Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN
Scienze della Terra, della Vita e dell'Ambiente

Ciclo 30°

Settore Concorsuale: 05/D

Settore Scientifico Disciplinare: BIO/09

Relation between Aryl Hydrocarbon Receptor and Interleukin-6 in a human breast cell model and in the *Cyp1a1/1a2/1b1(-/-)* triple-knockout mouse model

Presentata da: Rossella Dallo

Coordinatore Dottorato

Professor Giulio Viola

Supervisore

Professoressa Tiziana Guarnieri

Esame finale anno 2018

TABLE OF CONTENTS

Presentation of the study

The research work performed during my PhD investigates the relationship between the evolutionary conserved xenobiotic sensor Aryl Hydrocarbon Receptor (AHR) and the pathway of Interleukin-6 (IL6), a cytokine frequently involved in inflammation. The physiologic expression of both AHR and IL6 is tightly regulated by different mechanisms and dysregulations in the control of their activity have been associated with several adverse outcomes, such as cancer. Here, we analysed their mutual regulation in two different experimental models. The first is a human epithelial breast cell model with an IL6-driven acute inflammation induction. The results on cellular lines have been obtained at the University of Bologna and are described in this thesis as "project 1". The second one is a mice model, triple-knockout for the AHR target genes *Cyp1a1/1a2/1b1* (CYP1-KO). The experiments on mice have been conducted at the Karolinska Institutet during my experience abroad and are named in this manuscript as "project 2". Starting from current data demonstrating that AHR mediates the induction of IL6, the aim of this study was:

- to evaluate if, in a human epithelial breast cell model, inflammation driven by IL6 could, in turn, regulate the expression of AHR thus creating an interplay between AHR and IL6, and to evaluate thus whether AHR could assume a role in sustaining inflammation;
- to investigate whether the CYP1-KO mouse model, which is characterized by a potentiated activation of AHR, presented an induced expression of *Il6* or other inflammation-related features, or adverse effects in pathways in which AHR exerts physiological functions.

Our results indicate that: *i)* IL6 administration stimulates STAT3 and AHR expression through the activation of pSTAT3; *ii)* The induction of endogenous IL6 is regulated by IL6 administration and by AHR activation; *iii)* IL6 affects cell cycle progression and migration activity; *iv)* AHR/CYP1 disrupted signaling does not interfere with *Il6* expression, but affects *PpARγ* and *Ahr* transcription and mammary gland tissue morphology. These results demonstrate that, in the human epithelial breast cell line MCF10A, AHR could assume an active role in the complex puzzle of early steps of inflammation. In the CYP1-KO we did not identify an altered expression of *Il6* indicating that an altered CYP1/AHR signaling does not interfere with IL6-related pathway. However, in contrast, we could observe an altered expression of *PpARγ*, an important marker in adipogenesis.

List of abbreviations:

AHR   Aryl Hydrocarbon Receptor

IL6   Interleukin-6

STAT3   Signal Transducer and Activator of Transcription 3

pSTAT3   Phospho- Signal Transducer and Activator of Transcription 3

CPT   Cryptotanshinone

FICZ   6-formylindolo[3,2-*b*]carbazole

TCDD   2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

TNBC   Triple Negative Breast Cancer

TEBs   Terminal End Buds

PPARγ   Peroxisome Proliferator-Activated Receptor Gamma

1. INTRODUCTION

1. INTRODUCTION

Everyday most animals, including humans, are exposed to a myriad of chemicals through the food chain, air and water. Some of these molecules carry relevant information about the animal's environment, such as the presence of food or predators, whereas others are dangerous and toxic and therefore must be avoided or eliminated. Several mechanisms have been developed throughout evolution to recognize a wide range of chemical structures and to initiate appropriate/opportune responses. Large families of olfactory/chemosensory receptors can detect volatile and soluble chemicals in the environment (Mombaerts, 1999) and stimulate numerous neural pathways. In addition to this system, inducible enzymes and transporters have developed to mediate the biotransformation and elimination of the environmental toxic compounds (Brattsten, 1979; Nebert & Gonzalez, 1987). The enzymatic components of this system include the cytochrome P450 superfamily, and the sensory components involve receptors that regulate the expression of the biotransformation and transporter genes, in response to environmental chemicals. These receptors include the aryl hydrocarbon receptor (AHR), a member of the basic-helix-loop-helix (bHLH)–Per-ARNT-Sim (PAS) gene superfamily.

1.1 BHLH-PAS PROTEINS

Basic HLH (helix–loop–helix)–PER–ARNT–SIM (bHLH–PAS) proteins are a family of heterodimeric transcription factors found throughout all three kingdoms of life that sense and respond to environmental pollutants or to physiological signals through their two PAS domains and are involved in dimerization, DNA binding and signal transduction. Dimers are formed between a class I factor, that can be tissue restricted or activated in response to a stimulus, and a more ubiquitous or unregulated class II factor (figure 1).
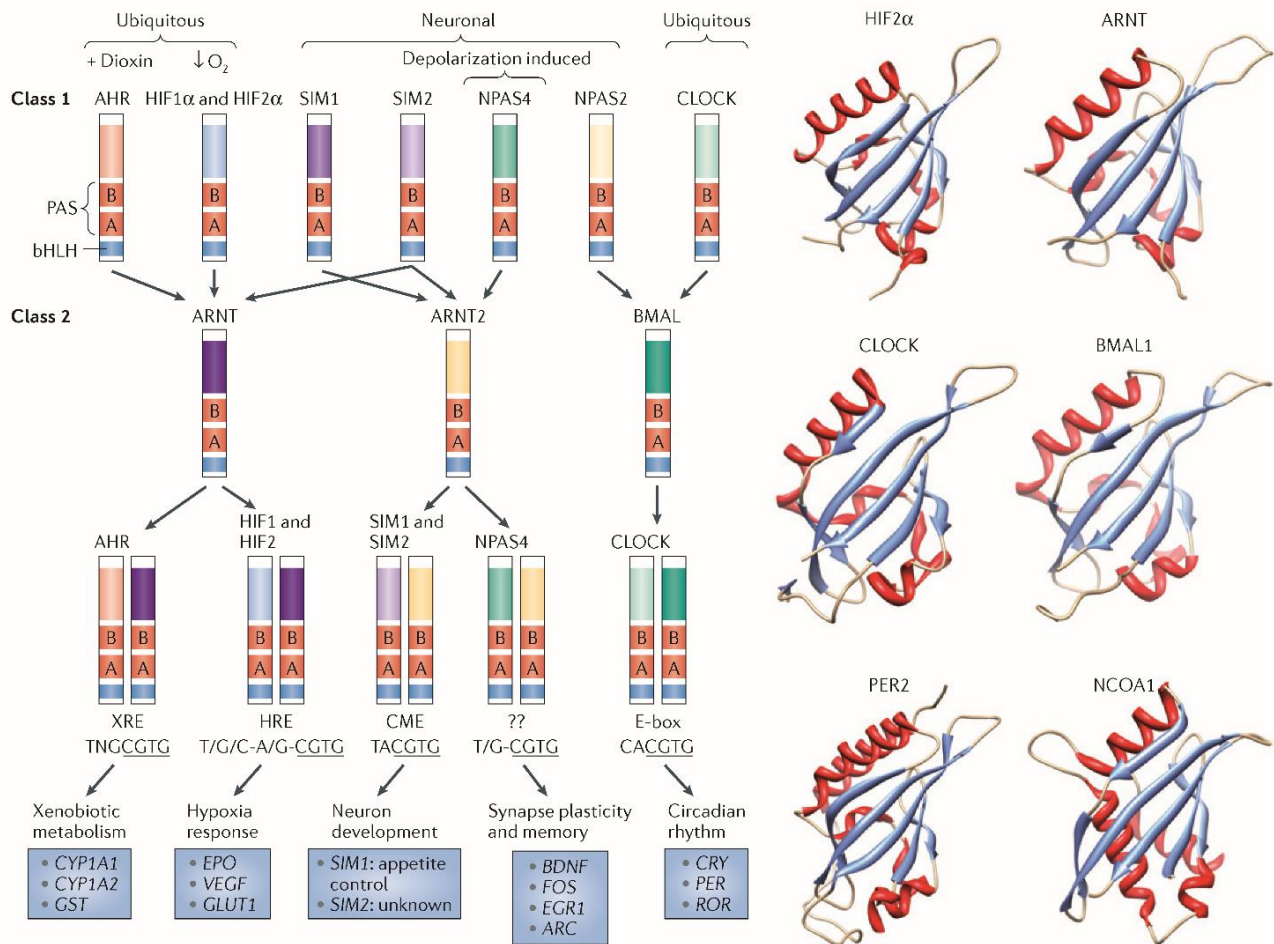
Figure 1. Roles and structures of class I and class II bHLH–PAS family members (Bersten, Sullivan, Peet, & Whitelaw, 2013)

These proteins have a broad range of roles in physiological processes related to homeostasis and to the stress response (Furness, Lees, & Whitelaw, 2007; McIntosh, Hogenesch, & Bradfield, 2010; Michaud et al., 2001; Ramamoorthi et al., 2011). An example of class I and class II interaction is the circadian locomotor output cycles kaput (CLOCK) and NPAS2 circadian rhythm factors, which dimerize with the protein brain muscle BMAL. This leads to the expression of genes encoding period (PER) and cryptochrome (CRY) proteins that subsequently translocate into the nucleus where they repress the transcriptional activity of BMAL and CLOCK proteins (McIntosh et al., 2010), activating thus transcriptional and translational feedback loops that comprise the core circadian clock machinery that control diurnal oscillation of physiological functions. The cycle takes approximately 24 hours to complete, thus describing the *circa* (near) *diem* (day) rhythm.

Another example of bHLH-PAS interaction is the dimerization between class I aryl hydrocarbon receptor (AHR) and class II AHR nuclear translocator (ARNT) proteins that induce transcription of genes for a range of biological outputs, such as detoxification, cellular homeostasis and immune responses.

1.2 ARYL HYDROCARBON RECEPTOR
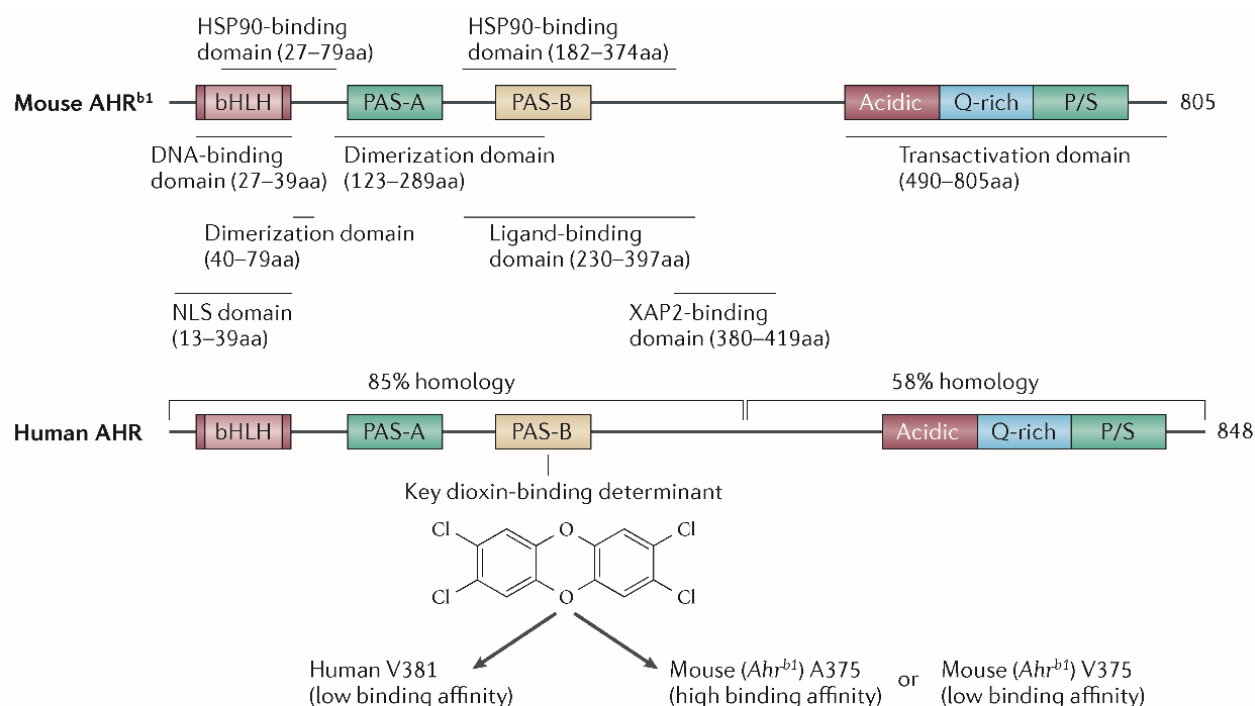
1.2.1 Background

Research in AHR began more than 50 years ago, after the earliest studies describing the induction of enzymes by foreign chemicals (Conney, Gillette, Inscoe, Trams, & Posner, 1959; Conney, Miller, & Miller, 1956, 1957) and the identification of the cytochrome P450 (Omura & Sato, 1962). The first evidentiary findings of AHR occurred in 1974 and 1976, when Poland and Nebert identified a cytoplasmatic factor that bound TCDD with high affinity, and called it aryl hydrocarbon receptor (A. Poland, Glover, & Kende, 1976; A. P. Poland, Glover, Robinson, & Nebert, 1974). Subsequently, in 1992 Ema and colleagues were able to clone the mouse *Ahr* gene (Ema et al., 1992) and in 1995 Fernandez-Salguero and coworkers (Fernandez-Salguero et al., 1995) created the first *Ahr(-/-)* knockout mouse line, a model that would become a valuable system with which to study the AHR-CYP1 pathway. A timeline of the AHR-signaling story is shown in table 1.

Table 1. Timeline of AHR history (Nebert, 2017)

Year	Discovery
1956	"Benzpyrene hydroxylase" activity induced by PAHs in rat liver
1962	Benzpyrene hydroxylase activity induced throughout rat GI tract by oral benzpyrene
1968	"AHH activity" assay designed; AHH induction by PAHs in fetal hamster cell cultures
1969	Differences in AHH inducibility by PAHs between inbred strains of mice
1970	PAH-induced AHH activity represents a cytochrome P450 (P1-450)
1972	PAH-induced AHH activity in B6 × D2 crosses exhibits predominantly Mendelian inheritance
1972	In utero AHH induction in fetuses by treatment of mother with PAHs
1973-1989	Importance of AHR-mediated CYP1 shown for cancer, mutagenesis, toxicity, and teratogenesis
1974	Evidence of AHR, based on dose-response curve: AHH induction as a function of TCDD concentration in B6 vs D2 inbred mouse strains
1976	Radiolabeled TCDD binding assay detects cytosolic AHR
1979	Development of benzo[a]pyrene-resistant clones in Hepa-1 cells: c1 & c37, c2, and c4
1980	AHR-mediated CYP1A1-induced sister-chromatid-exchange detected in GD7.5 embryo
1981, 1989	AHR-mediated P1-450 induction proposed to be involved in inflammatory process
1982	Intranuclear appearance of radiolabeled TCDD (AHR) asspcoated with P1-450 induction
1988, 1998	AHR-mediated CYP1A1 induction demonstrated in GD0.5 one-cell zygote
1991	Mouse Arnt gene cloned (human ARNT gene shortly thereafter)
1991	The term "bHLH/PAS" was first coined
1992	Mouse Ahr gene cloned (human AHR gene shortly thereafter)
1995	Creation of first of several Ahr(−/−) knockout mouse lines
1996	Standardized gene nomenclature for CYP genes in all species becomes well established
2005	AHR-CYP1 axis demonstrated in cultured ES cells
2008, 2013	Underscoring the importance of AHR-CYP1 axis in LM second-messenger pathways involving innumerable critical life events — in virtually every organ, tissue and cell-type

1.2.2 AHR structure

The human *AHR* gene consists of 11 exons encoded on chromosome 7 (Ema et al., 1994), the protein is composed of 848 amino acids and has a molecular mass of 96 kDa, while the murine ortholog consists of 11 exons encoded on chromosome 12 (Schmidt, Carver, & Bradfield, 1993). The protein has 805 amino acids and a molecular mass of 90 kDa (figure 2).

Figure 2. Mouse and human AHR (I. A. Murray, Patterson, & Perdew, 2014)

1.2.3 AHR ligands

The AHR is activated and/or bound by various classes of both endogenous and exogenous compounds. Among the endogenous are tryptophan derivatives, including FICZ (A. Rannug et al., 1987; U. Rannug et al., 1995; Y. D. Wei, Helleberg, Rannug, & Rannug, 1998), kynurenine (Heath-Pagliuso et al., 1998), fatty acid metabolites such as lipoxin A4 (Schaldach, Riby, & Bjeldanes, 1999) and tertapyrroles as bilirubin (Adachi et al., 2001), which have been isolated as natural ligands of AHR. Exogenous ligands are represented by xenobiotic compounds that can be either natural, such as the combustion-derived polycyclic aromatic hydrocarbon benzo[a]pyrene (B[a]P), or synthetic such as polychlorinated biphenyls (PCBs) and halogenated aromatic hydrocarbons

including 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (Denison & Nagy, 2003; Denison, Soshilov, He, DeGroot, & Zhao, 2011).

1.2.4 AHR mechanism

The molecular pathway leading to AHR activation by xenobiotics has been extensively studied (figure 3). In the absence of a ligand, the AHR resides in the cellular cytoplasm, bound to a molecular chaperone complex formed by two HSP90 molecules (Denis, Cuthill, Wikstrom, Poellinger, & Gustafsson, 1988), the 23-kDa co-chaperone p23 (Kazlauskas, Poellinger, & Pongratz, 1999), and the immunophilin-like AHR interacting protein AIP (also known as XAP2 or ARA9)(Ma & Whitlock, 1997). Upon ligand binding, the AHR protein undergoes conformational changes, the cofactors dissociate and the receptor translocates to the cellular nucleus where heterodimerizes with the class II bHLH protein ARNT (Probst, Reisz-Porszasz, Agbunag, Ong, & Hankinson, 1993). This heterodimer binds to a set of co-activators and/or corepressors and the resulting complex, being transcriptionally active, binds to XRE motifs containing the core bases 5'-GCGTG-3' located in the upstream regulatory region of target genes (Mimura, Ema, Sogawa, & Fujii-Kuriyama, 1999). DNA binding of the AHR/ARNT dimer leads to the recruitment of different histone acetyltransferases or histone modifiers that relax the local chromatin structure, reposition the nucleosomes and facilitate recruitment of components of the general transcription machinery.

