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The immunomodulatory role of heme oxygenase-1 during late pregnancy in mice

Dissertation

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1. Einleitung/Introduction

1.1. Problem

Numerous studies reveal that intrauterine growth restriction (IUGR) is associated with an increased prevalence of chronic diseases in later life, such as cardiovascular diseases (Barker and Osmond 1986, Osmond *et al.* 1993, Huxley et al. 2007), hypertension (Curhan *et al.* 1996a, Curhan *et al.* 1996b), diabetes mellitus type 2 (Hales and Barker 1992, Curhan *et al.* 1996b), obesity (Kensara *et al.* 2005), asthma (Nepomnyaschy and Reichman 2006), atopic dermatitis (Steffensen *et al.* 2000) and mental disorders (Indredavik *et al.* 2005, Betts *et al.* 2011, Lund *et al.* 2011). Barker was among the first to discover the relationship between low birth weight, as a result of poor maternal nutrition, and increased life time risk for coronary heart disease (Barker *et al.* 1989). He postulated the *fetal origin of adult disease hypothesis* (**Figure 1**), proposing that the fetal adaptions and developmental changes are permanently and increasing the risk to suffer from a chronic disease in adult life.

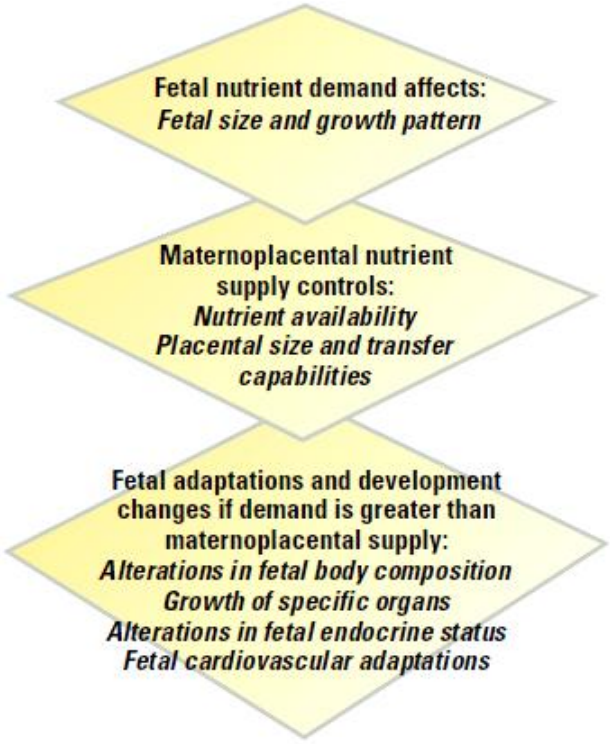


Figure 1 Illustration of the theory of Barker's fetal origin of adult disease also referred to as *fetal programming.* (Greenblum and McNamara 2010)

To date, Barker's hypothesis has been confirmed in clinical studies and animal models, which demonstrate negative short- and long-term consequences of different environmental challenges during pregnancy on offspring health. In addition to poor maternal nutrition, environmental factors such as exposure to smoking (Seller and Bnait 1995, Gilliland *et al.* 2003, Lannerö *et al.* 2006, Penn *et al.* 2007), alcohol (Sen and Swaminathan 2007, Zammit *et al.* 2009), pollution (Herr *et al.* 2011, Wood *et al.* 2011) and stress (Wright *et al.* 2004, Weinstock 2005, Sausenthaler *et al.* 2009) could be identified to trigger the incidence of chronic diseases in later life of the offspring, frequently accompanied by low birth weight/IUGR. This is also referred to as *fetal programming* (Gluckman *et al.* 2008). However, the underlying mechanisms that "program" the fetus and result in the increased vulnerability of the offspring to develop chronic diseases in later life still remain largely elusive. The growing incidence of diseases such as cardiovascular diseases, diabetes mellitus type 2 and the rise of IUGR during the last decade (Romo *et al.*

2009, Beard *et al.* 2009), as well as the changing role of women in the Western society with more and more women experiencing an increased stress perception (Solano *et al.* 2011) indicate the need to identify the mechanisms of fetal programming. This identification would allow the primary prevention of such risks and would offer approaches for therapeutic interventions to protect the developing fetus from negative consequences of prenatal environmental challenges and ensure a stable intrauterine environment. It can be proposed that a potentially important mediator of e. g. stress during pregnancy may be the placental expression of heme oxygenase-1 (HO-1). HO-1 is an enzyme expressed in most tissues under physiological conditions and involved in tissue homeostasis (Keyse and Tyrrell 1989, Ryter *et al.* 2006, Scott *et al.* 2007, Gozzelino *et al*. 2010). Moreover, it is susceptible to environmental factors (Motterlini *et al*. 2000, Ryter *et al*. 2006, Xiang *et al*. 2011). HO-1 is highly expressed in placenta throughout pregnancy (Watanabe *et al.* 2004, Zhao *et al.* 2009) and known to play a regulatory role in a number of inflammatory processes and act pregnancy-protective (Liu *et al.* 2001, Bainbridge and Smith 2005, Kobayashi *et al.* 2006, Orozco *et al.* 2007, Patil *et al.* 2008, Sheikh *et al.* 2011, Elmarakby *et al.* 2012, Sass *et al*. 2012).

1.2. Placenta and pregnancy

Human and mouse placenta: similarities and differences

Placenta is the first organ that is formed during mammalian embryogenesis and builds an interface between fetal and maternal environment. It is necessary for fetal growth and viability and stimulates maternal well-being throughout pregnancy. Mammals have developed different kinds of placentation, which differ in structure and composition of placental barrier tissues, separating maternal from fetal blood (Grosser 1909, 1927). Human placenta is composed of maternal and zygote-derived (fetal) tissues with a zygote-derived trophoblast layer, which comes into direct contact with the maternal blood, forming a so-called hemochorial placenta. The similarities between murine and human hemochorial placentas, allow translating the results from mouse models to explain human physiology. To overcome the limitations of working in an animal model, it is also necessary to consider the differences between both species (**Figure 2**). The duration of pregnancy in mice takes between 18 and 21 days.

Functions of the hemochorial placenta

The placenta is the crucial factor for fetal nutrient supply and creating a protective and stable environment for the developing fetus. This is achieved in various ways, involving different

types of feto-maternal interfaces, as has been summarized by Georgiades *et al*. (Georgiades *et al.* 2002). They described five principal placental functions:

1. Protection of the fetus from the maternal immune system.
2. Assurance of adequate fetal nutrient supply and toxic waste removal.
3. Adaptation of maternal vasculature and blood volume for optimal substances exchange.
4. Synthesis and secretion of hormones.
5. Maintenance of the pregnant uterus in the necessary physiological condition.

These functions can be affected by intrinsic and extrinsic, i.e. environmental, factors, demanding a placental adaptation helping to maintain the needed nutrient supply to the fetus, as a major determinant of intrauterine growth. Hence, these placental functions indicate the relevant role of placenta in fetal programming. Environmental challenges can lead through placental adaptation to changes in placenta, including size and morphological structure (Fowden *et al.* 2006) affecting fetal nutrient supply.

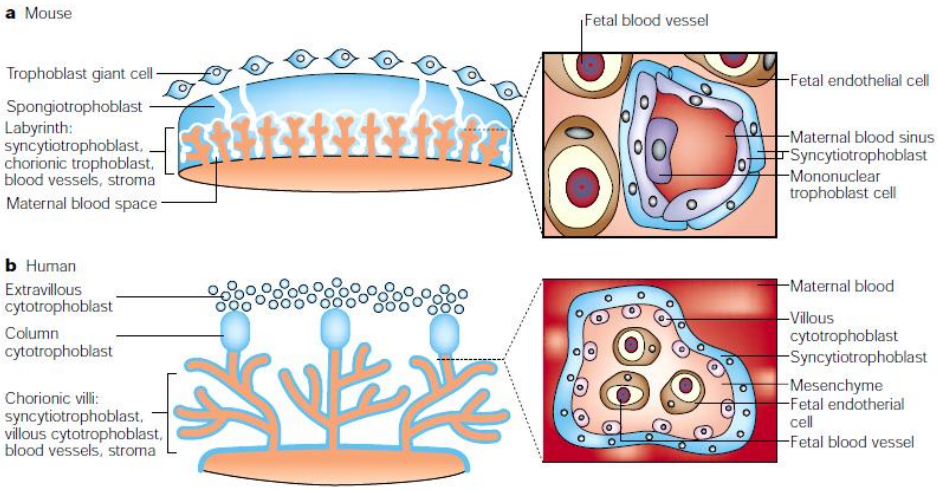


Figure 2 Comparative anatomy of the mouse (**a**) and human (**b**) placenta. The inset images show a cross-section through the feto-maternal interface and illustrate the number and type of cell layers between the maternal and fetal blood. (Rossant and Cross 2001)

Anatomy of the placenta

The biological structure of murine placenta with hemochorial interface, which develops around gestation day (gd) 10.5, is comparable to second and third trimester in human pregnancies (Georgiades *et al.* 2002). The following description of the anatomy of murine placenta serves to provide an overview and better understanding of the placentas used for experiment in the present thesis.

Mature placenta is of discoid shape and consists of distinct layers (**Figure 3**). On the fetal side there is the labyrinth layer where direct gaseous and nutrient exchange takes place

between fetal and maternal blood. Maternal blood flows in maternal blood spaces between highly interconnected villi, directly contacting the hemochorial interface, which is formed by trophoblast cells (fetal tissue). Centrally, the umbilical cord attaches to the labyrinth connecting fetal circulation with two arteries and a vein to the placenta and maternal circulation. The spongiotrophoblast cells build up the junctional zone with endocrine function. A line of trophoblast giant cells separates fetal placenta and maternal decidua. Decidua refers to modified endometrium where the placenta attaches to the uterus during pregnancy. It contains maternal vasculature reaching into labyrinth layer for substances exchange.

In mice, as a commonly used measurement indicating changes in placenta function, the labyrinth/junctional zone (L/JZ) ratio can be calculated. It is defined as the ratio of labyrinth and junctional zone layer sizes.

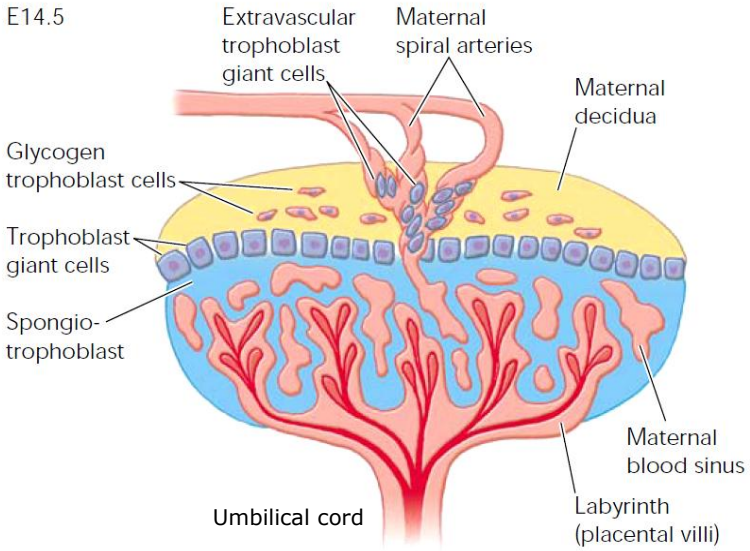


Figure 3 A schematic drawing of the anatomy of the mature murine placenta. (Watson and Cross 2005)

1.3. Immunological adaption to pregnancy

Pregnancy is considered as a very special immunological setting. Medawar was among the first to describe the unique immunology of the feto-maternal interface calling the fetus a semi-allograft, carrying foreign antigens and molecules from the father without being rejected by the maternal immune system (Medawar 1952). This is an immunological challenge for the pregnant mother and her immune system has to undergo several adaptational processes in order to prevent rejection of the growing fetus and ensure pregnancy success. The mechanisms contributing to fetal tolerance have been identified to some extent in human

pregnancies, but remain not fully understood. Human trophoblasts (fetal tissue in direct contact with maternal tissue) do not express the human leukocytes antigens (HLA) class II and the classical HLA-A and –B, but a specific array of non-classical class I molecules (HLA-E, -G), which both enables them to inhibit or evade the detection by maternal immune cells, such as cytotoxic ($CD8^+$) T, natural killer (NK) and dendritic cells (DCs). Also involved in immunological adaptation to pregnancy are decidual NK (dNK) cells, a unique subpopulation of NK cells that function as switchboards, licensed and educated to control trophoblast invasion, vascular remodeling, and to interact with DCs (Karimi *et al.* 2008) especially during the first trimester of pregnancy (**Figure 4**). DCs, which are largely present in decidua of normal pregnancy, not only induce NK cell activation, they also activate T helper cells (identified as $CD4^+$) through antigen presentation and promote expansion of T regulatory (Treg) cells, that have been characterized by their $CD4^+CD25^+$ phenotype and represent a third of all $CD4^+$ T cells in pregnancy. Treg cells and their suppressive nature, including inhibition of NK and T helper cell functions through i.e. IL-10, have proven to be essential for mediating maternal tolerance to the fetus during implantation since their depletion lead to pregnancy failure during early murine pregnancy (Aluvihare *et al.* 2004). Interestingly, Treg depletion in the second half of murine pregnancy does not affect fetal development (Shima *et al.* 2010), indicating a more moderate function in late pregnancy.

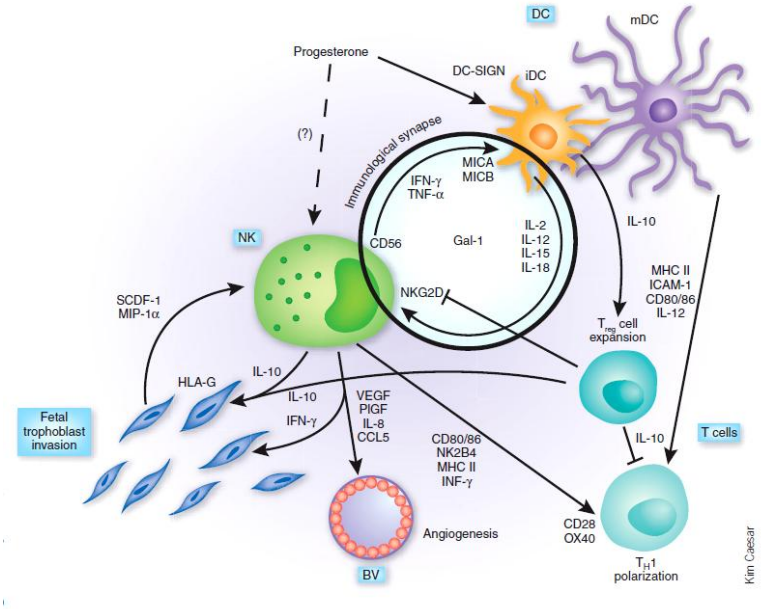


Figure 4 A schematic diagram illustrating the complex immunological crosstalk between different immune cells at the feto-maternal interface with the focus on decidual NK cells functioning as switchboards. (Karimi *et al.* 2008)

Role of $CD8^+$ T cells during pregnancy

$CD8^+$ T cells, cytotoxicity and pregnancy complications

Cytotoxic T cells are lymphocytes and belong to the $CD8^+$ subset of T cells, developing in the thymus. They can recognize specific antigens and kill cells by induction of programmed cell

death (apoptosis). Allograft rejection is largely mediated by activated $CD8^+$ T cells. To become activated, naïve T cells need to interact with antigen presenting cells (APCs), such as dendritic cells or macrophages, that are presenting specific antigens bound to MHC class I molecules or directly with the target cell. The binding to the antigen and MHC class I molecule via T cell antigen receptor (TCR) provides activation, together with co-receptors CD8 and CD28, which strengthen the activation signal. Activated $CD8^+$ T cells kill specifically target cells like, for instance, virus-infected cells, cancer cells or allografts, by the release of cytotoxic effector proteins (perforin and granzymes) or interaction of transmembrane proteins expressed on cytotoxic T and target cell (FASL/FAS). $CD8^+$ T cells are present at the feto-maternal interface but their function during normal pregnancy is unknown. Pregnancies with preeclampsia and abortion seem to be related to changes in $CD8^+$ T cell frequency and function (Joachim *et al.* 2001, Carbone *et al.* 2010, Molvarec *et al.* 2011), indicating that $CD8^+$ T cells are critically involved in regulating trophoblast invasion and pregnancy protection (Erlebacher *et al.* 2007).

Trophoblast recognition versus antigen recognition

Mechanisms of how decidual $CD8^+$ T cells are activated during pregnancy and promote fetal tolerance are controversial. Two distinct hypotheses have been expressed, involving trophoblast versus antigen recognition. Shao *et al.* (Shao *et al.* 2005) described previously unknown $CD8^+$ Treg cells in decidua, as a subset of $CD8^+$ T cells. They reported that human placental trophoblast cells could activate such $CD8^+$ Treg cells, which are not restricted to MHC class I molecules, but require a costimulation through carcinoembryonic antigen (CEA) expressed on trophoblasts in early pregnancy. The $CD8^+$ Tregs promote tolerance by suppression of T cell dependent B cell differentiation, as was shown in vitro, suggesting a potential protection of the fetus from destructive antibody effects in vivo. Erlebacher *et al*. (Erlebacher *et al.* 2007) proposed that fetal tolerance is ensured by $CD8^+$ T cells activated by indirect fetal antigen presentation through trophoblast cells, resulting in defective priming and clonal deletion. In vitro activated indirectly alloreactive T cells were also unable to attack the fetus. Further, no direct fetal antigen presentation occurs, leaving $CD8^+$ T cells, as major mediator of allograft rejection, less of a threat since they do not recognize but ignore the fetal allograft.

