutrophils to the endotelium has been also proved. (*Feinmark and Cannon, 1986*).

In addition to AA, 5-LO can metabolize (oxygenase activity) other PUFAs such as 5,8,11,14,17-eicosapentaenoic acid (EPA) leading to the formation of other pro-inflammatory mediators such as 5-Hydroxyeicosapentaenoic acid (5-HEPE) or can convert Docosahexaenoic acid (DHA) in pro-resolving mediators like D-resolvins .

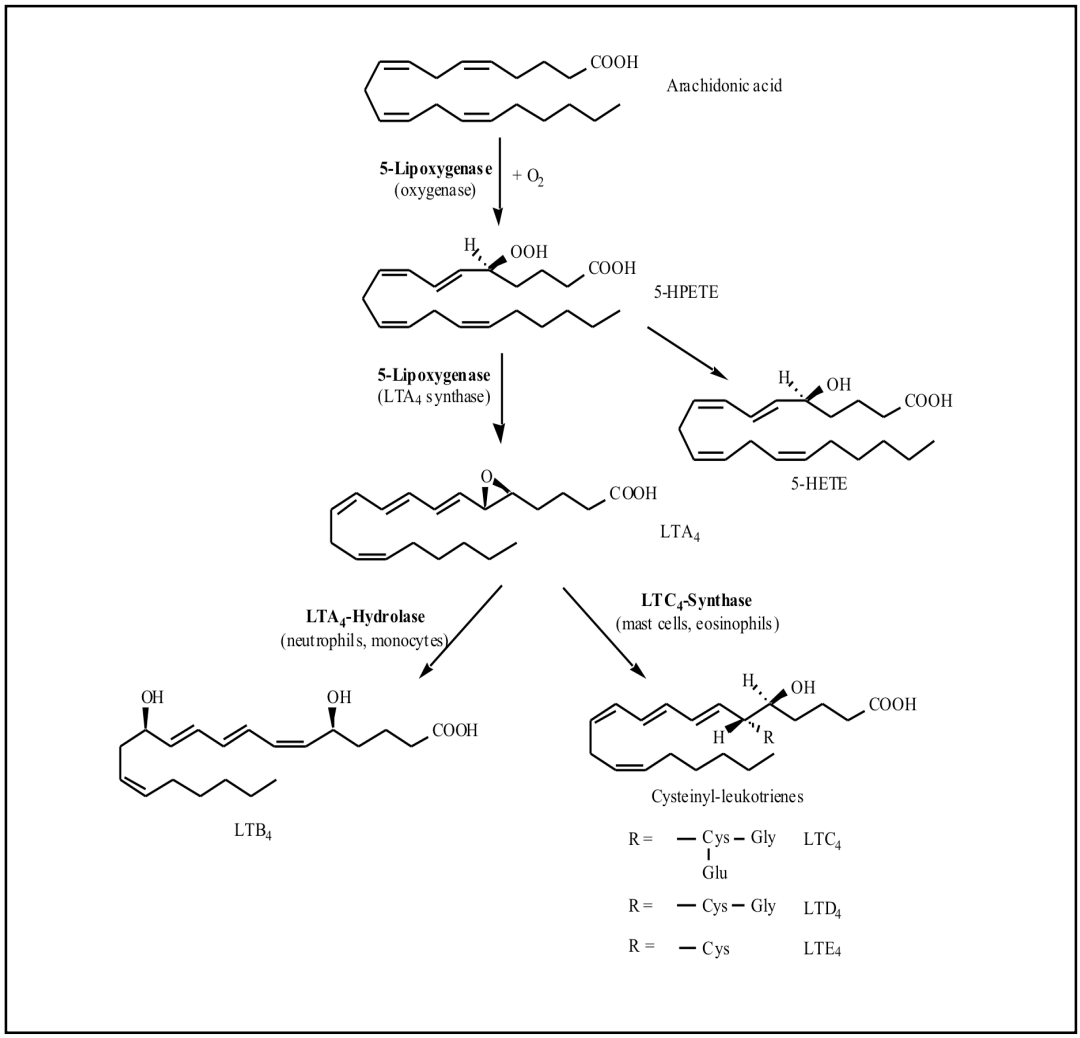


Fig.2 The leukotriene synthetic pathway (*Werz O., 2002*).

1.2.1.2 Regulation of 5-Lipoxygenase

LT biosynthesis is a multistep process tightly regulated at different levels and by several mechanisms (*Werz O., 2002*):

I) interaction of 5-LO with other proteins;

II) binding of 5-LO with small molecules, phospholipids and glycerides;

III) phosphorylation of 5-LO;

IV) intracellular localization of 5-LO.

I) Essentially, two proteins interact with 5-LO and modulate its activity: "coactosine-like protein" (CLP) and "5-LO activating protein" (FLAP).

CLP is a protein part of ADF/Cofilin group, which promotes actin polymerization. It binds 5-LO to the C2-Like domain and the association of these two proteins was proved by the similar migration patterns in Mono Mac cells as well as in neutrophils. Though CLP is not able to activate 5-LO alone, it can support Ca^{2+} activation of the enzyme leading to the formation of 5-HETE in absence of PC or working as a scaffold for the Ca^{2+} activated enzyme. When CLP is in combination with PC, the production of LTA_4 increases 3-4 folds. Moreover, it stabilize 5-LO enzyme and prevents its non-turnover inactivation (*Basavarajappa et al., 2014, Esser et al., 2009, Provost et al., 2001*).

The 5-LO activating protein "FLAP" ("Five Lipoxygenase Activating Protein") plays a pivotal role in the availability of the substrate and the

efficiency of 5-LO enzyme. FLAP is a 18kDa protein which is a member of the membrane-associated proteins in eicosanoid-and glutathione metabolism superfamily (MAPEG). This protein is essential for the production of LTs by 5-LO, without the FLAP activity there is no production of LTs. To date an indole-class of compounds, which inhibits selectively FLAP and, consequentially, LT production, has been identified. MK886 represents the first molecule identified by scientists at Merck-Frosst on 1986s, then, was individualized also a quinolone derived class with similar properties (*Evans, et al., 2008*).

II) Among small molecules, the effect of Ca^{2+} and ATP on 5-LO activity are the most studied. In particular, LT biosynthesis was observed after stimulation of neutrophils by Ca^{2+} ionophore (*Samulesson, 1979*), suggesting that the intracellular concentration of this divalent cation in the cell is relevant for 5-LO activity. Ca^{2+} binds to the N-terminal C2-like β-sandwich domain which is a hydrophobic phospholipid-binding present also in $cPLA_2$, thus this cation plays an important role in the translocation of these enzymes to the nuclear envelope (*Rouzer and Samuelsson, 1987*). Also Mg^{2+} is able to activate 5-LO *in vitro* (*Reddy et al., 2000*).

ATP represents another small molecule able to activate 5-LO. It has a direct binding on 5-LO and it has been shown that its stimulatory activity is strictly related to the number of phosphate in the molecule (AMP<ADP<ATP) (*Werz et al., 2002a*)

Membrane phospholipids have shown the function to increase 5-LO activity. In particular, PC is required for both basal and Ca^{2+} stimulated 5-LO activity *in vitro* (*Skorey and Gresser,1998*).

In addition to phospholipids, also glycerides could augment the catalysis of 5-LO *in vitro* also in absence of Ca^{2+}. In fact, it has been shown that the inhibition of the diacylglycerol-forming pathway involving phospholipase D (PLD) and phosphatidic acid phosphatase (PA-P) decreased 5-LO product synthesis as well as 5-LO translocation (*Albertet al., 2008*). Moreover, recently, a sex-related PLD activity has been shown in human monocytes with a consequent different LT production in male and female monocytes (*Pergola et al., 2011*).

III) 5-LO can be phosphorylated at three residues: a) Ser-271, by MAPKAP kinases; b)Ser-663 by ERK2; c)Ser-523 by Protein Kinase (PK)A catalytic subunit.

The phosphorylation status can influence not only its activation but also regulate the subcellular localization of this enzyme. In fact, the phosphorylation at Ser-271 and Ser-663 increase LT production in neutrophils, monocytes and lymphocytes B in presence or in absence of intracellular Ca^{2+}increment. On the contrary, phosphorylation to Ser-523 by PKA corresponds to a loss of 5-LO function.

IV) 5-LO is considered “a mobile enzyme” for its characteristic to migrate in the cell from the cytosolic fraction to the nuclear one, after stimulation. This event is strictly related to LT formation. In resting condition, this enzyme is located in the cytosol or in the nucleoplasm of the cell depending from the cell type, the phosphorylation status and the sex. In fact recently, it has been shown that 5-LO subcellular localization differs in male and female

neutrophils, in resting condition. In particular, in these cells, androgen causes a perinuclear localization of 5-LO due to ERK and, consequentially, a lower formation of 5-LO products after stimulation (*Pergola et al., 2008*) **Fig. 3**.

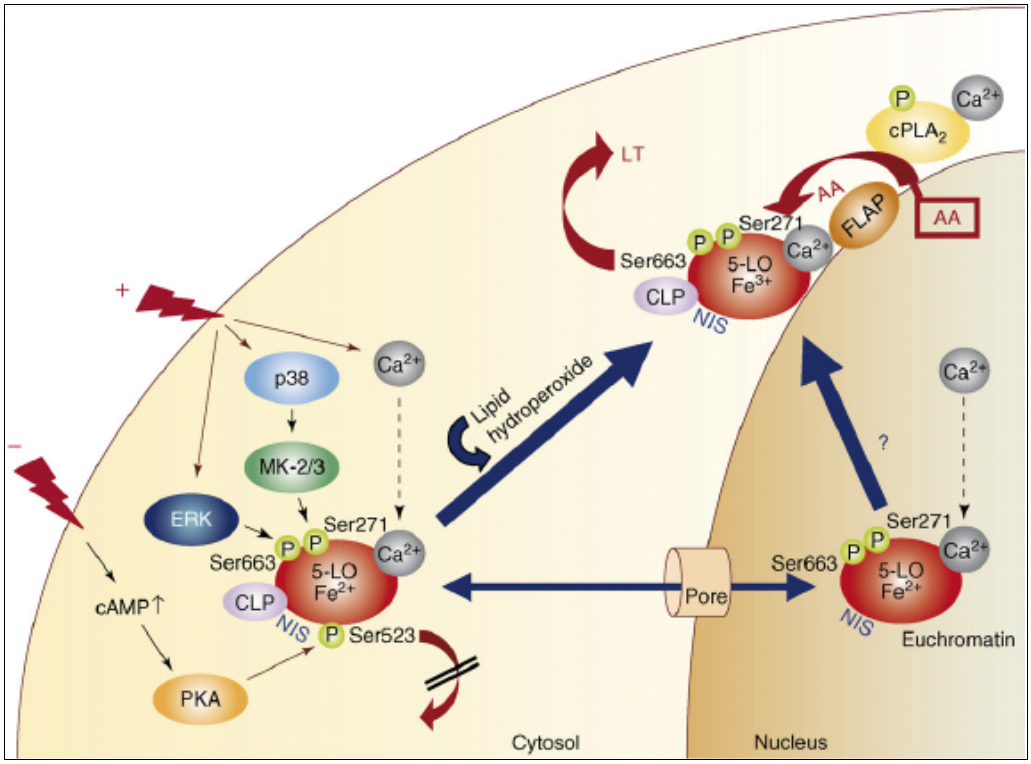


Fig. 3 5-LO activation in the cells. In the resting cell, 5-LO is localized in either the cytosol or a soluble compartment inside the nucleus. On activation, 5-LO migrates to the nuclear envelope, where $cPLA_2$ and FLAP aid 5-LO in LT biosynthesis. $cPLA_2$ liberates AA from phospholipids. Membrane-bound FLAP is thought to facilitate the transfer of AA to 5-LO. CLP can bind 5-LO. Nuclear import sequences (NIS) in the 5-LO sequence determine import into the nucleus. Stimuli of LT biosynthesis lead to an increase in Ca^{2+} and/or activation of MAPK. When 5-LO is activated, the iron is oxidized by LOOH from Fe^{2+} to Fe^{3+}. In 5-LO, Ser271 is phosphorylated by MK-2/3 downstream of p38 MAPK. ERK phosphorylates 5-LO on Ser663. An increase in cAMP levels activates PKA, which represses 5-LO activity through phosphorylation on Ser523 (*Radmark, O. et al., 2007*)

1.2.1.3 Physiopathological role of Leukotrienes

LT are lipid mediators involved in several immune/inflammatory related diseases. They act through the G protein-coupled receptors that are located on the outer plasma membrane of structural and inflammatory cells leading to different biological functions (e.g., smooth muscle cell contraction and neutrophil chemotaxis). In particular, LTB_4 and cys-LT have different receptors. There are two known receptors for LTB_4: the high affinity B leukotriene 1 receptor (BLT1) mainly expressed in leukocytes (*Yokomizo et al., 1997*) and the lower affinity (20 fold lower than BLT1 due to its affinity for 12- and 15-HETE) BLT2 receptor ubiquitously expressed. but its physiological activity are still unknown (*Yokomizo et al., 2000-2001*).

Concerning cys-LT receptors, to date at least two receptors are identified: cys-LT1 and cys-LT2. The first is the most studied one. It is expressed on a variety of both immune-competent cells and structural cells. Results with cys-LT1 antagonists both in clinical studies and in animal models of asthma suggest that many of the features of asthma (eosinophilic airway inflammation, bronchoconstriction, edema, goblet cell hyperplasia, and structural remodeling of the airway) are mediated by cys-LT1 signaling (*Peters-Golden, Henderson WR Jr, 2007*). The cys-LTs–cysLT1 pathway is also implicated in the pathogenesis of allergic rhinitis (*Peters-Golden , et al., 2006*). Cys-LT2 is co-expressed with cys-LT1 in many cell types including endothelium, eosinophils, mast cells, and macrophages. Although its role in immune-competent cells remains unclear, it has been studied for its important role on vascular permeability mediation (*Beller et al., 2004*). CysLT1 as well as 5-LO are important target for the treatment of asthma. In particular, montelukast, zafirlukast and pranlukast (cys-LT1 inhibitors) are available for clinical treatment of asthma. Also a

5-LO inhibitor, zileuton, has been used in the treatment of this disease, however its availability is limited due to hepatotoxicity.