The activity of AHR is controlled by negative feedback mechanisms, involving ubiquitination and proteasome-mediated degradation (McIntosh et al., 2010). Another mechanism is through the induction of the aryl hydrocarbon receptor repressor (AHRR) that competes with AHR for heterodimer formation with ARNT and prevents the AHR-ARNT binding to the xenobiotic-responsive elements (XREs) (Oshima, Mimura, Sekine, Okawa, & Fujii-Kuriyama, 2009). The clearance of its ligand FICZ is another feedback mechanism. Indeed, FICZ is an AHR ligand and activator, but it is also a good substrate for CYP1 enzymes. As a result, the clearance of FICZ by the CYP1 results also in the shutdown of AHR pathway (Wincent et al., 2009).
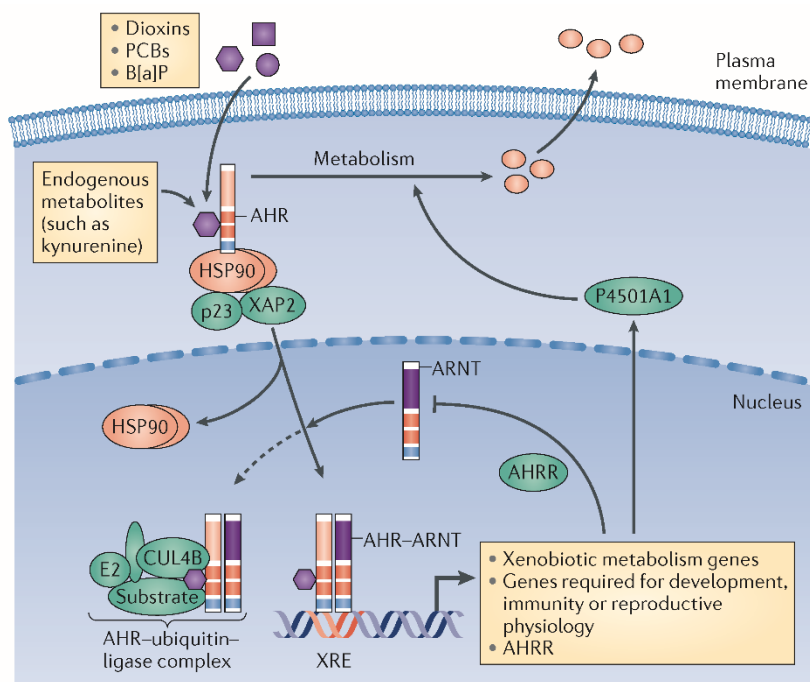
Figure 3. Ligand activation of AHR and control by negative feedback mechanisms (Bersten et al., 2013)

1.2.5 AHR functions

The AHR has been studied for several decades largely in relation to toxicology and pharmacology. It was originally identified as a transcription factor responsible for the induction of xenobiotic-metabolizing cytochrome P4501 (CYP1). Nowadays, research on AHR has expanded into different aspects of physiology, such as embryonic and fetal development, glucose and lipid metabolism, circadian rhythmicity, and immune, reproductive, cardiovascular and neural systems, (Nebert, 2017; Nebert & Karp, 2008). Increasing attention is given today to the role of AHR in tumorigenesis. Experimental data has reported the association between AHR and tumor onset, promotion and progression (Dietrich & Kaina, 2010; Gasiewicz, Henry, & Collins, 2008; Koliopanos et al., 2002), and several works evidenced constitutive AHR activation in different types of tumors (Dever & Opanashuk, 2012; Peng et al., 2009), especially in breast cancer (Eltom, Gasmelseed, & Saoudi-Guentri, 2006; Yang et al., 2005). In particular, a recent study demonstrated high levels of AHR expression in TNBC (Triple Negative Breast Cancer) and their metastases (Novikov et al., 2016). This cancer is characterized by high proliferation/metastatic grade and capacity to invade surrounding tissues, and by the absence of ER, Progesterone Receptor (PR) and Human EGF (Epidermal Growth Factor) Receptor 2 (HER2). Interestingly, in TNBC has been found both serum and tissue high levels of IL6 (Marotta et al., 2011; Sansone et al., 2007), one of the most involved cytokine in inflammation. Roles of AHR, in mutual interplay with IL6, has been explored in several

experimental models, and, in particular, Hollingshead group obtained an inflammatory state in MCF-7 breast and ECC-1 endocervical cancer cells, through the administration of IL-1B or phorbol 12-myristate 13-acetate (PMA), in combination with TCDD-induced activation of AHR (Hollingshead, Beischlag, Dinatale, Ramadoss, & Perdew, 2008). This treatment resulted in a prolonged stimulation of IL6 expression, which was also sustained by REL A, the p65 subunit of NF-Kb.

1.3 THE CYTOCHROME P450 (CYP) ENZIMES

The cytochrome P450 represents a family of anciently evolved isoenzymes that exist in bacteria, plants and animals, and are responsible for specific endogenous functions including the biosynthesis of steroid hormones, prostaglandins, bile acids (Nebert & Russell, 2002). They also constitute the major enzyme family capable of catalyzing the oxidative biotransformation of several drugs and xenobiotics (Zanger, Turpeinen, Klein, & Schwab, 2008). In humans, the CYP superfamily consists of 57 genes and 58 pseudogenes, in comparison to 108 functional and 88 pseudogenes in mice, and is divided into 18 families based on protein sequence homology (Nelson et al., 2004). Known also as monooxygenases, P450 enzymes include a heme-iron center in which molecular oxygen is bound to iron (Mason, 1957), and are located in the smooth endoplasmic reticulum of several tissues. The name P450 derives from the spectrophotometric Soret peak of light absorbance at 450 nm showed when reduced with NADPH and bound to CO (Omura & Sato, 1962).

1.3.1 CYP1 enzyme family

The CYP1 enzyme family, a member of the CYP superfamily, is inducible via the ligand-activated AHR, and includes three proteins: CYP1A1, CYP1A2, and CYP1B1 (Nelson et al., 2004). The *CYP1A1* and *CYP1A2* genes are located on chromosome 15q24.1 (on chromosome 9 in mice) and consist of seven exons and six introns, whereas *CYP1B1* consists of three exons located on chromosome 2p22.2 (and on chromosome 17 in mouse) (G. I. Murray, Melvin, Greenlee, & Burke, 2001; Nelson et al., 2004).

In humans, CYP1A1 induction occurs mostly in extrahepatic tissues such as pancreas, thymus, uterus, and small intestine (Ding & Kaminsky, 2003). CYP1A1 metabolizes planar substrates, including PAHs, β-naphthoflavone (Nebert, 2017) and tryptophan metabolites. CYP1A2 is abundantly expressed in liver (Schweikl et al., 1993) and metabolizes several molecules including caffeine and clinically important drugs (Gunes & Dahl, 2008; Zhou, Wang, Yang, & Liu, 2010).

CYP1B1, like CYP1A1, is expressed at high levels in extrahepatic tissue: mammary gland, prostate, uterus, spleen, thyroid, bone marrow, kidney (Bieche et al., 2007), metabolizes arylamines and environmental chemicals including PAHs and biphenyls and has been found overexpressed in tumors (G. I. Murray et al., 1997).

1.4 INFLAMMATION

Inflammation is the body's first response to internal and external harmful stimuli that eliminate the aggressor agent and restore the normal tissue physiology. The nature of the stimuli can be physical (injuries or radiation), chemical (poisons or toxins) and biological (pathogens like viruses, fungi or bacteria).

The clinical symptoms of inflammation were initially described by a physician in first century Rome, Aulus Cornelius Celsus, as *calor* (heat), *dolor* (pain), *rubor* (redness), and *tumor* (swelling). When tissue homeostasis is perturbed, a complex orchestration of events leads to the inflammatory process. Several cell types as macrophages and mast cells release mediators of inflammation such as cytokines, chemokines and histamine, which induces local vasodilation and increased regional blood flow to the inflamed area, microvascular permeability and the mobilization and infiltration of leukocytes at the site of injury (de Visser, Eichten, & Coussens, 2006).

When this process is maintained for a short period of time, it has therapeutic effects that terminate with the restoration of the pre-injury state, but if it becomes chronic and uncontrolled it can represent a pathophysiologic basis for many diseases such as diabetes, rheumatoid arthritis, Alzheimer's, pulmonary and cardiovascular pathologies, and cancer (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006; Medzhitov, 2010).

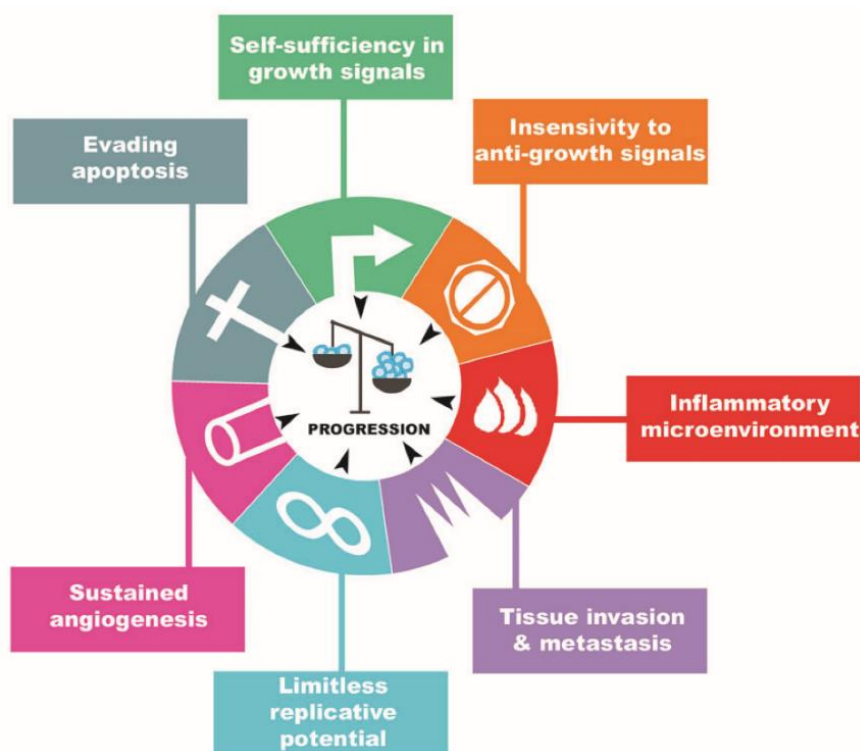### *1.4.1 Inflammation and cancer*

Carcinogenesis is the process in which a normal cell acquires a tumoral phenotype, and is divided in three steps: tumor initiation, tumor promotion and tumor progression (Karin & Greten, 2005).

In the first one, carcinogens induce irreversible changes on DNA, provoking the activation of oncogenes and/or the inactivation of suppressor genes. In the second one, risk factors, tumor promoters such as viruses, tobacco or chronic inflammation lead mutated cells to expand by reducing apoptosis and increasing cell proliferation (Coussens & Werb, 2002; Karin & Greten, 2005). Finally, invasion of adjacent tissue and development of metastasis characterize the last stage,

in which tumor can also accumulate other mutations and acquires tumorigenic proprieties (Karin & Greten, 2005).

In 1863, Rudolf Virchow first postulated the crucial link between chronic inflammation and cancer, by noting the presence of leukocytes in tumor biopsies. He proposed that an inflammatory context could promote a cellular environment that drives the development of carcinogenesis (Demaria et al., 2010; Mantovani, 2009).

Nowadays, it is a well-accepted paradigm that inflammation is linked to cancer, as evidenced from a wide range of lines of works in this context (Balkwill, Charles, & Mantovani, 2005; Balkwill & Mantovani, 2001; Coussens & Werb, 2002; Hussain, Hofseth, & Harris, 2003; Mantovani, Allavena, Sica, & Balkwill, 2008). Epidemiological data indicate that chronic inflammation predisposes one to different forms of cancer, and that over 25% of all cancers are related to chronic infections (Hussain & Harris, 2007), but the association between inflammation and cancer is not only restricted to increased risk for a subset of tumors. Indeed, an inflammatory component is represented by the microenvironment of most neoplastic tissues, including those not epidemiologically related to inflammatory processes. The microenvironment is supplied by bioactive molecules such as growth factor, survival factors, pro-angiogenic factors, and herein inflammation contributes to proliferation and survival of malignant cells, aggressiveness of the cancer, development of metastasis and angiogenesis, subversion of adaptive immunity and reduced response to chemotherapeutic agents. Accordingly, cancer-related inflammation has been included as a seventh hallmark of cancer, in addition to the previous six identified by Hanahan and Weinberg (Colotta, Allavena, Sica, Garlanda, & Mantovani, 2009; Hanahan & Weinberg, 2011) (figure 4).

Figure 4. Inflammation as the seventh hallmark of cancer (Colotta et al., 2009)

In the tumor microenvironment, the inflammation is characterized by the presence of macrophages, that secrete angiogenic factors and metalloproteinases (Mantovani et al., 2008) and leucocytes. Other involved cells comprise natural killer (NK), dendritic and mast cells, neutrophils, eosinophils and lymphocytes. These cells types produce several cytotoxic mediators as reactive oxygen intermediates (ROS), membrane perforating agents, serine and cysteine proteases, TNFα, interleukins, interferons (INFs), and enzymes such as cyclo-oxygenase-2 (COX-2), 5-lipoxygenase (5-LOX). In addition, inflammation increases the expression of NF-κB (nuclear factor κB), STAT3 (signal transducer and activator of transcription 3), AP-1 (activator protein 1) and HIF-1α (hypoxia-inducible factor 1α), transcription factors that control the regulation of genes involved in proliferation, growth and survival (Aggarwal, 2009; Coussens & Werb, 2002; Mantovani et al., 2008).

Among the inflammatory mediators included in the tumor microenvironment, cytokines represent a family of molecules which perform different cellular functions, but depending on concentrations and tissue specificity can exert both pro- and anti-tumorigenic proprieties (Balkwill & Mantovani, 2001). The cytokines most involved in inflammation are TNFα, IL4, IL10, IL13, IL1, IL15, IL17, IL23 and IL6.

1.5 IL6

1.5.1 IL6 pathway

IL6 is a pleiotropic cytokine that mediates a wide range of physiologic functions in immune regulation, hematopoiesis and oncogenesis (Ara & Declerck, 2010; Fielding et al., 2008; Kishimoto, 2006; Paul, 1991). Depending on cellular context, IL6 is able to signal through different protein kinase-dependent proliferation and anti-apoptosis cascades, among which the phosphatidylinositol-triphosphate kinase (PI-3K)/Akt pathway, the mitogen-activated protein kinase (MAPK) pathway and the most implicated in breast cancer Janus kinase (JAK)/signal transducer and activator of transcription-3 (STAT3) pathway (Hodge, Hurt, & Farrar, 2005) (figure 5).
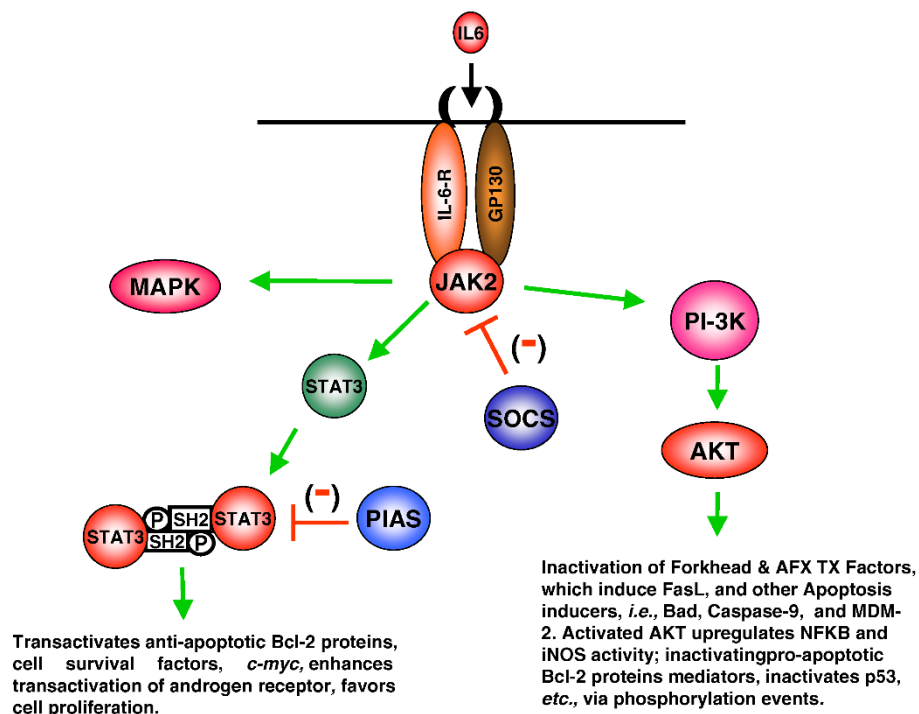

Figure 5. IL-6: A pleiotropic cytokine affecting proliferative and anti-apoptotic pathways (Hodge et al., 2005)

The IL6 receptor complex is formed by the ligand-binding protein IL6Rα (also called CD126) and the signal transducing component gp130 (or CD130). A family of tyrosine kinases, the Janus kinases (JAK), are associated constitutively with gp130 and activated upon IL6 binding (Heinrich et al., 2003; Heinrich, Behrmann, Muller-Newen, Schaper, & Graeve, 1998). The activation of JAKs leads to tyrosine phosphorylation of STAT3, and translocation of pSTAT3 from the cytoplasm to the nucleus, where it recognizes enhancer elements in the promoters of target genes and starts their transcription. The termination of the IL6/JAKs/STAT3 pathway is controlled by the SOCS

(suppressor of cytokine signaling) feedback inhibitors and PIAS (protein inhibitor of activated STAT) proteins (Heinrich et al., 2003).

1.5.2 STAT3

STAT3 represents the prime transcriptional regulator that mediates IL6 dependent cell growth, differentiation and survival signals (Heinrich et al., 1998; Yu & Jove, 2004). STAT3 belongs to the STAT family proteins composed by seven currently known members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (figure 6). Structurally STATs present DNA binding domains, dimerization domains and activation domains, and are responsible to convey signals from cytokines receptors to the nucleus (Yu & Jove, 2004).
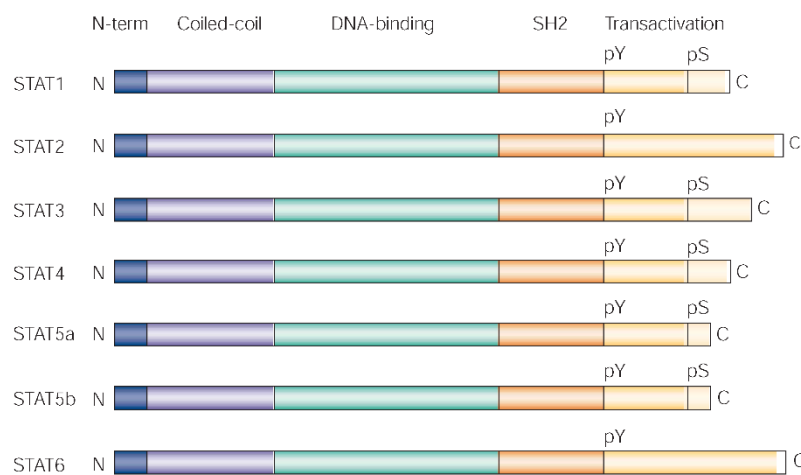
Figure 6. The STAT family of proteins (Yu & Jove, 2004)

In normal cells, the activity of STATs is tightly regulated by the presence or absence of the respective ligand, whereas in tumor cells cytokine receptors can become constitutively activated, for example by paracrine expression of their ligands (Heinrich et al., 2003; Hirano, Ishihara, & Hibi, 2000). Since STATs regulate the expression of genes involved in cell proliferation, apoptosis and immune response, permanent activation can have a role in the tumor cell. In fact, constitutively activated STATs has been reported in many tumors, and, in particular, STAT3 has the highest incidence of constitutive expression when compared with the other members (Hodge et al., 2005) (table 2).

