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Influence of prenatal acetaminophen use on maternal adaptation to pregnancy, fetal immune development and children's health.

Dissertation

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1. INTRODUCTION

The birth of a healthy baby is generally considered as a successful pregnancy. This success is achieved by an adequate maternal immune and endocrine adaptation to pregnancy resulting in continuation of fetal immune tolerance, placentation and timely fetal development [1]. The course of pregnancy is dependent on endogenous factors such as age, weight of mother or levels of pregnancy-related hormones and exogenous factors including environmental challenges. These challenges include psychological stress perception [2, 3], infections [4, 5] and medication such as acetaminophen [6]. The exogenous challenges may alter maternal adaptation to pregnancy and consequently the immune tolerance towards the fetus, leading to either pregnancy complications such as fetal loss, preterm labor and preeclampsia or an impaired fetal development.

1.1 Acetaminophen

Acetaminophen (N-acetyl-para-aminophenol, APAP, i.e. Paracetamol®, Tylenol®) is an over-the-counter medication, commonly used worldwide. Along with aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs), APAP is valued for its analgesic and antipyretic effects. In contrast to other NSAIDs, APAP has little anti-inflammatory activity. Although the mechanism of action could not be conclusively clarified yet, APAP is known to mainly inhibit the cyclooxygenase (COX). Further findings additionally propose that it is highly selective for COX-2 [7].

In general, APAP is well tolerated when used in therapeutic range [8, 9, 10]. However, APAP also accounts for almost 50% of all cases of acute liver failure in the United States, associated with a 30% mortality rate [11].

APAP is metabolized in the liver into toxic and non-toxic metabolites. When used in therapeutic doses, APAP is metabolized by three different metabolic pathways: glucuronidation, sulfation and N-hydroxylation and dehydration followed by glutathione (GSH) conjugation (Fig. 1.1 upper part). The final products of all path-

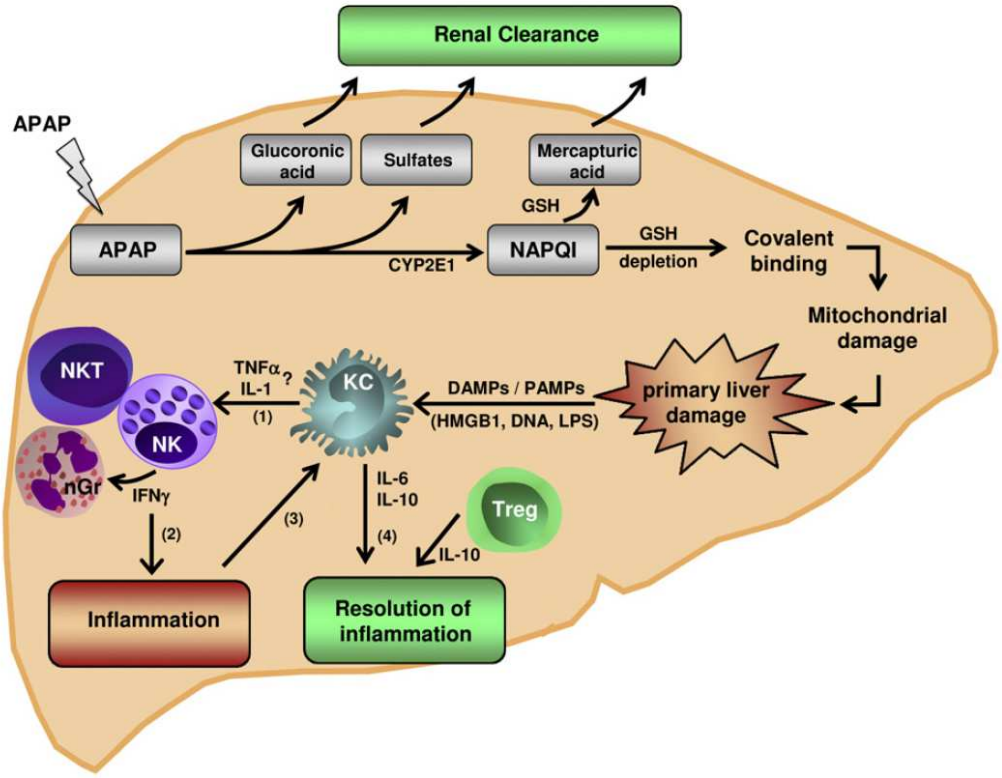


Fig. 1.1: Schematic overview depicting metabolism and liver toxicity of acetaminophen accompanied by the activation of the immune response upon primary liver injury. (APAP, acetaminophen; NAPQI, N-acetyl-p-benzoquinone imine; GSH, glutathione; DAMPs, damage-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; HMGB1, high mobility group box-1; LPS, lipopolysaccaride; KC, Kupffer cell; NK, natural killer cell; NKT, natural killer T cell; Treg, regulatory T cell; nGr, neutrophil granulocyte; NO, nitric oxide; TNFα, tumor necrosis factor-alpha; IL-1,-6,-10, interleukin-1,-6,-10; and IFNγ, interferon-gamma.) Figure taken from Thiele *et al.*, 2013 [6]

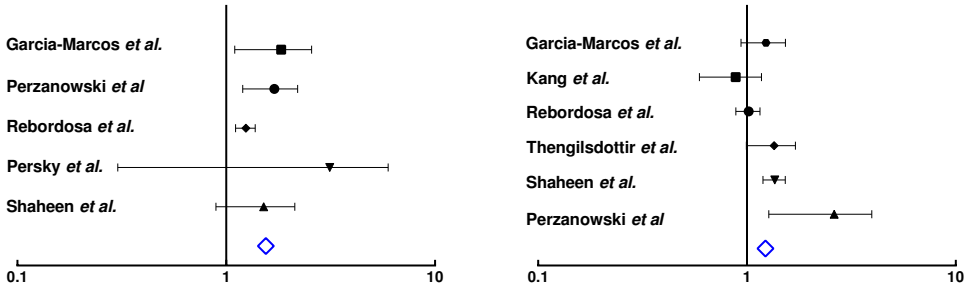
ways are inactive, non-toxic and are subsequently excreted via the kidneys. However, in the third pathway the toxic metabolite N-acetyl-pbenzoquinone imine (NAPQI) is produced by isoenzymes of cytochrome P450. This metabolite needs to be finally inactivated by conjugation with the sulfhydryl groups of hepatic GSH before it can be excreted as non-toxic mercapturic acid conjugate via the kidneys.

The formation of the toxic metabolite NAPQI represents the first crucial step in APAP-induced hepatotoxicity. Additionally a depletion of hepatic GSH occurs due to APAP overdose [12]. When finally the capacity of GSH is exceeded, NAPQI interacts with protein sulfhydryl groups leading to protein adduct formation, which

consequently results in a loss of activity of several liver enzymes [12], as well as the activation of death signaling pathways in hepatocytes, mitochondrial dysfunction and consequently hepatocellular necrosis (Fig. 1.1) [13, 14]. This has secondary effects on the innate immune response summarized in the lower part of Fig. 1.1 [15, 16, 17, 18, 19, 20, 21].
Hepatotoxicity caused by APAP overdose can finally lead to acute liver failure which became increasingly recognize as the number of cases due to APAP rose from 28% in 1998 to 51% in 2003 and reached similar values also in the following years [22, 23]. Most of these cases result from suicidal (44%) or unintentional (48%) overdose [22], also during pregnancy [24]. Since APAP is free merchantable, self-medication without consultation of a physician or pharmacists is the standard. Thereby, the 4 g/day recommendation can be easily exceeded - unintentionally - by taking different compounds simultaneously, due to product name confusions or misleading labels. Further, it has been shown that already the recommended dose can result in increased liver enzyme activity, e.g. alanine aminotransferase (ALT) arising from liver damage [25]. That emphasizes that APAP toxicity is not just a matter of acute overdose but can also be a consequence of repeated ingestion.

1.2 APAP and pregnancy

APAP is frequently taken by pregnant women [26], since it is generally considered to be 'safe' as harmful effects on the unborn child have not been described yet. Therefore, APAP is recommended by physicians as the medication of choice during pregnancy. The Food and Drug Administration (FDA) of the United States has classified APAP into 'Pregnancy Category B' supporting that APAP can be used without concerns for maternal health and fetal development [27]. It has been assessed that approximately 50% of pregnant women have used APAP at least once during pregnancy, although the frequency is higher in the United States (65%) compared to Europe (20-30%) [26, 28].
However, there have been a number of clinical trials revealing a potential threat of APAP to pregnancy since the occurrences of miscarriages, preterm birth and low birth weight could be linked to APAP use, although the results are rather conflicting [6]. Additionally, most of these studies focussed mainly on crude parameter such as the incidence of pregnancy complication and fetal malformations. Therefore, the impact of APAP on the maternal and fetal immune system remains elusive.



(a) Etminan *et al.*, 2009 [31] (b) Eyers *et al.*, 2011 [32]

Fig. 1.2: The association between APAP use in pregnancy and asthma in childhood have been addressed in epidemiological studies. The forest plots present the results of meta-analyses published by Etminan *et al.* [31] (a) and Eyers *et al.* [32] (b).

Since APAP crosses the placenta, it exposes the unprotected fetus to potential APAP-induced liver damage [29, 30]. A number of epidemiological studies have recently provided unambiguous evidence that the prenatal use of APAP is associated with an increased risk for the child to develop asthma later in life [31, 32, 33, 34]. Two meta-analyses reported a similar strength of association for the risk of asthma and wheezing due to APAP intake during pregnancy (Fig. 1.2) [31, 32]. Further, failure in neurodevelopment [35], appearance of attention-deficit/hyperactivity syndrome [28] and male infertility [36, 37] have been correlated to the prenatal use of APAP.

Despite all these epidemiological findings, experimental rodent models investigating the effects of APAP on fetal development and the impact of pregnancy on the APAP metabolism are sparse. Further, very high doses of APAP have been used in these studies and they share the same problem as many human studies by using rather crude read-out parameters such as survival, mortality, litter size, fetal loss and fetal weight [38, 39, 40, 41]. Remarkably, one of these studies could indeed show that the circumstance of being pregnant leads to increased hepatotoxicity, higher incidence of liver necrosis and increased serum ALT compared to non-pregnant controls [39].

1.3 Maternal adaptation to pregnancy

The maternal organisms has to cope with a number of changes in order to adapt to the developing pregnancy. Among others, this includes changes in the metabolism as well as in the endocrine and immune system.

A pregnant woman faces an immunological challenge as the fetus is semi-allogenic, but the maternal immune system has to tolerate the 'allograft' in order to prevent rejection of the fetus. On the other hand, the capacity of the immune system to cope with infectious antigen and noxious agents needs to be maintained. Therefore, a fine tuned maternal endocrine and immune adaptation to pregnancy is required to promote fetal tolerance and sustain fetal development [1].

Numerous factors are involved in the immune regulatory processes at the feto-maternal interface to prevent the rejection of the fetus and contribute to a successful pregnancy [42, 1]. Key features include the modulation of decidual natural killer (NK) cell function [43, 44] and the arrest of decidual dendritic cells (DC) in a tolerogenic state (tDC) [45, 46, 47]. Together they are responsible for the induction of effector T-cell apoptosis and expansion of $CD4^+$ regulatory T (Treg) cells in order to promote fetal tolerance [48, 49, 50, 51, 52]. Additionally, $CD8^+$ regulatory or suppressor T cells have been reported to contribute to fetal tolerance [53, 54, 55]. All these pathways are mediated by the increase of steroid hormones such as progesterone [56, 1].

Progesterone plays a pivotal role in pregnancy establishment and maintenance due to its endocrine and immunological effects [57]. Its function is enabled by the intracellular progesterone receptors (PR) A and B. These receptor isoforms have distinct function. PR-A is crucial for implantation and a selective knockout leads to an infertile phenotype in mice, while blocking of PR-B has no impact on fertility [58].

To date, insights on whether or not APAP affects maternal adaptation to pregnancy are still largely unknown. However, it is evident that APAP suppresses the humoral and cellular immunity although the studies suggesting this were carried out in non-pregnant mice [59, 60]. Moreover, it has been reported that even therapeutic doses of APAP reduce level of female sex hormones in humans [61]. In mice, APAP was shown to elicit anti-estrogenic responses including inhibition of progesterone receptor up-regulation in the uterus and in the liver [62]. Furthermore, it has recently been reported that APAP suppresses the release of prostaglandin E_2 which is involved in mediating the tolerogenic phenotype of DCs [63]. This goes in line with the observation that liver DCs undergo maturation upon APAP challenge [64].

The immune adaptation towards pregnancy is a vulnerable process and the impact of APAP needs to be elucidated. A putative disruption of the immune-regulatory processes not only affects the mother, but it might also affect the fetal-maternal interface.

1.4 Placenta

The placenta is responsible for maternal-fetal exchange of gas and nutrients, hormone production and maternal tolerance of feto-paternal antigens. Therefore, the placenta is essential to maintain a successful pregnancy and ensure fetal growth and development [65].
The placenta is mainly formed by trophoblast cells of extra-embryonic origin that differentiate into distinct cell types. Both mice and humans have a hemochorial placenta characterized by direct contact of maternal blood with the chorion [66]. However, there are several differences between a human and a mouse placenta that need to considered. In humans, decidualization of the uterus starts before conception [67] and the final placental structure becomes apparent already at day 21 of pregnancy. The main feature includes the chorionic villi, that are surrounded by the maternal blood in the intervillous space as early as from the 10^{th} -12^{th} week of pregnancy and consist of cytotrophoblast and syncytiotrophoblast cells surrounding the fetal vessels. [65]
In mice, placentation evolves from an initially choriovitelline to a chorioallantoic pattern at gestation day (gd) 11.5 [65]. A mouse placenta can be subdivided into a layer of maternal decidua, a junctional zone and a labyrinthine zone (Fig. 1.3) [68]. The labyrinth consists of trophoblastic septae and fetal blood vessels which are separated from the maternal blood by two syncytiotrophoblast layers and one cytotrophoblast layer. These layers also constitute the placental barrier [69]. The labyrinth is responsible for a sufficient exchange of gas and nutrients, but by means of the placental barrier it also restricts the crossing of toxins and pathogens as well as the unchecked diffusion of e.g. hormones or ions [70, 71, 72]. In both mouse and human placentas, the placental barrier becomes thinner with gestational age. Hereby the diffusion distance is minimized for passive exchange [73].
The junctional zone is composed of glycogen cells and spongiotrophoblastic cells, while trophoblastic giant cells form the border to the maternal decidua [65]. Although the function of each cell type is not fully clarified yet, the junctional zone is generally known to play an endocrine role [74, 68]. Human and mice placenta differ in respect to their endocrine function. In humans, progesterone is produced by the corpus luteum, which is stimulated by the human chorionic gonadotropin produced by the placental trophoblast cells [75]. After 8 weeks of gestation, the placental syncytiotrophoblast takes over progesterone production until parturition. Further,

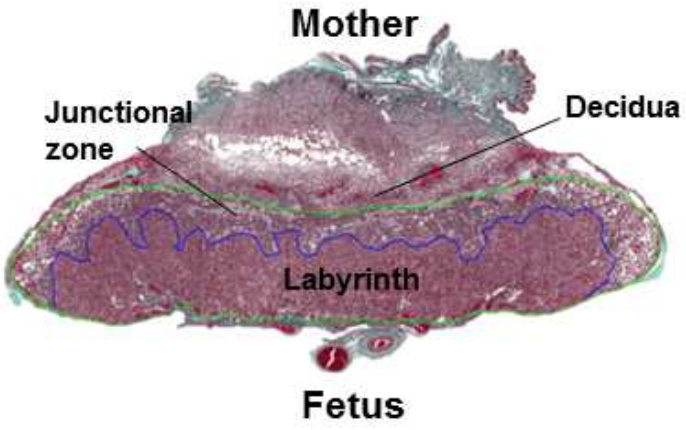


Fig. 1.3: A mouse placenta on gd 16.5 stained with Masson-Goldner trichrome staining: The chorioallantoic mouse placenta is composed of the decidua, the junctional zone and the labyrinth.

the human placental hormone function also includes estrogen, human chorionic somatomammotrophic hormone and human placental growth hormone [65].
In contrast to humans, progesterone is produced by the corpus luteum throughout gestation in mice [76]. Early in pregnancy, progesterone production is stimulated by prolactin which is secreted by the pituitary gland. On gd 8-9 placental trophoblast giant and spongiotrophoblast cells take over and produce placental lactogen (mPL) 1 and later mPL 2 which are both responsible for stimulating the progesterone production by the corpus luteum [77].
In conclusion, the placenta does not only provide nutrients and hormones but also acts as a barrier to protect the fetus. This barrier, however, seems to be leaky for APAP. This fact emphasizes the need to investigate the impact of APAP on fetal immune ontogeny.

1.5 Fetal immune ontogeny

APAP can cross the placenta [29, 30]. Therefore, the effects on the growing fetus need to be considered when evaluating the impact of APAP on pregnancy.
The concept of 'fetal programming' was proposed in 1989 leading to suggestion that the prenatal life determines postnatal life [78]. Following this, many epidemiological studies investigated the role of different environmental factors such as smoking [79], alcohol [80], maternal nutrition [81] and stress perception [82] on fetal development.

The idea underlying this is that fetal development and prenatal environmental conditions are inseparably linked to each other. Consequently, an interference in these processes can have long-lasting consequences for the child later in life.
One of these processes is the immune system responsible for protecting the host against pathogens and other substances which are potentially harmful. This is enabled by the ability of the immune system to distinguish between 'own' and 'foreign' cells. An impairment of the immune system can either lead to autoimmune diseases such as Multiple sclerosis [83] and type 1 diabetes [84] or to allergies [85]. The development of the immune system begins prenatally as a gradual process throughout gestation [86]. Key steps include the fetal immune organ development [87, 88] followed by hematopoietic stem cell (HSC) seeding and migration to the thymus and the bone marrow [89, 90]. Finally, immune cells of the innate and adaptive immune system need to undergo differentiation and maturation [91, 92]. Numerous similarities between murine and human immune development have been reported [93], although there are species-specific differences that need to be considered, e.g. gestational length and time of organogenesis.

1.5.1 Hematopoiesis

In mice, the initial hematopoietic stem cell locations is found in the extraembryonic yolk sac and the intraembryonic PAS (para-aortic splanchnopleura)/AGM (aorta-gonad-mesonephros) region. From gd 11.5, the hematopoiesis shifts to the fetal liver [95, 96], which is then the main HSC source until the bone marrow takes over shortly before birth (Fig. 1.4 upper part). The bone marrow remains the main source of hematopoiesis throughout life. The fetal spleen - playing a rather minor role as hematopoietic organ - starts producing HSCs around gd 15.5 [94].
HSC are characterized by their capacity of multi-potency and self-renewal [97]. This means that HSCs are able to replicate themselves in order to maintain their population [90]. Additionally, they are able to differentiate into progenitor cells for the myeloid and lymphoid lineages in order to give rise to either monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/ platelets and dendritic cells or to T cells, B cells and NK cells [98]. On the way of maturation and differentiation, HSC first lose their self-renewal capacity followed by a step-by-step loss of their lineage potential. This is necessary in order to become

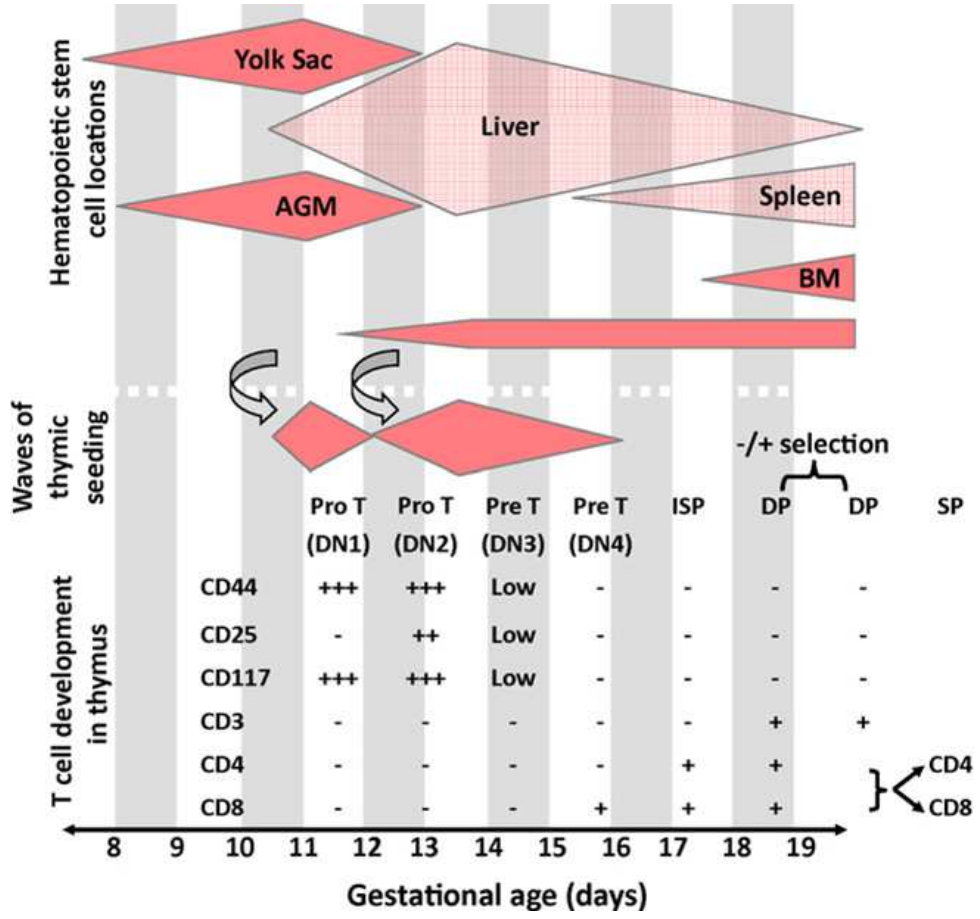


Fig. 1.4: Fetal immune ontogeny in mice. Upper part: Hematopoietic stem cell locations in fetal mice. Lower part: Liver-derived T cell progenitor cells seed the thymus and undergo maturation and selection processes. Figure taken from Solano *et al.*, 2011 [94]

a mature functional cell of a certain lineage [98]. In the mouse system, HSC and their subtypes can be identified by their cell surface marker, defined as Lineage negative (Lin^{neg}; lacking antigens for mature immune cell lines), $CD117^{+}$ (c-kit^{+}), Stem cell antigen 1 (Sca-1^{+}) and $CD34^{neg}$, together also referred to as LKS population, whereas multi-potent progenitors (MPPs) are characterized by Lin^{neg} c-kit^{+} Sca-1^{+} and $CD34^{+}$ [98].