Regulator $CD8^+$ T cells

Within the $CD8^+$ T cell population, naturally occurring Treg cells could be identified by the expression of $CD122^+$ (IL-2 receptor ß chain). In genetically CD122-deficient mice, their role for sustaining immune homeostasis has been demonstrated as the $CD8^+$ Treg cells reverted severe hyper-immunity due to abnormally activated T cells via regulating potentially

dangerous $CD8^+$ T cells (Rifa'i *et al.* 2004). To date, no information is available on the role of $CD8^+CD122^+$ T cells in pregnancy and fetal tolerance.

1.4. HO-1

Furthermore, immunomodulatory molecules such as Indoleamine 2,3-dioxygenase (IDO) (Hönig *et al.* 2004, Miwa *et al.* 2005) or Galectin-1 (Blois *et al.* 2007) are involved in reproductive success and, as Blois *et al.* demonstrated for Galectin-1, play a pivotal role in conferring feto-maternal tolerance through multiple mechanisms. Another potent immunomodulatory molecule is HO-1. Due to the many properties attributed to HO-1 (as described below) it has been proposed to affect pregnancy and normal function of placenta.

HO-1 is the rate-limiting enzyme that catalyzes the first step of the degradation of heme into biliverdin with the products carbon monoxide (CO) and ferrous iron (Fe^{2+}) at the expense of molecular oxygen and NADPH (**Figure 5**). The inducible enzyme is involved into tissue homeostasis and is susceptible to environmental factors. HO-1 is widely expressed in placenta during pregnancy, but its role in placental function is not well understood until today. In early pregnancy could be shown that immunological abortions are associated with local HO-1 down-regulation, while an up-regulation prevented abortion in mice (Zenclussen ML *et al.* 2006). Further, a down-regulation/deficiency of HO-1 could be detected in human placenta samples of mothers suffering from preeclampsia (Zenclussen AC *et al*. 2003).

HO-1 and its immune-modulatory properties

The tissue-protecting effects of HO-1 are mainly due to the products of heme metabolism (**Figure 5**). Biliverdin is converted to bilirubin by biliverdin reductase before it acts as physiological antioxidant and potent scavenger of reactive oxygen species (Stocker *et al.* 1987). Ferrous iron binds to ferritin, the primary intracellular protein for storing iron and is kept in a soluble and non-toxic form until recycled for further cell processes, such as heme synthesis. Fe^{2+} functions as a stimulator of the synthesis of ferritin and provides cytoprotection through the antioxidant capability of ferritin (Eisenstein *et al.* 1991, Balla et

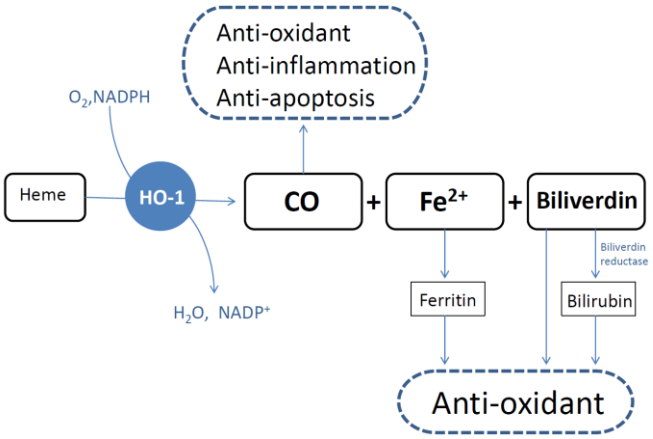


Figure 5 A schematic drawing of heme metabolism catalyzed by HO-1 with its cyto-protective products. (Modified after: Babusikova *et al. 2008)*

al. 1992). CO has multiple functions in different cellular mechanisms and acts amongst others anti-oxidative, anti-inflammatory and anti-apoptosis.

The role of HO-1 during pregnancy has not been completely discovered. In transplants, the up-regulation of HO-1 promotes graft acceptance (Soares *et al.* 1998, Pileggi *et al.* 2001). It is not known if the placenta adopted this mechanism for fetal tolerance during pregnancy. Findings in humans, where significantly higher amounts of HO-1 mRNA in myometrium of pregnant uterus were found compared to non-pregnant myometrium (Acevedo and Ahmed 1998) and high placental HO-1 expression in mice (Watanabe *et al.* 2004) suggest a relevant role of HO-1 for successful pregnancy. This hypothesis was strengthened by further reports such as low HO-1 expression in stress-triggered and IL-12-mediated murine abortions (Zenclussen AC *et al*. 2002) and increased fetal weight after injections of adenoviral HO-1 vector into pregnant rats (Kreiser *et al.* 2002). HO-1 removes cytotoxic free heme from cells and is thought to function pregnancy-protective through its metabolites (**Figure 5**). Their tissue-protecting effects grant protection of the fetus from oxidative injury and rejection. In addition HO-1 might be needed to supply the fetus with iron, an essential element for cellular growth and fetal development (Watanabe *et al.* 2004). CO, a potent vasodilator, impacts vascular tone in uterus and placenta, as was shown, e. g., by a CO-induced decrease of placental perfusion pressure (Bainbridge and Smith 2002), indicating CO having a part in the maintenance of adequate blood flow at the feto-maternal interface. Furthermore, CO is likely involved in the down-regulation of uterine contraction (via the HO-CO-pathway) (Watanabe *et al.* 2004). The functions of HO-1 and its metabolites during pregnancy remain a field of research.

HO-1 and $CD8^+$ T cells

HO-1 is also known to have potent immune regulatory functions by interacting directly with $CD8^+$ Treg cells (Andersen *et al.* 2009). In recent studies, a HO-1 specific $CD8^+$ Treg population was described in cancer patients, which were recruited by tumor cells via HO-1 expression. The cells do not express a certain phenotype and could only be characterized by their specificity. They show a strong inhibition of cytotoxicity, proliferation and cytokine release of other T cells resulting in a very effective local immune suppression, stronger than that promoted by common Treg cells, leading to tumor immunity. To date, the role of such HO-1 specific $CD8^+$ Treg cells in cell-mediated tissue protection during pregnancy has not been investigated yet.

1.5. Hypothesis and research objectives

We[1] propose that changes of placental HO-1 expression can mediate the effects of environmental challenges on pregnancy. As environmental challenge we employ a stress model simulating perceived psychological stress, which is a daily stressor for a growing number of women in the Western world and can affect fetal development.

Primary hypothesis

Stress challenges during late pregnancy lead to impaired pregnancy outcome.

Secondary hypothesis

Changes in HO-1 expression mediate the effects of stress during pregnancy via immune pathways.

Objectives

To address these hypotheses, the following objectives have been outlined

1. To identify if stress challenges during late pregnancy alter HO-1 expression on the protein level in the placenta.
2. To identify if stress challenges during late pregnancy alter HO-1 mRNA expression in the placenta.
3. To test if stress challenges during late pregnancy affect fetal development and reproductive outcome.
4. To determine the effect of reduced HO-1 expression at the feto-maternal interface on fetal weight and placental function.
5. To assess if fetal weight is modulated by an altered placental function.
6. To identify if stress challenges during late pregnancy alter HO-1 expression in maternal immune cells.
7. To determine if reduced expression of HO-1 affects maternal immune adaptation to pregnancy.

[1] Though all experiments have been carried out independently by myself, I see myself as part of the research group and therefore use *we* instead of *I* in the present thesis.

2. Material und Methoden/Materials and methods

2.1. Mice

6-8 week old virgin female BALB/c mice and 8-10 week old DBA/2J male mice were purchased from Charles River (Sulzfeld, Germany). 6-8 week old virgin female and male BALB/c mice heterozygous for a genetic deletion of the Hmox gene encoding for the enzyme HO-1 ($Hmox^{+/-}$) were obtained as a kind gift from T.Y. Tsui (Department for General Surgery, University Medical Center Hamburg-Eppendorf). All animals were maintained in the Animal Facility of University Medical Center Hamburg-Eppendorf with a 12 hours light/dark cycle. The mice were given mouse chow and water *ad libitum*. The mice were allowed to adjust to the facility for one week prior to mating. Animal care and experimental procedures were conducted according to University Hospital Hamburg-Eppendorf policies, conforming to ethical standards according to the German Animal Welfare Act (Deutsches Tierschutzgesetz). Ethical approval was given by State Authority of Hamburg (ORG_526, G10/067).

2.2. Maternal stress protocol and experimental setup

BALB/c females aged 8-10 weeks were mated with DBA/2J males. Mating was assessed upon the appearance of a vaginal plug, which was checked for every morning. The day of appearance of the plug was designated gestation day (gd) 0.5. Successful pregnancy was determined by the females' weight increase on gd 8.5 and 10.5. Pregnant dams were randomly divided into two groups: (1) control group and (2) stress group. Controls were kept undisturbed throughout pregnancy. The stress group was challenged by exposition to sound stress of 75 dB at 300 Hz emitted for 1 second, 4 times per minute by a mouse repellent device (Digger, X4-Life), placed in the mouse cage for 24 hours commencing between 9-11 a.m. on gd 12.5 and 14.5 (**Figure 6**).

$Hmox^{+/-}$ females aged 8-10 weeks were mated to $Hmox^{+/-}$ males (generated by Yet *et al.* 1999). Pregnant dams were kept undisturbed throughout pregnancy.

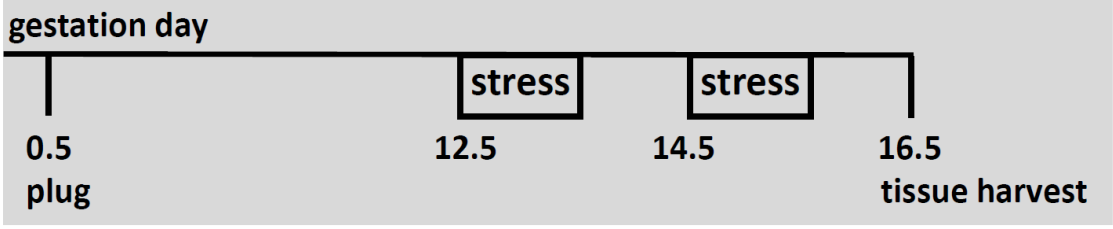


Figure 6 Experimental setup for prenatal stress intervention in the stress group.

2.3. Tissue preparation

2.3.1. BALB/c x DBA/2J mating

On gd 16.5 dams were anesthetized by carbon dioxide and oxygen inhalation and sacrificed by cervical dislocation. The inguinal lymph nodes, which drain the uteri, were collected and implantation sites were documented. The abortion rate was calculated as percentage of abortion sites from number of total implantation sites. Each uterus was rapidly placed in RNAlater® solution (Ambion) at room temperature (RT) to stabilize the RNA for quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). The fetuses were separated from the placenta and body weights were recorded. Tails and legs of the fetus were taken for further PCR/genomic analyses. Lymph nodes were placed into falcon tubes with phosphate buffered saline (PBS), on ice. Placenta samples and decidua were quickly dissected and kept in RNAlater®. After 22-24 hours the samples were moved to -20 °C storage.

2.3.2. Syngeneic BALB/c Hmox$^{+/-}$ mating

The uterus was placed in a petri dish filled with PBS. The fetuses were separated from the placenta and body weight was recorded. Tails and legs of the fetus were taken for PCR/genomic analyses. Lymph nodes were placed into a falcon tube with PBS, on ice. For immunohistochemistry the placenta samples covered with uterus were embedded in cryo-embedding medium TissueTek® (Sakura) and put on dry ice until frozen. The samples were then stored at -80 °C until further use.

2.4. Flow cytometry

2.4.1. Isolation of lymphocytes from inguinal lymph nodes

Tissue was processed to obtain single cell suspensions in FACS buffer (0.5 % BSA in PBS). Briefly, lymph nodes were mashed through a 40 µm filter. Cells were then washed with 8-15 ml ice cold PBS, spun at 450 g for 8 min at 4 °C and re-suspended in 500 µl FACS buffer. Trypan blue negative viable cells were counted using a hemocytometer. FACS analysis was used to further exclude potential dead cells and other debris based on granularity and size.

2.4.2. Flow cytometry protocol

$5x10^5$ isolated lymphocytes were added to a 96-well plate. Cells were spun for 4 min at 400 g and 4 °C, then resuspended in 50 µl solution of Fc Block (antibody anti-CD16/CD32 0.2 % in FACS buffer) to prevent non-specific binding. After 15 min of incubation on ice, cells were washed by addition of 150 µl FACS buffer spinning for 4 min at 400 g and 4 °C. 50 µl of the antibodies mix against specific surface antigens were added at optimized dilutions (**Table 1**),

to cells which did not require extracellular staining 50 µl FACS buffer was added. Cells were incubated on ice for 30 min in the dark and afterwards washed with 150 µl of FACS buffer and spun for 4 min at 400 g and 4 °C. Cells were then permeabilized for 20 min using 50 µl of CytoFix/CytoPerm (BD Biosciences), and washed twice with 150 µl of PermWash (BD Biosciences). Cells were spun for 4 min at 400 g and 4 °C. 50 µl of the solutions of antibodies against intracellular antigens were added as required, and cells were incubated on ice for 30 min in the dark. In wells where no intracellular staining was required, 50 µl of PermWash was added. After incubations cells were washed with 150 µl of PermWash and spun for 4 min at 400 g and 4 °C. All cells were then fixed by adding 200 µl of BD Stabilizing Fixative (BD Biosciences) to each well. Acquisition was performed using FACSCanto II (BD Biosciences), with compensation set using single-color stained samples. Data was analysed using FloJo software (FloJo, TreeStar) to investigate frequency and phenotype of maternal immune cell populations.

Antigen Specificity	Fluorochrome	Clone	Dilution	Company/catalog number
CD3e	APC	145-2C11	1:200	Biolegend/100311
CD279	FITC	J43	1:200	eBioscience/11-9985-82
CD122	PE	TM-b1	1:200	eBioscience/12-1222-81
CD28	PerCP-Cy5.5	37.51	1:200	eBioscience/45-0281-82
CD11c	PE-Cy7	HL3	1:200	BD Pharmingen/558079
CD8a	Pac Blue	53-6.7	1:200	Biolegend/100725
MHCII	FITC	14-4-4S	1:200	BD Pharmingen 553543
HO-1	PE	HO-1-2	1:100	Abcam/ab83214
CD11b	PerCP-Cy5.5	M1/70	1:200	BD Pharmingen/550993

Table 1 List of antibodies used to perform the flow cytometric analysis of leukocytes isolated from inguinal lymph node tissue.

2.5. Genomic PCR

2.5.1. DNA isolation

Fetal tails were collected on gd 16.5. Genomic DNA was isolated using the Tissue DNeasy Kit (Qiagen, Germany) according to the manufacturer's manual instructions.

2.5.2. Polymerase chain reaction (PCR) for fetal sex determination

Isolated genomic DNA was used for PCR analysis to determine fetal gender. PCR was employed to detect the presence or absence of the Y chromosome according to established protocol (Lambert *et al.* 2000) and described primer sequences (**Table 2**, Eurofins) were used. The 402 bp band (Sry) obtained identifies the male fetuses and the 544 bp product (IL3) confirms the correct amplification of the template DNA (**Figure 7**). A 100 bp DNA ladder (Invitrogen) was used as length standard.

Sry Primer 1 (20mer)	5'-TGGGACTGGTGACAATTGTC-3'
Sry Primer 2 (20mer)	5'-GAGTACAGGTGTGCAGCTCT -3'
IL3 Primer 1 (20mer)	5'-GGGACTCCAAGCTTCAATCA-3'
IL3 Primer 2 (20mer)	5'-TGGAGGAGGAAGAAAAGCAA -3'

Table 2 List of primer sequences used for fetal sex determination.

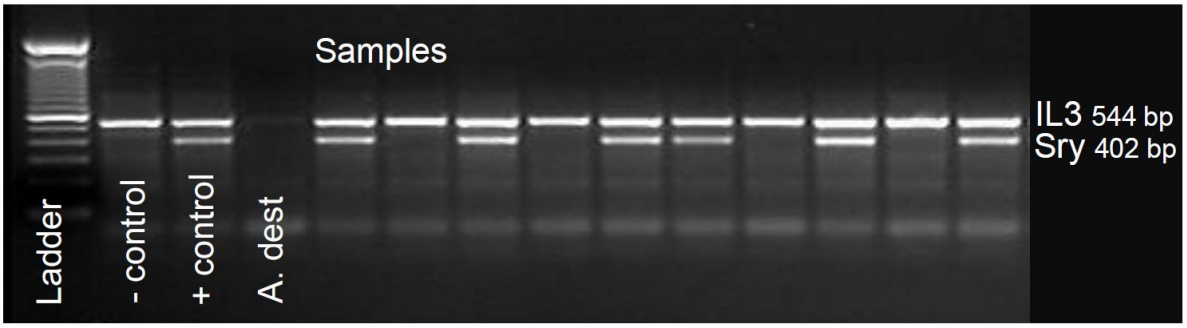


Figure 7 PCR amplification of fetal IL3 and Sry genes. Columns show the electrophoresis results for a female sample used as – (negative) control, a male sample (+ control) and fetal samples. Presence or absence of the Y chromosome was used to determine fetal sex.

