The effects of chorioamnionitis on liver inflammation in prenatal and postnatal sheep

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Eindverhandeling voorgedragen tot het bekomen van de graad master in de biomedische wetenschappen klinische moleculaire wetenschappen

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CONTENTS

LIST OF ABBREVIATIONS

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

Chol: Cholesterol

Endo: Endotoxin

FIRS: Fetal inflammatory response syndrome

GA: Gestational age

GST: Glutathione S-transferase

HDL: High Density Lipoprotein

H/E: Haematoxylin and eosin

HRP: Horseradish peroxidase

IA: Intra-amniotic

IL: Interleukin

LDL: Low Density Lipoprotein

LPS: Lipopolysaccharide

LTA: Lipoteichoic acid

MPO: Myeloperoxidase

NFκB: Nuclear factor kappa B

PBS: Phosphate Buffered Saline

POD: Peroxidase

Ppia: Peptidylprolyl isomerase A

Prot: Protein

TdT: Terminal deoxynucleotidyl Transferase

TG: Triglyceride

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TUNEL: Terminal transferase dUTP nick end labelled

PREFACE AND ACKNOWLEDGEMENT

After finishing my Junior Internship at the department of Molecular Genetics, Dr. Ronit Shiri-Sverdlov and Dr. Boris Kramer offered me the opportunity to continue the collaboration during my Senior Internship. Since preterm birth is a major problem worldwide and important information about this topic is still missing, this project drew my attention and I did not hesitate to continue working on this topic. Although the initial plan was to investigate the effect of maternal hypercholesterolemia on preterm birth, this thesis will discuss another topic concerning preterm birth. Despite a good preparation and an approved DEC-proposal, we could not start the planned experiments as our mice were infected with an *aspicularis* Worm infection at the CPV. To fill in the time period of my Senior Internship, I performed several projects concerning chorioamnionitis-associated preterm birth of which this thesis manuscript is the result.

Regardless of all the challenges and disappointments we had to deal with during my Senior Internship, Dr. Ronit Shiri-Sverdlov and Dr. Boris Kramer kept believing in me and supported all the activities in which I was enrolled. Therefore, I would like to thank them for all their support, understanding and patience. Veerle Bieghs has been a great teacher, not only concerning all the laboratory skills but also when I needed other advice I could count on her. I am extremely thankful for all her help and comments. Also Patrick van Gorp and Anne Custers gave me advice in daily research life, for which I want to thank them. I thank Dr. Marion Gijbels for her help in analyzing all immunohistochemical stainings and for her support during my internship period. In addition, I would like to thank Dr. Willem Voncken for critical reviewing several parts of the manuscript, Dr. Otto Bekers and Claudia Clorefice (Clinical Chemistry, University Hospital Maastricht) for helping me with performing the plasma measurements and Monique Vergouwe (Molecular Genetics) and Tim Wolfs (General Surgery, Maastricht University) for giving technical advice about the immunohistochemical stainings. Also thank you to all other people at the department of Molecular Genetics and the department of Paediatrics for offering me a great time during both my Junior and Senior Internship.

Besides all the people that have supported me on the work floor, I also have a family to be proud of. My family and boyfriend have supported me incredibly. I want to thank them for their understanding, patience and love. Without them I would have never come this far. They gave me the opportunity to do what I like and to develop in the person that I am at the moment. My boyfriend gave me the perseverance to reach my goals, without him this thesis manuscript would not be the same.

ABSTRACT

Introduction: Preterm delivery is a major problem in reproductive medicine today. At least 60 percent of all preterm births are associated with chorioamnionitis-induced fetal inflammatory response syndrome (FIRS). This syndrome is characterized by systemic inflammation and an elevation of pro-inflammatory mediators. Currently, the mechanisms whereby chorioamnionitis induces FIRS are poorly understood.

The liver is a main source for systemic inflammation and the major haematopoietic site during fetal life. Accordingly, we hypothesize that the liver is involved in the systemic inflammation associated with chorioamnionitis.

Material and methods: The effect of chorioamnionitis on *prenatal* livers was analyzed in ovine fetuses at the gestational age of 125 days. Chorioamnionitis was induced by intra-amniotic injection of 10 mg endotoxin or saline (control) either two days or two weeks before delivery. In addition, the effect of chorioamnionitis on *postnatal* livers was analyzed in lambs at the gestational age of 140 days and the postnatal age of eight weeks. Chorioamnionitis was induced by intra-amniotic injection of 10 mg endotoxin or saline (control) 30 days before delivery. Parameters of haematopoiesis, inflammation, lipid levels and cell damage were investigated in the livers of these prenatal and postnatal sheep.

Results: Already two days after the endotoxin injections, the number of hepatic myeloperoxidase (MPO)-positive cells was increased and prenatal hepatic mRNA levels of pro-inflammatory genes interleukin (IL)-1ß and tumor necrosis factor (TNF) were significantly higher compared to controls. Remarkably, two weeks after the endotoxin injections the prenatal livers were still inflamed and the levels of markers for liver damage (ALT and AST) were significantly elevated.

At term delivery, the number of hepatic CD3-positive and TUNEL-positive cells was increased and hepatic cholesterol and triglycerides were decreased in animals that received endotoxin compared to controls. At postnatal age of eight weeks, only a significant increase in hepatic triglycerides was observed in animals that received endotoxin compared to control animals.

Conclusion: These findings suggest that the prenatal hepatic effects, caused by chorioamnionitis, have an effect on postnatal life and stress the importance of further research concerning the underlying mechanisms of FIRS.

1. INTRODUCTION

Preterm delivery, which is defined as birth at less than 37 completed weeks of gestation, is a major problem in reproductive medicine today. The percentage of preterm deliveries has risen progressively over the last twenty years (figure 1) [1]. Currently, they account for 12–13 percent of deliveries in the USA and 5–9 percent in other developed countries [1, 2]. Irrespective of the progress in neonatal care, preterm delivery is still the most important cause of neonatal death and long-term neurological morbidity [1, 3, 4]. Approximately 75 percent of perinatal deaths occur among preterm babies [1]. Advances in medical technologies and therapeutic care have led to improved rates of survival among preterm infants. However, preterm babies that survive have a higher risk of morbidity [1].

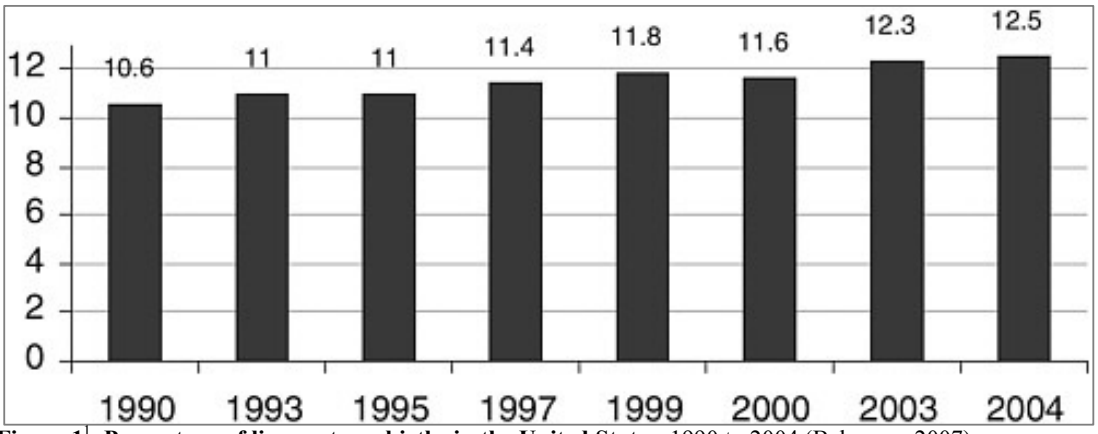


Figure 1[1]. Percentage of live preterm births in the United States, 1990 to 2004 (Behrman, 2007).