HSC migrate and colonize the fetal thymus during gestation in two waves as the thymus itself does not contain own stem cells. In mice, the first seeding of T cell progenitors starts on gd 10.5 followed by a second wave between gd 12.5 and 16.5 [99, 94].

1.5.2 T cell maturation

The maturation of T cells and their selection are crucial aspects of fetal immune ontogeny, in both humans and mice. Hereby, the thymus plays a pivotal role as its epithelial architecture facilitates growth, correct differentiation and T cell receptor repertoire selection of large numbers of immature thymocytes in order to generate functional, mature, self-MHC-restricted T cells [100].
In mice, common lymphoid progenitor cells originated from liver HSC, populate the thymus and become T cell precursors. These immature thymocytes express neither CD8 nor CD4 and are therefore known as the 'double negative' (DN) population. Following the expression of CD25 and CD44, four distinct DN subsets can be identified: DN1, DN2 (pro-thymocytes) and DN3, DN4 (pre-thymocytes) [101]. DN1 thymocytes express c-kit and CD44 and the T cell receptor (TCR) must be developed by V(D)J recombination [102]. This stage is followed by upregulation of CD25 and rearrangements of the TCRβ locus (DN2). During DN3, thymocytes stop expressing c-kit and CD44 while the TCR must produce an $\alpha\beta$ chain. Finally also CD25 is upregulated (DN4) and subsequently CD8 and CD4 are expressed and are then known as double-positive (DP) thymocytes. First, CD8 is expressed alone representing the intermediate single positive (ISP) thymocyte before CD4 is also upregulated [103, 94]. The process of 'positive selection' is characterized by changing expression of CD3 from pre-DP ($CD3^-CD4^+CD8^+CD44^-CD25^-$) to post-DP ($CD3^+CD4^+CD8^+CD44^-CD25^-$). In the final stage of T cell maturation, the negative selection, results in single positive (SP) thymocytes, expressing either CD4 or CD8. These cells are subsequently released from the thymus to the periphery (Fig. 1.4) [101, 99, 94].
Most of the thymocytes die during maturation by failing the selection process. Therefore apoptosis is an important part of this process occurring in mice between gd 15.5 and 18.5 in order to eliminate T cells responding to self-antigen and non-functional T cells and to ensure migration of T cells that recognize foreign antigen into the periphery [104].

In summary, given that APAP is hepatotoxic and the fetal liver is temporally the major source of HSCs in humans as well as in mice, before the HSC pool relocates to the bone marrow, the fetal immune ontogeny may be particularly vulnerable to APAP intake during pregnancy. Hence, challenging the fetal HSC pool in the liver

may result in an impaired fetal immune development as these progenitor cells then colonize the fetal thymus in order to undergo T cell maturation. This, in turn, may have long-term consequences for children's immunity and health. To date, insights need to be gained if the prenatal use of APAP may lead to such consequences.

2. OBJECTIVES

The main goal of this thesis is to evaluate the effects a non-toxic or a toxic single APAP dose have on the maternal liver, as well as the effects on maternal immune and endocrine adaptation to pregnancy. Moreover, the impact of prenatal APAP intake on pregnancy outcome on the one hand and on the other hand on fetal immune development in liver and thymus are to be investigated.

Therefore the following objectives have been defined:

- Establishment of a mouse model allowing to study the effects of prenatal APAP use
- Evaluation of liver toxicity and morbidity after APAP intake in pregnant mice compared to non-pregnant animals
- Investigation of the effects APAP has on the maternal immune system
- Analysis of placental morphology and endocrine responses due to APAP use
- Examination of the effect of APAP on the fetal stem cell reservoir and on T cell maturation

3. MATERIALS AND METHODS

3.1 Timed pregnancies

Six to eight weeks old C57Bl/6J female wild-type mice were purchased from Charles River (Sulzfeld, Germany). Upon a post-shipping acclimatization period of at least 7 days they were mated to either C57Bl/6J male mice (syngenic mating) or CBy.SJL(B6)-Ptprca/J male mice (allogenic mating). The presence of a vaginal plug in the morning was considered as gd 0.5. Maternal weight controls on gd 8.5 and 10.5 were conducted to confirm successful pregnancy. Females of the same age served as non-pregnant controls. Animals were kept under 12 h light/ dark circles and received food and water ad libitum. All experiments were performed in accordance with the animal ethics approval given by the State Authority of Hamburg ($G11/094\_APAP$).

3.2 APAP treatment

Sixteen hours prior to APAP treatment, pregnant and non-pregnant females were fasted to achieve equal glutathione levels. APAP was dissolved in sterile phosphate buffered saline (PBS, Gibco, Life Technologies GmbH, Darmstadt, Germany) and pregnant (on gd 12.5) and non-pregnant mice were injected intraperitoneally (i.p.) with APAP in the morning. Control animals received PBS only. Pregnant mice were sacrificed either on gd 13.5 or 16.5 for harvesting maternal and fetal tissue, tissue from non-pregnant mice was obtained 24 or 96 hours post-injection. A graphical summary of the APAP Mouse model is displayed in Fig. 3.1.

3.3 Weight assessment and food intake

For maternal weight assessment, a starting weight was documented before the food withdrawal on gd 11.5 and this value was considered as 100% to subtract out inter-individual differences in weight. The weight loss/gain calculation was performed

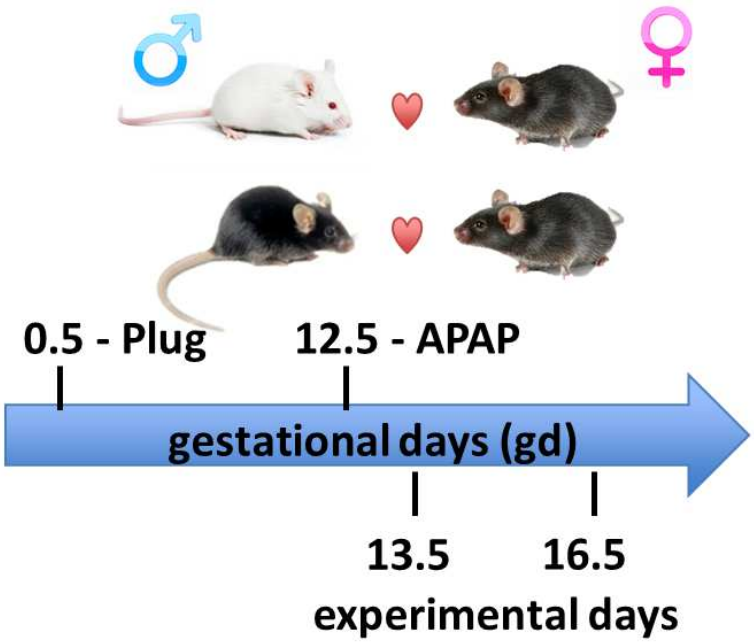


Fig. 3.1: Experimental setup: C57Bl/6J female mice were mated to either CBy.SJL(B6)-Ptprca/J male mice (allogenic mating, above) or to C57Bl/6J male mice (syngenic mating, below). Pregnant mice were injected intraperitoneally with APAP on gd12.5. Tissue harvesting was conducted either on gd 13.5 or on gd16.5.

based on weight measurements every 24 h. The food and water intake was quantified manually. After APAP injection, the mice received a known amount of food and water. Subsequently food and water were re-weighed on a daily basis and the consumption was calculated by difference.

3.4 Tissue harvesting

Mice were anesthetized lethally by intravenous injection of 150 mg/kg methohexital and 15 mg/kg heparin. Blood samples were collected by right ventricle cardiac puncture for plasma ALT and progesterone levels. Maternal liver and uterus-draining lymph node were harvested and kept in PBS on ice. Parts of the maternal liver were fixed in formalin for subsequent histology. The uterus was removed and number of implantations and abortions was documented to analyze pregnancy outcome in the respective pregnant mice. Fetuses were isolated from the embryonic membranes to determine fetal weight and the fetal tail was removed for gender determination. Subsequently, the fetuses were stored in biopsy cassettes (Tissue-Tek III Blue, Sakura

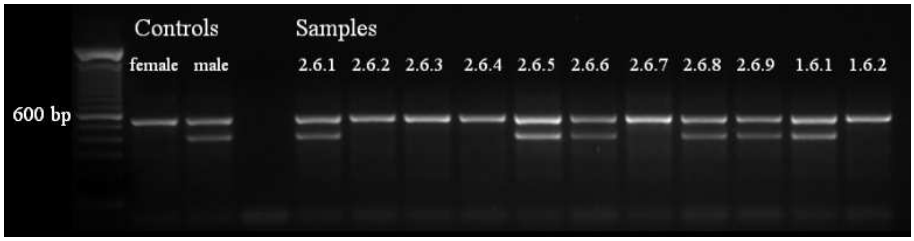


Fig. 3.2: Determination of fetal sex on an agarose gel after PCR amplification. The 544 bp band represents the IL3 gene (internal control) while the 402 bp band identifies male fetuses by the detection of the Sry gene.

Finetek Europe) on gd13.5 and in embedding cassettes 'Macro' (Carl Roth GmbH & Co. KG, Karlsruhe) on gd 16.5, incubated in Bouin solution (Sigma-Aldrich, St, Louis, USA) for 24 hours and then transferred into 70% ethanol.
Fetal liver and thymus were harvested from four fetuses per each litter on gd 16.5 using a S6E Stereo Zoom Mircoscope (Leica, Bensheim, Germany) and kept in cRPMI (RPMI Medium 1640 (Gibco, Life Technologies GmbH, Darmstadt, Germany) containing 10% FCS, 1% PenStrep and 1% L-Glutamine). Placentas taken on gd 13.5 and 16.5 respectively were embedded in biopsy cassettes and stored in 4% Formaldehyde solution (36.5-38%, Sigma-Aldrich, St, Louis, US) for 24 h before transfer into 1% Formaldehyde solution for long-term storage.

3.5 Polymerase Chain Reaction PCR)

PCR analysis was performed to determine the gender of the fetuses based on the presence or absence of the Y-chromosome according to a published protocol [105]. Therefor fetal tails were collected on gd 13.5 and 16.5 and DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) following the manufracturer's intructions. The detection of the IL3 gene (544 bp) serves as internal control while the detection of the Sry gene (402 bp) identifies male fetuses. The following primerpairs have been used: Sry locus 5'-TGGGACTGGTGACAATTGTC-3' and 5'-GAGTACAGGTGTGCAGCTCT-3', IL3 specific 5'-GGGACTCCAAGCTTCAAT CA-3' and 5'-TGGAGGAGGAAGAAAAGCAA-3'. The PCR program consists of 10 min inital denaturation at 94°C, 33 cycles of 94°C for 40 s, 50°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min.
Agarose gel electrophoresis was performed to detect the bands under UV light using the gel documentation system BioDoc II with the associated software BioDoc An-

alyze 2.0 by Biometra. For preparing the gel, 6 g agarose was dissolved in 200 ml TAE buffer and brought to boil in a microwave. After cooling, ethidium bromide (0,25 μg/μl) was added and subsequently samples could be pipetted in the empty wells. Electrophoresis was performed at voltage of 80 V.

3.6 Histology

3.6.1 Hematoxylin and eosin (H&E) stain

For histological analysis, maternal livers were fixed in 4% formalin and fetuses were fixed with Bouin solution prior to paraffin embedding. Sections were stained with hematoxylin and eosin following our standard protocol, briefly: deparaffinization using XEM (xylene substitude, d-Limonene $C_{10}H_{16}$, DiaTech GmbH & Co. KG, Bamberg, Germany) for 10-15 min twice followed by rehydration through a graded series of ethanol (Walter CMP GmbH & Co. KG, Kiel, Germany) and double distilled water. Subsequently slides were incubated with Hem alum solution acid according to Mayer (Roth GmbH & Co. KG, Karlsruhe, Germany) for 15 min and immersed with tap water for at least 15 min. Afterwards slides were incubated with Eosin (Waldeck GmbH & Co. KG, Münster, Germany) for 60 sec. Finally slides were dehydrated using 100% ethanol (3 x 2 min) and XEM (2 x 5 min) before they were covered with the mounting media 'Eukitt' (O. Kindler GmbH, Freiburg, Germany) and a cover glass.
Slides were scanned with a Mirax Midi Slide Scanner (Zeiss, Oberkochen, Germany). Nuclei appear purplish blue while the cytomplasma including cytoplasmic filaments in muscle cells, intracellular membranes, and extracellular fibres are stained pink.

3.6.2 Masson-Goldner trichrome staining

Placentas fixed in 4% formalin were embedded in paraffin and mid-sagittally cut in 4 μm sections using a microtome (SM2010R, Leica, Bensheim, Germany). Slides were deparaffinized and rehydrated using XEM and ethanol (see above).
Masson-Goldner trichrome staining was performed following standard protocol: slides were first incubated for 8 min using Weigert's iron hematoxylin staining solution (equal ratio of Ferric Hematoxylin solution A and B, Waldeck GmbH & Co. KG, Münster, Germany). Next, slides were shortly washed in distilled water and rinsed with tap water for 10 min and again washed in distilled water. Subsequently, slides

were incubated for 8 min in Goldner solution 1 (0.33 g Ponceau de Xylidine (Sigma-Aldrich, St, Louis, US), 0.1 g Acid Fuchsin (Sigma-Aldrich, St. Louis, US) and 3 ml acetic acid ad 500 ml Aqua dest.) followed by washing twice in 1% ethanoic acid (J.T.Baker, Center Valley, PA, US) for 30 sec. Incubation with Goldner solution 2 (20.0 g Molybdatophosphoric acid hydrate (Merck KGaA, Darmstadt, Germany) and 10.0 g Orange G (Roth GmbH & Co. KG, Karlsruhe, Germany) ad 500 ml Aqua dest.) is needed for 9 min (until destaining of the connective tissue). After another washing procedure of 3 x 30 sec in 1% ethanoic acid, slides were incubated with Goldner solution 3 (1.0 g Light Green SF yellowish (Merck KGaA, Darmstadt, Germany) and 1 ml acetic acid ad 500 ml Aqua dest.) for 3 min. Afterwards slides were again rinsed in 1% ethanoic acid for 30 sec and 2 min before they were dehydrate by an ascending ethanol series (70%, 96%, 100% for each 2-3 min) and XEM (twice 5 min). Finally sections were covered with the mounting media 'Eukitt' and a cover glass and also scanned with the Mirax Midi Slide Scanner.
Histomorphological analyses of placental areas were performed by two independent observers using Pannoramic Viewer (3DHistech Kft. Budapest, Hungary). Cell nuclei appear dark brown, cytoplasma and muscle are blick red while connective tissue appears green and erythrocytes show up bright orange.

3.7 Ex vivo Magnetic Resonance Imaging

Fetuses and placentas harvested on gd 16.5 were exposed to 4% gadolinium-based contrast agent (Multihance, Bracco, Germany) for 48 h for contrast enhancement and subsequently embedded in 1% agarose. MRI was conducted on a small animal MR scanner at 7.0 T (ClinScan, Bruker BioSpin, Germany) using a circularly polarized transmit/receive coil with an inner diameter of 40 mm and a resonator length of 40 mm. Data were acquired using a 3D-turbo spin echo sequence (flip-back pulse (RESTORE) 90°; TR, 200 ms; TE, 18 ms; FoV, 36 x 36 x 7.69 mm^3; matrix, 448 x 448 x 96; voxel size, 80 μ m) in sagittal orientation. Volumetric analysis of the placentas were performed using an open source image analysis software (OsiriX imaging software©, Pixmeo, Geneva, Switzerland).

3.8 Tissue processing

Single cell suspensions of maternal lymph nodes were obtained by passing the tissue through a cell strainer (Falcon Cell Strainer 40 μm, BD Bioscience, VWR, Germany). After centrifugation at 450 g for 8 min at 4°C, the cell pellet was resuspended in Fluorescence-activated cell sorter (FACS) buffer (1x PBS + 0.5 % BSA). The same cell isolation procedures were used for generating single cell suspensions from fetal liver with the modification that red blood cell (RBC) lysis was performed using 500 μl, RBC lysis buffer (eBioscience, SanDiego, CA, USA) after centrifugation. Single cell suspensions from fetal thymus were obtained by processing the tissue in a scratched petri dish and washing it through a cell strainer with cRPMI. After centrifugation, cells from fetal liver and thymus were resuspended in cRPMI. Number of viable leukocytes in all tissues was obtained by counting the cells using a Neubauer chamber upon adding Trypan Blue stain (0.4 %, Life Technologies GmbH, Darmstadt, Germany).

3.9 Flow cytometry

For flow cytometric analyses, 1.0x10^6 fetal liver cells and 0.5x10^6 fetal thymus or maternal lymph node cells respectively were used for staining of cell markers. Non-specific binding was blocked by rat anti-mouse CD16/CD32 Mouse Fragment crystallizable (Fc) Block (1:200, BD Bioscience) and Normal Rat Serum (1:100, eBioscience) for 15 min at 4°C. Subsequently, the cells were incubated with the respective antibodies for 30 min for surface and intracellular stainings. Antibodies used in this study are displayed in Tab. 3.1.
In the protocol for surface staining, 7-amino-actinomycin D (7AAD, 1:400, Biolegend) was used to identify dead cells. For intracellular stainings of FoxP3, cells were fixed and permeabilized using Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience, SanDiego, USA) and for intracellular stainings of INFγ Fixation/Permeabilization Buffer (eBioscience, SanDiego, USA) was used, respectively. Data were acquired using a BD LSRFortessa II (BD Biosciences) provided by the FACS Sorting Core Unit of the University Medical Center Hamburg-Eppendorf. Data analysis was conducted using FlowJo (Tree Star Inc., Ashland, OR, USA). Isotype controls and 'fluorescent minus one' (FMO) antibody staining were used to reliably identify positive and negative cell populations.

Tab. 3.1: Summary of antibodies including fluorochrome (FITC: Fluorescein Isothiocyanate, APC: Allophycocyanin; Cy: Cyanine, PE: R-phycoerythrin, BV: Brilliant Violet), clone, dilution and source (BD Pharmingen (BD, Heidelberg, Germany), eBioscience (SanDiego, CA, USA) and Biolegend (Fell, Germany)).

Antigen	Fluorochrome	Clone	Dilution	Source
Fetal liver				
anti-CD34	FITC	RAM34	1:200	BD
anti-CD45	APC-Cy7	30-F11	1:400	BD
mouse lineage antibody	APC		1:200	BD
anti-Ly-6A/E (Sca-1)	PE-Cy7	D7	1:100	eBioscience
anti-CD117	BV421	2B8	1:200	Biolegend
anti-CD135	PE	A2F10.1	1:100	BD
Fetal thymus				
anti-CD4	FITC	RM4-5	1:400	Biolegend
anti-CD25	APC	PC61	1:200	Biolegend
anti-CD3	PE-Cy7	145-2C11	1:200	Biolegend
anti-CD8	PE-Cy5	53-6.7	1:100	Biolegend
anti-CD44	Pacific Blue	IM7	1:200	Biolegend
anti-CD45	APC-Cy7	30-F11	1:400	BD
anti-FoxP3	PE	FJK-16s	1:200	eBioscience
Lymph node (CD4 Panel)				
anti-CD4	FITC	RM4-5	1:400	Biolegend
anti-CD45	APC-Cy7	30-F11	1:400	BD
anti-CD25	APC	PC61	1:200	Biolegend
anti-CD3	PE-Cy7	145-2C11	1:200	Biolegend
anti-CD8	Pacific Blue	53-6.7	1:100	Biolegend
anti-FoxP3	PE	FJK-16s	1:200	eBioscience
Lymph node (CD8 Panel)				
anti-CD4	FITC	RM4-5	1:400	Biolegend
anti-CD28	APC	37.51	1:100	Biolegend
anti-CD3	PE-Cy7	145-2C11	1:200	Biolegend
anti-CD8	Pacific Blue	53-6.7	1:100	Biolegend
anti-CD45	APC-Cy7	30-F11	1:400	BD
anti-CD122	PE	TM-b1	1:100	eBioscience
Lymph node (DC Panel)				
anti-MHC II	FITC	14-4-4S	1:100	BD
anti-CD86	APC	GL-1	1:200	Biolegend
anti-CD8	PE-Cy5	53-6.7	1:100	Biolegend
anti-CD11c	PE-Cy7	N418	1:100	Biolegend
anti-CD54	Pacific Blue	YN1/1.7.4	1:100	Biolegend
anti-CD3	APC-Cy7	145-2C11	1:200	Biolegend

Tab. 3.2: Continuation of summary of antibodies including fluorochrome, clone, dilution and source.

Antigen	Fluorochrome	Clone	Dilution	Source
Lymph node (NK Panel)				
anti-KLRG1	FITC	2F1/KLRG1	1:100	Biolegend
anti-CD49b	PerCP-Cy5.5	DX5	1:100	Biolegend
anti-NKp46	APC	29A1.4	1:100	Biolegend
anti-CD3	AF700	500A2	1:200	eBioscience
anti-CD69	BV421	H1.2F3	1:50	Biolegend
anti-LY49D	PE	4E5	1:50	Biolegend
anti-INFγ	PE-CF594	XMG1.2	1:50	BD
anti-CD27	PE-Cy7	LG.3A10	1:400	Biolegend
anti-CD11b	BV605	M1/70	1:200	BD

3.10 ALT measurement

Cardiac blood sample were centrifuged at 10.000 g for 20 min at 4°C and the supernatant plasma was immediately frozen at -20°C. ALT measurement was performed using COBAS INTEGRA®400 plus by Roche Diagnostics Ltd. (Rotkreuz, Switzerland).

