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RAMI EL-DAIRI

EFFECTS OF PIOGLITAZONE, β-NAPHTHOFLAVONE AND AFLATOXIN B1 ON GENE EXPRESSION PROFILES IN HUMAN TROPHOBLASTS *IN VITRO*

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ABSTRACT

Various chemicals such as pharmaceuticals and environmental contaminants can exert adverse effects on fetal development. The placenta is an endocrine organ that also regulates the passage of endogenous and xenobiotic compounds between the mother and the fetus during pregnancy. The human placenta has unique tissue-specific properties including a distinct set of transcription factors that are highly expressed in placenta such as peroxisome proliferator-activated receptor gamma (PPARγ) and aryl hydrocarbon receptor (AhR). PPARγ and AhR play vital roles in regulating placental functions including placental development and xenobiotic metabolism, respectively.

In this thesis, the effects of a PPARγ-agonist pioglitazone and an AhR-agonist β-naphthoflavone (BNF) were investigated on transcriptional responses in human primary trophoblasts to reveal their potential target genes and gene networks. In addition, the effect of aflatoxin B1 (AFB1) on gene expression profiles in trophoblasts was studied. AFB1 is a common food contaminant that causes acute and chronic toxicity and can be transferred and metabolized through the placenta. The trophoblasts were isolated from full term placentas after delivery and were exposed to pioglitazone, BNF or AFB1. A gene expression profiling analysis was performed, and Ingenuity Pathway Analysis (IPA) was used to identify the biological functions and regulatory networks among the dysregulated genes. Neither BNF nor AFB1 affected cell viability at the concentration selected for Gene expression array analysis as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The functionality of the

trophoblasts was confirmed by determinations of induced cytochrome P450 (CYP)1A1 mRNA levels and ethoxy-O-deethylase activity in BNF exposed trophoblasts in comparison to untreated control cells.

Gene expression analysis revealed that pioglitazone and BNF affected the regulation of especially those genes involved in cellular growth and invasion, the inflammatory response, as well as lipid metabolism and cholesterol uptake, whereas AFB1 affected genes related to placental growth, endocrine signalling, energy homeostasis and cell cycle regulation in primary trophoblasts. Pioglitazone was associated significantly with 69 altered genes (32 up- and 37 downregulated ± 2-fold), BNF 321 genes (64 up- and 257 downregulated ± 1.5-fold), and AFB1 170 genes (46 up- and 124 downregulated ± 1.5-fold) in primary trophoblasts when compared to control cells. There were only six genes, whose expression was changed in response to all three treatments: chorionic gonadotropin beta 1/2, CGB3, corticotropin releasing hormone, chorionic somatomammotropin hormone like 1, growth hormone 1 and pyruvate dehydrogenase. These genes are important regulators of pregnancy maintenance as well as placental and fetal growth.

In conclusion, in placental trophoblast cells, pioglitazone, BNF and AFB1 modulated gene expression each with its own distinct pattern. These studies represent novel information about placental gene expression and provide insights into how chemicals may disrupt normal placental physiological functions in pregnant women.

National Library of Medicine Classification: QS 645, QU 475, QU 550.5.G4, QV 600, WQ 210.5, WQ 212

Medical Subject Headings: Fetal Development/drug effects; Pioglitazone; beta-Naphthoflavone; Aflatoxin B1; Placenta; Trophoblasts; Transcription Factors; PPAR gamma; Receptors, Aryl Hydrocarbon; Gene Expression Regulation; Gene Expression Profiling; Oligonucleotide Array Sequence Analysis; In Vitro Techniques; Humans

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Pioglitatsonin β-naftoflavonin ja aflatoksiini B1:n vaikutukset geenien ilmentymiseen ihmisen istukan viljellyissä trofoblasteissa
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TIIVISTELMÄ

Monet kemialliset altisteet kuten lääkeaineet ja ympäristökemikaalit voivat vaikuttaa haitallisesti sikiön kehitykseen. Istukka on endokrinologinen eli hormoneja tuottava elin, joka säätelee sekä elimistön omien aineiden että vierasaineiden kulkeutumista äidin ja sikiön välillä raskauden aikana. Ihmisen istukalla on monia vain sille ominaisia piirteitä kuten kudosspesifinen geeninsäätelytekijöiden ilmentyminen. Istukassa runsaasti ilmentyviä geeninsäätelytekijöitä ovat esimerkiksi aryyli-hiilivetyreseptori (AhR) ja peroksisomi-proliferaattorin aktivoima reseptori gamma (PPARγ).

Tässä väitöskirjassa tutkittiin PPARγ-agonisti pioglitatsonin, AhR-agonisti β-naftoflavonin (BNF) ja homemyrkky aflatoksiini B1:n (AFB1) vaikutusta geenien ilmentymiseen viljellyissä ihmisen istukan trofoblasteissa. Trofoblastit eristettiin istukasta ja altistettiin pioglitatsonille, BNF:lle ja AFB1:lle. Laajamittaisen geenien ilmentymisanalyysin jälkeen geenien ilmentymisprofiilien muodostamia säätelyverkkoja ja signalointireittejä analysoitiin käyttämällä Ingenuity Pathway Analysis (IPA) -ohjelmistoa. BNF ja AFB1 eivät vaikuttaneet solujen elinkykyyn transkriptomiikka-analyysiin käytetyillä pitoisuuksilla. Trofoblastien toimintakyky varmistettiin BNF:llä altistetuissa soluissa mittaamalla soluista sytokromi P450 (CYP)1A1 geenin aktivaatiota ja etoksiresorufiini-O-de-etylaasientsyymin (EROD) aktiivisuuden nousua.

Geenien ilmentymisanalyysin perusteella pioglitatsoni ja BNF vaikuttivat ihmisen trofoblasteissa erityisesti solujen kasvuun ja invasoitumiseen, tulehdusvasteeseen sekä lipidimetaboliaan ja kolesterolin soluunottoon liittyvien

geenien säätelyyn, kun taas AFB1 vaikutti geeneihin, jotka osallistuvat istukan kasvuun, hormonaaliseen signalointiin, energiatasapainoon ja solusyklin säätelyyn. Pioglitatsoni vaikutti istukan trofoblasteissa merkitsevästi 69 geenin (32 ylöspäin ja 37 alaspäin ilmentynyttä geeniä, ≥2 kertainen muutos), BNF puolestaan vaikutti 321 geenin (64 ylöspäin- and 257 alaspäin ≥1.5 kertainen muutos), ja AFB1 170 geenin (46 ylöspäin- and 124 alaspäin ≥ 1.5-kertainen muutos) ilmentymiseen, kun altistettuja soluja verrattiin altistamattomiin soluihin. Ainoastaan kuuden geenin ilmentyminen muuttui merkittävästi kaikilla käsittelyillä. Nämä geenit olivat istukkahormonit β1/2 ja β3, kortikotropiinia vapauttava hormoni, istukan somatomammotropiinin kaltainen hormoni-1, kasvuhormoni-1 ja pyruvaattidehydrogenaasi, jotka ovat tärkeitä raskauden ylläpidon sekä istukan ja sikiön kasvun säätelijöitä.

Yhteenvetona voidaan todeta, että pioglitatsonin, BNF:n ja AFB1:n säätelemät geenien ilmentymisprofiilit istukan trofoblasteissa olivat keskenään erilaisia. Tämä tutkimus tuo uutta tietoa geenien ilmentymisestä istukassa ja lisää ymmärrystämme siitä, miten kemikaalit voivat häiritä istukan toimintaa raskausaikana.

Luokitus: QS 645, QU 475, QU 550.5.G4, QV 600, WQ 210.5, WQ 212

Yleinen suomalainen ontologia: sikiönkehitys; istukka; kemikaalit; kemialliset yhdisteet; altisteet; transkriptiotekijät; geenilekspressio; DNA-sirut; in vitro -menetelmä; ihminen

"Knowledge is life and a cure."

- Imam Ali Iben Abi Talib (KAW)

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قل إن صلاتي ونسكي ومحياي ومماتي لله رب العالمين لا شريك له

Say, “Indeed, my prayer, my rites of sacrifice, my living and my dying are for Allah, Lord of the worlds.
Holy Quran 6:162

Kuopio, June 2021
Rami El-Dairi

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I El-Dairi R, Huuskonen P, Pasanen M, Rysä J. Peroxisome proliferator activated receptor gamma (PPAR-γ) ligand pioglitazone regulated gene networks in term human primary trophoblast cells. Reproductive Toxicology 81:99-107, 2018.

II El-Dairi R, Huuskonen P, Pasanen M, Rysä J. Aryl hydrocarbon receptor (AhR) agonist β-naphthoflavone regulated gene networks in human primary trophoblasts. Reproductive Toxicology 96:370-379, 2020.

III El-Dairi R, Rysä J, Storvik M, Pasanen M, Huuskonen P. Aflatoxin B1 disturbs endocrine function, protein synthesis and energy homeostasis of human primary trophoblasts. (submitted).

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CONTENTS

ABBREVIATIONS

AFB1	Aflatoxin B1
AFL	Aflatoxicol
AFM1	Aflatoxin M1
AFBO	Aflatoxin B1-8,9-epoxide
AhR	Aryl hydrocarbon receptor
BaP	Benzo(a)pyrene
BNF	β-naphthoflavone
CEBPβ	CCAAT/enhancer binding protein beta
CGB	Chorionic gonadotropin beta
CRH	Corticotropin releasing hormone
CT	Cytotrophoblast
CYP	Cytochrome P450
E2	17β-estradiol
EVT	Extravillous cytotrophoblast
ER	Estrogen receptor
FAs	Fatty acids
GH	Growth hormone
ICM	Inner cell mass
IGF1	Insulin-like growth factor 1
IL	Interleukin
MRP	Multidrug resistance protien
NFκB	Nuclear factor kappa B
TNFα	Tumor necrosis factor alpha
PAH	Polycyclic aromatic hydrocarbon
PPAR	Peroxisome proliferator-activating receptor
SCT	Syncytiotrophoblast
SLC	Solute carrier
TG	Triglyceride
TCDD	2, 3, 7, 8-tetrachlorodibenzo-para-dioxin

1 INTRODUCTION

The human placenta is a transitory organ that is responsible for the growth and development of the fetus by providing immune protection, hormone production and the transfer of gases and nutrients between the maternal and fetal blood (Pijnenborg et al., 1980; Sadler, 2011). The placenta also creates a unique microenvironment for the fetus as it serves as a "barrier" that separates fetal blood from maternal blood. Storvik et al. (2014) analyzed the unique characteristics of the placental transcriptome and reported that a small number of xenobiotic metabolizing cytochrome P450 (CYP) enzymes are expressed in placenta. These include CYP19A1, which mainly converts androgens into estrogens, and CYP1A1, whose substrates include not only steroid hormones, but also many xenobiotics such as benzo(a)pyrene (BaP) and planar polycyclic aromatic hydrocarbons (PAHs) (Pelkonen and Nebert, 1982). In addition, a number of transcription factors whose co-expression correlated with CYP1A1, i.e. aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptor gamma (PPARγ), and CCAAT/enhancer binding protein beta (CEBPβ) were highly expressed in human placenta (Storvik et al., 2014).

PPARγ is a nuclear receptor (NR) and belongs to the steroid/thyroid/retinoid receptor super-family. In addition to its key role in adipose tissue regulation and insulin sensitization, PPARγ has a vital role in placental and fetal development as well as placental hormone production and secretion (Asami-Miyagishi et al., 2004; Barak et al., 1999; Guo et al., 2015; Janani and Kumari, 2015). PPARγ is also important in trophoblast differentiation as well as in the invasion processes and the maturation of extravillous and villous trophoblasts, (Barak et al., 1999; Fournier et al., 2002; Shalom-Barak et al., 2004). PPARγ also regulates the production and secretions of human chorionic gonadotropin (hCG) and vascular endothelial growth factor (VEGF) in the placenta as well as placental lipid transport and metabolism (Desvergne and Wahli, 1999; Schild et al., 2002; Tarrade et al., 2001). Since PPARγ plays a vital role in the placenta, and can be activated by naturally occurring ligands such as prostaglandins and eicosanoids, and also by synthetic ligands such as thiazolidinediones (Wieser et al., 2008), it is crucial to study PPARγ-regulated networks in the placenta.

AhR is a ligand-activated transcription factor that control a variety of developmental and physiological events such as xenobiotic metabolism (Crews, 1998; Gu et al., 2000). It has been shown that the AhR content varies across

tissues, cell types and developmental stages where AhR mRNA is highly expressed in placenta, lung, heart, pancreas, and liver, with lower levels of expression found in brain, kidney, and skeletal muscle (Carver et al., 1994; Kahl et al., 1980; Storvik et al., 2014). Because of its structure, AhR is highly susceptible to environmental planar toxicants such as dioxins. It is well known that AhR upregulates the most important xenobiotic metabolizing enzyme of the placenta, CYP1A1. Several exogenous xenobiotics, such as PAHs, polychlorinated biphenyls and dioxins that are found in various commercial products, exert their toxic effects through AhR mediated pathways (Detmar et al., 2008), therefore it is vital to investigate AhR induced alterations in gene expression.

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus* molds; these microorganisms contaminate important food crops such as rice, maize nuts, cereals, soybeans (Alshannaq and Yu, 2017) and can even be present in animal products such as meat, eggs and milk (Pier, 1992). Aflatoxins, especially aflatoxin B1 (AFB1) are known to cause both acute and chronic toxicity with the liver as their major target (Williams et al., 2004), possessing carcinogenic (Ostry et al., 2017), teratogenic (Gong et al., 2004; Smith et al., 2017), and immunosuppressive properties (Williams et al., 2004) as well as potential endocrine disruptive effects (Storvik et al., 2011). It has been reported that these toxins can be transferred and metabolized while passing through the placenta (Partanen et al., 2010) and can be found in breast milk, neonatal cord blood and serum of pregnant women (Lamplugh et al., 1988). Storvik et al. (2011) previously demonstrated that AFB1 causes disturbances in normal estrogen production in chorion carcinoma cells (JEG-3) and therefore, it was deemed important to study its gene regulatory effects in human placenta namely primary trophoblast cells in vitro.

Although it is evident that the feto-placental unit is exposed to various xenobiotics and environmental contaminants during pregnancy, the gene regulatory effects of many of these agents e.g. pioglitazone and β-naphthoflavone (BNF) in the placenta have not been investigated. These agonists were selected since pioglitazone is a PPARγ agonist in clinical use and BNF is a prototypical agonist of the AhR. Both PPARγ and AhR are highly expressed in the placenta and have a wide range of exogenous and endogenous agonists. Furthermore, it is also unclear whether there are alterations in gene expression caused by AFB1 in human placenta. Therefore, the main aim for this thesis was to investigate the alterations in the gene expression profile of human term placental trophoblasts in response to a PPARγ agonist pioglitazone, an AhR agonist, BNF and AFB1.

2 REVIEW OF THE LITERATURE

2.1 PLACENTA

2.1.1 Origin, structure, function

A fertilized human embryo forms blastomeres after undertaking multiple cell divisions. The blastomeres undergo a further set of divisions and give rise to blastocyst that are made up of the outer cell layer trophectoderm (TE); these cells are the origins of epithelial trophoblasts that give rise to the placenta, and the inner cell mass (ICM) that generates all the tissues in the embryo (for a review, see Marikawa and Alarcón, 2009). The TE of the blastocyst invades the uterine decidua and the proliferating ICM differentiate into a hypoblast (primitive endoderm) and the epiblast. The hypoblast gives rise to the primitive endoderm of the yolk sack, whereas epiblast is the cellular source of the three germ layers (endoderm, ectoderm, and mesoderm) that will form all of the adult tissues (for a review, see Nakamura et al., 2016).

The human placenta is a discoid organ of average 22 cm in diameter weighing about 500 g with a thickness at the center of about 2.5 cm. The placenta has two surfaces (chorionic plate and basal plate) with the intervillous space located between these plates; this contains 30-40 branched fetal villus trees (fetal lobules). The chorionic plate faces the fetus and it is attached to the umbilical cord, whereas the basal plate is embedded in the uterine endometrium. The chorionic plate is covered by the amnion that is made up of a single layered epithelium and an avascular connective tissue (amnionic mesenchyme). The chorionic vessels of chorionic mesenchyme are continuing with the umbilical cord vessels. The chorionic arteries branch to supply the villous trees whereas the chorionic veins that form the umbilical vein cross underneath the chorionic arteries and they are extensions of the veins of the villous trees. The terminal villi, which are formed from 20 weeks of gestation onwards and the final branches on the villous tree, are highly vascularized by fetal capillary networks and the principal sites of maternal-fetal exchange. The basal plate of the human placenta contains 10-40 elevated regions called lobes that contain one to four villous trees that arise from the chorionic plate and reach into the intervillous space (Figure 1) (for a review, see Gude et al. 2004).

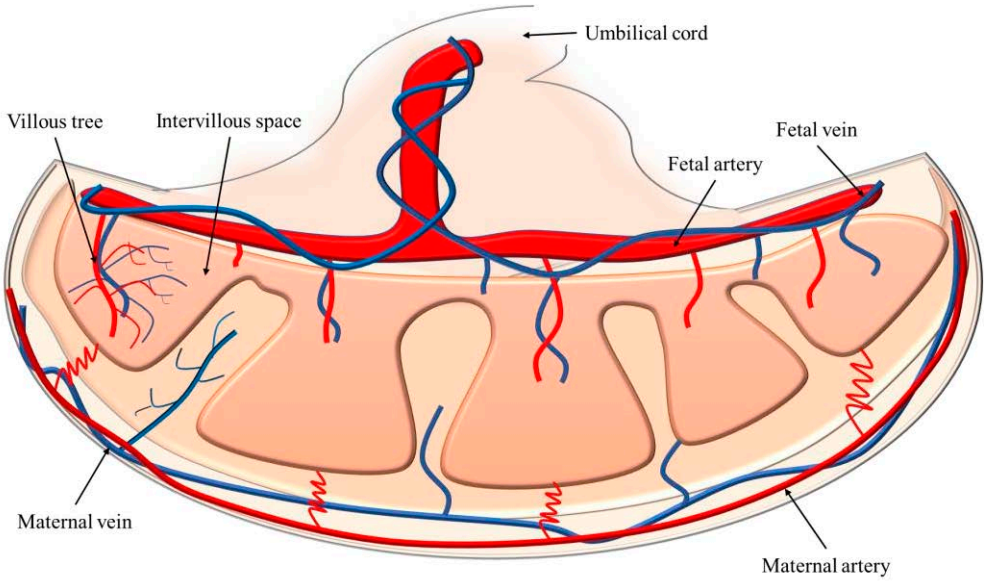

Figure 1. Illustration of the structure of human placenta.

The placenta is a transitory organ that maintains pregnancy by providing nutrient and oxygen uptake and synthesis and the exchange of various macromolecules between the mother and the fetus (Hay, 1994) (Figure 2). It has a role in immune protection and xenobiotic metabolism e.g. protecting the fetus from infections, chemical exposure, and maternal diseases (Gude et al., 2004). The placenta synthesizes many hormones, growth factors, cytokines, chemokines, and eicosanoids that regulate maternal and fetal physiology during pregnancy (Benyo et al., 1997; Ehsani et al., 2019; Gude et al., 2004). In addition, the placenta facilitates the transfer of maternal hormones, immunoglobulins, respiratory gases and nutrients (vitamins, carbohydrates, amino acids and lipids) that are necessary for fetal development (Garty et al., 1994; Gude et al., 2004; Schinkel and Jonker, 2003). On the other hand, compounds secreted by the placenta such as steroid hormones, prolactin, and placental lactogen to the mother evoke a variety of physiological changes including lactation, immune suppression, insulin resistance, pancreatic beta-cell expansion and insulin production (Gootwine, 2004; Newbern and Freemark, 2011).