1.1 Causes and consequences of preterm delivery

Preterm delivery is not evenly distributed among women and the causes of singleton preterm birth are incompletely understood. Clinically, the single greatest risk factor for spontaneous preterm birth is a maternal history of prior preterm birth [1]. When a woman has had a prior preterm delivery, her risk for repeated preterm delivery is some two- to fivefold higher, depending on the presence of other potential risk factors. Also premature rupture of membranes, multiple pregnancy intrauterine death, fetal and uterine indications and infections have been categorized as risk factors for preterm birth [1, 2, 5]. Chorioamnionitis for example, has been associated with 60 percent of preterm births [6]. Recent studies have indicated that there are also important, independent socio-economic, racial and familial genetic risk factors for the occurrence of preterm birth. For example, African-American women have about a two- to four-fold greater rate of preterm deliveries than Europeans, and a low social class increases twice the risk on preterm birth. Additional risk factors include low body weight, teenage

pregnancy, high maternal age, maternal stress, depression, substance and/or tobacco abuse, absence of prenatal care and periodontal disease. Maternal smoking throughout pregnancy is associated with about a two-fold risk for preterm birth and the increased risks associated with periodontal disease appear to most closely correlate with its severity [5].

Preterm delivered babies are at high risk for neonatal death, and even preterm babies that survive have a higher risk of morbidity [1, 3, 4]. Complications of preterm birth include neurological deficits, blindness, deafness and chronic lung disease. Survival and adverse cognitive, organ functional and motor outcomes are inversely related to gestational age; the highest rates of adverse outcomes are seen in the extremely preterm (28 weeks of gestational age) and severely preterm (28–31 weeks of gestational age) infants [2]. However, even late preterm infants born between weeks 32 and 36 of gestation have increased risk of adverse birth outcomes, such as feeding intolerance and abnormalities of all parts of the visual system [1, 2]. Later in childhood, the prematurely born infants have reduced motor, speaking, writing, mathematical and behavioural skills, compared to children born at term [2]. It is therefore not surprising that premature babies require specialized medical care that can become very expensive for family and society [3]. The prevention of preterm delivery is difficult and most interventions to halt labour are unsuccessful [5, 6]. Clinical interventions to reduce the incidence of preterm birth have largely been directed at targeting treatment for individual risk factors and at answering clinical questions rather than pathogenic mechanistic ones, and have not been very successful [7].

1.2 Chorioamnionitis and the associated fetal inflammatory response syndrome

Studies of the aetiology of spontaneous preterm birth indicate that they are likely the pathological outcomes of microbial activation of cellular components and mediators of an inflammatory pathway that results in onset of labour and membrane rupture [2]. Chorioamnionitis, defined as inflammation of the amniotic fluid and fetal membranes caused by bacteria-produced endotoxin, is such a pregnancy complication [8]. A variety of pathogens such as *Ureaplasma urelyticum*, *Escherichia coli* and *Streptococcus agalactiae*, have been associated with chorioamnionitis [2]. At least 60 percent of all preterm births below 25 weeks of gestation affect mothers that suffer from chorioamnionitis [6, 9]. Not only can chorioamnionitis result in preterm labour, but also significant risks to the fetus and neonate are associated with this complication [10]. Chorioamnionitis induces a fetal inflammatory response syndrome (FIRS), which is characterized by systemic inflammation and an elevation of pro-

inflammatory mediators [11]. It is believed that the production of these inflammatory mediators is involved in the preterm birth associated morbidity and even mortality [10, 12]. Currently, the mechanisms whereby chorioamnionitis induces FIRS are poorly understood. The current view is that during the course of ascending maternal intra-uterine infection, microorganisms may reach the deciduas and can cross intact membranes into the amniotic cavity where they can stimulate the production of inflammatory mediators by resident macrophages and other host cells. Microorganisms that than gain access to the fetus may elicit a systemic inflammatory response syndrome, referred to as FIRS (figure 2) [6, 11].

The diagnosis of chorioamnionitis is however a challenge. Indeed, in 70 percent of women that suffer from chorioamnionitis, the bacterial invasion of the membranes and placenta not necessarily induces a maternal inflammatory response [12, 13]. Consequently, the clinical course can be distinguished between a systemic inflammatory response of the mother with increased leukocyte counts and fever, and a clinically silent form with no symptoms in the mother. Both forms can result in preterm birth but the predominant form at early gestational age is clinically silent [6]. It has been reported that the incidence of the clinical silent form is up to 60 percent throughout different populations and is usually postnatal diagnosed by pathological reviewing the placenta [2,6].

Multiple explanations could clarify this distinction. First, the time course of chorioamnionitis relating to the invasion of microbes is not known [6]. Second, the interaction of the pathogens with the immune system of the host in chorioamnionitis is poorly understood. Moreover, the mechanism that might prevent or control the inflammatory response has not been studied in chorioamnionitis [6]. Third, the function of the immune system may be primed or modulated by previous exposures to pro-inflammatory agonists. For example, the immune system can be rendered tolerant after previous exposures to pro-inflammatory stimuli [14]. In addition, the effect of the indolent chorioamniotic infection on the function of the immune cells is not clear [15].

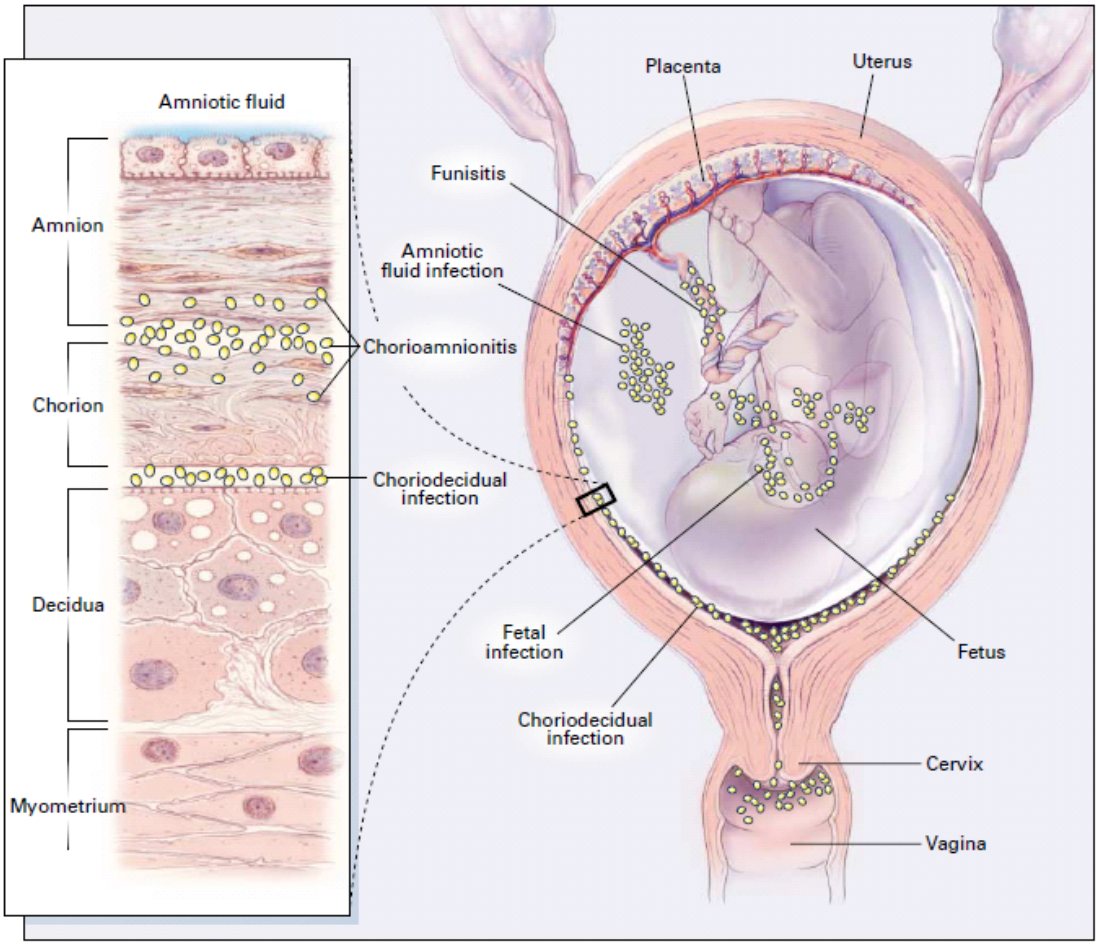


Figure 2[6]. Anatomy of the fetal compartment and associated pathogenesis of inflammation-induced preterm birth. Yellow circles represent bacteria. Bacterial colonization of the vagina occurs during pregnancy and the bacteria ascend through the uterine cervix without necessarily rupturing the membranes. The latter step might be due either to the adherence and virulence of the organism itself or to a deficiency in maternal genetic or immunological responses. The bacteria then replicate in the choriodecidual space. At this point, a maternal inflammatory response is initiated in 30 percent of the cases. The bacteria continue to propagate and cross into the placenta, and are subsequently transported into the fetus via the umbilical cord. At this stage, a fetal inflammatory response to the bacterial challenge might occur. Bacteria might also enter the amniotic cavity by crossing the amniotic membranes. Degradation of the amniotic membranes, a result of the inflammatory response, could assist in the transport of bacteria into the amniotic cavity. Bacteria in the amniotic cavity might enter the fetus via swallowing (Elovitz, 2004).