Table 2. Activated STAT transcription factors in tumor types (Hodge et al., 2005)

Tumour type Activated	STAT factor
Multiple myeloma	STAT3
Acute lymphocytic leukemia (ALL)	STATI, STAT5
Chronic lymphocytic leukemia (CLL)	STATI, STAT3
Acute myelogenous leukemia (AML)	STATI, STAT3, STAT5
Large granular lymphocyte leukemia (LGL)	STAT3
Chronic myelogenous leukemia	STAT5
Lung cancer	STAT3
Breast cancer	STAT3
Renal cancer	STAT3
Prostate cancer	STAT3
Pancreatic carcinoma	STAT3
Melanoma	STAT3
Colon carcinoma	STAT3
Gastric carcinoma	STAT3
Cervical cancer	STAT3
Ovarian cancer	STAT3
Hepatocellular carcinoma	STAT3
Head and neck cancers	STAT3

1.5.3 IL6 functions

Depending on cellular context and concentration, IL6 can exert both physiologic and pathologic roles. Among the physiologic functions, IL6 is involved in normal cell inflammatory processes, host immune defence mechanisms, developmental differentiation of lymphocytes, modulation of cellular growth, cell proliferation and cell survival and bone formation (figure 7) (Heinrich et al., 1998; Hodge et al., 2005; Kamimura, Ishihara, & Hirano, 2003; Steelman et al., 2004).
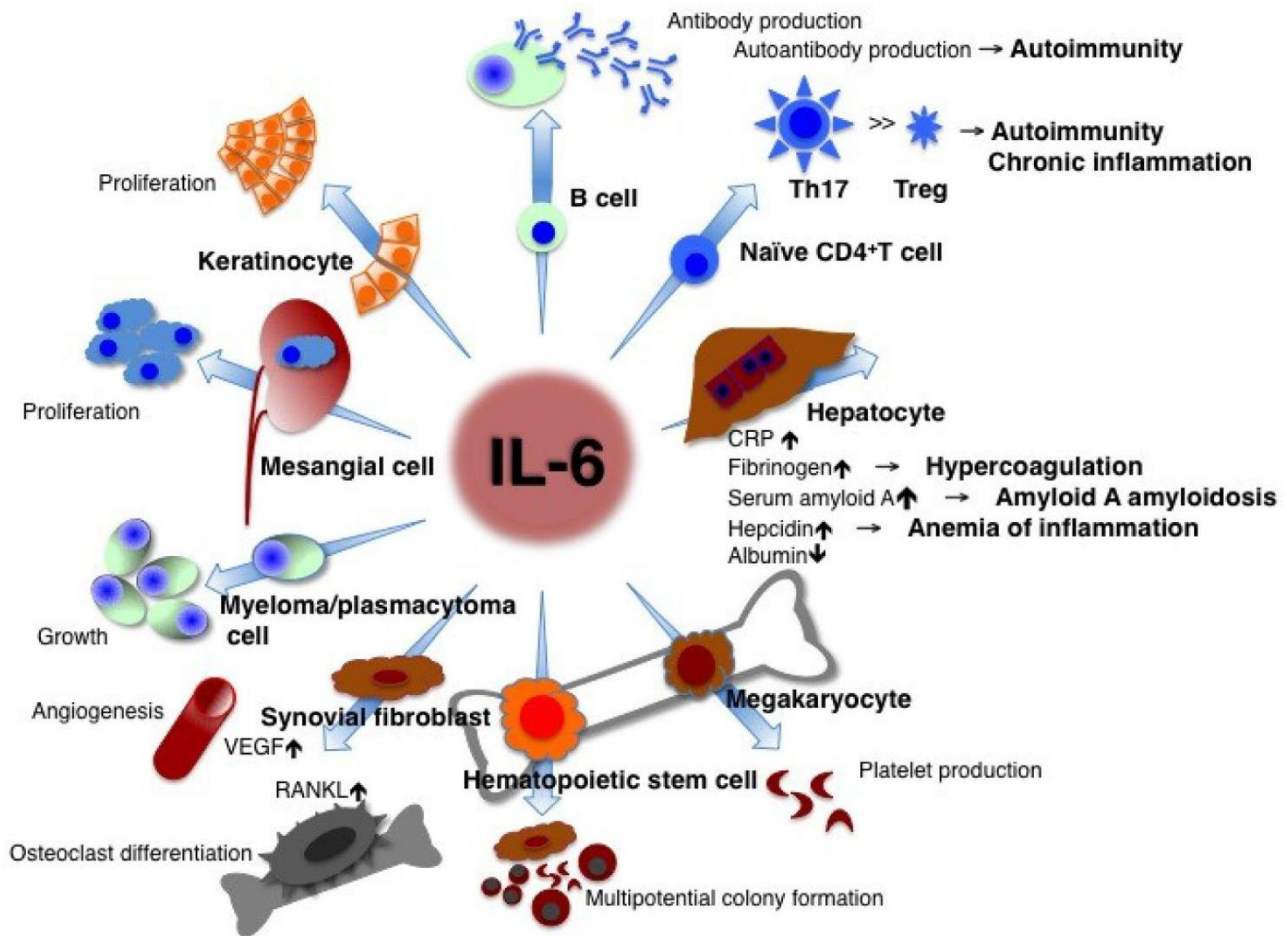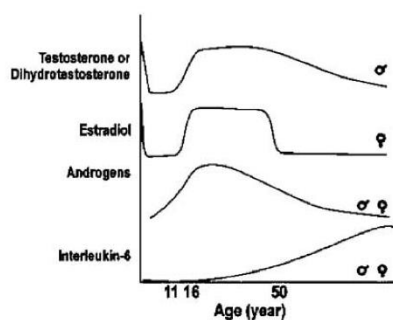
Figure 7. IL6 functions (Tanaka & Kishimoto, 2012)

IL6 is controlled by hormonal feedback mechanisms and age-related changes in the hormonal balance, such as the decrease in circulating estrogen and androgen, result in elevated IL6 levels. Aberrant IL6 expression is associated with different adverse outcomes, such as chronic inflammatory disorders, cardiovascular diseases and cancer (figure 8) (Dijsselbloem, Vanden Berghe, De Naeyer, & Haegeman, 2004).

Figure 8. IL6 gene expression and ageing (Dijsselbloem et al., 2004)

Elevated expression of IL6 has been implicated in a wide range of cancers, such as cervical cancer (L. H. Wei et al., 2003), colorectal cancer (Belluco et al., 2000), endometrial cancer (Bellone et al., 2005), lung cancer (Songur et al., 2004), ovarian carcinoma (Zakrzewska & Poznanski, 2001) and breast cancer (Garcia-Tunon et al., 2005; Salgado et al., 2003), and is associated with a poor prognosis in breast cancer (Bachelot et al., 2003; Salgado et al., 2003; Zhang & Adachi, 1999). Furthermore, high levels of IL6 have been demonstrated in the serum of breast cancer patients with respect to healthy donors and correlates with advanced breast tumor stages (Kozlowski, Zakrzewska, Tokajuk, & Wojtukiewicz, 2003). In the same manner, high serum and tissues levels of IL6 has been found in Triple Negative Breast Cancer (TNBC) (Marotta et al., 2011; Sansone et al., 2007). In a recent study, it has been studied the role of oncostatin M, a multifunctional cytokine that belongs to the IL6 group, in the regulation of AHR in human HepG2 hepatocarcinoma cells, and has been demonstrated that oncostatin M modulates AHR expression and activity, thus making inflamed cells more prone to bind to xenobiotics and environmental pollutants (Stobbe-Maicherski et al., 2013).
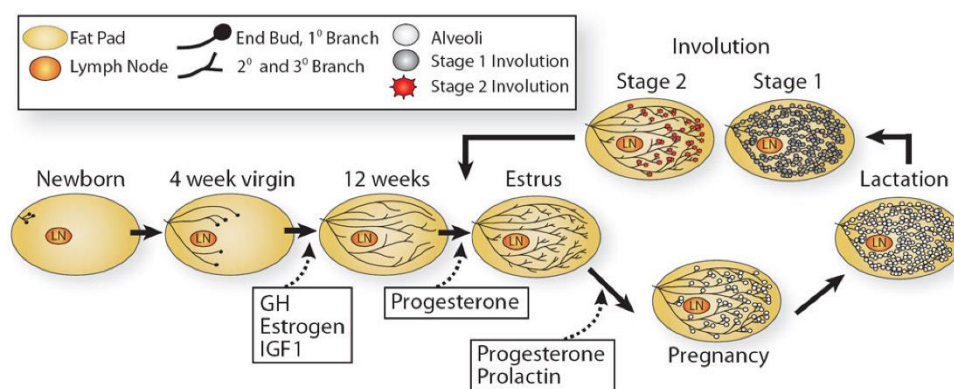
1.6 MAMMARY GLAND DEVELOPMENT

In mammals, the mammary gland consists of an elaborate network of branched epithelial ducts embedded within the mammary fat pat, a complex stroma containing adipocytes, fibroblasts, nerves, blood vessels and different immune cells (Polyak & Kalluri, 2010).

The mammary gland development occurs in three different and regulated stages: embryonic, pubertal and reproductive. In the embryonic stage, epithelial/mesenchymal interactions produce the rudimentary ductal structure present at birth, which remain quiescent until puberty (Macias & Hinck, 2012).

Prompted by high levels of estrogen and growth hormones such as insulin-like growth factor 1, puberty initiates branching morphogenesis, and the end of the rudimentary ducts proliferates and swells into different multilayered epithelial structures, known as terminal end buds (TEBs) (Hinck & Silberstein, 2005). These structures then undergo processes of elongation, bifurcation and lateral branching until reaching the limit of the fat pad, thereby forming a full epithelial tree (Sternlicht, Kouros-Mehr, Lu, & Werb, 2006).

Upon pregnancy, the luminal epithelium proliferates and differentiates into alveoli, which secrete milk during lactation. Massive apoptosis initiates the process of involution after lactation, removing up to 80% of the epithelium and remodeling back the gland to its pre-pregnancy state (figure 9).

Figure 9: Stages of postnatal mammary gland development (Macias & Hinck, 2012)

All the processes involved in driving mammary morphogenesis are orchestrated by molecular signals such as growth factors, tyrosine kinases, extracellular matrix molecules and proteases (McNally & Martin, 2011), thereby, alterations in signaling implicated in these interactions may impair the correct development of the mammary gland.

Among its physiologic functions, AHR has shown to be involved in the regulation of the cell cycle, cell proliferation and differentiation, and apoptosis (Gonzalez & Fernandez-Salguero, 1998). Accordingly, AHR signaling has shown to play a role in the mammary gland development. In fact, in *Ahr*$^{-/-}$ male mice, histology examinations using whole mount techniques have shown that the ducts do not fill the whole mammary fat pad, indicating an abnormal ductal development (Huang et al., 2016). Similar effects have also been observed in female mice where the AHR was shown to impact the mammary gland development. In their work, Hushka and colleges observed that in *Ahr*$^{-/-}$ mice the number of TEBs was reduced by 50% and there was an increase in blunt-ended terminal ducts during postnatal development (Hushka, Williams, & Greenlee, 1998). Furthermore, the activation of AHR by xenobiotic ligands seems to have a negative effect on milk production, resulting in the inability of the mice to nutritionally support their offspring (Vorderstrasse, Fenton, Bohn, Cundiff, & Lawrence, 2004). Adipocytes are required for the correct development and branching of TEBs. Importantly, AHR is known to crosstalk with peroxisome proliferator activated receptors (PPARs) that are involved in the development of adipose tissue and the maintenance of lipid and glucose homeostasis, processes important for the mammary gland development and function as well (Borland et al., 2014; Shaban et al., 2004).

2. AIMS

2. AIMS

Historically, there has been a large focus on describing adverse effects of AHR activation by environmental pollutants such as dioxins. However, how endogenous AHR signaling is regulated is still largely unknown. The aim of this study is to better understand the physiologic interplay between AHR and IL6, a cytokine frequently involved in inflammation. It was performed in two different experimental approaches: a human breast epithelial cell model (project 1) and a *Cyp1a1/1a2/1b1(-/-)* (CYP1-KO) triple-knockout mouse model (project 2).

Project 1:

The expression levels of inflammatory cytokine IL6 are strictly regulated by hormonal feedback mechanisms and are further progressively increasing with age, due to the menopause/andropause-associated disturbed hormonal balance. In addition, AHR is constitutively activated in a wide range of tumour types, among which the triple negative breast cancers that are characterized by both serum and tissue high levels of IL6. Given these findings, in our work we wondered if AHR could be regulated by means of IL6-driven inflammation. We started our work from the observation that AHR mediates the induction of IL6 in MCF-7 breast and ECC-1 endocervical cancer cells, and that oncostatin M, a cytokine that belongs to the interleukin-6 cytokine family, is able to induce AHR expression in a STAT3-dependent manner in human HepG2 hepatoma cells. We investigated thus whether in breast epithelial cell lines showing a triple negative phenotype, IL6-driven inflammation could instigate the expression of AHR, which, in turn, endows endogenous IL6 induction. Therefore, in the early stages of inflammation, AHR could assume an active role and sustain inflammation.

Project 2:

CYP1 enzymes play a crucial role in negative feedback mechanisms of AHR activation, and this physiologic regulation is of functional importance. CYP1-KO mice are characterized by a potentiated activation of AHR due to reduced ligand metabolism. Sustained activation of AHR has been linked to damaging effects in several biological processes, resulting in *e.g.* immunotoxicity, endocrine disruption, and development of cancer (Mitchell & Elferink, 2009; Moennikes et al., 2004). Previous studies have shown that ablation or inhibition of CYP1 enzymes results in AHR-dependent altered levels of cytokines, moreover AHR activation is able to induce *IL6* mRNA levels

and is linked with mammary gland cancer (Hollingshead et al., 2008). Recently, a critical function of the AHR-dependent CYP1 enzyme family in regulation of AHR functions in gut immunity was described (Schiering et al., 2017). The role of CYP1 enzymes in physiological functions of AHR in other tissues is however still largely unknown. In the mammary gland, AHR crosstalks with multiple signaling pathways that may impact mammary gland development and function. In particular, the aim of this study was to determine the impact of altered CYP1/AHR signaling on *Il6* expression and on pathways related to inflammation, cell proliferation and adipogenesis.

3. METHODS

3. METHODS

<u>Project 1:</u>

Cell culture and reagents

The human epithelial breast cancer MDA-MB-231 cell line was maintained in Dulbecco's Modified Eagle's Medium High Glucose (EuroClone) supplemented with 10% Fetal Bovine Serum (FBS, EuroClone), 100 U/mL penicillin, and 100 U/mL streptomycin. The human epithelial non-tumorigenic MCF10A cells were cultured with Dulbecco's Mem Nutrient Mix F12 (Euroclone) with 25 mM Hepes and L-Glutamine, 10% Fetal Bovine Serum, 100 U/mL penicillin and 100 U/mL streptomycin , 20 ng/ml EGF, 10 µg/ml bovine insulin and 0.5 µg/ml hydrocortisone. Both cell lines were maintained in a 37°C incubator with 5% CO2. Recombinant Human Interleukin-6 (IL6) was purchased from ImmunoTools (Friesoythe, Germany), 6-formylindolo[3,2-*b*]carbazole (FICZ) from Sigma Aldrich and 1,2,6,7,8,9-hexahydro-1,6,6-trimethyl- (R)-phenanthro(1,2-b)furan-10,11-dione (Cryptotanshinone) from Selleckchem (Houston, TX , USA).

Real Time

Total RNA from MCF10A and MDA-MB-231 was extracted with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) as described by the manufacturer's protocol. RNA purity and quality were defined spectrophotometrically by measuring the 260/280-absorbance ratio. The cDNA was synthesized from 1 µg of total RNA by use of Improm-II Reverse Transcription System (Promega, Madison, WI, USA). Real time polymerase chain reaction (RT-PCR) was performed on a Stratagene mx3000p thermocycler (Thermo Fisher Scientific, Waltham, MA USA), using a SYBR green supermix (Bio-Rad). The primer sequences were designed using the primer-blast online software and were as follows: for *β-ACTIN* reverse 5'-ATCTGGGTCATCTTCTCGCG-3', forward 5'-CAACTGGGACGACATGGAGA-3', for *STAT3* reverse 5'-CTCTTCCAGTCAGCCAGCTC-3', forward 5'-CGGAGAAGCATCGTGAGTGA-3', for *IL6* reverse 5'-AGTGCCTCTTTGCTGCTTTC-3', forward 5'-AGACAGCCACTCACCTCTTC-3', for *AHR* reverse 5'-AGTTATCCTGGCCTCCGTTT-3', forward 5'-CTTCCAAGCGGCATAGAGAC-3'.

Western Blot

Total proteins were extracted from cells using a cell lysis buffer containing 0,1 M NaCl, 0,01 M Tris, 0,001 M EDTA, 0,1% Triton. Equal amounts of proteins were loaded and electrophoresed through 10% polyacrylamide gels and were then transferred to nitrocellulose blotting membranes

(Amersham, USA). After being blocked for 1 hour using PBS with 0.05% Tween 20 and 5% milk powder, membranes were incubated with the following antibodies: rabbit polyclonal Ah Receptor, 96 kDa (Santa Cruz Biotechnology), rabbit Phospho-STAT3, 79-86 kDa (Cell Signaling Technology), mouse STAT3, 79-86 kDa (Cell Signaling Technology), mouse β-Actin, 42kDa (Santa Cruz Biotechnology). The following day the membranes were washed with PBS Tween and incubated for 1 hour with the appropriate HRP-conjugated secondary antibody. Blots were developed using enhanced chemiluminescence (Amersham ECL Detection Reagent), images were recorded with Chem Doc XRS+ (Biorad) and analyzed by Image-Lab 3.0 software.

ELISA assay

$5x10^3$ cells were seeded into 96-wells plates, cultured until confluent, and treated with increasing doses of IL6 (0, 5, 10, 20, 30 ng/ml) for 24 hours. Cells were abundantly washed with PBS, the medium was replaced and the following day the medium of every sample was collected and stored at -80°C for subsequent analyses. ELISA assay was performed according to the manufacturer's instructions (Biotechnology Komabiotech, Yeongdeungpo-gu, Seoul 07207, Korea). The plate was read at 450 nm wavelength by using a microplate reader spectrophotometer (Biorad, Hercules, USA) and results were calculated by using a standard curve.

EROD assay

MCF10A and MDA-MB-231 cells were seeded into 96-well plates at a concentration of $5x10^3$cells/well, incubated at 37°C and grown until over-confluent. Cells were treated with increasing concentrations of IL6 (0, 5, 10, 20, 30 ng/ml). The following day, EROD assay has been conducted by using the protocol described by Schiwy (Schiwy et al., 2015). 3 replicates for each experimental group were performed.

MTT cell viability assay

To evaluate the cell viability we performed the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Cells were seeded into 96-well plates at a concentration of $5x10^3$cells/well and incubated overnight. The following day, cells were treated with increasing concentrations of IL6 (0, 5, 10, 20, 30 ng/ml) with 3 replicates for each experimental group. After 24 hours, the medium was removed from each well, and cells were washed with PBS and incubated with 10 µl of MTT reagent for 3 hours at at 37˚C and then with 100 µl of solubilization solution to dissolve the formazan products. The quantity of formazan was

measured by recording changes in absorbance at 570 nm using a microplate reader spectrophotometer (Biorad, Hercules, USA) to calculate the proliferation rate.

Flow cytometric analysis

MCF10A and MDA-MB-231 cells were seeded at a density of $1{,}3 \times 10^5$ into 6-wells plates, and the following day were treated with IL6 0, 5, 10, 20, 30 ng/ml. After 24 hours cells were trypsinized, centrifuged, washed with PBS and fixed with 70% cold ethanol. After 20 minutes cells were centrifuged twice 5 minutes at 1500 rpm, resuspended in 500 µl of PBS containing 50 µg/ml RNase A and incubated at room temperature for 30 minutes. Cells were then centrifuged and resuspended in PBS containing 10 µg/ml propidium iodide and cell cycle was analyzed with FACS (BD FACSaria cell sorter, BD Biosciences, San Jose, CA, USA).

Wound healing assay

To analyze the cell migration we performed a wound healing assay. $1{,}3 \times 10^5$ cells were seeded into a 6-wells plate, incubated and cultured until confluent. Then, a straight scratch was made on the confluent monolayer using a 200 µl pipette tip. The wells were washed to remove the debris and cells were treated with IL6 at the concentration of 10 ng/ml in a serum free medium. The growth towards the center of the gap was monitored and images were captured at the beginning and at regular intervals at two time points during cell migration, after 24 and 48 hours. The average percentage of the covered wound area was measured and quantified.