3.11 Progesterone measurement

For progesterone measurement, plasma sample were diluted 1:4 using the Multi-Diluent 3 (Siemens Healthcare Diagnostic Ltd, Camberley, UK) and measured with a competitive immunoassay on the ADVIA Centaur XP Immunoassay System (Siemens Healthcare Diagnostic Ltd, Camberley, UK) using direct chemiluminescent according to manufacturer's instructions.

3.12 Statistical analysis

All results are expressed as means ± standard error of the mean (SEM). Means between groups were compared using one-way ANOVA and Bonferroni's post tests using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA).

4. RESULTS

4.1 Establishment of a mouse model allowing to study the effects of prenatal APAP use

Addressing the question raised by the epidemiological studies - showing an increased risk in the offspring for asthma if the mother took APAP - we saw a need to establish a suitable mouse model in order to investigate the impact of APAP on pregnancy and fetal development. A syngenic mating combination of C57Bl/6 mice was chosen to minimize interstrain variability. Furthermore, the time of APAP administration was set to gd 12.5 for the following reason: at this stage, hematopoiesis translocates from the extraembryonic yolk sac and the intraembryonic PAS (para-aortic splanchnopleura)/AGM (aorta-gonad-mesonephros) region to the fetal liver [95, 96], which subsequently becomes the main source for HSCs. HSCs have the ability of self-renewal and they differentiate into erythrocytes, myeloid cells and lymphocytes. Simultaneously, the thymus is colonized by these liver progenitor cells to undergo T cell maturation. Further, by then the full establishment of the hemochorial mouse placenta is completed.

Dose-Response-Experiments

For the successful establishment of an APAP mouse model the question of the adequate APAP dose had to be answered. In order to answer this question, timed pregnancy was initiated and different APAP doses between 50 mg/kg and 450 mg/kg were injected intraperitoneally (ip) in pregnant and non-pregnant mice. These APAP dosages are comparable to a dose of 4 mg/kg to 36 mg/kg in humans, according to the guidelines of the U.S. Department of Health and Human Services, Food and Drug Administration [106]. A detailed overview of the different doses used and their human equivalent is provided in Tab. 4.1. After 24 h liver damage was quantified by plasma ALT levels in both pregnant and non-pregnant mice and fetal weight was assessed.

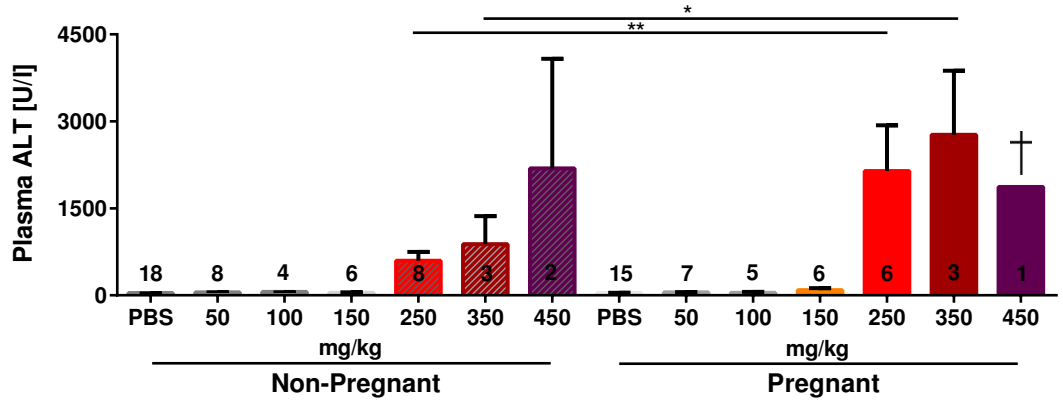


Fig. 4.1: Dose-Response-Experiment: Non-pregnant and pregnant mice on gd 12.5 were injected with different doses of APAP. Plasma alanine aminotransferase (ALT) levels were analyzed 24 h after APAP challenge. Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$, ** $p<0.01$.

The plasma ALT level is an indicator for liver damage, as it is normally located inside of the hepatocytes and can be detected in the blood when hepatocytes are damaged. In response to APAP elevated ALT levels were only observed at a dose of 250 mg/kg, regardless if the mouse was pregnant or not (Fig. 4.1). However, ALT levels of pregnant mice were significantly higher than those of the non-pregnant ones at the same dose (Fig. 4.1). This was supported by the histological analysis which showed necrosis of centrilobular hepatocytes in the livers of pregnant mice treated with 250 mg/kg while a comparable severity of necrosis was only observed when non-pregnant mice were treated with 450 mg/kg APAP (data not shown). Further,

Tab. 4.1: Conversion of animal doses of APAP used in the present study to human equivalent doses according to the guidelines of the U.S. Department of Health and Human Services, Food and Drug Administration (FDA).

dose in mice	equivalent dose in human	dose for 70 kg human
50 mg/kg	4 mg/kg	280 mg
100 mg/kg	8 mg/kg	560 mg
150 mg/kg	12 mg/kg	840 mg
250 mg/kg	20 mg/kg	1400 mg
350 mg/kg	28 mg/kg	1960 mg
450 mg/kg	36 mg/kg	2520 mg

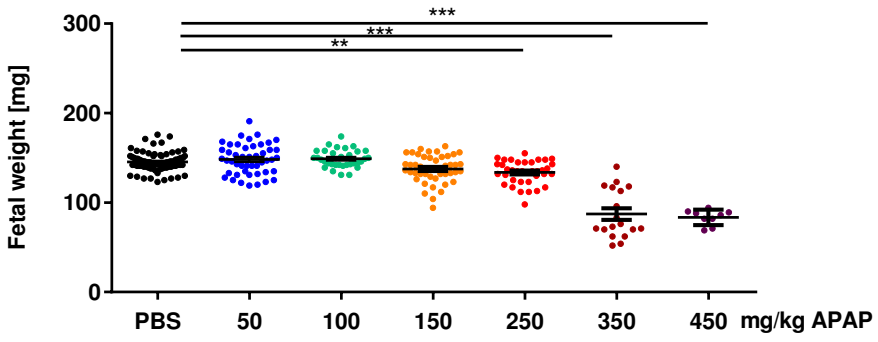


Fig. 4.2: Dose-Response-Experiment: Non-pregnant and pregnant mice on gd 12.5 were injected with different doses of APAP. The fetal weight was calculated on gd 13.5. Data are presented as scatter dot plots indicate mean ± SEM, ** $p<0.01$; *** $p<0.001$ versus PBS control animals.

non-pregnant mice tolerate a dose of 450 mg/kg while one pregnant mouse died within 24 h after APAP injection.

Changes in fetal weight due to APAP challenge were detected at a dose of 150 mg/kg and the weight was further significantly decreased with increasing APAP intake (Fig. 4.2). With the maximum dose of 450 mg/kg, the fetal weight was halved compared to the control group Fig. 4.2).

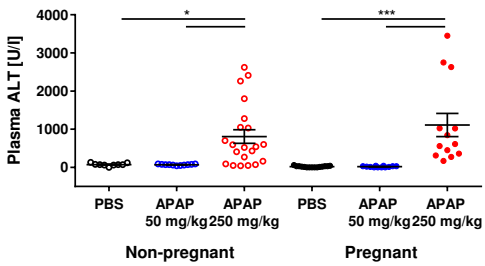
As a consequence, two doses were chosen for further investigation: the low dose of 50 mg/kg reflecting a non-toxic dose causing no liver damage and no effect on fetal weight and the high dose of 250 mg/kg resulting in severe liver damage in pregnant mice and decreased fetal weight.

4.2 Evaluation of liver toxicity and morbidity after APAP intake

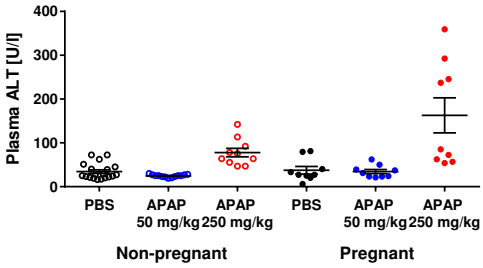
After agreement regarding the doses used, we initially focused on the maternal liver damage in comparison to non-pregnant mice and the reaction of the mice in response to APAP.

4.2.1 Plasma ALT levels and change in weight

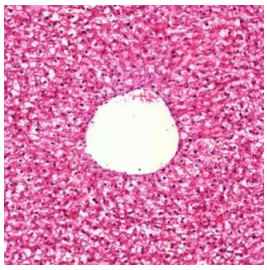
An allogenic mating combination was chosen as it reflects the natural way of reproduction. ALT levels were significantly increased in pregnant and non-pregnant animals 24 h after administration of 250 mg/kg APAP (Fig. 4.3 a) but did not show a similar big differences in ALT levels compared to the syngenic mating. However,



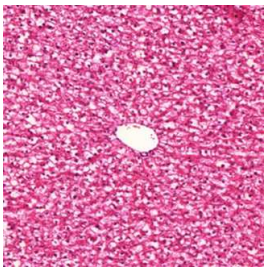
(a) 24 h after APAP challenge



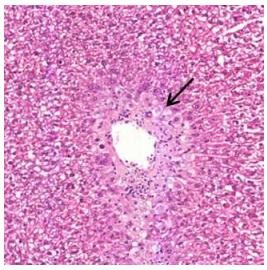
(b) 96 h after APAP challenge



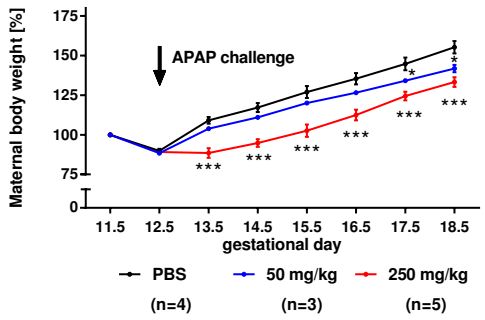
(c) PBS



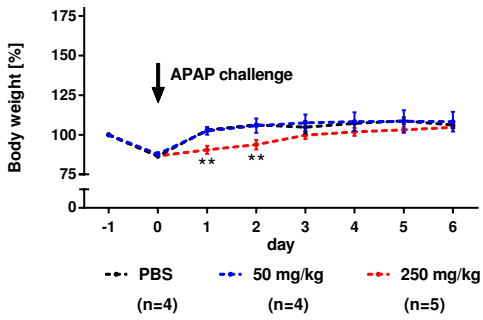
(d) 50 mg/kg APAP



(e) 250 mg/kg APAP



(f) pregnant



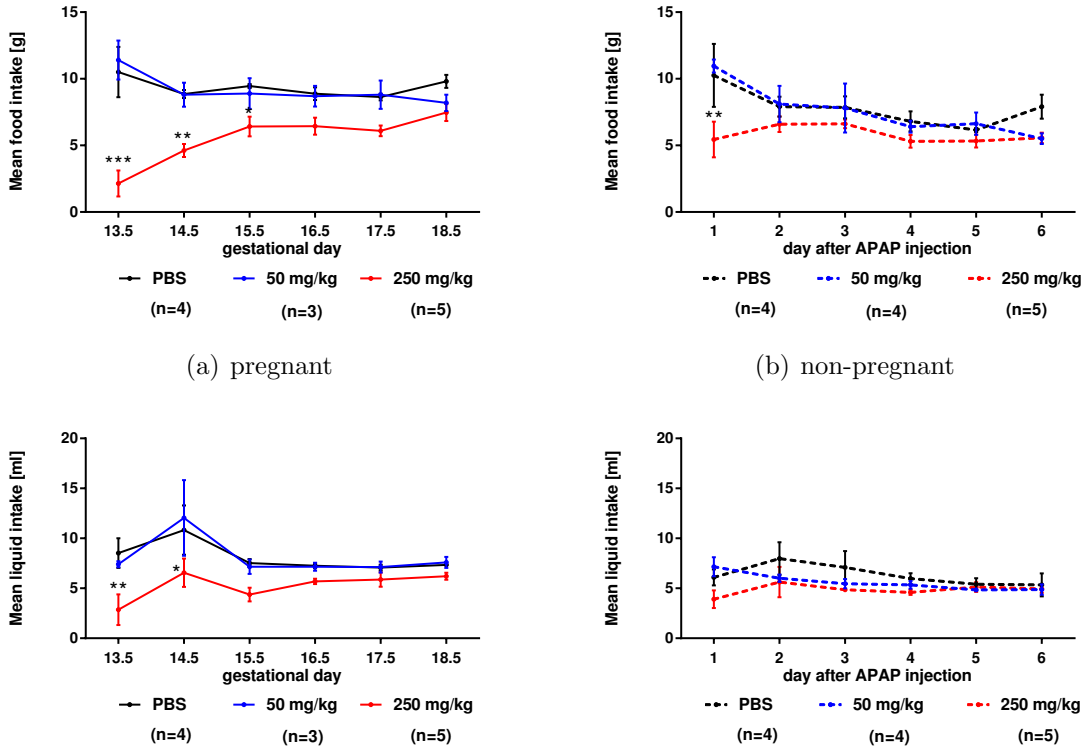
(g) non-pregnant

Fig. 4.3: Non-pregnant and pregnant mice on gd 12.5 were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP. ALT levels were analyzed 24 h (a) and 96 h (b) after APAP challenge. Representative pictures of paraffin tissue sections stained with H&E display maternal livers from pregnant mice treated with either PBS or 50 mg/kg or 250 mg/kg APAP (c-e). The black arrow points to a necrotic area of centrilobular hepatocytes around a vein. Further the body weight was assessed for pregnant (f) and non-pregnant mice (g). Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$, ** $p<0.01$; *** $p<0.001$. Dot colors are as followed: PBS (black), 50 mg/kg (blue) and 250 mg/kg (red).

after 96 h, plasma ALT levels of pregnant animals were still elevated compared to non-pregnant controls (Fig. 4.3 b). Liver histopathology revealed evidence for typically seen centrilobular necrosis 24 h after administration of 250 mg/kg APAP in pregnant mice which was absent in the 50 mg/kg treated or control pregnant females (Fig. 4.3 c-e).
In order to get an impression how the mice react to the APAP challenge, the weight gain/ loss after APAP injection was observed until birth of the offspring or 6 days post-injection in non-pregnant animals, respectively. It could be shown that the weight of pregnant and non-pregnant mice treated with 250 mg/kg is significantly decreased compared to the weight of the control animals (Fig. 4.3 f and g). However, while non-pregnant animals could regain the weight within three days, pregnant mice were not able to restore their weight to the level of the control animals until giving birth. Further, also in pregnant mice who received 50 mg/kg APAP, a slight decrement in weight could be observed, which is in contrast to non-pregnant animals treated with the same dose.

4.2.2 Food and water intake

For the purpose of providing an explanation for the weight loss especially in pregnant mice, the food consumption was quantified after APAP injection until birth or 6 days post-injection in case of non-pregnant animals. Administration of 50 mg/kg APAP revealed no alteration regarding food or water intake in both pregnant and non-pregnant animals (Fig. 4.4). In contrast, a sharp food drop was observed in all animals treated with 250 mg/kg APAP, but it was more severe in the case of pregnancy. The food intake of pregnant mice was reduced by 75% following APAP challenge (Fig. 4.4 a). Also the water uptake was decreased by more than half (Fig. 4.4 c). A recovery occured after 72 h, but levels of food and water intake shown by control animals were not reached until birth. Non-pregnant mice showed an altered eating and drinking behavior within the first 24 h after APAP injection, which was subsequently restored to levels of control mice, though.



(a) pregnant

(b) non-pregnant

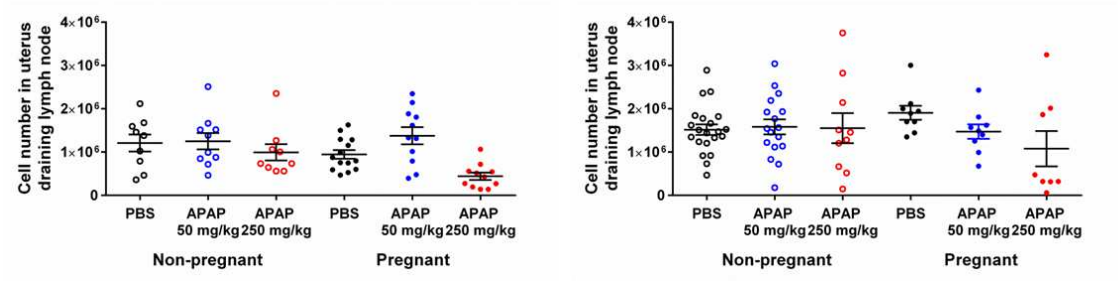
(c) pregnant

(d) non-pregnant

Fig. 4.4: Non-pregnant and pregnant mice on gd 12.5 were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP. Food and water intake was measured daily from the point of challenge until birth in case of pregnant mice (a and c) or til 6 days post-injection respectively (b and d). Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$, ** $p<0.01$.

4.3 Investigation of the effects APAP has on the maternal immune system

During pregnancy, a fine tuned maternal immune adaptation is necessary to promote fetal tolerance and sustain fetal development. Therefore, the maternal inguinal lymph node was investigated, as it is known to drain the uterus, in order to reveal potential modulations of the maternal immune system in response to APAP. With regard to mimic the maternal adaptation to semi-allogenic fetuses - due to the presence of paternal allo-antigens - experiments were performed using an allogenic mating combination.



(a) gd 13.5 (b) gd 16.5

Fig. 4.5: Non-pregnant and pregnant mice on gd 12.5 were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP. Cell counts were performed on lymph node-derived leukocytes 24 h (a) and 96 h (b) after APAP injection and the total cell number was calculated. Data are presented as scatter dot plots indicate mean ± SEM.

4.3.1 Cell number in uterus-draining lymph-node

During tissue processing, the total number of cells obtained from the uterus-draining lymph nodes of pregnant and non-pregnant mice was evaluated on gd 13.5 and 16.5, respectively.

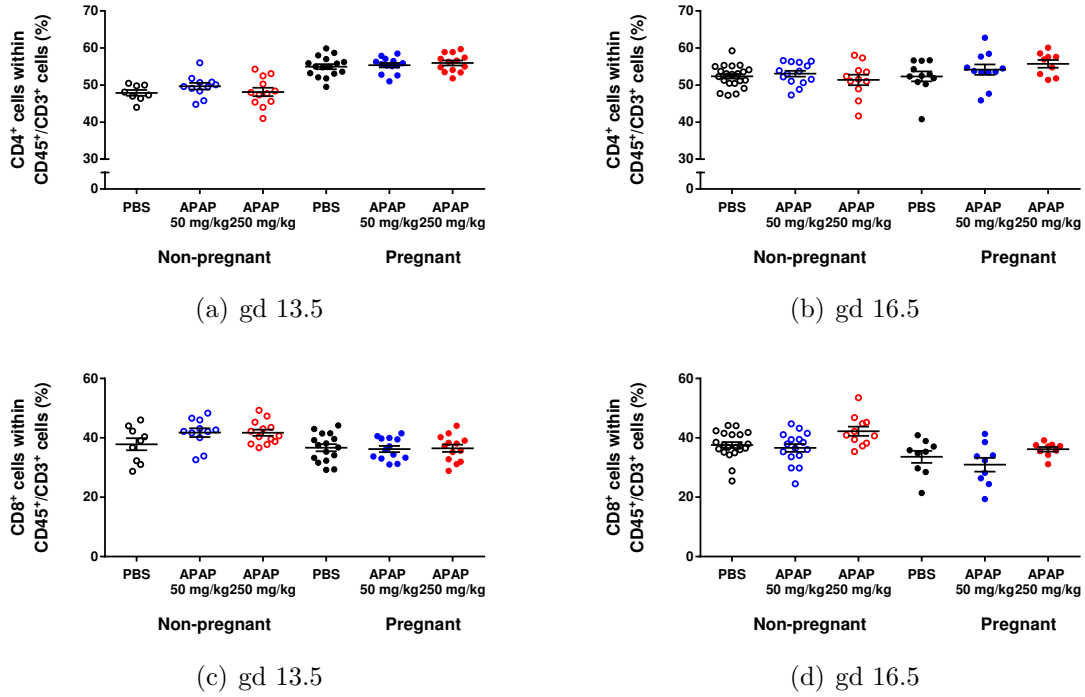
The analysis show that the cell numbers did not significantly differ between the groups on gd 13.5, although the cell number in lymph nodes from pregnant mice treated with 250 mg/kg APAP tended to decrease, compared to pregnant control animals (Fig. 4.5 a). On gd 16.5, total lymph node cells were dose-dependently decreased in pregnant mice, although levels of significance were not reached (Fig. 4.5 b). In non-pregnant mice, total lymph node cells were not significantly different between the different groups 4 days after APAP injection.

As a next step, phenotyping of the cells was performed using flow cytometry.

4.3.2 T cell populations

Flow cytometry phenotyping of uterus-draining lymph node leukocytes revealed a shift towards an increased frequency of $CD4^+$ T cells and a decreased frequency of $CD8^+$ T cells. However, no difference in response to APAP could be observed in $CD4^+$ and $CD8^+$ T cell frequencies between groups on gd 13.5 (Fig. 4.6 a and c) as wells as on gd 16.5 (4.6 b and d). Representative dots plots showing the distribution of $CD4^+$ and $CD8^+$ T cell subsets in pregnant and non-pregnant mice are displayed in Fig. 4.6 e and f.