An alternative hypothesis suggests that in most cases the bacteria do not reach the fetus and/or the amniotic cavity; instead, they remain in the choriodecidual space and elicit an inflammatory reaction in the decidua and placenta. In turn, inflammatory mediators (e.g. cytokines and chemokines) reach the fetal circulation and evoke a fetal response (Elovitz, 2004) [16].

1.3 Animal models concerning preterm birth and chorioamnionitis

In recent years, the use of animal models to elucidate the mechanisms of preterm birth has gained much interest. However, the choice of an appropriate animal model is not straightforward since many species differ from humans in the length of gestation, the number of fetuses, the type of placentation, the hormonal regulation of parturition and the timing of fetal organ maturation. For example, little mammalian species such as rabbits, rats and mice have short gestational periods with multiple fetuses and they experience equivalent maturational sequences only near term or postnatal [1]. Among all species, sheep and nonhuman primates are closest to human development, since the maturation of the lungs and cerebrum, two organs with important contributions to neonatal morbidity and mortality most closely parallel the development in humans (figure 3) [1, 17]. As would be expected, nonhuman primates represent a near-ideal species in which to study preterm birth; however, the cost and therefore restricted quantity of these species limits the use of this animal model [16].

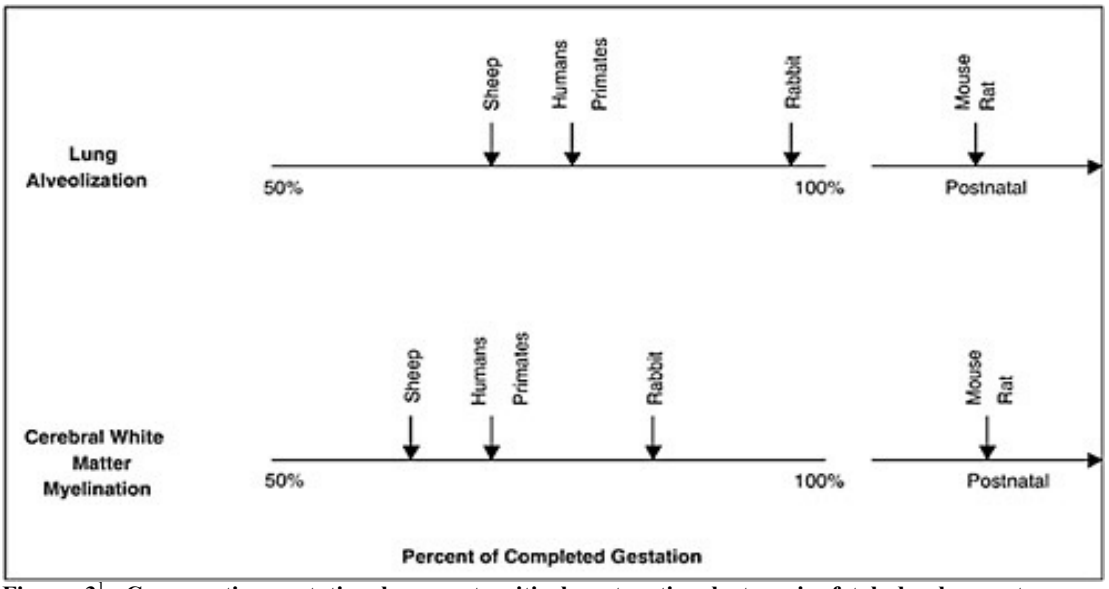


Figure 3[1]. Comparative gestational ages at critical maturational steps in fetal development among different species relevant to neonatal morbidity. Vertical arrows indicate when lung alveolar development or preoligodendrocyte development (oligodendrocytes are responsible for myelination) begins. This schema indicates that maturation of the lungs and cerebrum, two organs with important contributions to neonatal morbidity and mortality, in sheep and nonhuman primates most closely parallel development in humans. In contrast, rabbits and rodents experience equivalent maturational sequences only near term or postnatal, limiting their role in ascertaining the relationships among the causes of prematurity and its consequences (Behrman, 2007).

Although not every specie is equally appropriate to study preterm birth and different interventions had to be applied in order to induce preterm birth in these species, much can be learned about the mechanisms of preterm birth by using these animal models. Mice, rats, rabbits, sheep and nonhuman primates have all been utilised as models for infection-induced preterm delivery. In these animal models numerous infectious and inflammatory agents, including killed or live Escherichia coli, group B streptococcus, components of the cell wall of bacteria, IL-1 and atypical bacteria such as Ureaplasma, have been used. Which of these approaches better approximates what occurs in the human situation is not clear.

The use of live bacteria might mimic those cases of human preterm birth in which cultures of amniotic fluid test positive for bacteria [16]. However, more recent studies that have used PCR for detection, are suggesing that the presence of bacteria (in particular, atypical bacteria such as mycoplasma and ureaplasma) is higher in individuals with preterm labour than was previously demonstrated by bacteria culture alone. Although histological chorioamnionitis is evident in many spontaneous preterm deliveries, most of these preterm infants do not have positive blood cultures. These studies suggest that there is an inflammatory, but not an overtly infectious state, in the uterus, decidua or placenta [16].

The use of killed bacteria, components of the cell wall (LPS or LTA) or proinflammatory cytokines (IL-1) create an inflammatory state in the absence of an overt infection [16]. Regardless of the provocative stimuli or the route of administration, all these models have confirmed the central role of the inflammatory response and proinflammatory cytokines in infection-induced preterm birth [16].

Recently, our research group has established an animal model for chorioamnionitis [18]. In this model endotoxin, a lipopolysaccharide (LPS) from cell walls of gram negative bacteria, is used as provocative stimulus since it is a pro-inflammatory agonist that can be used to study shock and inflammatory responses [19]. When it is administered into the amniotic cavity, endotoxin can induce chorioamnionitis and inflammatory responses in human pregnancies and preterm infants. Since sheep are closest to the human development and more appropriate than nonhuman primates, fetal lambs are used to study the effects of intra-amniotic exposure to endotoxin [20].

It is also physiologically acceptable to use endotoxin as a proinflammatory stimulus, as it is often present in the gastrointestinal tracts of humans and animals [19]. Through infection, humans are constantly exposed to low levels of endotoxin. There are two factors that can contribute to an increased permeability of endotoxin from gastrointestinal tract into blood:

gastrointestinal distress and alcohol consumpition [21]. Inflammation will then be initiated in many cell types by signalling through toll-like receptor 4 (TLR-4) to activate nuclear factor (NF) kappa B, which in turn leads to the production of cytokines, chemokines and antimicrobial peptides. Activation of the toll-like pathway also induces surface expression of costimulatory molecules that are required for the induction of adaptive immune responses such as CD80 and CD86 [12].

1.4 Aim of the study and experimental design

Most studies in the field of chorioamnionitis and FIRS investigated the effect on lungs and brain [11, 12, 22]. However, in order to fully understand the mechanisms leading to the initiation of chorioamnionitis-induced FIRS, the involvement of the liver in the fetal systemic inflammatory response has to be studied.

Recent observations suggest that the liver plays an early causal role in the systemic inflammatory response evoked by different stimuli such as oxidative stress, lipopolysaccharide and dietary cholesterol [23-26]. Accordingly, **we hypothesize that the liver is involved in the fetal systemic inflammatory response evoked by chorioamnionitis**. To test this hypothesis, we examined whether intra-amniotic endotoxin injection in sheep induces prenatal inflammation in the fetal liver. Specifically, livers of premature fetuses were analyzed after caesarean section at a gestational age (GA) of 125 days (comparable with a GA of 28 weeks in humans). Both short term (two days) and long term (two weeks) effects of endotoxin-induced chorioamnionitis were studied. Parameters of haematopoiesis, inflammation, lipid levels and cell damage were investigated in the livers of these prenatal sheep.