2.5.3. PCR for determination of fetal HO-1 genotype

Isolated genomic DNA of the offspring of $Hmox^{+/-}$ mothers was used for PCR determination of the fetal HO-1 genotype. Two sets of primers were specially designed to differentiate wild type (wt) and mutants (**Table 3**, Tib Molbiol, Berlin). 1 µl DNA samples were prepared with 12.5 µl KAPA 2G Fast Ready Mix with Dye (Peqlab), 9.5 µl Diethylpyrocarbonate (DEPC) water (1 ml DEPC (Roth) in 1000 ml distilled water, autoclaved) and 1 µl of required primers in a dilution of 1:10. Amplification protocol for PCR was as follows: initial denaturation 95 °C for 2min, denaturation 95 °C for 45 sec, annealing 60 °C for 1 min, extension 72 °C for 30 sec, final extension 72 °C for 5 min. Wild type ($Hmox^{+/+}$), Heterozygote ($Hmox^{+/-}$) and Knock-out ($Hmox^{-/-}$) genotypes were analysed by gel electrophoresis using Agarose Sieve 3:1 (Biozym Scientific GmbH) in a concentration of 1.5 %. The gel was prepared by dissolving the appropriate amount of agarose in Tris-acetate-EDTA (TAE) buffer (Invitrogen) and boiling in a microwave. After cooling, 4 µl ethidium bromide solution 1 % (Invitrogen, 10 mg/ml) was added and the solution was cast into the gel tray containing a comb. After the gel had solidified, the comb was removed, creating empty wells. Finally the chamber was filled with TAE buffer, so that the gel was fully covered. A 100 bp DNA ladder (Invitrogen) was used as length standard. Electrophoresis was performed at voltage of 110 V. The detection of DNA

bands was performed under UV light using the gel documentation system BioDoc II with the associated software BioDoc Analyse 2.0 by Biometra.

Wild type Primer Forward (19mer)	5'-GGTGACAGAAGAGGCTAAG-3'
Wild type Primer Reverse (19mer)	5'-CTGTAACTCCACCTCCAAC-3'
Mutant Primer Forward (20mer)	5'-TCTTGACGAGTTCTTCTGAG-3'
Mutant Primer Reverse (19mer)	5'-ACGAAGTGACGCCATCTGT-3'

Table 3 List of primer sequences used for fetal HO-1 genotyping.

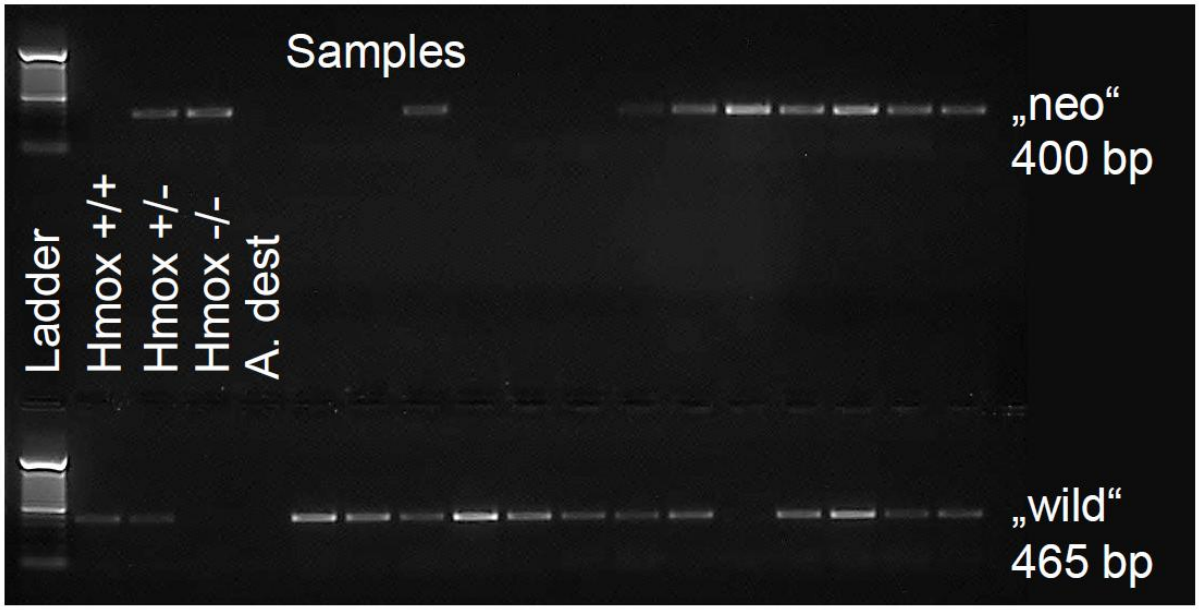


Figure 8 PCR amplification of fetal DNA. Samples represent $Hmox^{+/+}$, $Hmox^{+/-}$ and $Hmox^{-/-}$ DNA amplified with neo and wild primers. The 465 bp band (wild) shows the HO-1 gene. The 400 bp band (neo) obtained identifies the XhoI-BamHI neomycin resistance gene (neo) expression cassette, which is the mutated HO-1 gene. The presence of both bands in the gel electrophoresis indicates a $Hmox^{+/-}$ genotype, whereas only one 465 bp band obtained identifies $Hmox^{+/+}$ and only one 400 bp band identifies $Hmox^{-/-}$ genotype.

2.6. Copy DNA

2.6.1. RNA isolation

Placentas were kept in RNAlater® at -20 °C. The RNA isolation was carried out with the RNeasy Plus Universal Mini Kit (QIAgen) according to the manufacturer's manual. For tissue homogenization Precellys®24 (PeQlab), micro packaging vials (PeQlab) with ceramic beads (1.4 mm) (PeQlab) were employed. Up to 50 mg of placental tissue was placed into the vials and 900 ml Lysis Reagent (QIAzol) were added. Homogenization followed with 2 cycles each 20 sec at 5500 rpm, a break of 5 sec between cycles. Vials were put on ice immediately after homogenizing and RNA isolation was performed. All steps were carried out under sterile and RNase-free conditions.

2.6.2. Determination of RNA quantification and quality

2.6.2.1. RNA quantification

Concentration and purity of RNA was quantified using NanoQuant technology by Tecan (microplate reader infinite® M200, NanoQuant plate™, i-control™ software). Samples were measured in duplicates from a sample volume of 2 µm. The ratio of the absorbance at 260 and 280 nm (A260/280) was used to assess the purity of nucleic acids. For pure RNA, A260/280 is 2.0. The ratios observed in the processed samples ranged from 1.96-2.01.

2.6.2.2. RNA integrity and RNA integrity number (RIN)

Assessment of RNA integrity was performed by Agilent 2100 Bioanalyser. Agilent 2100 Bioanalyser Software and Agilent RNA 6000 Pico Kit were used according to the manufacturer's manual. Agilent RNA kits contain RNA Pico chips and reagents designed for analyses of RNA fragments by electrophoretically separation based on fragment size (**Figure 9**). The RNA integrity number (RIN) is a software tool designed to estimate the integrity of RNA samples. The software assigns an integrity number (1-10) to a sample by analyzing the entire electrophoretic trace of the total RNA sample, including the presence and absence of degradation products. A RIN of 10 means completely intact RNA, whereas a low RIN indicates degenerated RNA. Our samples were assigned a RIN of 7.3 or higher, indicating good conditions for qRT-PCR (Fleige and Pfaffl 2006).

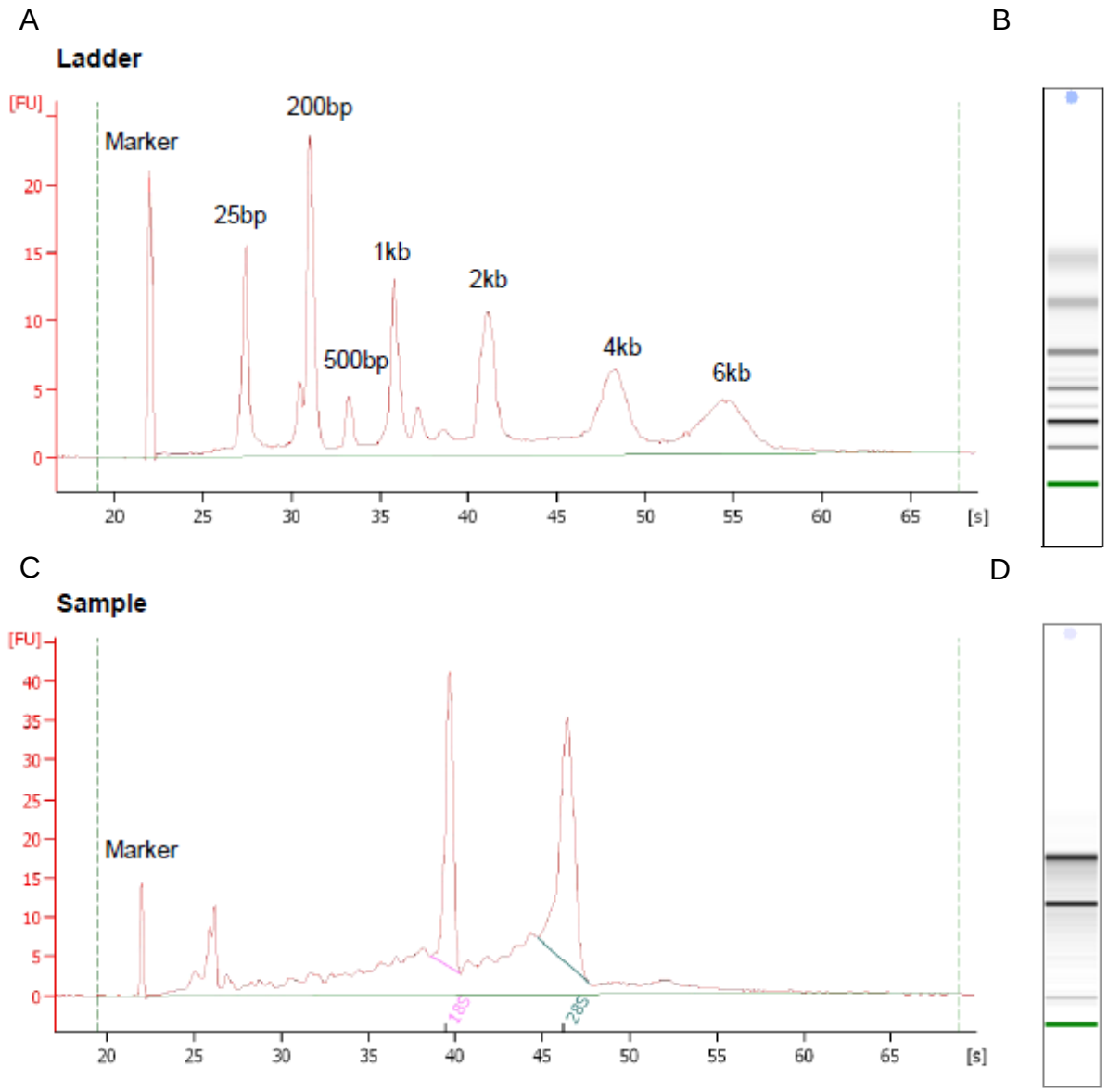


Figure 9 Electropherogram (A) and gel-like image (B) (bioanalyser scan) of the RNA 6000 Pico ladder. It contains six RNA fragments ranging in size from 0.2 to 6 kb at a total concentration of 1 ng/µl. A marker fragment is run with each of the samples to compensate for drift effects that may occur during the course of a chip run. Representative electropherogram (C) and gel-like image (D) of total RNA of murine placenta. Assigned RIN is 7.6. Second and third peak represent rRNA of 18 S and 28 S subunit, very moderate inter-peak noise is present. Small peak at 24-29 seconds represent 5 S and 5.8 S subunits, tRNA and small RNA fragments about 100 bp.

2.6.3. Testing purity of isolated RNA

2.6.3.1. PCR, gel electrophoresis and DNA digestion

Possible DNA contamination of the isolated RNA was tested by PCR and gel electrophoresis (Agarose 1.5 % (Rotigarose, Roth), 110 V, 45 min). The established protocol for

determination of HO-1 genotype was adapted, only wild type primers were used. No further changes were made to the protocol. The presence of a 465 bp band in the gel electrophoresis indicates DNA contamination of the RNA sample and requires purifying contaminated samples before using them in subsequent applications. DNA digestion was completed in all samples using DNA-free™ Kit (Applied Biosystems) containing Ambion® DNase Treatment and Removal Reagents according to the manufacturer's manual. RNase Inhibitor (RNaseOUT, Invitrogen) (40 U/µl) was added to the master mix to prevent loss of RNA due to contamination by RNases.

2.6.4. cDNA transcription

cDNA synthesis was accomplished using reverse transcription. 1 µg/µl RNA of each sample was pipetted into a sterile 0.5 ml eppendorf tube and filled up with DEPC water to a volume of 10 µl. 1 µl of desoxyribonucleotide triphosphates (dNTP) (Invitrogen) and 1 µl solution of random primers (0.25 µg/µl) (Invitrogen) were added and incubated for 5 min at 65 °C. Tubes were chilled on ice quickly afterwards. A master mix containing 4 µl 5X First-Strand buffer (Invitrogen), 2 µl 0.1 M dithiothreitol (DTT) (Invitrogen), 1 µl RNase inhibitor (RNaseOUT, Invitrogen) (40 U/µl) and 1 µl of the enzyme reverse transcriptase (Superscript II RT, Invitrogen) (200 U/µl) was pipetted into each sample. Samples were then incubated for 10 min at 25°C, 50 min at 42°C and 15 min at 70°C. The obtained cDNA was stored at -20°C until further use.

2.6.5. cDNA quantification

Concentration and purity of cDNA was quantified using NanoQuant technology by Tecan (microplate reader infinite® M200, NanoQuant plate™, i-control™ software). Samples were measured in duplicates from a sample volume of 2 µl. The ratio of the absorbance at 260 and 280 nm (A260/280) was used to assess the purity of nucleic acids. For pure DNA, A260/280 is 1.8. Our samples showed ratios between 1.66-1.71.

2.6.7 qRT-PCR

The qRT-PCR enables the simultaneous amplification and detection of specific DNA sequences in real-time. All experiments were performed with the Applied Biosystems StepOnePlus Real-Time PCR Systems using the corresponding software. For relative quantitation of the HO-1 gene the *comparative C_T method* (also referred to as the *$2^{-\Delta\Delta CT}$ method*) was employed (Schmittgen and Livak 2008). The comparative C_T method compares values obtained from two different RNA samples which are directly normalized to a housekeeping gene and presents data as *fold change* in gene expression. As housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was selected. Through a

standard curve in triplets of six different dilutions the efficiency of HO-1 and HPRT amplifications were shown to be 99.34 % (HO-1) and 96.95 % (HPRT) (meaning a standard curve slope of -3.338 and -3.397) and approximately equal (**Figure 10**).

The qRT-PCR was conducted with cDNA as template in 20 µl reactions. For detecting the HO-1 gene and HPRT gene, probes and different primer pairs were designed and ordered at TIB Molbiol (**Table 4**).

The following mixture was used for singleplex reaction of HPRT/HO-1: 10 µl 2x Gene Expression Master Mix (Applied Biosystems), 0.4 µl 20 µM F-primer, 0.4 µl 20 µM R-primer, 0.2 µl 20 µM probe and 7 µl DEPC water. 2 µl cDNA in required dilution were added last to reach a total amount of 30-50 µg cDNA in each well. Triplets were prepared for each sample and target.

All reactions were performed in 45 cycles and conditions were as follows: initial denaturation 50°C for 2 min and 95°C for 9 min, denaturation 95°C for 30 sec, annealing and extension 60°C for 60 sec.

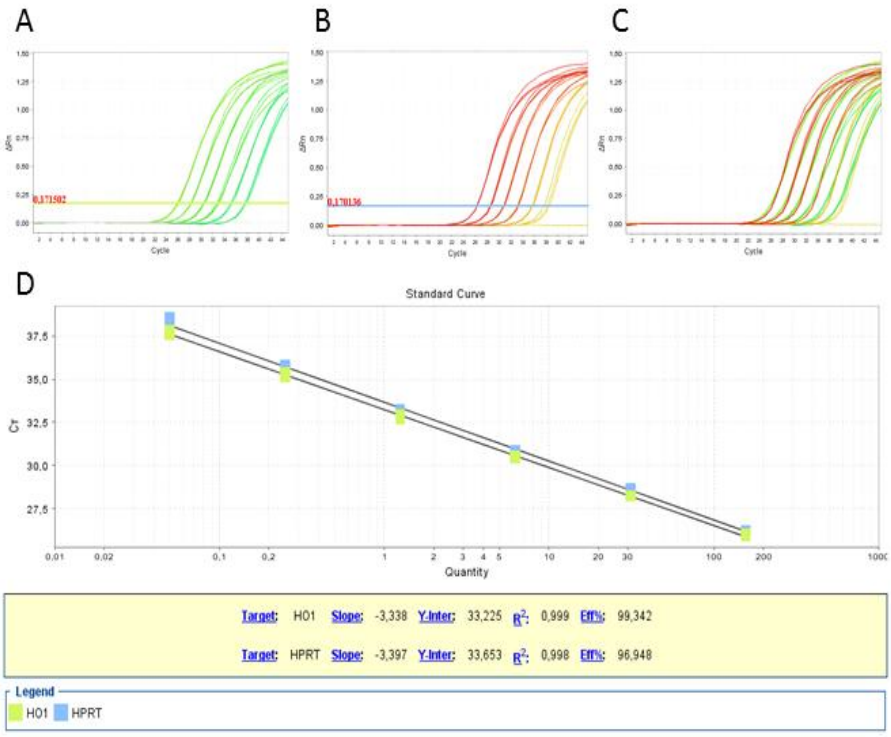


Figure 10 Amplification plots showing increases in fluorescence (*Δ*Rn, baseline-corrected normalized reporter) of HO-1 gene amplification (A), HPRT gene amplification (B) and HO-1 and HPRT gene amplification in one graph (C) plotted against PCR cycle number. Threshold is indicated in red numbers and through a horizontal line. Standard curves of HO-1 and HPRT gene amplification demonstrating efficiencies close to 100 %
(meaning a standard curve slope of -3.32) (D).