1.2.1.4 Cyclooxygenases

Prostaglandins (PG) are not stored free in tissues, but are formed by the action of the COX, or prostaglandin endoperoxide synthase (PGHS) in a two-step conversion of AA. First, the enzyme converts AA to a cyclic endoperoxide (PGG_2) by the action of cyclooxygenase (COX) activity adding the 15-hydroperoxy group. The hydroperoxy group of PGG_2 is reduced to the hydroxy group of PGH_2 by a peroxidase that uses a wide variety of compounds to provide the requisite pair of electrons. Both cyclooxygenase and hydroperoxidase activities are contained in the same dimeric protein molecule. These unstable intermediate products of AA metabolism by COX are then rapidly converted to the corresponding PG by specific isomerase enzyme, the nature of which is determined by the enzyme content of the tissue under consideration **Fig 4**.

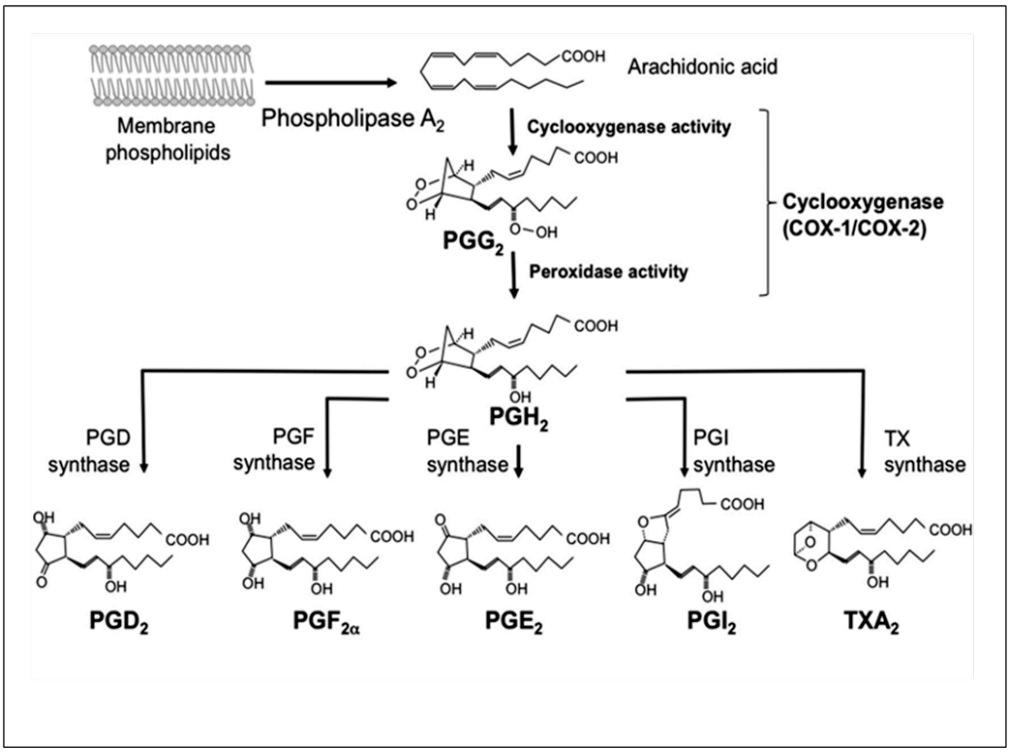


Fig. 4 Cyclooxygenase pathway and prostanoids (*Kawahara K. et al.,2015*)

COX was first purified from the sheep seminal vesicle, a prodigious source of the protein (*Miyamoto et al.,1976*) as a homodimer of approximate molecular mass of 70 kDa and subsequently cloned from the same tissue. COX is eme-dependent glycoprotein, is a dimer of identical subunits, so altogether, there are two cyclooxygenase active sites and two peroxidase active sites in close proximity. COX is embedded in the membrane of the nucleus and endoplasmatic reticulum of all mammalian cells except the erythrocytes. Ferrum atom in heme group is responsible of catalytic activity; in fact only when the ferrum is in the oxidation state III the COX is active.

Initially, it was thought that COX was a single enzyme that produced PG in most tissues and cell types. However, a number of studies have illustrated that COX activity is increased in certain inflammatory states and is induced in cells by pro-inflammatory cytokines and growth factors *in vitro* (*Sano et al., 1995*). Following these observations, extensive research in this field led to the discovery that two forms of COX exist, named COX-1 and COX-2. With the availability of the cDNA encoding the protein and specific antibodies, numerous studies were performed to evaluate the distribution, expression and regulation of the two COXs both *in vitro* and *in vivo*. The transcription of COX-1 yields a 2.7-kilobase (kb) mRNA that encodes a 576-residue, 65-kd protein. Conversely, the transcription of COX-2 yields a 4.5-kb mRNA that encodes a 70-kd protein with roughly 70-75% homology to the COX-1 protein. Using sequence analysis of human genomic DNA, researchers have concluded that the amino acids, important for catalysis by COX-1, are conserved and equally important for catalysis by COX-2. Evidences suggest that COX-1 and COX-2 are similar in structure and function but exist as 2 distinct enzymatic entities. They

have been defined as monotropic integral membrane proteins located primarily in the endoplasmic reticulum (COX-1) and perinuclear envelope (COX-2) (*Morita et al., 1995*).

The constitutive isoform (COX-1) is present in tissues such as the stomach, gut or kidney, where PG production plays a cytoprotective role in maintaining normal physiological processes, such as: platelet aggregation inhibition, inhibition of platelet and neutrophil adhesion, dilation of bronchial and vascular smooth muscle (mediated by PGI_2) and other effects mediated by PGE_2 (described later). By contrast, the inducible isoform of COX (COX-2) is only constitutive in parts of the kidney, and brain, in fetal membrane and in the uterus during pregnancy. In all other tissues and cells, COX-2 is inducible by variety of mitogens, tumour promoters and pro-inflammatory stimuli, including bacterial factors and cytokines. COX-2 is expressed in many cells including fibroblasts and macrophages. The COX-2 expression is regulated by mitogen-activated protein kinase (MAPK). They are a group of serine threonine kinases and the MAPK family in mammalian cells includes extracellular signal-regulated kinase-1 and –2 (Erk-1/2), the c-Jun NH2-terminal kinases (JNK) and p38 MAPK. Their actions on transcription factors, affect gene-regulated cellular responses by relaying growth, development, differentiation, and stress stimuli (*Garrington et al., 1999*).

COX-2 releases large quantities of pro-inflammatory PG at the site of inflammation. PG mediate various manifestations of the inflammatory response, including fever, hyperalgesia, increase in vascular permeability and oedema. In the intestine, PG influence motility and secretion and they stimulate diarrhoea by promoting chloride secretion and blocking sodium absorption in epithelial cells (*Sartor et al., 1991*). PG are involved in a number of inflammatory conditions and diseases, such as arthritis,

skin inflammation where PG potentiate the inflammatory actions of platelet-activating factor and asthma where PGD_2 mediate bronchoconstriction (*Henderson et al., 1991*).

Based on these differences, the concept of "pro-inflammatory COX-2" versus a "protective COX-1" rapidly emerged. However, in a model of carrageenan-induced pleurisy in rats it was concluded that COX-2 may be pro-inflammatory in early phase and may regulate resolution in the later phase of pleurisy, by generating an alternate set of PG such as those of the cyclopentenone family (PGD_2 and 15deoxy$\Delta^{12\text{-}14}PGJ_2$) (*Gilroy et al., 1999*).

1.2.1.5 Physiopathological role of PGE_2

Among PGs, PGE_2 is involved in several classic signs of the inflammatory process such as vasodilatation and increased micro vascular permeability, moreover it is responsible also of the pain because of its activity on peripheral sensory neurons as well as central sites (*Funk CD., 2001*).

PGE_2 is synthesized from PGH_2 by cytosolic PGE synthase (cPGES) or microsomal PGE synthase (mPGES-1 and mPGES-2) (*Samuelsson et al., 2007*). cPGES is constitutively and abundantly expressed in the cytosol of various tissues and cells and it requires glutathione as a cofactor (*Tanioka et al., 2000*). The role of cPGES and even its ability to form PGE_2 is controversial. cPGES seems capable of converting COX-1-derived but not COX-2-derived PGH_2 to PGE_2 in cells, particularly during the immediate PGE_2-biosynthetic response elicited by Ca^{2+}evoked stimuli. mPGES-1 is a member of the membrane-associated proteins involved in eicosanoid and glutathione metabolism superfamily, and like cPGES, it requires glutathione as cofactor (*Jakobsson et al., 1999*). mPGES-1 is a peri-nuclear protein that is markedly induced by cytokines and growth factors and down-regulated by anti-inflammatory glucocorticoids, as in the case of COX-2 (*Mancini et al., 2001; Thorén et al., 2000*). It is functionally coupled with COX-2 in marked preference to COX-1(*Murakami et al., 2000*). Constitutive expression of mPGES-1 in certain tissues and cell types was also reported.

PGE_2 acts through distinct G protein coupled receptors (EP-1-4) and mediate intracellular signals via cyclic nucleotide. EP-3 and 4 are the most expressed and represents the receptors with highest affinity for PGE_2.This prostanoid can exert both pro-inflammatory and anti-inflammatory activity. In particular, the binding of PGE_2

on EP-3 receptors on mast cell increases the vascular permeability as well as promotes leukocyte recruitment by induction of degranulation and IL-6 release. Moreover the binding of PGE_2 to EP-4 receptor on T cells facilitates Th1 differentiation and Th17 expansion (*Kawahara et al., 2014*). In addition to that, PGE2 can exert anti-inflammatory actions on innate immune cells, such as neutrophils, monocytes, and natural killer cells (*Harris et al., 2002*).

1.3 Sex-dependent differences in inflammatory diseases

Accumulate evidences show the relevance of the sex-dependent differences in the immune and inflammatory diseases. In particular, the female prevalence in autoimmune diseases represents an important task for many researchers and industries. This sex-bias seems to affect their development as well as their incidence. In particular, it has been reported that only after puberty, female have a predominant incidence of asthma disease compared to male suggesting an important role of androgens (*Zannolli and Morgese, 1997*). Moreover multiple sclerosis (MS) has an important higher incidence and severity in female compared to male (*Khalid R, 2014*). Also in animal models of MS, such as the Experimental Autoimmune Encephalomyelitis (EAE) in mice, sex-related differences are evident. In particular, Palaszynki *et* al., showed protective effects of androgens in this model. Female predominance has been also observed in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) with female to male prevalence ratios of 7-9:1 for SLE and 2-3:1 for RA (*Tedeschi et al., 2013*). LT plays a pivotal role in all of the mentioned disorders. Interestingly, it has been recently shown a sex-dependent difference in LT production in human blood immune cells. In particular, testosterone seems to exerts a protective role affecting 5-LO subcellular localization/activity in these cells with a consequent decreased LT biosynthesis. In fact, as described above, 5-LO subcellular localization as well as its phosphorylation by MAPK are crucial steps in LT biosynthesis. The mechanisms of action underlying this protective effect are, essentially, two:

- First, in neutrophils and in human whole blood testosterone affects 5-LO compartmentalization via ERKs activation. In these cells, in resting condition,

5-LO enzyme is located mostly in the cytosolic fraction then, upon stimulation, it migrates to the perinuclear one producing LT. The testosterone-dependent basal activation of ERK, confines 5-LO to a perinuclear compartmentalization (which is not active) in resting condition leading to less amount of this enzyme in the cytosolic fraction able to migrate and produce LTs (*Pergola et al., 2008*).

- Second, in monocytes, androgens cause ERK activation that leads to lower PLD activity (apparently attributable to the PLD1 isoform) and DAG levels, which are required for fully induced 5-LO activity and consequently LT biosynthesis

These androgen-dependent sex differences call for in-depth analysis of the impact of sex and androgens on immune cell biology and related pathophysiological processes (*Pergola et al., 2011*).

Intriguingly, also protective effect of female hormones in inflammatory diseases has been reported. In particular, as compared with age-matched men, pre-, but not post-, menopausal women have been shown to be protected against cardiovascular diseases, which may be associated with the vasoprotective and anti-inflammatory effects of estrogen (17b-estradiol) (*Kannel et al., 1995*). In addition to that, pre-menopausal women show a favorable prognosis of sepsis compared to men (*Schröder et al., 1995*). Moreover, the ability of the estrogen to inhibit the expression of adhesion molecules in primary lung endothelial cells causes a lower neutrophil afflux in this tissue during inflammation (*Villar et al., 2011*). On these bases, the elucidation of the mechanisms underlying the protective role of both female and male hormones in eicosanoid-related diseases represents the main aim of the present investigation.

2. AIM OF THE PRESENT INVESTIGATION

Eicosanoids, PGs and LTs, are key mediators of the inflammatory response. They are synthesized from AA through essentially two enzymes: COXs or 5-LO, respectively.

Among PGs, PGE_2 is the most ubiquitously produced PG under both physiological and pathophysiological conditions (*Ushikubi et al., 2000; Narumiya S., 2007*) and is long known as an immunosuppressant for its inhibition of production of inflammatory cytokines by macrophages (*Harris et al., 2007*). Moreover PGE_2 has shown immune-activator properties acting on the EP4 receptor (*Sakata et al., 2010*).

Besides PGs also LTs plays different and pivotal roles in the immune/inflammatory process. In particular, LTB_4 has strong chemotactic activity (*Yokomizo et al., 1997*) and cys-LT promote the smooth muscle cells contraction, activation of leukocytes and increase vascular permeability. Their biosynthesis is a thin regulated process which strictly depends on: a) interaction of 5-LO with other proteins (i.e FLAP); b) phosphorylation of 5-LO protein; c) subcellular localization of 5-LO.