Since the prenatal liver was adversely affected by endotoxin-induced chorioamnionitis, **we hypothesize that endotoxin-induced chorioamnionitis can also affect postnatal hepatic function.** Therefore, a second experimental set-up was designed where the livers were analyzed at either a gestational age of 140 days (= near term gestation in sheep) or a postnatal age of eight weeks. Mothers of both groups received an intra-amniotic injection of endotoxin or saline (control) 30 days before the delivery at 140 days gestational age. Again, parameters of haematopoiesis, inflammation, lipid levels and cell damage were investigated in the livers of these sheep.

2. MATERIALS AND METHODS

2.1 Animals, intra-amniotic injections and tissue processing at delivery

The present study was performed according to the guidelines of the Animal Care Committee of the University of Maastricht, which approved the protocol. Time-mated Texel ewes, bearing both singletons and twins, were randomly assigned to groups of five animals, to receive a single dose of 10 mg endotoxin (*Escherichia coli* 055:B5; Sigma Chemical, St. Louis, MO) or the equivalent dose of saline for control by ultrasound guided intra-amniotic injections [18].

In the first experimental design, prenatal effects of chorioamnionitis induced by intra-amniotic endotoxin injections were studied two days and two weeks before the GA of 125 days (figure 4A). The low GA of 125 days is comparable with a human GA of approximately 28 weeks. Term gestation in sheep is approximately 140 days. After caesarean section at GA of 125 days, a cord blood arterial sample was collected followed by a lethal injection of pentobarbital sodium via an umbilical vein. Pieces of the liver were either fixated in 4% formaldehyde or snap-frozen in liquid nitrogen for further analysis.

In the second experimental design, postnatal effects of chorioamnionitis induced by intra-amniotic endotoxin injections were studied at either a gestational age of 140 days or a postnatal age of eight weeks. Mothers of both groups received an intra-amniotic injection of endotoxin or saline (control) 30 days before the delivery at 140 days gestational age (figure 4B). After delivery at GA of 140 days, a cord blood arterial sample was collected. The lambs were sacrificed either at GA of 140 days or at postnatal age of eight weeks by a lethal injection of pentobarbital sodium via an umbilical vein. Pieces of the liver were snap-frozen in liquid nitrogen for further analysis.

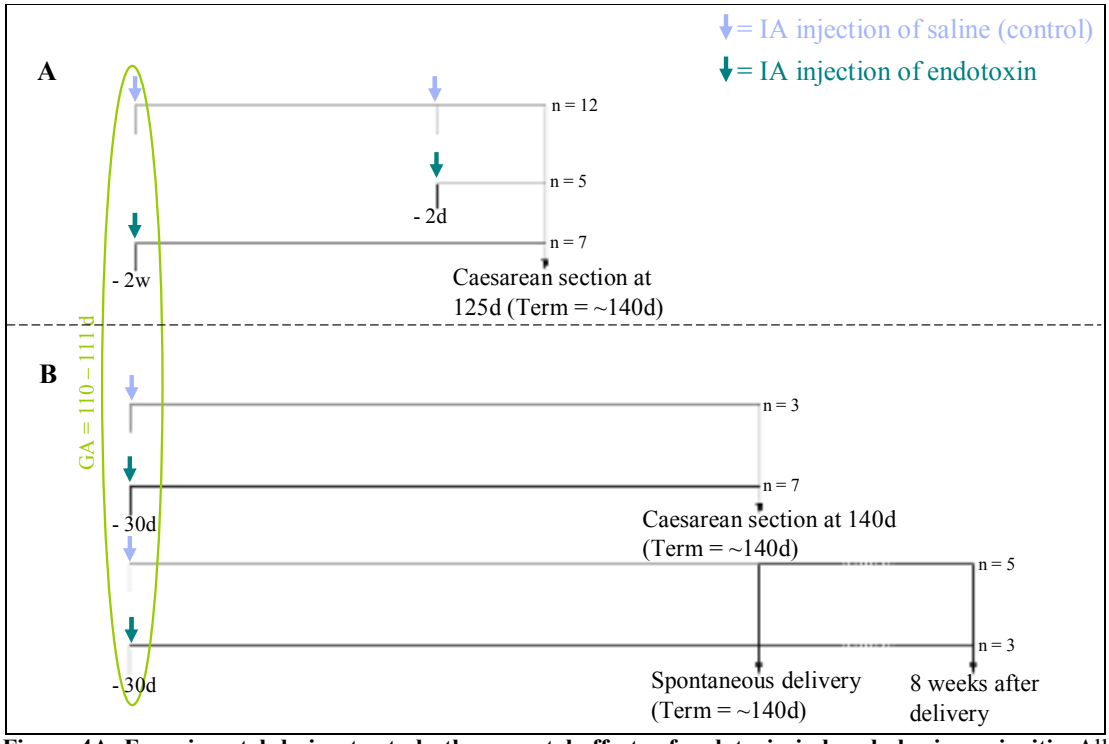


Figure 4A. Experimental design to study the prenatal effects of endotoxin-induced chorioamnionitis. All fetuses were analyzed after caesarean section at the gestational age (GA) of 125 days, which is comparable with the developmental age of 28 weeks in humans. Term gestation in sheep is approximately 140 days. Chorioamnionitis was induced by intra-amniotic endotoxin injection two days (2d) or two weeks (2w) before delivery. **Figure 4B. Experimental design to study the postnatal effects of endotoxin-induced chorioamnionitis.** Endotoxin was injected 30 days (30d) before delivery. Livers of lambs were analyzed either after caesarean section at gestational age (GA) 140 days (140d) or eight weeks after a spontaneous delivery. IA = intra-amniotic.

2.2 Immunohistochemistry and histological analysis

Liver tissue of animals with GA 125 days was fixed in 4% formaldehyde, embedded in paraffin and cut in 3 µm sections. Liver tissue of animals with GA 140 days or postnatal age eight weeks was snap-frozen in liquid nitrogen and cut in 7 µm sections. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide for 20 minutes. Nonspecific binding sites were blocked with serum. After antigen retrieval with DAKO Real Target Retrieval Solution (DAKO S2031, dilution 1:10; Glostrup, Denmark), CD3-positive T-lymphocytes were stained with a polyclonal rabbit anti-human CD3 antibody (DAKO A0452, dilution 1:200; California, USA) at 4°C overnight. Polyclonal rabbit anti-human MPO (DAKO A0398, dilution 1:500; California, USA) was used as primary antibody to identify MPO-positive cells. Unbound antibody was removed with phosphate buffered saline (PBS) and biotinylated polyclonal swine anti-rabbit IgG (DAKO E0353, dilution 1:200/1:500

respectively; California, USA) was applied as secondary antibody for 1 hour at room temperature. After adding StrepABComplex/HRP (DAKO KO377; Glostrup, Denmark), immunostaining was performed with 3-amino-9-ethylcarbazole (AEC) and sections were counterstained with haematoxylin (See: Appendix 1+2).

Haematoxylin and eosin (H/E) staining was performed on paraffin or frozen liver sections to evaluate the degree of inflammation and haematopoiesis (See: Appendix 3).

To investigate fat deposition in the liver, frozen 7 µm-thick sections were stained with Oil red O. Briefly, liver cryosections were fixed for 60 minutes in 3.7% formaldehyde solution and stained with 0.2% Oil red O in 60% triethyl-phosphate for 30 minutes. Sections were counterstained with haematoxylin, washed with running tab water and covered with coverslip using 10% glycerol in PBS (See: Appendix 4).