HO-1 F-primer (23mer)	5'- TgCTCgAATgAACACTCTggAgA -3'
HO-1 R-primer (19mer)	5'- TgCAggggCAgTATCTTgC -3'
HPRT F-primer (24mer)	5'- ggAATTTgAATCACgTTTgTgTCA -3'
HPRT R-primer (24mer)	5'- TTTTACTggCAACATCAACAggAC -3'
HO-1 probe	5'- 6FAM-ACgAAgTgACgCCATCTgTgAgggA—BBQ -3'
HPRT probe	5'- 6FAM-TTgCAgATTCAACTTgCgCTCAXTCTT—PH -3'

Table 4 List of primer and probe sequences used for qRT-PCR amplification of HO-1 and HPRT cDNA.

2.7. Histological methods

2.7.1. Preparation of frozen placenta sections

Frozen placenta tissues were cut into 8-10 µm histological sections using a cryotome (Microm, HM505 E). Tissue sections were placed on aminosilane-coated glass slides (GoldLine Microscope Slides, frosted, VWR international) and dried at RT. Some slides were stored in -20 °C for Masson Trichrome staining. The remaining slides were fixed in acetone for 10 min at -20 °C and stored at -20 °C after drying.

2.7.2. Immunohistochemical (IHC) detection of HO-1 in frozen placenta sections

Tissue sections were allowed to dry at RT and surrounded with PAP-pen (Dako). Slides were hydrated in Tris Buffered Saline (TBS) 0.05 M, 0.88 % NaCl, pH 7.5 (Trizma base (Sigma) 0.0074 M, sodium chloride 0.0435 M (Sigma-Aldrich), Trizma hydrochloride 0.15 M (Sigma)). Non-specific binding of the avidin biotinylated peroxidase complex and of the antibodies was blocked by the incubation of the sections with avidin and biotin blocking solutions (Vector) and Protein Blocking Agent (Immunotech) following manufacturer's instructions. The slides were incubated with the primary antibody solution (**Table 5**) in a humid chamber overnight at 4 °C. Slides were rinsed thoroughly. Endogenous peroxidase was blocked by incubation in 3 % H_2O_2 in methanol (Fischer) for 30 min. The slides were washed in TBS and incubated with the secondary antibody solution for 30 min at RT. As amplification system, we used avidin biotinylated peroxidase complex (ABC-PO, VECTASTAIN®, Vector) 1:100 in TBS for 30 min, which binds to the biotinylated secondary antibody. The signal was detected by incubating the samples in a solution of H_2O_2 and diaminobenzidine (DAB Chromogen, Dako) in TBS which stains the positive cells in brown colour. This was followed by a light counterstaining with 0.1% Meyer's haematoxylin (Roth). Slides were rinsed in running tap water followed by dehydration in ethanol 80 %, 90 %, 96 % and 100 %, and HistoClear (substitute for xylene,

National Diagnostics) twice for 5 min. The slides were mounted using vitroglut and cover slips. Slides were examined using a Zeiss Axioscope light microscope and photo documentation was performed with a digital image analysis system (Zeiss KS400).

Solution	Antigen Specificity	Origin	Conjugated	Dilution factor	Diluent	Company/catalog number
1st antibody	HO-1	Rabbit	-	1:100	1% FCS in TBS	Epitomics/1922-1
2ry antibody	Rabbit IgG	Goat	Biotin	1:200	2% FCS 4% MNS in TBS	Jackson-Immunotech/800-367-5296

Table 5 Antibody solutions used for IHC detection of HO-1. FCS= fetal calf serum; MNS= mouse normal serum.

2.7.3. Masson-Goldner trichrome staining of frozen placenta sections

Masson-Goldner trichrome staining kit (VWR international) was used to visualize the different components of placental tissue. The kit consists of three different staining solutions. Azophloxine and phosphotungstic acid Orange G solutions stain components such as muscle, cytoplasm and erythrocytes. Light green SF solution counterstains connective tissue whereas Weigert's iron hematoxylin (Waldeck GmbH&Co. KG, Division Chroma) stains the nuclei.

Placental tissue cryosections were allowed to dry at RT and fixed for 1 hour in Formalin 10 % in PBS. Subsequently, sections were fixed overnight in Bouin's solution (RT) in order to intensify the colors and increase the contrasts between the tissue components. Slides were washed in tap water and rinsed briefly in distilled water. Tissue sections were dehydrated twice in ethanol 70 %. Weigert's solution was prepared following manufacturer instruction and applied to the tissue sections. Slides were rinsed in tap water followed by acetic acid 1 % in destilled water. Azophloxine staining solution (Goldner 1) was applied and incubated for 10 min. Slides were rinsed again in 1 % acetic acid and incubated in phosphotungstic acid Orange G (Goldner 2). After washing in acetic acid 1 % the slides were placed into Light green SF solution for 2 min. Finally, the slides were rinsed in acetic acid 1 % and dehydrated in ethanol 70 %, 96 %, twice in ethanol 100 %, and twice in HistoClear (substitute for xylene, National Diagnostics). The slides were mounted using vitroglut and cover slips.

2.8. Statistical analyses

For statistical analyses, continuous outcomes were calculated as the mean and SEM, which were used for data presentation. One-way analyses of variance were carried out for normally

distributed data and Bonferroni corrected level of significance for the number of tests performed was used. For not normally distributed data, the independent two sample Mann-Whitney U-tests was used, for analyses of three or more samples Kruskal-Wallis test was employed. Level of significance was set at a p-value of < 0.05.

All statistical analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

3. Ergebnisse/Results

3.1. HO-1 protein is expressed in all placental layers on gd 16.5.

Evaluation of HO-1 expression in placental tissue sections stained by immunohistochemistry for HO-1 showed that HO-1 protein is expressed in placenta on gd 16.5. Single HO-1 positive cells can be identified in labyrinth, junctional zone layers and also in decidua and myometrium (maternal tissue layers) (**Figure 11 A-C**). To assess if HO-1 expression was altered by stress, cells expressing HO-1 were quantified in the IHC stained slides. The semi-quantitative technique did not allow for differences in placentas of control and stress challenged dams to reach levels of significance. The number of positive cells was calculated per mm² (**Figure 11 G**, **Table 6**); the placental sizes were similar in both groups (**Figure 11 H, Table 7**).

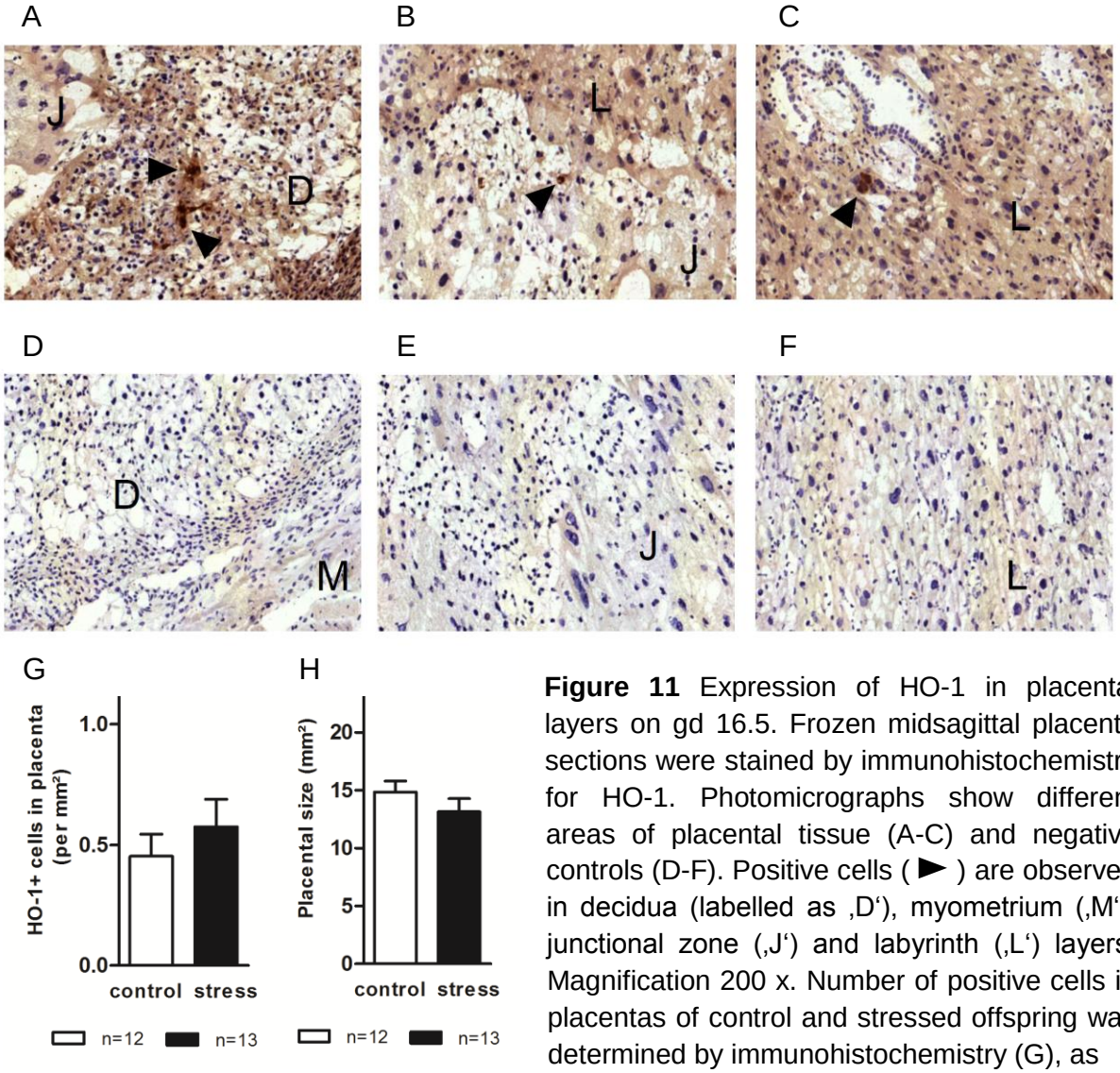


Figure 11 Expression of HO-1 in placental layers on gd 16.5. Frozen midsagittal placenta sections were stained by immunohistochemistry for HO-1. Photomicrographs show different areas of placental tissue (A-C) and negative controls (D-F). Positive cells (►) are observed in decidua (labelled as ‚D'), myometrium (‚M'), junctional zone (‚J') and labyrinth (‚L') layers. Magnification 200 x. Number of positive cells in placentas of control and stressed offspring was determined by immunohistochemistry (G), as well as placental size (H). Bars represent the mean ± SEM. The differences between groups did not reach significance (T-Test).

	Control group (1)		Stress group (2)	
	Mean	SE	Mean	SE
Feto-maternal interface	0.972	0.186	1.065	0.208
Decidua	2.226	0.683	3.738	1.007
Junctional zone	0.25	0.025	0.238	0.137
Labyrinth	0.589	0.124	0.511	0.091
Myometrium	3.91	1.044	4.484	1.241
L/JZ-ratio	2.649	0.129	2.478	0.124

Table 6 Total number of HO-1$^+$ cells (per mm²) in zones of the feto-maternal interface of the offspring, *p < 0.05.

	Control group (1)		Stress group (2)	
	Mean	SE	Mean	SE
Feto-maternal interface	20.391	1.5	18.301	1.043
Decidua	5.479	0.36	4.239	0.601
Junctional zone	3.73	0.14	4.019	0.331
Labyrinth	10.764	0.736	9.854	0.479
Myometrium	2.359	0.354	1.767	0.224

Table 7 Size of the zones at the feto-maternal interface (in mm²) of the offspring, *p < 0.05.

3.2. HO-1 mRNA in placenta decreases in response to stress.

Considering that immunohistochemistry, as a semi-quantitative method, only yields an approximation of the total HO-1 protein in placenta, we decided to do further quantitative testing. qRT-PCR was carried out to identify if changes in HO-1 expression after stress challenge can be found on RNA level in placenta. qRT-PCR results unveil that stress challenge during pregnancy leads to a slight significant decrease of HO-1 mRNA level on gd 16.5 (**Figure 12**).

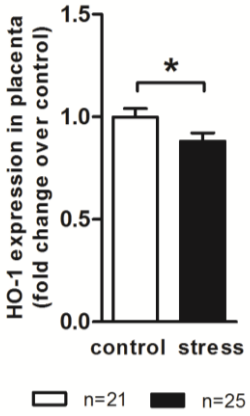


Figure 12 HO-1 expression in placenta, assessed by qRT-PCR. Fold change over control expression was calculated using the comparative C_T method. HPRT was used as endogenous control. Values are displayed as mean ± SEM. *p < 0.05, T-Test.

3.3. Prenatal stress challenge in late pregnancy impairs fetal development, but does not affect reproductive outcome.

Next, we examined if prenatal stress impacts on fetal weight, which was used as a measure for fetal development. Fetal body weight was recorded in two separate experiments and found to be reduced in response to stress (**Figure 13 A, F**), one of them showing a significant reduction (**Figure 13 A**). The pregnancy data of both experiments show that stress challenge does not affect abortion rates (**Figure 13 C, H**) or implantations (**Figure 13 G**). However, a significant increase in number of implantations can be observed in one experiment (**Figure 13 B**).

3.4. Reduced HO-1 expression leads to impaired fetal development and effects reproductive outcome.

To determine if the effect of stress on fetal development was a result of HO-1 reduction in placenta, we used genetically engineered $Hmox^{+/-}$, which are heterozygote for the deletion of HO-1 gene. Similarly to prenatally stressed fetuses $Hmox^{+/-}$ and $^{-/-}$ offspring showed significantly reduced body weight compared to BALB/c wild type fetuses (**Figure 14 A**). This difference was not due to decreased maternal weight in $Hmox^{+/-}$ females. On the contrary, body weight (recorded on gd 0.5) was even significantly higher in $Hmox^{+/-}$ females, compared to wild type BALB/c of similar age (**Figure 14 B**). Further, low HO-1 expression also affects reproductive outcome. Implantations (**Figure 14 C**) and abortion rate (**Figure 14 D**) were significantly increased in $Hmox^{+/-}$ compared to BALB/c wild type mice. Interestingly, litter sizes of syngeneic wild type and $Hmox^{+/-}$ breeding did not significantly differ from each other (**Figure 14 E**), as observed over a period of 12 months. Gender distributions of the offspring found *in utero* (**Figure 14 F**) on gd 16.5 and of the life offspring (**Figure 14 G**) are similar. The frequencies of $Hmox^{+/+}$, $^{+/-}$ and $^{-/-}$ genotype in the offspring (*in utero* or in born pups) did not adjust to Mendel estimated distribution. The yield of $Hmox^{-/-}$ offspring is less than 10% in males and females and does not come close to the expected Mendelian 25%, neither in life offspring, nor *in utero*.