Statistics

All statistical tests were performed using the GraphPad Prism software Version 6.0. Data are presented as mean ±SEM. Statistical comparisons were performed using One sample t test and a value of $P < 0.05$ was considered statistically significant.

Project 2:

Mice model

Mammary gland tissues from C57BL/6 mice were used. We compared the WT phenotype (CYP1-WT) with the Cyp1a1/1a2/1b1(-/-) triple-knockout (CYP1-KO), generated by crossing the Cyp1a1/1a2(-/-) double-knockout mouse with the Cyp1b1(-/-) single-knockout mouse (Dragin et al., 2008). All mice were bred in the Francis Crick Institute animal facility (London, UK) under specified pathogen-free conditions and all tissue analyses were performed at Karolinska Institutet (Stockholm, Sweden). All animal procedures were conducted under a Project Licence granted by the UK Home Office. For all the experiments, dissected fat pads containing the mammary gland tissues from a total of 24 mice were used: 12 females, of which 6 CYP1-KO and 6 CYP1-WT, and 12 males, of which 6 CYP1-KO and 6 CYP1-WT.

RNA extraction and Real time

For the gene expression analysis we extracted the total RNA from tree different mammary gland tissues from each mouse; one left and one right thoracic and the right abdominal mammary glands. Thus in total for each gene we evaluated expression in 18 separate tissues per sex from each genetic model. Total RNA was extracted using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen) following manufacturer's instruction and one microgram of total RNA was reverse-transcribed using iScript™ Reverse Transcription Supermix for RT-qPCR (BioRad). Quantitative real time PCR was performed in a 96-well format 7500 Real Time PCR System (Thermo Fisher Scientific) using TaqMan™ Universal Master Mix II and gene-specific TaqMan™ probes (Thermo Fisher Scientific). Quantification of each gene was calculated using the delta-delta CT method, normalized to *B2m* housekeeping gene and expressed relatively to WT control.

Histology and immunohistochemistry

For the immunohistochemistry and the histological analysis, the mammary gland tissues were formalin-fixed, paraffin-embedded and sectioned at the microtome in slices of 3 µm each. Ki67 expression (Rabbit, Cell Signaling) was assessed by the immune-peroxidase method, and hematoxylin eosin protocol was used for the histology. For each mouse we sectioned one abdominal mammary gland, so in total we examined 12 females (6 CYP1-KO and 6 CYP1-WT) and 12 males (6 CYP1-KO and 6 CYP1-WT).

Statistics

For the gene expression analysis, differences between groups were analyzed using the two-way analysis of variance (ANOVA). Experimental data are expressed as the mean ± SD. *P*-values less than 0.05 were considered statistically significant and less than 0,001 highly significant. Statistical analyses were performed using GraphPad Prism version 6.0.
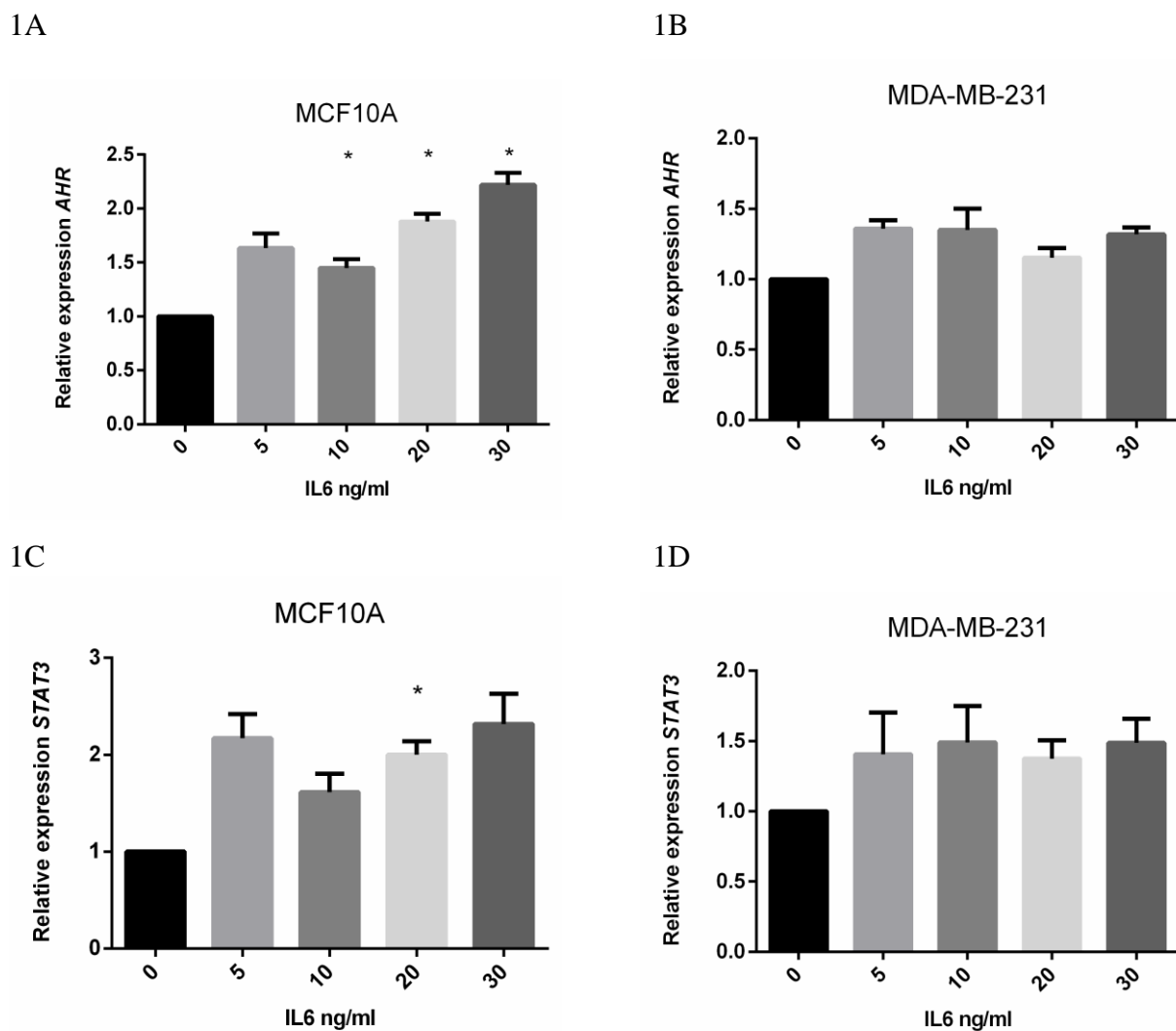
4. RESULTS

4. RESULTS

Project 1:

## 4.1 IL6 administration stimulates STAT3 and AHR expression through the activation of pSTAT3.

Previous experimental data demonstrate that, in human HepG2 hepatocarcinoma cells, the pleiotropic interleukin IL6-type cytokine oncostatin M stimulates AHR expression through STAT3 (Stobbe-Maicherski et al., 2013). Accordingly, with this study, we investigated if IL6 could modulate AHR induction in human breast epithelial MCF10A and breast cancer cell line MDA-MB-231. To do this, we administrated increasing concentrations of IL6, and we evaluated *AHR* gene expression after 24 hours of IL6 treatment. Real time analysis showed a slight enhancement in MDA-MB-231 cells, and a significant increase of *AHR* expression in MCF10A cells (fig. 1A, 1B). We then analyzed *STAT3* mRNA expression and we observed a similar increasing trend in both cell lines (fig. 1C, 1D). Thereafter, in order to investigate the phosphorylated form of STAT3 and whether the increase in *AHR* and *STAT3* expression was confirmed also at the protein level, we evaluated these markers by western blot. Similarly, after 24 hours of IL6 treatment, we observed an enhanced expression of AHR, STAT3 and pSTAT3 proteins in both cell lines (fig. 1E, 1F). These data suggest a link between STAT3 activation and AHR induction. Consequently, in order to further investigate their association, we inhibited STAT3 phosphorylation and evaluated the AHR expression. We used Cryptotanshinone (CPT) as inhibitor, a natural quinoid diterpene isolated from the roots of the plant Salvia miltiorrhiza Bunge. Specifically, this molecule strongly inhibits the STAT3 phosphorylation at the Tyr705, and prevents the dimerization of STAT3. As expected, following administration of increasing concentrations of CPT, we observed a dose-dependent decrease of pSTAT3 expression, and, interestingly, an AHR decreased expression simultaneously occurred, supporting the dependence of AHR expression from STAT3 activation (fig. 1G, 1H).
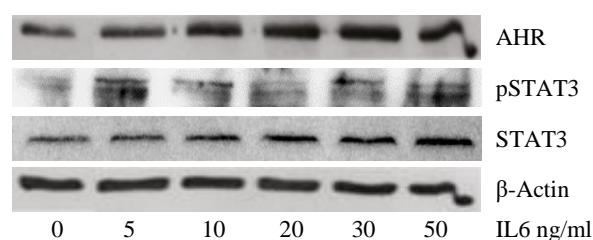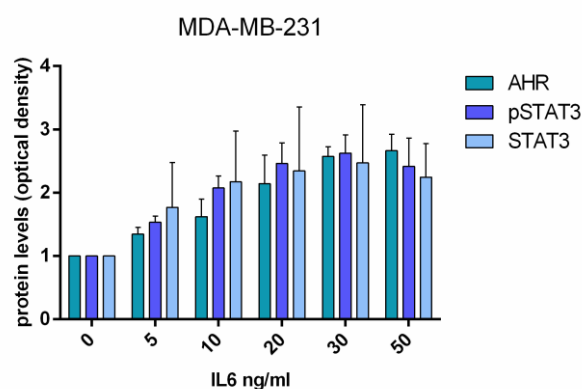
1A

1B

1C

1D

Figure 1 (A, B, C, D). Effects of IL6 on gene expression. Cells were treated with 0, 5, 10, 20, 30 ng/ml of IL6. After 24 hours transcriptional levels of *AHR* and *STAT3* were analyzed. Relative mRNA expression was measured by qRT-PCR using 2(-ΔΔCt) method (n=3). The results are expressed as mean±SEM; *p<0.05
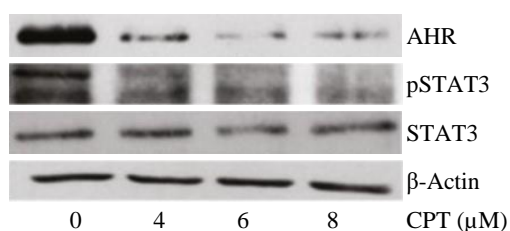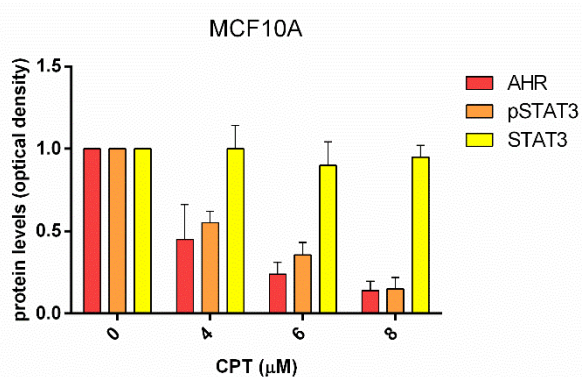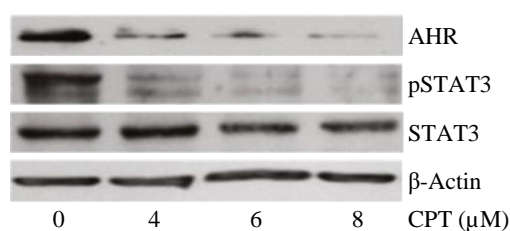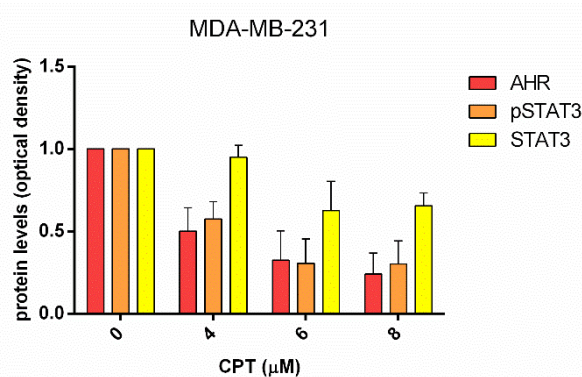
1E
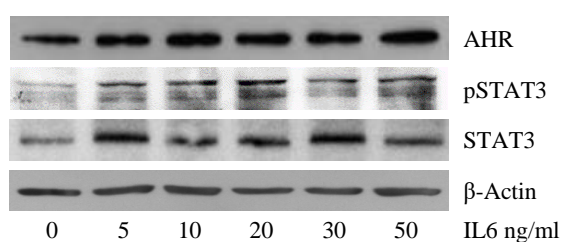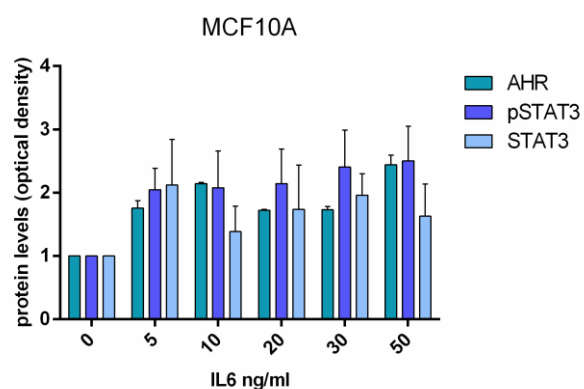
1F

1G

1H

Figure 1. (E, F) Effects of IL6 on protein expression by western blot analysis. Bar graphs and representative gel blots of AHR, pSTAT3, STAT3 proteins. Cells were treated with 0, 5, 10, 20, 30, 50 ng/ml of IL6. After 24 hours, protein expression was analyzed. Each band was quantified using densitometry and normalized to the respective β-Actin (n=3). The results are expressed as mean±SEM. **(G, H) Inhibitory effects of Cryptotanshinone (CPT) on AHR and pSTAT3 expression.** Bar graphs and representative gel blots of AHR, pSTAT3 and STAT3 proteins. Cells were treated with 0, 4, 6, 8, µM of CPT. After 24 hours protein levels were analyzed. Each band was quantified using densitometry and normalized to the respective β-Actin (n=3). The results are expressed as mean±SEM.

4.2 The induction of endogenous IL6 is regulated by IL6 administration and by AHR activation.

To determine if the administration of exogenous IL6 could generate an auto-inflammatory loop, we evaluated whether IL6 could, on the other hand, enhance the production of endogenous IL6. In Hollingshead's work, activation of *IL6* gene and the secretion of the cytokine as a consequence of AHR stimulation has been shown (Hollingshead et al., 2008).

Initially, we evaluated *IL6* mRNA after 24 hours of exogenous IL6 administration. We observed in both cell lines a dose dependent increasing trend in *IL6* expression (fig. 2A, 2B). Then, through ELISA test, we investigated the endogenous IL6 secretion after 24 hours from the removal of increasing doses of exogenous IL6 (fig. 2C, 2D). Similarly, in both cell lines, endogenous IL6 secretion was still present and it was proportional to the exogenous inflammatory stimulus. We could expect this result as the establishment of a sustained secretion of IL6 could be a consequence of the increased gene expression of *IL6*, and required some time to be released in the medium.

In order to evaluate the expression levels of *AHR* and inflammatory parameters in post-inflammatory state following the suspension of the IL6 administration, we analyzed their gene expression after removing the IL6 stimulus. Interestingly, two different patterns were observed in the two cell lines, in particular, in MCF10A, *IL6* and *STAT3* slightly decreased in their expression (fig. 2E), suggesting a gradual shutdown of the endogenous inflammation. In MDA-MB-231, these inflammatory parameters slightly enhanced their expression (fig. 2F). Interestingly, in both cell lines we observed a significant reduction of AHR.

In order to evaluate the possible contribution of AHR in the expression of *IL6*, we decided to treat cells with 6-formylindolo [3,2-*b*] carbazole (FICZ), a tryptophan metabolite and a high affinity AHR ligand, and to analyze the *IL6* mRNA expression once that AHR was activated. We observed that in MCF10A, the activation of the receptor (FICZ+/IL6-) caused a significant increased *IL6* gene expression (fig. 2G), and that the activation of the receptor, in conjunction with IL6 administration (FICZ+/IL6+) induced a more pronounced expression of *IL6*, suggesting that both AHR activation both IL6-induced inflammation cooperate to the increase endogenous *IL6* expression.

2A

MCF10A

2B

MDA-MB-231

2C

MCF10A

2D

MDA-MB-231
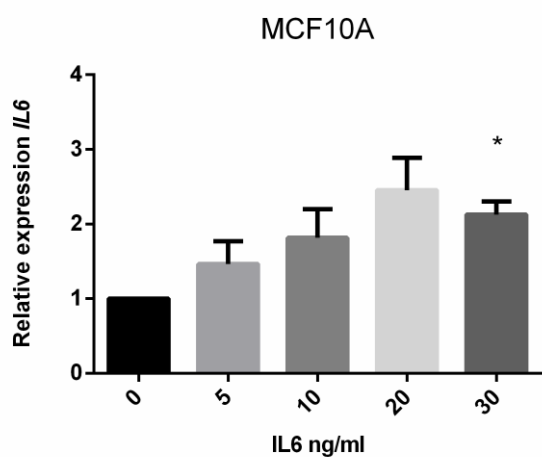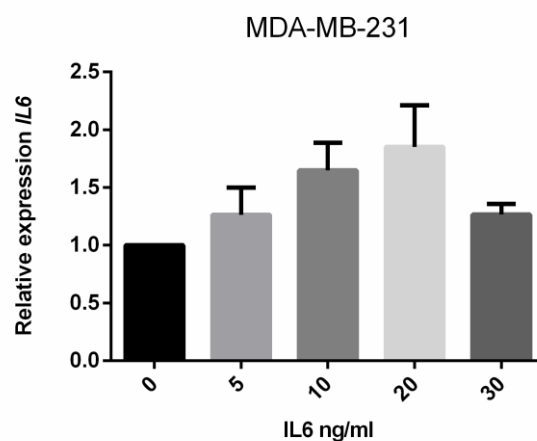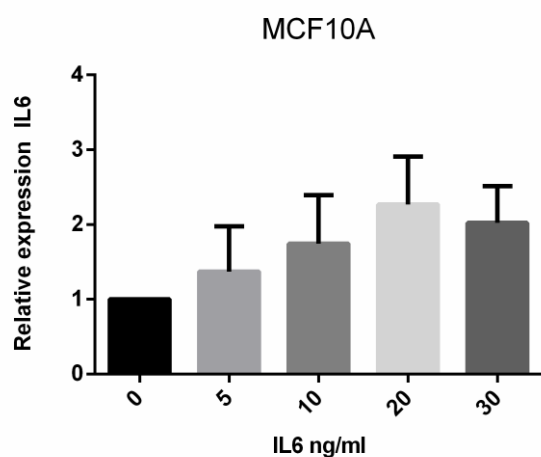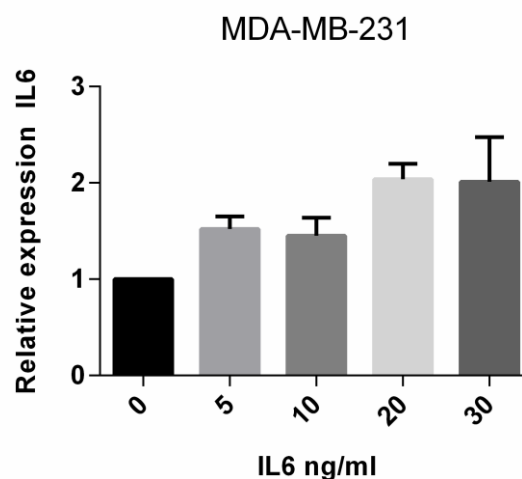
Figure 2. (A, B) Effects of IL6 on gene expression. Cells were treated with 0, 5, 10, 20, 30 ng/ml of IL6. After 24 hours, transcriptional levels of *IL6* were analyzed. Relative mRNA expression was measured by qRT-PCR using 2(-ΔΔCt) method (n=3). The results are expressed as mean±SEM; *p<0.05. **(C, D) Evaluation of IL6 secretion.** After treating the cells with increasing doses of IL6 (0, 5, 10, 20, 30 ng/ml) for 24 hours, cells were washed with PBS and incubated with new culture medium. The following day, IL6 secretion was analyzed by ELISA assay (n=3). The results are expressed as mean±SEM.