Subsequently, the impact of APAP on $CD4^+$ T cell subset was analyzed. It could



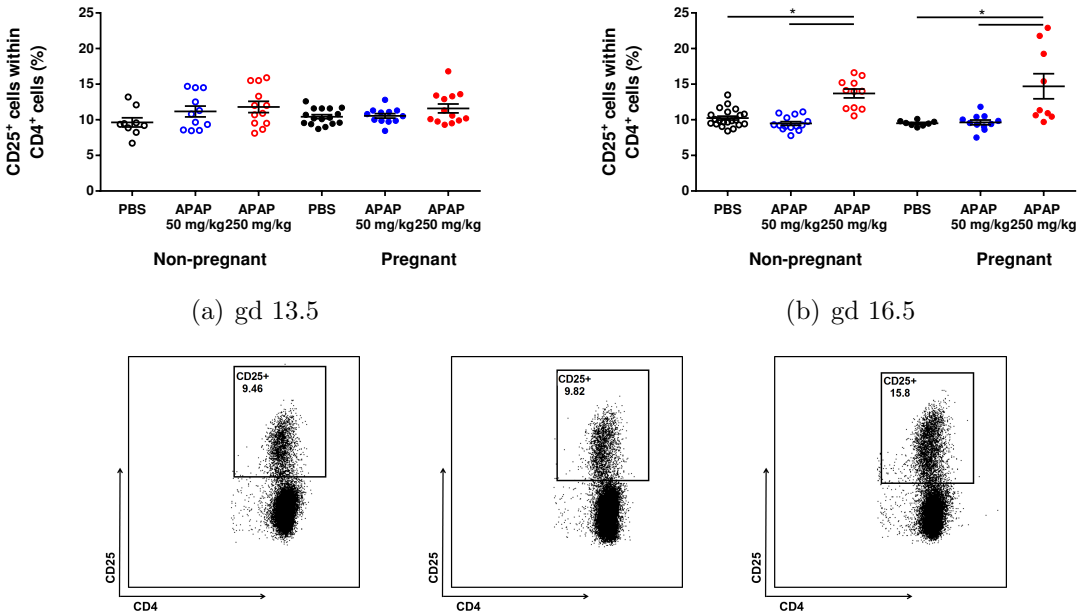
(a) gd 13.5 (b) gd 16.5

(c) gd 13.5 (d) gd 16.5

Fig. 4.6: Non-pregnant and pregnant mice on gd 12.5 were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP. Flow cytometry was performed on lymph node-derived leukocytes 24 h and 96 h after APAP injection. Graphs present the frequencies of $CD4^+$ (a+b) and $CD8^+$ T cells (c+d). Data are presented as scatter dot plots indicate mean ± SEM. Representative dot-plots show $CD4^+$ and $CD8^+$ T cell subsets of pregnant (e) and non-pregnant (f) mice of the control group.

be shown that the frequency of activated $CD4^+$ T cells, defined by the additional expression of CD25, was not modulated on gd 13.5 in response to APAP (Fig. 4.7 a). However, on gd 16.5, the frequency of activated $CD4^+$ T cells was significantly increased in response to 250 mg/kg APAP, while no changes could be observed after treatment with 50 mg/kg (Fig. 4.7 b). Representative dot plots of pregnant mice of the respective treatment groups are shown in Fig. 4.7 c-e.

Another $CD4^+$ T cell subpopulation that was analyzed in the uterus-draining lymph node are Treg cells, which are defined by the expression of FoxP3 on $CD4^+$ T cells. On gd 13.5, a significant increase in Treg cells could be observed in pregnant mice treated with 250 mg/kg APAP, while in non-pregnant animals treated with the same dose, the frequency remained unchanged (Fig. 4.8 a). No changes could be determined after challenge with 50 mg/kg APAP in both, pregnant and non-pregnant mice.



(a) gd 13.5 (b) gd 16.5

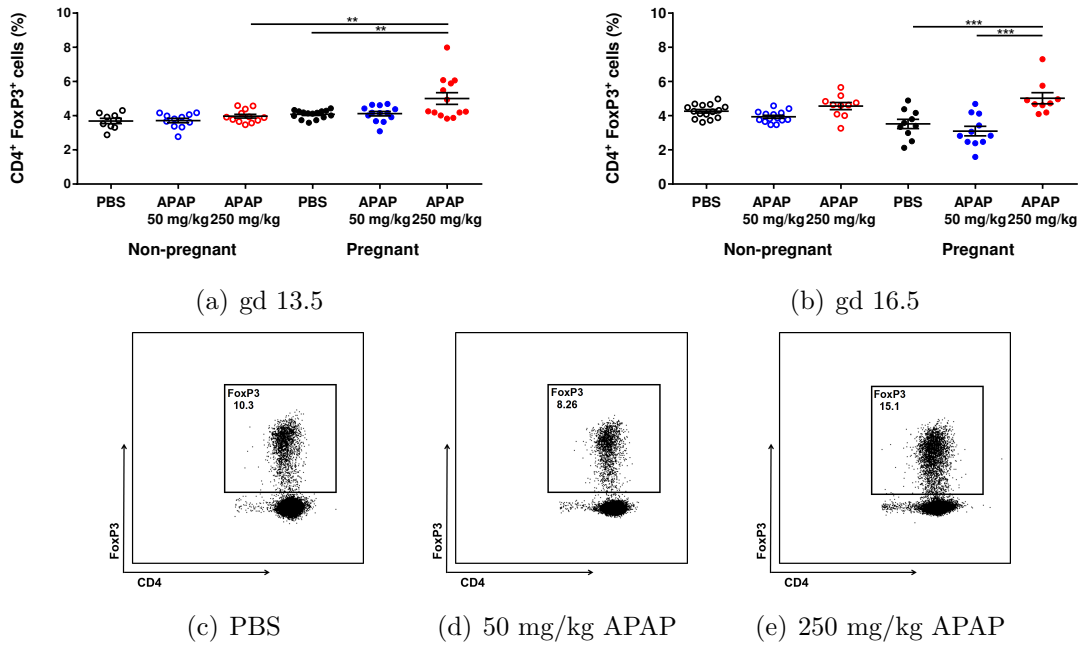
(c) PBS (d) 50 mg/kg APAP (e) 250 mg/kg APAP

Fig. 4.7: Non-pregnant and pregnant mice on gd 12.5 were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP. Flow cytometry was performed on lymph node-derived leukocytes 24 h and 96 h after APAP injection. Graphs present the frequencies of activated CD4 T cells on gd 13.5 (a) and 16.5 (b). Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$. Representative dot-plots show $CD25^+$ T cell subsets of pregnant mice of the respective groups (c-e).

On gd 16.5, the increased frequency of Treg cells upon treatment with 250 mg/kg APAP administration seen on gd 13.5 was even more profound (Fig. 4.8 b). Additionally mice treated with 50 mg/kg APAP showed a slightly decreased frequency of Treg cells. In constrast, Treg frequencies of non-pregnant mice remained unaltered after APAP challenge, irrespective of the dose. Representative dot plots are displayed from pregnant mice of the respective groups (Fig. 4.8 c-e).

Next, CD8 subpopulations in uterus-draining lymph nodes were investigated. On gd 13.6, a pregnancy-related increase in $CD8^+CD122^+$ T cell frequency could be observed in pregnant control mice, compared to non-pregnant controls. However, no significant differences in response to APAP could be determined between groups (Fig. 4.9 a).

In contrast, on gd 16.5 the $CD8^+CD122^+$ T cell frequency was significantly increased after challenge with 250 mg/kg APAP. In response to 50 mg/kg APAP a slightly



(a) gd 13.5 (b) gd 16.5

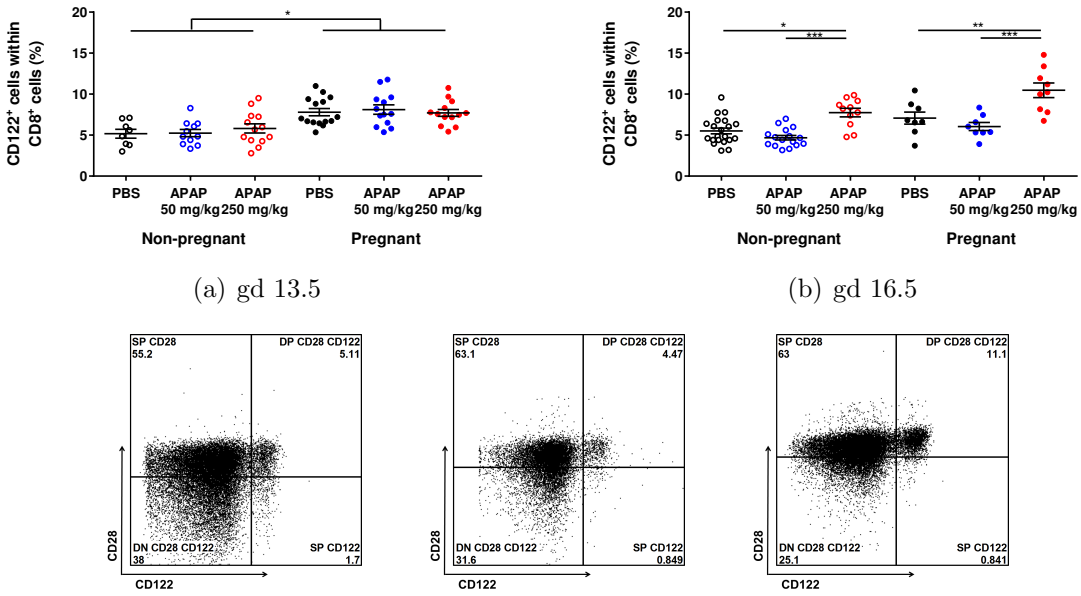
(c) PBS (d) 50 mg/kg APAP (e) 250 mg/kg APAP

Fig. 4.8: Non-pregnant and pregnant mice on gd 12.5 were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP. Flow cytometry was performed on lymph node-derived leukocytes 24 h and 96 h after APAP injection. Graphs present the frequencies of regulatory CD4 T cells (Tregs) on gd 13.5 (a) and 16.5 (b). Data are presented as scatter dot plots indicate mean ± SEM, ** $p<0.01$, *** $p<0.001$. Representative dot-plots show FoxP3$^+$ T cell subsets of pregnant mice of the respective groups (c-e).

decreased CD8$^+$CD122$^+$T cell frequency was revealed. However, no difference could be seen between pregnant and non-pregnant mice (Fig. 4.9 b). Representative dot plots of the respective groups are shown for pregnant mice (Fig. 4.9 c-e).
Furthermore, the frequencies of CD8$^+$ CD28$^-$ T cells was analyzed, but they remained unaltered after APAP administration in pregant and in non-pregnant mice (data not shown).

4.3.3 Dendritic cell populations

Another cell population assessed were DCs as they play an important role in mediating tolerance towards the fetus.
The frequency of DCs, defined as CD11c$^+$, was generally increased in pregnant mice compared to non-pregnant controls, but remained unaltered by APAP administration (Fig. 4.10 a). However, the pregnancy-related decrease of CD11c$^+$ mature DCs, assessed by the co-expression of MHC class II, was abolished in lymph nodes of preg-



(a) gd 13.5 (b) gd 16.5

(c) PBS (d) 50 mg/kg APAP (e) 250 mg/kg APAP

Fig. 4.9: Non-pregnant and pregnant mice on gd 12.5 were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP. Flow cytometry was performed on lymph node-derived leukocytes 24 h and 96 h after APAP injection. Graphs present the frequencies of $CD122^+$ $CD8^+$ T cells on gd 13.5 (a) and 16.5 (b). Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Representative dot-plots show $CD122^+$ $CD8^+$ T cell subsets of pregnant mice oh gd 16.5 of the respective groups (c-e).

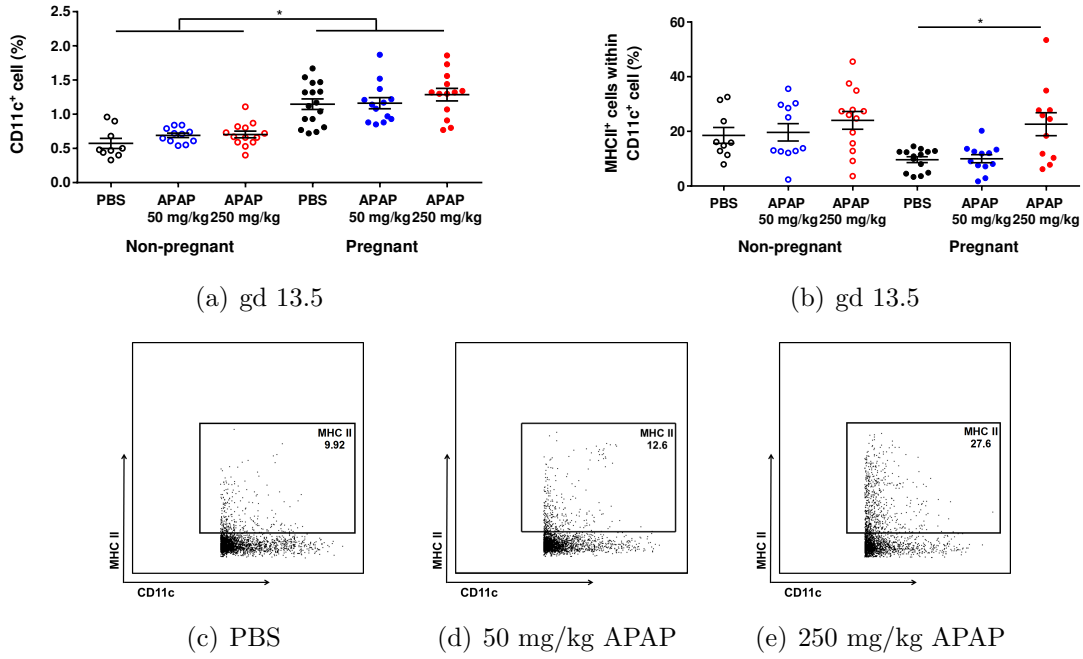
nant mice treated with 250 mg/kg APAP (Fig. 4.10 b). Representative dot-plots show the expression of MHC class II of pregnant mice on gd13.5 of the respective groups.

On gd 16.5, the frequency of DCs remained unchanged and the increased frequency of mature DCs in response to 250 mg/kg APAP was no longer detectable (data not shown).

4.3.4 Natural killer cell population

Another population investigated in the uterus-draining lymph node of pregnant and non-pregnant mice were NK cells, as they play a beneficial role in placentation.

On gd 13.5, the frequency of NK cells, defined by the expression of NKp46, was observed to be slightly increased in pregnant control mice. Further, 250 mg/kg APAP provoked an increase of the NK cell population in non-pregnant and pregnant mice,



(a) gd 13.5 (b) gd 13.5

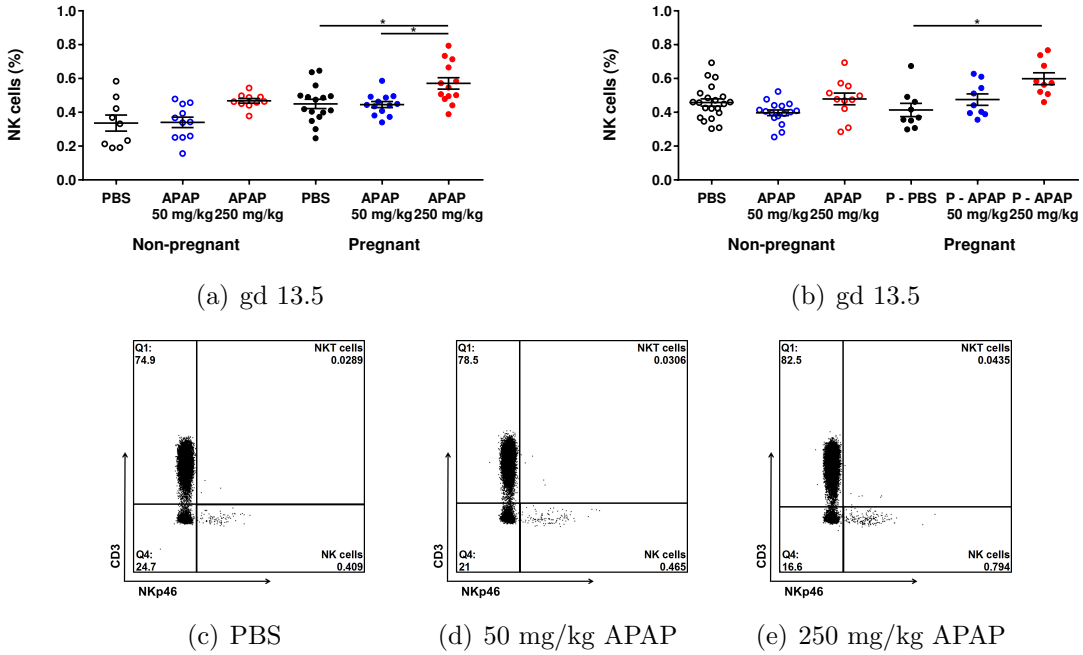
(c) PBS (d) 50 mg/kg APAP (e) 250 mg/kg APAP

Fig. 4.10: Non-pregnant and pregnant mice on gd 12.5 were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP. Flow cytometry was performed on lymph node-derived leukocytes 24 h and 96 h after APAP injection. Graphs present the frequencies of dendritic cells, defined as $CD11c^+$, (a) and the additional expression of MHC class ll (b) on gd 13.5. Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$. Representative dot-plots show MHC class II subsets of pregnant mice (gd13.5) of the respective groups (c-e).

although level of significance was only reached in pregnant animals (Fig. 4.11 a). This APAP-induced increase of NK cells was still present on gd 16.5 in pregnant mice. In contrast, non-pregnant animals showed equal frequencies of NK cells in all group 4 days after injection of PBS or APAP, respectively (Fig. 4.11 b). Representative dot-plots display the NK cell population of pregnant mice on gd13.5 (Fig. 4.11 c-e).

Subsequently, the maturation of NK cells was assessed by analyzing the additional expression of CD49b (DX5). On gd 13.5, the frequency of mature NK cells was shown to be significantly increased in pregnant compared to non-pregnant animals, both treated with PBS. This increase was further enhanced by APAP treatment with 50 mg/kg. (Fig. 4.12 a). In contrast, the dose of 250 mg/kg was observed to have an opposite effect as the frequency of mature NK cells was decreased.

Further, the proliferation activity of NK cells was determined by the expression of CD69. It could be revealed that APAP induces a dose-dependent proliferation of



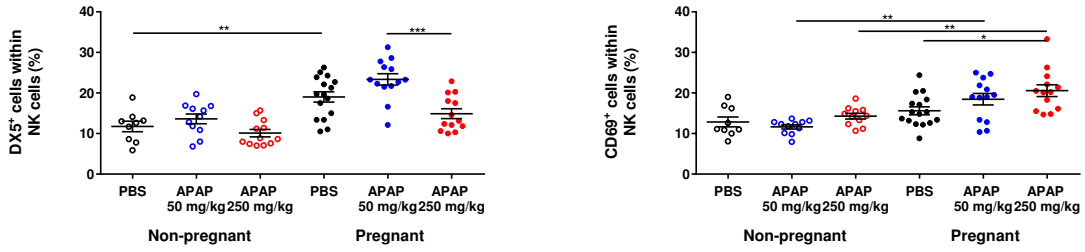
(a) gd 13.5 (b) gd 13.5

(c) PBS (d) 50 mg/kg APAP (e) 250 mg/kg APAP

Fig. 4.11: Non-pregnant and pregnant mice on gd 12.5 were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP. Flow cytometry was performed on lymph node-derived leukocytes 24 h and 96 h after APAP injection. Graphs present the frequencies of natural killer cells, defined as NKp46$^+$, on gd 13.5 (a) and gd 16.5 (b). Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$. Representative dot-plots show NKp46$^+$ subset of pregnant mice (gd13.5) of the respective groups (c-e).

NK cells, which gained significant levels when 250 mg/kg APAP were given. In contrast, non-pregnant animals did not show this CD69 co-expression due to APAP challenge (Fig. 4.12 b).

In summary, for maintaining a successful pregnancy and promoting fetal tolerance, the maternal immune system has to adapt to the 'semi-allogenic' fetus. The results obtained from the analysis of the uterus-draining lymph node showed that APAP interferes with these adaptational processes. It could be shown that APAP provokes an increase of CD4$^+$CD25$^+$ T cells. Further, APAP induced an elevation of CD8$^+$ CD122$^+$ T cells. An effect, specifically observed in pregnant animals, was an increase of CD4$^+$ Treg cells in response to APAP intake. Moreover, APAP abolished the pregnancy-related decrease of mature dendritic cells. Finally, APAP caused an increment of NK cells which was more prominent in pregnant animals. Additionally, APAP modulated the maturation status of NK cells and the proliferation activity



(a) gd 13.5 (b) gd 13.5

Fig. 4.12: Non-pregnant and pregnant mice on gd 12.5 were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP. Flow cytometry was performed on lymph node-derived leukocytes 24 h and 96 h after APAP injection. Graphs present the frequencies of mature natural killer (NK) cells, defined by the additional expression of DX5 (a) and proliferating NK cells assessed by co-expression of CD69 (b) on gd 13.5. Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$., ** $p<0.01$, *** $p<0.001$

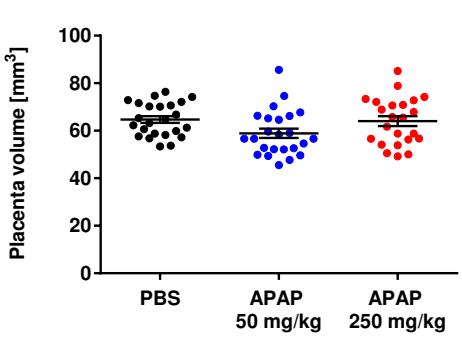
in pregnant mice, whereas these populations remained unaltered in non-pregnant animals.