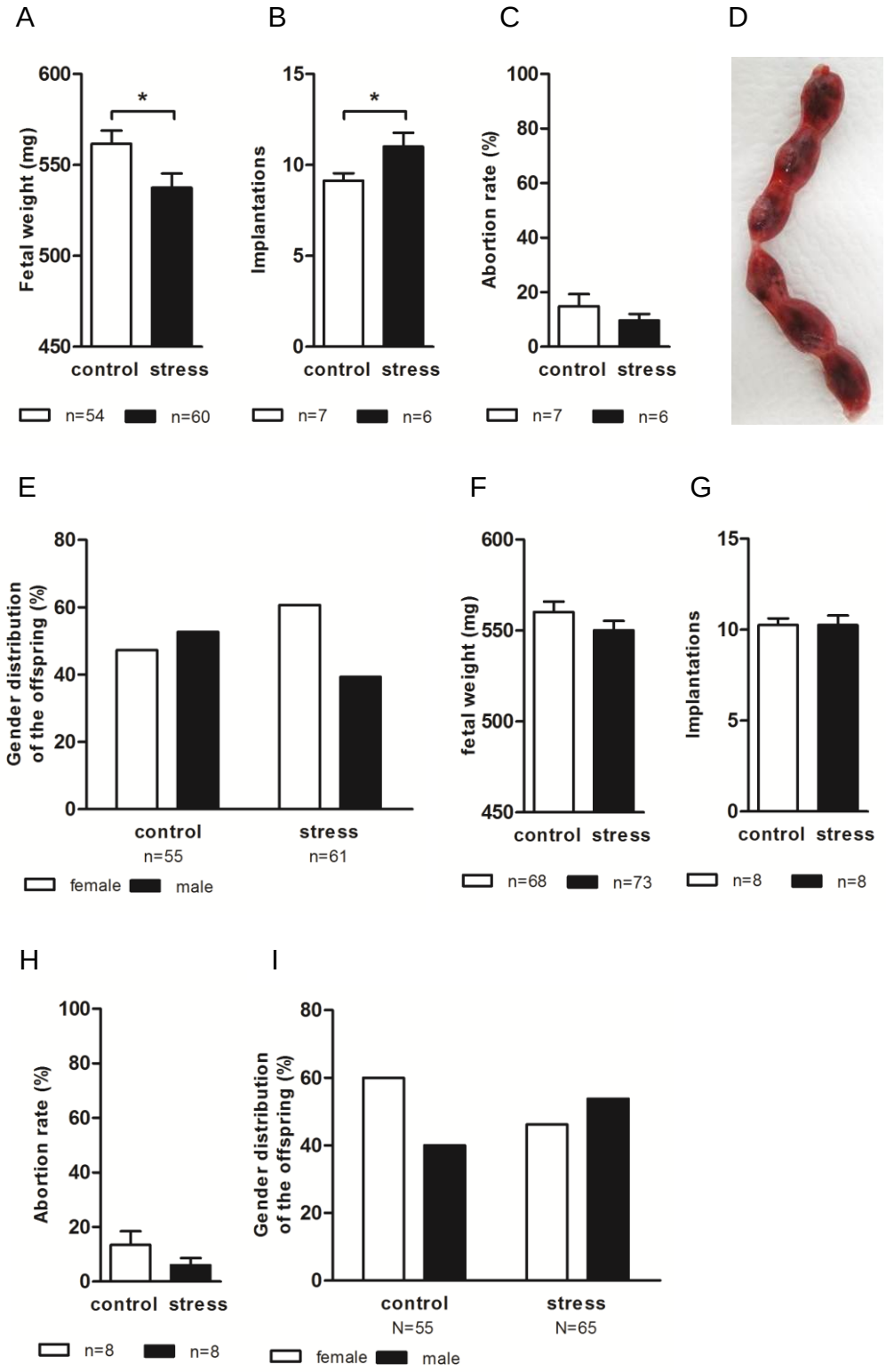


Figure 13 Animal data of two independent experiments using BALB/c control and stressed mice (A-E and E-H), showing fetal body weight of control and stressed offspring (A, F), abortion rates (C, H) and implantations (B, G). (D) shows a photograph of six feto-placental units, pictured as found in vivo. By PCR the frequency of females and males was determined in each experiment (E, H). Values are displayed as mean ± SEM. *p < 0.05, T-Test.

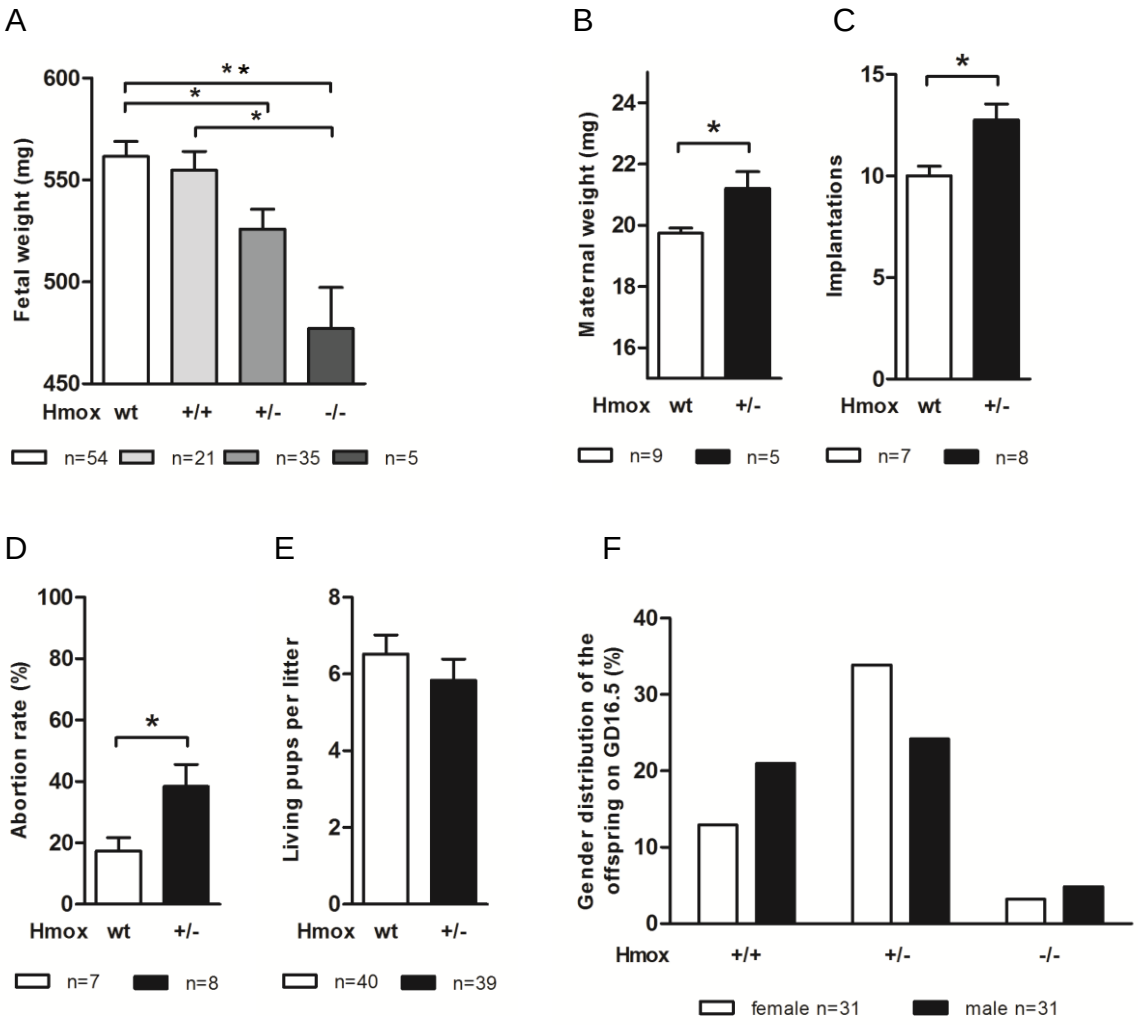


Figure 14 Animal data of genetically engineered BALB/c Hmox$^{+/-}$ breeding in comparison to BALB/c wild type. Shown are fetal body weight of wild type and Hmox$^{+/+}$, $^{+/-}$ and $^{-/-}$ offspring (A), maternal weight on gd 0.5 (B) and reproductive outcome, including implantations (C), abortion rate (D) and litter size (E). By separate PCRs the frequency of HO-1 genotype and sex was determined of the offspring found *in utero* on gd 16.5 (F) and the born life offspring (G). Bars represent the mean ± SEM. *p < 0.05, **p < 0.01, ANOVA, Bonferroni's Mulitple Comparison Test (A); T-Test (B, C, D, F); Mann Whitney U Test (E).

3.5. Impaired fetal development in Hmox$^{+/-}$ and $^{-/-}$ offspring is related to changes in placental function.

In order to unveil if HO-1 induced changes in fetal weight are related to changes in placental function, we analysed histological placenta sections stained for Masson-Goldner trichrome. Reduced HO-1 expression results in smaller placentas in Hmox$^{+/-}$ and even more in $^{-/-}$ offspring (**Figure 15 A**). Conversely, the L/JZ ratio increases in Hmox$^{+/-}$ and $^{-/-}$ offspring (**Figure 15 B**). These results suggest that observed HO-1 induced changes in fetal weight are related to changes in placenta function.

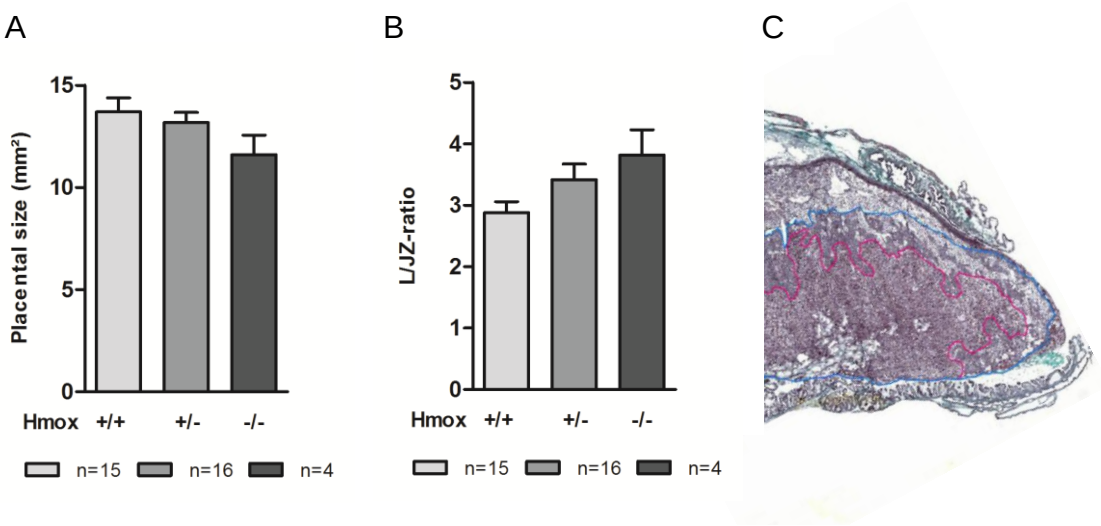


Figure 15 Analyses of histological placenta sections stained for Masson-Goldner trichrome. Placental size (A) and L/JZ ratio (B) were measured using the program Mirax Viewer. C shows a photomicrograph of a representative placental tissue section with measurements for placenta size (blue line) and L (pink line). Values are displayed as mean ± SEM. The differences between groups did not reach levels of significance (ANOVA).

3.6. Reduced HO-1 expression affects maternal immune adaptation to pregnancy.

In the next step, flow cytometry analyses of immune cells from maternal lymph nodes were performed to identify if stress challenge affects maternal immune adaptation to pregnancy. Stress challenge decreased HO-1 expression in maternal immune cells from uterus draining lymph nodes to similar levels than in Hmox$^{+/-}$ dams (**Figure 16 A**). HO-1 is largely produced in T and dendritic cells (**Figure 16 B**). The frequency of CD8^{+} T cells in lymphocytes increased upon stress challenge and even more in Hmox$^{+/-}$ dams (**Figure 16 D**). Interestingly, the CD122^{+}CD8^{+} T regulatory cells, analysed within the CD8^{+} population, conversely decreased in stressed and Hmox$^{+/-}$ dams (**Figure 16 F**).

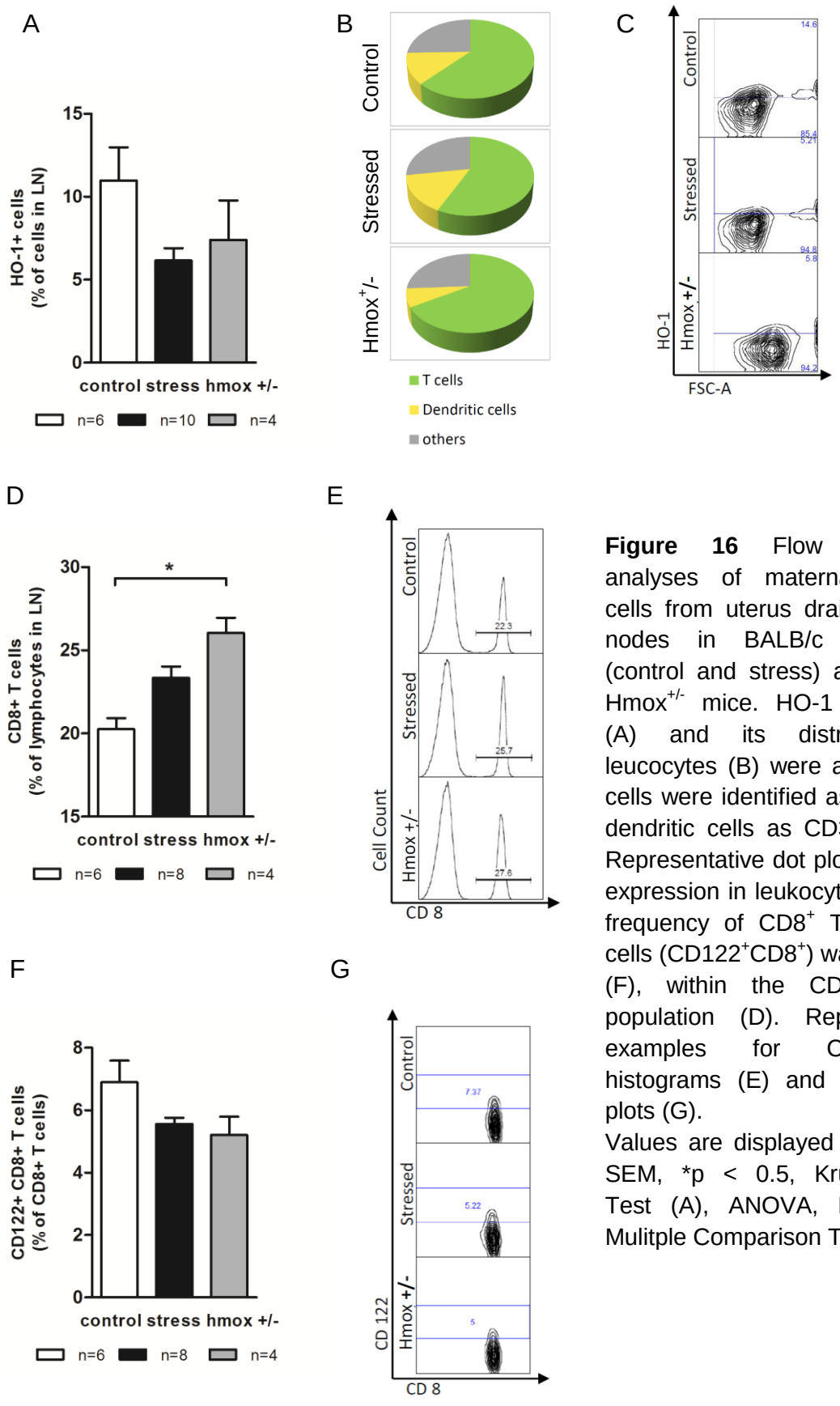


Figure 16 Flow cytometric analyses of maternal immune cells from uterus draining lymph nodes in BALB/c wild type (control and stress) and BALB/c Hmox$^{+/-}$ mice. HO-1 expression (A) and its distribution in leucocytes (B) were assessed. T cells were identified as CD3^{+} and dendritic cells as CD3^{neg}CD11c^{+}. Representative dot plots for HO-1 expression in leukocytes (C). The frequency of CD8^{+} T regulatory cells (CD122^{+}CD8^{+}) was analysed (F), within the CD8^{+} T cell population (D). Representative examples for CD8^{+} cell histograms (E) and CD122 dot plots (G).
Values are displayed as mean ± SEM, *p < 0.5, Kruskal-Wallis Test (A), ANOVA, Bonferroni's Mulitple Comparison Test (D, F).

4. Diskussion/Discussion

The tissue-protecting effects and involvement of HO-1 in a number of inflammatory processes have been described in many tissues. Resulting from extensive research in the field of transplantation, HO-1 could be identified as a protective gene promoting graft acceptance (Soares *et al.* 1998, Pileggi *et al.* 2001). Only little is known about the functions of HO-1 during pregnancy and its role in fetal tolerance. In the present study, we were able to show that reduced HO-1 expression induced by stress or in $Hmox^{+/-}$ mice results in altered immune adaptation to pregnancy, characterized by increased $CD8^+$ T cells, but decreased $CD8^+$ Treg cells (identified by CD122 expression). This coincides with altered placental function and signs for fetal growth restriction in prenatally stressed and $Hmox^{+/-}$ and $^{-/-}$ offspring. Thus, we propose a critical role for HO-1 during late pregnancy as a mediator for cytotoxic $CD8^+$ T cell activity at the feto-maternal interface. Our results suggest that upon activation by placental HO-1 specific $CD8^+$ Treg cells (Andersen *et al*. 2009) can inhibit local inflammation by suppressing cytotoxic $CD8^+$ T cell activity (Rifa'i *et al.* 2004). Thus, in healthy pregnancies $CD8^+122^+$ Treg cells would support fetal development and placental function. In pregnancies with down-regulated placental HO-1 expression, e.g., in response to environmental challenges, such as stress, less $CD8^+$ Treg cells are found resulting in increased $CD8^+$ T cell cytotoxicity and proliferation, which we assume promotes the observed altered placental function and impaired fetal development. The latter, measured by fetal weight, not only predicts a reduced short-term survival of newborns (Chen *et al.* 2011, Cnattingius *et al.* 1998), but also long-term health outcome, as has been demonstrated with respect to the concept of fetal programming in numerous studies in mice and humans, indicating high vulnerability throughout whole pregnancy.