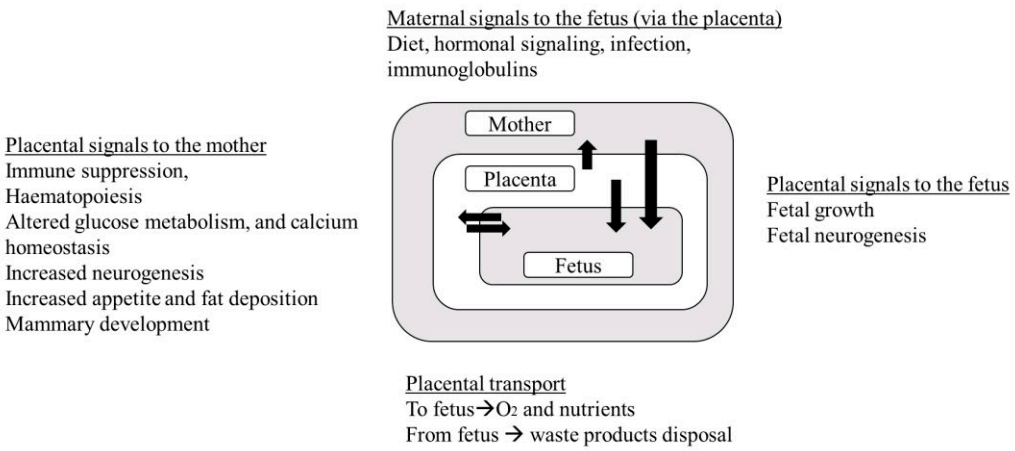

Figure 2. Schematic representation of transport and signalling between developing fetus and the maternal circulation in the placenta.

2.1.2 Histopathology

The human placenta is made up of different cell types and tissues as a result of the fusion of maternal uterine tissue and fetal trophoblast cells (Figure 3). The polar mononucleated trophoblast that makes up the TE and lies over the ICM is responsible for making the attachment to the uterine epithelium (Okae et al., 2018). Soon after the attachment, the trophoblasts undergo a syncytial fusion and form oligonucleated syncytiotrophoblasts (SCTs); the remaining mononuclear progenitor trophoblasts are called cytotrophoblasts (CTs) (for a review, see Stejskalova and Pavek, 2011). The maternal blood is in direct contact with the apical plasma membrane of the SCTs of the microvillous, whereas the basal membrane of SCTs is in contact with the stroma of the villous tree.

Placental fibrinoids containing extracellular matrix proteins that originated from the extravillous trophoblasts (EVTs) cover the intervillous surface of the chorionic and basal plates and the placental villi (Frank et al., 1994; Huppertz et al., 1996). The vascular network of the placenta starts to form at week 2 of gestation, originating from the primitive villus mesenchyme (Asahara et al., 1999) and around week 4 of gestation, there is a mature functional structure of placental villi that surrounds fetal blood, facilitating the uptake of nutrients and oxygen from maternal blood (Boss et al., 2018). Placental macrophages (Hoffbauer cells) appear on the 5th week of gestation and originate from the mesenchymal cells in the villous stroma (Demir et al., 1989). Hoffbauer cells play an important role in placental development such as in vasculogenesis and angiogenesis (Reyes and Golos, 2018). Fetal vessels are covered by non-fenestrated endothelium

throughout the gestation. Furthermore, the luminal diameter of the placental vessels is controlled by paracrine and autocrine factors since there is no neural innervation in the placenta (Huppertz, 2008).

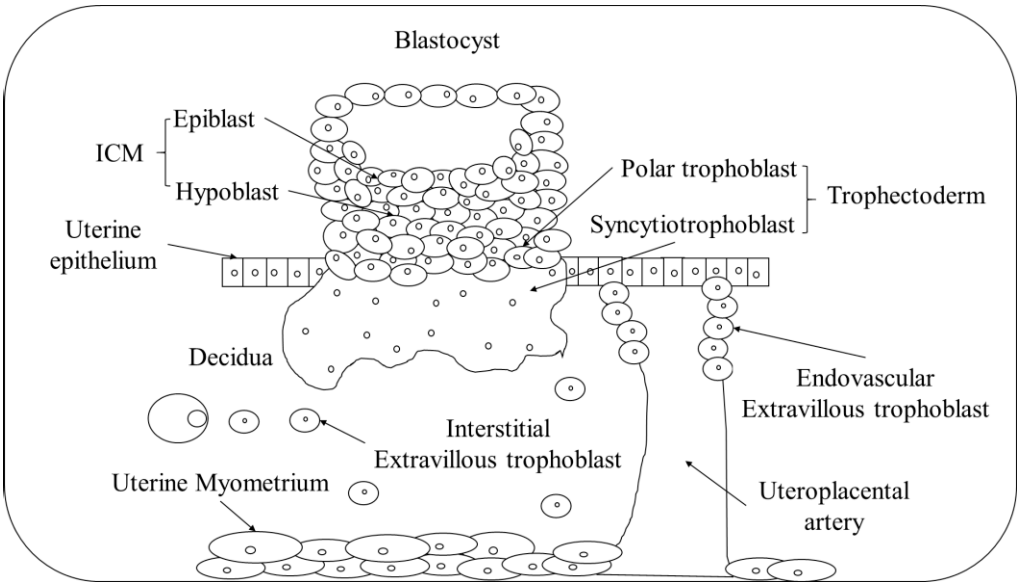

Figure 3. Blastocyst invasion of the uterine wall. The trophectoderm is the outer cell layer of the blastocyte and it surrounds the inner cell mass (ICM). The trophectoderm will give rise to polar cytotrophoblasts that will fuse and form syncytiotrophoblasts. Cytotrophoblasts will penetrate the syncytiotrophoblasts and form the columns of the extravillous cytotrophoblast that in turn will invade the uterine decidua and the myometrium and form the endovascular and interstitial trophoblast.

2.1.3 Trophoblasts

The human trophoblast cells are made up of three major trophoblast subpopulations, CTs, extravillous cytotrophoblasts and SCTs. CT cells divide and fuse to form terminally differentiated SCTs that surround the conceptus that is fully embedded by the decidual stroma (Pötgens et al., 2002). SCTs are highly polarized epithelial cells covering the microvilli of the villous tree on its apical border; these cells are involved in several placental functions such as active transport, peptide and steroid hormones synthesis, and protection against xenobiotics (Ockleford and Menon, 1977). The SCTs are the primary site of the feto-maternal exchange of nutrients, waste products and hormones (Chatuphonprasert et al., 2018). CTs penetrate the layer of SCTs and form columns

of EVTs that in turn form the cytotrophoblastic shell that is located at the interface of the feto-maternal space. EVTs then invade the uterine decidua and remodel the maternal blood vessels; these cells are referred to as endovascular trophoblasts (Gude et al., 2004). The EVTs that invade the myometrium and promote an expansion of the villous region and the recruitment of maternal blood vessels are called interstitial trophoblasts. Interstitial trophoblasts will terminally differentiate and become multinucleated placental bed giants cells that become deeply embedded in the decidua (Kaufmann et al., 2003) (Figure 4).

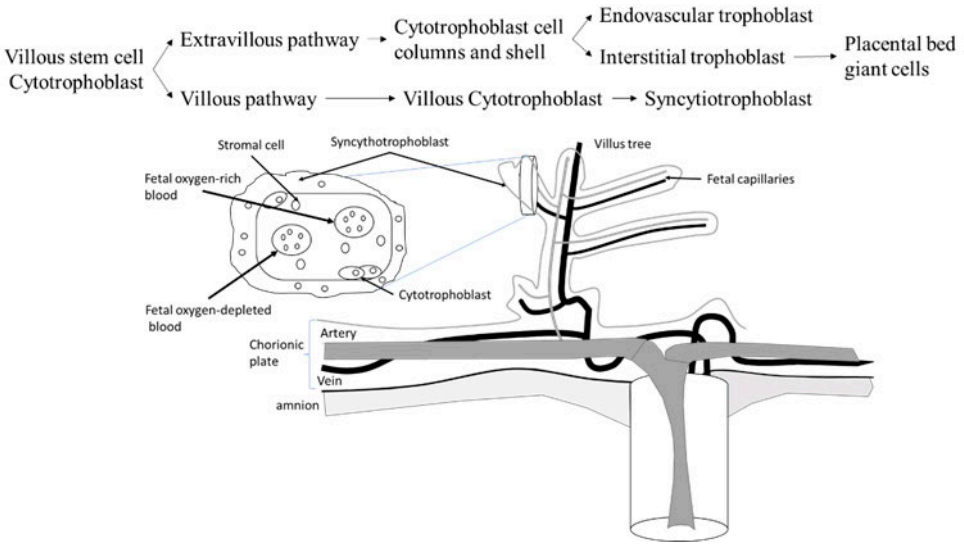

Figure 4. Placental villous tree (fetal lobules) bathed in maternal blood in the intervillous spaces. Human placenta contains 30-40 villous trees. The maternal blood is in direct contact with apical plasma membrane of the microvilli of the syncytiotrophoblast, whereas the basal membrane of syncytiotrophoblast is in contact with the stroma of the villous tree. The syncytiotrophoblast cells are the primary site of the feto-maternal exchange of nutrients, waste products, and hormones. The cross-sectional view of the villous tree reveals the syncytiotrophoblast cell layer with the remaining cytotrophoblast cells surrounding the fetal chorionic vessels.

2.2 PLACENTAL TRANSCRIPTOME STUDIES

The placental transcriptome i.e., the entire set of RNA transcripts from the genome of placental cells in any given state, displays a unique expression profile since it contains a collection of mRNA species that are not present in any other human organ (Storvik et al., 2014). The unique profile of the placental transcriptome

attributable to it reflects the gene expression of hormones, enzymes and proteins that are exclusively expressed in the placental tissue. For instance, human placental alkaline phosphatase (PALP), chorionic somatomammotropin hormone 1 (CSH1) (Gaspard et al., 1980), endogenous retrovirus group W member 1 (ERVWE1) (Frendo et al., 2003), lectin, galactoside-binding, soluble, 13 (LGALS13) (Huppertz et al., 2008) and human chorionic gonadotropin subunit beta (hCGβ) (Frendo et al., 2003) are all exclusively expressed in the placenta. PALP plays a role in syncitial fusion (Guilbert et al., 2002) whereas CSH1 stimulates lactation and is involved in fetal growth and metabolism (Newbern and Freemark, 2011). HCGβ, which is expressed by the syncytiotrophoblasts, plays an important role in CT differentiation into SCT (Pidoux et al., 2007). Frendo et al. (2003) has demonstrated the direct involvment of ERVWE1 in human trophoblast cell fusion and differentiation whereas Gadde et al., (2018) reported that a low expression of LGALS13 could be useful as an early marker of preeclampsia.

There are rather few transcriptome studies that have investigated the term placental transcription profile. For instance, placental RNA-seq analysis revealed that the placenta contained 228,004 transcripts, and the expression levels fragments per kilobase million (FPKM) was about 39,000 transcriptional active regions (Majewska et al., 2017). A gene ontology analysis of the RNA-seq data hinted at the biological processes associated with the category of reproduction such as placental development and embryo implantation and development (Majewska et al., 2017). In addition, a transcriptome-wide profile of 200 term human placentas revealed an enrichment in functional processes related to growth and development, including cellular respiration, transcriptional activity, and signal transduction (Deyssenroth et al., 2017).

On the other hand, various placental gene expression profiling studies have compared normal versus preeclamptic placental gene expression profiles from different gestation periods. These studies have identified differentially expressed genes, hormones, growth factors, cytokines, and microRNAs that play significant roles in angiogenesis, hypertension, inflammation, immune response, and placental growth and development (Keelan and Mitchell, 2007; Founds et al., 2011; Kaartokallio et al., 2015; Brew et al., 2016; Leavey et al., 2016; Gunel et al., 2017).

Furthermore, the placental transcriptome is developmental stage-dependent, as well as showing sex -, and cell-type dependency. Placental mRNA expression in the first and second trimester is enriched with transcripts that reflect the placental growth and maturation; these are involved in cell cycle, metabolism, mitosis, and cell communication and adhesion (Mikheev et al., 2008; Uusküla et al., 2012). On

the other hand, term placental villi are bathed in maternal blood and maternal immune cells, and microarray data has revealed that term placental transcripts are involved in oxidative stress response, immune response, and focal adhesion (Saben et al., 2014). Sood et al. (2006) and Kim et al. (2012) detected significant differences in gene expression between female and male placentas with a higher gene expression of immune regulators in the placentas of female fetuses. These two research groups also reported significant differences in gene transcription between different placental subregions (decidua, amnion, and chorion). The maternal side of the placenta (decidua) was found to express genes involved with hormone and cytokine production, highlighting the important role of decidua in placental growth and the maternal immune response to the placenta. These genes were different from those expressed in the amnion, which is the innermost layer of the fetal membrane and an epithelial tissue lining the amniotic cavity. Kim et al. (2012) noted that in the amnion there was an enrichment of genes involved in cell adhesion and a higher expression of epithelial splicing regulatory protein 1 (ESRP1), an epithelial splicing regulator while Sood et al. (2006) reported a high expression of Mucin 1, a protein that confers antibacterial and anti-adhesive properties to the amnion. Gene expression analysis of chorion, the outer layer of the fetal membrane, showed gene clusters involved in tissue remodelling, cell adhesion, synthesis of cytokines, and vascular development of the placentas (Kim et al., 2012; Sood et al., 2006).

2.3 PLACENTAL HORMONES

The human placenta is a hormonal tissue that synthesizes a wide range of steroid and peptide hormones that regulate the development of the feto-placental unit (Handwerger and Freemark, 2000). The human placenta secretes various hormones such as human chorionic somatomammotropin (hCSH), estrogen, progesterone, GH, hCG, leptin, relaxin and corticosteroids (Costa, 2016; Gootwine, 2004; Hirasawa et al., 2000; Marshall et al., 2017; Newbern and Freemark, 2011; Schanton et al., 2018). Table 1 is a summary of the main placental hormones along with their function in placenta. Here, the focus will be placed on estrogen and GH.

Table 1. The function and localization of the main placental hormones.

Placental hormone	Function	Localisation	References
Cortisol	Essential for normal fetal development and tissue maturation Placental 11β-HSD1 regenerates cortisol from inactive cortisone	Extravillous trophoblasts, endothelial cells lining fetal capillaries in terminal villi	Räikkönen et al., 2015; Stirrat et al., 2018
human Chorionic gonadotropin	Promotes angiogenesis and stimulates trophoblast invasion Maintains the production of progesterone until the placenta itself produces it Plays a role in myometrial quiescence and local immune tolerance	Extravillous trophoblasts, syncytiotrophoblasts	Handschuh et al., 2007; Fournier, 2016
human Chorionic somatomammotropin hormone	Promotes fetal growth and survival Placental growth Metabolic adaptors during pregnancy	Villous syncytiotrophoblasts	Chen et al., 1989; Martial, 1996; Newbern and Freemark, 2011
Growth hormone	Plays a role in fetal growth and placental development Maternal adaptation to pregnancy	Syncytiotrophoblasts	Caufriez et al., 1993; Lacroix et al., 2002; Pérez-Ibave et al., 2014
Estrogen	Placental energy and cholesterol homeostasis Promotes fetal and placental development Endometrial maturation and differentiation Maintenance of utero-placental blood flow	Syncytiotrophoblasts	Vrtačnik et al., 2014; Costa, 2016; Chatuphonprasert et al., 2018
Progesterone	Pregnancy maintenance Provides suitable uterine environment for fetal development Suppresses the maternal immune system	Syncytiotrophoblasts	Pepe and Albrecht, 1995; Tuckey, 2005

Leptin	Modulation of thermogenesis and angiogenesis Induces trophoblast cells proliferation and survival Plays a key role in placental adaptation to stimuli such as growth hormones, steroid hormones, hypoxia, and pro-inflammatory signals	Trophoblasts	Dos Santos et al., 2015; Schanton et al., 2018
Relaxin	Relaxation of the pubic symphysis during parturition Contributes to the regulation of the systemic vasculature and its adaptations during early pregnancy Initiating decidualization in preparation for implantation	Decidua, placental trophoblasts	Gunnersen et al., 1996; Marshall et al., 2017

2.3.1 Estrogen

Estrogen is a steroid hormone that is involved in placental energy and cholesterol homeostasis, carbohydrate and lipid metabolism, proliferation of the mammary epithelium, maintenance of utero-placental blood flow, regulation of skeletal homeostasis, electrolyte balance, and endometrial maturation and differentiation (Costa, 2016; Vrtačnik et al., 2014). Estrogen mediates its function by binding to and activating estrogen receptors (ERα or ERβ), either initiating a gene expression cascade or by activating signalling pathways (Costa, 2016; Vrtačnik et al., 2014). After estrogen binding to ERα or ERβ in the cytosol, the ERs dimerize (either as homo- or heterodimers) and bind to estrogen response elements (ERE) that are located in or near to the promotors of the target genes.

On the other hand, estrogen also influences several signalling pathways and transcription factors such as insulin-like growth factor 1 (IGF1), cyclin D1, sp1 transcription factor, nuclear factor kappa B (NFκB), CCAAT/enhancer binding protein beta (C/EBPβ), choline acetyltransferase (ChA), and signal transducer and activator of transcription 5 (STAT5) (Vrtačnik et al., 2014). IGF1 is an essential regulator of normal fetal and postnatal growth and plays significant physiological roles in every organ and tissue during fetal and postnatal life (Pérez-Ibave et al., 2014). Cyclin D1 is expressed in the stroma of the placental villi and in the nuclei of SCTs and plays a key role in the regulation and progression of the cell cycle (De Falco et al., 2004). NFκB is an important regulator of the immune response,

inflammation, immune development and trophoblast invasion (Mitchell et al., 2016; Xue et al., 2020). Sp1 transcription factor plays vital role in the expression of the *glucose transporter 1* gene and regulates the concentration of glucocorticoids that are essential for fetal development (Li et al., 2011; Okamoto et al., 2001). C/EBPβ is an important transcriptional regulator in adipocyte differentiation by transactivating the expression of C/EBPα and PPARγ. C/EBPα and PPARγ are two master transcription factors for terminal adipocyte differentiation (Guo et al., 2015); these receptors are key regulators of placental development, and trophoblast invasion and differentiation (Canettieri et al., 2012; Fournier et al., 2011; Schaiff et al., 2000). ChA activity was detected in human placenta where it plays a role in the uptake of amino acids (Morris, 1966; Rowell and Sastry, 1981). STAT5 (STAT5A and STAT5B) has important roles in the invasion and syncytialization of trophoblast cells, in the maintenance of normal immune functions, as well as in the regulation of vital cellular functions such as proliferation, differentiation, and survival (Gupta et al., 2016; Rani and Murphy, 2016).

There are different forms of estrogens present in the human placenta, i.e. estrone (E1), 17β-estradiol (E2), estriol (E3) and estetrol (E4) although estradiol (E2) is the most abundant form of estrogen and it is known to play a significant role during pregnancy by promoting the implantation of the embryo and stimulating the growth and differentiation of the endometrium (Groothuis et al., 2007; Loriaux et al., 1972). Estrogen is produced by the ovaries and the testes, but during early pregnancy, the corpus luteum of the ovaries is the main source of estrogen. However, in the later stages of pregnancy, placental SCTs are major sites for estrogen production with a secretion rate of a peak at 100-120 mg/day at term (Chatuphonprasert et al., 2018; Loriaux et al., 1972; Simpson and MacDonald, 1981). Placental CTs mainly express ERα whereas SCTs mainly express Erβ (Bechi et al., 2006; Bukovsky et al., 2003). Estrogen production (Figure 5) in the placenta requires the orchestrated cooperation between the mother, the placenta and the fetus to ensure the synthesis and transport of several precursors (i.e. 16α-OH-dehydroepiandrosterone sulphate (16α-OH-DHEAS), dehydroepiandrosterone sulphate (DHEAS), and androstenedione) and enzymes such as steroid sulphatase, 3β-HSD, 17β-HSD, and aromatase (Levitz and Young, 1977; Nelson and Bulun, 2001). DHEAS is produced by the maternal and fetal adrenal glands where it is first converted to 16α-OH-DHEAS by a fetal liver enzyme, CYP3A7, before being acted upon by placental aromatase to form E3. DHEAS can be also desulfated by steroid sulfatase to DHEA that in turn is converted by 3β-HSD into androstenedione in the

SCTs. In the placenta, esterone can be formed from androstenedione; esterone is changed into E2 by aromatase and 17β-HSD respectively. Androstenedione can also be used to make testosterone which then can produce E2 by the actions of 17β- HSD and aromatase respectively (Simpson et al., 1994; Vrtačnik et al., 2014).