2E

MCF10A

2F

MDA-MB-231

2G

MCF10A

2H

MDA-MB-231
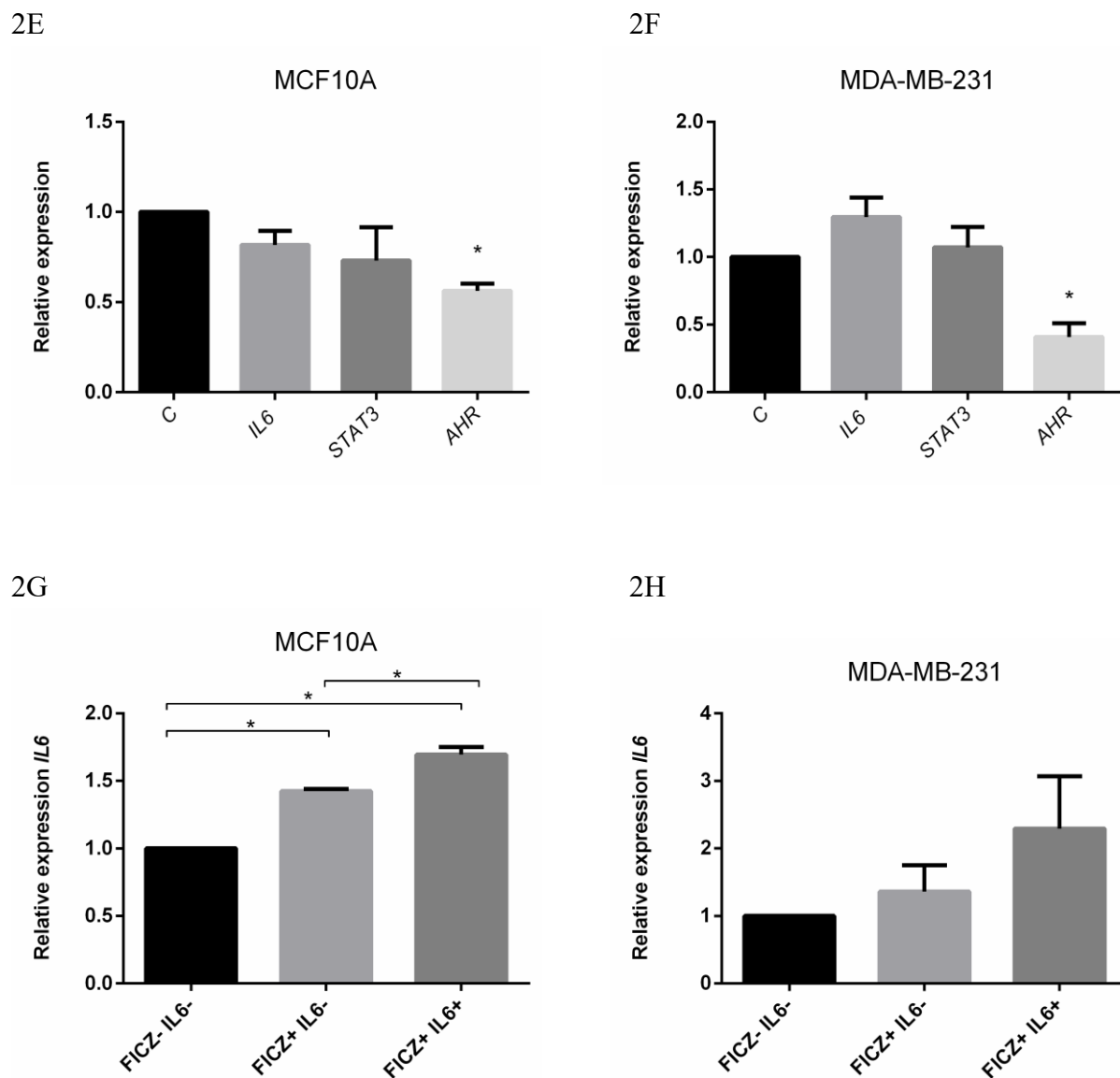
Figure 2. (E, F) Transcriptional levels after IL6 treatment, by qRT-PCR. Cells were treated with IL6 10 ng/ml. The following day, IL6 was removed, the cells were washed in PBS and incubated with new culture medium without IL6 for 24 hours. Relative mRNA expression of *IL6*, *STAT3* and *AHR* was measured by qRT-PCR using 2(-ΔΔCt) method (n=3). The results are expressed as mean±SEM; *p<0.05. **(G, H) Effects of FICZ and IL6 on gene expression.** Cells were treated with IL6 10 ng/ml and/or FICZ 50 nM for 24 hours and *IL6* gene expression was quantified. Relative mRNA expression was measured by qRT-PCR using 2(-ΔΔCt) method (n=3). The results are expressed as mean±SEM; *p<0.05.

4.3 IL6 affects cell cycle progression and migration activity.

To evaluate the role of AHR under inflammatory conditions, we treated cells with increasing concentrations of IL6 and subsequently we performed the 7-ethoxy-resorufin-O-deethylase (EROD) assay. This test is employed in the evaluation of the induction of an AHR-induced xenobiotic-metabolizing enzyme, the AHR target gene cytochrome P-450 (CYP)1A1. In both cell lines, the treatment with IL6 which, as anticipated, endowed the increase of *AHR* gene expression, did not cause significant changes in the CYP1A1 activity (fig. 3A, 3B). We could therefore exclude any role in toxicological signaling and hypothesize its possible role in proliferation, cell cycle progression and migration.

We then performed the MTT, a colorimetric assay utilized to measure the cell viability. In this test, the MTT tetrazole is reduced to purple formazan in living cells and the absorbance of each well quantified by a spectrophotometer provides the respective cell metabolic activity. We did not observe significant variations in the cell viability at any IL6 concentrations (fig. 3C, 3D) and as this result could be interpreted as a clue of cell cycle block, we decided to analyze the cell cycle progression through flow cytometry. Both cell lines were treated with usual increasing concentrations of IL6, and cell cycle was assessed with propidium iodide. In MCF10A, we did not find an effect at the 5 ng/ml concentration (data not shown) and instead, we observed a significant arrest in the cell cycle progression at the concentrations of 10, 20 and 30 ng/ml of IL6 (fig. 3E). In fact, non treated (NT) cells exhibit a G1 value of 49,2, and the treated ones a value of 61 (at 10 ng/ml), of 61,4 (at 20 ng/ml) and of 57 (at 30 ng/ml). Conversely, in MDA-MB-231 we could not evidence any alteration in the cell cycle progression after administration of IL6, as illustrated in the plot in fig. 3E in which the overlapping of the cell phases picks of the not treated and treated cells is shown.

We then evaluated the cell migration with a scratch test. We chose the 10 ng/ml dose of IL6 to treat cells since it is the concentration starting from which we could observe an effect in the cell cycle progression. Interestingly, we found an increase in the migration activity in both cell lines, statistically significant in the MCF10A (fig. 3F, 3G).

3A

3B

3C

3D
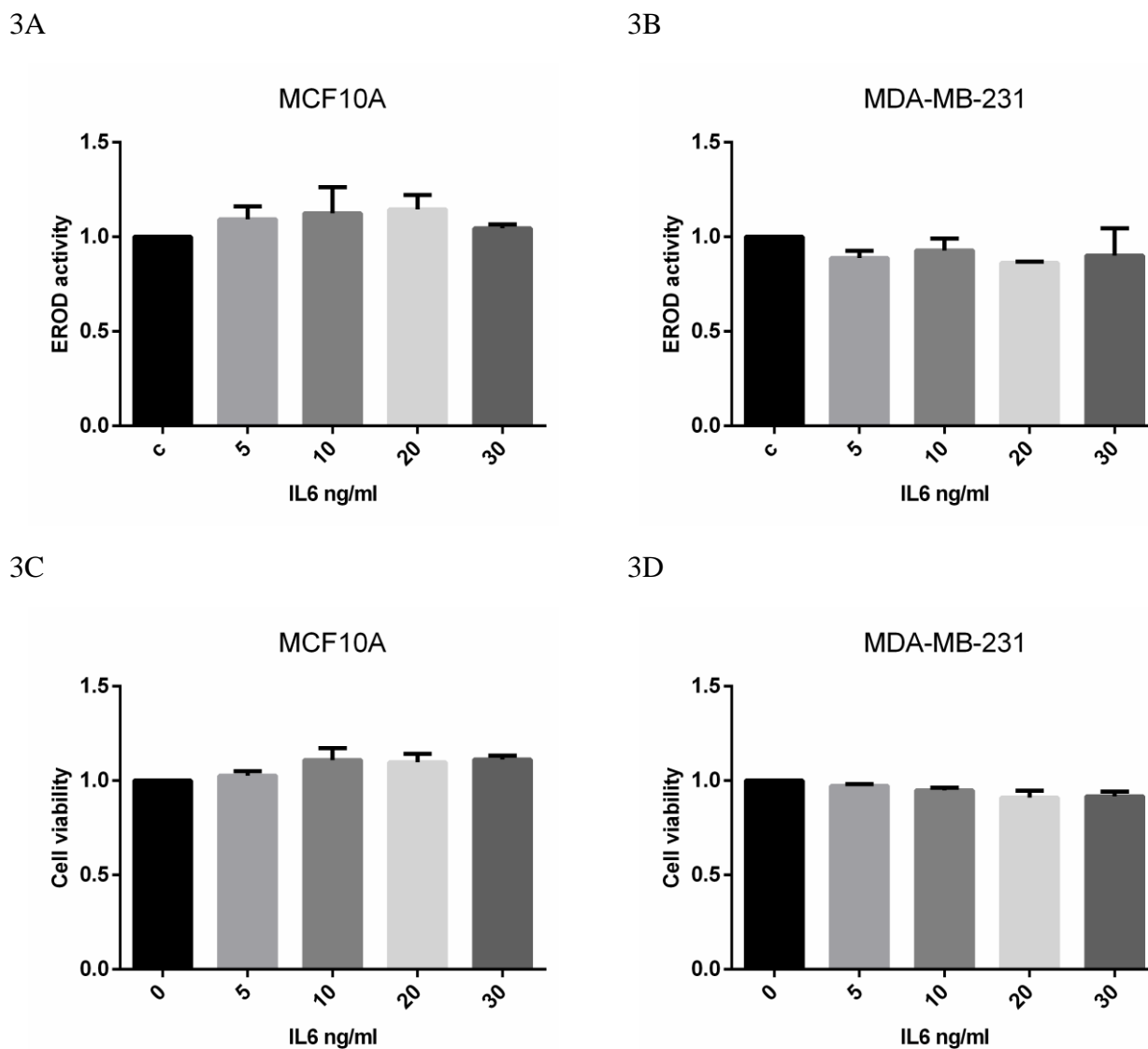
Figure 3. (A, B) Effects of IL6 on enzymatic activity by EROD assay. Cells were treated with 0, 5, 10, 20, 30 ng/ml of IL6 and the following day the induction of the xenobiotic-metabolizing enzyme cytochrome P-450(CYP)1A1 was quantified through spectrometry (n=3). The results are expressed as mean±SEM. **(C, D) Effects of IL6 on cell viability by MTT assay.** Cells were seeded and treated with 0, 5, 10, 20, 30 ng/ml of IL6 for 24 hours. Cells were treated with MTT reagent and solubilization solution and the absorbance levels were measured through spectrophotometry (n=3). The results are expressed as mean±SEM.

3E
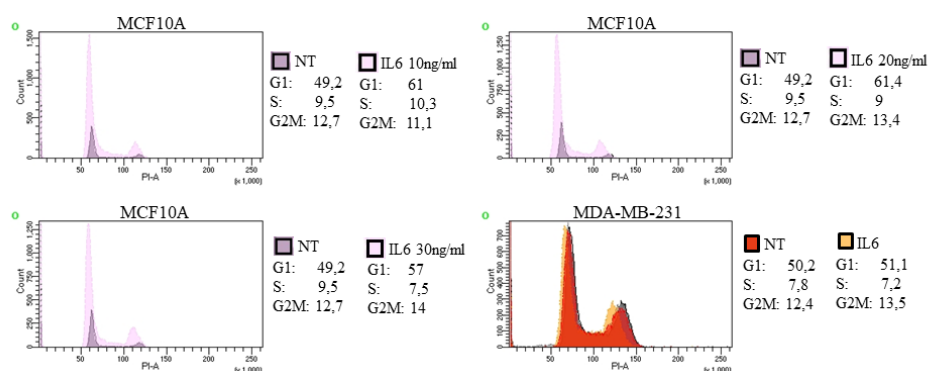
3F
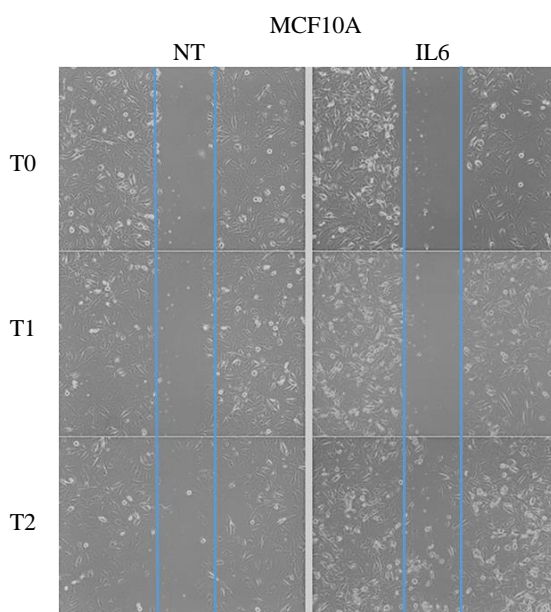
3G
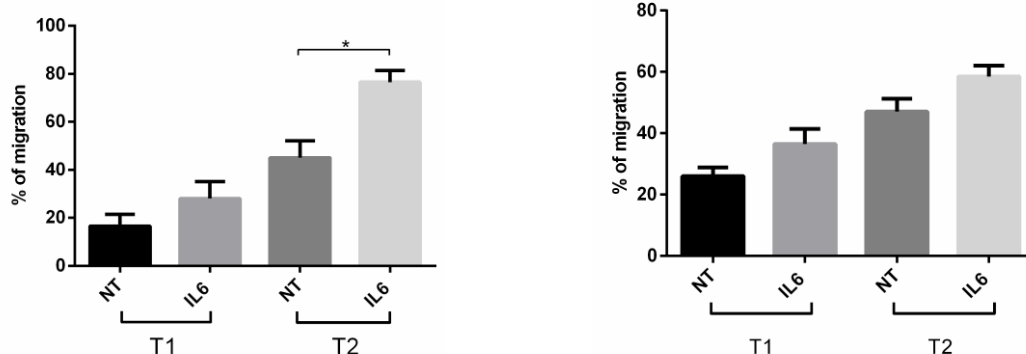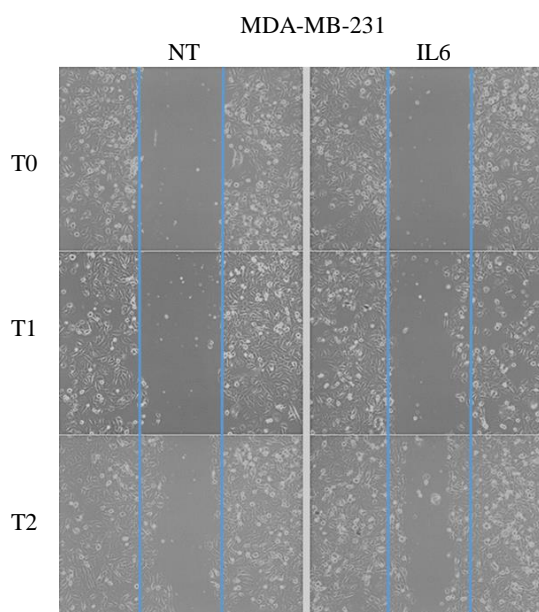
Figure 3. (E) MCF10A and MDA-MB-231 cell cycle analysis with PI. After treating cells with 0, 5, 10, 20, 30 ng/ml of IL6, cell cycle distribution was analyzed through FACS flow cytometer. Plots show the G1, S and G2M phases. Compared with non-treated control (NT), MCF10A cells treated with 10, 20, 30 ng/ml of IL6 were arrested in G1 phase. No differences in cell cycle distribution in treated and control MDA-MB-231 were seen, as shown in the overlapping of the cell phases picks of the control and treated cells. **(F, G) Effects of IL6 on migration using a wound healing assay.** Cells were scratched with a 200 pipette tip and the migration was observed at 0 hours (T0), 24 hours (T1) and 48 hours (T2), in cells treated with 10ng/ml of IL6 (IL6) and in control non-treated cells (NT). Average percentage of the covered wound area was quantified (n=3). The results are expressed as mean±SEM; *, $p < 0.05$.

Project 2:

4.4 AHR/CYP1 disrupted signaling affects *Pparγ* and *Ahr* transcription and mammary gland tissue morphology

To explore the meaning of the loss of the CYP1 enzymatic activity and the subsequent effects on inflammatory and adipogenesis-related markers and on cell proliferation pathways, we employed three different approaches. First, gene expression analyses of *Ahr*, *Il6* and *Pparγ* through quantitative real time PCR were performed. Then, histological analyses of the mammary gland tissues from CYP1-KO and CYP1-WT mice were conducted. Both male and female mice were included to determine sex-dependent effects in addition to the genotype-dependent effects. Last, we investigated the expression of the proliferation marker Ki67, through immunohistochemistry in order to evaluate whether there were effects on cell proliferation and in the development of the mammary gland.

From the gene expression results, in female mice we could observe a highly significant downregulation of *Pparγ* in the CYP1-KO genotype, compared to the WT (fig. 4A), while no effects on *Il6* expression were observed. A tendency toward increased *Ahr* expression was observed in female CYP1-KO mice, although not in a statistically significant way (*p-value = 0,0597*). Furthermore, this indication is strengthened by the fact that the analysis of *Ahr* expression in each separate mammary gland tissue shows a significant enhancement of *Ahr* mRNA in one of three thoracic mammary glands (fig. 4B). In male mice, we did not find a significant difference in *Ahr* or *Il6* expression, and, above all, no effects in the expression of *Pparγ* was observed, neither in the separate mammary gland tissues (not shown) or pooled data (fig. 4C).