Sex-dependent difference in autoimmune/inflammatory diseases is at glance in the scientific community. In fact, there is a well-known sex-bias in asthma incidence which is clearly related to the changes in male hormones pre and after puberty (*Zannolli et al., 1997*). In addition to that, female are more susceptible to develop autoimmune diseases like multiple sclerosis (*Khalid R., 2014*). To date, little is known about the molecular reason of this sex difference. Recently, it has been demonstrated that the LT biosynthesis in human immune cells (monocytes, neutrophils and in the human whole blood) is sex-biased. In particular, these cells,

coming from male donors, produce less LT levels than the female, after *in vitro* stimulation (*Pergola et al., 2008 and 2011)*. This difference in LT production is related to the effect of testosterone on 5-LO enzyme compartmentalization that affects its catalytic activity and this represents an important finding in the light of the well known sex-biased incidence of several immune diseases (i.e. asthma). Moreover, recently, a sex difference of 5-LO expression in the brain tissue of multiple sclerosis patients has been shown. In particular, there is an higher expression of this enzyme in female, but no data about LT levels are available to date. A beneficial effect by androgens has been observed also in other LT-related diseases, like atherosclerosis and cardiovascular diseases (CVD). In fact, although the incidence of these diseases is higher in males than in females, testosterone is a protective factor against atherosclerosis (*Malkin et al., 2003*), and men with coronary artery disease have lower levels of androgens than men with normal coronary angiograms (*English et al., 2000*).

On the contrary, few is known about sex differences in PGE_2 release, despite there is a sex bias in the incidence and in the outcomes of many inflammatory/autoimmune diseases PG- related (i.e. ulcerative colitis or sepsis *Tragnone et al., 1996; Schroeder et al., 1998*).

In the light of these remarks, the aim of the present investigation was to evaluate the differences in eicosanoid biosynthesis in male and in female using *in vivo* models of acute inflammation. Moreover, in order to investigate the molecular mechanisms related to these differences, as *in vitro* system, we isolated the immune cells responsible of eicosanoid production.

3. MATERIALS AND METHODS

3.1 Materials

Nycoprep was from PAA Laboratories (Linz, Austria). Ca^{2+}-ionophore A23187, AA, PGB_1, and EDTA were from Sigma (Deisenhofen, Germany). Enzyme immunoassay (EIA) kits were from Cayman Chemical Company (Aurogene, Rome, Italy). [^{3}H-PGE2] was from PerkinElmer Life Sciences (Milan, Italy). All other reagents and compounds were obtained from Sigma–Aldrich (Milan, Italy).

3.2 *In vivo* models of acute inflammation

3.2.1 Animals

Male and female CD-1 mice (8–9 weeks old, Charles River, Calco, Italy) and Wistar male and female rats (300 g, Harlan, Milan, Italy) were housed in a controlled environment (21±2◦C) and provided with standard rodent chow and water. All animals were allowed to acclimate for four days prior to experiments and were subjected to 12h light – 12h dark schedule. Experiments were conducted during the light phase.

The experimental protocols were approved by the Animal Care Committee of the University of Naples Federico II in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116/92) as well as with the European Economic Community regulations (Official Journal of E.C. L 358/112/18/1986).

3.2.2 Zymosan-induced peritonitis in mice

Zymosan-induced peritonitis in mice is a model of acute inflammation useful to study all the steps that characterize this process. In particular, zymosan injection in the peritoneal cavity of the mice induces the development of the typical hallmarks of the inflammatory process such as increased vascular permeability, oedema, leukocyte influx and release of inflammatory mediators (LT and PG).

3.2.2.1 Induction of peritonitis

Peritonitis was induced in mice by i.p. injection (0.5 mL) of zymosan (2 mg mL^{-1} in saline, boiled and washed; Sigma, Milan, Italy). At selected time points after zymosan injection (0, 15, 30, 60, 120, 180, 240, 360 and 480 min), mice were euthanized and peritoneal exudates were collected using 2 mL of phosphate buffered saline (PBS). The cells were counted using a light microscope in a Burker's chamber after vital trypan blue staining. Exudates were centrifuged at 20,000 × g for 20 min at 4°C and supernatants and cell pellets were collected and frozen at −80 ◦ C for measurements of eicosanoids (supernatants) and the evaluation of myeloperoxidase (MPO) activity and western blotting analysis of protein expression (cells, see next paragraphs). In one set of experiments peritonitis was induced only in female mice, according to what previously described, 30 min after the injection of 5α-dihydrotestosterone (0.5 mg kg^{-1} 5α-DHT in 2% DMSO) or vehicle (DMSO 2% in saline). At selected time points after zymosan injection (0, 15, 30, 240 min), mice were euthanized and peritoneal exudates were collected using 2 mL of PBS and the supernatants of the peritoneal exudates were stored at -80°C for LT measurement (see next paragraphs).

3.2.2.2 Vascular permeability

Evans blue dye (40 mg kg^{-1}, 0.3 mL; Sigma, Milan, Italy) was injected into the tail vein followed by an i.p. injection of zymosan (0.5 mL; 2 mg mL^{-1}). After 0, 15, 30, 60, 120, 180 and 240 min, mice were euthanized and peritoneal exudates were collected using PBS (0.1 mL g^{-1} of body weight). After centrifugation, supernatants (diluted 1:3) were analyzed for Evans Blue bound to plasma albumin by reading at 610 nm in a plate reader (MultiskanGo, Thermo Scientific).

3.2.2.3 Mieloperoxydase activity

The use of Mieloperoxydase (MPO) activity, as an index of neutrophil infiltration, is well documented (*Bradley et al.,1982*) . Cell pellets were disrupted by sonication in 2 mL PBS (50 mM, pH 6) with 0.5% hexadecyltrimethylammonium bromide, freeze-thawed three times and centrifuged (32,000 × g, 20 min) to collect supernatants which were used in MPO assay, performed as described previously (*Bradley et al.,1982*) .. Briefly, 20 µL of samples were added to a 96 well plate and the reaction was initiated by the addition of 0.2 mL of assay buffer containing 0.167 mg mL^{-1} of o-dianisidine and 0.0005% hydrogen peroxide. The rate of change of absorbance was monitored in kinetic mode by a plate reader (IMarkmicroplate Reader, Bio-Rad, Segrate, Milan, Italy). Levels of MPO in samples were determined from the calibration curve using human neutrophil MPO as reference standard. The levels of MPO were expressed as U mL^{-1}.

3.2.2.4 Blood collection and plasma preparation

Blood was collected by intracardiac puncture immediatly after killing mice with CO_2. In particular, the puncture was made through the insertion of 1mL syringe with a needle of 22 gauge in the heart. Plasma was obtained by centrifugation of the whole blood (597 x g at 4 °C, 15 minutes).

Plasma testosterone was measured with a commercially available KIT EIA (Cayman Chemical Company,Aurogene, Rome, Italy).

3.2.2.5 Mouse gonadectomy

To investigate the impact of sex hormones, male mice were orchidectomized (ORCH group) and allowed to recover for 5 weeks. This time period ensures the metabolization of sex hormones so that they are no longer in the blood, and it is sufficient to allow the turnover of immune cells generated under the influence of this reproductive hormone (*Rettew et al., 2008; Scotland et al., 2011*) . In particular, orchidectomy was performed through scrotal incision. Sham groups were performed through the similar procedures, except that the gonads were not removed. After 5 weeks, peritonitis was induced as previously reported and mice were sacrificed at 15 or 30 min (the peak time) for the measurement of LTC_4 or LTB_4 in the exudates, respectively.

3.2.2.6 Determination of 5-LO and COX products

The peritoneal exudate levels of LTC_4, LTB_4 were measured by EIA kits according to manufacturer's instructions (Cayman Chemical; Aurogene, Rome, Italy), while PGE_2 by radioimmunoassay (RIA) (PerkinElmer Life Sciences, Milan, Italy; Sigma, Milan, Italy) and expressed as ng mL^{-1}.

In one set of experiments, the amount of LTs content in the exudates was measured by ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis in Prof. Oliver Werz's laboratory at the Department of Pharmacy of the "Friederich Schiller Universitaet" of Jena, Germany. In particular the supernatants of peritoneal exudates were extracted by solid phase extraction on RP18-columns containing a polymeric reversed phase after conditioning the column with 1 ml 100% methanol and 1 mL H_2O. After washing the columns two times with 0.5 mL H_2O, eicosanoids were eluted with 300 µL 100% methanol. UPLC-MS/MS analyses were carried out on an Acquity UPLC BEH C18 column (1.7 µm, 2.1×50 mm, Waters, Milford, MA, USA) using an AcquityTM UPLC system (Waters) and a QTRAP 5500 Mass Spectrometer (AB Sciex, Darmstadt, Germany) equipped with a Turbo V™ Source and electrospray ionization (ESI) probe. Automatic peak integration were performed with Analyst 1.6 software (AB Sciex, Darmstadt, Germany) using Intelli Quan default settings. Data were normalized on the internal standard PGB_1.

3.2.2.7 Preparation of total cell lysates

Total cell lysates were prepared by lysis for 15 min at 4°C with lysis buffer (20 mM HEPES, pH 7.6, 1.5 mM $MgCl_2$, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF), 15 µg mL^{-1} soybean trypsin inhibitor, 3 µg mL^{-1} pepstatin A, 2 µg mL^{-1} leupeptin, 1% Nonidet P-40, 20% glycerol, 50 mM NaF). Lysates were centrifuged (12 000 xg, 15 min 4°C), the supernatants were collected and stored at -80 °C.

3.2.2.8 Western blotting analysis

Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Segrate, Milan, Italy). The samples were mixed with 4X gel loading buffer (250 mM Tris, 8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 10 mg mL^{-1} of bromophenol blue), heated for 6 min at 95°C. Each sample was loaded and analyzed by electrophoresis on a 10% SDS-polyacrylamide gel. Correct loading of the gel and transfer of proteins to the nitrocellulose membrane was confirmed by Ponceau staining. After electroblot to nitrocellulose membrane (Whatman Protran, Dassel, Germany), membranes were blocked with 0.1 % PBS/Tween containing 5% Milk for 1 hour at room temperature. Membranes were washed and then incubated with primary antibody overnight at 4°C. COX-1, COX-2 (Cell Signalling, Aurogene, Rome, Italy) and β-actin antibodies were used as 1:1000 dilution. After the incubation, the membranes were washed six times with 0.1% PBS-Tween and were incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies diluted 1:10,000 in 0.1% PBS-Tween containing 5% non-fat dry

milk. The membranes were washed and protein bands were detected by an enhanced chemiluminescence system.

3.2.2 Carrageenan-induced pleurisy in rats

Rats were anesthetized with 4% enflurane mixed with 0.5 l/min O_2, 0.5 l/min N_2O and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected, and saline (0.2 mL) or 1% (w v^{-1}) λ-carrageenan type IV (0.2 mL) was injected into the pleural cavity. The skin incision was closed with a suture, and the animals were allowed to recover. At the selected time points (2-4-8 hours) after carrageenan injection, animals were sacrificed by CO_2 inhalation. Pleural exudate was collected by injection in the cavity of 2mL of saline solution, after centrifugation (800 x g for 10 min) the cells were counted by Burcher´s chamber and supernatants were frozen at -80°C. In one set of experiment, rats were pre-treated with 1,5 mg/kg Mk886 (Caymann- chemical, Aurogene, Rome, Italy) 30 minutes prior pleurisy induction. Animals were sacrificed 4 hours after pleurisy induction. The amount of PGE_2 in the supernatant of centrifuged exudates was measured by RIA. Results are expressed as the total amount of PGE_2 measured in the pleural exudate of one rat (nanograms per rat).

3.2.3 PAF-induced shock in mice

PAF C-16 was dissolved in chloroform and stored at -20°C. The PAF working solution was freshly prepared directly before use. To this aim, chloroform was evaporated under N_2, and PAF was dissolved in 0.9% saline solution containing 0.25% bovine serum albumin. Mice were challenged with 30 μg kg^{-1}; 100 μg kg^{-1}; 200 μg kg^{-1} PAF in a

volume of 200 µL via a tail vein injection. Death (determined by cessation of breathing) was then recorded over a period of 2 h. All animals surviving the 2-h test session were euthanized by CO_2 inhalation.

3.3 *In vitro* models of inflammation

3.3.1 Murine resident peritoneal macrophages

3.3.1.1 Collection of resident peritoneal macrophages

Resident peritoneal macrophages (PM) were obtained by lavage of the peritoneal cavity of female and male naive and sham mice or from ORCH mice with 7 mL of cold Dulbecco's modified Eagle's medium (DMEM) with heparin (5U mL^{-1}). PM were then centrifuged at 500×g 4°C for 5 min, resuspended at 1×10^7 cells mL^{-1} and 0.5 mL of cell suspension were added to a 15 mL conical polypropylene tube for each experimental condition. Cells were then incubated at 37°C with ionophore A23187 (4.5 mL, final concentration 2.5µM, 15 min), zymosan (4.5 mL, final concentration 13 particles per cell, 30 min) or vehicle (0.25% dimethyl sulfoxide in DMEM for A23187 and DMEM for zymosan). EDTA at a final concentration of 4 mM was added at the end of incubation time to A23187-stimulated cells; in one set of the experiment, PM from female mice were pre-treated with 5α-DHT (10nM, 15 min, 37°C) and PM from male mice were pre-treated with β-estradiol (100nM,15 min, 37°C) piror stimulation. All samples were then centrifuged at 500 × g at 4 ◦ C for 5 min. Supernatants were collected and frozen at −80°C for measurements of LTC_4 and PGE_2 by EIA (Cayman Chemical Company; Aurogene, Rome, Italy) and RIA (PerkinElmer Life Sciences, Milan, Italy;

Sigma, Milan, Italy) respectively. The cell pellets were washed by resuspending in ice-cold PBS, centrifuged and collected and frozen at −80°C for 5-LO evaluation.

3.3.1.2 Preparation of cell lysates

Total cell lysates were prepared as previously described (see paragraph 3.2.2.7). Subcellular fractionation was carried out by mild-detergent lysis (0.1% NP40) according to Werz and colleagues (*Pergola et al., 2008*). Isolated PM were suspended in ice-cold NP-40-lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1%NP-40, 1 mM PMSF, 60 µg mL^{-1} soybean trypsin inhibitor and 10 µg mL^{-1} leupeptin), vortexed (3 × 5s), kept on ice for 10 min, and centrifuged (1250 µg, 10 min, 4°C). Resultant supernatants (non-nuclear fractions) were transferred to a new tube whereas the pellets (nuclear fractions) were resuspended in ice-cold relaxation buffer (50 mM Tris-HCl, pH 7.4,250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 60 µg mL-1 soybean trypsin inhibitor, 10 µg mL^{-1} leupeptin). Both nuclear and non-nuclear fractions were centrifuged (1250 µg, 10 min, 4°C) for further purification. Lysis of cells and integrity of nuclei were confirmed by light microscopy with trypan blue exclusion. Nuclei in relaxation buffer were disrupted by sonication (3 ×5 s).