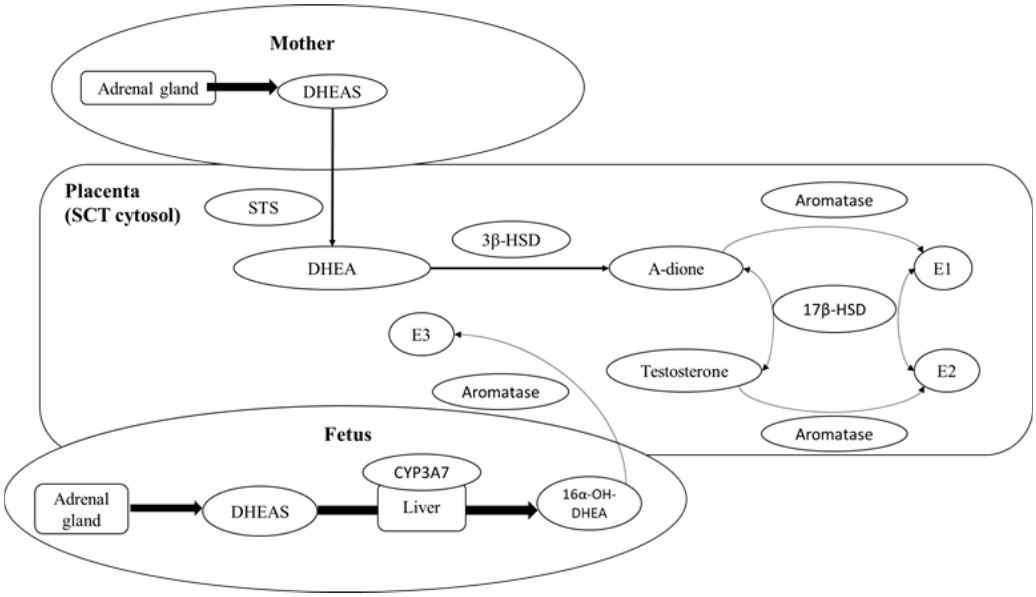

Figure 5. Estrogen biosynthesis pathways between the mother, the placenta, and the fetus. 3β-HSD, 3-Beta-hydroxy-delta (5)-steroid dehydrogenase; 16α-OH-DHEAS, 16α-Hydroxy-dehydroepiandrosterone sulphate; 17β-HSD, Hydroxysteroid 17-beta dehydrogenase; A-dione, Androstenedione; CYP3A7, Cytochrome P450 family 3 subfamily A member 7; Aromatase (CYP19A1); DHEAS, Dehydroepiandrosterone sulphate; E1, estrone; E2, 17β-estradiol; E3, estriol; E4, estetrol; SCT, syncytiotrophoblast; STS, Steroid Sulfatase.

2.3.2 Growth hormone

The human genome contains five growth hormone (GH) genes that form a cluster in chromosome 17; four of those five genes, (hGH-V, hCS-A, hCS-B, and hCS-L), are expressed in the placental villous tree and one (hGH-N) in the pituitary gland (Hirt et al., 1987; MacLeod et al., 1992; Palmetshofer et al., 1995). GH plays a significant role in fetal growth, placental development, maternal adaptation to pregnancy e.g. it can stimulate maternal IGF1 production resulting in ensuring the availability of glucose and amino acids to the fetus (Caufriez et al., 1993; Lacroix et al., 2002; Pérez-Ibave et al., 2014). SCTs synthesize placental GHs (Cooke and Liebhaber, 1995). Placental growth hormone receptors (Frankenne et al., 1992) and the

expression of GH are induced during fetal development between gestation weeks 12 and 20, subsequently the levels stabilize until delivery (MacLeod et al., 1992). During pregnancy, the production of maternal pituitary GH is silenced, whereas placental growth hormone variant 2 (hGH-V) becomes the predominant GH in the mother's blood (Handwerger and Freemark, 2000).

2.4 PLACENTAL INFLAMMATORY RESPONSE

The human placenta and the associated fetal and maternal membranes express virtually all known cytokines. Placental trophoblasts, fetal hofbauer cells and stromal cells in the placenta were reported to secrete different patterns of inflammatory cytokines throughout the gestation period (Steinborn et al., 1998). Cytokines are key components of the paracrine/autocrine communication network within the feto-maternal interface and are required to ensure a successful pregnancy (Bowen et al., 2002b). Placental cytokines play a vital role in parturition, including the expulsion of the fetus by evoking uterine contractions, membrane rupture, and dilation of the cervix (Bowen et al., 2002a). Furthermore, cyokines are important in the maternal-placental immune cross-talk, trophoblast invasion, differentiation, proliferation and apoptosis, placental growth, angiogenesis, metabolic, and endocrine homeostasis (Keelan and Mitchell, 2007). Table 3 summarizes the functions of selected important cytokines in the placenta.

Table 3. Selected important list of cytokines and their function in the placenta.

Cytokine	Function	References
Proinflammatory cytokines		
Interleukin 1α Interleukin 1β	Labor, uterine contraction, fetal membrane remodeling and rupture. Trophoblast differentiation, estrogen production, and labor	Bowen et al., 2002a Benyo et al., 1997; Nilkaeo et al., 2000
Interleukin 6	Labor, cervical ripening, fetal membrane remodeling and rupture, and fetal growth	Gunn, 1996; Paradowska et al., 1997; Bowen et al., 2002b; Ehsani et al., 2019
Tumor necrosis factor α	Regulate trophoblast growth and proliferation, regulate placental hormone production, immunoprotection, parturition, myometrial contractility and fetal membrane rupture	Garcia-Lloret et al., 1996; Keelan et al., 1997; Hansen et al., 1999; Bowen et al., 2002a

Anti-inflammatory cytokines		
IL1 receptor antagonist	Protective role against maternal inflammation, myometrial contraction, regulation of placental hormones	Bowen et al., 2002b; Girard et al., 2010; Prutsch et al., 2012
Interleukin 4	Labor, myometrial contraction	Bry and Lappalainen, 1994; De Moraes-Pinto et al., 1997
Interleukin 10	Myometrium contraction, protective role in the placenta	Hennessy et al., 1999
Chemokines		
Interleukin 8	Play crucial role during delivery and in feto-maternal immune defense, fetal membranes remodeling and rupture	Shimoya et al., 1999; Bowen et al., 2002a; Kang et al., 2007;
Macrophage inflammatory protein 1	Activate immune cells in the placenta, promote myometrial contractions	Ehsani et al., 2019 Dudley et al., 1995; Dudley et al., 1996
Monocyte chemoattractant protein 1	Cervical ripening, proliferation, and activation of immune cells	Sennström et al., 2000
Adipokines		
Leptin	Placental angiogenesis, inflammatory response	Domali and Messinis, 2002; Lappas et al., 2005b, 2005a
Resistin	Placental metabolic homeostasis, insulin resistance	Keelan and Mitchell, 2007; Lappas et al., 2005b
Adiponectin	Insulin sensitivity and placental inflammatory response	Chen et al., 2006; Whitehead et al., 2006; Lappas et al., 2005b
Interferons		
Interferon α/β/γ	Immune protection, suppress maternal immune system, and induce trophoblast apoptosis	Zdravkovic et al., 1994; Paradowska et al., 1997; Keelan and Mitchell, 2007

2.5 PLACENTAL TOXICOKINETICS

2.5.1 Metabolism

Placental metabolism and transporters are unique and very different from their counterparts in the liver or other extrahepatic tissues. Placental metabolism is mostly focused on the synthesis of hormones (Myllynen et al., 2007). Phase I and phase II xenobiotic metabolising enzymes catalyse the biotransformation of lipophilic substances into water-soluble and excretable metabolites. Phase I reactions function mainly by adding or removing polar functional groups to xenobiotics and consist of several reductases, dehydrogenases, oxidoreductases. The main phase I metabolism functional enzymes in human placenta are CYPs, acetylcholinesterase, butyrylcholinesterase, alcohol dehydrogenase, aldehyde dehydrogenase, epoxide hydrolase, and monoamine oxidase (Chen et al., 1976; Cizkova and Tauber, 2018; Hahn et al., 1993; Sanchis and Guerri, 1986).

There are 57 human CYP genes mainly expressed in the liver; these are divided into 18 families and play a role in metabolizing exogenous drugs and chemicals and metabolising endogenous substrates such as fat-soluble vitamins, sterols, or prostanoids (Nelson et al., 2004; Seliskar and Rozman, 2007). Fifteen human CYPs, primarily belonging to the CYP1, CYP2, CYP3 families, are involved in xenobiotic metabolism and the majority of them are concentrated in the endoplasmic reticulum of the liver (Seliskar and Rozman, 2007). Other CYPs such as CYP11, CYP17,CYP19 and CYP2E1, are involved in steroid biosynthesis (Seliskar and Rozman, 2007). In extrahepatic tissues such as the kindey, lung and the placenta, the CYP expression profile differs from that present in the liver (Hakkola et al., 1998). For instance, human term placenta express only a few CYP enzymes that are vital for the metabolism of xenobiotics and also the biosythesis and catabolism of steroids and fatty acids (FAs) (Myllynen et al., 2007; Storvik et al., 2014; Zhang and Yang, 2009). Placental CYP enzyme expression was also shown to be dependent on the gestation stage. In the first trimester, immunological or RT-PCR detection identified the expression of CYP1A1, CYP1A2, CYP2C, CYP2D6, CYP2E1, CYP2F1, CYP3A4, CYP3A5, CYP3A7, and CYP4B1. On the other hand, at term, the expression profile had become more restricted with the tissue only expressing CYP1A1, CYP2E1, CYP2F1, CYP3A3/4, CYP3A5, and CYP4B1. These different expression patterns may reflect the critical role of the placenta in metabolism during the earlier stages of organogenesis and embryogenesis (Hakkola et al., 1996a, 1996b).

CYP1A1 is the major enzyme involved in the metabolism of many exogenous and endogenous molecules and plays a key role in the bioactivation of pro-carcinogens and pro-teratogens, such as arylamines and PAHs. CYP1A1 mainly catalyzes BaP metabolism to an epoxide intermediate, which is further metabolised by epoxide hydrolase to the ultimate carcinogen B(a)P-7,8-diol-9,10-epoxide-2 (Androutsopoulos et al., 2009; Stejskalova and Pavek, 2011). CYP1A1 was shown to be expressed in the placenta (Pasanen et al., 1988). Constitutive CYP1A1 activity is very low, however, its expression was significantly upregulated in placentas from smoking mothers (Huuskonen et al., 2008).

As compared to the liver, CYP19A1 is highly expressed in the placenta; in fact, the placenta is the tissue where CYP19A1 has the highest mRNA expression in the human body (Vähäkangas et al., 2019). CYP19A1 is responsible for estradiol production by catalyzing the conversion of A-dione to esterone and for converting testosterone into estardiol (Chatuphonprasert et al., 2018; Seliskar and Rozman, 2007). In addition to endogenous substrates such as cortisol and glucocorticoids, CYP19A1 can also act on several exogenous substrates such as AFB1, bisphenol A (BPA), diethystilbesterol, methadone, and lindane. These substrates affect the catalytic activity of CYP19A1 and cause either its induction or inhibition in the placenta (for a review, see Vähäkangas et al., 2019). Furthermore, the expression of CYP19A1 was shown to have a positive correlation with the expression of many transcription factors that are highly expressed in the placenta and play a role in hormonal regulation and estrogen catabolism, highlighting the vulnerability of the placenta to endocrine disruptors (Storvik et al., 2014).

CYP2E1, that is expressed in term placenta (Vieira et al., 1998), metabolises ethanol, generating reactive oxygen species (ROS) and hydroxyethyl radicals which can trigger oxidative stress (Gemma et al., 2006; Wang et al., 2009). Oxidative stress and ROS have been associated with the development of intrauterine growth retardation (IUGR) and preeclampsia (Aouache et al., 2018; Takagi et al., 2004). Furthermore, alcohol intake during any stage of pregnancy, perhaps due to the by-products generated by CYP2E1, increases the risk of fetal alcohol syndrome, mitochondrial damage, lipid peroxidation, placental vasoconstriction, maternal metabolic disorders, and fetal growth retardation (Gupta et al., 2016; Lee et al., 2020).

The biotransformation reactions performed by phase II enzymes are generally considered as the detoxifying step in drug metabolism by conjugating the polar xenobiotics produced by phase I enzymes through glucuronidation, esterification, methylation, sulfation, acetylation, and glutathione and amino acid conjugation

(Iyanagi, 2007). The liver is the main site of expression of most of the phase II drug metabolizing enzyme families such as UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione S-transferases (GSTs) and methyltransferases (Jancova et al., 2010). On the other hand, extrahepatic tissues such as the placenta contain a few of the phase II enzymes (Huuskonen et al., 2008) such as GST (Alparslan and Daniş, 2015) mainly GSTA4 (Storvik et al., 2014), NATs (Derewlany et al., 1994), SULT1A and SULT2B (Jancova et al., 2010; Stanley et al., 2001) and various UGTs such as UGT2B4, 2B7, 2B10, 2B11 and 2B1 (Collier et al., 2002; Reimers et al., 2011).

2.6 PLACENTAL TRANSCRIPTION FACTORS

2.6.1 Aryl hydrocarbon receptor

The AhR is a ligand-activated transcription factor that belongs to the basic helix-loop-per-Arnt-Sim (bHLH–PAS) superfamily; it is highly expressed in various organs including placenta, ovaries and lungs. Upon ligand binding, AhR translocates to the nucleus and binds to its heterodimer partner, aryl hydrocarbon receptor nuclear translocator (ARNT), and then the complex binds to the dioxin response element (DRE) (Schmidt and Bradfield, 1996) (Figure 6).

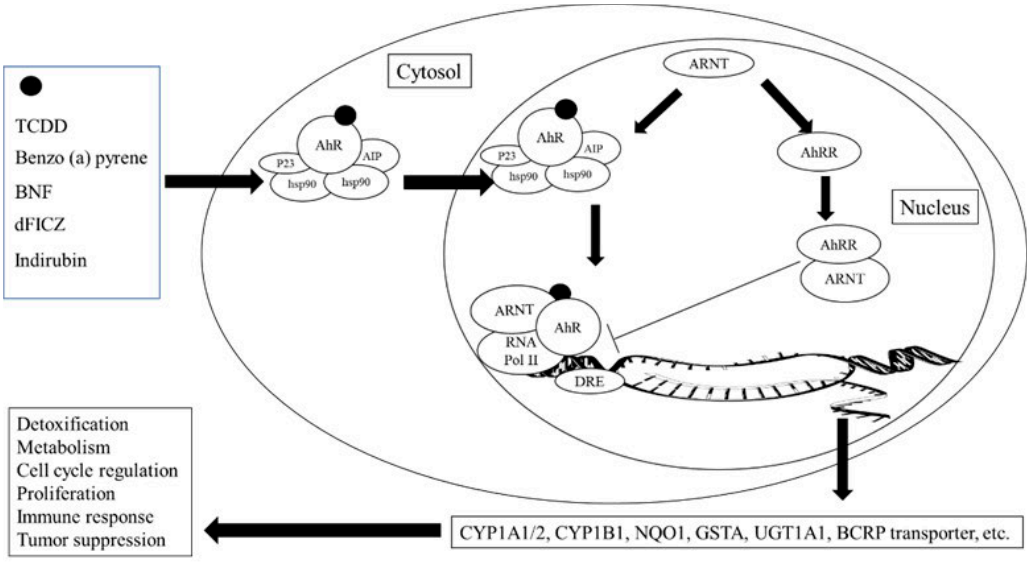

Figure 6. AhR activation by an agonist leads to chaperone dissociation and AhR-ARNT heterodimerization and binding of the dioxin response element (DRE). The AhR-activated gene-battery is involved in the detoxification of environmental contaminants and carcinogens, metabolism, oxidative stress responses, cell cycle regulation, proliferation, immune response, and tumor suppression. AhR, Aryl hydrocarbon receptor; AHRR, Aryl-hydrocarbon receptor repressor; AIP, Aryl hydrocarbon receptor interacting protein; ARNT, Aryl hydrocarbon receptor nuclear translocator; BCRP, Placenta-specific ATP-binding cassette transporter; BNF, β-naphthoflavone; CYP1A1/2, Cytochrome P450 family 1 subfamily A member 1/2; CYP1B1; Cytochrome P450 family 1 subfamily B member 1; dFICZ, diformylindolo (3,2-b) carbazole; GSTA, Glutathione S-transferase alpha; HSP90, Heat shock protein 90; NQO1, NAD(P)H quinone dehydrogenase 1; UGT1A1, UDP glucuronosyltransferase family 1 member A1.

DRE occurs in the promoters of genes that encode for many xenobiotic metabolizing enzymes such as CYP1A1/2, CYP1B1, NAD(P)H-dependent quinone oxyreductase-1 (NQO1), GSTA, and UGT1A1. DRE seems to be involved also in the genes coding for cytokines such as IL17A/F, IL10, IL21, IL22, and RORγt, playing a role in the differentiation and function of the immune cells (Apetoh et al., 2010; Veldhoen et al., 2008). The AhR-activated gene-battery is involved in the detoxification of environmental contaminants and carcinogens, metabolism, oxidative stress responses, cell cycle regulation, proliferation, immune responses, and tumor suppression (Arnold et al., 2010; Miao et al., 2005; Tsuji et al., 2012; Vondracek et al., 2011; Yeager et al., 2009). AhR and ARNT are highly expressed in term placenta, but on the other hand, AhR repressor (AHRR) and inducible CYP1A1

are expressed in the placenta but at a lower degree when compared to the levels in other tissues (Pavek and Smutny, 2014). A representative list of AhR-activated placental genes and their functions is shown in Table 4**.**

Table 4. A representative list of the function of the AhR-regulated placental genes.

Target genes	**Gene function**	**References**
	Metabolism	
CYP1A1	Xenobiotic metabolism	Stejskalova and Pavek, 2011
CYP1B1	Metabolism of xenobiotics, steroid hormones, EVT proliferation, migration, and invasion	Li et al., 2017; Wu et al., 2019
COX	Mitochondrial respiratory chain	Vondracek et al.,2011; Kalpage et al., 2020
SLC2A1	Glucose transport across the placental barrier	Jansson and Powell, 2009; Sutter et al., 2019
	Development and growth	
CYP19A1	Estrogen biosynthesis	Chatuphonprasert et al., 2018
SERPINE1	Trophoblast invasion and migration	Kisanga et al., 2018; Brauze et al., 2019
WNT5A	Human trophoblast invasion	Chen et al., 2016; Wang et al., 2016
ADM	Implantation and vascular remodeling of spiral arteries	Portal-Nunẽz et al., 2012;
FOS	Trophoblast cell invasion.	Matson and Caron, 2014 Peng et al., 2015; Cirillo et al., 2019
	Protein synthesis, cell cycle and motility	
SERPINB2	Modulation of proteostasis	Lee et al., 2015; Brauze et al., 2019
GADD45A	DNA damage, growth suppression	Wang et al., 1999; Esakky et al., 2014
ACTA2	Cellular migration	Rico-Leo et al., 2013
	Immune response and placental development	
IL1β	Stromal cell differentiation and autoregulator of decidualization in humans, inflammatory response	Frank et al., 1995; Terao et al., 1998; Lahoti et al., 2014
S100A8	Role in embryo development and maternal deciduum vasculature, inflammatory response	Yoon et al., 2006; Baker et al., 2011; Schiopu and Cotoi, 2013
TGFβ1	Human trophoblast cell invasion and proliferation, immune response	

TNF *INFγ*	Trophoblast invasion, proinflammatory cytokine	Forbes et al., 2010; Zhou et al., 2014; Morikawa et al., 2016; Yi et al., 2018
IL22	Proliferation and migration of human placenta-derived mesenchymal stromal cells, endometrial vasculature remodelling, angiogenesis at implantation sites, maintenance of the decidua	Wen et al., 2018; Hou et al., 2019 Murphy et al., 2009; Hao and Whitelaw, 2013; Domínguez-Acosta et al., 2018
	Inflammatory response	Xue et al., 2016

ACTA2, Actin Alpha 2; ADM, Adrenomedullin; CYP1A1, Cytochrome P450 family 1 subfamily A member 1; CYP1B1, Cytochrome P450 family 1 subfamily B member 1; CYP19A1, Cytochrome P450 family 19 subfamily A member 1; COX, Cytochrome C Oxidase; FOS, Fos Proto-Oncogene; GADD45, Growth Arrest And DNA-Damage-Inducible 45; IL, Interleukin; INFγ, Interferon gamma; S100A8, S100 Calcium binding protein A8; SERPINB2, Serpin family B member 2; SERPINE1, Serpin family E member 1; SLC2A1, Solute carrier family 2 member 1; TGFβ1, Transforming growth factor beta 1; TNF, Tumor necrosis factor; WNT5A, Wnt family member 5A.