We did not find any difference in the proliferation marker Ki67 between the females, males, CYP1-KO and CYP1-WT (fig. 4D), while, from the histological analysis, we could evidence some alterations in the morphology of the tissue. In female mice, we found a clear difference in the number of ductal structures embedded in the whole fat pad, where the CYP1-KO presented a lower number of ducts compared with the CYP1-WT control (fig. 4E). This characteristic was exhibited in three of the six CYP1-KO female mice, *i.e.* in 50% of the total number of the samples. Another interesting feature that we observed was the difference in the composition of the liquid inside the ductal structures. In the female CYP1-KO mice the secretions were more abundant and apparently fattier, with a lipid composition (fig. 4F).

4A

4B

4C
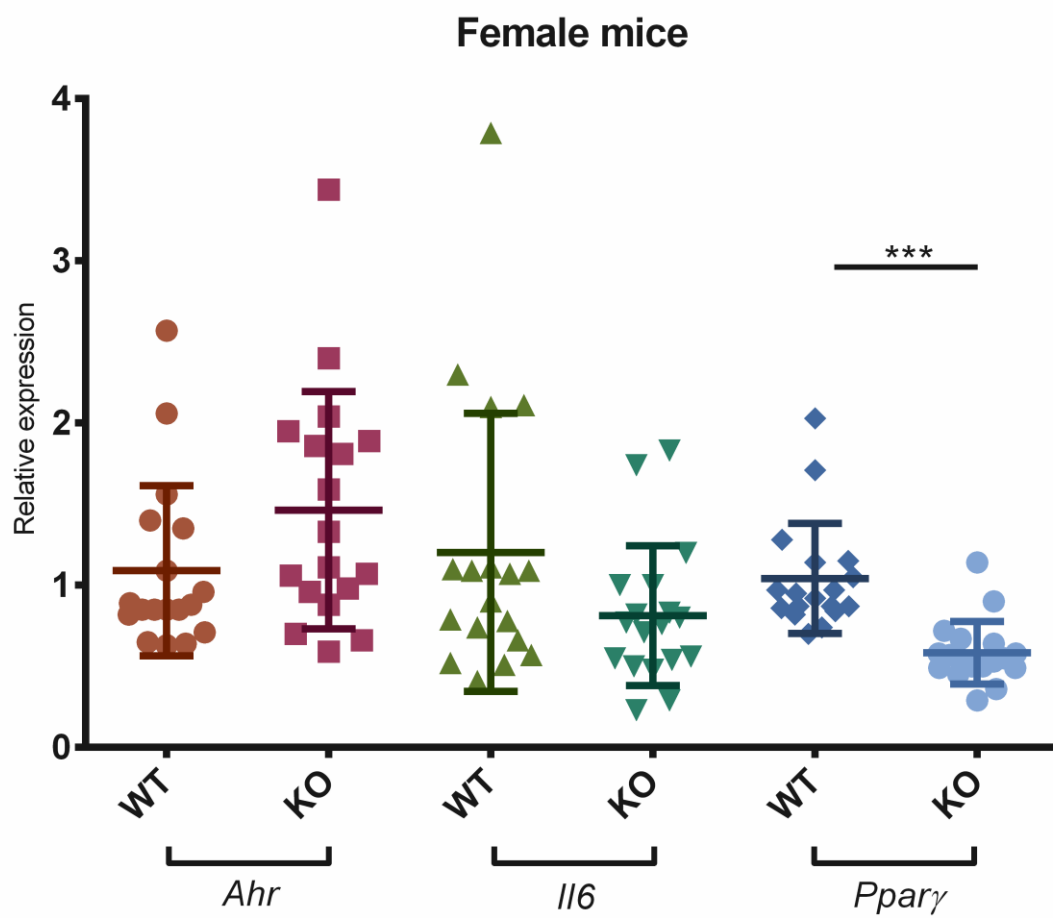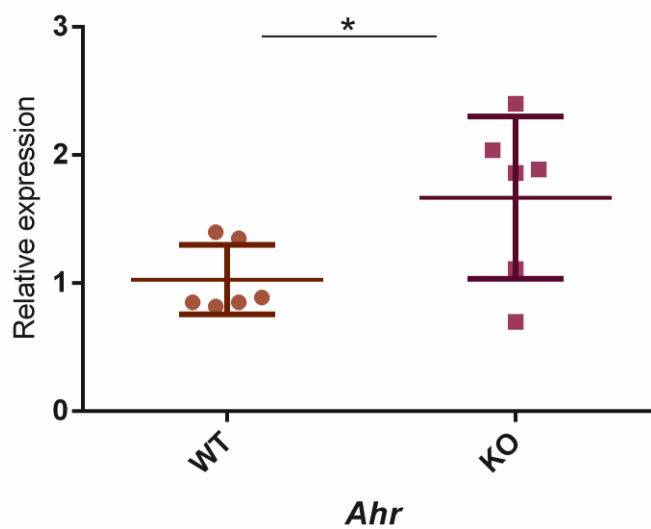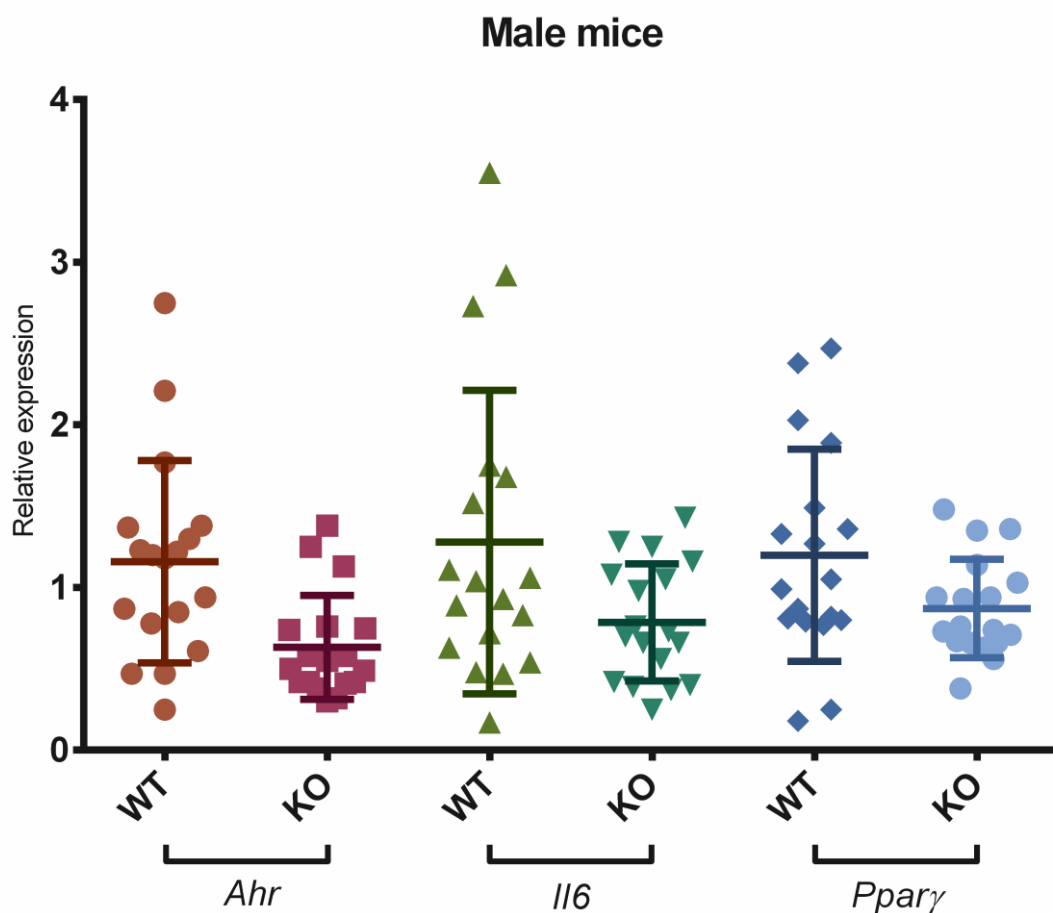
Male mice

Figure 4 (A, B, C). Effects of altered CYP1/AHR signaling on gene expression. (A) *Ahr*, *Il6*, *Pparγ* gene expression in mammary gland tissues from female mice, CYP1-WT and CYP1-KO (n=18 mammary glands from 6 mice). Error bars represent mean ± S.D. ***$P < 0.001$, calculated by two-way ANOVA. **(B)** *Ahr* gene expression in one of the thoracic mammary glands (n=6 mammary glands from 6 mice), from female CYP1-WT and CYP1-KO. Error bars represent mean ± S.D. *$P < 0.05$ calculated by two-way ANOVA. **(C)** *Ahr*, *Il6*, *Pparγ* gene expression of mammary gland tissues in male mice, CYP1-WT and CYP1-KO (n=18 mammary glands from 6 mice). Error bars represent mean ± S.D.

4D

Female CYP1-WT
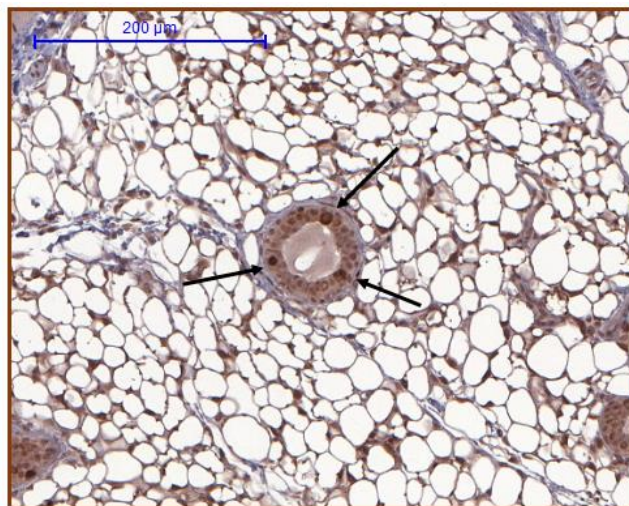
Female CYP1-KO
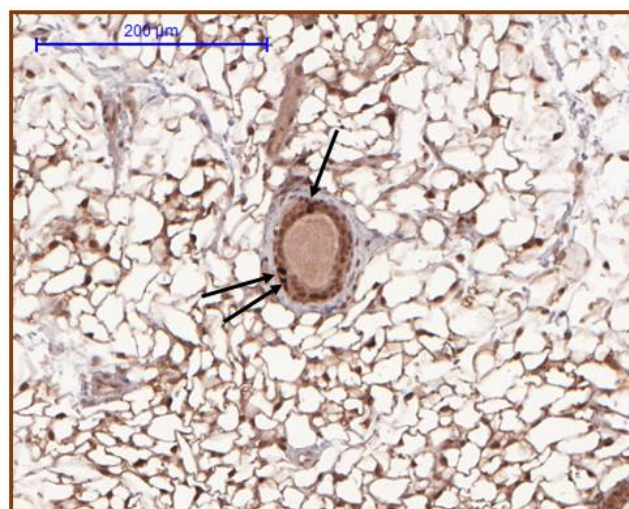
Figure 4 (D). Effects of altered CYP1/AHR signaling on proliferation. Ki67 expression in female CYP1-WT and CYP1-KO assessed by the immune-peroxidase method. Scale bar 200 µm**.**

4E

Female CYP1-WT
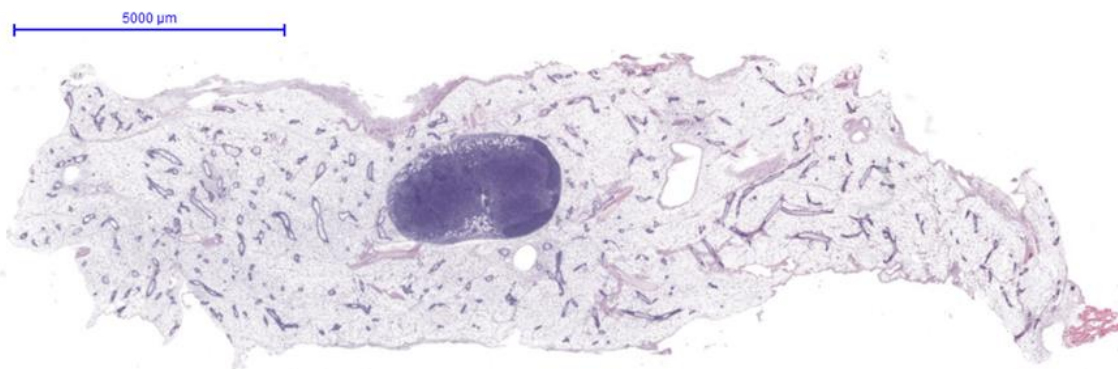
Female CYP1-KO
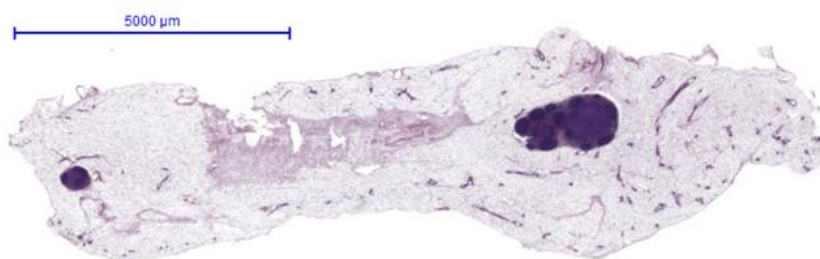
Figure 4 (E). Effects of altered CYP1/AHR signaling on morphology and proliferation. Histological analysis of representative female CYP1-WT and CYP1-KO mammary gland tissues showing reduced number of ductal structures in female CYP1-KO compared to CYP1-WT. Hematoxylin eosin staining, scale bar 5000 µm.

4F

Female CYP1-WT
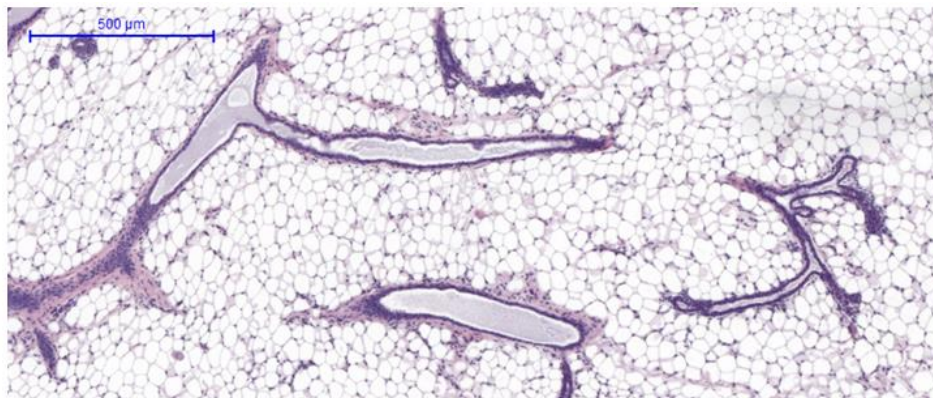
Female CYP1-KO
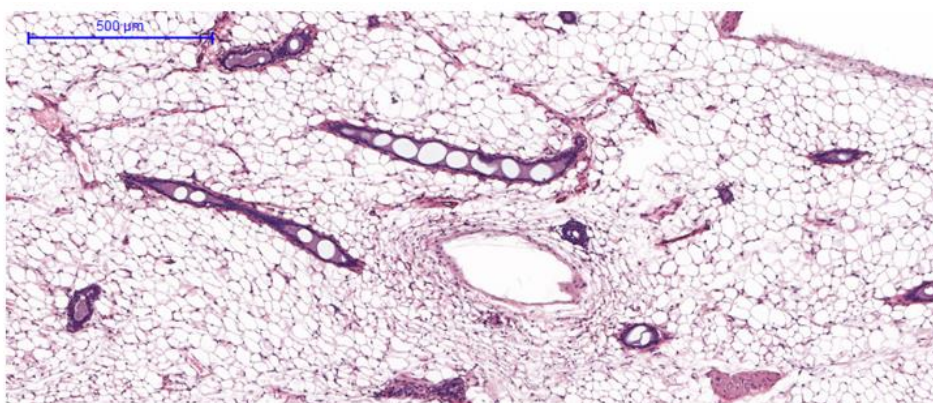
Figure 4 (F). Effects of altered CYP1/AHR signaling on morphology. Histological analysis of representative female CYP1-WT and CYP1-KO mammary gland tissues showing the apparent diverse composition of the secretion inside the ducts. Hematoxylin eosin staining, scale bar 500 µm.

5. DISCUSSION

5. DISCUSSION

In <u>project 1</u>, we provided some pieces of evidence suggesting the participation of AHR to IL6-driven acute inflammation in human breast cells. The focus of the research was to investigate a possible connection between the environmental biosensor AHR and inflammation, which today is demonstrated to be one of the most influencing risk factors in the development of cancers. This issue is of pivotal importance, as the consequences of a mutual interplay between AHR and IL6 could represent one of the first steps in leading to chronic inflammation. Shedding light on this topic is even more urgent in those types of cancers that do not currently have therapies. In this respect, we decided to use in our study the MCF10A cell line, a non-tumoral human breast cell line that does not express HER2, ER and PR. These markers, which support the growth of steroid positive breast cancers, are absent in the TNBC phenotype. Due to the absence of these pharmacological targets, TNBC cannot be treated with hormonal therapy (aromatase inhibitors or tamoxifen) or with trastuzumab, which is an antagonist of HER2 receptor. Aging is characterized by a menopause/andropausa-associated alteration of the physiologic hormone balance and, in particular, a decrease of estrogen and androgen was observed. Interestingly, this rapid decline correlates with the progressive increase of the inflammatory cytokine IL6, both in serum and breast tissues (Papanicolaou, Wilder, Manolagas, & Chrousos, 1998), thus increasing the inflammatory state that is considered a risk factor for cancer. Not surprisingly, the incidence of BC rises in the second middle of life, in the pre- or post-menopausal period, when sexual steroid hormones gradually decrease. In this context, the participation of AHR to the IL6-driven inflammatory pathway, which instigates BC, has been frequently described (Goode, Pratap, & Eltom, 2014; Jin, Lee, Pfent, & Safe, 2014). Because of this, AHR has often been proposed as a candidate marker which can play a role in the development of TNBC. Furthermore, it is recognized that AHR might influence tumor development, and an increased activation of AHR is likely to enhance basal AHR activity in carcinogenesis, especially given the systemic exposure to AHR agonist ligands derived from emission pathways such as wood burning, hospital waste incineration but also from the food chain and microbial sources.

The tight link between inflammation and AHR expression has been efficaciously showed by Hollingshead group (Hollingshead et al., 2008). They in fact evidenced an enhancement of *IL6* expression in MCF-7 breast and ECC-1 endocervical cancer cells when inflammation was combined with AHR stimulation. More recently, Stobbe-Maicherski and colleagues (Stobbe-Maicherski et al., 2013) showed the STAT3 dependent stimulation of AHR in HepG2 cells treated

with Oncostatin M, a cytokine belonging to the interleukin-6 family. Starting from these data, we tried to understand if in a human epithelial breast cell model showing a triple negative phenotype, IL6-driven inflammation could instigate the expression of AHR, which, in turn, endows endogenous *IL6* expression. Ultimately, we hypothesized an auto-inflammatory loop driven by IL6 administration at the beginning and sustained by AHR afterwards. This issue is of pivotal importance, as modulation of inflammation could explain the diverse individual susceptibly to some environmental ligands of AHR, which have an impact on human health.

To test our hypothesis, we initially generated an IL6-driven acute inflammatory experimental design in both MCF10A and MDA-MB-231, by treating cells for 24 hours with increasing doses of IL6. We confirmed Stobbe-Maicherski's results, as the expression of AHR mRNA and protein showed an IL6 dose-dependent increase. STAT3 mRNA and protein expression increased consistently, as did the phosphorylated form pSTAT3. These results highlight the activation of the pathway IL6/STAT3 in both cell lines and, intriguingly, draw attention to the concomitant activation of AHR.