3.3.1.3 Western blotting 5-LO analysis

Protein concentration and western blotting were performed as previously described (see paragraph 3.2.2.8). After electroblot to nitrocellulose membrane (Whatman Protran, Dassel, Germany), membranes were blocked with 0.05% PBS/Tween containing 5% BSA for 2 h at room temperature. Membranes were washed and then incubated with

primary antibody overnight at 4°C. 5-LO (AK-7, 1551, affinity purified, Karolinska Institutet, Stockholm, Sweden) and β-actin (Cell-Signalling, Aurogene, Rome, Italy) antibodies were used as 1:1000 dilution. After the incubation, the membranes were washed six times with 0.1% PBS-Tween and were incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies diluted 1:10000 in 0.1% PBS-Tween containing 5% non-fat dry milk. The membranes were washed and protein bands were detected by an enhanced chemo-luminescence system.

3.3.2 Human neutrophils

3.3.2.1 Isolation and stimulation of human neutrophils

Leukocyte concentrates, prepared from freshly withdrawn peripheral blood of healthy adult human donors who had not taken any anti-inflammatory drugs for the last 10 days, with consent, were obtained from the Institute of Transfusion Medicine at University Hospital Jena, Germany. Neutrophils were isolated as previously described (*Pergola et al., 2008*). In brief, neutrophils were isolated from leukocyte concentrate with a multi step procedure: 1) dextran sedimentation; 2) centrifugation on Nycoprep; 3) hypotonic lysis of erythrocytes. Finally, neutrophils were suspended in ice-cold PBS containing 0.1% glucose (PG buffer) and counted by Vi-CELL™ XR. For PG production, $5x10^6$ neutrophils, from female and male donors, respectively, were suspended in PG buffer containing 1mM $CaCl_2$ (PGC buffer) and stimulated with 0.5 μM A23187 (4 hours 37°C) in presence or absence of 10 μM AA. Then, neutrophils were stimulated with 0.5 μM

A23187 (4 hs 37°C) after a pre-treatment with 30 nM Mk886 (15 min 37°C). In other set of experiments, cells were stimulated with 10 μM AA (10 min 37°C).The reaction was stopped on ice and samples were centrifuged (12,000 xg/5min/4°C), PGE_2 levels in the supernatants were measured with ELISA kit (BIOTREND, Germany).

3.4 Statical analysis

The results are expressed as mean ± standard error (SE) of the mean of *n* observations, where *n* represents *in vivo* the number of animals whether *in vitro* the number of experiments performed on different days. Triplicates were used for the various *in vitro* treatment conditions. In the western blot, the figures shown are representative of at least three experiments.

For the statistical evaluation of the data Student´s t test for paired observations was applied. A *p* value <0.05 (*), <0.01 (**) or <0.001 (***) was considered significant.

4. RESULTS

4.1 Sex–dependent LT biosynthesis and related inflammatory reactions in zymosan-induced peritonitis in mice: *in vivo* and *in vitro* analysis

(Data of this thesis have been published: "Pharmacological Research 87 (2014): 1-7" Rossi A., Pergola C., Pace S., Rådmark O., Werz O., Sautebin L.)

LTs are lipid mediators involved in several autoimmune-inflammatory diseases. They are produced during the early phase of the inflammation and are essentially responsible for the increase of the vascular permeability (cys-LT) and the recruitment of the inflammatory cells (LTB_4) (*Kolaczkowska et al., 2002*).

4.1.1 *In vivo* evaluation of inflammatory reaction and LT biosynthesis

4.1.1.1 Sex differences in inflammatory reaction: vascular permeability

Intraperitoneal injection of zymosan resulted in an increased vascular permeability as one of the primary steps initiating inflammation leading to plasma leakage and swelling (*Kolaczkowska et al., 2002*). A similar time-course in the increase of vascular permeability in male and female with a peak at 60 min is evident (**Fig. 5**). However, significant ($p<0.05$ $n=8$ at 30 and 60 min) sex-dependent difference was observed in the amount of dye extravasation that was higher in female *vs* male mice, particularly at 30 and 60 min after zymosan challenge (**Fig. 5**).

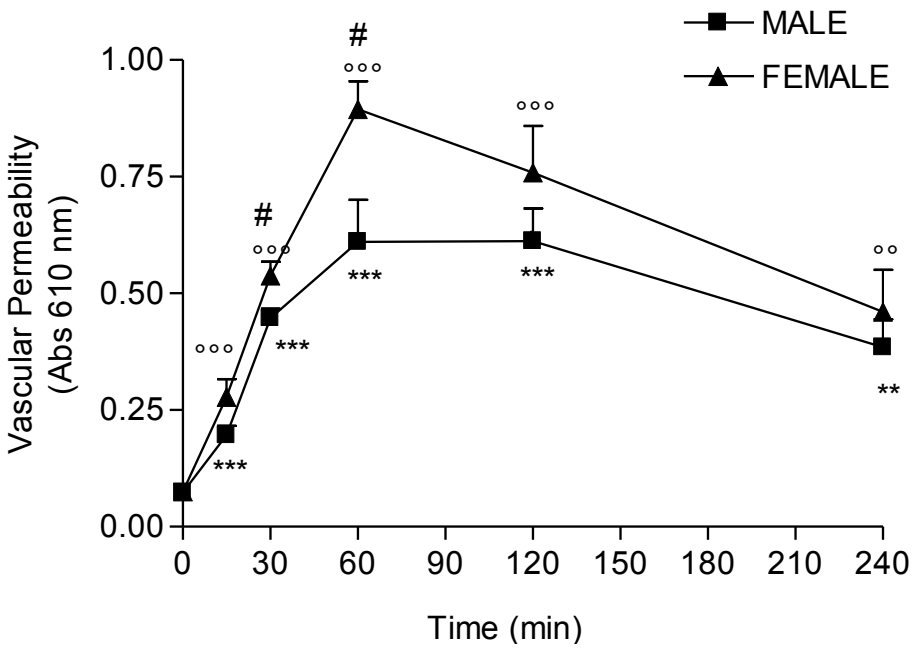


Fig.5 caption. Sex-dependent differences in vascular permeability in zymosan-induced peritonitis in mice. Mice were treated with zymosan (*i.p.*) and sacrificed at the indicated time points. Time zero (T0) corresponded to untreated mice. Values represent means ± S.E.M; *n*=8 mice; °°p<0.01; °°°p<0.001 female *vs* female T0; **p<0.01; ***p<0.001 male *vs* male T0; #p<0.05; ##p<0.01 female *vs* male

4.1.1.2 Sex differences in inflammatory reaction: leukocyte recruitment in the peritoneal cavity

After the induction of the peritonitis there is a time-dependent increase of the afflux of leukocyte in the peritoneal cavity (*Cash et al., 2009*). In fact, the cell recruitment (**Fig. 6A**) under the effect of the released chemo-attractive factors (i.e. LTs) became significant at 120 min (from 1.24 $x10^6 \pm 0.09$ of cell in the cavity at time zero to 2.26 $x10^6 \pm 0.36$ at 120 min after zymosan for female, $p<0.05$, *n=10-8*; and from 1.35 $x10^6 \pm 0.11$ of cell in the cavity at time zero to 2.84 $x10^6 \pm 0.42$ at 120 min after zymosan for male, $p<0.05$, *n=10-8*) and reached a peak at 240 min ($p<0.001$, *n=16* for both sexes). Interestingly, at the time-peak the number of peritoneal cells was significantly ($p < 0.05$) higher in female (13.01 $x10^6 \pm 1.17$ cell in the cavity of female mice and 9.87 $x10^6 \pm 0.82$ in the cavity of male) than in male mice, which was accompanied by an higher MPO activity (measured as neutrophil marker) in female (4.340±0.270 UmL^{-1}, *n=12* female; 3.220±0.290 UmL^{-1}, *n=13* male; $p < 0.01$ **Fig. 6B**).

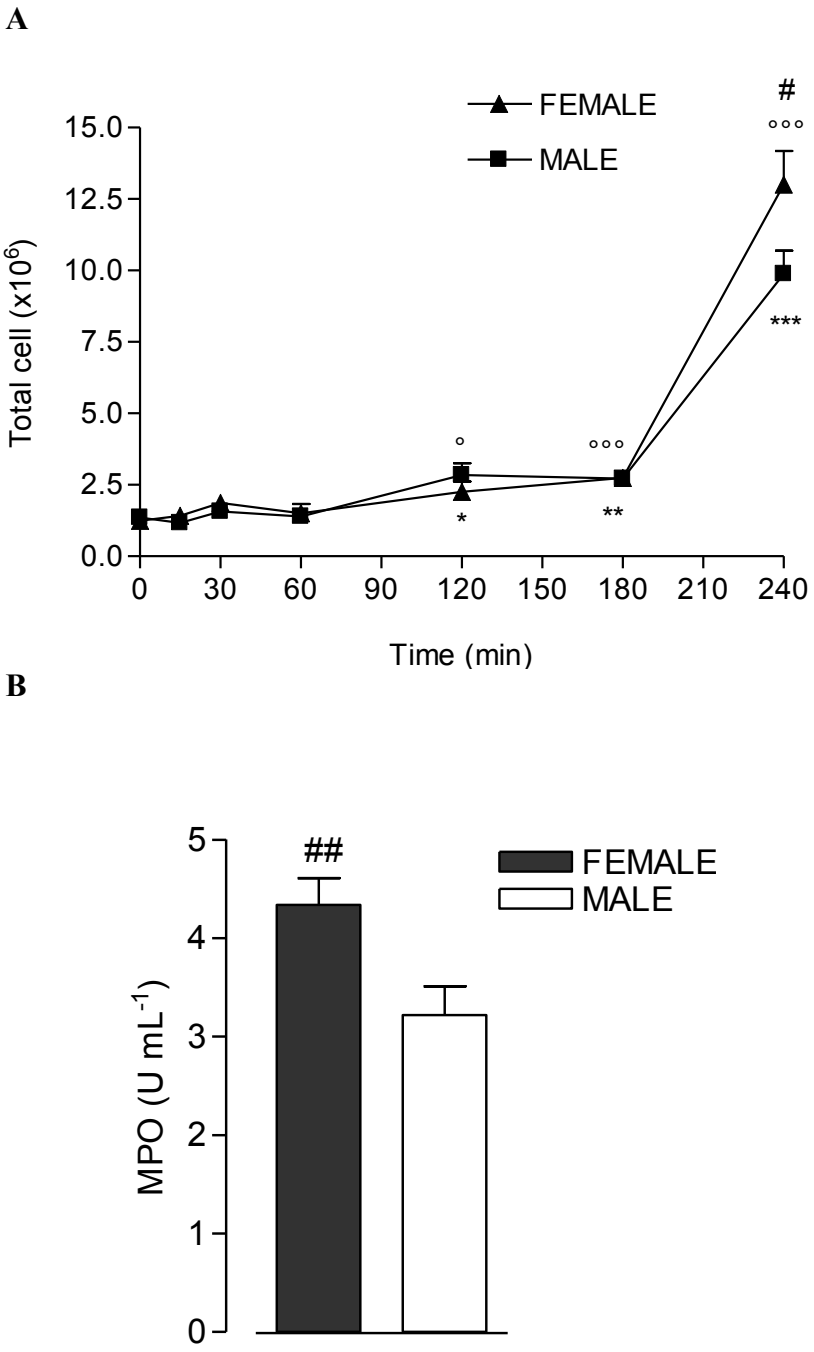


Fig.6 caption. Sex-dependent differences in peritoneal cell influx (A) and MPO activity (B) in zymosan-induced peritonitis in mice. Mice were treated with zymosan (*i.p.*) and sacrificed at the indicated time points. Time zero (T0) corresponded to untreated mice. Values represent means ± S.E.M; (A) *n*=10 mice/T0;15 min; *n*=13 mice/30 min; *n*=13-15 mice/60 min; *n*=8 mice/120 min; 180 min for cell influx; *n*=16 mice/240 min; (B) *n*=12 mice/240 min. °p<0.05; °°p<0.01; °°°p<0.001 female *vs* female T0; *p<0.05; **p<0.01; ***p<0.001 male *vs* male T0; #p<0.05; ##p<0.01 female *vs* male

4.1.1.3 Sex differences in LT production in the peritoneal exudate

First, the time-course of LTC_4 production which represents the main metabolite of 5-LO (*Kolaczkowska et al., 2002; Rao et al.,1994)* in the peritoneal exudate following zymosan challenge was evaluated. In both sexes there was a slight increase in the first 15-30 min after zymosan injection, with a rapid decrease after 60 min. Though the similar kinetic, LTC_4 levels were higher in the exudate from female mice (15 min: 149.78 ± 21.49 ng mL^{-1} *n=15* for female and 67.64 ± 20.06 ng mL^{-1} *n=14* for male, $p<0.01$; 30 min: 137.48 ± 19.16 ng mL^{-1} *n=13* for female and 63.75 ± 15.01 ng mL^{-1} *n=11* for male, $p<0.01$; 60 min: 47.04 ± 10.4 ng mL^{-1} *n=14* for female and 20.79 ± 4.18 *n=13* for male, $p<0.05$ **Fig. 7A)**.

Due to the important chemo attracting action of LTB_4 its levels were measured as well in the exudates. As expected its production was much more lower compared to LTC_4 and interestingly the kinetic different. In particular, after zymosan injection there was a biphasic trend in LTB_4 release with a significant ($p<0.01$) sex-difference during at 30 min (30 min: 0.293±0.032 ng mL^{-1} *n=13* for female and 0.149±0.0310 ng mL^{-1} *n=11* for male). No sex differences were found in the late increase (120-180 min) of LTB_4 (**Fig.7B**).