4.4 *Analysis of placental morphology and endocrine responses due to APAP use*

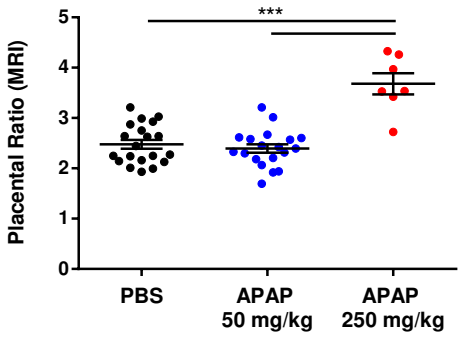
The placenta represents the interface between mother and fetus and its functions are crucial to ensure fetal growth and development. Placental functional area are of particular interest, e.g. the labyrinth, where the exchange of gas and nutrients between mother and fetus. The junctional zone represents a source of pregnancy-related hormones. Therefore, placental morphology was investigated with respect to APAP challenge.

4.4.1 MRI analysis of placenta

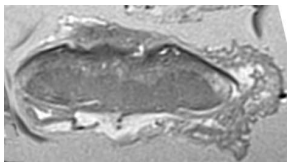
In a first approach, the placentas obtained on gd16.5 from dams treated with either PBS or APAP were analysed by MRI. Volumetric analysis revealed similar placental volumes, irrespective of the prenatal treatment (Fig. 4.13 a). Subsequently also the volume of the functional zones was determined and the placental ratio (labyrinth/ junctional zone) was calculated (Fig. 4.13 b). A morphological distinction of functional areas was not possible in 16 of 23 examined placentas exposed to 250 mg/kg APAP. Here, the placental tissue appeared very inhomogeneous and disintegrated.



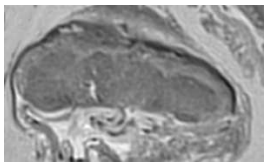
(a) gd 16.5



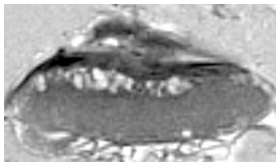
(b) gd 16.5



(c) PBS



(d) 50 mg/kg APAP



(e) 250 mg/kg APAP

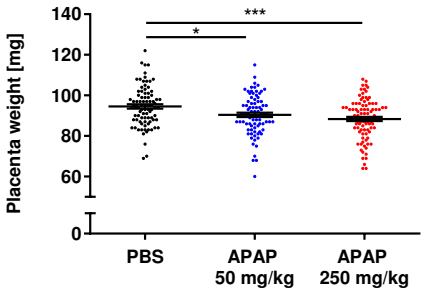
Fig. 4.13: Pregnant mice were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5 and MRI analysis was performed on gd 16.5. Graphs present the placental volume (a) and the the placental ratio of labyrinth/ junctional zone (b). Data are presented as scatter dot plots indicate mean ± SEM, *** $p<0.001$. Representative MRI images display placentas exposed to either PBS or 50 mg/kg or 250 mg/kg APAP (c-e). The labyrinth appears darker in the MRI while the junctional zone appears brighter.

Furthermore, some samples showed vacuoles in the junctional zone, which appear as white in the MRI image (Fig. 4.13 e). Consequently, the placental ratio could be calculated for 7 samples. In these samples the ratio was shown to be increased. This resulted from an increased labyrinth combined with a decreased junctional zone.

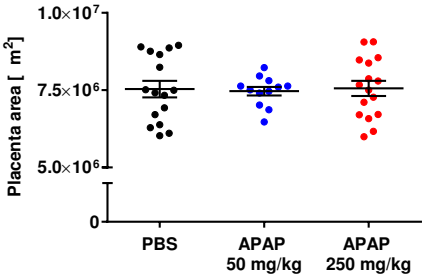
4.4.2 Histological analysis of placenta

In a second approach to asses placental functional areas, Masson's trichrome staining was performed. Histomorphological analysis was conducted to confirm the results obtained from MRI. Moreover, possible limitations associated with MRI imaging were aimed to overcome, e.g. morphological distinction of labyrinth and junctional zone, especially of the placentas exposed to 250 mg/kg APAP.

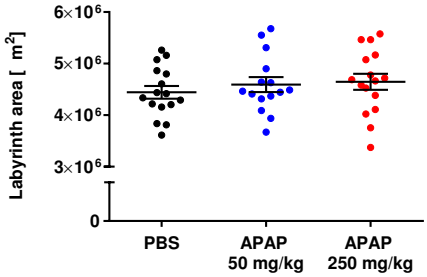
Upon tissue harvesting the placental weight was measured on gd 13.5. The analysis showed a slight, but significant decline in weight, which was observed to be dose-dependent (Fig. 4.14 a). However, histomorphological analysis revealed no



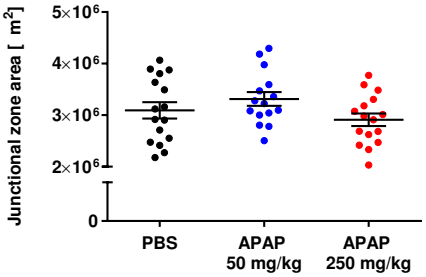
(a) Placental weight



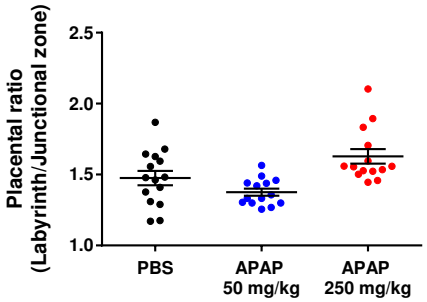
(b) Placental area



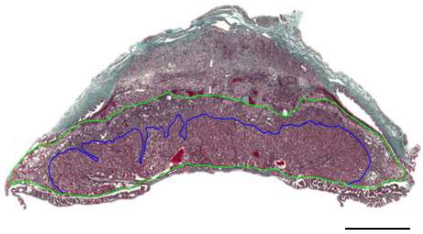
(c) Labyrinth area



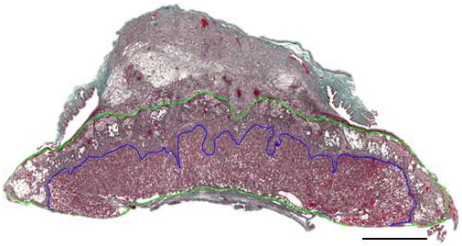
(d) Junctional zone area



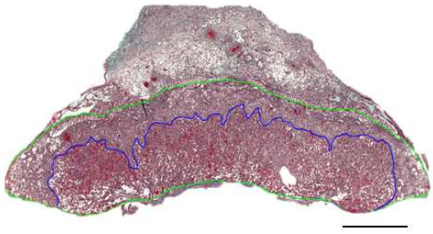
(e) Placental Ratio



(f) PBS



(g) 50 mg/kg



(h) 250 mg/kg

Fig. 4.14: Pregnant mice were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5 and placental histomorphology was performed on gd 13.5. Placental weight (a) was evaluated as well as the overall surface area of the placenta (b), the labyrinth (c) and the junctional zone (d). Placental ratio was calculated (e) and representative pictures display placental mid-sagittal tissue sections (f-g). Blue lines indicate the labyrinth while the area between green and blue lines indicate the junctional zone. Black lines denote 1000 μm. Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$, *** $p<0.001$.

difference regarding overall surface area between PBS and APAP treated placentas (Fig. 4.14b). Also, the area of the labyrinth showed no significant differences, although the mean labyrinth area was slightly increased (Fig. 4.14 c). The junctional zone was increased in response to 50 mg/kg APAP, while no difference were observed after 250 mg/kg APAP (Fig. 4.14 d). Consequently, the calculated placental ratio was decreased following 50 mg/kg APAP and increased after exposure to 250 mg/kg (Fig. 4.14 e). Representative pictures of placental mid-sagittal tissue sections from gd 13.5 are shown in (Fig. 4.14 f-h). The overall placenta surface areas is marked in green, the border between labyrinth and junctional zone is shown in blue.

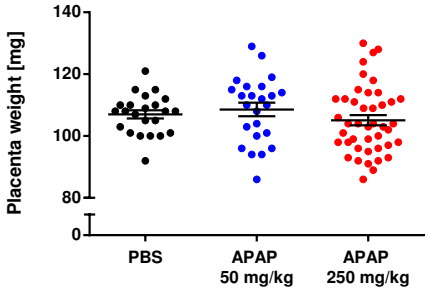
On gd 16.5, placental weight was no longer altered due to APAP (Fig. 4.14 a). The calculation of placental area revealed no significant differences between the groups (Fig. 4.14 b). However, a slight decrease in response to 50 mg/kg and a minor increase due to 250 mg/kg APAP could be observed. Evaluating this more closely, an increase of the labyrinth due to 250 mg/kg APAP (Fig. 4.14 c) and a significant decline of the junctional zone after treatment with 50 mg/kg APAP was detected (Fig. 4.14 d).

Subsequently, calculating the labyrinth/junctional ratio for gd 16.5, placentas from both APAP treated groups showed a significantly increased ratio (Fig. 4.14 e). Noteworthy was that alterations induced by 50 mg/kg APAP largely affected the junctional zone, as this area initially increased within 24 h but 4 days after injection significantly decreased. Conversely, placental alterations induced by 250 mg/kg APAP resulted in an enlarged labyrinth while the junctional zone was reduced on both gd 13.5 and 16.5. Representative pictures of placental mid-sagittal tissue sections from gd 16.5 are shown in (Fig. 4.15 f-h).

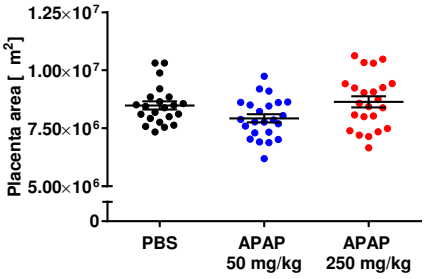
4.4.3 Plasma progesterone levels

Since the placenta is a source of pregnancy-related hormones, it was then evaluated if the placental changes observed upon APAP treatment affected the production of pregnancy-related hormones. Blood plasma was obtained from the heart in order to determine levels of progesterone on gd 13.5 and gd 16.5, respectively.

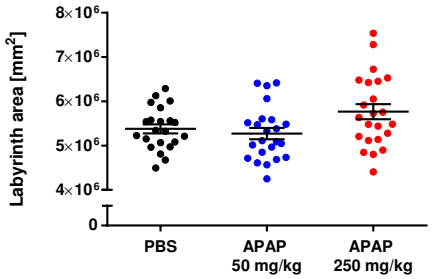
A dose-dependent decrease of progesterone in response to APAP, compared to control animals, was observed on gd 13.5, and level of significance was reached upon treatment with 250 mg/kg APAP (Fig. 4.16 a). On gd 16.5, plasma progesterone



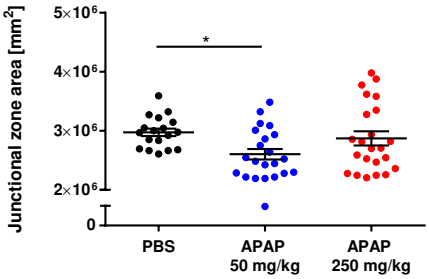
(a) Placental weight



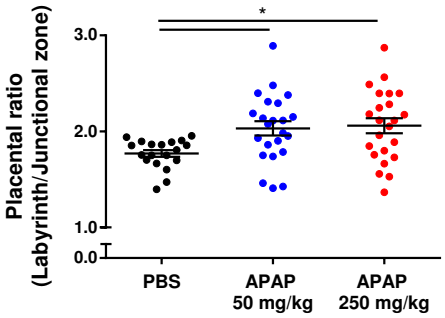
(b) Placental area



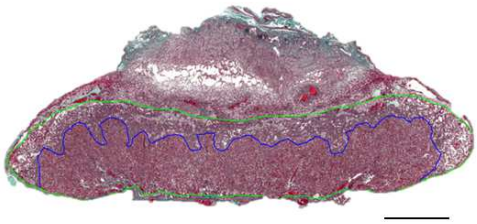
(c) Labyrinth area



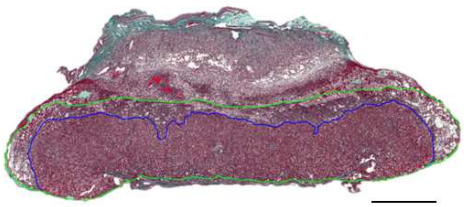
(d) Junctional zone area



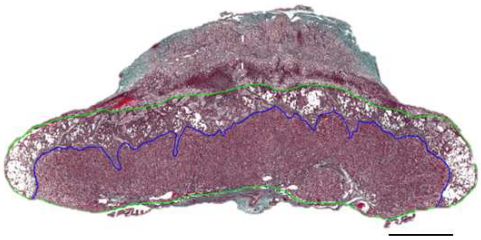
(e) Placental Ratio



(f) PBS

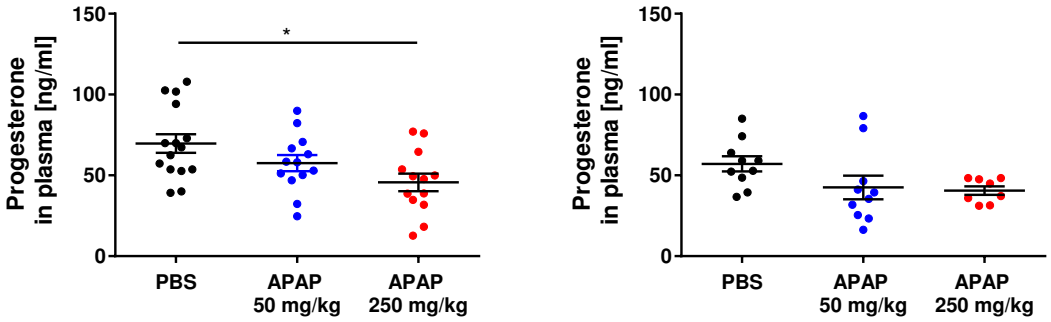


(g) 50 mg/kg



(h) 250 mg/kg

Fig. 4.15: Pregnant mice were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5 and placental histomorphology was performed on gd 16.5. Placental weight (a) was evaluated as well as the overall surface area of the placenta (b), the labyrinth (c) and the junctional zone (d). Placental ratio was calculated (e) and representative pictures display placental mid-sagittal tissue sections (f-g). Blue lines indicate the labyrinth while the area between green and blue lines indicate the junctional zone. Black lines denote 1000 μm. Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$.



(a) gd 13.5 (b) gd 16.5

Fig. 4.16: Pregnant mice were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5. Maternal plasma progesterone levels were evaluated on gd 13.5 (a) and gd 16.5 (b). Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$.

levels were generally lower compared to gd 13.5, irrespective of the treatment. However, the decline in response to APAP seen on gd 13.5 was still present in animals treated with either 50 mg/kg or 250 mg/kg APAP (Fig. 4.16 b).

In summary, the fetal-maternal interface represents a functional area nourishing the fetus and producing hormones to maintain tolerance and subsequently a successful pregnancy. It could be shown that APAP affects the morphology of the placenta which resulted in an altered placental ratio of labyrinth and junctional zone. Further, maternal progesterone levels were decreased in response to APAP.

4.5 Pregnancy outcome

In a next step, pregnancy outcome following APAP administration was evaluated. Therefore, the number of implantations in the uterus was documented as well as the number of abortions in order to calculate the individual abortion rate. Experiments were performed on gd 13.5 and 16.5 using the syngenic as well as the allogenic mating combination. Representative photos of implantations in the uterus are shown in Fig. 4.17.

It could be observed that APAP has no influence on the number of implantations as shown in Tab. 4.2. As observed on gd 13.5, the syngenic mating allows more implantations than the allogenic mating. However, on gd 16.5 both mating show 8-9 implantations irrespective of the treatment.

(a) gd 13.5 (b) gd 16.5

Fig. 4.17: Representative photos of implantations in the uterus obtained on gd 13.5 (a) and 16.5 (b). The black arrow points to an abortion.

Further, no significant differences with regard to fetal loss in response to APAP could be determined (Tab. 4.2). Although it appears as if the abortion rate on gd 13.5 after syngenic mating is dose-dependently increased, this could not be confirmed when using the allogenic mating. Additionally, on gd 16.5 fetal loss rate was below 5 % for both syngenic and allogenic mating, independent of APAP treatment.

4.6 Examination of the effect of APAP on fetal HSC reservoir and on T cell maturation

4.6.1 Fetal weight

When studying the impact of a prenatal challenge, a first hint for an impaired fetal development is given by the fetal weight. Assessments of fetal weight happened prenatally on gd 13.5, gd 16.5 and postnatally one day after birth. As the results obtained from syngenic and allogenic mating have been equal, I refrain from showing both in order to focus on the allogenic mating combination.
The results showed a significant, dose-dependently decline of fetal weight 24 h after administration of APAP (Fig. 4.18 a). This fetal weight loss was still prominent 96 h after injection of 250 mg/kg APAP, whilst fetuses exposed to 50 mg/kg APAP

Tab. 4.2: Pregnancy outcome: Implantations and abortion rate remained unaltered due to APAP challenge observed on gd 13.5 and 16.5 using either a syngenic (C57Bl/6 x C57Bl/6) or an allogenic mating combination (C57Bl/6 x CBy.SJL(B6)-Ptprca/J) [mean ± sem (N)].

	syngenic		**allogenic**	
	gd 13.5	**gd 16.5**	**gd 13.5**	**gd 16.5**
implantations				
PBS	9.5 ± 0.2 (17)	8.8 ± 0.6 (8)	8.3 ± 0.3 (19)	8.0 ± 0.3 (12)
50 mg/kg	9.2 ± 0.2 (12)	8.4 ± 0.4 (8)	7.7 ± 0.3 (17)	8.6 ± 0.3 (11)
250 mg/kg	10.0 ± 0.4 (8)	8.5 ± 0.4 (8)	8.4 ± 0.2 (17)	8.2 ± 0.4 (9)
abortion rate				
PBS	7.8 ± 2.0 (17)	4.7 ± 3.6 (8)	7.4 ± 2.1 (19)	3.1 ± 1.6 (12)
50 mg/kg	9.8 ± 3.3 (12)	4.2 ± 2.9 (8)	1.3 ± 3.9 (17)	0.0 ± 0.0 (11)
250 mg/kg	15.0 ± 3.7 (8)	4.6 ± 3.1 (8)	4.6 ± 1.7 (17)	2.8 ± 1.9 (9)

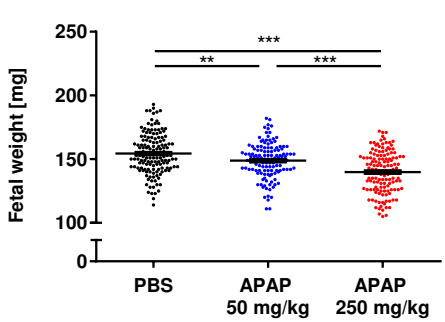
could regain the weight of control fetuses. (Fig. 4.18 b). Representative examples for fetuses harvested on gd 16.5 are shown in Fig. 4.18 d.
After birth, the weight of the newborns prenatally treated with 250 mg/kg APAP was still significantly decreased compared to the offspring of dams treated with either PBS or 50 mg/kg APAP (Fig. 4.18 c).

4.6.2 Fetal liver

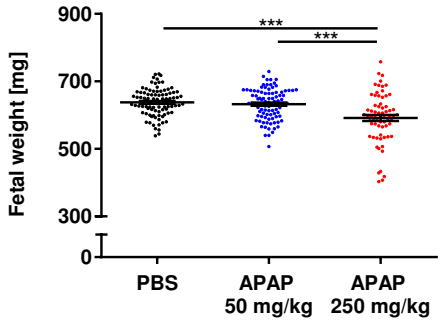
Given that APAP is hepatotoxic and the fetal liver is the major source of HSCs during fetal development, this organ is of particular interest in the present setting. Therefore the fetal liver was investigated on gd 13.5 and 16.5 using both mating combinations.

Histology

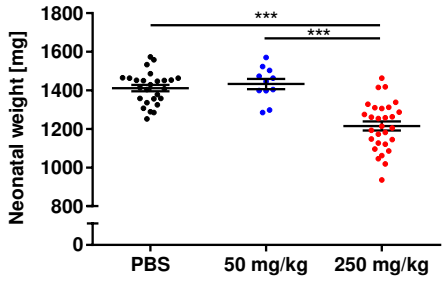
First, the fetuses were collected and embedded in paraffin on gd 13.5 in order to evaluate the morphology of the fetal liver due to APAP challenge 24 h after injection. Therefore, H&E staining was performed on transverse sections in lower thoracic region in the fetuses obtained from allogenic mating.
It was not possible to identify any histological signs for liver damage due to APAP challenge. Even the treatment with the toxic dose of 250 mg/kg did not cause liver necrosis as seen in the maternal liver. The hematopoiesis happening during this



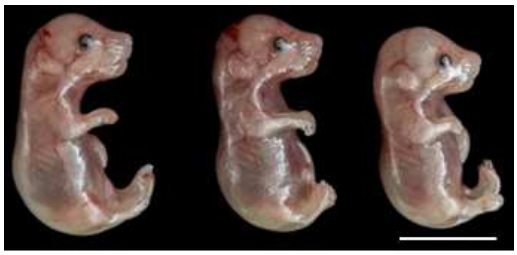
(a) gd 13.5



(b) gd 16.5



(c) postnatal day 2



(d) gd 16.5

Fig. 4.18: Pregnant mice were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5. Fetal weight was assessed on gd 13.5 (a) and gd 16.5 (b) as well as the weight of the newborns (c). Data are presented as scatter dot plots indicate mean ± SEM, ** $p<0.01$, *** $p<0.001$. A representative picture from fetuses of gd 16.5 are shown in (d), white line denotes 10 mm.

time is represented excessively and may overlay morphological changes.
As a second step, ALT levels were measured in plasma obtained from fetal blood on gd 16.5, and ALT levels remained below the physiological limit of 100 U/l in all groups (data not shown). Hence, no measurable liver damage could be detected with our approach in the fetal liver in response to APAP using classical parameter such as histomorphology and ALT assessments.