Given the correlation between AHR and pSTAT3 expression, we decided to evaluate AHR protein after inhibiting the STAT3 pathway, using Cryptotanshinone, a specific inhibitor of the phosphorylation of STAT3. Intriguingly, we could find a dose dependent correlation in the decrease of pSTAT3 and of AHR, confirming a tight link between the two pathways.

Thereafter, starting from Hollingshead's data, we tested the possible establishment of an auto-inflammatory loop which produces supplementary, endogenous IL6. We firstly analyzed the expression of *IL6* mRNA in inflamed cells, where we showed that it increased in both cell lines. Consistently, IL6 secretion was enhanced. This result is particularly interesting, especially when considering that tumoral MDA-MB-231 cell line displays an inflammatory phenotype which is endowed by endogenous IL6 expression (Faggioli et al., 1996). Conversely, in our experimental model, MCF10A is a non-transformed cell line which, following an acute inflammatory stimulation, turns to an inflammatory phenotype which stimulates AHR expression.

In post-inflammatory state, we observed two different behaviors of the cell lines. In MCF10A, we observed the concomitant reduction of *AHR*, *IL6* and *STAT3*, while in MDA-MB-231 *AHR* decreased, and *IL6* and *STAT3* slightly increased. These results suggest that AHR could be a critical mediator in inflammatory conditions in non tumorigenic cells, as it can exacerbate *IL6* production only when inflammation occurs. In post-inflammatory state, when the exogenous administration of IL6 is lacking, its expression decreases, together with a reduction of endogenous *IL6* and *STAT3* endogenous expression. This suggests the need of a chronic inflammation stimulus to maintain the

loop, and AHR could be considered as a sort of inflammatory amplifier and not an inducing factor. In contrast, in MDA-MB-231, the decrease of *AHR* expression coupled to *IL6* and *STAT3* increase, indicate that in these cells AHR is only a participant in a complex framework where the leading actor is IL6-induced inflammation.

In understanding to what extent AHR could sustain *IL6* endogenous expression, we focused on the cytokine induction after AHR activation. We treated cells with FICZ, a powerful AHR agonist, and once AHR was activated, we confirmed an increased *IL6* expression in both cell lines, although in MDA-MB-231 the increase was not significant. This effect is even more remarkable in combination with IL6 treatment, suggesting a reinforcement between the two molecules in exerting the effect.

We were then interested in evaluating if IL6 could modulate the oxidative stress, since AHR is a mediator of the cellular toxicological effects, however we did not find a correlation between IL6-driven inflammation and EROD activity. We could thus exclude any role in toxicological signaling.

Certainly, AHR and IL6 regulate physiologic functions such a viability, cell cycle and migration. In our experimental conditions, we did not observed differences in cell viability, in both cell lines, while the results on the IL6 effects on migration and cell cycle progression were unexpected, in fact in MCF10A the enhanced migration caused by IL6 was associated with an arrest in G1 phase of the cell cycle. This is an interesting finding since the deregulation of the cell cycle is one of the key events in the development of a malignancy, and IL6 can induce a change in the phenotype of the non-tumoral MCF10A cells from a proliferative state to an invasive one, indicating that proliferation is not always associated with invasion. This step is not required in MDA-MB-231 cell line, as its phenotype is tumoral and invasive and, for this reason, does not need a slowing of cell cycle to acquire migratory abilities. In a recent study, a FICZ-induced cell cycle arrest in LoVo cells has been demonstrated (Yin et al., 2016) and we then suggest that the increase of AHR expression induced by IL6 administration tended to arrest MCF10A cell cycle in G1 phase. Nonetheless, further studies are needed to confirm it. In conclusion, this study sheds light on the mutual interplay between IL6/STAT3 pathway and AHR and on the role of this transcription factor in IL6-driven inflammation in breast cells. Altogether, we demonstrated that in the complex puzzle of early steps of inflammation, in MCF10A cell line AHR could assume an active role and sustain inflammation. This ability, along with its role as a sensor for xenobiotics, could explain the high incidence of inflammatory diseases associated to AHR ligands exposition in humans.

Project 2:

AHR represents a ligand dependent transcription factor activated by xenobiotics and natural compounds such as tryptophan metabolites, and it is involved in several physiological functions. Hence, dysregulation of the AHR pathway can affect different biological processes. The regulation of AHR is controlled by feedback mechanisms with the AHR-regulated cytochrome P4501 (CYP1) enzymes, which was recently shown to be critical for immune homeostasis in the gut (Schiering et al., 2017). Several studies have described a role for AHR signaling in the mammary gland development, and, in the present work, we showed that impaired CYP1/AHR-feedback signaling affects *Pparγ* and *Ahr* transcription and mammary gland ducts morphology.

We found no changes in *Il6* expression in female or male CYP1-KO mice, and from the histological analysis no inflammatory phenomena were observed, such as infiltration of macrophages or other types of inflammatory cells, suggesting that although the CYP1-KO mouse model presents increased levels of AHR activation, altered CYP1/AHR signaling does not interfere with IL6-related inflammatory pathways.

In the female CYP1-KO mice, we observed a highly significant downregulation of *Pparγ*, and a reduced number of epithelial ductal structures. These effects could be linked, since *Pparγ* is a central regulator of the adipocytes development, and adipocytes are required for the correct development and maintenance of the mammary ductal architecture. The mammary gland epithelial tissue is embedded in the fat pad, a heterogeneous stroma containing different amount of both white and brown adipose tissue, and it has been shown that selective ablation of mammary adipocytes results in the block of the formation and branching of TEBs (Landskroner-Eiger, Park, Israel, Pollard, & Scherer, 2010). Moreover, mice lacking white adipose tissue show mammary ducts unable to grow and branch normally (Couldrey et al., 2002). Thus, we could hypothesize that the downregulation of *Pparγ* could be involved in the altered ductal structures. However, from the immunohistochemical analysis, no defects in proliferation were observed, and, a reduced number of ductal structures was only observed in the half of the total number of samples. To further investigate this connection, we are analyzing RNA sequencing data in order to evaluate alterations in expression of *Pparγ* upstream or downstream genes and to get a more comprehensive view on pathways affected. In addition, more analysis should be performed, such as bromodeoxyuridine (BrdU) assay to evaluate proliferation, or TUNEL test to study apoptosis, or whole mounts to better recognize the ductal structures; more investigation aimed at understanding the mechanisms that lead

to the downregulation of *Pparγ* in the female CYP1-KO is required. Given the lipid appearance of the liquid inside the ducts in the female CYP1-KO, we could hypothesize that the altered *Pparγ* expression may be involved also in the difference of the secretions, but more detailed studies would be necessary to investigate it.

In male mice, no downregulation of *Pparγ* was observed, suggesting a different mechanism and role of CYP1 in regulation of this adipogenesis-related marker compared to female mice.

In the female CYP1-KO mice, a significant upregulation of *Ahr* in one of the thoracic mammary glands was also observed. Pooling all the *Ahr* expression data from the three different mammary gland tissues from each mouse, the significance is lost, but the p-value still remains close to the significance level. To date, it is not very well known how the expression of AHR itself is regulated, and there are contradictory studies regarding this topic. However, among the investigated mechanisms, it is known that AHR activation induces the transcription of *Ahr*, which could explain the upregulation observed in our study.

Taken together, these results show the effects of disrupted CYP1/AHR-feedback signaling on mammary gland. Every day, we are exposed to chemicals which may act as CYP1 inhibitors, as well as AHR activators, mostly derived from endogenous sources, but also from industrial emission pathways, the diet and microbial sources. Thus, an altered CYP1/AHR pathway could represent a novel toxic mode of action leading to impaired physiological processes.

Finally, the CYP1-KO mouse model provides a very good in vivo model to investigate the physiological roles of CYP1 enzymes. However, additional experiments need to be performed to better understand the molecular mechanisms underlying the CYP1-mediated effects. Firstly, the same parameters evaluated in this study could be repeated in mice with permanent knockout of Ahr (Ahr(-/-), AHR-KO). This mouse model has no functional AHR and, consequently, all downstream genes regulated by AHR are affected, including the CYP1 enzymes. The comparison among the CYP1-KO and the AHR-KO mice models should allow the differentiation between AHR-dependent functions and AHR-regulated CYP1-dependent functions. To further discriminate between AHR- and CYP1-dependent functions, a third comparison using a recently developed Cyp1a1-overexpressing mouse model could be performed (Schiering et al., 2017). Moreover, to better evaluate morphodynamic effects and changes on the organization of the ductal structures and the branching of the terminal end buds, whole mounts of the mammary fat pads should be performed. In parallel, since AHR has recognized roles in the immune and endocrine systems, it is of interest to

extend the analysis performed in this study to other tissues, such as pituitary-, adrenal-, and thyroid glands, pancreas, liver, white- and brown adipose, ovary and testis.

6. REFERENCES

6. REFERENCES

Adachi, J., Mori, Y., Matsui, S., Takigami, H., Fujino, J., Kitagawa, H., . . . Matsuda, T. (2001). Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. *J Biol Chem, 276*(34), 31475-31478. doi: 10.1074/jbc.C100238200

Aggarwal, B. B. (2009). Inflammation, a silent killer in cancer is not so silent! *Curr Opin Pharmacol, 9*(4), 347-350. doi: 10.1016/j.coph.2009.06.018

Aggarwal, B. B., Shishodia, S., Sandur, S. K., Pandey, M. K., & Sethi, G. (2006). Inflammation and cancer: how hot is the link? *Biochem Pharmacol, 72*(11), 1605-1621. doi: 10.1016/j.bcp.2006.06.029

Ara, T., & Declerck, Y. A. (2010). Interleukin-6 in bone metastasis and cancer progression. *Eur J Cancer, 46*(7), 1223-1231. doi: 10.1016/j.ejca.2010.02.026

Bachelot, T., Ray-Coquard, I., Menetrier-Caux, C., Rastkha, M., Duc, A., & Blay, J. Y. (2003). Prognostic value of serum levels of interleukin 6 and of serum and plasma levels of vascular endothelial growth factor in hormone-refractory metastatic breast cancer patients. *Br J Cancer, 88*(11), 1721-1726. doi: 10.1038/sj.bjc.6600956

Balkwill, F., Charles, K. A., & Mantovani, A. (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell, 7*(3), 211-217. doi: 10.1016/j.ccr.2005.02.013

Balkwill, F., & Mantovani, A. (2001). Inflammation and cancer: back to Virchow? *Lancet, 357*(9255), 539-545. doi: 10.1016/s0140-6736(00)04046-0

Bellone, S., Watts, K., Cane, S., Palmieri, M., Cannon, M. J., Burnett, A., . . . Santin, A. D. (2005). High serum levels of interleukin-6 in endometrial carcinoma are associated with uterine serous papillary histology, a highly aggressive and chemotherapy-resistant variant of endometrial cancer. *Gynecol Oncol, 98*(1), 92-98. doi: 10.1016/j.ygyno.2005.03.016

Belluco, C., Nitti, D., Frantz, M., Toppan, P., Basso, D., Plebani, M., . . . Jessup, J. M. (2000). Interleukin-6 blood level is associated with circulating carcinoembryonic antigen and prognosis in patients with colorectal cancer. *Ann Surg Oncol, 7*(2), 133-138.

Bersten, D. C., Sullivan, A. E., Peet, D. J., & Whitelaw, M. L. (2013). bHLH-PAS proteins in cancer. *Nat Rev Cancer, 13*(12), 827-841. doi: 10.1038/nrc3621

Bieche, I., Narjoz, C., Asselah, T., Vacher, S., Marcellin, P., Lidereau, R., . . . de Waziers, I. (2007). Reverse transcriptase-PCR quantification of mRNA levels from cytochrome (CYP)1, CYP2 and CYP3 families in 22 different human tissues. *Pharmacogenet Genomics, 17*(9), 731-742. doi: 10.1097/FPC.0b013e32810f2e58

Borland, M. G., Krishnan, P., Lee, C., Albrecht, P. P., Shan, W., Bility, M. T., . . . Peters, J. M. (2014). Modulation of aryl hydrocarbon receptor (AHR)-dependent signaling by peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) in keratinocytes. *Carcinogenesis, 35*(7), 1602-1612. doi: 10.1093/carcin/bgu067

Brattsten, L. B. (1979). Ecological significance of mixed-function oxidations. *Drug Metab Rev, 10*(1), 35-58. doi: 10.3109/03602537908993900

Colotta, F., Allavena, P., Sica, A., Garlanda, C., & Mantovani, A. (2009). Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis, 30*(7), 1073-1081. doi: 10.1093/carcin/bgp127

Conney, A. H., Gillette, J. R., Inscoe, J. K., Trams, E. R., & Posner, H. S. (1959). Induced synthesis of liver microsomal enzymes which metabolize foreign compounds. *Science, 130*(3387), 1478-1479.

Conney, A. H., Miller, E. C., & Miller, J. A. (1956). The metabolism of methylated aminoazo dyes. V. Evidence for induction of enzyme synthesis in the rat by 3-methylcholanthrene. *Cancer Res, 16*(5), 450-459.

Conney, A. H., Miller, E. C., & Miller, J. A. (1957). Substrate-induced synthesis and other properties of benzpyrene hydroxylase in rat liver. *J Biol Chem, 228*(2), 753-766.

Couldrey, C., Moitra, J., Vinson, C., Anver, M., Nagashima, K., & Green, J. (2002). Adipose tissue: a vital in vivo role in mammary gland development but not differentiation. *Dev Dyn, 223*(4), 459-468. doi: 10.1002/dvdy.10065

Coussens, L. M., & Werb, Z. (2002). Inflammation and cancer. *Nature, 420*(6917), 860-867. doi: 10.1038/nature01322

de Visser, K. E., Eichten, A., & Coussens, L. M. (2006). Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer, 6*(1), 24-37. doi: 10.1038/nrc1782

Demaria, S., Pikarsky, E., Karin, M., Coussens, L. M., Chen, Y. C., El-Omar, E. M., . . . Lotze, M. T. (2010). Cancer and inflammation: promise for biologic therapy. *J Immunother, 33*(4), 335-351. doi: 10.1097/CJI.0b013e3181d32e74

Denis, M., Cuthill, S., Wikstrom, A. C., Poellinger, L., & Gustafsson, J. A. (1988). Association of the dioxin receptor with the Mr 90,000 heat shock protein: a structural kinship with the glucocorticoid receptor. *Biochem Biophys Res Commun, 155*(2), 801-807.

Denison, M. S., & Nagy, S. R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol, 43*, 309-334. doi: 10.1146/annurev.pharmtox.43.100901.135828

Denison, M. S., Soshilov, A. A., He, G., DeGroot, D. E., & Zhao, B. (2011). Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicol Sci, 124*(1), 1-22. doi: 10.1093/toxsci/kfr218

Dever, D. P., & Opanashuk, L. A. (2012). The aryl hydrocarbon receptor contributes to the proliferation of human medulloblastoma cells. *Mol Pharmacol, 81*(5), 669-678. doi: 10.1124/mol.111.077305

Dietrich, C., & Kaina, B. (2010). The aryl hydrocarbon receptor (AhR) in the regulation of cell-cell contact and tumor growth. *Carcinogenesis, 31*(8), 1319-1328. doi: 10.1093/carcin/bgq028

Dijsselbloem, N., Vanden Berghe, W., De Naeyer, A., & Haegeman, G. (2004). Soy isoflavone phyto-pharmaceuticals in interleukin-6 affections. Multi-purpose nutraceuticals at the crossroad of hormone replacement, anti-cancer and anti-inflammatory therapy. *Biochem Pharmacol, 68*(6), 1171-1185. doi: 10.1016/j.bcp.2004.05.036

Ding, X., & Kaminsky, L. S. (2003). Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu Rev Pharmacol Toxicol, 43*, 149-173. doi: 10.1146/annurev.pharmtox.43.100901.140251

Dragin, N., Shi, Z., Madan, R., Karp, C. L., Sartor, M. A., Chen, C., . . . Nebert, D. W. (2008). Phenotype of the Cyp1a1/1a2/1b1(−/−) Triple-Knockout Mouse. *Mol Pharmacol, 73*(6), 1844-1856. doi: 10.1124/mol.108.045658

Eltom, S. E., Gasmelseed, A. A., & Saoudi-Guentri, D. (2006). The aryl hydrocarbon receptor is over-expressed and constitutively activated in advanced breast carcinoma. *Cancer Res, 66*(8 Supplement), 408.

Ema, M., Matsushita, N., Sogawa, K., Ariyama, T., Inazawa, J., Nemoto, T., . . . Fujii-Kuriyama, Y. (1994). Human arylhydrocarbon receptor: functional expression and chromosomal assignment to 7p21. *J Biochem, 116*(4), 845-851.

Ema, M., Sogawa, K., Watanabe, N., Chujoh, Y., Matsushita, N., Gotoh, O., . . . Fujii-Kuriyama, Y. (1992). cDNA cloning and structure of mouse putative Ah receptor. *Biochem Biophys Res Commun, 184*(1), 246-253.

Faggioli, L., Costanzo, C., Merola, M., Bianchini, E., Furia, A., Carsana, A., & Palmieri, M. (1996). Nuclear factor kappa B (NF-kappa B), nuclear factor interleukin-6 (NFIL-6 or C/EBP beta) and nuclear factor interleukin-6 beta (NFIL6-beta or C/EBP delta) are not sufficient to activate the endogenous interleukin-6 gene in the human breast carcinoma cell line MCF-7. Comparative analysis with MDA-MB-231 cells, an interleukin-6-expressing human breast carcinoma cell line. *Eur J Biochem, 239*(3), 624-631.

Fernandez-Salguero, P., Pineau, T., Hilbert, D. M., McPhail, T., Lee, S. S., Kimura, S., . . . Gonzalez, F. J. (1995). Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. *Science, 268*(5211), 722-726.

Fielding, C. A., McLoughlin, R. M., McLeod, L., Colmont, C. S., Najdovska, M., Grail, D., . . . Jenkins, B. J. (2008). IL-6 regulates neutrophil trafficking during acute inflammation via STAT3. *J Immunol, 181*(3), 2189-2195.

Furness, S. G., Lees, M. J., & Whitelaw, M. L. (2007). The dioxin (aryl hydrocarbon) receptor as a model for adaptive responses of bHLH/PAS transcription factors. *FEBS Lett, 581*(19), 3616-3625. doi: 10.1016/j.febslet.2007.04.011

Garcia-Tunon, I., Ricote, M., Ruiz, A., Fraile, B., Paniagua, R., & Royuela, M. (2005). IL-6, its receptors and its relationship with bcl-2 and bax proteins in infiltrating and in situ human breast carcinoma. *Histopathology, 47*(1), 82-89. doi: 10.1111/j.1365-2559.2005.02178.x

Gasiewicz, T. A., Henry, E. C., & Collins, L. L. (2008). Expression and activity of aryl hydrocarbon receptors in development and cancer. *Crit Rev Eukaryot Gene Expr, 18*(4), 279-321.

Gonzalez, F. J., & Fernandez-Salguero, P. (1998). The aryl hydrocarbon receptor: studies using the AHR-null mice. *Drug Metab Dispos, 26*(12), 1194-1198.