Cell apoptosis was determined by evaluating terminal transferase dUTP nick end labeled (TUNEL) cells using a commercially available kit (In Situ Cell Death Detection Kit, Roche Applied Science, Mannheim, Germany). Cryosections were fixed during 20 minutes at room temperature with 4% paraformaldehyde. Slides were washed in PBS for 30 minutes. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide for 10 minutes at room temperature. Afterwards, slides were permeabilized with 0.1% Triton X-100 for 2 minutes on ice. The slides were incubated with 50 µl Terminal deoxynucleotidyl Transferase (TdT) enzyme in a humidity chamber for 1 hour at 37°C to add deoxynucleotide to the free 3 -OH end of DNA breaks characteristic of apoptotic cell death. Then, slides were washed in three changes of PBS for 1 minute per wash. 50 µl horse-radish peroxidase (POD) was applied to the slides and incubated in a humidity chamber for 30 min at 37°C, to convert the fluorescence based TUNEL detection into a colorimetric labeling suited for transmission light microscopy. Afterwards, slides were again washed in three changes of PBS for 1 minute each. 250 µl peroxidase substrate was applied and allowed to stain for 5 to 15 minutes at room temperature. Slides were then washed in demineralized H2O for 5 minutes. Tissues were counterstained in haematoxylin for 3 seconds at room temperature (timing depends on age and use of haematoxylin). Slides were washed in running tab water and for 1 minute in demineralized H2O, dehydrated in graded changes of alcohols, cleared in 2 changes of xylene, and mounted in entellan using a coverslip (See: Appendix 5).

All stained sections were photographed at 200x magnification using a Nikon digital camera DMX1200 and ACT-1 v2.63 software from Nikon Corporation. For the CD3 and MPO immunohistochemical stainings, cell numbers were counted in six randomly selected microscopical views and were noted as cells/mm². For the H/E- and TUNEL-staining, whole

sections were analyzed for inflammation and apoptosis respectively, and scored in a blinded manner by a specialized animal pathologist. The H/E-sections from each animal were scored as zero if they had no inflammatory cells present in tissue, one for a few inflammatory cells, two for moderate cell infiltration, three for a large number of inflammatory cells and four if inflammation was spread all over in the tissue. The sections were also scored for haematopoiesis. This scoring scale went also from zero to four and is based on the number hematopoietic cells present in the liver tissue. The same scoring scale was used for the TUNEL stained sections, based on the amount of TUNEL-positive cells.

2.3 RNA extraction and RT-PCR

Total RNA was isolated from frozen liver samples by using Tri-reagent of Sigma, as previously described [27]. All applications were done according to manufacturer's protocols. Briefly, liver samples were homogenized in 1.0 ml Tri-reagent with the MiniBeadBeater. To ensure complete dissociation of nucleoprotein complexes, the samples were allowed to stand for 5 minutes on ice in the Tri-reagent. Additionally, phase separation was performed by adding 200 µl of chloroform per 1 ml of Tri-reagent, shaking for 15 seconds and incubating for 15 minutes on ice. This was followed by centrifugation at 13.2 rpm for 15 minutes and removal of the aqueous phase. RNA precipitation was performed by adding 0.5 ml isopropanol per ml Tri-reagent used in sample preparation, incubating on ice for 10 minutes and centrifugation at 13.2 rpm for 10 minutes at 4°C, resulting in formation of a pellet. The RNA pellet was washed by the addition of 0.5 ml 70% ethanol, vortexing and centrifugation at 13.2 rpm for 5 minutes at 4°C. After removing the supernatant and air-drying the pellet for a short period, the RNA was re-dissolved by adding RNase-free water. The pellet was dissolved in 200 µl RNase-free water by pipetting (See: Appendix 6). Quality and quantity of the RNA were determined with the Nanodrop ND-1000 (Witec Ag, Littau, LU). The final preparation of RNA is free of DNA and proteins when it has an OD 260/280 ratio of ≥ 1.7.

Reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, USA) according to manufacturer's instructions. Real-time PCR was performed on a Bio-Rad MyIQ with the IQ5 v2 software using the IQ SYBR Green Supermix with fluorescein (Bio-Rad, Hercules, USA) and 10 ng of cDNA. For each gene a standard curve was generated with a serial dilution of a liver cDNA pool. Primers for target genes interleukin (IL)-1β, IL-8, tumor necrosis factor (TNF) and glutathione S-transferase (GST), were developed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA, USA) using default settings. To standardize for the amount of cDNA, cyclophylin A (peptidylprolyl

isomerase A, ppia) was used as housekeeping gene. Primer sequences are given in table 1. The liver real-time PCR results were obtained using the relative standard curve method.

Table 1. Primers for real-time PCR performed on total RNA, isolated from lamb liver samples.

Primer	Forward	Reverse
ppia*	TTATAAAGGTTCCTGCTTTCACAGAA	ATGGACTTGCCACCAGTACCA
IL-1β	AGAATGAGCTGTTATTTGAGGTTGATG	GTGAGAAATCTGCAGCTGGATGT
IL-8	TTCCAAGCTGGCTGTTGCT	GTGGAAAGGTGTGGAATGTGTTT
TNF-α	CATCTTCTCAAGCCTCAAATAACAA	TGCGAGTAGATGAGGTAAAGCCC
GST	CTACATTGCCACCAAATACAACCT	AATCTGCCACCCCCTCTGA

* housekeeping gene

2.4 Plasma parameters

Alanine aminotransferase (ALT) as well as aspartate aminotransferase (AST), triglycerides, total cholesterol and HDL-cholesterol have been determined on a Beckman Coulter Synchron LX20 PRO Clinical Chemistry analyzer (Beckman Coulter, Fullerton, USA).

The ALT and AST activity are determined by an enzymatic rate method. The rate of change in absorbance, which is caused by the enzymatic reaction, is directly proportional to the activity of ALT and AST in the sample and is used by the Synchron system to calculate and express the ALT and AST activity.

Both triglyceride and cholesterol concentration were measured by a time-endpoint method. The direct HDL Cholesterol method is a homogeneous assay without the need for any offline pre-treatment or centrifugation steps. The method depends on a unique detergent which solubilizes only the HDL lipoprotein particles and releases HDL cholesterol to react with cholesterol esterase and cholesterol oxidase in the presence of chromogens, to produce a color product. More specifically, cholesterol esterase hydrolyzes cholesterol esters to free cholesterol and fatty acids. Free cholesterol is oxidized to cholestene-3-one and hydrogen peroxide by cholesterol oxidase. Peroxidase catalyzes the reaction of hydrogen peroxide with 4-aminoantipyrine and phenol to produce a colored quinoneimine product. The same detergent also inhibits the reaction of the cholesterol enzymes with LDL, VLDL, and chylomicrons lipoproteins by adsorbing to their surfaces. A polyanion contained in the reagent enhances the selectivity for HDL cholesterol assay by complexing LDL, VLDL, and chylomicrons lipoproteins. The HDL reagent is used to measure the cholesterol concentration by a time-endpoint method. The system monitors the change in absorbance at 560 nanometers. This change in absorbance is directly proportional to the concentration of

cholesterol in the sample and is used to calculate and express the HDL-cholesterol (HDL-C) concentration.

The triglyceride concentration is measured by a similar method. Namely, triglycerides in the sample are hydrolysed to glycerol and free fatty acids by the action of lipase. A sequence of three coupled enzymatic steps using glycerol kinase, glycerophosphate oxidase and horseradish peroxidase causes the oxidative coupling of 3,5-dichloro-2-hydroxybenzenesulfonic acid with 4-aminoantipyrine to form a red quinoneimine dye.

LDL-cholesterol (LDL-C) has been calculated by the Friedewald equation. This equation estimates LDL-cholesterol from the measurements of total cholesterol, triglycerides and HDL-cholesterol;

$$LDL - C = total\ cholesterol - \left(\frac{triglyceride}{2.2} + HDL - C\right)$$

2.5 Hepatic lipid analysis

Approximately 50 mg of frozen liver tissue was homogenized for 30 seconds at 50 rpm in a closed tube with 5.0 mm glass beads and 1.0 ml SET buffer (Sucrose 250mM, EDTA 2mM and Tris 10mM). Complete cell destruction was done by two freeze-thaw cycles and three times passing through a 27-gauge syringe needle and a final freeze-thaw cycle. Protein content was measured using the BCA method (Pierce, Rockford, USA). Triglyceride (TG) and cholesterol (Chol) content were measured according to manufacturer's instructions on a Benchmark 550 Micro-plate reader (Bio-Rad, Hercules, USA). Concentration of TG or Chol are expressed relative to the protein (prot) content of the liver sample (See: Appendix 7).