Flow cytometry

In order to further analyse the fetal liver, we harvested the fetal liver on gd 16.5. The phenotype of fetal liver cells was assessed by flow cytometry with a special focus on HSCs defined as Lin^{neg} $CD34^{neg}$ c-kit^{+}, and $Sca\text{-}1^{+}$ and multi-potent progenitor cells defined as Lin^{neg} c-kit^{+} $Sca\text{-}1^{+}$ and $CD34^{+}$.

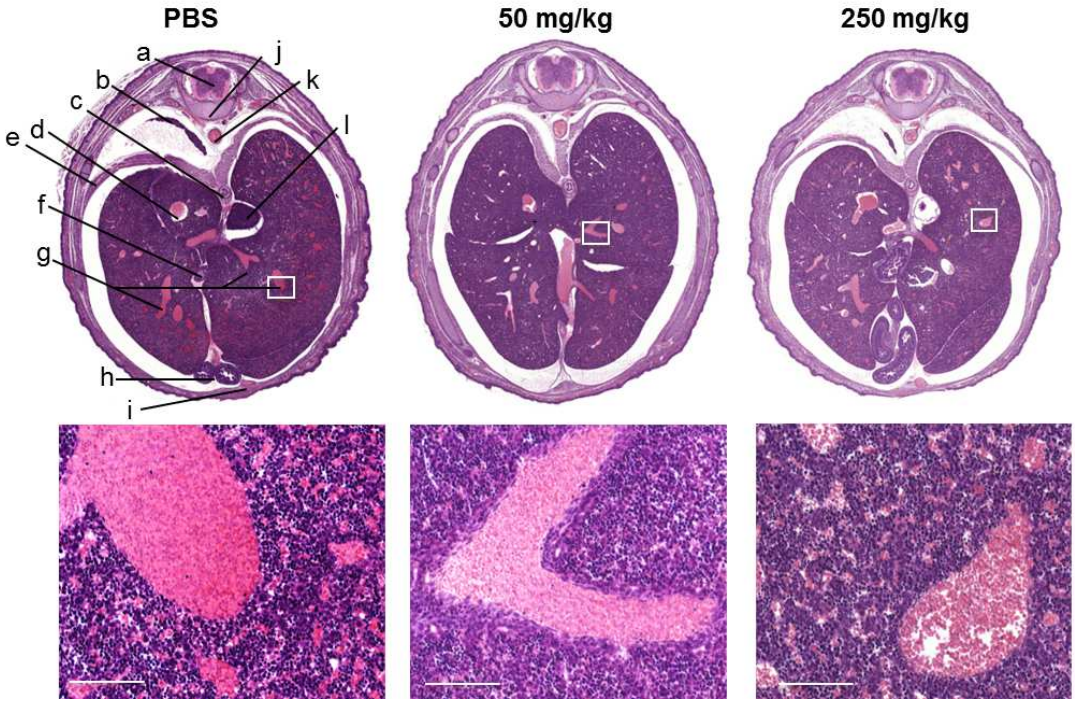
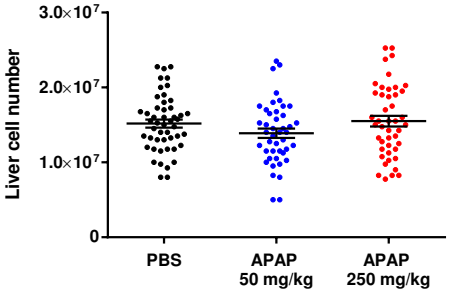


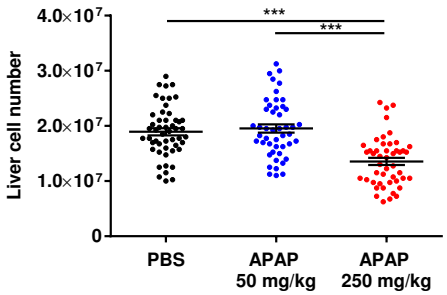
Fig. 4.19: Histology of fetal liver: Pregnant mice were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5. H&E staining was performed on transverse sections in lower thoracic region of fetuses obtained on gd 13.5 from the respective treatment groups: PBS (left), 50 mg/kg APAP (center) and 250 mg/kg APAP (right). Morphological structures are described in the photomicrograph of a PBS treated fetus. a: spinal cord, b: caudal border of right lung lobe, c: oesophagus, d: intra-hepatic inferior vena cava, e: diaphragm, f: bile and bile duct, g: branches of hepatic veins, h: loops of small intestine, i: umbilical vein, j: cartilage primordium, k: thoracic descending aorta, l: caudate liver lobe. White squares indicate areas of liver tissue and hepatic veins selected for presentation at higher magnification below. White lines denote 100 μm.

During the preparation of a stained single cell suspensions, the total number of cells was counted. No difference in total cell number was observed due to APAP challenge when the mice were mated allogenically (Fig. 4.20 a). In contrast, after syngenic mating a decline of fetal liver cells could be observed in response to 250 mg/kg APAP (Fig. 4.20 b).

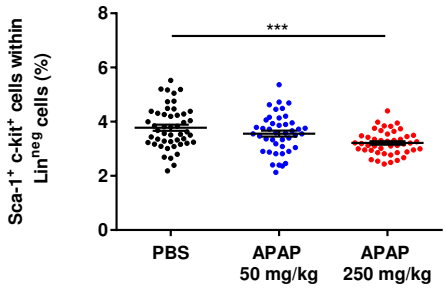
However, flow cytometry analysis revealed equal results for both matings. Hence I confine myself to show the result obtained from the allogenic mating. The frequency of HSCs was significantly reduced in response to 250 mg/kg APAP (Fig. 4.20 c), but was not affected when exposed to 50 mg/kg. In contrast, both doses of APAP produced an effect on the frequency of multi-potent progenitor cells (MPPs) which was



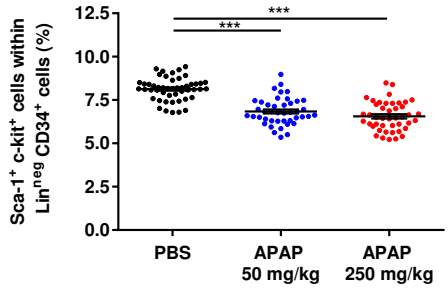
(a) Liver cell count (allogenic mating)



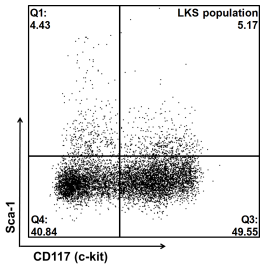
(b) Liver cell count (syngenic mating)



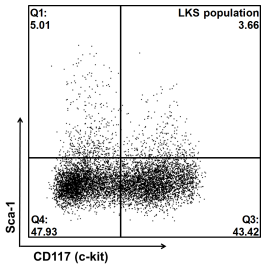
(c) LKS



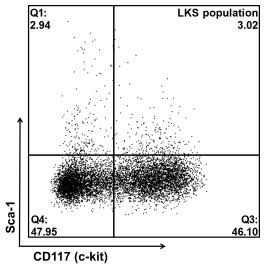
(d) MMP



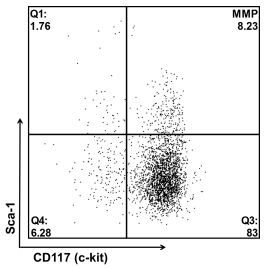
(e) PBS



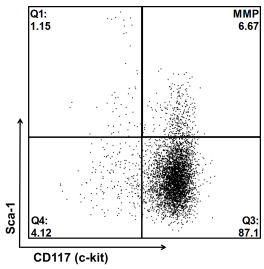
(f) 50 mg/kg APAP



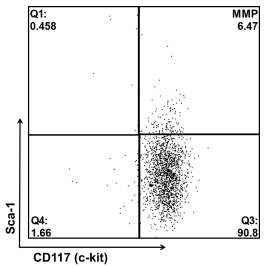
(g) 250 mg/kg APAP



(h) PBS

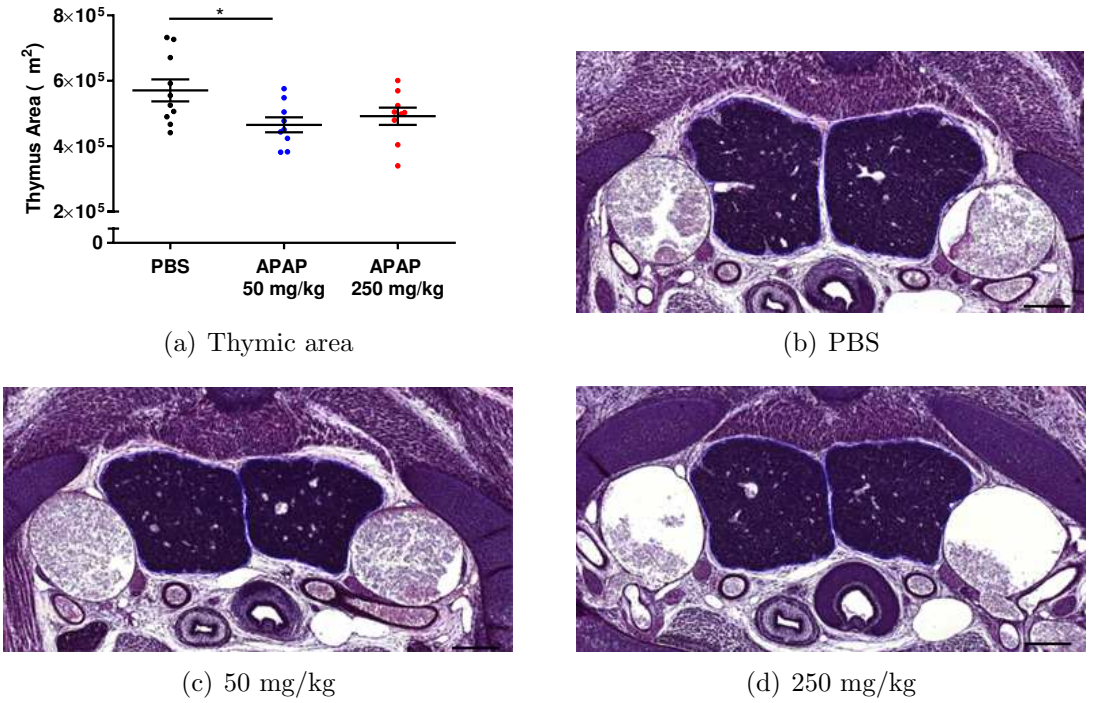


(i) 50 mg/kg APAP



(j) 250 mg/kg APAP

Fig. 4.20: Flow cytometry analysis of fetal liver on gd 16.5: Pregnant mice were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5. Total cell number were determined from allogenic (a) and syngenic mating (b). Frequencies of HSCs defined as Lin^{neg} c-kit^{+} Sca-1^{+} (c) and of multi-potent progenitor cells (MPPs) defined as Lin^{neg} CD34^{+} c-kit^{+} Sca-1^{+} cells (d) were determined by flow cytometry. Data are presented as scatter dot plots indicate mean ± SEM, *** p<0.001.Representative dot plots are presented below for HSCs (e-g) and MPPs (h-j) respectively. Dot plots show the frequency of Sca-1^{+} and c-kit^{+} cells within the Lin^{neg} or Lin^{neg} CD34^{+} population.



(a) Thymic area

(b) PBS

(c) 50 mg/kg

(d) 250 mg/kg

Fig. 4.21: Histology analysis of fetal thymus on gd 16.5: Pregnant mice were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5. Transverse tissue sections in upper thoracic region of fetuses were stained with H&E and thymic area was calculated. Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$. Representative photomicrographs display transverse histological tissue sections of the thymus of fetuses exposed to PBS, 50 mg/kg and 250 mg/kg APAP (b-d).

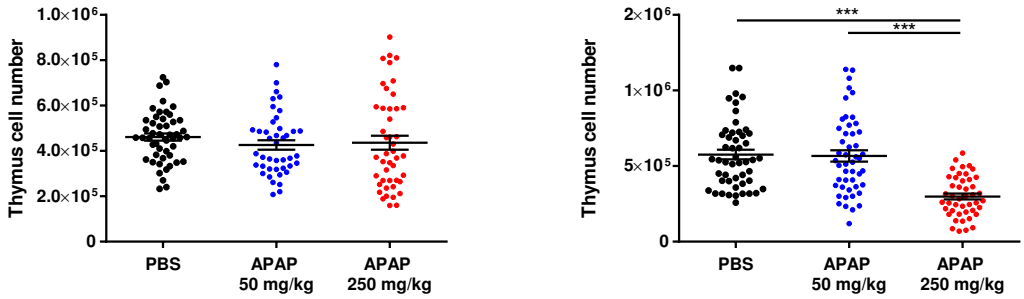
shown to be decreased compared to control animals (Fig. 4.20 d). Representative dot plots for HSCs and MPPs are shown in Fig. 4.20 e-g and h-j, respectively.

4.6.3 Fetal thymus

Subsequently the fetal thymus was analysed as liver-derived progenitor cells seed into the thymus and undergo T cell differentation. Hence, the thymus has a pivotal role in fetal T cell development.

Histology

The fetal thymus was only examined on gd 16.5, because of its small size on gd 13.5. Fetuses obtained from allogenic mating were collected and embedded in paraffin to evaluate the size in response to prenatal APAP exposure. Therefore, transverse



(a) Thymus cell count (allogenic mating)

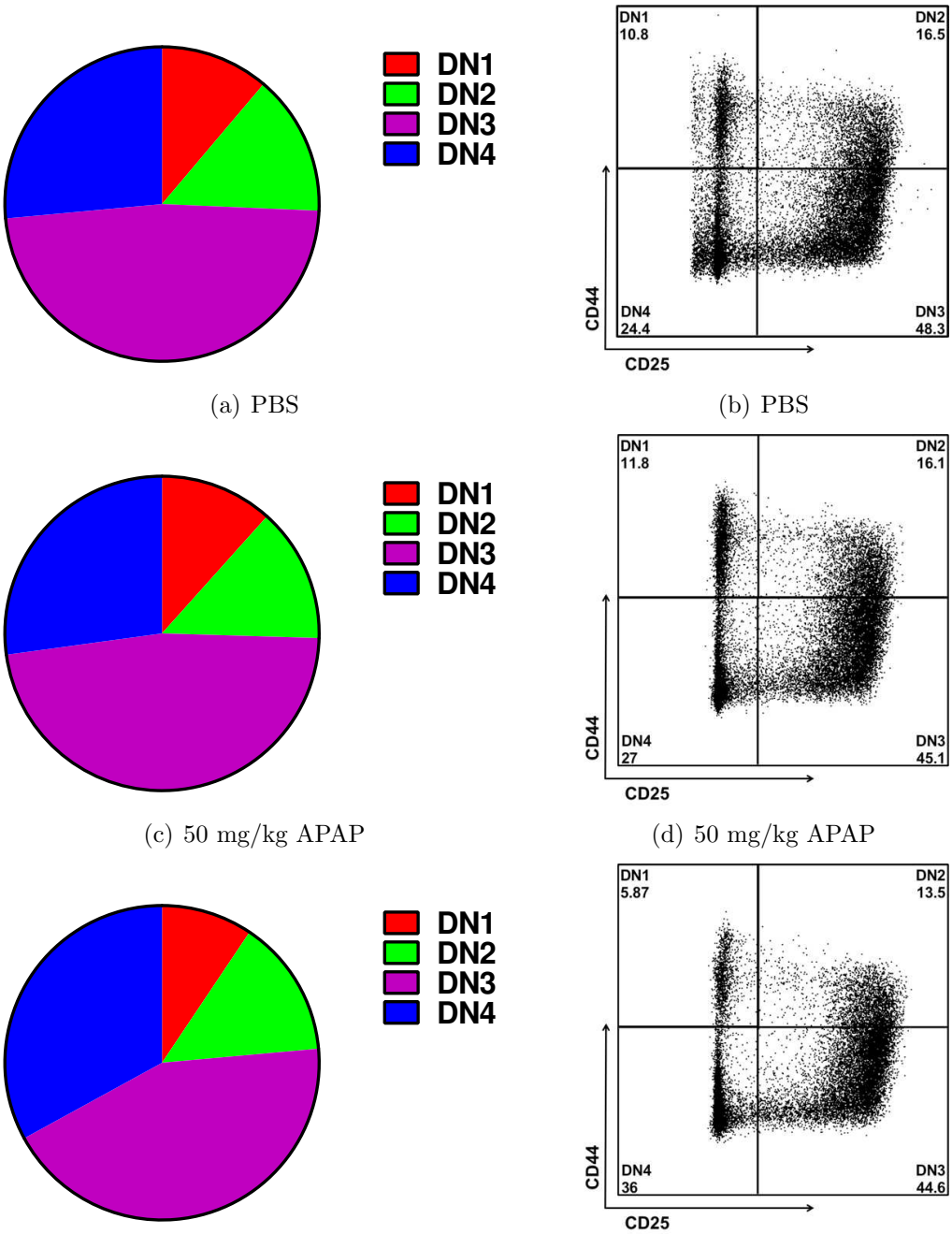
(b) Thymus cell count (syngenic mating)

Fig. 4.22: Analysis of fetal thymus on gd 16.5: Pregnant mice were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5. Total cell number were determined from allogenic (a) and syngenic mating (b). Data are presented as scatter dot plots indicate mean ± SEM, *** $p<0.001$.

histological tissue sections of the higher thoracic region were stained by H&E.
It could be shown that the transversal area of fetal thymus sections are decreased in response to APAP independent of the dose that was administrated (Fig. 4.21 a), although level of significance was only reached in case of 50 mg/kg APAP application. Representative figure of H&E stained cross section of fetal thymuses are shown in Fig. 4.21 b-d.

Flow cytometry

Following histological analysis, the fetal thymus was also investigated applying flow cytometry in order to determine the distinct maturation steps during T cell development and the impact of APAP on this process. Therefore, the fetal thymus was harvested on gd 16.5 using both syngenic and allogenic mating.
First, a single cell suspension was preparated and the total cell number of the fetal thymus was calculated. It could be shown that the total cell number did not differ between the groups when mating the mice allogenically (Fig. 4.22 a). In contrast, using a syngenic mating combination, the cell number of the fetal thymus was significantly decreased in response to 250 mg/kg APAP compared to control animals. The challenge with 50 mg/kg APAP had no implication for thymic cell number (Fig. 4.22 b).
However, the mating combination used, had no impact on the phenotype of the thymic cells analysis, as results obtained from flow cytometry revealed comparable results for both matings. Therefore, I will restrict myself to present the result ob-



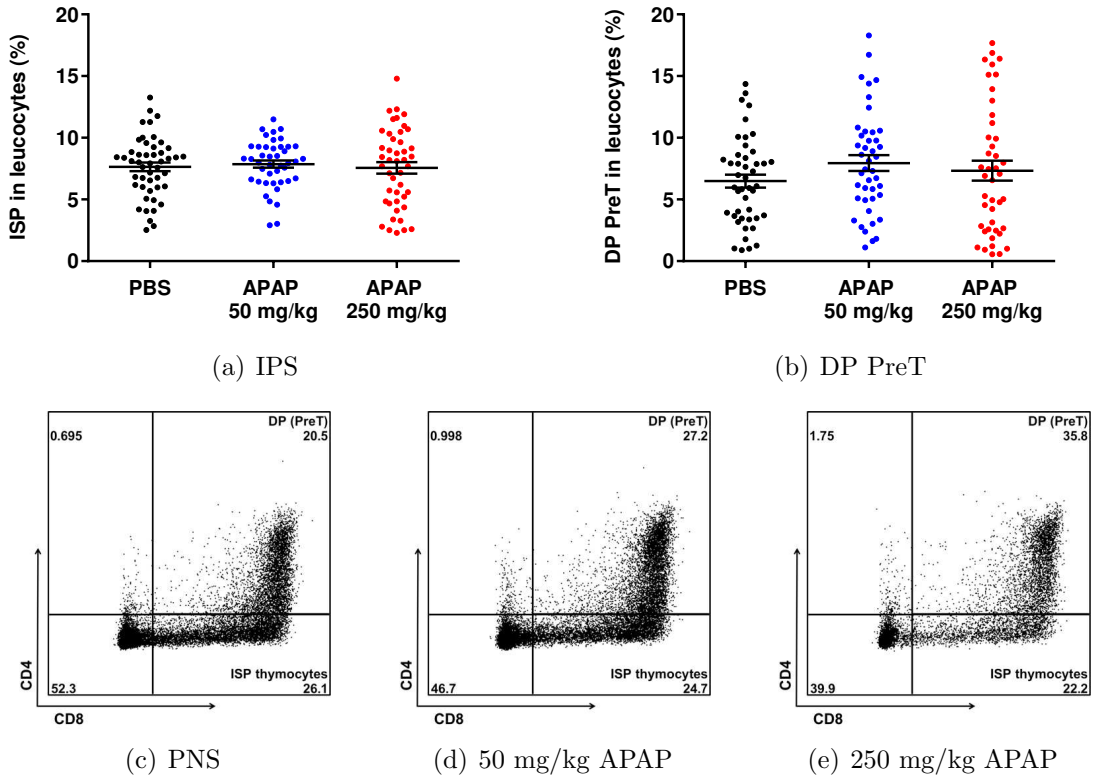
(a) PBS (b) PBS

(c) 50 mg/kg APAP (d) 50 mg/kg APAP

(e) 250 mg/kg APAP (f) 250 mg/kg APAP

Fig. 4.23: Flow cytometry analysis of fetal thymus on gd 16.5: Pregnant mice were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5. Frequencies of double negative (DN) populations have been determined in fetuses exposed to PBS (a), 50 mg/kg APAP (c) and 250 mg/kg APAP (e). Data are presented as pie charts. Representative dot plots are presented on the right side for the respective groups (b,d and f).

tained from allogenic mating starting with the most immature, the double negative (DN) populations, to finally come to the most mature, the single positive $CD4^+$ and $CD8^+$ T cells respectively.
The DN population 1-4 are defined by their different expression of CD25 and CD44. The treatment with 50 mg/kg APAP did not result in a shift within the four DN populations compared to control animals (Fig. 4.23 a and c). However, the application of 250 mg/kg APAP induced a significant decrease of DN1 and DN3 while DN4 could benefit from this (Fig. 4.23 e). The frequency of DN2 remained unaffected by APAP. The obtained frequencies of DN populations are displayed as pie charts for the respective treatment groups and representative dot plots are shown in Fig. 4.23. In conclusion, these results suggest a more mature phenotype after APAP challenge.
The next maturation steps include the intermediate single positive (ISP), expressing just CD8, and the double positive thymocytes (pre-DP) expressing both CD4 and CD8 but not CD3. The analysis revealed equal frequencies of these population, irrespective of the treatment. However, it was noticeable that both populations, specially the DP preT, are subject to variation as frequencies ranked from 0% to almost 20 % (Fig. 4.24 a and b). Representative dot plots of the respective groups are displayed in Fig. 4.24 c-e.
After positive selection pre-DP become post-DP, characterized by additional expression of CD3. The frequency of this maturation step was dose-dependent decreased following APAP challenge, although level of significance were not reached due to high variation within the samples (Fig. 4.25 a).
Following negative selection thymocytes still express CD3, including either CD4 or CD8. These single positive (SP) populations were also shown to be decrease in response to APAP. This decrease is minor in terms of SP CD8 thymocytes (Fig. 4.25 b), but significant in the case of SP CD4 (Fig. 4.25 c). However, these values are widely spread. Representative dot plots showing these three populations are displayed in Fig. 4.25 d-f. In conclusion, these results suggest a more immature phenotype after APAP challenge.
Finally, given that CD3 is a marker of maturation during T cell development, this population was analysed separately within all thymocytes. Flow cytometry analysis revealed a decrease of $CD3^+$ cells in fetuses exposed to 250 mg/kg APAP indicating a more immature thymocyte's phenotype (Fig. 4.26 a). Representative dot plots showing the $CD3^+$ population of the respective treatment are displayed in Fig. 4.26 b-d.