2.6.1.1 Aryl hydrocarbon receptor ligands

Synthetic ligands such as β-naphthoflavone (BNF), PAHs, halogenated hydrocarbons, and natural AhR agonists such as 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), indoxyl sulfate, and 6-formylindolo-(3,2-b)-carbazole (FICZ) have been shown to bind to inactive AhR that resides in the cytoplasm. After these synthetic or natural ligands bind to AhR, the complex translocates to the nucleus where it binds to the promoters of its target genes (for a review, see Tian et al., 2015).

PAHs are formed during the incomplete combustion of organic compounds such as tobacco, food, and wood. PAHs are both mutagenic and carcinogenic (Grova et al., 2006). PAHs have contaminated food because of the dispersal of airborne PAH-containing particles from polluted air or soil gaining access to the surfaces of plants (Directorate, 2002). Fish and mussels can also be exposed to PAHs from contaminated water (Lutz et al., 2006). PAH metabolites have been detected in the milk of exposed mothers during pregnancy and lactation (Grova et al., 2002), and they are suspected to be endocrine disruptors acting like hormones (Gozgit et al., 2004). Furthermore, PAHs were reported to interfere with fetal development and the maintenance of pregnancy (Drwal et al., 2019).

TCDD or 2, 3, 7, 8-tetrachlorodibenzo-para-dioxin, a halogenated aromatic hydrocarbon and a group 1 human carcinogen as classified by the International

Agency for Research on Cancer (IARC) in 1997, is the most potent inducer of CYP1A1 (Whitlock, 1989). TCDD exposure induces a plethora of biological responses, including immunotoxicity, liver damage, wasting syndrome, disruption of normal hormonal signalling pathways, impairments in development, fertilization, reproduction, endocrine, nervous, and immune systems (Giesy et al., 2002). Exposure to TCDD may also cause changes in the sex ratio and the compound has been implicated in tumor promotion and carcinogenesis (Mandal, 2005; Warner et al., 2011).

Although several endogenous AhR ligands such as ITE and indoxyl sulfate have been identified (Schroeder et al., 2010; Song et al., 2002), the regulation of the function of AhR by these ligands is poorly understood and hence should be investigated. Furthermore, there is a wide range of exogenous AhR activators, including omeprazole, cyprodinil, leflunomide, that are widely used in agriculture or as pharmaceuticals (Fang et al., 2013; Murray and Perdew, 2008; O'Donnell et al., 2010). Omeprazole, a proton pump inhibitor used by pregnant women in the treatment of acid reflux, was reported to cross the placenta and showed therapeutic potential against preeclampsia (Ching et al., 1986; Cluver et al., 2015). Cyprodinil, a fungicide used worldwide in agriculture, was shown to be an AhR activator, a potential endocrine and (extracellular signal-regulated kinase) ERK signalling pathway disrupter (Fang et al., 2013). Leflunomid, a pyrimidine synthesis inhibitor which is used in the treatment of rheumatoid arthritis, has been claimed to exert embryotoxic and teratogenic effects, mainly leading to growth retardation and craniofacial and skeletal malformations in animal studies (Levy et al., 2016).

2.6.1.2 β-Naphthoflavone, BNF

β-naphthoflavone (BNF), or 5,6-benzoflavone, is a synthetic derivative from naturally occurring flavonoid compounds (Vyas et al., 1983). BNF is a prototypical potent AhR agonist, and it is an inducer of some phase I enzymes such as CYP1A1/2 (Pretti et al., 2001) and phase II enzymes such as UGT, NQO1 and GST (Lněničková et al., 2018). However, AhR activation does not induce phase II enzymes in the placenta (Huuskonen et al., 2008) unlike the situation in the liver (Kessler and Ritter, 1997).

2.6.2 Peroxisome proliferator-activated receptors

PPARs (peroxisome proliferator-activated receptors) are ligand-activated NRs that belong to the steroid/thyroid/retinoid receptor super-family (Desvergne and Wahli, 1999). The three PPAR isoforms (PPARα/β/γ) form a heterodimer with 9-cis retinoid x receptor (RXR) that binds to their corresponding PPAR response elements (PPARE); this process occurs mainly in hepatocytes and adipocytes (Krey et al., 1993). PPARs are involved in several physiological processes e.g. inflammation, maintenance of homeostasis, transport, uptake, intracellular binding, activation and catabolism of FAs (Ahmadian et al., 2013) (Figure 7). There are three PPAR isotypes: PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3) (Chen et al.,1993; Zhu et al., 1993).

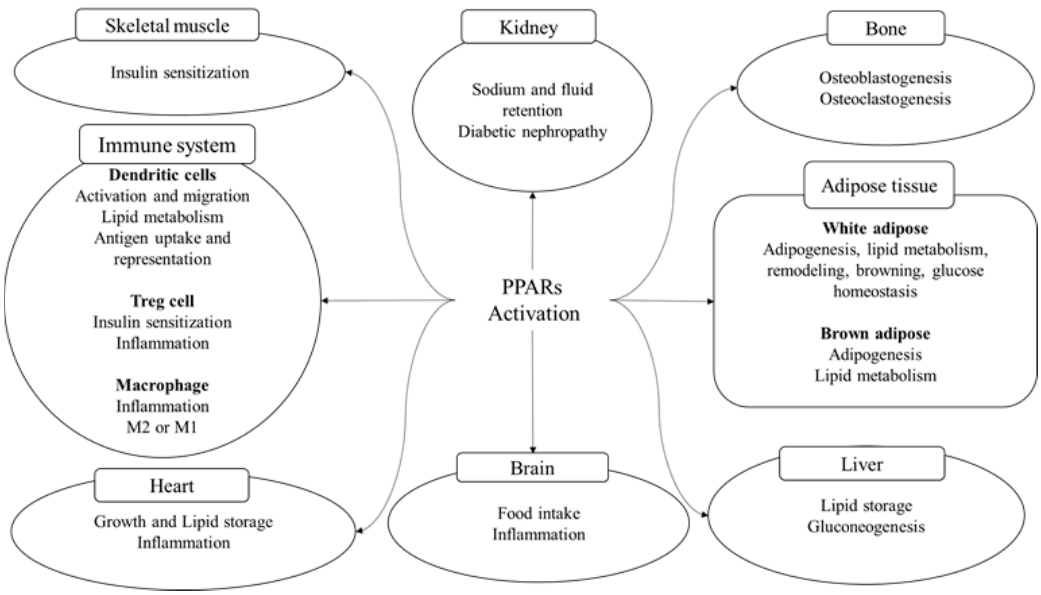

Figure 7. Pleiotropic functions of ligand-activated peroxisome proliferator-activated receptors (PPARs) in different organs and tissues, adapted from (Ahmadian et al., 2013).

Saturated and unsaturated FAs and eicosanoids bind to PPARα/β/γ, with PPARα having the highest affinity (Desvergne and Wahli, 1999). PPARβ/δ modulates lipid and carbohydrate metabolism, cell proliferation and differentiation, as well as inflammatory responses (Yao et al., 2017). The human PPARG gene (*hPPARG*) is located on chromosome number 3 at a position close to the *retinoic acid receptor* (*RARB*) and *thyroid hormone receptor* (*TR*) (Dreyer et al., 1992; Zhu et al., 1995). The

human *PPARG* gene exists in two isoforms (two mRNA splicing variants), hPPARG1 and hPPARG2 (Mukherjee et al., 1997). *PPARG1* is expressed in nearly all cells, whereas *PPARG2* is expressed mainly in adipose tissue (Mukherjee et al., 1997). *PPARG* is abundant in white and brown adipose tissue where it plays a key role in the regulation of adipogenesis, although low levels PPARG mRNA are expressed in skeletal muscles, liver, and heart (Janani and Kumari, 2015). There are several co-factors such as steroid receptor coactivator 1 (SRC-1), CREB binding protein (CBP), E1A-associated factor (P300), PPAR-G coactivator (PGC); PPAR-binding protein (PBP), thyroid receptor-associated protein 220 (TRAP220), vitamin D receptor interacting protein 230 (DRIP230), receptor interaction protein 140 (RIP140), AR-associated protein 70 (ARA70), which play a role in co-activating PPARG. There are also other PPARG interacting molecules that are co-repressor proteins such as silencing mediator for retinoid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (N-CoR) which mediate a repressive signal by interacting with the non-liganded PPARG (for a review see, Reddy et al., 2010).

PPARG is highly expressed in the placenta where it is expressed at a ten-fold higher level than that present in liver (Storvik et al., 2014). Target genes of PPARγ and their functions in the placenta are shown in Table 5. These genes such as *Acyl-CoA synthase and oxidase*, *FA binding protein* (*FAB*), *FA transport protein* (*FATP*) and *FA translocase* (*CD36*) are involved in glucose transport, and activation, uptake, and the transport of FA in blood and across the cell membrane (Grygiel-Górniak, 2014). Other PPARγ target genes such as *activator protein 1*, *NFκB*, *tumor necrosis factor alpha* (*TNFA*), *interleukin 1 beta* (*IL1B*) and *interferon gamma* (*INFG*) play vital roles in trophoblast invasion and placental development (Antonelli et al., 2010; Lee et al., 2019; Xue et al., 2016; Zheng et al., 2018).

Table 5. A representative selection of PPARγ-regulated placental genes and their function.

Target genes	Gene function	References
Fatty acid transport and binding		
Lipoprotein lipase	Fatty acid release	Schoonjans et al., 1996;
Fatty acid transport protein	Fatty acid transport	Huter et al., 1997
Fatty acid transporter	Fatty acid transport across cell membrane	Martin et al., 1997; Lager et al., 2016
Fatty acid binding protein 4	Intracellular fatty acid binding	Motojima et al., 1998; Lager et al., 2016
		Tontonoz et al., 1994; Li et al., 2017
Fatty acid metabolism		
Acyl-CoA synthase	Fatty acid activation	Schoonjans et al., 1995; Fisher et al., 2019
Glucose transport		
Glucose transporter type 4	Intracellular glucose transport	Wu et al., 1998; Stanirowski et al., 2017
Inflammatory response		
Hemioxygenase 1	Antioxidant and anti-inflammatory functions	Kaartokallio et al., 2014; Cho et al., 2018
Trophoblast invasion and placental development		
Activator protein 1	Trophoblast invasion	Jiang et al.,1998; Zheng et al., 2018
Signal transducer and activator of transcription	Trophoblast invasion and syncytialization	Jiang et al., 1998; Gupta et al., 2016
Nuclear factor κB	Trophoblast invasion	Jiang et al.,1998; Xue et al., 2020
Tumor necrosis factor α	Trophoblast invasion, negative regulators of trophoblast growth	Yui et al., 1994; Jiang et al.,1998; Lee et al., 2019
	Trophoblast invasion	Jiang et al.,1998; Lee et al., 2019
Interleukin 1β	Trophoblast invasion, negative regulators of trophoblast growth	Yui et al., 1994; Antonelli et al., 2010; Lee et al., 2019
Interferon γ	Trophoblast invasion	
	Trophoblast invasion	
Interleukin 6	Trophoblast invasion, motility	

Human chorionic gonadotropin	Maintaining normal pregnancy by promoting the trophoblasts expression of Bcl-xL	Jiang et al.,1998; Sharma et al., 2016
Vascular endothelial growth factor	Endometrial tissue remodelling and trophoblast invasion	Handschuh et al., 2009 Lash et al., 2003; Terrasi et al., 2013
Chemokine (C-X-C Motif) ligand 16	Endometrial tissue remodelling Trophoblast transendothelial migration	Postea et al., 2008; Fan et al., 2019 Chen and Khalil, 2017;
Matrix metalloproteinase 9	Trophoblast invasion	Gao et al., 2017
Matrix metalloproteinase 7	Trophoblast invasion	Shen et al., 2007; Chen and Khalil, 2017
Mucin 1		Thirkill et al., 2007
Cyclooxygenase 2		Subbaramaiah et al., 2001; Yi et al., 2018
Transforming growth factor β		Caniggia et al., 1999; Poleni et al., 2007

2.6.2.1 PPARγ ligands

Natural and synthetic PPARγ ligands are utilized as therapeutic interventions in glucose-linked disorders. PPARγ is activated by natural ligands such as unsaturated fatty acids (linolenic, eicosatetraenoic, docohexaenoic, arachidonic, palmitoleic, oleic, nervonic) saturated fatty acids (capric, lauric, myristic, palmitic, stearic, arachidic, and behenic), eicosanoids, dicarboxylic fatty acids, 15-deoxy prostaglandin (PG) J2, and PG J2. FAs are the precursors of the eicosanoids and are a critical component of the membrane lipids that maintain cellular and organelle integrity as well as being important intracellular mediators of gene expression. Fatty acids are also essential for fetal growth and development, especially for the fetal brain and the retina (Duttaroy, 2009). The human placenta is the major source of PGs during pregnancy (Mitchell et al., 1982). During pregnancy, PGs are converted to essential PPARγ ligands and they play a key role in fetal organ development, fetal hypothalamic–pituitary–adrenal axis stimulation (Duttaroy, 2009). PGs also play a role in parturition including the maintenance of the blood flow in the uterus and the placenta, activation of myometrial contractility and cervical ripening, and inhibition of fetal breathing and movement at the time of birth (Challis, 1998; Challis et al., 2002).

On the other hand, PPARγ is also activated by synthetic ligands such as farglitazar, S26948, INT131 and thiazolidinediones including troglitazone,

rosiglitazone, ciglitazone and pioglitazone (for reviews, see Desvergne and Wahli, 1999; Grygiel-Górniak, 2014; Janani and Kumari, 2015). Pioglitazone is still on the market, whereas troglitazone, and rosiglitazone were withdrawn. Ciglitazone is used as a prototypical PPARγ agonist and was reported to inhibit plasmin-induced proinflammatory human peripheral monocyte activation via the modulation of p38 MAP kinase activity (Syrovets et al., 2002). Ciglitazone also inhibited cigarette smoke induced inflammatory responses in human middle ear epithelial cells (Jun et al., 2012). Troglitazone is a drug used to treat type 2 diabetes (an anti-hyperglycemic compound) which acts primarily by decreasing insulin resistance (Plosker and Faulds, 1999). Troglitazone was reported to attenuate hypoxia-induced injury in cultured term human trophoblasts (Elchalal et al., 2004). Troglitazone also significantly reduced the release of lipopolysaccharide stimulated IL6, IL8, and TNFA in decidual and myometrial macrophages in C57BL/6J mice (Lappas et al., 2002a). Maternal exposure to rosiglitazone decreased the feto-placental weight and the thickness of the trophoblast layer and labyrinthine vasculature (Duttaroy, 2009). In vitro exposure to rosiglitazone prevented a placental injury by blocking NFκB mediated inflammation (Kadam et al., 2019; Xu et al., 2016). Furthermore, rosiglitazone has been reported to augment the antioxidant response and to promote the survival of human trophoblasts (HR et al. 2019) and in C57BL/6J mice it was able to prevent preterm birth by reducing the level of inflammation and upregulating antioxidative response (HR et al., 2019; Kadam et al., 2017).

2.6.2.2 Pioglitazone

Pioglitazone is an antidiabetic compound that can activate the intracellular PPARγ, while decreasing the metabolic and vascular aspects of insulin resistance (Kliewer et al., 1992; Smith, 2001). Pioglitazone alters the transcription of several of the genes influencing carbohydrate and lipid metabolism, resulting in altered amounts of protein synthesis i.e. it evokes extensive metabolic changes (Smith, 2001). Pioglitazone increases glycemic control in type 2 diabetics by increasing insulin sensitivity through its action on PPARγ1 and PPARγ2, and it also exerts effects on lipid metabolism via its effects on PPARα (Hanefeld, 2001; Smith, 2001). The results of these interactions include an increase in the numbers of glucose transporters 1 and 4, a reduction in the levels of free fatty acids, enhanced insulin signalling, a reduced TNFA gene expression and finally a remodelling of adipose tissue (Waugh et al., 2006). Together, these can increase glucose uptake and utilization in the

peripheral organs and decrease gluconeogenesis in the liver, thereby reducing insulin resistance (Desvergne and Wahli, 1999; Smith, 2001) (Figure 8).

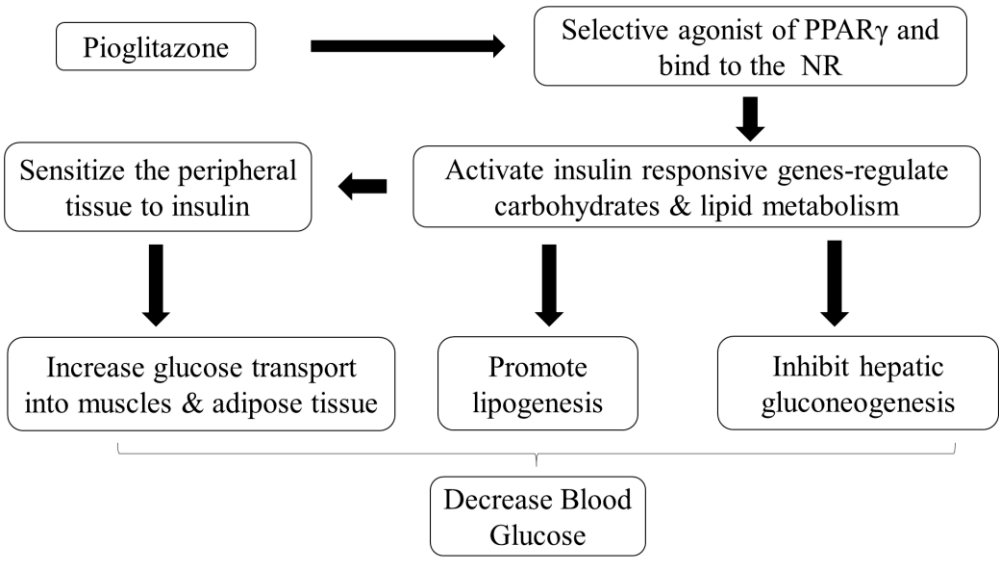

Figure 8. Pioglitazone improves glycemic control in type 2 diabetics by increasing insulin sensitivity through its action at PPARγ. NR, Nuclear receptor.

2.7 EFFECTS OF CHEMICAL EXPOSURE DURING PREGNANCY

The exposure to environmental chemicals can cause alterations to fetal programming and increase the risk of diabetes, cardiovascular disease, neurodevelopment and cancers later in adulthood (Cooke, 2014; Govarts et al., 2016). Several investigators have described the drastic effects of various environmental contaminents such as dioxins, polychlorinated biphenyls (PCB), organophosphates, BPA and methyl mercury on fetal development and growth (Grandjean et al., 2001; Lv et al., 2019; Nghiem et al., 2019). Nghiem et al. (2019) reported that human prenatal exposure to dioxin affected neuronal activity and the functional connectivity between different brain regions. Furthermore, prenatal exposure to PCB is associated with neurobehavioral effects in humans (Gladen and Rogan, 1991; Grandjean et al., 2001). PCBs are commonly used as plasticizers and flame retardants in consumer products and have been shown to cause adverse developmental effects in children who had been prenatally exposed to organophosphates (Doherty et al., 2019) Furthermore, prenatal exposure to methyl mercury was shown to cause dramatic effects on the development of the pancreas (Schumacher and Abbott, 2017). Mycotoxins contaminate 25% of the

global cereal harvest according to the Food and Agricultural Organization (FAO) (WHO, 1999). Mycotoxins, especially AFB1, pose a great risk to human health; AFB1 is a major risk component in the products imported to the European Union (European Commission, 2006). AFB1 is carcinogenic, with the liver as the major target organ but AFB1 can pass by the liver and be metabolised by the placenta and it has been detected in the fetal circulation (Partanen et al., 2010). Maternal exposure to AFB1 may impair fetal growth via potential maternal systemic inflammation, impaired placental growth, or an elevation of placental cytokines (For a review, see Smith et al., 2017).