2.6 Data analysis

Data were statistically analyzed using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Comparisons between controls and endotoxin-injected groups at GA of 125 days were performed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison tests for post-hoc analyses. Groups at GA of 140 days or postnatal age of eight weeks were compared using two-tailed non-paired t-tests. Scoring of haematopoietic, inflammatory and apoptotic cells was calculated by Pearson's χ^2-test. Results are presented as means ± SEM and a p-value < 0.05 was considered as statistically significant. * indicates significant differences between saline and endotoxin-injected groups. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

3. RESULTS

3.1 Prenatal hepatic effects of endotoxin-induced chorioamnionitis

To test the hypothesis that the prenatal liver is involved in the systemic inflammatory response evoked by chorioamnionitis, we examined whether intra-amniotic endotoxin injection in sheep induces prenatal inflammation in the fetal liver. Livers of premature fetuses were analyzed after caesarean section at a gestational age (GA) of 125 days. Both short term (two days) and long term (two weeks) effects of endotoxin-induced chorioamnionitis were studied. Parameters of haematopoiesis, inflammation, cell damage and lipid levels were investigated in the livers of these prenatal sheep.

3.1.1 Inflammation and haematopoiesis based on histology

To investigate the effect of endotoxin-induced chorioamnionitis on haematopoiesis and inflammation, H/E staining was performed. The liver sections from each animal were scored from zero to four, based on the amount of haematopoietic and inflammatory cells.

Liver sections of animals with GA 125 days showed no differences in inflammatory cell infiltration between control animals and animals that received endotoxin two days before delivery. However, an increased level of infiltrated inflammatory cells was detected two weeks after endotoxin exposure (figure 5A, C-E). There were no significant differences in haematopoiesis between the three groups (figure 5B, C-E).

To define the specificity of the infiltrated inflammatory cells, immunohistochemical stainings against T-lymphocytes (CD3) and myeloperoxidase (MPO) were performed.

Consistent with the observations of the H/E staining, CD3-positive T-lymphocytes showed a significant increase in the livers of animals that received endotoxin two weeks before delivery at GA of 125 days (figure 6A). Remarkably, most CD3-positive T-lymphocytes were positioned in the haematopoietic clusters (figure 6C-E). To further characterize the inflammation, we used immunohistochemical detection of MPO (figure 6B + F-H), since MPO is an important enzyme involved in the processes of reactive oxygen generation and inflammation [28]. Interestingly, the number of MPO-positive cells was increased both two days and two weeks after the endotoxin injections compared to control (figure 6B). Unlike the CD3-positive T-lymphocytes, the MPO-positive cells were not located in between the haematopoietic clusters (figure 6F-H).

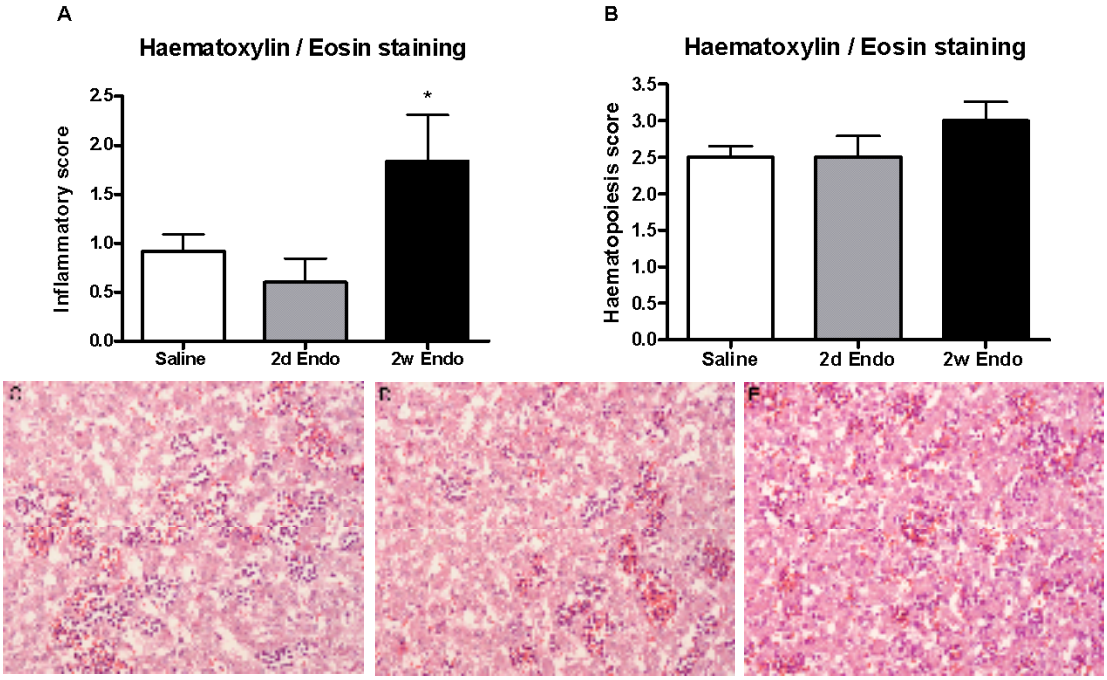
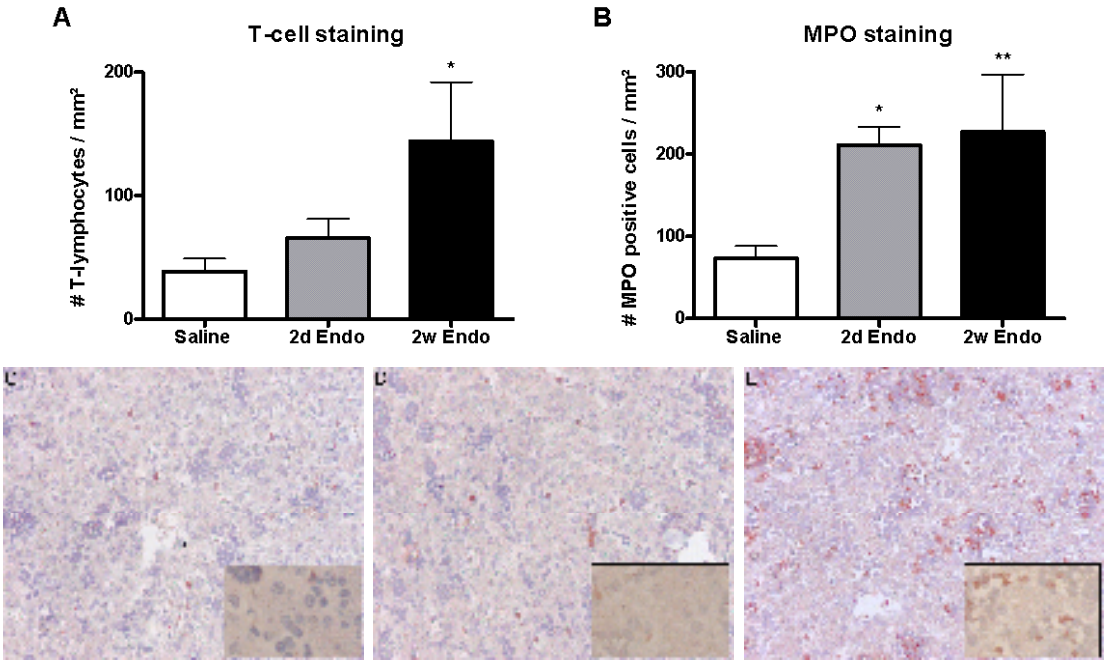


Figure 5. Hepatic inflammation and haematopoiesis based on H/E staining in animal groups with GA 125d. (A) Representative inflammatory score of haematoxylin- and eosin- (H/E) stained liver sections from control and endotoxin (endo) exposed animals. **(B)** Representative score for haematopoiesis of H/E-stained liver sections from control and endotoxin (endo) exposed animals. **(C-E)** Representative pictures (200x magnification) of the H/E-staining for the saline, two days (2d) and two weeks (2w) endotoxin exposed animals respectively. * Significant different from saline injected group.