With our present work, we were able to build on to previous findings proposing that perceived stress during late pregnancy can lead to impaired pregnancy outcome in mice and humans (Knackstedt *et al.* 2005, Pincus-Knackstedt *et al.* 2006, Sausenthaler *et al.* 2009) with signs for fetal growth restriction, i. e., reduced fetal body weight. Further, we detected a significant difference in placental HO-1 mRNA levels of prenatally stressed offspring compared to control, indicating a modulation of HO-1 in response to the environmental challenge of stress. No differences were found in matters of HO-1 protein expression, as assessed by IHC, probably because this semi-quantitative method is not sensitive enough. Evaluation of HO-1 protein expression in control placentas, showed positive staining in placenta and decidua cells, as has been reported for rat, guinea pig, human and mouse before (Ihara *et al.* 1998, Odrcich *et al*. 1998, Barber *et al.* 2001, Lyall *et al.* 2000, Zenclussen AC *et al.* 2002). For all dams in our study, reproductive data were documented. In one of our experiments, we observed significantly increased implantation rates in stressed mice compared to control.

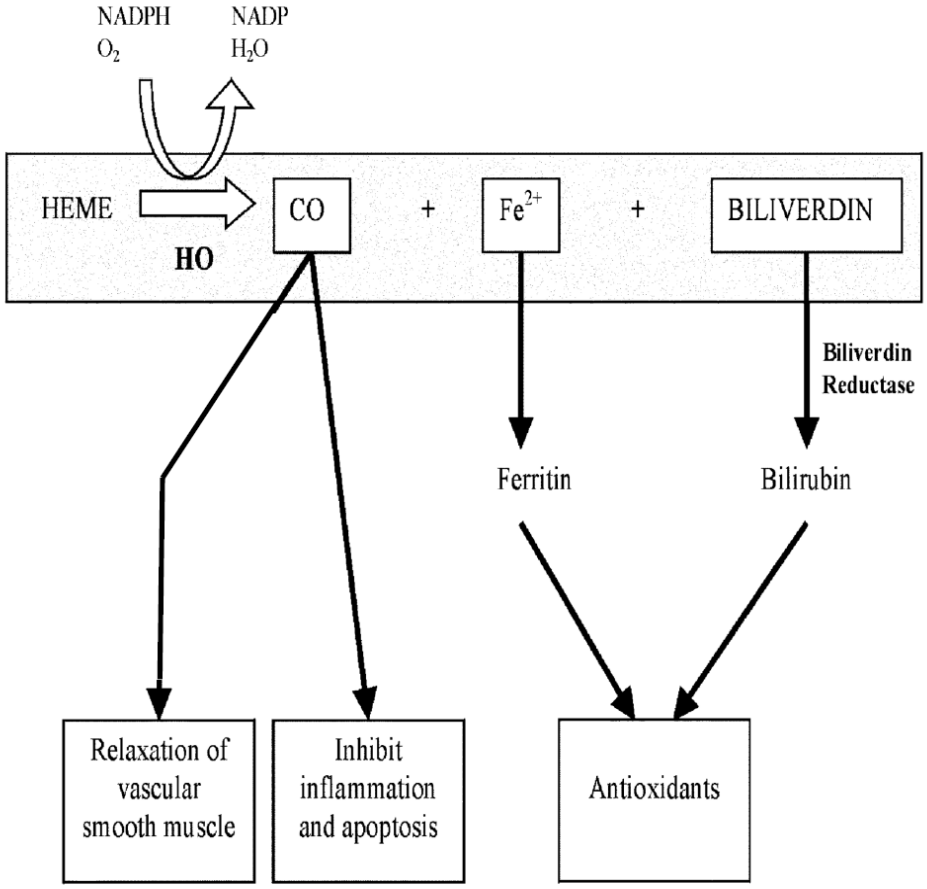
Applied stress challenges to late pregnancy on gd 12.5 and 14.5 do not interfere with implantation as no increased fetal loss rate can be observed, in contrast to stress exposure during peri-implantation period which has high levels of fetal loss despite having similar implantations (Blois *et al.* 2005). Therefore, repeating the experiment with a larger number of mice would most certainly lead to equal implantation rates.

By the use of transgenic $Hmox^{+/-}$ mice (generated by Yet *et al.* 1999) we were able to gain insight into the role of HO-1 *in vivo* in terms of pregnancy and breeding outcome. Genotyping surviving offspring, resulting from heterozygote matings (n=39) over a period of 12 months showed a low yield of $Hmox^{-/-}$ mice, less than the predicted 25% according to Mendel's laws, confirming previous observations (Poss and Tonegawa 1997a, Yet *et al.* 1999, Zhao *et al.* 2009, Zenclussen ML *et al.* 2011). Our findings *in utero* on gd 16.5 showed similar genotype distribution, indicating a high number of intrauterine deaths of offspring with $Hmox^{-/-}$ genotype in early pregnancy (Zhao *et al*. 2009). Consistent to that are the significantly increased abortion rates, which we calculated in $Hmox^{+/-}$ dams, compared to wild type dams. Interestingly, in our experiments the average pup number per litter of $Hmox^{+/-}$ dams did not differ much from litter sizes seen in wild type matings despite high abortion rates and contrary to previous findings (Zhao *et al.* 2009). This could be a result of the increased implantation rates in $Hmox^{+/-}$ dams compensating for the high fetal losses. Recent evidence suggests a key role for HO-1 in the ovary and reports less oocytes and poor fertilization in HO-1 deficient mice (Zenclussen ML *et al.* 2012) which stands in contrast to our observation of significantly larger numbers of total implantations in $Hmox^{+/-}$ mice. Reasons for these unequal findings could maybe be strain-dependent or due to other factors, such as fluctuating hormone levels influencing HO-1 function in the ovary. From the work of Poss and Tonewaga (Poss and Tonewaga 1997a, 1997b) we know that HO-1 knockout mice suffer from anemia, accumulating tissue iron especially in liver and kidneys and systemic inflammation in late adulthood, accompanied by weight loss. Until early adulthood, the $Hmox^{-/-}$ mice appear to be slightly smaller than $Hmox^{+/-}$ and $^{+/+}$ mice. On the basis of these results we expected to see a decrease in body size/weight of $Hmox^{+/-}$ mice compared to wild type mice. However, we observed in our study that $Hmox^{+/-}$ mice of $Hmox^{+/-}$ mothers showed significantly higher body weight in early adulthood than wild type mice born by wild type mothers. This could either be a result of the defective iron metabolism in HO-1 deficient mice and chronic inflammation with e. g. hepatosplenomegaly and/or early kidney damage due to heme and iron accumulation leading to water retention, or the $Hmox^{+/-}$ mice could have developed possibly even intrauterine a permanent mechanism for (over-) compensating the HO-1 deficiency of the heterozygote mother and themselves.

In 1999, the first known human case of HO-1 deficiency was reported by Yachie *et al*. (Yachie *et al.* 1999) who analysed the patient's HO-1 gene and revealed deletions in

maternal and paternal alleles resulting in a complete deficiency of functional HO-1 production. The HO-1 deficient boy presented with clinical symptoms including growth retardation, enhanced systemic inflammatory reactions, reticuloendothelial dysfunction, anemia, asplenia and nephropathy. He died at the age of six years. The presentation of this human case with a short survival stands in contrast to findings in $Hmox^{-/-}$. The mice seem to be less affected by oxidative stressors and suffer predominantly from iron metabolic disorders (Kawashima *et al*. 2002, Yachie *et al*. 1999) with a long survival of up to 22 months (Poss and Tonegawa 1997a). Comparing the clinical symptoms of HO-1 deficient mice and human (Kawashima *et al*. 2002, Yachie *et al*. 1999) not only allows a broader understanding of the pathology of reduced HO-1 expression, it also indicates the clinical relevance of HO-1 e. g. in terms of human pregnancy. The past medical history of the patient's healthy mother reveals two intrauterine fetal deaths (Koizumi 2007), possibly due to her heterozygous HO-1 genotype. Detection of down-regulated HO-1 expression in spontaneous abortions in humans (Zenclussen AC *et al.* 2003) support these findings and emphasize relevance of HO-1 in human pregnancies, with similar evidence found in mice (Zenclussen AC *et al*. 2002, Zenclussen AC *et al*. 2005, Zenclussen ML *et al*. 2006). Further, HO-1 with its known role in maternal vascular tone and fetal hemodynamic functions (Zhao *et al*. 2008) in healthy pregnancies has been identified to be highly involved in the pathophysiology of pregnancy disorders, such as preeclampsia (PE) (reviewed by Bainbridge and Smith 2005) (**Figure 17**), manifesting with a maternal syndrome, including hypertension and proteinuria, and fetal growth restriction, perinatal death and preterm birth. Yet, the underlying mechanisms require identification and data on placental HO-1 expression in PE pregnancies are still controversial. However, an attenuating effect of HO-1 induction could be shown on hypertension in pregnant rats (George *at el*. 2011a) and on the basis of evaluation of HO expression in infarct-damaged placentas of pre-eclamptic human pregnancies a low overall HO function can be postulated (Lash *et al*. 2002, Bainbridge and Smith 2005) with less antioxidant metabolites at the feto-maternal interface, leading to restrictions in placental function and the clinical picture of PE.

Considering our present results, we can speculate that these complications are also related to a failure to establish fetal tolerance through via HO-1 activated $CD8^+$ Treg cells and consequent induction of $CD8^+$ T cell proliferation and cytotoxicity (Molvarec *et al.* 2011). The proposed involvement of HO-1 in the development of PE raises the question if pregnant transgenic $Hmox^{+/-}$ dams suffer from PE, which to our best knowledge has not been investigated yet and could simply be detected i.e. by taking blood pressure of pregnant mice. The transgenic Hmox-1 deficient mice would represent a new approach for further investigating the role of HO-1 in PE.



Healthy pregnancy	**Maintenance of adequate blood flow through uterine and placental blood vessels**		**Inhibition of excessive activation of inflammatory and apoptotic cascades in placental tissue**	**Maintenance of a healthy oxidant-antioxidant balance within the placenta**	
PE pregnancy	**Decreased and oscillating blood flow through placental blood vessels**		**Heightened activation of inflammatory and apoptotic cascades in placental tissue**	**Increased levels of ROS coupled with a decrease in antioxidant capacity within the placenta**	

Figure 17 Heme degradation pathway and the physiological roles of its metabolic breakdown products during healthy and PE pregnancy. (Modified after: Bainbridge and Smith 2005)

In addition to mutations of HO-1 gene alleles in humans, a GT-repeat polymorphism in the promoter region of the gene has been described, with the length directly associated to transcription in response to oxidative stress (Lavrovsky et al. 1994) and hence, associated to the cytoprotective effect, as could be shown in cardiovascular disease (Exner *et al*. 2001, Kaneda *et al*. 2002, Chen *et al*. 2002, Bai *et al*. 2010). It is very likely that the investigated polymorphism also affects pregnancy and placental HO-1 expression, as Denschlag *et al.* demonstrated an association of the HO-1 gene polymorphism in woman with idiopathic recurrent miscarriage (Denschlag *et al.* 2004).

Taking into account all information on HO-1 in pregnancy with its mediator function for $CD8^+$ T cell activity, its modulation occurring in abortions, PE and in response to environmental challenges such as stress and genetic variances, HO-1 may be proposed as a pregnancy-associated biomarker for prediction of such pregnancy pathologies and characterization of susceptible women enabling prophylactic/early treatment. To effectively achieve clinical application of HO-1 as biomarker, further research is necessary, e. g. the identification of normal range of serum HO-1 levels, marking a low risk to develop complications during pregnancy, or the investigation of the relationship between serum HO-1 levels and the severity of pregnancy disorders, such as severe PE which has been shown to be associated with elevated serum HO-1 levels ante- and postpartum, compared to mild pre-eclampsic and normotensive pregnant women (Vitoratos *et al*. 2011).

The observed key functions of HO-1 in creating a stable and protective intrauterine environment further indicate its putative role as a therapeutic target in complicated pregnancies. With regard to the present results, it is tempting to reason that in pregnancies with deteriorating placental function resulting from reduced placental HO-1 expression, e.g. due to genetic or environmental conditions, HO-1 up-regulation could lead to ameliorated placental function and prevent negative consequences, such as the fetal programming of (adult) diseases and fetal growth restriction. In abortions, where HO-1 down-regulation associated with increased Th1/Th2 cytokine production was reported, the up-regulation of HO-1 has been proposed to be pregnancy-protective and avoid abortion onset (Zenclussen AC *et al*. 2005). Additionally, George *et al*. recently proposed a novel therapeutic activity of HO-1 induction for PE treatment in pregnant rats (George *et al*. 2011a, 2011b, George and Arany 2012, George *et al.* 2012). This is a promising finding, since to date, there is no causal treatment known for PE which is a major cause for fetal-maternal morbidity and mortality worldwide (World Health Report 2005). Recent studies, pointing out the potential of CO as therapy for pregnancy complications, further reaffirm the idea of HO-1 as therapeutic target, for CO has been found to mimic the protective effects of HO-1 activity at the feto-maternal interface (Zenclussen ML *et al.* 2011, El-Mousleh *et al.* 2012). Through increased numbers of activated $CD8^+$ Treg cells and anti-oxidative metabolites at the feto-maternal interface, HO-1

induction would represent a way to strengthen natural protection and defense mechanisms of the body.

There are various stimuli that induce HO-1, including heme and cobalt protoporphyrin (CoPP) (Ryter *et al.* 2006). Most of them cause oxidative stress in cells and are toxic in high amounts, such as heme. CoPP, a potent and effective inducer of HO-1 has been employed in many rodent models and *in vitro,* demonstrating protective effects through HO-pathway, and considered as a potential therapeutic agent in situations where up-regulation of HO-1 is desired (Shan *et al.* 2007). However, this was criticized at once and adverse effects of CoPP known from animal models and cell cultures were listed for why CoPP could not be used in clinical studies (Schmidt 2007), raising the question which of the noted HO-1 inducers could be safe for application in human, especially during pregnancy with most substances crossing the placenta. Considering approved pharmacological drugs with rare and in detail characterized side effects and the ability to induce HO-1, such as statins (Kim *et al.* 2012, Li *et al.* 2012), Aspirin® (Grosser *et al.* 2003) and votile anesthetics (Hoetzel *et al.* 2002, Hoetzel and Schmidt 2010), seemed like a more promising approach for clinical application. But the use of such drugs has not yet been investigated to an adequate extent in pregnant women and (contradictory) evidence on possible teratogenicity could not fully establish safe use during pregnancy (Kozer *et al.* 2003, Kenis *et al.* 2005, James *et al.* 2008, Palanisamy *et al.* 2011a, Wang *et al.* 2012, Kong *et al.* 2012a, 2012b).

Another approach to avoid pregnancy complications that are based on low HO-1 expression could be a prophylactic approach. For we know that perceived stress during pregnancy decreases HO-1 expression significantly, pregnant women should prevent daily hassles and stress, which turns into a challenge with more and more women focusing on advanced education and job opportunities. Support can be provided by specialists in the field of complementary and alternative medicine, who offer e.g. progressive relaxation programs (Bastani *et al.* 2005, 2006), mindful yoga (Beddoe *et al.* 2009), music, massage and aroma therapy (Hodgson *et al.* 2007) which all have been shown to decrease maternal stress and anxiety during pregnancy. In women with genetic mutations or relevant polymorphism for HO-1 gene, stress prevention and alleviation through alternative therapies might not suffice. To avoid pregnancy complications and the lack of via HO-1 activated $CD8^+$ Treg cells, treatment by adoptive-cell-transfer could be an option. Adoptive immunotherapy is a promising approach to induce anti-tumor immunity in cancer patients (Dudley and Rosenberg 2003, Sun *et al*. 2010) and could be performed in pregnant women through isolating $CD8^+$ Treg cells, their *ex vivo* expansion and activation and subsequent autologous transfusion, assuming the cells reach the placenta and suppress local cytotoxicity.

To expand and clarify knowledge of the role of HO-1 during pregnancy, examining parameters that possibly interact with HO-1, such as maternal age, body weight, fetal HO-1

expression and hormones is necessary. In our experiments we measured serum levels of progesterone, a pivotal and pregnancy-supportive hormone whereof only subtle deficits in serum levels can affect maternal adaptation to pregnancy, resulting in reduced birth weight (Mucci *et al.* 2004, Hartwig *et al.* 2012), to unravel possible effects on HO-1 function. The serum progesterone levels turned out to be similar for control and $Hmox^{+/-}$ dams (data not shown) indicating that progesterone neither affects HO-1 expression, nor cytotoxic $CD8^+$ T and Treg cell function.

In summary, our results indicate a role for $CD8^+122^+$ T cells in supporting fetal development and placental function. Our insights expand the proposed role of HO-1 during pregnancy of which the expression is reduced in response to stress. Reduced HO-1 expression leads to altered immunological adaptation to pregnancy via HO-1, a mechanism potentially contributing to fetal programming and the pathogenesis of certain pregnancy disorders. Thus, these results have relevance for both basic science and clinical contexts, providing a platform for further investigations that could lead to prophylactic and curative treatment to ensure stable conditions for the fetus to develop and prevent low birth weight/IUGR, which is protective for short- and long-term health.

5. Zusammenfassung/Summary

The inducible enzyme heme oxygenase-1 (HO-1) plays a regulatory role in a number of inflammatory processes. It catabolizes the degradation of heme and acts as an anti-oxidant and cytoprotectant through its products biliverdin, carbon monoxide and free iron. In the placenta, HO-1 is highly expressed and assumed to mediate pregnancy-protective effects, but its function throughout pregnancy is not well understood.

Our objective was to examine if a decreased HO-1 expression, e.g., upon stress challenges during late murine pregnancy or in heterozygous HO-1 knockout mice (further referred to as $Hmox^{+/-}$) affect fetal development and maternal immune adaptation.