2.7.1 Mycotoxins

Mycotoxins are secondary fungal metabolites that are toxic to animals including humans (Rodrigues et al., 2011). Mycotoxins (e.g. aflatoxin B1, ochratoxin, fusarium and sterigmatocystin) are produced by three major genera of fungi, Aspergillus, Penicillium and Fusarium (for a review, see Pleadin et al., 2019). Mycotoxins have caused various outbreaks with thousands of cases reported of premature mortality e.g. due to cancer, liver and kidney diseases (Paterson and Lima, 2010). The aflatoxin outbreaks in several countries such as India and Kenya, have been associated with elevated mortality rates and acute hepatites (Lewis et al., 2005; Paterson and Lima, 2010). On the other hand, many cases of nephropathy were reported in regions of the Balkan peninsula and North African countries due to an ochratoxin A endemic (Malir et al., 2016). There were gastroenteritis cases in China due to the ingestion of wheat contaminated with deoxynivalenol (Paterson and Lima, 2010). Several epidemiological studies have revealed that zearalenone contamination can cause significant effects on human health including premature puberty in preteen children, human cervical cancer and premature thelarche. Zearalenone was also reported to interfere with conception, ovulation, implantation, fetal development, and the viability of newborn animals (Paterson and Lima, 2010).

Mycotoxins pose a significant risk not only to human health, but also to the feto-placental unit. Maternal exposure to mycotoxins during pregnancy may have detrimental effects on pregnancy outcomes including an increased risk of preterm birth and late-term miscarriage (for a review, see Kyei et al., 2020). For instance, maternal exposure to aflatoxin impaired intrauterine fetal growth, promoted neonatal jaundice, and increased the risk of perinatal death and preterm birth. On the other hand, maternal exposure to fusarium mycotoxins was associated with

hypertensive emergencies in pregnancy and with neural tube defects (Kyei et al., 2020). Deoxynivalenol, T-2 toxin, and zearalenone, that are naturally occurring by-products of fusarium mycotoxins, were shown to alter the expression and structure of junctional proteins and to be able to induce IL6 release in a human placental BeWo cell line (Toutounchi et al., 2019). IL6 was reported to regulate hCG release from human trophoblasts (Nishino et al., 1990).

Many of the mycotoxins or their metabolites have been shown to penetrate through the placenta. For example, the AFB1 metabolite; aflatoxicol, was reported to cross the placenta after being formed by placental enzymes (Partanen et al., 2010). Ochratoxin was demonstrated to have the ability to cross the placenta in rodents and to be present in the milk of female rabbits (Galtier, 1991). There is no data on whether sterigmatocystin can pass through the placenta, however, sterigmatocystin can be a precursor of AFB1 or it can be a metabolic end product produced by Aspergilli (Kobayashi et al., 2018) and therefore it can be considered as a potential threat to cross the placental barrier.

2.7.1.1 Aflatoxin B1

Aflatoxins are secondary metabolites produced by Aspergillus Flavus and A. parasiticus and at least sixteen different enzmes are involved in their synthesis (Yu et al., 1995). These fungi grow in the soil and can contaminate crops such as cereals, soybeans, rice, maize, and nuts (Alshannaq and Yu, 2017) and their metabolites can contaminate animal products such as eggs, meat, and milk (Pier, 1992). The ideal growth of these mycotoxins occurs in hot and humid areas such as Sub-Saharan Africa e.g. Kenya and in many densely populated South Asian countries such as India (Rushing and Selim, 2019) (Figure 9).

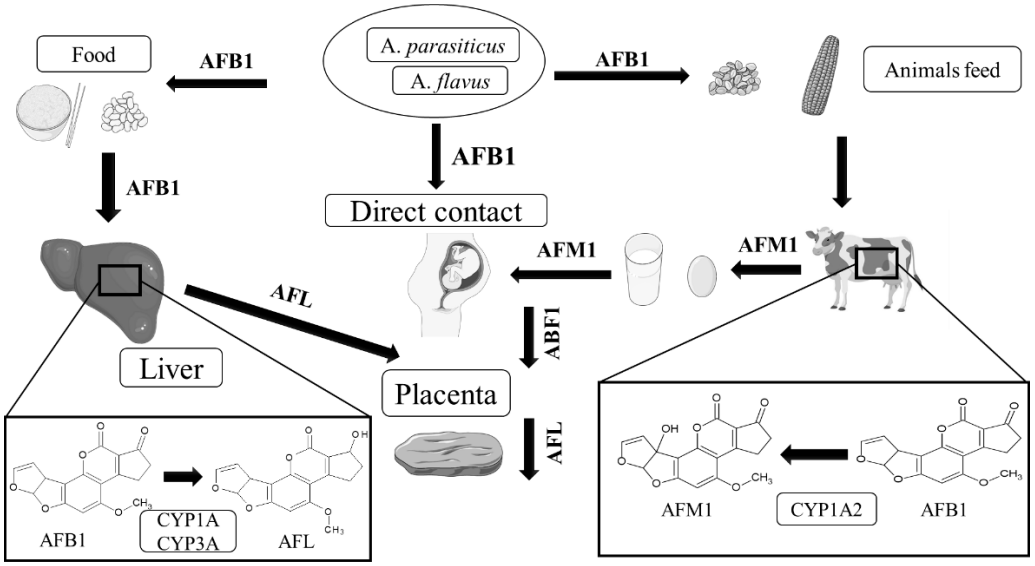

Figure 9. Aflatoxin B1 (AFB1) contamination sources that reaches the placenta either after direct human contact to A. parasiticus or A. flavus, ingesting food (beans or corn or others) contaminated with AFB1 or eating animal products that contain AFB1 or its metabolites. AFB1, Aflatoxin B1; AFL, Aflatoxicol; AFM1, Aflatoxin M1; CYP1A, Cytochrome P450 family 1 subfamily A; CYP3A, Cytochrome P450 family 3 subfamily A.

Aflatoxins, especially AFB1, are known to cause acute and chronic toxicity, with the liver being their major target organ (Williams et al., 2004); these toxins possess carcinogenic (Ostry et al., 2017), teratogenic (Gong et al., 2004; Smith et al., 2017), immunosuppressive properties (Williams et al., 2004) and potentially also endocrine disruptive effects (Storvik et al., 2011). These toxins can be transferred and metabolized through the placenta and can be detected in breast milk, neonatal cord blood and serum of pregnant women.

AFB1 is mainly metabolized in the liver, especially by CYP enzymes, into the carcinogenic epoxide, aflatoxin B1-8,9-epoxide (AFBO), and also into much less potent hydroxylated metabolites such as aflatoxin Q1 (AFBQ1), aflatoxicol (AFL), and aflatoxin M1 (AFM1) (Figure 10). AFBO is formed by CYP3A4 and CYP1A2 and the carcinogenicity of AFBO is attributable to the fact that AFBO is highly electrophilic and thus is capable of forming DNA and protein adducts (Rushing and Selim, 2019; Ueng et al., 1995). AFQ1 is also produced by CYP3A4 and it can be detected in human feces and urine of exposed individuals (Gallagher et al., 1996; Mykkänen et al., 2005). AFM1 is a AFB1-hydroxylated metabolite produced by CYP1A2; it has been identified in the urine of AFB1-exposed animals and humans

(Langouët et al., 1995; Mykkänen et al., 2005). AFM1 was also detected in the milk of humans and animals and was shown to be carcinogenic as it could form DNA adducts (Giovati et al., 2015; Hsieh and Hsieh, 1987). AFL, on the other hand, is produced by NADPH reductase and can be converted back to AFB1 by the same enzyme. Even though AFL is found in the urine and breast milk, AFL also can pass through the placental barrier making it the sole AFB1-metabolite that can cause a significant effect on the feto-placental unit (Partanen et al., 2010).

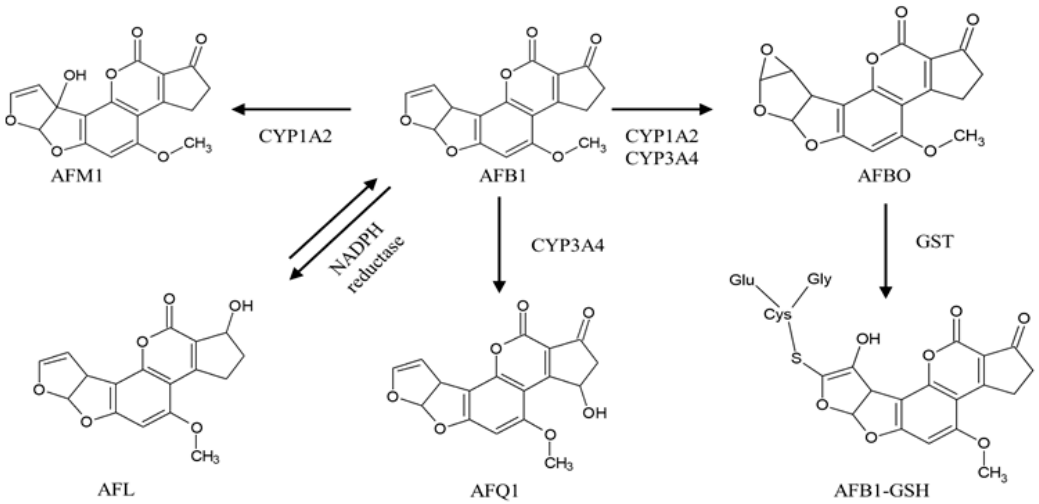

Figure 10. Aflatoxin B1 metabolic pathways and the enzymes that are involved in the process. AFB1, Aflatoxin B1; AFB1-GSH, AFB1-glutathione conjugate; AFBO, Aflatoxin B1-8,9-epoxide; AFL, Aflatoxicol; AFM1, Aflatoxin M1; AFQ1, Aflatoxin Q1; CYP1A2, Cytochrome P450 Family 1 Subfamily A Member 2; CYP1A2, Cytochrome P450 Family 3 Subfamily A Member 4; GST, Glutathione S-transferase.

3 AIMS OF THE STUDY

The main hypothesis of this study is that chemicals and environmental contaminants can affect placental gene expression and thus may change the hormonal and physiological functions of the human placenta.

More specifically, the aims of this study are:

1. To determine PPARγ regulated gene networks using pioglitazone-treated human primary trophoblasts.
2. To characterize AhR target genes using BNF-treated human primary trophoblasts.
3. To identify transcriptomic responses after AFB1 exposure which will be characterized in placental primary trophoblast cells.

4 SUBJECTS AND METHODS

The original publications are referred here by the Roman numerals (I-III).

4.1 PLACENTAL SAMPLES (I-III)

In total, twelve placentas were obtained for the three studies from Kuopio University Hospital after a cesarean section. The studies were approved by the Research Ethics Committee, Hospital District of Central Finland (dated 15.11.2011) and are part of the studies being carried out by the Kuopio Birth Cohort consortium (www.KuBiCo.fi). Informed written consent was obtained from all individual participants and experiments were conducted according to the principles of the Declaration of Helsinki. The full-term placentas (Figure 11) were from uncomplicated pregnancies. All the mothers were non-smokers. Due to confidentiality reasons, not all clinical data could be accessed. There are relatively few deliveries in the hospital, and information on ethnicity/chronic diseases of patients or newborns' weight could allow the identification of the mother/child.

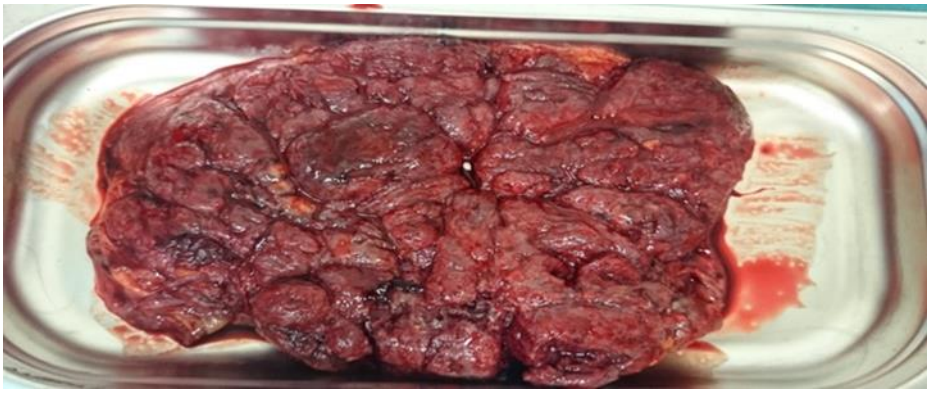

Figure 11. Chorionic plate (maternal side) of human placenta.

4.2 ISOLATION AND CELL CULTURE OF PRIMARY HUMAN TROPHOBLAST CELLS (I-III)

The isolation of primary trophoblasts was started within 30 minutes after delivery and conducted according to Petroff et al. (2006), with minor modifications. Cotyledons (3-4) were detached from the uteral side of the placenta. Then it was

dissected and tough membranes, blood clots and viers were removed. The soft villous materials were rinsed with NaCl solution and further dissected, and the tissue homogenate was incubated using digestion enzymes: Penicillin/Streptomycin/Amphotericin B (Cellgro), foetal bovine serum (Gibco), L-glutamine (Cellgro), trypsin (Gibco) and deoxyribonuclease 1 from bovine pancreas (DNase I, Sigma Aldrich) in Iscove's Modified Dulbecco's Medium (IMDM) (Lonza) cell culture medium. After repeating the digestion process three times, cytotrophoblast cells were separated with isotonic Percoll (Sigma Aldrich) gradients and transferred to 6-well plates (Nunc) in a Cell-IQ incubator (Chip-Man Technologies Ltd) at +37 °C and cultured for 72 hours in an IMDM cell culture medium. Typically the cell yield was 1.25 x 10^6 cells per gram of placental tissue. The purity of the isolated CT cells was monitored by flow cytometry and it was typically 96% (study I, Supplemental Figure 1).

On day four, placental cells were treated with 20 μM pioglitazone, 25 μM BNF or 1 μM AFB1 (all from Sigma-Aldrich) (six duplicates) and kept in Cell-IQ incubator (Chip-Man Technologies Ltd.) at +37 °C for 72 hours. The compounds were diluted with dimethyl sulfoxide (DMSO) (Sigma Aldrich) and the (DMSO) was used as negative control. The total cell, cytotrophoblast and syncytiotrophoblast counts were monitored throughout the cell culture using the Cell IQ imaging system (Figure 12).

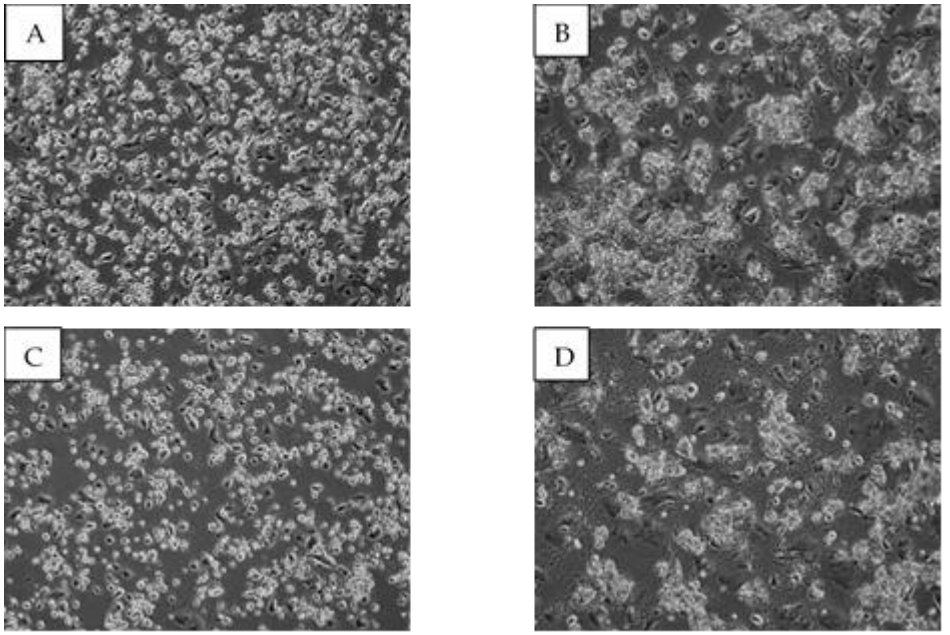

Figure 12. Representative figures of cultured primary trophoblast cells. Trophoblasts were treated with 5 μM β-naphthoflavone (BNF) or 0.25% (dimethyl sulfoxide) DMSO as a control for 72 hours. (A) DMSO, 0 h) B) DMSO (72 h) C) BNF (0 h) D) BNF (72 h).

4.3 TOTAL RNA EXTRACTION (I-III)

In studies I and II, total RNA was extracted with a GenElute Kit (Sigma Aldrich) and in study III with a Qiagen All Prep mini kit (Qiagen Ltd.) followed by DNase I treatment (Ambion Turbo DNA-Free Kit, Ambion/Life Technologies). The quality and integrity of the isolated RNA was monitored by Agilent RNA 6000 Nano assays (Agilent Technologies) according to the manufacturer's instructions. All the RNA Integrity Number (RIN) values were above 9.0, indicating high-quality RNA with little degradation.

4.4 GENE EXPRESSION ARRAY (I-III)

First, RNA samples for gene expression arrays were labelled using the IlluminaR Total PrepT RNA Amplification Kit (Life Technologies) according to the manufacturer's instructions, with 350 ng of RNA per sample. The quality of all cRNA samples was controlled on 2100 Bioanalyzer RNA nano chips (Agilent) and quantified using a Nanodrop 2000 spectrometer (Thermo Scientific). A total of 750 ng of each sample was used in the Direct Hybridization Assay Workflow (Illumina)

using HumanHT12_V4 (I-II) or HumanHT12 (III) expression beadchips (Illumina). The bead-arrays were scanned using a HiScan instrument (Illumina). The work was carried out in the Core Facility of the Estonian Genome Center, University of Tartu.

Probe intensity and detection data were obtained using Illumina BeadStudio™ software, and further processed with GeneSpring™ GX 12.6 software (Agilent Technologies) (I-II) or using Chipster software (CSC, Finland) (III). In studies I-II, the raw expression data was quality assessed, log2-transformed, and subjected to background adjustment and normalization. Genes were defined as differentially expressed if the fold-change was at least ±2-fold for pioglitazone and ±1.5-fold for BNF-treated primary trophoblasts as compared to respective controls and if it was also statistically significant ($p < 0.05$, One-way ANOVA and Tukey's HSD post-hoc test followed by the Benjamini and Hochberg false discovery rate). In study III, the differentially expressed genes were selected when the difference in the normalized expression was statistically significant ($p < 0.05$, Student's t-test with adjustment by the Benjamini-Hochberg multiple testing correction) and the fold-change between the AFB1 treated group was at least 1.5-fold as compared to the respective controls. The complete data sets will be available at the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus (GEO) database and gene expression profiling data comply with the Minimum Information About a Microarray Experiment (MIAME) standard. The data can be obtained from the GEO database with the accession number GSE103993 (I-II) and GSE153590 (III).

4.5 QUANTITATIVE REAL-TIME (QRT)-PCR (I-III)

Complementary DNA (cDNA) was synthesized from single-stranded RNA via reverse transcription with the first-strand cDNA synthesis technique (M-MuLV reverse transcriptase, Fermentas) then stored at -80°C. RT-q-PCR was performed using Taqman Real Time PCR assays (Thermo Fisher Scientific), using 2 ug of total RNA in each synthesis (Huuskonen et al., 2008). The assay was performed using an ABI prism 7500 instrument (Applied Biosystems, Foster City, CA) and the detection was performed using genes with FAM (carboxyfluorescein)-labeled TaqMan probes. The complete list of TaqMan PCR assays used are shown in Table 6. From each sample, duplicate technical replicates were analysed, and the gene expression was normalized with β-actin measured from the same samples using the ΔΔCT method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

Table 6. Targets of qRT-PCR assays.