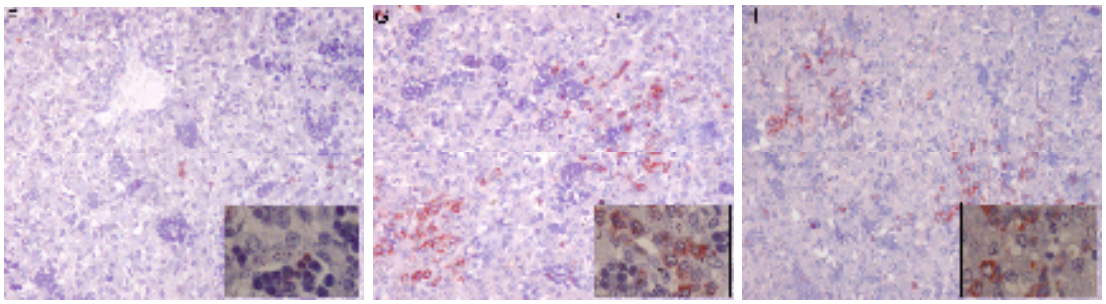


Figure 6. Hepatic inflammation based on liver immunohistochemistry in animal groups with GA 125d. **(A+B)** Liver paraffin sections were stained against T-lymphocytes (CD3) and myeloperoxidase (MPO) respectively. Scale expressed as amount of cells per mm². **(C-E)** Representative pictures (200x magnification, inset: 400x magnification) of the CD3 staining for the saline, two days (2d) and two weeks (2w) endotoxin exposed animals respectively. **(F-H)** Representative pictures (200x magnification, inset: 400x magnification) of the MPO staining for the saline, two days and two weeks endotoxin exposed animals respectively. * Significant different from saline injected group.

3.1.2 Inflammation based on gene expression profiling

To investigate the hepatic response to endotoxin-induced chorioamnionitis in more detail, mRNA expression of genes involved in inflammation was determined by RT-PCR. We assessed induction of interleukin (IL)-1β as a transmigrator of leukocytes, tumor necrosis factor (TNF)-alpha as a stimulator of the acute phase reaction [29] and interleukin (IL)-8 as a chemokine that mediates neutrophil recruitment [30].

The mRNA levels for pro-inflammatory cytokines TNF and IL-1β were induced rapidly in the prenatal liver of animals that were sacrificed two days after the endotoxin injection. Two weeks after the endotoxin exposure, cytokine levels went back to basal levels (figure 7A+B). Surprisingly, the mRNA levels for the neutrophil recruiter IL-8 did not change in the fetal liver after fetal exposure to endotoxin (figure 7C).

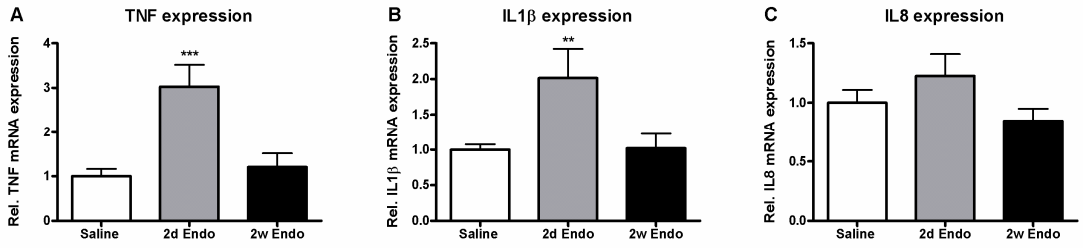
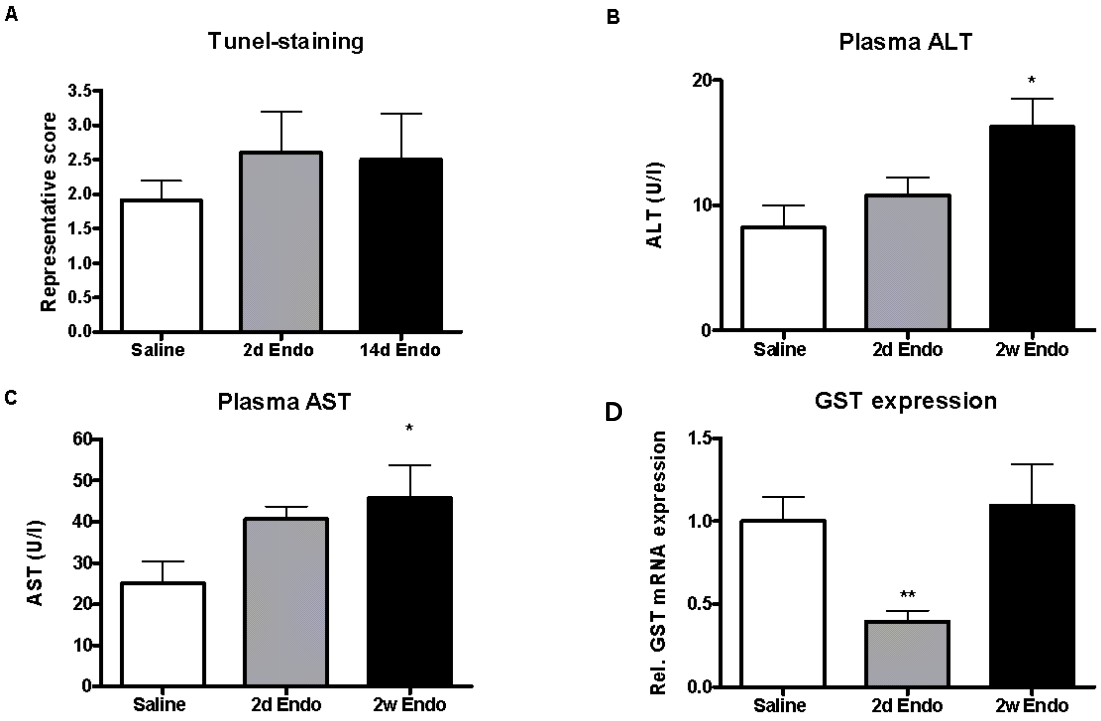


Figure 7. Hepatic gene expression analysis with quantitative reverse-transcription PCR. Cytokine mRNA levels are normalized to cyclophylin A. **(A-C)** Relative gene expression of TNF, IL-1β and IL-8 in the prenatal liver two days (2d) and two weeks (2w) after endotoxin (endo) compared to saline control. * Significant different from saline injected group.

3.1.3 Induced liver damage after endotoxin-induced chorioamnionitis

To assess whether the inflammatory response in the liver is associated with increased cell death, the level of apoptotic cells was established. In addition, the level of plasma alanine aminotransferase (ALT) was determined to reflect the damage to hepatocytes. To avoid the risk of false-positive results due to the extrageneous sources of plasma ALT activity, additional markers for liver function, such as plasma aspartate aminotransferase (AST) levels and mRNA levels of the anti-oxidant glutathione S-transferase (GST), were measured. Even though plasma AST levels are considered as a less specific biomarker of liver function, as AST is also localized in heart, brain and skeletal muscle, plasma AST levels can give additional information about the liver damage. Also GST expression can be used to give additional information about the liver, since the expression is restricted to liver and kidney [31]. Although a significant effect on the amount of apoptotic cells could not be observed (figure 8A, E-G), both plasma ALT and AST measurements showed a significant increase two weeks after endotoxin-induced chorioamnionitis (figure 8B+C). Moreover, decreased expression of GST mRNA was observed two days after prenatal exposure to endotoxin. Two weeks after the endotoxin injection, the mRNA expression went back to basal levels (figure 8D). This is in line with previous observations in the animal group of GA 125 days concerning the pro-inflammatory cytokines.