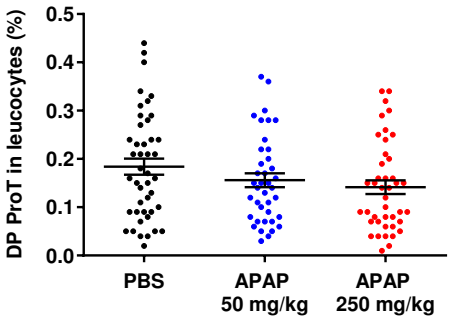


(a) IPS (b) DP PreT

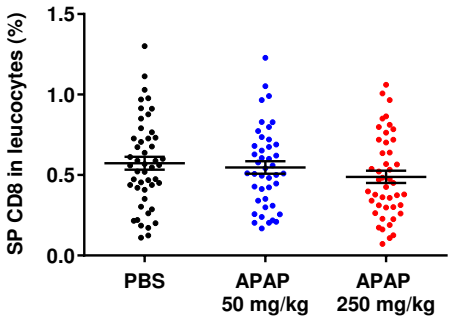
(c) PNS (d) 50 mg/kg APAP (e) 250 mg/kg APAP

Fig. 4.24: Flow cytometry analysis of fetal thymus on gd 16.5: Pregnant mice were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5. Frequencies of intermediate single positive thymocytes (a) and double positive thymocytes (b) have been determined. Data are presented as scatter dot plots indicate mean ± SEM. Representative dot plots are presented below for the respective groups (c-e).

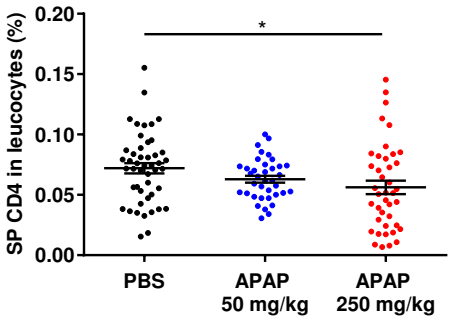
In summary, fetal development, especially the fetal immune development, is a vulnerable process prone to exogenous challenges. Hereby the fetal liver and the thymus play an important role to either supply HSCs or to ensure successful T cell maturation. It could be shown that implantation and abortion rate remain unaffected by APAP. However, the fetal weight was decreased after APAP challenge and in case of 250 mg/kg APAP this decrease could not be recovered till birth. The fetal liver, the main HSC source until birth, is not impaired in the same way as the maternal liver, as typical centrilobular necrosis could not been detected. Nevertheless, APAP induced a decrease in frequency of HSCs and MMPs as shown by flow cytometry analysis. Finally, APAP revealed to have an impact on the T cell maturation process in the fetal thymus. While maturation seems to be accelerated before the positive



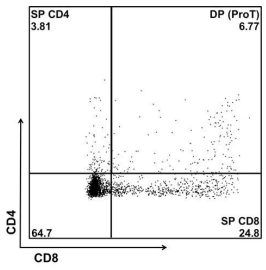
(a) DP ProT



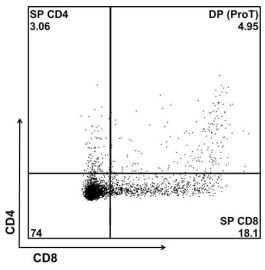
(b) SP CD8



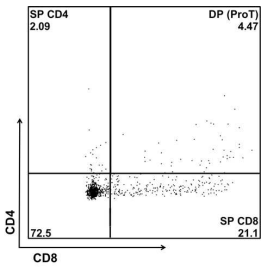
(c) SP CD4



(d) PBS

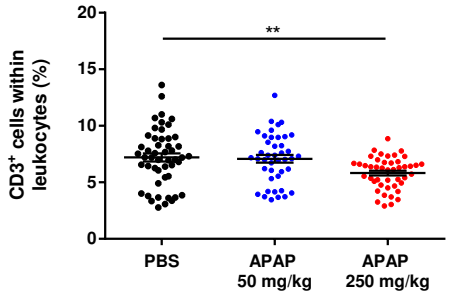


(e) 50 mg/kg APAP

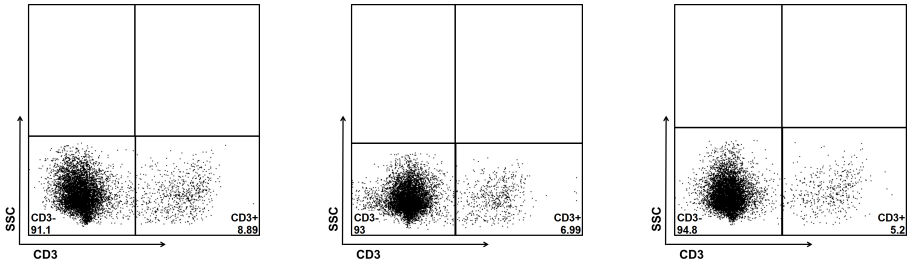


(f) 250 mg/kg APAP

Fig. 4.25: Flow cytometry analysis of fetal thymus on gd 16.5: Pregnant mice were treated with either PBS or 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5. Frequencies of double positive thymocytes (a), single positive (SP) CD8 (b) and SP CD4 (c) have been determined. Data are presented as scatter dot plots indicate mean ± SEM, * $p<0.05$. Representative dot plots are presented below for the respective groups (d-f).



(a) Frequency CD3 cells



(b) PBS (c) 50 mg/kg APAP (d) 250 mg/kg APAP

Fig. 4.26: Flow cytometry analysis of fetal thymus on gd 16.5: Pregnant mice were treated with either PBS, 50 mg/kg APAP or 250 mg/kg APAP on gd 12.5. Frequencies of $CD3^+$ cells have been determined. Data are presented as scatter dot plots indicate mean ± SEM, ** $p<0.01$. Representative dot plots are presented below for the respective groups (b-d).

selection, it seems to be delayed as the frequency of $CD3^+$ cells is decreased later on.

5. DISCUSSION

The goal of this thesis project was to evaluate the impact of a single non-toxic and a single toxic APAP dose on pregnant mice in comparison to non-pregnant ones, especially on liver damage and morbidity caused by APAP challenge. Further, I investigated the effects of APAP on the maternal immune and endocrine adaptation to pregnancy and the fetal-maternal interface. Finally, I studied the consequences of maternal APAP intake on pregnancy outcome and fetal immune development in liver and thymus.
I here provide evidence that prenatal application of APAP in mice causes maternal liver damage and increased morbidity of pregnant animals. Further, prenatal APAP affects the immune and endocrine adaptation to pregnancy as Treg cells are increased, the tolerogenic state of DCs is abolished and the plasma progesterone levels are decreased. Further, placental changes have been observed resulting in an increased placental ratio of labyrinth to junctional zone. Subsequently, fetal development and immune ontogeny were significantly impaired by prenatal APAP, mirrored by reduced fetal weight, lower frequency of HSCs and MMPs in the fetal liver and a delay in T cell development in the fetal thymus.

The need of such investigation was motivated by the lack of studies addressing the effects of APAP during pregnancy. Animal studies are rather sparse and the available studies used high doses [41] and administration throughout gestation [38, 40]. Further these studies had rather gross readout parameter such as survival, litter size, fetal weight and morphological changes. Although there are many human epidemiological study revealing an association between APAP and various diseases, e.g. failure in neurodevelopment, attention-deficit/hyperactivity syndrome, male infertility and asthma [107], animal models to investigate the underlying molecular and cellular mechanisms are most widely missing. Moreover, APAP is still recommended by physicians to be the only 'safe' medication to treat fever and pain during pregnancy and, even more serious, APAP is an over-the-counter medication

and therefore frequently taken without contacting a physician. Further, APAP is often a component of a compound, but this is often not clearly recognized by people leading to simultaneous intake. This raises the probability that the recommended dose limit of APAP is unintentionally exceeded and hepatotoxicity is caused. Therefore, the US Food and Drug Administration (FDA) has recently recommended that manufacturers need to limit the APAP content of each unit dose to 325 mg and demanded safety labeling changes including a new boxed warning [108].

For the mouse model we were able to develop here in order to unveil possible the mechanisms involved in the increased risk of asthma in the children later in life [32], we chose a time point at mid-gestation, gd 12.5. This has already been successfully used for investigating the impact of prenatal stress on the susceptibility of murine offspring toward airway inflammation [109]. Further, in the primary target of APAP, the liver, hematopoiesis occurs during prenatal life starting at gd 11.5 [94]. Additionally, the fetal thymus is colonized by liver progenitor cells to undergo T cell maturation. Hence, both the liver and the thymus may be particularly susceptible towards APAP induced toxicity.
The applicability of our mouse model was proven by challenging pregnant mice prenatal with APAP or PBS respectively and allowing them to give birth. The offspring was raised and at 6-8 weeks, they were challenged with an OVA-sensitization mouse model of allergic airway inflammation in order to examine the sensitivity of offspring to allergic asthma [110]. We observed that the offspring prenatally exposed to APAP showed an increased frequency of infiltrated leukocytes including eosinophils. Eosinophils are used as an indicator of inflammatory airway response, and were used to compare Bronchoalveolar Lavage (BAL) of control and APAP-treated offspring by cytospin assay. Additionally performed flow cytometric analysis of the BAL independently confirmed this increased severity of airway inflammation. An increased frequency of Siglec-F^+ eosinophils was detected in cells isolated from the airways in adult offspring upon prenatal APAP treatment. Also histological analysis demonstrated that the number of eosinophil and leukocyte infiltrates in lung parenchyma of OVA-sensitized and OVA-challenged mice was also increased in the offspring of mothers prenatally treated with APAP [110]. These findings confirm that our model is indeed suitable to mirror the results from epidemiological studies showing an increased risk for asthma in response to prenatal APAP, as experimentally induced asthma-like symptoms could be provoked after sensitizing adult offspring with OVA.

However, some limitations need to be considered when translating the findings into humans. First of all, the way of application is the main difference, as APAP is normally ingested orally and is here administered ip. As a consequence the bioavailability is almost similar to intravenous and therefore increased than after oral application. However, given that we are not interested in the antipyretic and analgesic potency APAP may have, but on its effect on the liver, the first liver passage is of particular importance. In this respect, it could be shown that oral and ip application are not that different. The only difference observed is that in the case of ip administration the absorption happens at the peritoneum and after oral application the absorption happens from the gastro-intestinal tract, but in both cases the drug is transported via the portal vein directly into the liver. The advantage of ip injection in contrast to oral application by gavage, is an accurate, reliable, convenient dosing possibility with reproducible results needed to achieve a successful readout in drug research. Further, ip injection is less stressful for the mice in comparison the other available administration routes.

Another critically point is the fact that the mice were fasting 16 h prior APAP administration which may be mostly uncommon in humans. This fasting was performed to achieve equal GSH levels and exclude GSH as a possible confounder for experimental results. However, it is known that fasting depletes GSH stores [111] and given that GSH is needed to inactivate the toxic metabolite of APAP, NAPQI, it is not surprising that fasting enhances the hepatotoxicity of APAP [112]. Hence, this point has to be considered when evaluating the results obtained in our study. Although, as APAP is generally taken when a person is not feeling well, having fever or pain, a fasting-like situation may be found sometimes.

The APAP dosages we administered were calculated based on the body weight, which is generally higher in pregnant mice. Hence, one might argue that pregnant mice received a higher dose of APAP. However, the liver of pregnant mice is increased in proportion to the body weight due to physiological changes to support fetal growth and development, which appears to equalise the higher APAP dose [39]. Here we used two different single APAP doses. The dose of 50 mg/kg APAP can be considered as therapeutic, hereby reflecting a medication frequently taken during human pregnancy. The second dose, 250 mg/kg APAP is above the single dose limit recommended during pregnancy, thus it can reflect an overdose which is not rare to be taken during pregnancy [113].

Serum biomarkers of liver injury include levels of ALT, aspartate aminotransferase (AST) and bilirubin. Noticeable, ALT levels have been different with respect to the mating combination used. Female mice syngenically mated with C57Bl/6 males have shown significantly higher ALT values compared to non-pregnant animals. Further, 450 mg/kg APAP induced a mortality rate of up to 35% in pregnant animals 24 h upon administration of APAP, whilst a 100% survival was seen in response to this dose in non-pregnant mice [110]. Subsequently it could be concluded that pregnant mice are more susceptible towards APAP-induced hepatotoxicity. This increased hepatotoxicity of APAP was also described in pregnant Crl:CD-1 (ICR) BR Swissmice, mirrored by an increased serum ALT activity, higher incidence of liver necrosis and greater mortality [39]. In contrast, female mice allogenically mated to CBy.SJL(B6)-Ptprca/J male mice having a Balb/c background did not show significantly elevated ALT levels compared to non-pregnant control, although ALT values were slightly higher in pregnant mice 24 h after administration of 250 mg/kg APAP. Hence, it could be that the maternal system may be able to cope better with liver inflammation in pregnancies that are immunologically in a more tolerogenic state as a more profound immune adaptation was mounted.

In addition, liver metabolism might be altered in pregnant mammals. Indeed, pregnant mice showed an increased hepatic GSH level than non-pregnant animals that declined in both groups following the administration of APAP. This reduction of hepatic GSH level was much greater in pregnant mice than in non-pregnant controls [110]. Further, particularly in pregnant animals increased levels of AST and bilirubin could be determined [110]. Additionally an altered regulation of hepatic phase II metabolism in mice during pregnancy has been shown [114]. In detail, this study demonstrated downregulation of hepatic glucuronidation as UDP-glucuronosyltransferases were shown to be decreased by 40 to 80% in pregnant dams. Further, an upregulation of sulfation was reported as sulfotransferases were demonstrate to be increased 100 to 500% at various time points during pregnancy. Among others these phase II enzymes are cross regulated by the estrogen receptor alpha (ERα). Hence, the study showed that the expression and activity of the hepatic ERα receptor was enhanced in pregnant mice [114].

In humans, absorption and disposition of APAP were shown not to be not affected by pregnancy [115]. However, an increased half-life of APAP was described during pregnancy [115]. That matches our observation that the mean ALT level 96 hours

after APAP injection of 250 mg/kg was still elevated in pregnant compared to non-pregnancy mice. Hence, an attenuated metabolism might result in higher steady state levels of the toxic metabolite NAPQI which could be detoxified by GSH prior to protein binding, but future studies are needed to clarify this issue.
After hepatic GSH is depleted, NAPQI leads to the formation of protein adducts. Irrespective of the finding regarding ALT, AST and bilirubin, we were not able to detect a significant difference in the amount of liver APAP adducts between pregnant and non-pregnant mice. We quantified the protein band of 37 kDa of the APAP adducts by Western blots [110]. This could be due to the observation that in the pregnant mice the levels of GSH were much higher compared to non-pregnant controls [110]. This could increase the capacity for GSH conjugation and hereby avoid an elevated protein adduct formation.
As a last point, increased susceptibility of pregnant mice towards APAP-induced hepatotoxicity was further supported by increased morbidity. Pregnant mice showed a massive weight loss after APAP challenge and food and water consumption was almost non-existent or very low. This means that pregnant animals suffer more than non-pregnant ones from pathologies of acute liver damage. Given that fasting reinforces hepatotoxicity, the circumstances of avoiding food and water result in the fact that health effects on mother and certainly also on the fetus are worsen.

Hepatocellular necrosis leads to the activation of the innate immune response. This includes upregulation of inflammatory cytokins and activation of immune cells [13, 16]. Indeed, we could demonstrate a significant increase of infiltrating $Gr1^{+}CD11b^{+}$ neutrophils into the liver of pregnant versus non-pregnant mice [110]. The involvement of neutrophils in APAP hepatotoxicity is supported by a study depleting neutrophils by an *in vivo* treatment with anti-Gr-1 antibody [116]. Treated mice were significantly protected against APAP-induced liver injury, as shown by reduced serum ALT levels and centrilobular hepatic necrosis and improved mouse survival. The authors concluded that accumulated neutrophils in the liver contribute to the progression and severity of APAP-induced liver injury [116]. In contrast, neutrophils were also associated to contribute to host defense and injury resolution following APAP overdose in mice and humans [117].
Responsible for the accumulation of neutrophil in the liver of APAP-treated mice are assumed to be NK and NKT cells [20]. This study reported that depletion of both - NK and NKT cells - detected by anti-NK1.1 significantly protected mice

from APAP-induced liver damage, proven by decreased serum ALT level, improved survival, decreased hepatic necrosis, inhibition of messenger RNA expression for interferon (IFN) γ, Fas ligand and several chemokines and decreased neutrophil accumulation in the liver. Also, hepatic NK and NKT cells were identified as the major source of IFNγ [20]. Hence, it was concluded that NK and NKT cells may promote the inflammatory response of liver innate immune system, thus contributing to the severity and progression of liver injury downstream of the metabolism of APAP and depletion of reduced GSH in hepatocytes [20]. Given that NK frequencies in the uterus-draining lymph node were increased in response to APAP in pregnancy mice, I suggest an evaluation of hepatic NK cells in pregnant mice in order to possibly uncover an association of increased neutrophils and NK cells in the liver.
Furthermore, also macrophage infiltration into the liver was shown to contribute to hepatotoxicity upon challenge with APAP [118]. As the C-C chemokine receptor (CCR) 5 is related to recruitment of macrophages to sites of inflammation, this study made use of CCR5 knockout (KO) mice. Hepatic damage was reported to be larger in CCR5 WT animals compared to KO animals. Additionally, infiltration of macrophages into the liver was shown to be reduced in CCR5 KO mice. This was accompanied by a decreased inflammatory responses and APAP-caused hepatotoxicity was significantly blocked by pretreatment of gadolinium chloride to inhibit macrophage activity [118]. As alterations in APAP metabolism were excluded due to equal levels of cytochrome P450 2E1 between CCR5 WT and KO animals, the authors concluded that macrophage recruitment into the inflammatory sites significantly facilitate APAP-mediated hepatotoxicity and CCR5 gene deletion protects from APAP-induced liver injury [118].
In summary, the innate immune systems plays a prominent role for the severity of APAP-induced liver damage. Further, studies in non-pregnant animals demonstrated that APAP suppresses the cellular and humoral immunity. It was shown that antibody production was suppressed and B-cell frequency in splenocytes was significantly decreased by APAP administration [60]. Also lymphocyte loss was reported in the spleen, thymus and liver draining lymph nodes after administration of a hepatotoxic dose of APAP [119]. In this context it is crucial to understand that a pregnant woman is already facing an immunological challenge due to the semi-allogenic fetus.

During pregnancy the maternal immune system have to mount a contradictory de-

mand. On one hand, the 'semi-allogenic' fetus needs to be tolerated by the maternal immune system and rejection need to be prevented. Furthermore, the fetus needs to be nourished. On the other hand, the capacity to respond against infection and noxious agent has to be maintained throughout gestation to ensure maternal and fetal survival. Therefore, the maternal immune system and also the maternal endocrine system need to adapt to the process of pregnancy.

One key feature of this maternal immune adaption is the arrest of DCs in a tolerogenic state [120, 121, 45, 46, 47]. The analysis of the uterus-draining lymph node revealed that prenatal administration of APAP induces an up-regulation of the maturation marker MHC class II on DCs. It is indicative that APAP may interfere the tolerogenic immune millieu. In this context, liver DCs are reported to regulate intrahepatic inflammation in chronic liver disease and may also modulate the hepatotoxic effects of APAP [122]. Indeed, it was shown later that the DC immune-phenotype was altered due to APAP challenge, as liver DC were demonstrated to expressed higher MHC class II, costimulatory molecules, and Toll-like receptors [64]. Further, this study reported that liver DC have the particular capacity to prevent NK cell activation and induced neutrophil apoptosis [64]. These data suggest a protective function of liver DC against APAP toxicity which is further supported by findings that the depletion of liver DCs is associated with exacerbated hepatotoxicity [64]. Taken together, an APAP-challenged immune system may provoke the maturation of DCs to impede hepatotoxicity, but in case of pregnancy, this might lead to a loss of fetal tolerance. Therefore, I suspect a contradictory demand that should be clarified between the need of DCs in a tolerogenic state to ensure fetal tolerance on the one hand, and on the other hand the requirement of mature DCs to prevent APAP-induced hepatotoxicity. However, evidence for DC cell modulation in response to APAP are rather minor.