Gene	Symbol	TaqMan Assay
Beta-actin	BACT	4316315E
C-C motif chemokine ligand 3	CCL3	Hs00234142_m1
CD36 molecule	CD36	Hs00169627_m1
C-X-C motif chemokine ligand 8	CXCL8	Hs00174103_m1
Cytochrome P450 family 1 subfamily A member 1	CYP1A1	Hs00153120_m1
Cytochrome P450 family 11 subfamily A member 1	CYP11A1	Hs00194153_m1
Cytochrome P450 family 19 subfamily A member 1	CYP19A1	Hs00240671_m1
Fatty acid binding protein	FABP	Hs01086177_m1
Growth differentiation factor 15	GDF15	Hs00171132_m1
Hemioxygenase 1	HMOX1	Hs01110250_m1
Hydroxysteroid 11 beta dehydrogenase 2	HSD11B2	Hs00388669_m1
Hydroxysteroid 17 beta dehydrogenase 1	HSD17B1	Hs01561219_m1
Interleukin 1beta	IL1B	Hs00174097_m1
Leptin	LEP	Hs00174877_m1
Lipoprotein lipase	LPL	Hs00173425_m1
Matrix metallopeptidase 7	MMP7	Hs01042796_m1
Matrix metallopeptidase 9	MMP9	Hs00234579_m1

Microsomal glutathione S-transferase-1	MGST1	Hs00220393_m1
S100A8	S100A8	Hs00374264_g1
Wnt family member 5A	WNT5A	Hs00998537_m1

4.6 INGENUITY PATHWAY ANALYSIS (I-III)

The statistically differentially expressed genes were mapped into functional networks, upstream regulators, and diseases and functions with Ingenuity Pathway Analysis (IPA) software (Qiagen, USA) as previously described (Rysä et al., 2018). Shortly, the list of differentially expressed genes and the values of their fold change were uploaded into IPA. Then a core analysis was performed using the following parameters: only the set of diferentially expressed genes by GeneSpring-sofware mapped to the IPA database, direct and indirect relationships included, endogenous chemicals included, and confidence=experimentally observed. This software generates networks and canonical pathways based on the connectivity and the biological relationships among those differentially expressed genes. The IPA-analysis calculates whether there is an over-representation of significantly up- or down-regulated genes and a particular functional annotation compared to what could be expected by chance alone as measured with a Fischer's exact test ($p < 0.05$). In addition, IPA generates an activation "Z-score" for each category. A positive "Z-score" ≥2 is given if the gene changes have led to a statistically significant increase of biological function or disease. Vice versa, a negative "Z-score" ≤2 is given if the gene changes have led to a statistically significant decrease of biological function or disease. An illustrative summary of the gene expression profiling method used in this thesis is shown in Figure 13.

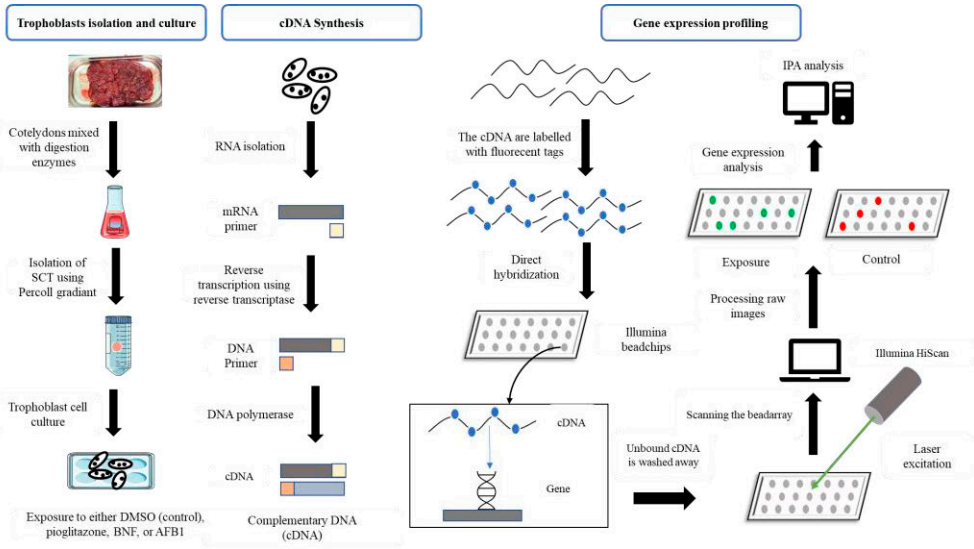

Figure 13. Schematic representation of a gene expression profiling experiment.

4.7 ETHOXYRESORUFIN-O-DEETHYLASE (EROD) ASSAY (I-II)

The catalytic activity of CYP1A1 of term trophoblasts was assessed by using the ethoxy-O-deethylase (EROD) assay according to Burke et al. (1985). The isolated human primary trophoblasts were incubated with 5 μM BNF for 24 h in the follwing set-up: ($3.5x10^6$ cells/well) were plated in 6-well plates and incubated in triplicates). Exposure was stopped by changing the BNF medium to a fresh one containing 0.2 μM of 7-ethoxyresorufin (the substrate of EROD assay) or DMSO-vehicle for controls. Fluorescent detector (Shimadzu RF5000, Kyoto, Japan) was hourly used to moniter the EROD-activity using EX530 EM585 wavelengths to point out time-dependent CYP1A1 activity, reported as pmol/min*10^6 cells. A standard curve (1-1000 nM) was used to normalize the 7-OH-resorufin concentration after the subtraction of the background fluorescence.

4.8 CELL VIABILITY (II-III)

The cytotoxicity of BNF and AFB1 was assessed with 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (Sigma-Aldrich) as previously described by Storvik et al. (2011). Human primary trophoblasts (5 x 10^5 cells/well) were incubated with 0.25 -100 μM of BNF (study I) or 0.01- 3 μM of AFB1 (study II) for 24 h at 37°C and then the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent (Sigma-Aldrich) and was added for 4 h. After

the incubation, sodium dodecyl sulfate (SDS) (Sigma-Aldrich) – N,N-Dimethylformamide (DMF) (Sigma-Aldrich) was added and the plate was maintained overnight at 37 °C. The optical density was measured using a BioTek ELx800 reader (BioTek) at a wavelength of 570 nm. The results were expressed as a percentage of the control cells exposed to 0.25% DMSO to the same concentration used as the solvent for BNF and AFB1.

4.9 STATISTICAL ANALYSIS (I-III)

In the MTT test, the significance of the differences between exposures and respective controls was analyzed by one-way ANOVA followed by Tukey's multiple comparison post-hoc test. In functional assays and RT-qPCR, the statistical difference between two groups was analyzed by Student's t-test. All the statistical analyses were done by using GraphPad Prism software. A value of $p < 0.05$ was considered statistically significant.

5 RESULTS

The most relevant findings from the original articles are presented and discussed here. Tables and figures in original publications are referred to with the Roman numerals I-III with the corresponding numbers as in the original articles.

5.1 CELL VIABILITY AND FUNCTIONALITY OF THE CELLS

First, the purity of isolated human primary trophoblasts was studied by flow cytometry; the purity of the isolated CTs was 96%. Next, in order to investigate the sensitivity of human term trophoblasts to BNF and AFB1, the cell viability of primary trophoblasts was measured with the MTT-assay after they were treated with increasing doses of BNF (0.25-100 μM) (study II) and AFB1 (0.01-3 μM) (study III) for 24 h (Figure 14). None of the tested concentrations of BNF had any effects on cell viability, whereas 3 μM AFB1 decreased the cell viability significantly (87% as compared to controls; $p < 0.05$).

Then the functionality of the cells in response to pioglitazone and BNF treatments was studied by measuring CYP1A1 mRNA levels and evaluating EROD activity. Quantitative RT-PCR results revealed that BNF treatment increased CYP1A1 mRNA levels (130-fold, $p < 0.05$) as compared to controls. In addition, the EROD activity was measured in primary trophoblasts that had been treated with 5 μM BNF (24 h) and compared with 0.25% DMSO-treated control cells (study I-II). BNF exposure evoked a significant increase in the EROD activity ($p < 0.001$) in comparison to the very low EROD activity of the control cells.

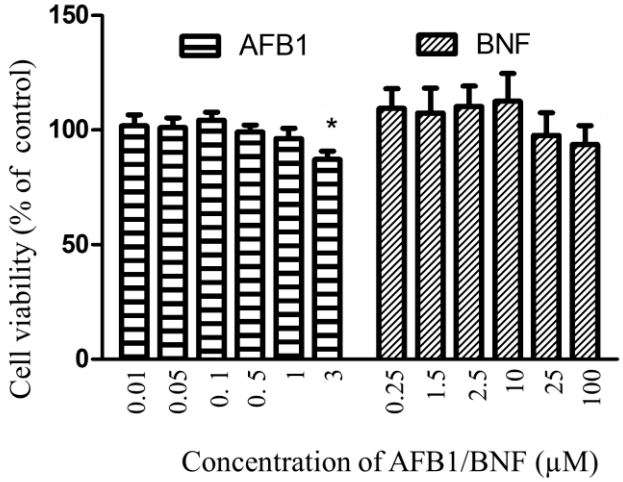

Figure 14. Effect of aflatoxin B1 (AFB1) and β-naphthoflavone (BNF) on the viability of primary trophoblasts. The MTT-assay was used to assess the cell viability in AFB1- and BNF-treated primary human trophoblasts (24 h), expressed as a percentage of control cells with 0.25% dimethyl sulfoxide (DMSO). The columns represent the mean ± SD of relative values from four individual experiments. *p < 0.05 one-way ANOVA followed by Tukey's multiple comparison post-hoc test.

5.2 GENE EXPRESSION ANALYSIS

Gene expression profiling was utilised to characterize the gene regulatory effects of pioglitazone, BNF and AFB1 in human primary trophoblas and was performed on Illumina expression beadchips. Pioglitazone dysregulated 69 genes (32 up- and 37 downregulated ± 2-fold, $p < 0.05$) in primary trophoblasts when compared to control cells. However, when a threshold of a 1.5-fold difference in gene expression levels was selected between pioglitazone treated trophoblasts and control cells, then 284 differentially expressed genes were observed to be dysregulated (135 upregulated and 149 downregulated, $p < 0.05$). BNF, on the other hand, caused a dysregulation of 321 genes (64 up-, and 257 down-regulated ±1.5-fold, $p < 0.05$) when compared to controls in human primary trophoblasts. AFB1 treatment of placental trophoblasts led the dysregulation of 170 genes (46 up- and 124 downregulated, ±1.5-fold, $p < 0.05$) when compared to controls. Figure 15 shows the number of genes altered in primary trophoblasts in response to either pioglitazone, BNF, or AFB1. The confirmatory RT-qPCR-analyses (studies I-II) of selected differentially expressed genes were in line with the results of gene expression profiling (study I, Table 1; study II, Supplementary Table 1).

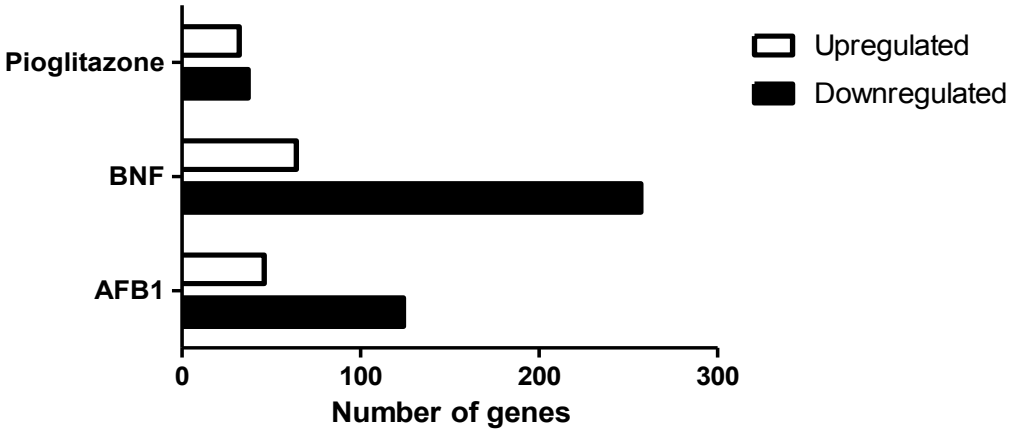

Figure 15. Number of genes altered in primary trophoblast in response to either pioglitazone, β-naphthoflavone (BNF), or aflatoxin B1 (AFB1).

A Venn diagram was produced to describe the overlap of significantly up- or down-regulated genes in response to pioglitazone, BNF, and AFB1 in primary trophoblasts (Figure 16). The overlapping six genes among the three studies were chorionic gonadotropin beta 1/2 (CGB1/CGB2), CGB3/5/8, CRH, CSHL1, GH1, and pyruvate dehydrogenase (PDK4). These genes are involved in pregnancy via the maintenance and regulation of placental and fetal growth. BNF did display some similarities with pioglitazone and AFB1 driven gene responses whereas pioglitazone- and AFB1 induced quite distinct gene expression patterns in primary trophoblasts.

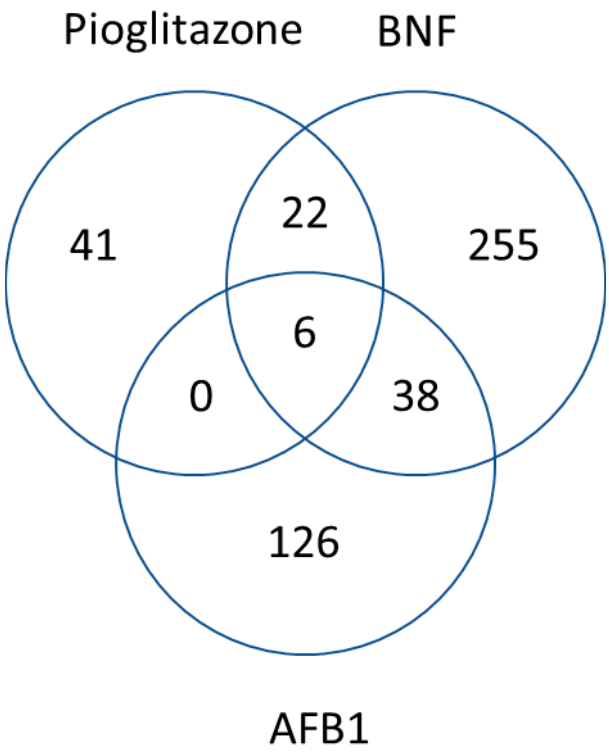

Figure 16. Venn diagram of the number of differentially expressed genes that are in common between pioglitazone-(more than 2-fold, $p < 0.05$), β-naphthoflavone (BNF)- (more than 1.5-fold, $p < 0.05$), or aflatoxin B1 (AFB1)- (more than 1.5-fold, $p < 0.05$) treated primary trophoblasts.

However, the expression of the overlapping genes that differentially changed in response to pioglitazone, BNF and AFB1 did not change in the same direction in response to the exposure to these three different agents (Table 7). All the six genes were upregulated in pioglitazone-treated term trophoblasts, whereas they were all downregulated in AFB1-treated term trophoblasts. PDK4 was the only gene upregulated in BNF-treated trophoblasts among the six common differentially expressed genes.

Table 7. The overlapping upregulated and downregulated differentially expressed genes in pioglitazone-, BNF-, AFB1-treated term trophoblasts.

Overlapping genes	Pioglitazone	BNF	AFB1
CGB1/2	Upregulated	Downregulated	Downregulated
CGB3/5/8	Upregulated	Downregulated	Downregulated
CRH	Upregulated	Downregulated	Downregulated
CSHL1	Upregulated	Downregulated	Downregulated
GH1	Upregulated	Downregulated	Downregulated
PDK4	Upregulated	Upregulated	Downregulated

CGB, Chorionic gonadotropin beta; CRH, Corticotropin releasing hormone; CSHL1, Chorionic somatomammotropin hormone like 1; GH1, Growth hormone 1; PDK4, Pyruvate dehydrogenase.

The gene expression analysis revealed that many of the top upregulated genes in pioglitazone-treated primary trophoblasts were involved in carbohydrate and lipid metabolism such as fatty acid binding protein 4 (FABP4), PDK4, Perilipin 2 (PLIN2) and retinol dehydrogenase 10 (RDH10) whereas many of the top downregulated genes were implicated in inflammatory and immunomodulatory responses including C-C motif chemokine ligand 3 (CCL3), C-X-C motif chemokine ligand 8 (CXCL8), CCL3 like 1 (CCL3L1), and CCL4L1. On the other hand, in BNF-treated term trophoblasts, many of the top upregulated genes were involved in xenobiotic metabolism e.g., CYP1A1 and CYP1B1 whereas many of the top downregulated genes have a role in placental growth and development e.g., placenta enriched 1 (PLAC1), CSHL1, CSH2, and pappalysin 1 (PAPPA1). Several upregulated genes in AFB1-treated trophoblasts are known to play key roles in protein synthesis and in the regulation of the cell cycle such as ribosomal protein S27 like (RPS27L), cyclin dependent kinase inhibitor 1A (CDKN1A), RNA polymerase II subunit A (POLR2A), ribosomal protein S19 (RPS19), TP53 regulated inhibitor of apoptosis 1 (TRIAP1), and growth arrest and DNA damage inducible alpha (GADD45A) whereas many of the top downregulated genes have functions related to placental development and endocrine activities including CSH1, GH1, and CSHL1. The top 10 up- and down-regulated genes in primary trophoblasts in response to pioglitazone, BNF, or AFB1 are shown in Figure 17.

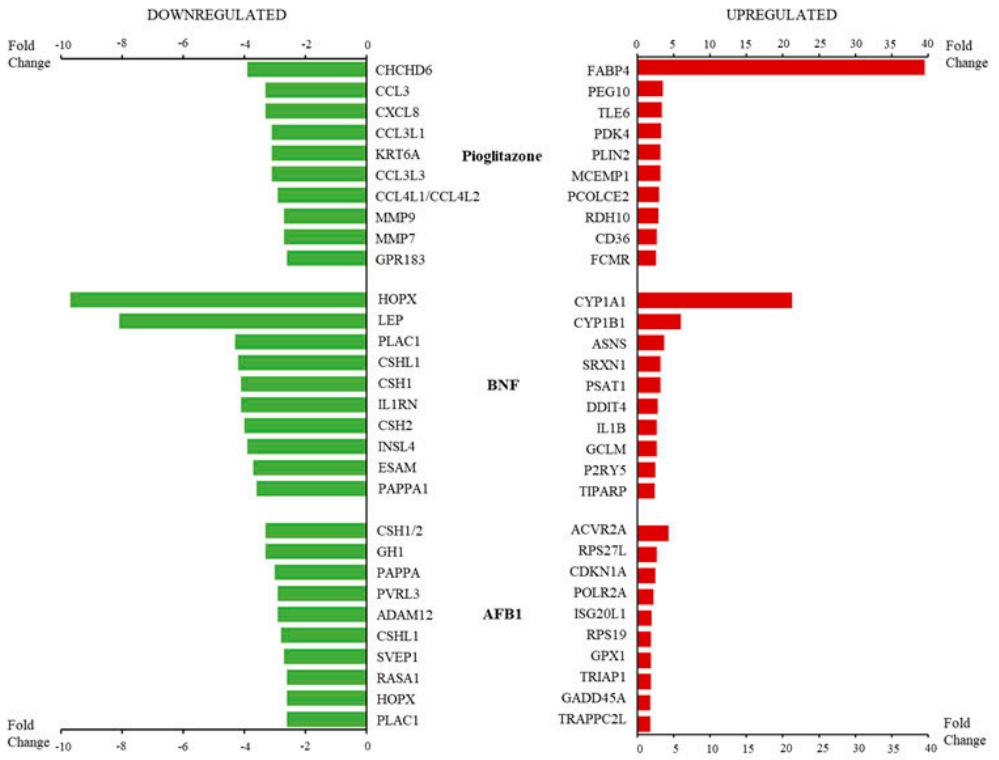

Figure 17. Top 10 up- and down-regulated differentially expressed genes in response to pioglitazone, β-naphthoflavone (BNF) or aflatoxin B1 (AFB1) in human primary trophoblasts.

5.3 MOLECULAR PATHWAY ANALYSIS

5.3.1 Canonical pathways

To reveal the most significant canonical pathways altered in primary trophoblasts in response to pioglitazone, BNF, or AFB1, an IPA functional analysis was performed (Table 8). There were many pathways related to the immune response among the most significant predicted canonical pathways in both pioglitazone and BNF treated trophoblasts. To confirm the reliability of the analysis, the AhR signalling pathway was observed as being one of the most significant pathways in BNF-treated primary trophoblasts. Based on the canonical pathways, it was evident that AFB1 was regulating a different set of genes than pioglitazone and BNF, since in primary trophoblasts, AFB1 caused the dysregulation of many genes involved in growth hormone signalling and cellular proliferation and development.

Table 8. Top five most significant canonical pathways altered in human primary trophoblasts in response to pioglitazone, β-naphthoflavone (BNF), or aflatoxin B1 (AFB1). Ingenuity pathway analysis showing the canonical pathways that were most significant in the data sets ($p < 0.05$ (i.e., - log10 ≥ 1.3)), Fisher's Exact test.