DBA/2J-mated BALB/c females were exposed to 24 hour sound stress on gestation days (gd) 12.5 and 14.5. On gd 16.5 placental tissue and uterus-draining lymph nodes (LN) were collected, fetal weight was documented. Placental HO-1 expression was analysed by immunohistochemistry and quantitative real time-PCR. $Hmox^{+/-}$ mice were syngeneically mated. On gd 16.5 tissue was collected and fetal HO-1 genotype and weight was recorded. Placental tissue sections were stained with Masson's trichrome to study placental function. Phenotype and frequency of maternal immune cells harvested from uterus-draining LN were identified by flow cytometry. For statistical analyses, continuous outcomes were checked for normal distribution. If data was normally distributed, one-way analyses of variance were carried out. For not normally distributed data, Mann-Whitney U-tests were used. Level of significance was set at a p-value of 0.05.

Stress challenges led to a reduced fetal weight. Further, stress challenge lowered HO-1 tissue expression at the feto-maternal interface and significantly decreased HO-1 expression largely in T and dendritic cells among maternal leucocytes from LN, along with an increase in $CD8^+$ T cells. The increased frequency of $CD8^+$ T cells inversely correlated with a reduced expression of CD122 on $CD8^+$ T cells in stressed mice, compared to control. $CD8^+CD122^+$ T cells are presumed to have regulatory, pregnancy-protective functions.

Similarly, an increased frequency of $CD8^+$ T cells and a reduction of $CD122^+$ could be detected on $HO\text{-}1^{+/-}$ dams, compared to wild type (wt) controls. Lower fetal weight was observed in $Hmox^{+/-}$ and $^{-/-}$ offspring, compared to wt fetuses of the same litter. Placental size was slightly decreased in $^{+/-}$ offspring when compared to wt. This was accompanied by altered ratios of functional placental areas (labyrinth/junctional zone).

Our results show that reduced HO-1 expression induced by stress or present in $Hmox^{+/-}$ mice results in an impaired immune adaptation to pregnancy, characterized by increased $CD8^+$ T cells and decreased $CD8^+$ regulatory T cells, which are pregnancy-protective functions and of which generation is presumably induced by placental HO-1. This coincides with altered placental function and signs for fetal growth restriction. Reduced HO-1 expression could

thereby have detrimental short- and/or long-term effects on offspring development and health. To prevent these effects pharmacological interventions could target HO-1 to modulate immune tolerance during pregnancy.

Some of these data have been included in a manuscript which has been submitted to the Journal of Clinical Investigation on December 3, 2012.[2]

[2]María Emilia Solano, Mirka Katharina Kowal, Chressen Catherina Much, Huang Ho, Caitlin Jago, Denys deCatanzaro, Khalil Karimi, Victoria J. Parker, Kurt Hecher, Petra Clara Arck Regulation of fetal development by $CD8^+$ T cells via progesterone and heme oxygenase-1-dependent pathways. Journal of Clinical Investigation (JCI).

6. Abkürzungsverzeichnis/List of abbreviations

APC	antigen presenting cell
CD	cluster of differentiation
cDNA	copyDNA
CO	carbon monoxide
CoPP	cobalt protoporphyrin
DC	dendritic cell
DEPC	diethylpyrocarbonate
DNA	desoxyribonucleic acid
dNK	decidual natural killer
Fe^{2+}	ferrous iron
gd	gestation day
HLA	human leukocytes antigen
HO-1	heme oxygenase-1
HPRT	hypoxanthine-guanine phosphoribosyltransferase
IL	interleukin
IUGR	intrauterine growth restriction
L/JZ ratio	labyrinth/junctional zone ratio
mRNA	messengerRNA
NADPH	nicotinamide adenine dinucleotide phosphate
NK	natural killer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	preeclampsia
qRT-PCR	quantitative real time-PCR
RIN	RNA integrity number
RNA	ribonucleic acid
RT	room temperature
TAE	tris-acetate-EDTA
TBS	tris buffered saline
Treg	T regulatory
wt	wild type

7. Literaturverzeichnis/References

Acevedo CH, Ahmed A (1998) Hemeoxygenase-1 inhibits human myometrial contractility via carbon monoxide and is upregulated by progesterone during pregnancy. J Clin Invest; 101(5):949-55.

Andersen MH, Sørensen RB, Brimnes MK, Svane IM, Becker JC, thor Straten P (2009) Identification of heme oxygenase-1-specific regulatory $CD8^+$ T cells in cancer patients. J Clin Invest; 119(8): 2245-56.

Aluvihare VR, Kallikourdis M, Betz AG (2004) Regulatory T cells mediate maternal tolerance to the fetus. Nat Immunol; 5(3):266-71.

Babusikova E, Jesenak M, Durdik P, Dobrota D, Banovcin P, (2008) Exhaled carbon monoxide as a new marker of respiratory diseases in children. J Physiol Pharmacol; 59 Suppl 6:9-17.

Bai CH, Chen JR, Chiu HC, Chou CC, Chau LY, Pan WH (2010) Shorter GT repeat polymorphism in the heme oxygenase-1 gene promoter has protective effect on ischemic stroke in dyslipidemia patients. J Biomed Sci; 17: 12.

Bainbridge SA, Smith GN (2005) HO in pregnancy. Free Radic Biol Med; 38(8): 979-88.

Balla J, Balla G, Nath K, Vercellatti GM (1992) Endothelial cell heme oxygenase and ferritin induction by heme proteins: a possible mechanism limiting shock damage. Clin Res; 40:323A.

Barber A, Robson SC, Myatt L, Bulmer J, Lyall F (2001) Heme oxygenase expression in human placenta and placental bed: reduced expression of placenta endothelial HO-2 in preeclampsia and fetal growth restriction. FASEB J; 15: 1158– 68.

Barker DJ, Osmond C (1986) Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet; 1:1077-81.

Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ (1989) Weight in infancy and death from ischaemic heart disease. Lancet; 2(8663):577-80.

Bastani F, Hidarnia A, Kazemnejad A, Vafaei M, Kashanian M (2005) A randomized controlled trial of the effects of applied relaxation training on reducing anxiety and perceived stress in pregnant women. J Midwifery Womens Health; 50(4): e36-40.

Bastani F, Hidarnia A, Montgomery KS, Aguilar-Vafaei ME, Kazemnejad A (2006) Does relaxation education in anxious primigravid Iranian women influence adverse pregnancy outcomes?: a randomized controlled trial. J Perinat Neonatal Nurs; 20(2): 138-46.

Beard JR, Lincoln D, Donoghue D, Taylor D, Summerhayes R, Dunn TM, Earnest A, Morgan G (2009) Socioeconomic and maternal determinants of small-for-gestational age births: patterns of increasing disparity. Acta Obstet Gynecol Scand; 88(5):575-83.

Beddoe AE, Paul Yang CP, Kennedy HP, Weiss SJ, Lee KA (2009) The effects of mindfulness-based yoga during pregnancy on maternal psychological and physical distress. J Obstet Gynecol Neonatal Nurs; 38(3): 310-9.

Betts KS, Williams GM, Najman JM, Alati R (2011) The association between birth weight and anxiety disorders in young adults. J Anxiety Disord; 25(8): 1060-7.

Blois SM, Ilarregui JM, Tometten M, Garcia M, Orsal AS, Cordo-Russo R, Toscano MA, Bianco GA, Kobelt P, Handjiski B, Tirado I, Markert UR, Klapp BF, Poirier F, Szekeres-Bartho J, Rabinovich GA, Arck PC (2007) A pivotal role for galectin-1 in fetomaternal tolerance. Nat Med; 13(12): 1450-7.

Carbone J, Gallego A, Lanio N, Chean C, Navarro J, Sarmiento E (2010) Peripheral blood $CD8^{+}DR^{+}$ T-cell count: a potential new immunologic marker of unexplained recurrent abortion. Fertil Steril; 94(1):360-1.

Chen YH, Lin SJ, Lin MW, Tsai HL, Kuo SS, Chen JW, Charng MJ, Wu TC, Chen LC, Ding YA, Pan WH, Jou YS, Chau LY (2002) Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. Hum Genet; 111(1): 1-8.

Chen HY, Chauhan SP, Ward TCS, Mori N, Gass ET, Cisler RA. (2011) Aberrant fetal growth and early, late, and postneonatal mortality: an analysis of Milwaukee births, 1996–2007. Am J Obstet Gynecol; 204:261.

Cnattingius S, Haglund B, Kramer MS (1998) Differences in late fetal death rates in association with determinants of small for gestational age fetuses: population based cohort study. BMJ; 316(7143): 1483-7.

Curhan GC, Chertow GM, Willett WC, Spiegelman D, Colditz GA, Manson JE, Speizer FE, Stampfer MJ (1996a) Birth weight and adult hypertension and obesity in women. Circulation; 94(6): 1310-5.

Curhan GC, Willett WC, Rimm EB, Spiegelman D, Ascherio AL, Stampfer MJ (1996b) Birth weight and adult hypertension, diabetes mellitus, and obesity in US men. Circulation; 94(12): 3246-50.

Denschlag D, Marculescu R, Unfried G, Hefler LA, Exner M, Hashemi A, Riener EK, Keck C, Tempfer CB, Wagner O (2004) The size of a microsatellite polymorphism of the haem oxygenase 1 gene is associated with idiopathic recurrent miscarriage. Mol Hum Reprod; 10(3): 211-4.

Dudley ME, Rosenberg SA (2003) Adoptive-cell-transfer therapy for the treatment of patients with cancer. Nat Rev Cancer; 3(9): 666-75. Review.

Eisenstein RS, Garcia-Magol B, Petingell W, Munro MN (1991) Regulation of ferritin and heme oxygenase synthesis in rat fibroblasts by different form of iron. Proc Natl Acad Sci USA; 88:688– 692.

Elmarakby AA, Jessica Faulkner J, Baban B, Sullivan JC (2012) Induction of Hemeoxygenase-1 Reduces Renal Oxidative Stress and Inflammation in Diabetic Spontaneously Hypertensive Rats. Int J Hypertens; 2012: 957235.

El-Mousleh T, Casalis PA, Wollenberg I, Zenclussen ML, Volk HD, Langwisch S, Jensen F, Zenclussen AC (2012) Exploring the potential of low doses carbon monoxide as therapy in pregnancy complications. Med Gas Res; 2(1): 4.

Erlebacher A, Vencato D, Price KA, Zhang D, Glimcher LH (2007) Constraints in antigen presentation severely restrict T cell recognition of the allogeneic fetus. J Clin Invest;117(5):1399-411.

Exner M, Schillinger M, Minar E, Mlekusch W, Schlerka G, Haumer M, Mannhalter C, Wagner O (2001) Heme oxygenase-1 gene promoter microsatellite polymorphism is

associated with restenosis after percutaneous transluminal angioplasty. J Endovasc Ther; 8(5): 433-40.

Fleige S, Pfaffl MW (2006) RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med; 27(2-3): 126-39.

Fowden AL, Ward JW, Wooding FPW, Forhead AJ, Constancia M (2006) Programming placental nutrient transport capacity. J Physiol; 572.1: 5–15.

George EM, Arany M, Cockrell K, Storm MV, Stec DE, Granger JP (2011a) Induction of heme oxygenase-1 attenuates sFlt-1-induced hypertension in pregnant rats. Am J Physiol Regul Integr Comp Physiol;301(5):R1495-500.

George EM, Cockrell K, Aranay M, Csongradi E, Stec DE, Granger JP (2011b) Induction of heme oxygenase 1 attenuates placental ischemia-induced hypertension. Hypertension; 57(5): 941-8.

George EM, Arany I (2012) Induction of heme oxygenase-1 shifts the balance from proinjury to prosurvival in the placentas of pregnant rats with reduced uterine perfusion pressure. Am J Physiol Regul Integr Comp Physiol; 302(5): R620-6.

George EM, Colson D, Dixon J, Palei AC, Granger JP (2012) Heme Oxygenase-1 Attenuates Hypoxia-Induced sFlt-1 and Oxidative Stress in Placental Villi through Its Metabolic Products CO and Bilirubin. Int J Hypertens; 2012: 486053.

Georgiades P, Ferguson-Smith AC, Burton GJ (2002) Comparative Developmental Anatomy of the Murine and Human Definitive Placentae. Placenta; 23, 3–19.

Gilliland F, Behane K, Li Y, Rappaport E, Peters J (2003) Effects of early onset asthma and in utero exposure to maternal smoking on childhood lung function. Am J Respir Crit Care Med; 167(6): 917-24.

Gluckman PD, Hanson MA, Cooper C, Thornburg KL (2008) Effect of in utero and early-life conditions on adult health and disease. N Engl J Med; 359:61-73.

Gozzelino R, Jeney V, Soares MP (2010) Mechanisms of cell protection by heme oxygenase-1. Annu Rev Pharmacol Toxicol; 50: 323-54.

Greenblum MD, McNamara DJ (2010) Destiny's Child: Fetal Origin of Adult Disease. Nutrition News for Health Care Providers. The Egg Nutrition Center, Washington D.C., USA [Online im Internet.] URL: http://www.sph.umn.edu/pdf/enhs/downloads/events/NR_Fetal_Origins.pdf [Stand: November 21, 2012, 11:38]

Grosser O (1909) Vergleichende Anatomie und Entwicklungsgeschichte der Eihäute und der Placenta mit besonderer Berücksichtigung des Menschen, Braumüller, Vienna.

Grosser O (1927) Frühentwicklung, Eihautbildung und Placentation des Menschen und der Säugetiere. In: Deutsche Frauenheilkunde, Geburtshilfe, Gynäkologie und Nachbargebiete in Einzeldarstellungen, Vol. 5, Jaschke RT, ed. Bergmann, Munich.

Grosser N, Abate A, Oberle S, Vreman HJ, Dennery PA, Becker JC, Pohle T, Seidman DS, Schröder H (2003) Heme oxygenase-1 induction may explain the antioxidant profile of aspirin. Biochem Biophys Res Commun; 308(4): 956-60.

Hales CN, Barker DJ (1992) Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia; 35(7):595-601.

Hartwig IR, Pincus MK, Diemert A, Hecher K, Arck PC (2012) Sex-specific effect of first-trimester maternal progesterone on birthweight. Hum Reprod. 2013 Jan;28(1):77-86. doi: 10.1093/humrep/des367. Epub 2012 Oct 9.

Herr CE, Ghosh R, Dostal M, Skokanova V, Ashwood P, Lipsett M, Joad JP, Pinkerton KE, Yap PS, Frost JD, Sram R, Hertz-Picciotto I (2011) Exposure to air pollution in critical prenatal time windows and IgE levels in newborns. Pediatr Allergy Immunol; 22(1 Pt 1): 75-84.

Hodgson DM, Nakamura T, Walker AK (2007) Prophylactic role for complementary and alternative medicine in perinatal programming of adult health. Forsch Komplementmed; 14(2): 92-101.

Hönig A, Rieger L, Kapp M, Sütterlin M, Dietl J, Kämmerer U (2004) Indoleamine 2,3-dioxygenase (IDO) expression in invasive extravillous trophoblast supports role of the enzyme for materno-fetal tolerance. J Reprod Immunol; 61(2): 79-86.

Hoetzel A, Geiger S, Loop T, Welle A, Schmidt R, Humar M, Pahl HL, Geiger KK, Pannen BH (2002) Differential effects of volatile anesthetics on hepatic heme oxygenase-1 expression in the rat. Anesthesiology; 97(5): 1318-21.

Hoetzel A, Schmidt R (2010) Regulatory role of anesthetics on heme oxygenase-1. Curr Drug Targets; 11(12): 1495-503. Review.

Huxley R, Owen CG, Whincup PH, Cook DG, Rich-Edwards J, Smith GD, Collins R (2007) Is birth weight a risk factor for ischemic heart disease in later life? Am J Clin Nutr; 85(5): 1244-50.

Ihara N, Akagi R, Ejiri K, Kudo T, Furuyama K, Fujita H (1998) Developmental changes of gene expression in heme metabolic enzymes in rat placenta. FEBS Lett; 439: 163–7.

Indredavik MS, Vik T, Heyerdahl S, Kulseng S, Brubakk AM (2005) Psychiatric symptoms in low birth weight adolescents, assessed by screening questionnaires. Eur Child Adolesc Psychiatry; 14(4): 226-36.

James AH, Brancazio LR, Price T (2008) Aspirin and reproductive outcomes. Obstet Gynecol Surv; 63(1): 49-57.

Joachim RA, Hildebrandt M, Oder J, Klapp BF, Arck PC (2001) Murine stress-triggered abortion is mediated by increase of $CD8^+$ TNF-$alpha^+$ decidual cells via substance P. Am J Reprod Immunol; 45(5): 303-9.

Kaneda H, Ohno M, Taguchi J, Togo M, Hashimoto H, Ogasawara K, Aizawa T, Ishizaka N, Nagai R (2002) Heme oxygenase-1 gene promoter polymorphism is associated with coronary artery disease in Japanese patients with coronary risk factors. Arterioscler Thromb Vasc Biol; 22(10): 1680-5.

Karimi K, Blois SM, Arck PC (2008) The upside of natural killers. Nat Med; 14(11):1184-5.

Kawashima A, Oda Y, Yachie A, Koizumi S, Nakanishi I (2002) Heme oxygenase-1 deficiency: the first autopsy case. Hum Pathol; 33(1): 125-30.