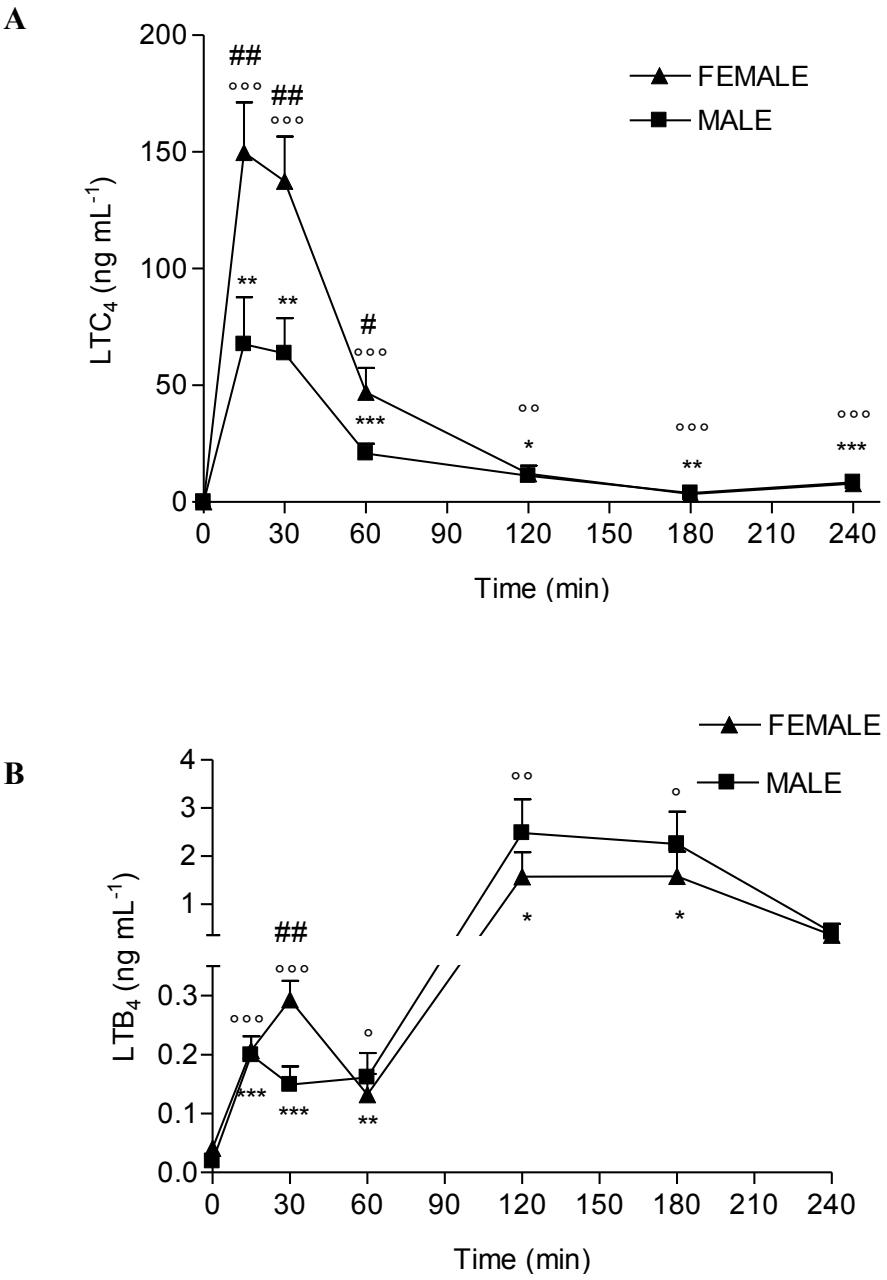


Fig.7 caption. Sex-dependent differences in LT biosynthesis in zymosan-induced peritonitis in mice. (A, B) Mice were treated with zymosan (*i.p.*) and sacrificed at the selected time points. Time zero (T0) corresponded to untreated mice. Values represent means± S.E.M; *n*=8 mice/T0; *n*=14-15 mice/15 min; *n*=11-14 mice/30 min; *n*=13-14 mice/60 min; *n*=8 mice/120 min, 180 min and 240 min. °°p<0.01; °°°p<0.001 female *vs* female T0; *p<0.05; **p<0.01; ***p<0.001 male *vs* male T0; #p<0.05 female *vs* male at time 60 min; ##p<0.01 female time 15 and 30 *vs* male time 15 and 30.

4.1.1.4 Effect of circulating sex hormones on LT biosynthesis

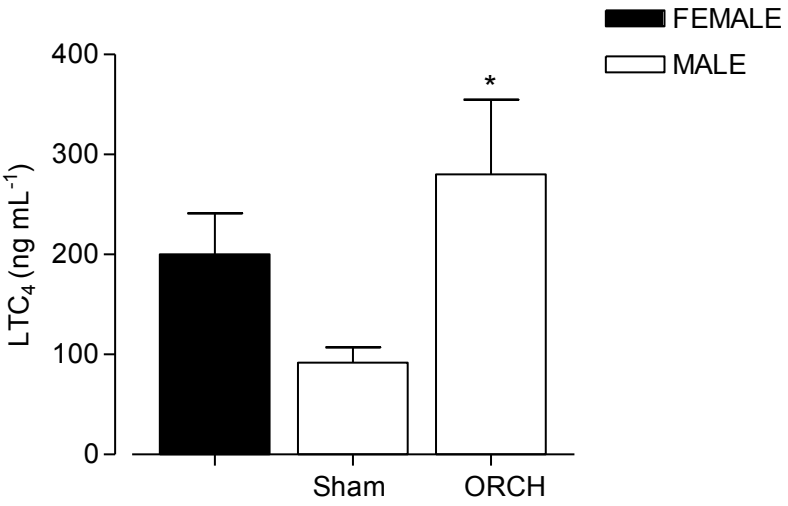
In order to evaluate the effect of the male sex hormones on LT production in zymosan-induced peritonitis, gonads from adult male mice were surgically removed. Five weeks after this procedure the levels of testosterone in operated mice (ORCH: 0.050 ± 0.006 ng mL^{-1} *n=9*) were similar to the levels in female mice (female: 0.055 ± 0.008 ng mL^{-1} *n=10*) and both significantly ($p<0.05$) lower than un-operated male mice (Sham male: 2.00 ± 0.7 ng mL^{-1} *n=9*) (**table 1**). Lower levels of testosterone in ORCH mice than in sham male mice corresponded to significant ($p<0.05$) higher levels of LTC_4 (Sham male: 91.70±15.6 ng mL^{-1}; ORCH: 280.00±74.70 ng mL^{-1} **Fig. 8A**) and LTB_4 at 15 min (Sham male: 0.185±0.028 ng mL^{-1}; ORCH: 0.290±0.032 ng mL^{-1}). Orchidectomized mice showed levels of LT similar to female (female: LTC_4 200.3±40.9 ng mL^{-1} *n=8*; female: LTB_4 0.275±0.03 ng mL^{-1} *n=7*) suggesting a negative role of testosterone in the production of these metabolites (**Fig. 8B**).

Table 1. Plasma Testosterone levels (ng mL^{-1})

Male	2.00±0.7
ORCH	0.050±0.006*
Female	0.055±0.00*

Values represent mean± S.E.M; *n*=9-10 mice/group. *p<0.05 ORCH *vs* male sham

A



B

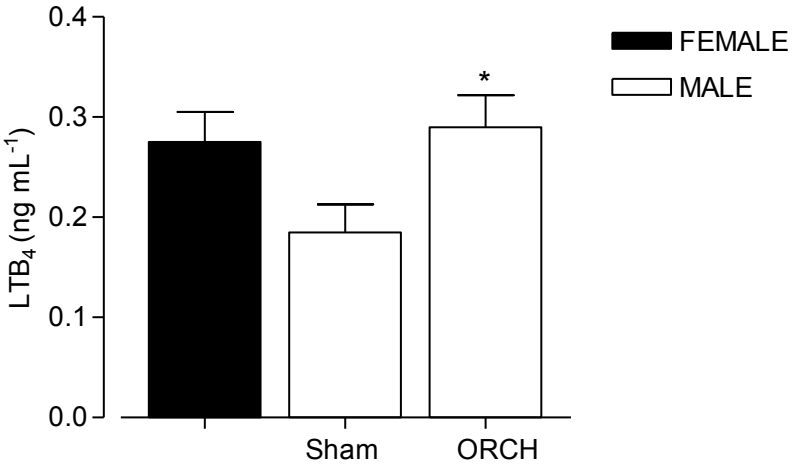


Fig.8 caption. Effects of orchidectomy on LT biosynthesis in zymosan-induced peritonitis in mice. ORCH and sham-operated mice were sacrificed at 15 (LTC_4, A) and 30 min (LTB_4, B) after zymosan (*i.p.*) treatment, respectively. Values represent mean± S.E.M; *n*=8-9 mice/time. *p<0.05 ORCH *vs* male sham.

4.1.1.5 *In vivo* effect of testosterone on LT production

To better understand the negative role of testosterone on LT biosynthesis, adult female mice were pre-treated with testosterone (0.5 mg kg^{-1} 5α-DHT in 2% DMSO) or vehicle (2% DMSO in saline) 30 min before the zymosan injection. The time course of LTC_4 production showed a significant ($p<0.001$) reduction at 30 min (vehicle: 0.343±0.105 LTC_4/PGB_1; 5α-DHT 0,5 mg/kg 0,261±0,0339 LTC_4/PGB_1; *n=5*) in female mice pre-treated with testosterone compared to female mice treated with vehicle (**Fig.9**).

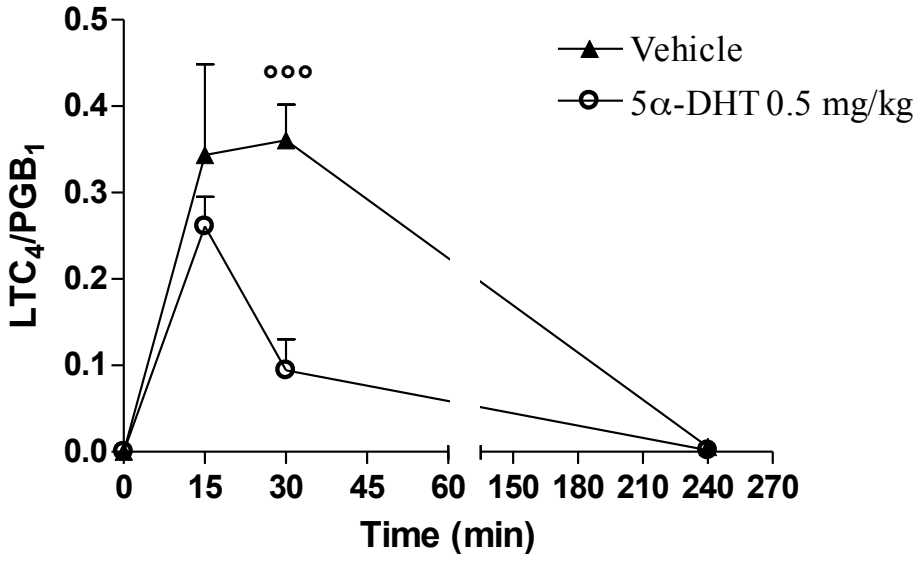


Fig.9 caption. Effects of 5α-DHT pre-treatment on LTC_4 biosynthesis in zymosan-induced peritonitis in female mice. 5α-DHT and vehicle pre-treated female mice were treated with zymosan (*i.p.*) and sacrificed at the selected time points. Time zero (T0) corresponded to untreated mice. Values represent mean± S.E.M; *n*=5 mice/time. °°°p<0.05 5α-DHT female mice *vs* vehicle.

4.1.2 *In vitro* investigation of sex differences in LT biosynthesis

4.1.2.1 Sex differences in LT production in PM

During the first 60 minutes after zymosan injection PM are the cell responsible for LTC_4 production (*Kolaczkowska et al., 2002; Rao et al.,1994*) thus we collected these cells in order to investigate *in vitro* the reason for the *in vivo* observed sex difference. First, to confirm the sex difference in LTC_4 production *in vitro*, we stimulated PM in suspension (collected at 4°C to keep the *in vivo* statement) with zymosan (13 particles/cell corresponding to the *in vivo* condition, 30 min 37°C) or with A23187 (2.5 μM , 15 min 37°C). After stimulation, PM from female produced significant higher levels of LTC_4 compared to PM from male, treated side-by-side with A23187 (female: 204,8±20,9 ng mL^{-1}; male: 123,1±14,6 ng mL^{-1} $p<0.05$ *n=4* **Fig. 10A**) or zymosan (female: 21.3±1 ng mL^{-1}; male: 8,5±0,6 ng mL^{-1}; $p<0.01$ *n=2* **Fig. 10B**). The observed sex difference was not present in PGE_2 release after stimulation with both stimuli (**Fig. 11A,B**) excluding that the observed sex difference in LTC_4 formation was dependent to a diverse AA release from the cells as well as that sex difference involved also the COX pathway in PM. Next, we analyzed if the difference in the LTC_4 production between activated female and male PM was accompanied by different 5-LO protein levels or subcellular localization. Intriguingly, while the basal 5-LO protein expression was not different between sexes (OD 5-LO/β-actin female: 1±0,05; male: 0,96±0,05; *n=3* **Fig. 12A**), a difference in 5-LO subcellular localization was evident. In fact, 5-LO was found mainly in the non-nuclear fraction of un-stimulated PM from female mice (OD 5-LO/β-actin % of female: 78,6±3,64;

n=4) whereas in PM from male mice (isolated and treated side-by-side with female PM) 5-LO was equally expressed in both the non-nuclear and in the nuclear fraction of resting cells (OD 5-LO/β-actin % of male: 21,4±3,64; *n=4*), indicating a sex-dependent localization of the 5-LO enzyme. The stimulation of PM with A23187 (**Fig. 12B**) and zymosan (**Fig. 12C**) induced the translocation of cytoplasmic 5-LO to the nuclear fraction in both sexes being however the migrated active fraction in male lower than in female .