Substance	Biological Function	-log$_{10}$(P-value)
Pioglitazone	Granulocyte adhesion and diapedesis	8.7
	Agranulocyte adhesion and diapedesis	7.1
	Atherosclerosis signalling	4.5
	Bladder cancer signaling	4
	Communication between innate and adaptive immune cells	3.9
BNF	Interferon signaling	7.6
	Granulocyte adhesion and diapedesis	7.6
	Agranulocyte adhesion and diapedesis	5.6
	Communication between innate and adaptive immune cells	4.5
	Aryl hydrocarbon receptor signalling	3.7
AFB1	Growth hormone signaling	5.1
	Cholecystokinin/Gastrin-mediated signalling	3.9
	UVB-Induced MAPK signaling	3.5
	Unfolded protein response	3.4
	mTOR signaling	3.4

5.3.2 Biological functions and gene networks

An IPA analysis was used to identify the most significant biological functions within the differentially expressed genes altered in human primary trophoblasts in response to pioglitazone, BNF, and AFB1 (Table 9). Pioglitazone-dysregulated genes were implicated in various biological functions such as lipid metabolism, and cellular movement and development. Cellular movement and development were also among the top five affected in BNF and AFB1 treated trophoblasts. According to the IPA analysis, both pioglitazone and BNF regulated genes were

related to lipid metabolism whereas BNF and AFB1 regulated genes were involved in cell death and the survival of primary trophoblasts.

Table 9. Top five biological functions of differentially expressed genes altered in pioglitazone- β-naphthoflavone- (BNF), or aflatoxin B1 (AFB1)-treated primary trophoblasts generated by Ingenuity Pathway Analysis (IPA). The p-value reflects the likelihood that the association between a set of genes in every dataset and a related biological function would be significant [p-value < 0.05 (i.e., - $\log_{10} \geq 1.3$), Fisher's Exact test].

Substance	**Biological Function**	**-log10(P-value)**
Pioglitazone	Lipid metabolism	8.9
	Molecular transport	8.9
	Small molecule biochemistry	8.9
	Cellular movement	8.7
	Cellular development	7.0
BNF	Cellular movement	19.7
	Cell death and survival	14.9
	Cell-to-cell signaling and interaction	12.5
	Cellular development	9.5
	Lipid metabolism	7.9
AFB1	Cell death and survival	10.8
	Cellular movement	8.0
	Cellular development	7.8
	Cellular growth and proliferation	7.8
	Cell-to-cell signalling and interaction	6.2

Furthermore, IPA was used to evaluate the effects of pioglitazone, BNF, and AFB1 to reveal the most significant gene networks that were generated based on the connectivity and the biological relationships among those differentially expressed genes. The top five most significant gene networks in primary trophoblasts are shown in Table 10. Lipid metabolism, cellular growth and

proliferation, organ system development and function were among the most significant gene networks altered in pioglitazone-treated term trophoblasts. On the other hand, BNF caused alterations in the gene networks related to lipid metabolism, cell cycle, and inflammatory response and finally AFB1 had evoked alterations in the gene networks involved in endocrine system signalling, cell cycle and DNA repair and lipid metabolism in primary trophoblasts.

Table 10. Top five molecular networks of differentially expressed genes altered in primary trophoblasts in response to pioglitazone, β-naphthoflavone (BNF), and aflatoxin B1 (AFB1).

Substance	Molecular network
Pioglitazone	Lipid metabolism, molecular transport, small molecule biochemistry
	Cellular growth and proliferation, organ morphology, reproductive system development and function
	Immunological disease, infectious diseases, cell morphology
	Cardiovascular system development and function, cellular movement, hematological system development and function
	Embryonic development, nervous system development and function, organ development
BNF	Lipid metabolism, small molecule biochemistry, vitamin, and mineral metabolism
	Organ morphology, organismal development, organismal injury, and abnormalities
	Cell cycle, cancer, organismal injury, and abnormalities
	Inflammatory disease, inflammatory response, organismal injury, and abnormalities
	Reproductive system development and function, cell-mediated immune response, cellular movement
AFB1	Endocrine system development and function, organ morphology, reproductive system development and function
	Cancer, cell-to-cell signalling and interaction, organismal injury, and abnormalities

Cell cycle, cell death and survival, nervous system development and function.
Cell cycle, cellular compromise, DNA replication, recombination, and repair
Lipid metabolism, small molecule biochemistry, vitamin, and mineral metabolism

5.3.3 Upstream regulators

We then searched for the most significant upstream regulators within the differentially expressed genes in pioglitazone-, BNF-, and AFB1-treated primary trophoblasts. IPA analysis tool was utilised. Several upstream regulators such as TNFα, lipopolysaccharide, STAT3 and heparin binding EGF like growth factor (HBEGF) were predicted to be inhibited, and only one upstream regulator, lipoprotein lipase, was predicted to be significantly activated in pioglitazone-treated primary trophoblasts. On the other hand, several upstream regulators such as CD3, suppressor of cytokine signalling 1 (SOCS1) were predicted to be activated, whereas transforming growth factor beta 1 (TGFβ1), IL6, and epidermal growth factor (EGF) were predicted to be inhibited within the differentially expressed genes in the BNF-treated primary trophoblasts. Finally, in response to AFB1, IPA predicted the activation of upstream regulators such as TP53, and several transcription factors including forkhead Box O (FOXO), RUNX family transcription factor (RUNX), and the inhibition of AhR and AR in human term trophoblasts. Table 11 summarizes the top most significant endogenous upstream regulators predicted to be activated or inhibited in primary trophoblasts in response to pioglitazone, BNF, or AFB1.

Table 11. The top upstream regulators predicted by ingenuity pathway analysis (IPA) to be activated/inhibited in human primary trophoblasts in response to either pioglitazone, β-naphthoflavone (BNF), or aflatoxin B1 (AFB1). The p-value was calculated using a Fisher's Exact Test to determine the significance of the overlap ($p < 0.05$ [i.e., $- \log_{10} \geq 1.3$] between the regulator and stretch responsive genes. Only functional annotations (activated or inhibited) that obtained a significant regulation z-score (>2) are presented.

Substance	Direction of regulation	Upstream regulator
Pioglitazone	activation	LIPL
	inhibition	TNF, STAT3, LPS, HBEGF, EHF
BNF	activation	KRAS, CD3, SOCS1, IRF4, TRIM24
	inhibition	TGFB1, IFNA2, IL6, ERBB2, EGF
AFB1	activation	TFRC, TP53, FOXO, RUNX3, IKZF1
	inhibition	AGT, AR, DHT, AHR, Creb

AGT, Angiotensinogen; AhR, Aryl hydrocarbon receptor; AR, Androgen receptor; Creb, CAMP responsive element binding protein; DHT, Dihydrotestosterone EGF, Epidermal growth factor; EHF, ETS homologous factor; ERBB2, Erb-B2 receptor tyrosine kinase 2; FOXO4, Forkhead Box O4; HBEGF, Heparin binding EGF like growth factor; IFNA2, Interferon alpha 2; IKZF1, IKAROS family zinc finger 1; IL, Interleukin; IRF4, Interferon regulatory factor 4; LIPL, Lipoprotein lipase; LPS, Lipopolysaccharide; RUNX, RUNX family transcription factor; STAT, Signal transducer and activator of transcription; SOCS1, Suppressor of cytokine signalling 1; TFRC, Transferrin receptor; TGFB1, Transforming growth factor beta 1; TNF, Tumor necrosis factor; TRIM24, Tripartite motif containing 24; TP53, Tumor protein P53.

6 DISCUSSION

Maintaining normal pregnancy requires an orchestrated series of critical processes such as trophoblast invasion and differentiation, tissue remodelling, changes in metabolism as well as the development and maintenance of placental function. The intrauterine phase of development is not only important for the optimal development of the fetus but also for the individual's susceptibility to chronic diseases in adult life (for a review, see Vähäkangas et al., 2019). Various exogenous xenobiotics have been reported to affect placental physiology and molecular pathways (for a review, see Vähäkangas et al., 2019). Any alterations to placental functions and placental gene expression profile, either by environmental contaminants, or by exogenous xenobiotics, might have direct consequences on the feto-placental unit and pregnancy outcomes.

This thesis studied the gene expression profiles of human term trophoblasts in response to a PPARγ-agonist, pioglitazone, an AhR-agonist, BNF or to a mycotoxin, AFB1. It was observed that pioglitazone and BNF dysregulated genes that play key roles in cellular growth and invasion, placental lipid metabolism and cholesterol uptake as well as in the inflammatory response, whereas AFB1 dysregulated genes that are involved in placental growth, energy homeostasis, endocrine signalling, and cell cycle regulation in human term trophoblasts.

6.1 THE ROLES OF PPARG, AHR AND AFB1 IN PLACENTAL DEVELOPMENT

6.1.1 Pioglitazone exposure is associated with genes that decrease invasion and promote differentiation of primary trophoblasts

The gene expression analysis revealed that pioglitazone dysregulated genes related to placental development. For instance, cellular growth and proliferation, organ morphology, reproductive system development and function were among the main altered gene networks in pioglitazone-treated trophoblasts. Furthermore, a functional analysis of the differentially expressed genes showed that cellular development, growth, proliferation and maintenance were among the main biological functions affected by pioglitazone in term primary trophoblasts. PPARγ has been shown to control directly or indirectly several genes that are involved in these processes such as MUC1, CXCL16, MMP7,and MMP9. MUC1, a gene that was

downregulated by pioglitazone, has been reported to be involved in trophoblast invasion and trans-endothelial migration (DeSouza et al., 1999; Shalom-Barak et al., 2004; Thirkill et al., 2007). Interestingly, consistent with this study's findings, MUC1 gene expression has been demonstrated to be inhibited by PPARγ agonists, however, this inhibition reduced trophoblast adhesion to uterine endothelial cells and also blocked the transendothelial migration of trophoblasts (Shen et al., 2007; Wang et al., 2010). CXCL16 was one of the downregulated genes seen after exposure to pioglitazone; a similar response was also reported by Lehrke et al., (2007). The inhibition of CXCL16 was shown to inhibit endometrial decidualization and proliferation and invasion of the first-trimester human trophoblast cells (Huang et al., 2006; Mei et al., 2019). Pioglitazone also downregulated MMP7 and MMP9 in trophoblasts. Both of these enzymes have been reported to promote trophoblast invasion, cervical ripening, fetal membrane remodelling and rupture, and moderators of extensive extracellular matrix remodelling in placenta (Bowen et al., 2002a). In addition, the inhibition of their expression and activity was negatively associated with the onset of labour and rupture of the fetal membranes (Bowen et al., 2002a; Marx et al., 2003; Roth and Fisher, 1999; Shen et al., 2007). Furthermore, pioglitazone also caused a downregulation of heme oxygenase 1 (HMOX1). While HMOX1 is better known for its anti-oxidant and anti-hypertensive properties, and cytoprotective effects, it plays a role in the regulation of trophoblast invasion and placental function (Lyall et al., 2000; McCarthy et al., 2011).

All in all, these findings are in line with the literature which highlights the significant role of PPARγ in early placental development by decreasing trophoblast invasion and promoting their differentiation (Asami-Miyagishi et al., 2004; Fournier et al., 2002; Segond et al., 2013; Tarrade et al., 2001; Wieser et al., 2008). This has been supported by studies conducted in Pparγ knockout mice which caused placental dysfunction and interfered with terminal differentiation of the trophoblast and placental vascularization, leading to severe myocardial thinning and death of the fetuses (Barak et al., 1999; Kubota et al., 1999). Study I demonstrated that the PPARγ agonist, pioglitazone, regulated genes promoting differentiation and repressed the invasion of human trophoblasts.

6.1.2 BNF exposure is associated with genes that decrease trophoblast proliferation, invasion, and migration

The functional *in silico* analysis of the genes whose expression changed significantly in response to BNF revealed that cellular movement, and development, and cell-to-cell signalling and interaction were among the main molecular and celluar functions altered in primary trophoblasts. The pathway analysis of differentially expressed genes in response to BNF predicted a significant decrease in cellular invasion, migration, and movement of primary trophoblats. For example, the AhR-agonist, BNF, decreased the expression of several genes that are crucial in cellular development, death, and survival in term trophoblasts. Those downregulated genes, such as CSH1, PLAC1, and CSHL1, are known to be highly expressed in the placenta (Chen et al., 1989; Fagerberg et al., 2014). CSH1 and CSHL1 are members of the somatotropin/prolactin family of hormones, and participate in the regulation of several complex physiological processes, including growth and metabolism (Pérez-Ibave et al., 2014). CSH1 and PLAC1 were previously shown to be downregulated by another AhR-agonist, TCDD (Fracchiolla et al., 2011). PLAC1 expression was reported to be increased during trophoblast differentiation, an effect localizing primarily to the differentiated syncytiotrophoblasts (Massabbal et al., 2005). PLAC1 was also shown to positively regulate placentation and oocyte meiosis and fertilization (Shi et al., 2018). Furthermore, the level of leptin was also significantly downregulated by BNF in term trophoblasts. In the placenta, leptin plays a significant role in reproduction and fertility, and has been implicated in implantation, angiogenesis, embryonic development, as well as trophoblast proliferation and survival (Cervero et al., 2005; Dos Santos et al., 2015; Henson and Castracane, 2006; Schanton et al., 2018). The outcomes of the study are in line with a previous experiment with EVT cell lines, describing TCDD's effects on fetal and placental growth (Fukushima et al., 2012). In conclusion, the decrease in expression of these genes in response to BNF is predicted to inhibit proliferation, invasion, and the migration of term trophoblasts.

6.1.3 AFB1 exposure is associated with genes that suppress primary trophoblasts movement, growth, and proliferation

The identification of canonical pathways related to differentially expressed genes in response to AFB1 predicted a significant inhibition of several canonical pathways involved in cellular growth and proliferation such as growth hormone signalling, insulin secretion signaling pathways, and VEGF family ligand-receptor

Interactions. The gene expression analysis also indicated that AFB1 downregulated CSH2, CSHL1, GH1, CRH, and PLAC1 in term trophoblasts. This downregulation is predicted to decrease important biological functions such as growth hormone signalling, cellular movement, development, growth and proliferation, and cell-to cell signalling and interactions. CSH, CSHL1, and PLAC1 have an important role in placental development as described earlier. It was reported that placental growth hormone is specifically expressed in the SCT layer of the human placenta where it promotes fetal growth, placental development, and maternal adaptation to pregnancy (Alsat et al., 1998, 1997; Pérez-Ibave et al., 2014). Previous studies also have demonstrated similar findings and reported that the inhibition of GH signalling was among the possible mechanisms behind the growth impairment observed in neonates whose mothers had been exposed to AFB1 during pregnancy (Rushing and Selim, 2019). CRH is a hormone mainly expressed by the placenta, and its inhibition has been reported to suppress implantation, trophoblast invasion and parturition (Sasaki et al., 1987; Challis et al., 2000; Bamberger et al., 2006; You et al., 2014). In conclusion, AFB1 downregulated many genes that could affect cellular movement, growth, and proliferation of term trophoblasts.

6.2 PPARG, AHR, AND AFB1 HAVE A ROLE IN PLACENTAL LIPID METABOLISM

6.2.1 Pioglitazone exposure is associated with genes that promote the uptake and transport of fatty acids in primary trophoblasts

The analysis of the gene list of differentially expressed genes in response to pioglitazone revealed that lipid metabolism, molecular transport, small molecule biochemistry were among the top gene networks that had been altered in term trophoblats. PPARγ was implicated in regulating those genes involved in lipid transport and metabolism, an integral part of fetal development. Pioglitazone upregulated fatty acid binding protein (FABP4 and FABP5), very low density lipoprotein receptor (VLDLR) and lipase g (LIPG) in human term trophoblasts. An upregulation of FABP expression was also previously reported after PPARγ activation, although in that case in human monocytes (Pelton et al., 1999). FABP regulates intracellular lipid accumulation in human trophoblasts by facilitating lipid uptake and trafficking and plays a crucial role in cellular growth, proliferation, as well as regulating transporters of FAs and angiogenic genes that are critical for the

establishment of the placental circulation and thus for the normal feto-placental growth and development (Basak et al., 2018; Scifres et al., 2011). Similar to the findings of this study, LIPG expression was also shown to be induced in response to PPARγ-activators (Schoonjans et al., 1996). Furthermore, the upregulation of VLDLR in trophoblasts was also reported by Tao et al., (2010) i.e. that the PPARγ agonist pioglitazone upregulated VLDLR expression in dose- and time-dependent manners in primary human and mouse preadipocytes. VLDLR plays a key role in triglyceride (TG) metabolism, signal transduction, angiogenesis and tumor growth (Takahashi et al., 2004), whereas LIPG facilitates the transfer of lipids from the mother to the fetus and it has been reported to be dysregulated in placental endothelial cells sampled from mothers with intrauterine growth-restricted pregnancies (Gauster et al., 2007). The analysis of the altered genes in response to pioglitazone also revealed that LPL was among the most significant upstream regulators that was predicted to be activated. LPL increases the availability of FAs by catalyzing the hydrolysis of the triacylglycerol into FAs (Mead et al., 2002). All these findings reveal that pioglitazone can cause an upregulation of the expression of genes mediating the increased uptake and transport of fatty acids in primary trophoblasts. The upregulation of placental FABP4, incomplete invasion, and an excessive proliferation of placental trophoblast have been described as being several of the main pathological features present in the placentas of women with preeclampsia (Redline and Patterson, 1995; Redman and Sargent, 2005; Yan et al., 2016).

6.2.2 BNF exposure is associated with genes that promote cholesterol uptake and prostaglandin biosynthesis from FAs and in human term trophoblasts

An interesting finding of study II was that one of the top molecular networks underpinning the BNF regulated gene response was disturbed lipid metabolism. The increase of genes such as CYP1A1, CYP1B1, IL1B and the decrease of PDK4, LPL, leptin in response to BNF would be predicted to increase lipid concentrations in primary trophoblasts. In line with current findings, the previous studies by Biljes et al. (2015) and Dou et al. (2019) reported that AhR deficient mice showed an impairment of lipid metabolism, highlighting the critical role of AhR in regulating PPARγ stability and suppressing adipocyte differentiation.

Several genes that are in involved in lipid metabolism such as CYP1A1, CYP1B1, and IL1B were upregulated, whereas S100A8, LPL, CRH, PAPPA, and leptin were downregulated in the response to BNF. The altered expression of these genes

would be predicted to lead to the induction of FAs and arachidonic acid (AA) hydrolysis and cholesterol uptake in primary trophoblasts. CYP1A1 and CYP1B1 are known target genes of AhR (Brauze et al., 2017). CYP1A1 was reported to catalyse the metabolisms of AA, eicosapentaenoic acid and long chain FAs (Arnold et al., 2010; Schwarz et al., 2004). Human CYP1B1 has been implicated in the metabolism of retinoids and AA (Choudhary et al., 2004). Consistent with the study findings, the levels of IL1B and S100A8 mRNA were reported to be increased via AhR-mediated activity (DiMeglio et al., 2014; Tamaki et al., 2004). IL1B also increased the production of prostaglandin E2 from FAs in both rodent and human fibroblasts (Attur et al., 2002; Roshak et al., 1996) and also in human osteoblasts (Brechter and Lerner, 2007). S100A8 was shown to be involved in the metabolism of AA (Kerkhoff et al., 1999) and it can impair cholesterol efflux (Chellan et al., 2014). LPL inhibition was shown to prevent the hydrolysis of lipids and fatty acid biosynthesis (Kinnunen and Ehnolm, 1976), transport of TGs (Bensadoun, 1991), and efflux of cholesterol (Zhang et al., 2014). CRH also was shown to induce fat loss and increase energy expenditure as well as activating brown fat thermogenesis (LeFeuvre et al., 1987; Cheng et al., 2011). Furthermore, CRH is an important metabolic regulator since it promoted white adipose tissue lipolysis (Toriya et al., 2010). PAPPA1 that was downregulated in the gene list, was shown to decrease cholesterol efflux (Tang et al., 2012) and the downregulation correlated significantly with serum cholesterol and the change in levels of TGs observed in obese children (Woelfle et al., 2011). In this study, the gene expression of leptin was downregulated in trophoblasts in response to BNF, and was shown to play a key role in modulating food intake and energy homeostasis as well as in the regulation of fatty acid metabolism (Hynes and Jones, 2001; Atkinson et al., 2002). For example, leptin has been shown to deplete TGs in the pancreatic islets of rats (Shimabukuro et al., 1997) and was reported also to be involved in the catabolism of fatty acids and decreasing the efflux of cholesterol from human macrophages (Hongo et al., 2009).