Treg cells are privotal for the acquisition and promotion of fetal tolerance during pregnancy [52, 1]. Frequencies of $CD4^+$ $FoxP3^+$ Tregs were significantly induced in uterus-draining lymph nodes of dams treated with 250 mg/kg APAP. As Tregs are critically involved in limiting the inflammatory response [123], I think, this increase of Treg in uterine-draining lymph nodes of APAP treated dams could show a protective mechanism against immune response triggered by APAP administration that the animals utilized to have a successful pregnancy. Treg execute their function by producing interleukin (IL)-10 [124]. IL-10 levels are elevated in women during early pregnancy and remain elevated throughout gestation. It acts as a suppressor

of active maternal immunity in order to allow acceptance of the fetal allograft [125]. However, not only CD4 Tegs cells produce Il-10, but also $CD8^{+}CD122^{+}$ Treg cells and herbey IL-10 mediates the regulatory activity of these cells [126]. Remarkably, also the frequency of these CD8 Treg cells was increased upon APAP challenge, which was more profound in pregnant animals. I assume that both taken together may contribute to the maintenance of tolerance to ensure fetal survivial. In the context of colitis, it was shown, that a mixure of $CD8^{+}$ Tregs together with $CD4^{+}$ Tregs can suppress colitis far more effective than single Tregs, indicating a synergistic effect of these Tregs [127].

Maternal adaptation to pregnancy is also supported by the modulation of NK cell function, particulary in the decidua [44]. It was shown that murine mature NK (mNK) cells present at the feto-maternal interface, identified as $CD27^{low}$ $DX5^{+}$ $CD3^{neg}$, have low cytotoxic capacity, produce higher amounts of IFN γ and expresses functional homologs of human NK cell immunoglobulin-like receptors. Furthermore, bone marrow-derived mNK ameliorate the rate of fetal loss when adoptively transferred into alymphoid RAG2 mice [44]. However, in the liver APAP-induced hepatocyte cell death is followed by the activation of NK cells promoting hepatotoxicity by overproduction of IFN γ [20] which mediates Th1-related immune response and airway hyper-responsiveness, neutrophilic and eosinophilic inflammation and airway remodeling in a chronic asthma model [128]. Further, NK cells also produce IL-4 which is critical for asthma development as it contributes to intricate pathways that result in inflammation and tissue damage [129]. In the uterus draining lymph node an elevated frequency of NK cells was observed in pregnant animals treated with 250 mg/kg APAP along with an increased proliferation activity. The maturation status due to 50 mg/kg APAP was induced in pregnant mice, while after administration of 250 mg/kg APAP DX5 expression was not significantly changed. However, given that the frequency of NK cells was already elevated due to 250 mg/kg APAP, the number of mature NK cells is increased in comparison the pregnant control animals. The extent to which this could be linked to a supportive role of NK cells regarding fetal tolerance or to which this has to been seen as an adverse effect remains open. In general, the frequency of NK cells in the uterus-draining lymph node is rather low and due to the results obtained there I propose a more in-depth investigation of NK cells in the decidua due to APAP.

All processes involved in maternal adaptation to pregnancy are induced by tolerogenic stimuli such as progesterone. Progesterone contributes to the establishment of

a protective immune milieu, to placental formation [57, 130] and mediates fetal immune tolerance and successful pregnancy progression by supporting the immature, tolerogenic phenotype of dendritic cells [121]. Progesterone measurements performed on pregnant mice showed that prenatal APAP reduced the pregnancy-hormone progesterone which is supported by a study describing that even therapeutic doses of APAP lower female sex hormone levels [61]. Declined progesterone levels may consequently affect the phenotype of maternal immune cells, especially DCs. However, also APAP-induced suppression of prostaglandin E_2 by inhibition of cyclooxygenase-2 could promote maturation of DC [7] as well as IFN γ produced by NK cells [131]. The dose-dependent decrease of progesterone upon APAP administration could also result from anti-estrogenic responses to APAP, such as the inhibition of progesterone receptor up-regulation in the uterus, which was observed in immature female mice after APAP treatment [62]. Moreover, the decrease in progesterone could also be a consequence of APAP-induced placental damage. We could demonstrate that APAP affected the area supporting maternal hormone production, the junctional zone [65], which was decreased in response to APAP. Although the function of this zone is not fully clarified yet [132], it was shown to be essential for fetal survival as demonstrated by the MASH2-/- mouse that lacks this zone and is lethal for the fetus around gd 10.5 [133]. Additionally essential for development of the junctional zone in the placenta is the homologous to the E6-AP Carboxyl Terminus (HECT) domain E3 ubiquitin ligase, Hectd1 [134]. Hectd1 is enriched in trophoblast giant cells and a disruption results in mid-gestation lethality and intrauterine growth restriction. A Hectd1 mutant placenta shown a reduction of number and nuclear size of trophoblast giant cells which leads to a decreased expression of Placental lactogen-1 and -2 [134]. Subsequently, this may result in a decreased progesterone production by the corpus luteum as stimulation from the placenta is missing [77]. Finally, this study demonstrated an increase in immature uterine NK cells in the maternal decidua of the Hectd1 mutant placenta [134]. Hence, Hectd1 may also contribute to maintain tolerance by promoting maturation of NK cells in the decidua. Taken together, the impact of APAP on the expression of Hectd1 could be of particular interest. APAP was shown to decrease progesterone and to provoke a decreased junctional zone. Hence, APAP may target Hectd1 expression. As a consequence, less number of trophoblast giant cells may result in a decreased expression of Placental lactogen which in turn may interfere with progesterone production in the corpus luteum.

Importantly, in human pregnancies, progesterone is produced by the placenta. Therefore further studies are needed to test if APAP treatment affects placental function and progesterone levels during human pregnancy. Further, it still remains to be investigated if APAP-induced inflammation [135] can also affect the ovary and facilitate local monocyte infiltration in the ovaries. Monocytes have been shown to regulate ovarian steroidogenesis [136], hence, APAP may cause decrease of progesterone via increased monocyte inflammation into ovaries in mice. In contrast, also a decrease of macrophages migration into the developing corpora lutea may be responsible for reduced progesterone levels as in the ovaries of macrophage-depleted mice the expression of steroidogenesis genes Star, Cyp11a1, and Hsd3b1 was diminished [136]. Therefore, the impact of APAP on the ovaries needs to be clarified, since APAP medication in humans may not only affect pregnancy progression, but also have yet unknown effects on female fertility.

Decreased levels of progesterone have been associated with a lower birth weight in humans, especially in female children [137]. A decreased fetal weight in response to APAP was observed on gds 13.5 and 16.5 and also in newborns, whereby it remains to be elucidated if this reduced fetal weight is due to low levels of maternal progesterone or a direct effect of APAP on the fetus. The latter possibility may result from the ability of APAP to cross the placental barrier [29]. However, also the massive weight loss of pregnant mice along with the food refusal indicate that the animals suffer from pathologies of acute liver damage. Therefore, it could be considered as a cofounder for a specific effect of APAP on fetal and neonatal weight loss.
In the fetus, APAP is metabolized by the fetal liver by conjugation with sulfate and glutathione but not with glucuronic acid. Detoxification by glutathione conjugation was reported to be approximately ten times slower than by adult liver, although an increase in acetaminophen oxidation with fetal age was also reported. Hence, the fetus is capable of catalyzing acetaminophen through oxidation, and therefore remains at risk should a large dose of the drug cross into the fetal circulation [138]. On the other hand young children are more resistant to acetaminophen hepatotoxicity attributable to biochemical differences. This includes that sulfation predominates over glucuronidation, probably contributing to less formation of toxic intermediates. In addition, infants have a greater capacity to synthesize glutathione, thereby inactivating toxic metabolites of acetaminophen more effectively than adults [139]. However, an increased risk to develop asthma due to APAP is reported for prenatal

exposure as well as for and APAP usage within the first year of life [31].
APAP was injected on gd 12.5, concurrently with the translocation of hematopoiesis to the fetal liver [95, 96]. Hence, the fetal liver may be particularly susceptible to hepatotoxicity, resulting in the decline of HSCs and hematopoietic MPPs as described here. HSCs have self-renewal capacity and they can originate into different blood cell types such as erythrocytes, myeloid cells and lymphocytes. An impairment of the HSC pool may affect the maturation of the lymphoid cell lineage and hereby harm the fetal immune ontogeny. Liver-derived T cell progenitor cells seed the thymus in two waves and undergo a complex process of differentiation and selection to generate mature, self-MHC-restricted T cells [94]. Damage to the HSC pool in the liver may therefore have consequences for the T cell repertoire. Indeed, we observed variations in different stage of T cell maturation in the thymus. Initially T cell development seems to be accelerated as the frequency of DN4, the most mature stage of the DN populations, was elevated in fetuses exposed to 250 mg/kg APAP. However, after positive selection T cell development was shown to be delayed as post-DPs were shown to be dose-dependently decreased following APAP challenge. This became even more clear after negative seletion, when SP CD4 T cell were significantly reduced due to APAP administration. Overall, a decreased frequency of $CD3^+$ cells in the thymus was observed, which suggests a delayed T cell maturation or changes in the selection processes. Nevertheless, we cannot be assured yet which point during the maturation process is impaired as the results we obtained for the different maturation and selection steps show high variability. It can be speculated that gd 16.5 may be too early for the investigation of the thymus as apoptosis, an important part of the selection process, proceed in mice between gd 15.5 and 18.5. Hence, I suggest that additional analyses are needed and gd 18.5 may be a suitable time point as also the developing bone marrow may be of major interest to investigate, because HSCs leave the liver and start migrating there on gd 17.5. The findings of an impaired immune ontogeny in response to prenatal APAP may now provide a justification for the association between prenatal APAP use and childhood asthma [34]. Clearly, future research aiming to confirm causality between the delayed fetal immune maturation and subsequent asthma risk are needed, as well as the identification or exclusion of an APAP-induced damage of the fetal lung.

Notably, a considerable variability was observed in the experimental outcomes upon APAP administration. I can exclude that this variability was present due to a

sex-specific APAP response, because female and male fetuses have been identified. However, this analysis did not affect the overall outcomes of the results presented here (data not shown). Therefore, we refrained to split the results by sex of the offspring. Beyer and colleagues proposed that genetic differences as well as epigenetic modifications may influence the susceptibility towards APAP, as they reported a considerable degree of animal-to-animal variability with regard to the extent of APAP-induced liver injury in C57Bl/6J mice [140]. Moreover, not only intra-strain variability with regard to APAP-toxicity has been reported, but also two thirds of 36 mouse strains exhibited variable degrees of liver damage [141]. Hence, the allogenic mating combination we used in this study may have influenced the impact and variability of APAP on mother and fetus. In humans, a variability of APAP-associated liver damage has also been described, e.g. in individuals carrying the CYP3A5 rs776746 A [142]. Moreover, individuals homozygous for the CD44 rs1467558 A allele were observed to have elevated serum liver enzyme levels after consumption of high doses of APAP for up to 2 weeks [142]. No data are available to date to indicate whether pregnancy aggravates APAP-induced liver damage in these patients. Clearly, genetic differences would not play a central role in our experimental setting which employs an inbreed mouse strain but we can not exclude an impact of differential epigenetic modifications among individuals on our results.

Finally, it remains open whether the observation seen in the fetus are due to a direct effect of APAP on the fetus or indirect effects via maternal liver injury. Although the fact that APAP can cross the placenta may support a direct fetal responses to prenatal APAP treatment and liver injury is not absolutely necessary to provoke change in the HSC pool, other mechanisms could also be considered. One possible mechanism could be the maternal microchimerism which is described as the presence of maternal cells in fetal and offspring's organs. Maternal microchimeric cells are naturally acquired during pregnancy and lactation and can persist until adulthood [143, 144]. The importance of maternal microchimerism is increasingly recognized, as microchimerism can induce tolerance as shown in allograft transplantation. Recipients transplanted with tissue from the mother were more protected from acute rejection rate, graft failure after 6 months and graft-versus-host disease (GVHD) in comparison to those transplanted with paternal tissue [145, 146]. In contrast, higher prevalence or frequencies of maternal microchimeric cells were associated with various auto-immune diseases [147, 148, 149] and, most interesting with asthma [150].

Hence I suggest that APAP may alter maternal microchimerism. Prenatal APAP challenge causes liver inflammation and decreases maternal plasma levels of progesterone. Due to the importance of progesterone, this decrease may result in an altered immune response to a less tolerogenic state causing an inflammatory environment at the fetal-maternal interface. This may facilitate the migration of maternal cell into the fetus and these cells may exhibit an altered, more inflammatory phenotype. As a consequence, fetal immune organs such as liver, thymus, spleen and bone marrow are exposed to these altered microchimeric cells and the immune ontogeny in the fetus is impaired. This includes frequency of HSCs and their migration ability, thymic seeding as well as T cell maturation and selection. As T cell are long-lived, prenatal APAP use might have long-lasting consequences on children's immunity and account for an increased risk for immune disease such as asthma.

In summary, the presented data of this thesis provide evidence that caution is required when using APAP during pregnancy, as it may interfere with maternal adaptation to pregnancy, induce placental damage and impairs fetal immune maturation as demonstrated in mice. Clearly, abstention from APAP medication during pregnancy may also be associated with negative effects on the unborn child, e.g. when maternal fever is not reduced, as shown in animal studies [151] and in human studies [152].
A recently published systematic review and meta-analysis reported an association between fever during pregnancy and an increased risk of adverse health impacts for the child. Strongest evidence was demonstrated for neural tube defects, congenital heart defects and oral clefts. Theses birth defects were shown to have pooled estimates between a 1.5- and nearly 3-fold increased risk due to maternal fever during the first trimester [152]. The impact of maternal fever in respect to asthma has only been addressed in 3 studies. Two of these studies reported an approximately twofold increased risk of asthma in children exposed to maternal fever [153, 154] whereas another study did not find an association with wheezing, eczema, or atopic sensitization [155]. Unfortunately, all studies did not provide any information on medication use. Hence, it remains somehow elusive if the reported risk was actually due to maternal fever then rather due to the use of medication such as APAP. However, there are other studies providing evidence suggesting a protective effect of antipyretic medications as the risk for an adverse outcome was reduced or even eliminated [152].

To my opinion, more studies are needed to estimate if the benefits of a reduced temperature outweigh the potential risk of APAP use during pregnancy and *vice versa*. Given that there is a lack of alternatives to treat pain or fever during pregnancy - aspirin and ibuprofen are not recommended - insights from longitudinal pregnancy cohorts are urgently require, in which the use of APAP is thoroughly documented and linked to fetal immune maturation, evaluated e.g. via cord blood assessments of HCS subsets and T cell maturation. These insights may then lead to a re-evaluation for prenatal APAP use recommendations. At present, I think, it is needed to draw the attention of gynaecologists and pharmacists to the potential risk of APAP. They are in close contact to pregnant women and have their confidence. Hence, it is their responsibility to inform pregnant women about the risks of APAP to dispel the assumption that APAP is 'safe' and can be used without worries for maternal and fetal health. This is particularly crucial for children at risk for asthma because of genetic predisposition.

6. SUMMARY

Acetaminophen (APAP, i.e. Paracetamol®or Tylenol®) is an over the counter drug, and hence, often self-medicated to treat fever or pain and generally recommended to pregnant women by their physicians. Recent epidemiological studies revealed an association between prenatal APAP use and an impaired children's health, i.e. an increased risk for asthma. Thus, it was the aim of the present thesis project to establish a mouse model in order to study the effects of prenatal APAP use on liver toxicity and morbidity, the maternal immune and endocrine adaptation to pregnancy and placental morphology. Additionally, the impact on pregnancy outcome and fetal immune development have been investigated.

Syngenic and allogenic-mated C57Bl/6J female mice were injected intraperitoneally with APAP (50 or 250 mg/kg) or PBS on gestational day 12.5, non-pregnant females served as controls. Pregnant mice were sacrificed either on gd 13.5 or 16.5, tissue from non-pregnant mice was obtained 24 or 96 hours post-injection.

APAP induces liver toxicity in pregnant and non-pregnant female mice mirrored by significantly increased plasma alanine aminotransferase levels. Liver histopathology revealed evidence for typical centrilobular necrosis 24 h after administration of 250 mg/kg APAP. In uterus-draining lymph nodes of pregnant dams, the frequencies of mature dendritic cells and regulatory T cells were significantly increased. Progesterone levels were significantly decreased in plasma of dams injected with APAP along with morphological changes of placental structure, mirrored by an increased placental ratio of labyrinth and junctional zone. Whilst litter size and fetal loss remained unaltered, a reduced fetal weight and a lower frequency of hematopoietic stem cells in the fetal liver were observed upon APAP. T cell maturation process in the fetal thymus was delayed shown by a reduced frequency of $CD3^+$ cells.

These data provide strong evidence that APAP interferes with maternal immune and endocrine adaptation to pregnancy, affects placental function and impairs fetal immune development. The latter may have long-lasting consequences on children's immunity and account for the increased risk for asthma observed in humans.

7. ZUSAMMENFASSUNG

Acetaminophen (APAP, Paracetamol®) ist ein freiverkäufliches Medikament und wird oft eigenverantwortlich bei Fieber oder leichten Schmerzen eingesetzt, auch in der Schwangerschaft. Epidemiologische Studien konnten einen Zusammenhang zwischen pränataler APAP Einnahme und einem erhöhtem Asthma-Risiko aufzeigen. Daher war es das Ziel der vorliegenden Arbeit, ein Mausmodell zu etablieren, um die Auswirkungen pränatalen APAP Einnahme studieren zu können.
Syngen und allogen verpaarte C57Bl/6J-Mäuse wurden intraperitoneal mit APAP (50 oder 250 mg/kg) oder PBS am Gestationstag 12.5 injiziert, nicht-schwangere Mäuse dienten als Kontrollen. Die Gewebeentnahme erfolgte an den Schwangerschaftstagen 13.5 und 16.5 bzw. 24 bzw. 96 Stunden nach der Injektion.
Der durch APAP verursachte Leberschaden wurde durch erhöhte Alanin-Aminotransferase-Konzentrationen im Blutplasma widergespiegelt. Histologische Untersuchungen der Leber zeigten zudem typische nekrotische Areale im Bereich der Zentralvenen 24 h nach Gabe von 250 mg/kg APAP. Im Uterus-drainierenden Lymphknoten von schwangeren Mäusen wurden erhöhte Frequenzen von reifen dendritischen Zellen und regulatorischen T-Zellen nachgewiesen. Der Progesteron-Spiegel im Blutplasma schwangerer Mäuse, die mit APAP behandelt wurden, war signifikant verringert und die Plazenta zeigte morphologischen Veränderungen. Während Implantationen und Abortrate nach APAP-Gabe unverändert blieben, konnten ein vermindertes fetales Gewichts und eine geringere Frequenz von hämatopoetischen Stammzellen in der fetalen Leber beobachtet werden. Die T-Zell-Reifung im fetalen Thymus war verlangsamt, bestätigt durch eine verminderte Frequenz von CD 3^+ Zellen.
Diese Daten weisen stark darauf hin, dass die pränatale APAP Einnahme die immunologische und endokrine Anpassung an die Schwangerschaft beeinflusst, die Funktion der Plazenta stört und die Entwicklung und Reifung des fetalen Immunsystems beeinträchtigt. Letzteres könnte langfristige Folgen für das kindliche Immunsystem haben und ein Grund für das erhöhte Asthma-Risiko liefern, das bei Kindern beobachtet wurde.

8. LIST OF ABBREVIATIONS

AGM	aorta-gonad-mesonephros
ALT	alanine aminotransferase
APAP	Acetaminophen (N-acetyl-para-aminophenol)
APC	Allophycocyanin
AST	aspartate aminotransferase
BAL	Bronchoalveolar Lavage
bp	base pair
BV	Brilliant Violet
CAR	constitutive androstane receptor
CCR	C-C chemokine receptor
CD	cluster of differentiation
COX	cyclooxygenase
Cy	Cyanine
DAMPs	damage-associated molecular patterns
DCs	dendritic cells
DN	double negative
DP	double positive
ERα	estrogen receptor alpha
FACS	Fluorescence-activated cell sorter
FDA	Food and Drug Administration of the United States
FITC	Fluorescein Isothiocyanate
FMO	fluorescent minus one
gd	gestation day
GSH	glutathione
GVHD	graft-versus-host disease
H&E	Hematoxylin and eosin
HECT	homologous to the E6-AP Carboxyl Terminus
HSC	hematopoietic stem cell

IFNγ	interferon-gamma
IL	interleukin
IP	intraperitoneally
ISP	intermediate single positive
KO	knockout
Lin	lineage
LPS	lipopolysaccaride
min	minute(s)
mNK	mature killer cells
mPL	mouse placental lactogen
MPPs	multi-potent progenitor cells
MRI	Magnetic Resonance Imaging
NAPQI	N-acetyl-p-benzoquinone imine
nGr	neutrophil granulocyte
NK	natural killer cells
NKT	natural killer T cells
NSAIDs	nonsteroidal anti-inflammatory drugs
PAMPs	pathogen-associated molecular patterns
PAS	para-aortic splanchnopleura
PCR	Polymerase Chain Reaction
PE	R-phycoerythrin
PPARα	peroxisome proliferator-activated receptor alpha
PR	progesterone receptor
PXR	pregnane X receptor
RBC	red blood cell
Sca-1	stem cell antigen 1
SEM	standard error of the mean
SP	single positive
TCR	T cell receptor
tDC	tolerogenic dendritic cells
TNFα	tumor necrosis factor-alpha
Treg	regulatory T cell
XEM	xylene substitude
7AAD	7-amino-actinomycin D

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10. CURRICULUM VITAE

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