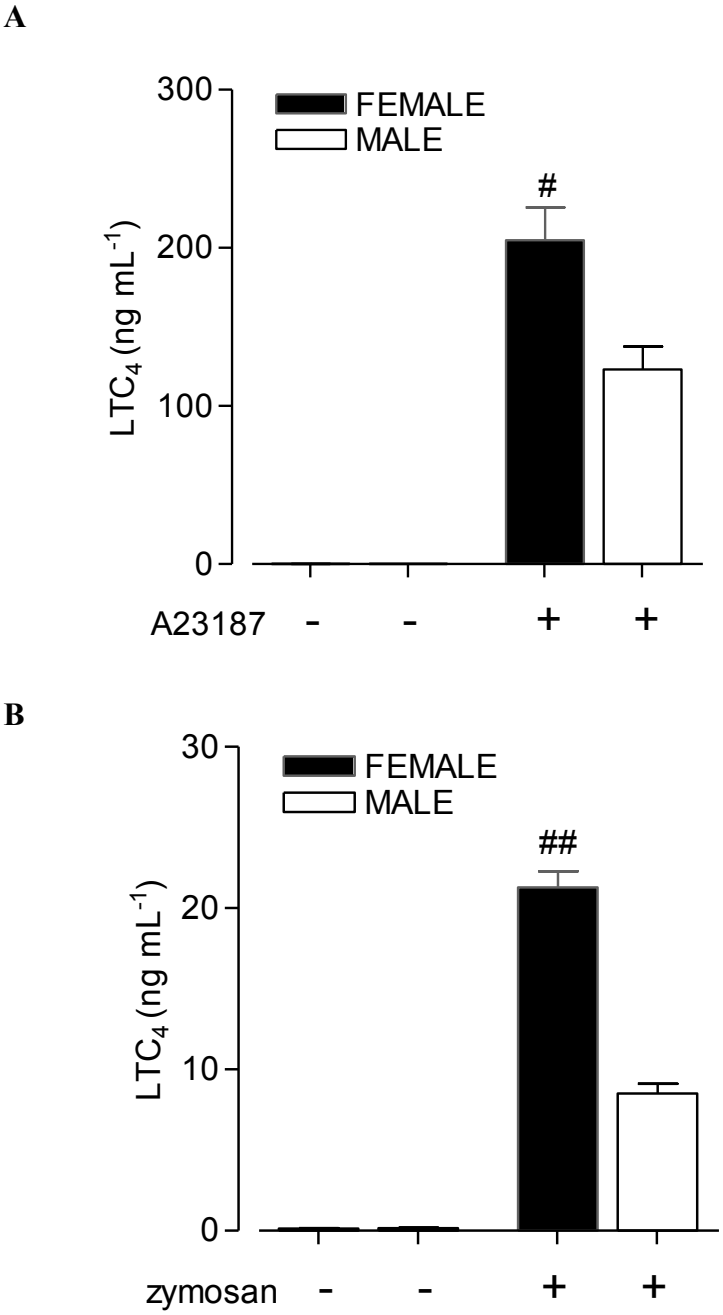
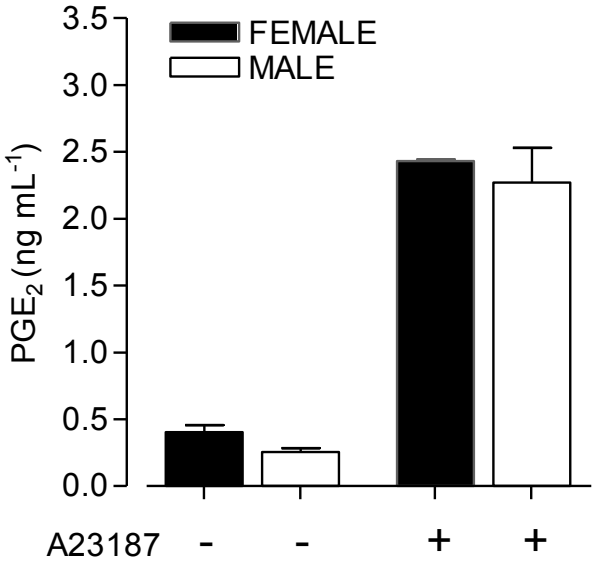


Fig.10 caption. Sex-dependent differences in LTC_4 biosynthesis in mouse peritoneal macrophages. LTC_4 formation in resident PM induced by (A) A23187 (2.5 μM; 15 min, 37°C) or (B) zymosan (13 particles $cell^{-1}$; 30 min, 37°C). Data are expressed as means ± S.E.M from *n*=4 (A23187) or *n*=2 (zymosan) independent experiments performed in triplicates, each. #p<0.05; ##p<0.01 stimulated PM from female *vs* stimulated PM from male mice.

A



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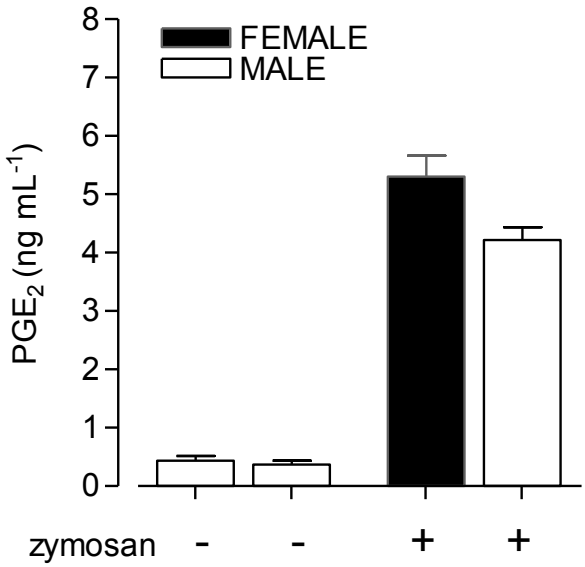
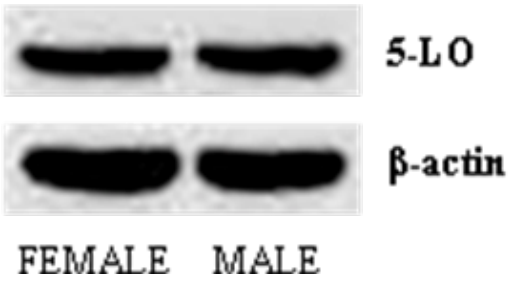


Fig.11caption. PGE_2 biosynthesis in mouse peritoneal macrophages is not sex-dependent. PGE_2 formation in resident PM induced by (A) A23187 (2.5 μM; 15 min, 37°C) or (B) zymosan (13 particles $cell^{-1}$; 30 min, 37°C). Data are expressed as means ± S.E.M from *n*=4 (A23187) or *n*=2 (zymosan) independent experiments performed in triplicates, each.

A



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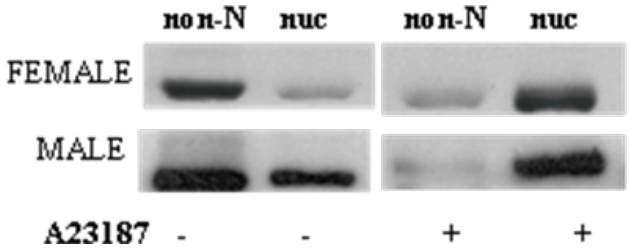


C

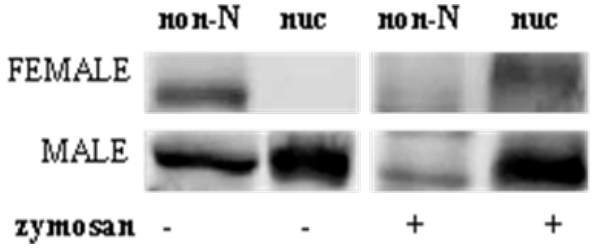
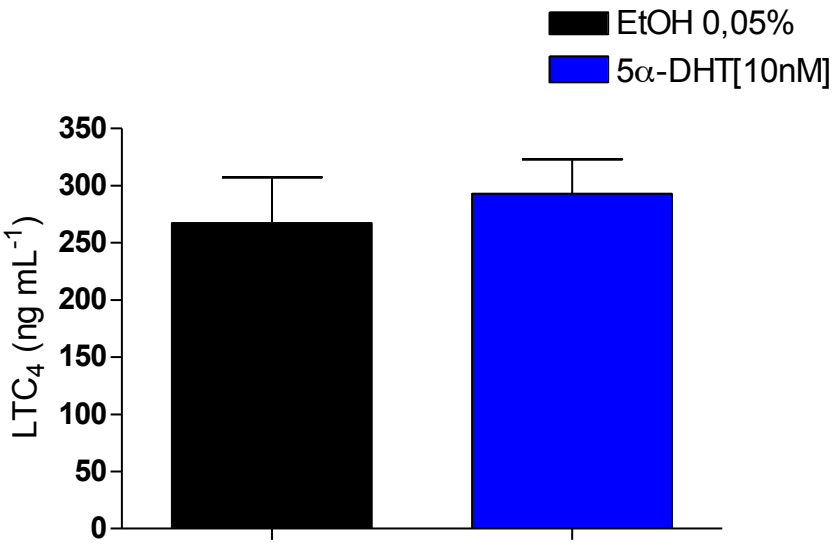


Fig.12caption. 5-LO protein expression and subcellular localization (A) 5-LO protein expression in resident PM was analyzed by immunoblotting of total cell lysates. Results are representative of 3 independent experiments. (B, C) Subcellular localization of 5-LO in resident PM from female and male mice induced by A23187 (2.5 μM; 15 min, 37°C) or zymosan (13 particles $cell^{-1}$; 30 min, 37°C) analyzed by immunodetection of 5-LO in the nuclear (Nuc) and non-nuclear (Non-N) fractions of mild-detergent (0.1% Nonidet P-40)-lysed cells. Data are representative of 2-4 independent experiments.

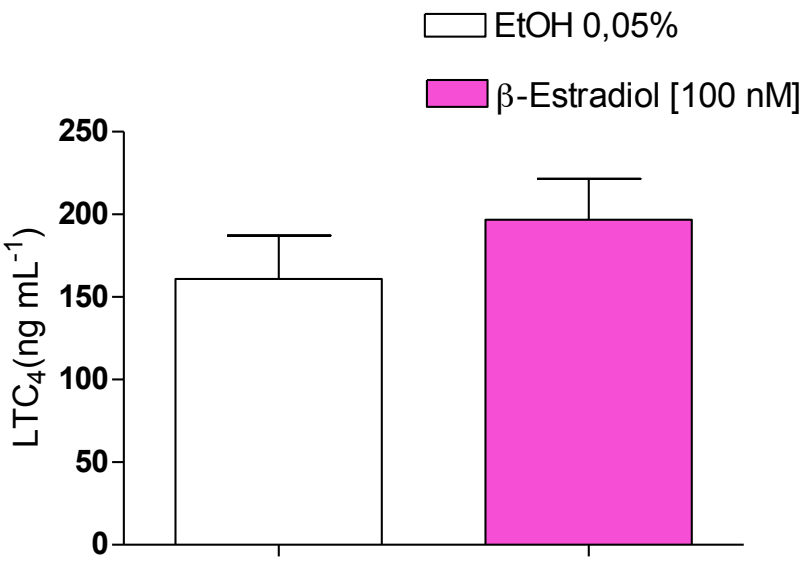
4.1.2.2 Effect of sex hormones on LT production in PM

Primarily, to evaluate the role of sex hormones in the sex-dependent production of LTC_4 in PM, we pre-treated the cells from female mice with 5α-DHT (10 nM; 30 min, 37 ◦C **Fig.13A**), and the cells from male mice with or 17β-estradiol (100 nM; 30 min, 37 ◦C **Fig.13B**). After A23187 stimulation, no significant effect of sex hormones in LTC_4 production was found. To confirm the hypothesis that the sex difference was due to the *in vivo* actions of male reproductive steroid hormones, we collected PM from ORCH mice and sham mice. After stimulation with A23187 (2.5 μM; 15 min, 37 ◦C), PM from ORCH produced a significant ($p<0.05$) (sham male: 56,90±1,45 ng mL^{-1}; ORCH: 73,50±4,40 ng mL^{-1}; *n=3* **Fig.13C**) higher amount of LTC_4 compared to PM from sham male mice.

A


EtOH 0,05%
5α-DHT[10nM]
LTC4 (ng mL-1)
350
300
250
200
150
100
50
0

B


EtOH 0,05%
β-Estradiol [100 nM]
LTC4(ng mL-1)
250
200
150
100
50
0

C

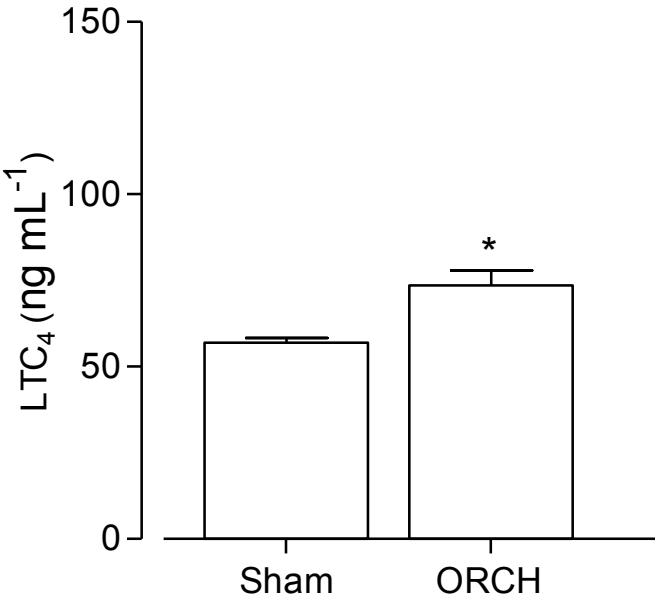


Fig.13 caption. Effects of hormones on LTC_4 production in stimulated-peritoneal macrophages from male mice. LTC_4 formation in resident PM from (A)female and (B)male mice *in vitro* pretreated with hormones. (C) LTC_4 formation in resident PM from orchidectomized (ORCH) and sham-operated mice induced by A23187 (2.5 μM; 15 min, 37°C). Data are expressed as means ± S.E.M from *n*=3 independent experiments performed in triplicates, each. *p<0.05, stimulated PM from ORCH mice *vs* stimulated PM from sham male mice

4.2 Sex–dependent PG biosynthesis and related inflammatory diseases in different models of acute inflammation:

As reported above, accumulated clinical evidences show that several inflammatory diseases are sex-biased. Similarly to what reported for LTs, PGs play a pivotal role in several disorders such as circulating shock (*Schröder et al., 1998*) or ulcerative colitis (*Tragnone et al., 1996*). Interestingly, in these diseases, male present higher severity and incidence. However, to date, little is known about sex difference in PG production, and this prompted us to study PG production in both sexes by the use of selected experimental models of acute inflammation.