Kenis I, Tartakover-Matalon S, Cherepnin N, Drucker L, Fishman A, Pomeranz M, Lishner M (2005) Simvastatin has deleterious effects on human first trimester placental explants. Hum Reprod; 20(10): 2866-72.

Kensara OA, Wootton SA, Phillips DI, Patel M, Jackson AA, Elia M (2005) Fetal programming of body composition: relation between birth weight and body composition measured with dual-energy X-ray absorptiometry and anthropometric methods in older Englishmen. Am J Clin Nutr.; 82(5): 980-7.

Keyse SM and Tyrrell RM (1989) Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. Proc Natl Acad Sci U S A; 86(1): 99–103.

Kim KJ, Kim KS, Kim NR, Chin HS (2012) Effects of Simvastatin on the Expression of Heme Oxygenase-1in Human RPE Cells. Invest Ophthalmol Vis Sci; 53(10): 6456-64.

Knackstedt MK, Hamelmann E, Arck PC (2005) Mothers in stress: consequences for the offspring. Am J Reprod Immunol; 54(2): 63-9.

Kobayashi H, Takeno M, Saito T, Takeda Y, Kirino Y, Noyori K, Hayashi T, Ueda A, Ishigatsubo Y (2006) Regulatory role of heme oxygenase 1 in inflammation of rheumatoid arthritis. Arthritis Rheum; 54(4): 1132-42.

Kong FJ, Ma LL, Hu WW, Wang WN, Lu HS, Chen SP (2012a) Fetal exposure to high isoflurane concentration induces postnatal memory and learning deficits in rats. Biochem Pharmacol; 84(4): 558-63.

Kong FJ, Tang YW, Lou AF, Chen H, Xu LH, Zhang XM, Lu HS (2012b) Effects of isoflurane exposure during pregnancy on postnatal memory and learning in offspring rats. Mol Biol Rep. 2012 Apr; 39(4): 4849-55.

Koizumi S (2007) Human heme oxygenase-1 deficiency: a lesson on serendipity in the discovery of the novel disease. Pediatr Int; 49(2): 125-32.

Kozer E, Costei AM, Boskovic R, Nulman I, Nikfar S, Koren G (2003) Effects of aspirin consumption during pregnancy on pregnancy outcomes: meta-analysis. Birth Defects Res B Dev Reprod Toxicol; 68(1): 70-84.

Kreiser D, Nguyen X, Wong R, Seidman D, Stevenson D, Quan S, Abraham N, Dennery PA (2002) Heme oxygenase-1 modulates fetal growth in the rat. Lab Invest; 82(6): 687-92.

Lambert JF, Benoit BO, Colvin GA, Carlson J, Delville Y, Quesenberry PJ (2000) Quick sex determination of mouse fetuses. J Neurosci Methods; 95(2): 127-32.

Lannerö E, Wickman M, Pershagen G, Nordvall L (2006) Maternal smoking during pregnancy increases the risk of recurrent wheezing during the first years of life (BAMSE). Respir Res; 7: 3.

Lash GE, McLaughlin BE, MacDonald-Goodfellow SK, Smith GN, Brien JF, Marks GS, Nakatsu K, Graham CH (2003) Relationship between tissue damage and heme oxygenase expression in chorionic villi of term human placenta. Am J Physiol Heart Circ Physiol; 284(1): H160-7.

Lavrovsky Y, Schwartzman ML, Levere RD, Kappas A, Abraham NG (1994) Identification of binding sites for transcription factors NF-kappa B and AP-2 in the promoter region of the human heme oxygenase 1 gene. Proc Natl Acad Sci USA; 91(13): 5987-91.

Li M, Liu Y, Shi H, Zhang Y, Wang G, Xu J, Lu J, Zhang D, Xie X, Han D, Wu Y, Li S (2012) Statins inhibit pulmonary artery smooth muscle cell proliferation by upregulation of HO-1 and p21WAF1. Naunyn Schmiedebergs Arch Pharmacol; 385(10): 961-8.

Liu Y, Zhu B, Luo L, Li P, Paty DW, Cynader MS (2001) Heme oxygenase-1 plays an important protective role in experimental autoimmune encephalomyelitis. Neuroreport; 12(9): 1841-5.

Lund LK, Vik T, Skranes J, Brubakk AM, Indredavik MS (2011) Psychiatric morbidity in two low birth weight groups assessed by diagnostic interview in young adulthood. Acta Paediatr; 100(4):598-604.

Lyall F, Barber A, Myatt L, Bulmer J, Robson S (2000) Hemeoxygenase expression in human placenta and placental bed implies a role in regulation of trophoblast invasion and placental function. FASEB J; 14: 208– 19.

Medawar P (1952) Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. Symp Soc Exp Biol; 7:320–338.

Miwa N, Hayakawa S, Miyazaki S, Myojo S, Sasaki Y, Sakai M, Takikawa O, Saito S (2005) IDO expression on decidual and peripheral blood dendritic cells and monocytes/macrophages after treatment with CTLA-4 or interferon-gamma increase in normal pregnancy but decrease in spontaneous abortion. Mol Hum Reprod; 11(12): 865-70.

Molvarec A, Shiozaki A, Ito M, Toldi G, Stenczer B, Szarka A, Nakashima A, Vásárhelyi B, Rigó J Jr, Saito S (2011) Increased prevalence of peripheral blood granulysin-producing cytotoxic T lymphocytes in preeclampsia. J Reprod Immunol; 91(1-2):56-63.

Motterlini R, Foresti R, Bassi R, Green CJ (2000) Curcumin, an antioxidant and anti-inflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress. Free Radic Biol Med; 28(8): 1303-12.

Mucci LA, Lagiou P, Hsieh CC, Tamimi R, Hellerstein S, Vatten L, Adami HO, Cnattingius S, Trichopoulos D (2004) A prospective study of pregravid oral contraceptive use in relation to fetal growth. BJOG; 111(9): 989-95.

Odrcich MJ, Graham CH, Kimura KA, McLaughlin BE,Marks GS, Nakatsu K, Brien JF (1998) Heme oxygenase and nitric oxide synthase in the placenta of the guinea pig during gestation. Placenta; 19: 509– 16.

Orozco LD, Kapturczak MH, Barajas B, Wang X, Weinstein MM, Wong J, Deshane J, Bolisetty S, Shaposhnik Z, Shih DM, Agarwal A, Lusis AJ, Araujo JA (2007) Heme oxygenase-1 expression in macrophages plays a beneficial role in atherosclerosis. Circ Res; 100(12): 1703-11.

Osmond C, Barker DJ, Winter PD, Fall CH, Simmonds SJ (1993) Early growth and death from cardiovascular disease in women. BMJ; 307(6918): 1519-24.

Palanisamy A, Baxter MG, Keel PK, Xie Z, Crosby G, Culley DJ (2011) Rats exposed to isoflurane in utero during early gestation are behaviorally abnormal as adults. Anesthesiology; 114(3): 521-8.

Patil K, Bellner L, Cullaro G, Gotlinger KH, Dunn MW, Schwartzman ML (2008) Heme oxygenase-1 induction attenuates corneal inflammation and accelerates wound healing after epithelial injury. Invest Ophthalmol Vis Sci; 49(8): 3379-86.

Penn AL, Rouse RL, Horohov DW, Kearney MT, Paulsen DB, Lomax L (2007) In Utero Exposure to Environmental Tobacco Smoke Potentiates Adult Responses to Allergen in BALB/c Mice. Environ Health Perspect; 115(4): 548-55.

Pincus-Knackstedt MK, Joachim RA, Blois SM, Douglas AJ, Orsal AS, Klapp BF, Wahn U, Hamelmann E, Arck PC (2006) Prenatal stress enhances susceptibility of murine adult offspring toward airway inflammation. J Immunol; 177(12): 8484-92.

Poss KD, Tonegawa S (1997a) Heme oxygenase 1 is required for mammalian iron reutilization. Proc Natl Acad Sci USA; 94(20): 10919-24.

Poss KD, Tonegawa S (1997b) Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci USA; 94(20): 10925-30.

Pileggi A, Molano RD, Berney T, Cattan P, Vizzardelli C, Oliver R, Fraker C, Ricordi C, Pastori RL, Bach FH, Inverardi L (2001) Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved in vivo function after transplantation. Diabetes; 50(9):1983-91.

Rifa'i M, Kawamoto Y, Nakashima I, Suzuki H (2004) Essential roles of $CD8^{+}CD122^{+}$ regulatory T cells in the maintenance of T cell homeostasis. J Exp Med; 200(9):1123-34.

Romo A, Carceller R, Tobajas J (2009) Intrauterine growth retardation (IUGR): epidemiology and etiology. Pediatr Endocrinol Rev; 6 Suppl 3:332-6.

Rossant J, Cross JC (2001) Placental development: lessons from mouse mutants. Nat Rev Genet.; 2(7):538-48.

Ryter SW, Alam J, Choi AM (2006) Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev; 86(2): 583-650.

Sass G, Barikbin R, Tiegs G (2012) The multiple functions of heme oxygenase-1 in the liver. Z Gastroenterol; 50(1): 34-40.

Sausenthaler S, Rzehak P, Chen CM, Arck P, Bockelbrink A, Schäfer T, Schaaf B, Borte M, Herbarth O, Krämer U, von Berg A, Wichmann HE, Heinrich J; LISA Study Group (2009) Stress-related maternal factors during pregnancy in relation to childhood eczema: results from the LISA Study. J Investig Allergol Clin Immunol; 19(6): 481-7.

Schmidt R (2007) Cobalt protoporphyrin as a potential therapeutic agent? FASEB J; 21(11): 2639; author reply 2640.

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc; 3(6): 1101-8.

Scott JR, Chin BY, Bilban MH, Otterbein LE (2007) Restoring HOmeostasis: is heme oxygenase-1 ready for the clinic? Trends Pharmacol Sci; 28(5): 200-5.

Seller MJ, Bnait KS (1995) Effects of tobacco smoke inhalation on the developing mouse embryo and fetus. Reprod Toxicol; 9(5): 449-59.

Sen B, Swaminathan S (2007) Maternal prenatal substance use and behavior problems among children in the U.S. J Ment Health Policy Econ; 10(4): 189-206.

Shan Y, Lambrecht RW, Donohue SE, Bonkovsky HL (2006) Role of Bach1 and Nrf2 in up-regulation of the heme oxygenase-1 gene by cobalt protoporphyrin. FASEB J; 20(14): 2651-3.

Shao L, Jacobs AR, Johnson VV, Mayer L. (2005) Activation of $CD8^+$ regulatory T cells by human placental trophoblasts. J Immunol; 174(12):7539-47.

Sheikh SZ, Hegazi RA, Kobayashi T, Onyiah JC, Russo SM, Matsuoka K, Sepulveda AR, Li F, Otterbein LE, Plevy SE (2011) An anti-inflammatory role for carbon monoxide and heme oxygenase-1 in chronic Th2-mediated murine colitis. J Immunol; 186(9): 5506-13.

Shima T, Sasaki Y, Itoh M, Nakashima A, Ishii N, Sugamura K, Saito S (2010) Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice. J Reprod Immunol; 85(2):121-9.

Soares MP, Lin Y, Anrather J, Csizmadia E, Takigami K, Sato K, Grey ST, Colvin RB, Choi AM, Poss KD, Bach FH (1998) Expression of heme oxygenase-1 can determine cardiac xenograft survival. Nat Med;4(9):1073-7.

Solano ME, Jago C, Pincus MK, Arck PC (2011) Highway to health; or How prenatal factors determine disease risks in the later life of the offspring. J Reprod Immunol; 90(1): 3-8.

Steffensen FH, Sørensen HT, Gillman MW, Rothman KJ, Sabroe S, Fischer P, Olsen J (2000) Low birth weight and preterm delivery as risk factors for asthma and atopic dermatitis in young adult males. Epidemiology; 11(2): 185-8.

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN (1987) Bilirubin is an antioxidant of possible physiological importance. Science; 235: 1043– 1046.

Sun Z, Shi L, Zhang H, Shao Y, Wang Y, Lin Y, Li X, Bai C (2011) Immune modulation and safety profile of adoptive immunotherapy using expanded autologous activated lymphocytes against advanced cancer. Clin Immunol; 138(1): 23-32.

Vitoratos N, Papakonstantinou K, Deliveliotou A, Economou E, Panoulis C, Hassiakos D, Creatsas GK (2011) Antepartum and postpartum serum heme oxygenase-1 levels in preeclamptic and normotensive pregnant women. In Vivo; 25(3): 445-50.

Wang Y, Cheng Y, Liu G, Tian X, Tu X, Wang J (2012) Chronic exposure of gestation rat to sevoflurane impairs offspring brain development. Neurol Sci; 33(3): 535-44.

Watanabe S, Akagi R, Mori M, Tsuchiya T and Sassa S (2004) Marked Developmental Changes in Heme Oxygenase-1 (HO-1) Expression in the Mouse Placenta: Correlation Between HO-1 Expression and Placental Development. Placenta; 25:387–395.

Watson ED, Cross JC (2005) Development of Structures and Transport Functions in the Mouse Placenta. Physiology (Bethesda); 20: 180-193.

Weinstock M (2005) The potential influence of maternal stress hormones on development and mental health of the offspring. Brain Behav Immun; 19(4): 296-308.

Wood RA, Bloomberg GR, Kattan M, Conroy K, Sandel MT, Dresen A, Gergen PJ, Gold DR, Schwarz JC, Visness CM, Gern JE (2011) Relationships among environmental exposures, cord blood cytokine responses, allergy, and wheeze at 1 year of age in an inner-city birth cohort (Urban Environment and Childhood Asthma study). J Allergy Clin Immunol; 127(4): 913-9.e1-6.

Wright RJ, Finn P, Contreras JP, Cohen S, Wright RO, Staudenmayer J, Wand M, Perkins D, Weiss ST, Gold DR (2004) Chronic caregiver stress and IgE expression, allergen-induced proliferation, and cytokine profiles in a birth cohort predisposed to atopy. J Allergy Clin Immunol; 113(6): 1051-7.

World Health Report 2005: Make every mother and child count. Geneva: World Health Organization (WHO), 2005.

Xiang Y, Liu G, Yang L, Zhong JL (2011) UVA-induced protection of skin through the induction of heme oxygenase-1. Biosci Trends; 5(6): 239-44.

Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S (1999) Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. J Clin Invest; 103(1): 129-35.

Yet SF, Perrella MA, Layne MD, Hsieh CM, Maemura K, Kobzik L, Wiesel P, Christou H, Kourembanas S, Lee ME (1999) Hypoxia induces severe right ventricular dilatation and infarction in heme oxygenase-1 null mice. J Clin Invest; 103(8): R23-9.

Zammit S, Thomas K, Thompson A, Horwood J, Menezes P, Gunnell D, Hollis C, Wolke D, Lewis G, Harrison G (2009) Maternal tobacco, cannabis and alcohol use during pregnancy and risk of adolescent psychotic symptoms in offspring. Br J Psychiatry; 195(4):294-300.

Zhao H, Wong RJ, Doyle TC, Nayak N, Vreman HJ, Contag CH, Stevenson DK (2008) Regulation of maternal and fetal hemodynamics by heme oxygenase in mice. Biol Reprod; 78(4): 744-51.

Zhao H, Wong RJ, Kalish FS, Nayak NR, Stevenson DK (2009) Effect of Heme Oxygenase-1 Deficiency on Placental Development. Placenta; 30: 861–868.

Zenclussen AC, Joachim R, Hagen E, Peiser C, Klapp BF, Arck PC (2002) Heme oxygenase is downregulated in stress-triggered and interleukin-12-mediated murine abortion. Scand J Immunol; 55(6): 560-9.

Zenclussen AC, Lim E, Knoeller S, Knackstedt M, Hertwig K, Hagen E, Klapp BF, Arck PC (2003) Heme oxygenases in pregnancy II: HO-2 is downregulated in human pathologic pregnancies. Am J Reprod Immunol; 50(1): 66-76.

Zenclussen AC, Sollwedel A, Bertoja AZ, Gerlof K, Zenclussen ML, Woiciechowsky C, Volk HD (2005) Heme oxygenase as a therapeutic target in immunological pregnancy complications. Int Immunopharmacol; 5(1): 41-51.

Zenclussen ML, Anegon I, Bertoja AZ, Chauveau C, Vogt K, Gerlof K, Sollwedel A, Volk HD, Ritter T, Zenclussen AC (2006) Over-expression of heme oxygenase-1 by adenoviral gene transfer improves pregnancy outcome in a murine model of abortion. J Reprod Immunol; 69(1):35-52.

Zenclussen ML, Casalis PA, El-Mousleh T, Rebelo S, Langwisch S, Linzke N, Volk HD, Fest S, Soares MP, Zenclussen AC (2011) Haem oxygenase-1 dictates intrauterine fetal survival in mice via carbon monoxide. J Pathol; 225(2): 293-304.

Zenclussen ML, Jensen F, Rebelo S, El-Mousleh T, Casalis PA, Zenclussen AC (2012) Heme oxygenase-1 expression in the ovary dictates a proper oocyte ovulation, fertilization, and corpora lutea maintenance. Am J Reprod Immunol; 67(5): 376-82.

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9. Lebenslauf

Entfällt aus datenschutzrechtlichen Gründen.

10. Eidesstattliche Versicherung

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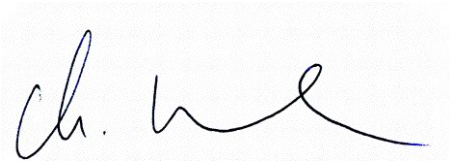

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