In summary, BNF can increase the levels of FAs as well as disrupting AA metabolism and elevating cholesterol uptake while decreasing cholesterol efflux in human trophoblasts. Alterations in cholesterol content during pregnancy have been associated with aberrant placental development, intrauterine growth restriction and altered steroidogenesis, leading to fetal or neonatal death (Christiansen-Weber et al., 2000). Furthermore, impairments in cholesterol uptake and efflux in human trophoblasts were associated with endothelial dysfunction of

the macro- and the micro-vascular vessels of the placenta as well as in the development of atherosclerosis in the foetal aorta (Fuenzalida et al., 2020).

6.2.3 AFB1 exposure is associated with genes that disrupt lipid metabolism and decrease glucose uptake in term trophoblasts

The gene expression analysis showed that AFB1 upregulated the expression of genes that caused hyperlipidaemia, and impaired glucose tolerance as well as decreasing the transport of D-glucose in term trophoblasts. The analysis also revealed that lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism were among the main gene networks altered in AFB1-treated trophoblasts. Previously, work done on different *in vitro* and *in vivo* experimental models also reported that AFB1 caused changes in various endogenous metabolic pathways, including cell membrane-associated metabolism, the tricarboxylic acid cycle, glycolysis,and metabolism of lipids, and amino acids (Baldwin and Parker, 1985; Cheng et al., 2017; Kiessl, 1986).

AFB1 downregulated the levels of CSH1 and CSHL1 hormones in term trophoblasts. These hormones play a vital role in fetal growth through the regulation of maternal carbohydrate, lipid and protein metabolism by increasing the availability of glucose and amino acids to the fetus (Handwerger and Freemark, 2000; Kim and Felig, 1971). Furthermore, in term primary trophoblasts, AFB1 downregulated the expression of several genes such as solute carrier family 2 member 1 (SLC2A1), leptin, low density lipoprotein (LDLR) and insulin induced gene 1 (INSIG1) which are involved in carbohydrate and lipid metabolism and energy production. SLC2A1 is a major transporter in glucose uptake and its downregulation will lead to a decrease in glucose availability to the cell (Mueckler and Makepeace, 2008). Leptin influences satiety, adiposity and regulates whole-body energy homeostasis (Henson and Castracane, 2006; Houseknecht and Portocarrero, 1998); the effect of downregulation of leptin was described in the previous chapter. The LDLR is responsible for the uptake of LDL cholesterol; in addition, it plays a key role not only in glucose homeostasis but also in inflammation (Dato and Chiabrando, 2018) whereas INSIG1 was reported to play an important role in the regulation of lipid metabolism by inhibiting the synthesis of cholesterol and FAs (Dong and Tang, 2010). The downregulation of LDLR and INSIG1 would be predicted to lead to higher blood cholesterol levels. Hence, the analysis demonstrated that AFB1 would be likely to evoke disturbances in lipid metabolism as well as decreasing of glucose uptake in term trophoblasts. The

downregulation of insulin signalling and glucose transport would be anticipated to have serious consequences on the feto-placental unit since placental transport is the only supply route of glucose for the fetus (Di Simone et al., 2009) and glucose is the primary substrate for fetal energy metabolism (Landon and Gabbe, 2011).

6.3 THE ROLES OF PPARG AND AHR IN THE PLACENTAL INFLAMMATORY RESPONSE

6.3.1 Pioglitazone exposure is associated with genes that decrease the inflammatory response in human trophoblasts

The pathway analysis of gene expression data revealed that the inflammatory response was among the most significant phenomena that were influenced by the PPARγ-agonist pioglitazone in primary trophoblasts. The downregulation of several genes with pro-inflammatory properties such as IL8, TNFα, NFκB, leptin, and CCL3, CXCL10 and CXCL16 would be expected to have detrimental effects on the pregnancy. It has been shown that NFκb activates several pro-inflammatory cytokines such as IL8 and TNFα and leptin in placenta (Lappas et al., 2005a). In addition to their critical role in the inflammatory state of the pregnancy, these cytokines are involved in the initiation and progression of human labor and delivery. PPARγ was reported to exert anti-inflammatory effects during pregnancy by regulating the activities of various gene targets such as IL6, TNF α, HMOX1, leptin and MUC1 (Bilban et al., 2009; Lappas et al., 2002a; Shalom-Barak et al., 2004; Tarrade et al., 2001). Elevated concentrations of IL8 and TNFα have also been associated with preeclampsia and were previously shown to be also downregulated by PPARγ (Christiaens et al., 2008; Lappas et al., 2005a, 2002a, 2002b; Sattar et al., 1996). CCL3, CXCL10 and CXCL16 were claimed to increase the recruitment of leukocytes and to trigger an acute inflammatory state (Estensen et al., 2015; Gotsch et al., 2007; Holder et al., 2011; Wolpe et al., 1988) and thus a downregulation of these chemokines will ultimately repress the inflammatory response. These findings are supported by the literature with several investigators describing the immunosuppressive effects of PPARγ in many different cell types such as macrophages chondrocytes, kupffer cells, microglia, and BV2 microglial cells (Choi et al., 2017; Luo et al., 2017; Qu et al., 2017; Zhang et al., 2018; Huang et al., 2019). In conclusion, pioglitazone decreased the gene expression of pro-inflammatory cytokines that could lead to poorer responses to combat the attack of infective agents during pregnancy.

6.3.2 BNF exposure is associated with genes that repress the inflammatory and immune responses in primary trophoblasts

Study II showed that the AhR exerts an anti-inflammatory role in the placenta in which BNF dysregulated various cytokines involved in the inflammatory response. Previous studies have similarly described the anti-inflammatory effects of AhR activation. It is thought that AhR ligands can suppress immune and inflammatory responses by inhibiting the production of pro-inflammatory mediators (Benson and Shepherd, 2011). The exposure to macrophages to the AhR-agonist, BaP was reported to decrease the secretion of IL1β, IL6 and CXCL1 by the cells (Riemschneider et al., 2018). Fueldner et al. (2018) reported that AhR may have an inhibitory role on the immune system that may lead to impaired pathogen clearance and the establishment of a chronic infection. Furthermore, Domínguez-Acosta et al. (2018) revealed that AhR activation has reduced pro-inflammatory cytokine levels that play a vital role in immune responses.

The analysis of BNF-treated trophoblasts revealed the downregulation of several genes that are vital for the immune response causing an anti-inflammatory response. BNF downregulated the expression of receptor interacting serine/threonine kinase (RIPK2), colony stimulating factor 1 receptor (CSF1R), CCL5, MMP9, CCL3, Leptin and CRH. For instance, HMOX1 has been shown to be involved in the inflammatory response (Takao et al., 2011) by modulating the production of INFβ (Tzima et al., 2009). RIPK2 is involved in NFκB/JNK signalling and in the generation of the proinflammatory cytokine IL1β (Thome et al., 1998). CSF1R is an important regulator of monocyte development, proliferation, recruitment, activation, and differentiation from bone marrow cells, and it also mediates microglia chemotaxis (Dai et al., 2002; Lenda et al., 2003; Auffray et al., 2009; Lelli et al., 2013). CCL5 is a chemokine that increases the activation of macrophages (Villalta et al., 1998) and chemotaxis in many immune cell types such as leukocytes (Oppenheim and Yang, 2005), monocytes (Struyf et al., 1998), human dendritic cells (Sato et al., 2000), eosinophils (Cheung et al., 2008), T-lymphocytes (Roth et al., 1998). In addition to the involvement of the MMP9 protein in the degradation of the extracellular matrix, MMP9 can mediate an inflammatory response (Vermaelen et al., 2003). CRH regulates IL6 mRNA expression during inflammation (Venihaki et al., 2001), contributes to the inflammatory response (Benou et al., 2005), and increases the activation of microglia (Kritas et al., 2014). In summary, the AhR-agonist, BNF, downregulated the expression of many cytokines that could

indicate that there would be a decrease of the intensity of the inflammatory response during pregnancy.

6.4 AFB1 EXPOSURE IS ASSOCIATED WITH GENES THAT SUPPRESS ENDOCRINE SIGNALLING AND CELL CYCLE REGULATION IN TERM TROPHOBLASTS

The inspection of the genes whose expression changed significantly in response to AFB1 indicated that several canonical endocrine signalling pathways such GH signalling, estrogen receptor signalling, insulin secretion signaling, CRH signaling and VEGF family ligand-receptor Interactions pathways would be predicted to be inhibited in primary trophoblasts. Other investigators have also described the effect of AFB1 on endocrine signalling and reported that AFB1 is a potential endocrine disruptor (Storvik et al., 2011), that could impair the androgen biosynthetic pathway (Adedara et al., 2014), alter CRH mRNA expression, and affect cellular growth, cell cycle progression, interfere with DNA damage checkpoints, and inhibit DNA repair systems and thus it might alter the morphogenesis and maintenance of the placenta (Wang et al., 2016; Yin et al., 2016; Weng et al., 2017; Yip et al., 2017).

Among the differentially expressed genes altered in term trophoblasts, AFB1 downregulated CSH1, GH1, epidermal growth factor receptor (EGFR), placental growth factor (PGF), CDKN1A, and nitric oxide synthase 3 (NOS3) are predicted to suppress endocrine signalling. The roles of CSH1, GH, and CRH in endocrine signalling were already discussed in previous chapters. In addition to its role in cell division and differentiation, EGFR was reported to be expressed by villous SCT and CT, and to play a key role in trophoblast hormone secretion, proliferation and differentiation (Maruo and Mochizuki, 1987; Bulmer et al., 1989; Amemiya et al., 1994). PGF is also important in trophoblast growth, and it also exerts angiogenic effects on the feto-placental circulation (Chau et al., 2017). Furthermore, it was reported that PGF regulates thyroid hormone signalling, where decreased levels of PGF in umbilical cord blood were associated with an increased risk of fetal hypothyroxinemia (Korevaar et al., 2014). NOS3, a gene that was also downregulated in trophoblasts in response to AFB1, plays key role in angiogenesis and was reported to inhibit the release of CRH and NOS3 is also associated with increased VEGF gene expression (Costa et al., 1993; Saleh et al., 2015).

The IPA analysis also predicted that estrogen signalling would be among the main pathways to be inhibited by AFB1 in term human trophoblasts. CYP19A1 was

one of the downregulated genes in primary trophoblasts in response to AFB1. CYP19A1 is highly expressed in the placenta as compared to the liver (Vähäkangas et al., 2019). CYP19A1 is the rate-limiting enzyme for estradiol production by catalyzing the conversion of A-dione to esterone and converting testosterone into estardiol, and its downregulation will significanlly suppress estradiol biosynthesis (Chatuphonprasert et al., 2018; Seliskar and Rozman, 2007).

Furthermore, based on IPA analysis, it seems that AFB1 suppresses cellular growth and cell cycle progression by dysregulating genes such as FOS, adrenomedullin (ADM), CDKN1A, and GADD45A in human primary trophoblasts. FOS, a gene that was downregulated in AFB1 treated trophoblasts, is an oncogene that plays a significant role in cell proliferation, and promotes cell cycle entry and progression (Brown et al., 1998; Kovary and Bravo, 1991). ADM mRNA levels were also downregulated in response to AFB1. ADM has been reported to regulate cellular proliferation, differentiation, growth, apoptosis and migration in different cell types (Shichiri and Hirata, 2003; Ouafik et al., 2009). The gene expression of CDKN1A or P21, and GADD45A was upregulated in response to AFB1. Both genes were reported to block cell cycle progression and DNA repair (Kleinsimon et al., 2018), and were demonstrated to be upregulated by AFB1 inducing a cell cycle arrest in human kidney and liver cell lines (Huang et al., 2019; Josse et al., 2012) . In conclusion, the findings of study III confirmed that AFB1 caused cell cycle arrest and repressed the cellular DNA repair system that could well lead to genotoxic stress. The transcriptional repression of DNA repair genes is a major feature of cellular senescence (Collin et al., 2018). The persistent damage to DNA and the promotion of senescence in the placenta would be predicted to exert detrimental effects on the developing embryo (Singh et al., 2020).

6.5 COMMON GENE EXPRESSION RESPONSES BETWEEN PIOGLITAZONE-, BNF-, AND AFB1-TREATED TROPHOBLASTS

A comparison analysis of the three datasets, revealed that there are six genes that were dysregulated in common, in pioglitazone-, BNF-, and AFB1-treated term trophoblasts i.e. CGB1/CGB2, CGB3, CRH, CSHL1, GH1, and PDK4. CGB is a placental hormone produced primarily by SCT and plays a role in the maintenance of pregnancy, and trophoblast differentiation (Nwabuobi et al., 2017; Pidoux et al., 2007). CRH is a placental hormone which is important in blastocyte implantation, trophoblast invasion and parturition (Sasaki et al., 1987; Challis et al., 2000; Bamberger et al., 2006; You et al., 2014). CSHL1 and GH1 have important roles in

placental growth, lipid and carbohydrate metabolism, and maternal adaptation to pregnancy, as already discussed in previous chapters. PDK4 is a mitochondrial protein that is an important regulator of glucose and FA metabolism and homeostasis (Abbot et al., 2005; Kulkarni et al., 2012). The expression of the PDK4 gene was upregulated by pioglitazone and BNF but downregulated by AFB1. The upregulation of PDK4 has been reported to be associated with increased oxidation of FAs whereas its inhibition decreased their oxidation and inhibited the glucagon-mediated expression of gluconeogenic genes (Park et al., 2018; Pettersen et al., 2019).

Furthermore, based on the IPA analysis, both AhR and PPARγ were predicted as being two of the most important upstream regulators of AFB1 dysregulated genes. AhR activity was observed to be one of the most extensively inhibited upstream regulators by AFB1 in placenta. Previous studies have reported that AFB1 caused significant increases in the levels of CYP1A1/A2, AhR, mRNA expression in human primary hepatocytes (Mary et al., 2015) as well as an AhR-mediated increase of CYP1A1 activity and transcription in H4IIE rat hepatoma cells (Ayed-Boussema et al., 2012). Furthermore, it was reported that the activation AFB1 induced by AhR can cause a potential disruption of several cellular functions such as cellular differentiation, adhesion, proliferation, and interference with the immune response (Arenas-Huertero et al., 2019). Interestingly, AhR was also reported to downregulate PPARγ. AhR was shown to regulate adipocyte differentiation by targeting PPARγ proteasomal degradation. AhR overexpression also reduced the stability of PPARγ and suppressed adipocyte differentiation, whereas AhR knockdown stimulated adipocyte differentiation in 3T3-L1 cells (Dou et al., 2019).

6.6 STUDY LIMITATIONS

In this thesis, pioglitazone, BNF and AFB1 were used to identify PPARγ-, AhR-, and AFB1- target genes in term trophoblasts. The main limitation of this thesis is that the gene expression patterns in all studies were examined only at one time point and with one concentration which means that it was not possible to evaluate if there was a dose dependent relationship in the alterations seen in the expression levels of the examined genes. However, the lifespan of the trophoblasts after delivery does not make it possible to conduct any long-lasting experiments using lower pioglitazone, BNF, or AFB1 doses. Interestingly, the relatively high doses used in the three studies did not affect either the viability or the functionality of the trophoblast cells. In fact, even AFB1 did not affect significantly the cell viability

up to a concentration of 1 μM. Nevertheless, high doses of BNF (25 μM) and AFB1 (1 μM) were used in this study as compared to other trials described in the literature in order to reflect "the worst-case scenario" of potential exposure to these compounds. There is no "physical" concentration for BNF, since it is a toxic substance. In several studies, a 5 μM concentration of BNF was used (Stejskalova et al., 2011; Wu et al., 2016). However, according to the cell viability analysis, the dose used in this study was not cytotoxic. Liver is the target organ of AFB1 toxicity and therefore, when tested in hepatic cells, much lower concentrations have been used e.g. 0.05 and 0.25 μM AFB1 (Josse et al., 2012). According to the cell viability assay, the trophoblasts seem to be less sensitive to the effects of AFB1, and consequently, a higher dose was used. For pioglitazone, the highest daily therapeutic dose of 45 mg (therapeutic indication by European Medical Agency) was used. Furthermore, since the gene expression profiling was done in primary trophoblasts isolated from twelve term placentas, the results cannot be generalised. Since the number of placentas is minimal and each placenta represents one individual, it is evident that the genetic background for the study is too restricted to make a true population-based analysis. Another limitation of the study was that a confirmatory RT-qPCR analysis was not performed to verify the gene expression array results of the AFB1-treated term trophoblasts.

Pioglitazone was chosen even though rosiglitazone is nearly 10 times more potent as an activator of human PPARγ (Shalom-Barak et al., 2004). However, there is some degree of receptor selectivity in favour of pioglitazone as it binds to a lesser extent to *hPPARα* than rosiglitazone (Sakamoto et al., 2000). In addition, pioglitazone is the only thiazolidine on the market in the European Union at the moment. BNF was chosen over TCDD, the most potent ligand of AhR (Van den Berg et al., 2006), in order to avoid exposure of the investigators to TCDD, classified as a definite human carcinogen (IARC, 1997).

In addition to the classical mechanism through which they activate NRs, PPARγ ligands have several alternative mechanisms (Peraza et al., 2006). AhR ligands can also exert different biological effects and the gene expression in response to these ligands is not uniform. For instance, TCDD increased the (TGFβ2), CYP1B1, and TGFβ -related genes in AhR (-/-) cells indicating that TCDD possesses more biological effects that are not AhR-mediated (Guo et al., 2004). For example, Hrubá et al. (2011) reported differences in the transcriptome of LNCaP cells treated with either BaP or TCDD. These authors reported that 19 % of the differentially expressed genes from the data set were BaP-selective but more, 29 %, of the differentially expressed genes were TCDD-selective.

Gene expression profiling studies only provide assumptions and associations and not causality. Thus, further experimental studies will be needed to determine the functional consequences of the findings emerging from this study. For instance, studies involving an analysis of PPARγ or AhR responses, will be needed to clarify whether PPARγ or AhR directly or indirectly regulate the expression of pioglitazone or BNF dysregulated genes, respectively. Other studies exploiting PPARG or AhR siRNA, antagonists or by applying a luciferase assay using PPARG or AhR binding elements could be also enlightening.

Term trophoblasts are not a suitable model to study the early stages of the developing placenta. Hence, first-trimester cells could be used to study the effects on early pregnancy. One example would be the HTR-8/SVneo cell line that was derived by transfecting the cells that grew out of chorionic villi explants of human first-trimester placenta. In addition, villus explants or placental organoids could be used to study the effects of these kinds of substances. Finally, whether these outcomes can be directly transferred to the function of the feto-placental unit *in vivo* is not known and will have to be the subject of future work.

7 CONCLUSIONS

The aim of this study was to examine the placental gene expression profiles, and to conduct a gene expression analysis in order to reveal the most significant molecular networks, and the main biological functions affected in term primary trophoblasts in response to pioglitazone, BNF, or AFB1. Human primary trophoblast cells were demonstrated to represent a relevant and reliable research model, especially in the fields of placental unit and fetal growth.

The main outcomes of this thesis were:

1 When term trophoblasts were exposed to the PPARγ-agonist pioglitazone, many of the genes that were altered repressed cellular invasion and inflammatory responses, but promoted cellular growth, differentiation, and uptake, and the transport of fatty acids. These molecular functions play a crucial role in pregnancy through placental maturation.

2 The genes affected by the AhR-agonist, BNF, are known to decrease the immune response, as well as the proliferation, invasion, and migration of trophoblasts. In contrast, there was evidence of BNF regulated genes that augmented lipid metabolism and cholesterol uptake in term trophoblasts. This aberrant placental lipid metabolism and disturbed immune response might have significant consequences on the feto-placental unit.

3 The AFB1 regulated genes were predicted to suppress endocrine signalling, cell cycle progression, and placental growth in term trophoblasts. AFB1 also disturbed genes regulating energy homeostasis by disrupting lipid metabolism as well as impairing glucose tolerance and decreasing the transport of glucose in term trophoblasts. AFB1 is a common food contaminant in various parts of the world. Maternal exposure to AFB1 during pregnancy might significantly alter placental functions which could have significant detrimental consequences on the likelihood of a successful outcome of her pregnancy.

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RAMI EL-DAIRI

Human placenta is an exchange organ between mother and fetus. Any alteration to placental functions might affect fetal growth and development. This study provides novel information on placental gene expression profiles, the most significant molecular networks, and the main biological functions affected in the placenta in response to chemical substances.

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