Goode, G., Pratap, S., & Eltom, S. E. (2014). Depletion of the aryl hydrocarbon receptor in MDA-MB-231 human breast cancer cells altered the expression of genes in key regulatory pathways of cancer. *PLoS One, 9*(6), e100103. doi: 10.1371/journal.pone.0100103

Gunes, A., & Dahl, M. L. (2008). Variation in CYP1A2 activity and its clinical implications: influence of environmental factors and genetic polymorphisms. *Pharmacogenomics, 9*(5), 625-637. doi: 10.2217/14622416.9.5.625

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674. doi: 10.1016/j.cell.2011.02.013

Heath-Pagliuso, S., Rogers, W. J., Tullis, K., Seidel, S. D., Cenijn, P. H., Brouwer, A., & Denison, M. S. (1998). Activation of the Ah receptor by tryptophan and tryptophan metabolites. *Biochemistry, 37*(33), 11508-11515. doi: 10.1021/bi980087p

Heinrich, P. C., Behrmann, I., Haan, S., Hermanns, H. M., Muller-Newen, G., & Schaper, F. (2003). Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J, 374*(Pt 1), 1-20. doi: 10.1042/bj20030407

Heinrich, P. C., Behrmann, I., Muller-Newen, G., Schaper, F., & Graeve, L. (1998). Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J, 334 (Pt 2)*, 297-314.

Hinck, L., & Silberstein, G. B. (2005). Key stages in mammary gland development: The mammary end bud as a motile organ. *Breast Cancer Res, 7*(6), 245-251. doi: 10.1186/bcr1331

Hirano, T., Ishihara, K., & Hibi, M. (2000). Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. *Oncogene, 19*(21), 2548-2556. doi: 10.1038/sj.onc.1203551

Hodge, D. R., Hurt, E. M., & Farrar, W. L. (2005). The role of IL-6 and STAT3 in inflammation and cancer. *Eur J Cancer, 41*(16), 2502-2512. doi: 10.1016/j.ejca.2005.08.016

Hollingshead, B. D., Beischlag, T. V., Dinatale, B. C., Ramadoss, P., & Perdew, G. H. (2008). Inflammatory signaling and aryl hydrocarbon receptor mediate synergistic induction of interleukin 6 in MCF-7 cells. *Cancer Res, 68*(10), 3609-3617. doi: 10.1158/0008-5472.can-07-6168

Huang, B., Butler, R., Miao, Y., Dai, Y., Wu, W., Su, W., . . . Gustafsson, J. (2016). Dysregulation of Notch and ERα signaling in AhR(−/−) male mice. *Proc Natl Acad Sci U S A, 113*(42), 11883-11888. doi: 10.1073/pnas.1613269113

Hushka, L. J., Williams, J. S., & Greenlee, W. F. (1998). Characterization of 2,3,7,8-tetrachlorodibenzofuran-dependent suppression and AH receptor pathway gene expression in the developing mouse mammary gland. *Toxicol Appl Pharmacol, 152*(1), 200-210. doi: 10.1006/taap.1998.8508

Hussain, S. P., & Harris, C. C. (2007). Inflammation and cancer: an ancient link with novel potentials. *Int J Cancer, 121*(11), 2373-2380. doi: 10.1002/ijc.23173

Hussain, S. P., Hofseth, L. J., & Harris, C. C. (2003). Radical causes of cancer. *Nat Rev Cancer, 3*(4), 276-285. doi: 10.1038/nrc1046

Jin, U. H., Lee, S. O., Pfent, C., & Safe, S. (2014). The aryl hydrocarbon receptor ligand omeprazole inhibits breast cancer cell invasion and metastasis. *BMC Cancer, 14*, 498. doi: 10.1186/1471-2407-14-498

Kamimura, D., Ishihara, K., & Hirano, T. (2003). IL-6 signal transduction and its physiological roles: the signal orchestration model. *Rev Physiol Biochem Pharmacol, 149*, 1-38. doi: 10.1007/s10254-003-0012-2

Karin, M., & Greten, F. R. (2005). NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol, 5*(10), 749-759. doi: 10.1038/nri1703

Kazlauskas, A., Poellinger, L., & Pongratz, I. (1999). Evidence that the co-chaperone p23 regulates ligand responsiveness of the dioxin (Aryl hydrocarbon) receptor. *J Biol Chem, 274*(19), 13519-13524.

Kishimoto, T. (2006). Interleukin-6: discovery of a pleiotropic cytokine. *Arthritis Res Ther, 8 Suppl 2*, S2. doi: 10.1186/ar1916

Koliopanos, A., Kleeff, J., Xiao, Y., Safe, S., Zimmermann, A., Buchler, M. W., & Friess, H. (2002). Increased arylhydrocarbon receptor expression offers a potential therapeutic target for pancreatic cancer. *Oncogene, 21*(39), 6059-6070. doi: 10.1038/sj.onc.1205633

Kozlowski, L., Zakrzewska, I., Tokajuk, P., & Wojtukiewicz, M. Z. (2003). Concentration of interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10) in blood serum of breast cancer patients. *Rocz Akad Med Bialymst, 48*, 82-84.

Landskroner-Eiger, S., Park, J., Israel, D., Pollard, J. W., & Scherer, P. E. (2010). Morphogenesis of the developing mammary gland: stage-dependent impact of adipocytes. *Dev Biol, 344*(2), 968-978. doi: 10.1016/j.ydbio.2010.06.019

Ma, Q., & Whitlock, J. P., Jr. (1997). A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Biol Chem, 272*(14), 8878-8884.

Macias, H., & Hinck, L. (2012). Mammary Gland Development. *Wiley Interdiscip Rev Dev Biol, 1*(4), 533-557. doi: 10.1002/wdev.35

Mantovani, A. (2009). Cancer: Inflaming metastasis. *Nature, 457*(7225), 36-37. doi: 10.1038/457036b

Mantovani, A., Allavena, P., Sica, A., & Balkwill, F. (2008). Cancer-related inflammation. *Nature, 454*(7203), 436-444. doi: 10.1038/nature07205

Marotta, L. L., Almendro, V., Marusyk, A., Shipitsin, M., Schemme, J., Walker, S. R., . . . Polyak, K. (2011). The JAK2/STAT3 signaling pathway is required for growth of CD44(+)CD24(-) stem cell-like breast cancer cells in human tumors. *J Clin Invest, 121*(7), 2723-2735. doi: 10.1172/jci44745

Mason, H. S. (1957). Mechanisms of oxygen metabolism. *Science, 125*(3259), 1185-1188.

McIntosh, B. E., Hogenesch, J. B., & Bradfield, C. A. (2010). Mammalian Per-Arnt-Sim proteins in environmental adaptation. *Annu Rev Physiol, 72*, 625-645. doi: 10.1146/annurev-physiol-021909-135922

McNally, S., & Martin, F. (2011). Molecular regulators of pubertal mammary gland development. *Ann Med, 43*(3), 212-234. doi: 10.3109/07853890.2011.554425

Medzhitov, R. (2010). Inflammation 2010: new adventures of an old flame. *Cell, 140*(6), 771-776. doi: 10.1016/j.cell.2010.03.006

Michaud, J. L., Boucher, F., Melnyk, A., Gauthier, F., Goshu, E., Levy, E., . . . Fan, C. M. (2001). Sim1 haploinsufficiency causes hyperphagia, obesity and reduction of the paraventricular nucleus of the hypothalamus. *Hum Mol Genet, 10*(14), 1465-1473.

Mimura, J., Ema, M., Sogawa, K., & Fujii-Kuriyama, Y. (1999). Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. *Genes Dev, 13*(1), 20-25.

Mitchell, K. A., & Elferink, C. J. (2009). Timing is everything: consequences of transient and sustained AhR activity. *Biochem Pharmacol, 77*(6), 947-956. doi: 10.1016/j.bcp.2008.10.028

Moennikes, O., Loeppen, S., Buchmann, A., Andersson, P., Ittrich, C., Poellinger, L., & Schwarz, M. (2004). A constitutively active dioxin/aryl hydrocarbon receptor promotes hepatocarcinogenesis in mice. *Cancer Res, 64*(14), 4707-4710. doi: 10.1158/0008-5472.can-03-0875

Mombaerts, P. (1999). Seven-transmembrane proteins as odorant and chemosensory receptors. *Science, 286*(5440), 707-711.

Murray, G. I., Melvin, W. T., Greenlee, W. F., & Burke, M. D. (2001). Regulation, function, and tissue-specific expression of cytochrome P450 CYP1B1. *Annu Rev Pharmacol Toxicol, 41*, 297-316. doi: 10.1146/annurev.pharmtox.41.1.297

Murray, G. I., Taylor, M. C., McFadyen, M. C., McKay, J. A., Greenlee, W. F., Burke, M. D., & Melvin, W. T. (1997). Tumor-specific expression of cytochrome P450 CYP1B1. *Cancer Res, 57*(14), 3026-3031.

Murray, I. A., Patterson, A. D., & Perdew, G. H. (2014). Aryl hydrocarbon receptor ligands in cancer: friend and foe. *Nat Rev Cancer, 14*(12), 801-814. doi: 10.1038/nrc3846

Nebert, D. W. (2017). Aryl hydrocarbon receptor (AHR): "pioneer member" of the basic-helix/loop/helix per-Arnt-sim (bHLH/PAS) family of "sensors" of foreign and endogenous signals. *Prog Lipid Res, 67*, 38-57. doi: 10.1016/j.plipres.2017.06.001

Nebert, D. W., & Gonzalez, F. J. (1987). P450 genes: structure, evolution, and regulation. *Annu Rev Biochem, 56*, 945-993. doi: 10.1146/annurev.bi.56.070187.004501

Nebert, D. W., & Karp, C. L. (2008). Endogenous functions of the aryl hydrocarbon receptor (AHR): intersection of cytochrome P450 1 (CYP1)-metabolized eicosanoids and AHR biology. *J Biol Chem, 283*(52), 36061-36065. doi: 10.1074/jbc.R800053200

Nebert, D. W., & Russell, D. W. (2002). Clinical importance of the cytochromes P450. *Lancet, 360*(9340), 1155-1162. doi: 10.1016/s0140-6736(02)11203-7

Nelson, D. R., Zeldin, D. C., Hoffman, S. M., Maltais, L. J., Wain, H. M., & Nebert, D. W. (2004). Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics, 14*(1), 1-18.

Novikov, O., Wang, Z., Stanford, E. A., Parks, A. J., Ramirez-Cardenas, A., Landesman, E., . . . Sherr, D. H. (2016). An Aryl Hydrocarbon Receptor-Mediated Amplification Loop That Enforces Cell Migration in ER-/PR-/Her2- Human Breast Cancer Cells. *Mol Pharmacol, 90*(5), 674-688. doi: 10.1124/mol.116.105361

Omura, T., & Sato, R. (1962). A new cytochrome in liver microsomes. *J Biol Chem, 237*, 1375-1376.

Oshima, M., Mimura, J., Sekine, H., Okawa, H., & Fujii-Kuriyama, Y. (2009). SUMO modification regulates the transcriptional repressor function of aryl hydrocarbon receptor repressor. *J Biol Chem, 284*(17), 11017-11026. doi: 10.1074/jbc.M808694200

Papanicolaou, D. A., Wilder, R. L., Manolagas, S. C., & Chrousos, G. P. (1998). The pathophysiologic roles of interleukin-6 in human disease. *Ann Intern Med, 128*(2), 127-137.

Paul, W. (1991). IL-6: a multifunctional regulator of immunity and inflammation. *Jpn J Cancer Res, 82*(12), 1458-1459.

Peng, T. L., Chen, J., Mao, W., Liu, X., Tao, Y., Chen, L. Z., & Chen, M. H. (2009). Potential therapeutic significance of increased expression of aryl hydrocarbon receptor in human gastric cancer. *World J Gastroenterol, 15*(14), 1719-1729. doi: 10.3748/wjg.15.1719

Poland, A., Glover, E., & Kende, A. S. (1976). Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. *J Biol Chem, 251*(16), 4936-4946.

Poland, A. P., Glover, E., Robinson, J. R., & Nebert, D. W. (1974). Genetic expression of aryl hydrocarbon hydroxylase activity. Induction of monooxygenase activities and cytochrome P1-450 formation by 2,3,7,8-tetrachlorodibenzo-p-dioxin in mice genetically "nonresponsive" to other aromatic hydrocarbons. *J Biol Chem, 249*(17), 5599-5606.

Polyak, K., & Kalluri, R. (2010). The role of the microenvironment in mammary gland development and cancer. *Cold Spring Harb Perspect Biol, 2*(11), a003244. doi: 10.1101/cshperspect.a003244

Probst, M. R., Reisz-Porszasz, S., Agbunag, R. V., Ong, M. S., & Hankinson, O. (1993). Role of the aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon (dioxin) receptor action. *Mol Pharmacol, 44*(3), 511-518.

Ramamoorthi, K., Fropf, R., Belfort, G. M., Fitzmaurice, H. L., McKinney, R. M., Neve, R. L., . . . Lin, Y. (2011). Npas4 regulates a transcriptional program in CA3 required for contextual memory formation. *Science, 334*(6063), 1669-1675. doi: 10.1126/science.1208049

Rannug, A., Rannug, U., Rosenkranz, H. S., Winqvist, L., Westerholm, R., Agurell, E., & Grafstrom, A. K. (1987). Certain photooxidized derivatives of tryptophan bind with very high affinity to the Ah receptor and are likely to be endogenous signal substances. *J Biol Chem, 262*(32), 15422-15427.

Rannug, U., Rannug, A., Sjoberg, U., Li, H., Westerholm, R., & Bergman, J. (1995). Structure elucidation of two tryptophan-derived, high affinity Ah receptor ligands. *Chem Biol, 2*(12), 841-845.

Salgado, R., Junius, S., Benoy, I., Van Dam, P., Vermeulen, P., Van Marck, E., . . . Dirix, L. Y. (2003). Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. *Int J Cancer, 103*(5), 642-646. doi: 10.1002/ijc.10833

Sansone, P., Storci, G., Tavolari, S., Guarnieri, T., Giovannini, C., Taffurelli, M., . . . Bonafe, M. (2007). IL-6 triggers malignant features in mammospheres from human ductal breast

  carcinoma and normal mammary gland. *J Clin Invest, 117*(12), 3988-4002. doi: 10.1172/jci32533

Schaldach, C. M., Riby, J., & Bjeldanes, L. F. (1999). Lipoxin A4: a new class of ligand for the Ah receptor. *Biochemistry, 38*(23), 7594-7600. doi: 10.1021/bi982861e

Schiering, C., Wincent, E., Metidji, A., Iseppon, A., Li, Y., Potocnik, A. J., . . . Stockinger, B. (2017). Feedback control of AHR signalling regulates intestinal immunity. *Nature, 542*(7640), 242-245. doi: 10.1038/nature21080

Schiwy, A., Brinkmann, M., Thiem, I., Guder, G., Winkens, K., Eichbaum, K., . . . Hollert, H. (2015). Determination of the CYP1A-inducing potential of single substances, mixtures and extracts of samples in the micro-EROD assay with H4IIE cells. *Nat Protoc, 10*(11), 1728-1741. doi: 10.1038/nprot.2015.108

Schmidt, J. V., Carver, L. A., & Bradfield, C. A. (1993). Molecular characterization of the murine Ahr gene. Organization, promoter analysis, and chromosomal assignment. *J Biol Chem, 268*(29), 22203-22209.

Schweikl, H., Taylor, J. A., Kitareewan, S., Linko, P., Nagorney, D., & Goldstein, J. A. (1993). Expression of CYP1A1 and CYP1A2 genes in human liver. *Pharmacogenetics, 3*(5), 239-249.

Shaban, Z., El-Shazly, S., Abdelhady, S., Fattouh, I., Muzandu, K., Ishizuka, M., . . . Fujita, S. (2004). Down regulation of hepatic PPARalpha function by AhR ligand. *J Vet Med Sci, 66*(11), 1377-1386.

Songur, N., Kuru, B., Kalkan, F., Ozdilekcan, C., Cakmak, H., & Hizel, N. (2004). Serum interleukin-6 levels correlate with malnutrition and survival in patients with advanced non-small cell lung cancer. *Tumori, 90*(2), 196-200.

Steelman, L. S., Pohnert, S. C., Shelton, J. G., Franklin, R. A., Bertrand, F. E., & McCubrey, J. A. (2004). JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia, 18*(2), 189-218. doi: 10.1038/sj.leu.2403241

Sternlicht, M. D., Kouros-Mehr, H., Lu, P., & Werb, Z. (2006). Hormonal and local control of mammary branching morphogenesis. *Differentiation, 74*(7), 365-381. doi: 10.1111/j.1432-0436.2006.00105.x

Stobbe-Maicherski, N., Wolff, S., Wolff, C., Abel, J., Sydlik, U., Frauenstein, K., & Haarmann-Stemmann, T. (2013). The interleukin-6-type cytokine oncostatin M induces aryl hydrocarbon receptor expression in a STAT3-dependent manner in human HepG2 hepatoma cells. *Febs j, 280*(24), 6681-6690. doi: 10.1111/febs.12571

Tanaka, T., & Kishimoto, T. (2012). Targeting Interleukin-6: All the Way to Treat Autoimmune and Inflammatory Diseases. *Int J Biol Sci, 8*(9), 1227-1236. doi: 10.7150/ijbs.4666

Vorderstrasse, B. A., Fenton, S. E., Bohn, A. A., Cundiff, J. A., & Lawrence, B. P. (2004). A novel effect of dioxin: exposure during pregnancy severely impairs mammary gland differentiation. *Toxicol Sci, 78*(2), 248-257. doi: 10.1093/toxsci/kfh062

Wei, L. H., Kuo, M. L., Chen, C. A., Chou, C. H., Lai, K. B., Lee, C. N., & Hsieh, C. Y. (2003). Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. *Oncogene, 22*(10), 1517-1527. doi: 10.1038/sj.onc.1206226

Wei, Y. D., Helleberg, H., Rannug, U., & Rannug, A. (1998). Rapid and transient induction of CYP1A1 gene expression in human cells by the tryptophan photoproduct 6-formylindolo[3,2-b]carbazole. *Chem Biol Interact, 110*(1-2), 39-55.

Wincent, E., Amini, N., Luecke, S., Glatt, H., Bergman, J., Crescenzi, C., . . . Rannug, U. (2009). The suggested physiologic aryl hydrocarbon receptor activator and cytochrome P4501 substrate 6-formylindolo[3,2-b]carbazole is present in humans. *J Biol Chem, 284*(5), 2690-2696. doi: 10.1074/jbc.M808321200

Yang, X., Liu, D., Murray, T. J., Mitchell, G. C., Hesterman, E. V., Karchner, S. I., . . . Sherr, D. H. (2005). The aryl hydrocarbon receptor constitutively represses c-myc transcription in human mammary tumor cells. *Oncogene, 24*(53), 7869-7881. doi: 10.1038/sj.onc.1208938

Yin, J., Sheng, B., Han, B., Pu, A., Yang, K., Li, P., . . . Yang, H. (2016). The AhR is involved in the regulation of LoVo cell proliferation through cell cycle-associated proteins. *Cell Biol Int, 40*(5), 560-568. doi: 10.1002/cbin.10592

Yu, H., & Jove, R. (2004). The STATs of cancer--new molecular targets come of age. *Nat Rev Cancer, 4*(2), 97-105. doi: 10.1038/nrc1275

Zakrzewska, I., & Poznanski, J. (2001). [Changes of serum il-6 and CRP after chemotherapy in patients with ovarian carcinoma]. *Pol Merkur Lekarski, 11*(63), 210-213.

Zanger, U. M., Turpeinen, M., Klein, K., & Schwab, M. (2008). Functional pharmacogenetics/genomics of human cytochromes P450 involved in drug biotransformation. *Anal Bioanal Chem, 392*(6), 1093-1108. doi: 10.1007/s00216-008-2291-6

Zhang, G. J., & Adachi, I. (1999). Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma. *Anticancer Res, 19*(2b), 1427-1432.

Zhou, S. F., Wang, B., Yang, L. P., & Liu, J. P. (2010). Structure, function, regulation and polymorphism and the clinical significance of human cytochrome P450 1A2. *Drug Metab Rev, 42*(2), 268-354. doi: 10.3109/03602530903286476