4.2.1 *In vivo* evaluation of inflammatory reaction and PG biosynthesis

4.2.1.1 Sex-dependent differences in PG biosynthesis in zymosan-induced peritonitis in mice

Zymosan injection resulted in a marked production of PGs in the peritoneal exudates of male and female mice. Both sexes presented the same kinetics in PG production. There were no sex differences in PGE_2 production during the first hour, while it occurred in the late phase of the inflammatory reaction sustained by neutrophils (*Cash et al., 2009*) starting at 60 minutes and becoming significant (male: 1,69±0,155 ng mL^{-1} *n=29*; female: 1,09±0,108 ng mL^{-1} *n=28*; $p<0.01$ **Fig.14**) at 240 min.

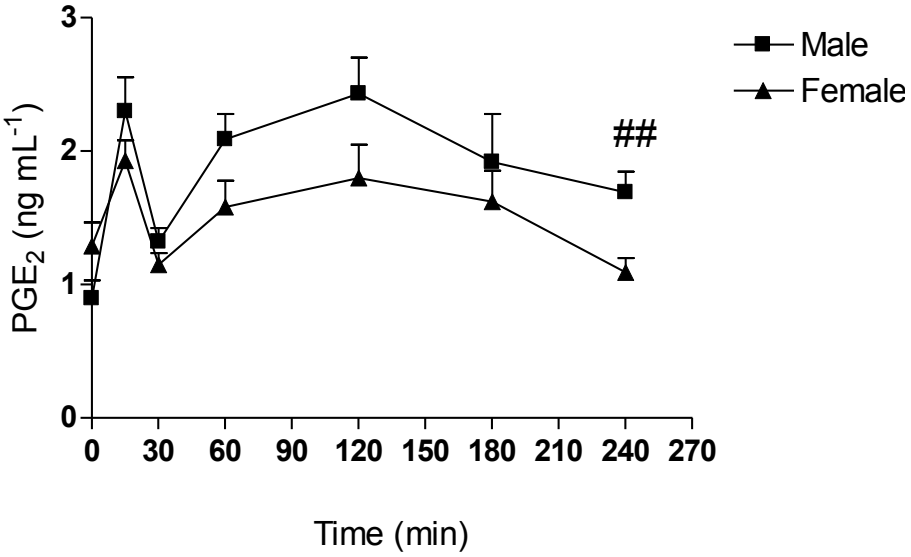


Fig.14 caption. Sex-dependent differences in PGE_2 biosynthesis in zymosan-induced peritonitis in mice. Mice were treated with zymosan (*i.p.*) and sacrificed at the selected time points. Time zero (T0) corresponded to untreated mice. Values represent means± S.E.M; *n*=23 mice/T0; *n*=24 mice/15 min; *n*=24 mice/30 min; *n*=25 mice/60 min; *n*=12 mice/120 min, *n*=11 180 min and *n*=29 240 ##p<0.01 male time 240 min *vs* female time 15 time 240 min.

4.2.1.2 Sex-biased PG biosynthesis in carrageenan-induced pleurisy in rats

Next, we analyzed if the observed sex difference in PG production was species-specific. To this aim we utilized a well known model of acute inflammation, carrageenan-induced pleurisy in rats, where PGs play a pivotal role (*Moore et al., 2010*). The injection of carrageenan induced a time-dependent production of PGs in the thoracic cavity starting after 120 min with a peak at 240 min and a decrease in the next 480 min, but still higher than the control animals. Significant ($p<0,05$) higher PGE_2 levels were found in the pleural exudates of male rats compared to the female (male 4 hours: 1.180±0.110 ng mL^{-1} *n=6*; female 4 hours: 0.8600±0.090 ng mL^{-1} *n=6*; male 8 hours: 0.377±0.055 ng mL^{-1} *n=6*; female 8 hours: 0.2230±0.0280 ng mL^{-1} *n=6* **Fig.15**) in the late times of the inflammatory response.

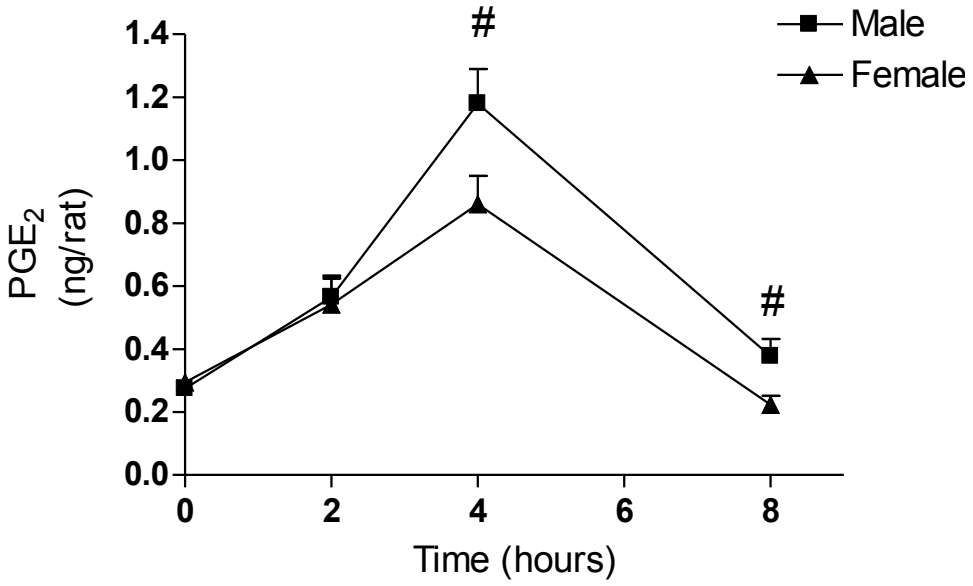


Fig.15 caption. Sex-dependent differences in PGE_2 biosynthesis in carrageenan-induced pleurisy in rats. Rats were treated with carrageenan by an intrapleural injection and sacrificed at the selected time points. Time zero (T0) corresponded to untreated rats. Values represent means± S.E.M; *n*=6 mice/T0; *n*=13 rats/2 hours; *n*=6 rats/4 hours ; *n*=6 rats/8 hours. #p<0.05 male time 4 and 8 hours *vs* female time 4 and 8 hours.

4.2.1.3 Sex difference in PG related-disease: the mouse PAF-induced mortality

Clinical studies show that circulating-shock induced mortality in human has an higher incidence in male compared to female (*Schroeder et al, 1998*). Interestingly, Becker and colleagues demonstrated the pivotal role played by PGs in PAF-induced mortality in mice. On these bases, we selected PAF-induced mortality as third model of acute inflammation to evaluate the PG-mediated sex difference. PAF injection caused a dose-dependent death in mice during the 2 hours of observation with sex-biased difference. The administration of PAF at the dose of 100 μg/kg resulted in a significant higher mortality in male mice compared to the female (p value= 0,001; 90% of deaths 27 out 30 male; 52% of deaths 15 out 29 female). The sex difference was abolished by the higher dose of 200 μg/kg of PAF (88% of deaths 43 out 49 male; 75% of deaths 43 out 57 **table 2**).

PAF [µg/kg]	sex	dead	total	mortality	p value
30	M	2	10	20	
	F	0	10	0	0.24
100	M	27	30	90	0.0013
	F	15	29	52	
200	M	43	49	88	
	F	43	57	75	0.0848

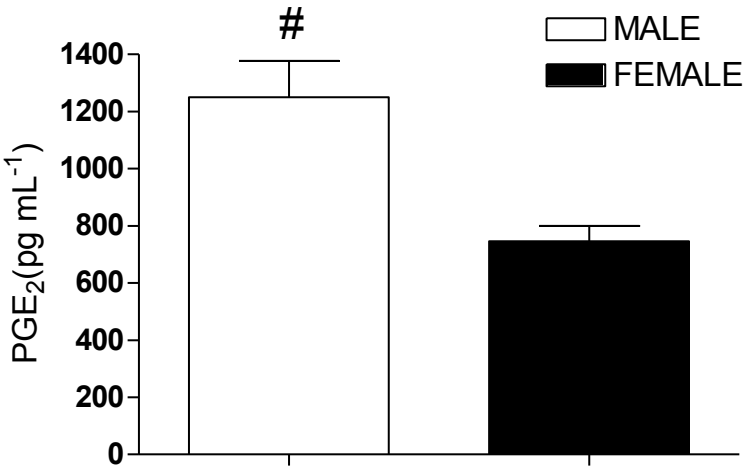
Table 2 caption. **Sex difference in mouse PAF-induced mortality.** Mice were treated *i.v.* with PAF (30 µg/kg; 100 µg/kg; 200 µg/kg) and observed for 2 hours. p value results from Fisher's exact test one tailed.

4.2.2 *In vitro* evaluation of PG biosynthesis

4.2.2.1 Capacity of PGE_2 production in human neutrophils is sex-dependent and receptor-independent

Neutrophils are the immune cells involved in the late phase of the inflammatory reaction, which are recruited in the inflammation site by chemo-attractant factors (i.e. LTB_4, *Yokomizo et al., 1997*) produced by the resident cells (*Dimasi et al., 2013*). To investigate a) the reason of the observed sex difference in PGE_2 production in the late phase of inflammation and b) in order to study if this difference is present also in human species, we isolated neutrophils from the blood of female and male donors and stimulated them with 0.5 μM A23187 to skip any receptor involvement. After *in vitro* stimulation with 0.5 μM A23187 (37°C; 4 hours), PGE_2 production was significantly ($p<0.05$) higher in male compared to female (male: 1250±127 pg $mL^{-1;}$ female: 746±53.5 pg mL^{-1}; *n=3* **Fig. 16A**). The addition of exogenous AA abolished the sex difference suggesting that this effect was due to the conversion of the endogenous substrate, the AA (male: 711±190 pg mL^{-1} ; female: 684±116 pg mL^{-1} **Fig. 16B**).

A



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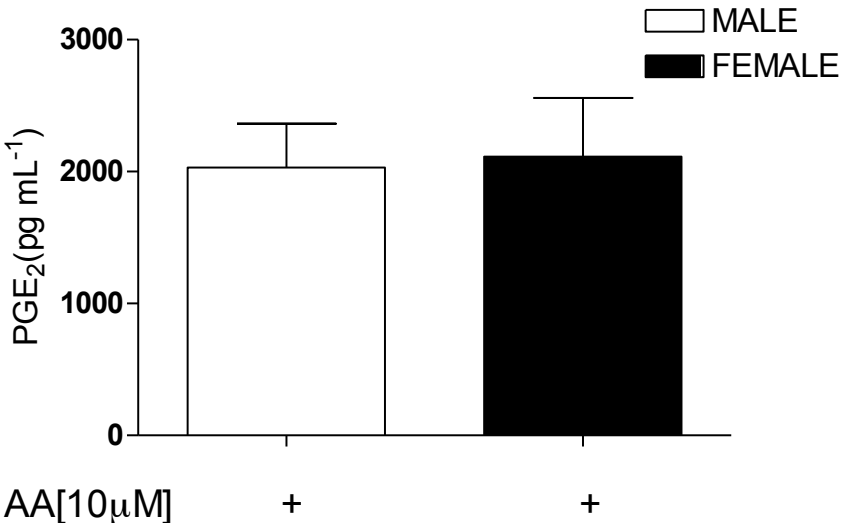


Fig.16 caption. Sex differences in PGE_2 biosynthesis in human neutrophils. PGE_2 formation in human neutrophils induced by (A) A23187 0.5 μM and (B) A23187 0.5 μM + AA 10 μM (37°C). Data are expressed as means ± S.E.M from *n*=3 independent experiments performed in triplicates, each. #p<0.05, male vs female, t-test.

4.2.3 Effect of 5-LO pathway inhibition on AA metabolism via COX enzymes

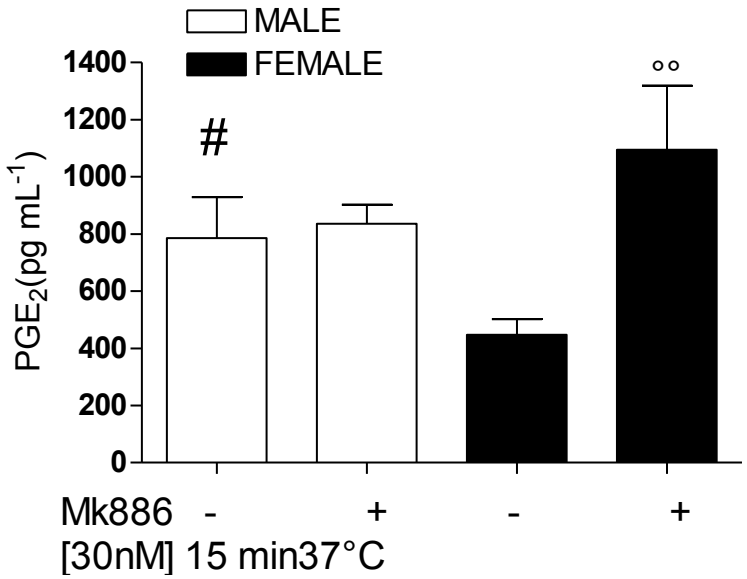
a) *In vitro* : human neutrophils

On the basis of the existent sex difference in arachidonic acid metabolism by 5-LO enzyme in human neutrophils leading to a higher production of LTs in female (*Pergola et al., 2008*), , we hypothesized that this event could have been responsible for the observed PGE_2 sex diverse biosynthesis (higher in male). Thus, we shifted the AA metabolism through the COX pathway by a pre-treatment of the cells with MK886 (30 nM 15 min 37°C), a FLAP inhibitor. After that, neutrophils were stimulated with 0.5 μM A23187 (4 hours, 37°C) for PGE_2 production. The blockage of 5-LO enzyme did not affect PGE_2 production in male neutrophils while significantly ($p<0.01$) increased the female levels, abolishing the sex difference (male DMSO: 786±144 pg mL^{-1} ; male MK886: 835±67 pg mL^{-1} ; female DMSO: 448±54,5 pg mL^{-1} ; female MK886: 1094±225 pg mL^{-1} *n=3* **Fig. 17A**) .

b) *In vivo*: in carrageenan-induced pleurisy in rats

To correlate the *in vitro* data on human neutrophils with the *in vivo* data, we pre-treated rats with MK886 (1,5 mg/kg, 30 min before pleurisy induction). Also *in vivo* MK886 was able to increase significantly ($p<0,05$) PGE_2 production only in female rats and to abolish the sex difference (male DMSO: 1.087±0.061 ng mL^{-1}; male MK886: 1.089±0.1 ng mL^{-1}; female DMSO: 0.881±0.057 ng mL^{-1}; female MK886: 1.18±0.12 ng mL^{-1} **Fig.17B**).

A



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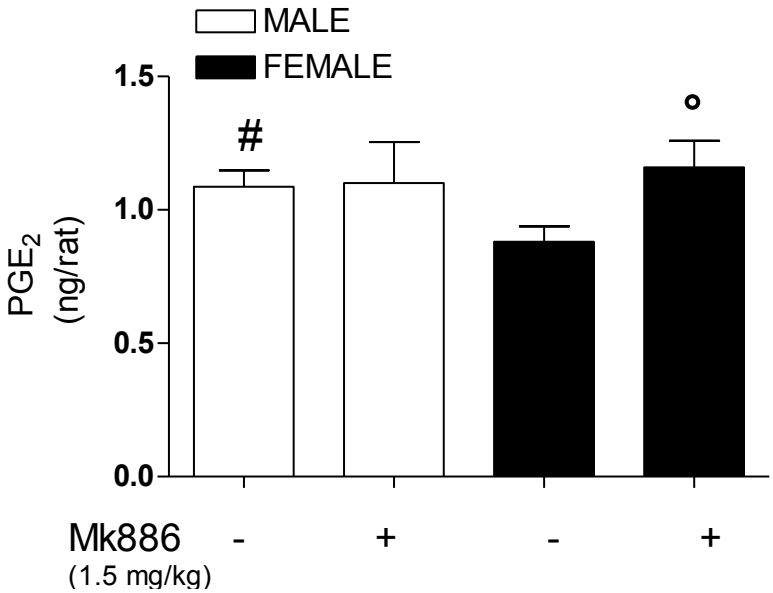

Fig.17 caption. Effect of MK886 pretreatment on PGE_2 biosynthesis. PGE_2 formation in human neutrophils induced by (A) A23187 0.5 μM in male and female neutrophils pre-treated with MK886 and (B) in carrageenan-induced pleurisy in rats pretreated with MK886 . Data are expressed as means ± S.E.M from (A) *n*=3 independent experiments performed in triplicates, each or *n*=28-6 rats ##p<0.01, #p<0.05male vs female, °° p<0.01, °p<0.05 untreated female vs mk886 treated female.

5. DISCUSSION

The scientific community is more and more interested in the sex differences in the inflammatory/immune diseases (*Whitacre CC., 2001*), but little is known to date about the mechanisms involved. In this thesis we have investigated the sex differences in eicosanoid biosynthesis, pivotal mediators in several autoimmune/inflammatory disorders with a well known sex bias, such as asthma (*Osman M, 2003*), multiple sclerosis (*Khalid R., 2014*) or sepsis shock (*Schroeder J et al., 1998*). Eicosanoid biosynthesis starts with the release of AA from the membrane phospholipids through the action of $cPLA_2$ then, the free AA can be substrate of different pathways and among them the 5-LO activity leads to the formation of LTs whereas COX activity to the formation of prostanoids. These processes can be regulated by different factors and recently sex has been addressed as one of them. In particular, Pergola and colleagues have shown that LT biosynthesis in human whole blood as well as in human immune cells (i.e. monocytes and neutrophils) is higher in female than male. This difference was due to the ability of testosterone to regulate 5-LO subcellular localization (neutrophils) or its activation status (monocytes) (*Pergola et al., 2008; Pergola et al., 2011*). In this thesis we show for the first time, that the sex difference in eicosanoid biosynthesis is present also in *in vivo* models of acute inflammation.

First, it was evaluated if the observed sex difference in LT biosynthesis was present also in an *in vivo* model of acute inflammation related to LTs such as zymosan-induced peritonitis in mice (*Rao et al., 1994*). After peritonitis induction, LTC_4 and LTB_4 levels were significantly higher in the inflamed peritoneum of female than male mice, which was followed and/or accompanied by sex differences

in relevant inflammation parameters measured in terms of vascular permeability and neutrophil infiltration. In particular, vascular permeability represents one of the primary steps in the inflammatory reaction that is a prerequisite for the leakage of plasma into the inflamed tissue. The *i.p.* injection of zymosan in mice induced an inflammatory reaction characterized by early vascular permeability and steady increase of protein leakage into the peritoneum in the first 60 min. Interestingly, these events were more robust in female than in male mice, implying a more pronounced inflammatory response in female animals. Vascular permeability represents, however, a highly regulated multistep process mediated by inflammatory mediators, and cys-LTs are particularly recognized as potent vascular-regulating agents through the interaction with the $cysLT_1$ receptor (*Griswold et al., 1991; Maekawa A. et al., 2002*). We observed that LTC_4 levels rapidly raised after zymosan injection (peaking at 15-30 min) and were significantly higher in female mice, suggesting that the sex differences in vascular permeability could be due, at least in part, to the different LT biosynthesis. Previous studies showed that the dominant peritoneal leukocyte population in the early phase of the inflammatory response in CD1 mice are the resident macrophages (about 95%, *Doherty et al., 1985*), which are known to mainly synthesize LTC_4 as 5-LO product (*Kolaczkowska et al., 2002; Rao et al., 1994*). In fact, it has been reported that macrophage depletion caused a profound decrease of LTC_4 production and vascular permeability in zymosan-induced peritonitis (*Kolaczkowska et al.,2002*). Moreover, cell numbers as well as MPO activity in the peritoneum (which is an index of neutrophil infiltration, another LT-related event) were significantly higher in female than in male mice after 240 min. Supporting the delayed cell recruitment in the peritoneal cavity, LTB_4 levels were found higher in the peritoneal cavity of female mice 15 minutes after the

zymosan injection, suggesting that the sex-difference in LT biosynthesis was due to the resident peritoneal cell stimulated by zymosan.

Previous studies demonstrated that the lower production of LT in male human immune cells such as neutrophils and monocytes (*Pergola et al., 2008; Pergola et al., 2011*) was due to the ability of testosterone to regulate 5-LO subcellular localization (in neutrophils) or its activation status (in monocytes) leading to a the sex-biased LT formation. On these bases, the effect of testosterone was investigated also in our *in vivo* model. First, to understand the role of this sex hormone on LT biosynthesis, we surgically removed the gonads from male mice and induced the peritonitis five weeks after (time necessary to deplete testosterone levels in the blood and to obtain the turnover of peritoneal resident cells). Interestingly, the higher production of LT in orchidectomized mice compared to the sham one accompanied by a lower plasma testosterone levels, led us to hypothesize that the circulating male hormone was responsible also for the *in vivo* sex difference in LT biosynthesis. Supporting this theory, the pre-treatment of female mice with testosterone significantly reduced LT levels in the exudates after peritonitis induction. Then, PM were selected as *in vitro* model to investigate the reason behind this sex differences because has been previously reported that in CD1 mice resident peritoneal cells are 95% macrophages (*Kolaczkowska et al.,2002*) and the higher production of LTC_4 in female PM after A23187 and zymosan stimulation strongly supported this choice. The absence of a sex difference in LT biosynthesis after *in vitro* pre-treatment of PM with hormones as well as the higher production of LTC_4 in PM from orchidectomized mice support the role of the circulating testosterone in the whole organism. In human cells the inhibitory activity of testosterone was related to a lack in 5-LO enzyme activity strictly connected to its subcellular localization.

Intriguingly, a substantial sex difference was found in 5-LO subcellular localization in PM from male and female mice according to the previous findings in human neutrophils (*Pergola et al., 2008*). In fact, the higher levels of 5-LO enzyme in the nuclear fraction of unstimulated male PM resulted in a lower trafficable 5-LO enzyme leading to less LT production. Interestingly, no sex differences were found in the total expression of 5-LO enzyme. Note that the levels of the COX-derived PGE_2 were instead equal in PM from male and female mice as well as in the first 60 minutes after zymosan injection where the PM are the cells responsible for its production. Surprisingly, a sex-biased PGE_2 formation was found in the late phase of the inflammatory response. In particular, 2 hours after peritonitis induction, a trend in higher production of PGE_2 from male mice was evident and it became significant at 4 hours. Interestingly, starting from 2 hours after the zymosan injection, there was a significant increase of the cell afflux in peritoneal cavity of both sexes that raised a peak at 4 hours where the cell population is mostly composed by infiltrated neutrophils, as confirmed by MPO levels. Notably, the same events were present in another model of acute inflammation such as carrageenan-induced pleurisy in rats. In fact, after carrageenan injection, a time-dependent production of PGE_2 with a peak at 4 hours was evident and, interestingly, with a similar kinetic for both sexes. According to the peritonitis data, higher levels of PGE_2 were present in male rat exudates in the late phase of the inflammatory reaction, measured (4 and 8 hours after pleurisy induction), mainly supported by neutrophils as shown by Mathieu J. and colleagues (*Mathieu J. et al., 1990*).

Moreover, higher mortality in male mice after PAF-induced shock strictly highlighted the higher capability of male neutrophils to produce PGs. In fact, as previously shown (*Becker et al., 1993 and Xiaoming et al., 1991*), inhibition of PG

production as well as neutrophil depletion both decrease the PAF- induced mortality in mice.

The *in vivo* evidence of the sex biased PG production by neutrophils represents an important finding in the light of the clinical outcomes that show that several inflammatory disorders in which PGs play a pivotal role (i.e ulcerative colitis or sepsis) have an higher incidence in male compared female (*Tragnone et al., 1996; Schroeder et al., 1998)*. Since neutrophils represent the cells present in the inflammatory infiltrate, in order to investigate if the sex difference in PG biosynthesis is present also in human, a dextran-based method combined with a differential centrifugation on Nycoprep cushions was used for the isolation of neutrophils from blood of female and male donors (*Pergola et al., 2008*). To induce PGE_2 production we selected calcium ionophore in order to skip the receptor involvement in the possible sex bias in the light of previous findings showing an higher expression of toll-like receptor 4 in male neutrophils as well as their higher susceptibility to interferon gamma stimulation (*Aomatsu et al., 2013*) . After stimulation, male neutrophils produced higher levels of PGE_2 than female. Interestingly, this sex difference was abolished by the addition of exogenous AA. Taking together these two data highlight the diverse AA metabolism by the two sexes, independently from receptor involvement. As mentioned above, AA can be metabolized either by COX for prostanoid biosynthesis or by 5-LO for LT production. On the basis of the existent sex difference in 5-LO product formation in male and female neutrophils (higher in females) (*Pergola et al., 2008*), we have hypothesized that the diverse production of PGE_2 could strictly depend on AA shunt from 5-LO to COX enzymes. In particular, the blockage of 5-LO activity by pre-treatment with Mk886, a FLAP inhibitor, increased PGE_2 formation in female cells,

abolishing the sex difference. These data support the theory that the sex difference in PGE_2 production was due, at least in part, to the already shown sex-biased metabolization of AA via 5-LO, being the production of LTs higher in female than in male. The low enzymatic activity of 5-LO in male, due to the different subcellular localization, enhances the substrate availability for COX leading to a higher production of PGE_2. Intriguingly, this conclusion was supported by the *in vivo* abolishment of the sex difference in PGE_2 biosynthesis in rats after Mk886 pre-treatment in the pleurisy model.

The results of this thesis clearly demonstrate that sex is an important factor that influences inflammation independently from the stimuli or species. and that it should be considered in the development of therapeutic strategy to achieve a sex-tailored treatment of sex-biased diseases.

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APPENDIX 1: ABBREVIATIONS

AA	arachidonic acid
BSA	bovine serum albumine
Ca-ion	calcium-ionophore A23187
CaMK	Ca^{2+} /calmodulin-dependent protein
CLP	coactosin-like protein
COX	cyclooxygenase
cPLA$_2$	cytosolic phospholipase A_2
CYP	cytochrome p450
cys-LT	cysteinyl leukotriene
CVD	cardiovascular diseases
DAG	diacylglycerol
DBD	DNA binding domain
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	ethylenediamine-tetraacetic acid
EPA	5,8,11,14,17-eicosapentaenoic
ERK	extracellular signal-regulated kinase
FLAP	5-lipoxygenase activating protein
GPCR	G-protein coupled receptor
HETE	hydroxyeicosatetraenoic acid

12-HHT	12-hydroxy-5,8,10-heptadecatrienoic acid
HpETE	hydroperoxy-6-trans-8,11,14-cis-eicosatetranoic acid
IP3	inositol 1,4,5-trisphosphate and diacylglycerol (DAG)
LM	lipid mediators
LO	lipoxygenase
LPS	lipopolysaccharide Epstein Barr Virus (EBV),
LT	leukotriene
LX	lipoxins
MAPEG	membrane-associated proteins in eicosanoid-and glutathione metabolism
MAPK	mitogen activated protein kinase
MPO	myeloperoxidase
MS	multiple sclerosis
NF-κB	nuclear factor-κB
Non-N	non nuclear fraction
NP40	Nonidet P-40
Nuc	nuclear fraction
ORCH	orchidectomized mice
O.D.	optical density
PAF	platelet activating factot
PBS	phosphate buffered saline
PC	phosphatidylcholine
PG	prostaglandin

PGC buffer	PBS-glucose-Ca^{+2} buffer (see materials and methods)
PGES	PGE synthase
PGHS	prostaglandin endoperoxide synthase
PI3K	phosphatidylinositol 3-OH kinase
PKA	protein kinase A
PKC	protein kinase C
PL	phospholipase
PM	resident peritoneal macrophages
PMA	phorbol 12-myristate 13-acetate
PMNL	polymorphonuclear leukocytes
PMSF	phenylmethyl sulfoniyl fluoride
PUFA	polyunsaturated fatty acid
RIA	radioimmunoassay
RA	rheumatoid arthritis
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematosus
$sPLA_2$	secretory phospholipase A_2
UPLC-MS/MS	ultra performance liquid chromatography tandem mass spectrometry
WB	western blot