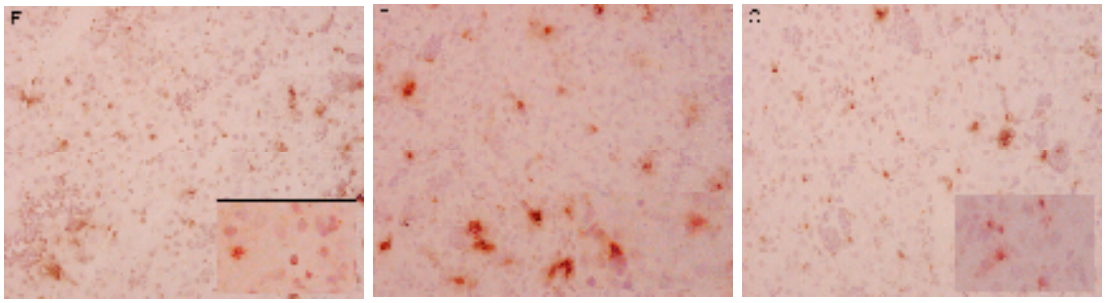


Figure 8. Quantification of liver damage. (A) Representative apoptotic score of TUNEL-positive cells from control and endotoxin (endo) injected animals. **(B+C)** Plasma levels of alanine aminotransferase activity (ALT) and aspartate aminotransferase activity (AST) respectively. **(D)** Relative mRNA expression of glutathione S-transferase (GST) two days (2d) and two weeks (2w) after endotoxin (endo) compared to control animals. **(E-G)** Representative pictures (200x magnification, inset: 400x magnification) of the TUNEL-staining for the saline, two days and two weeks endotoxin exposed animals respectively. * Significant different from saline injected group.

3.1.4 Lipid analysis after endotoxin-induced chorioamnionitis

Since the liver has indispensable functions in lipid homeostasis [32] and a correlation exists between lipid levels and inflammation [33], we assessed the potential effect of hepatic inflammation on both hepatic and plasma lipid levels. Biochemical analysis was performed to investigate the effect of intra-amniotic endotoxin exposure on lipid levels in the prenatal liver. Two days after endotoxin exposure, preterm delivered lambs showed hepatic cholesterol levels that tended to increase compared to control levels (p=0.06). Two weeks after endotoxin injections, hepatic cholesterol went back to basal levels (figure 9A). Furthermore, hepatic triglyceride levels were not affected by exposure to endotoxin (figure 9B), what is also confirmed by the Oil red O staining (data not shown).

In addition, plasma lipids were monitored. Interestingly, both total cholesterol and triglyceride levels were increased two days after endotoxin-induced chorioamnionitis (figure 9C+D). The increase in cholesterol corresponds with increased HDL and LDL levels, although only HDL levels were significantly increased two days after endotoxin-induced chorioamnionitis.

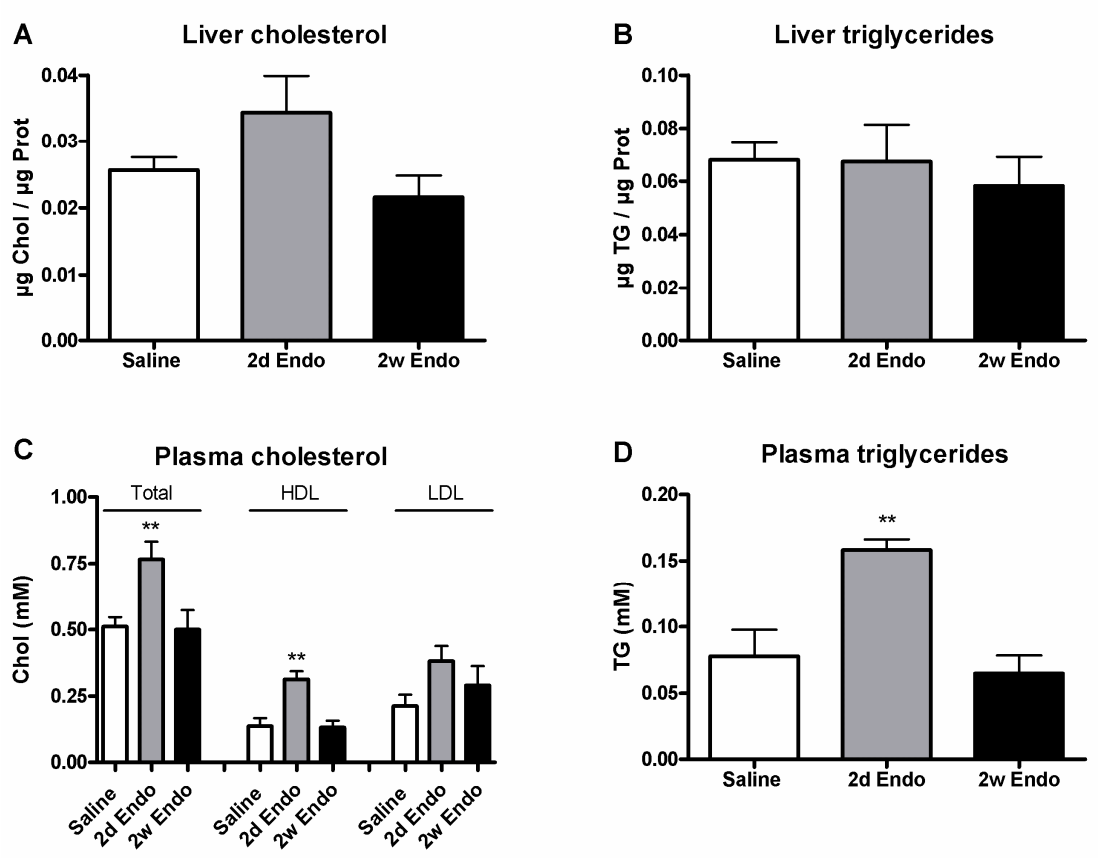


Figure 9. Liver and plasma lipid levels of saline and endotoxin (endo) exposed animals at GA 125d. (A-B) Liver cholesterol (Chol) and triglyceride (TG) levels of saline and endotoxin (endo) exposed animals. Hepatic lipid levels are corrected for their protein (Prot) content. **(C)** Plasma total Chol, HDL and LDL levels. **(D)** Plasma TG levels. * Significant different from saline injected group.

3.2 Postnatal hepatic effects of endotoxin-induced chorioamnionitis

Based on the adverse effects seen on the prenatal liver after endotoxin-induced chorioamnionitis, we hypothesize that endotoxin-induced chorioamnionitis can also affect liver function in postnatal life. To test this hypothesis, livers were studied either at a gestational age of 140 days or at a postnatal age of eight weeks. Mothers of both groups received an intra-amniotic injection of endotoxin or saline (control) 30 days before the delivery at 140 days gestational age. Parameters of haematopoiesis, inflammation, cell damage and lipid levels were investigated in the livers of these postnatal sheep.

3.2.1 Inflammation and haematopoiesis based on histology

To have a general overview of the inflammatory effect of endotoxin-induced chorioamnionitis, H/E staining was performed. The liver sections from each animal were scored from zero to four, based on the amount of inflammatory cells. Since haematopoiesis was not abundant anymore in these postnatal livers (figure 11A-D), no scoring was performed according to the amount of haematopoietic cells.

Based on the H/E-staining, liver sections of postnatal animals showed no differences in inflammatory cell infiltration between control animals and animals that received endotoxin 30 days before delivery (figure 10A). However, compared to the animals with GA 125 days, these postnatal livers seems to be more affected in general (figure 5C-E and figure 11A-D).

To gain more insight into the specificity of the infiltrated inflammatory cells, immunohistochemical stainings against T-lymphocytes (CD3) and myeloperoxidase (MPO) were performed.

Only a significant increase in CD3-positive T-lymphocytes could be observed in the animals that were born near term (GA 140 days) (figure 10B + figure 11 E-H). Both in the animal group with GA 140 days and in the animal group with postnatal age eight weeks, the number MPO-positive cells was not changed compared to control animals (figure 10C + figure 11 I-L).

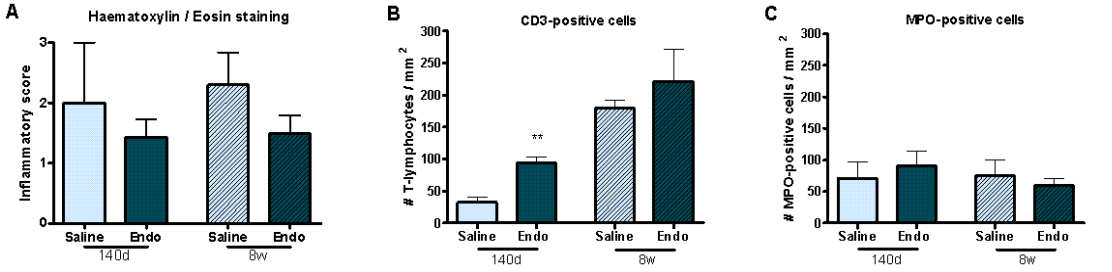
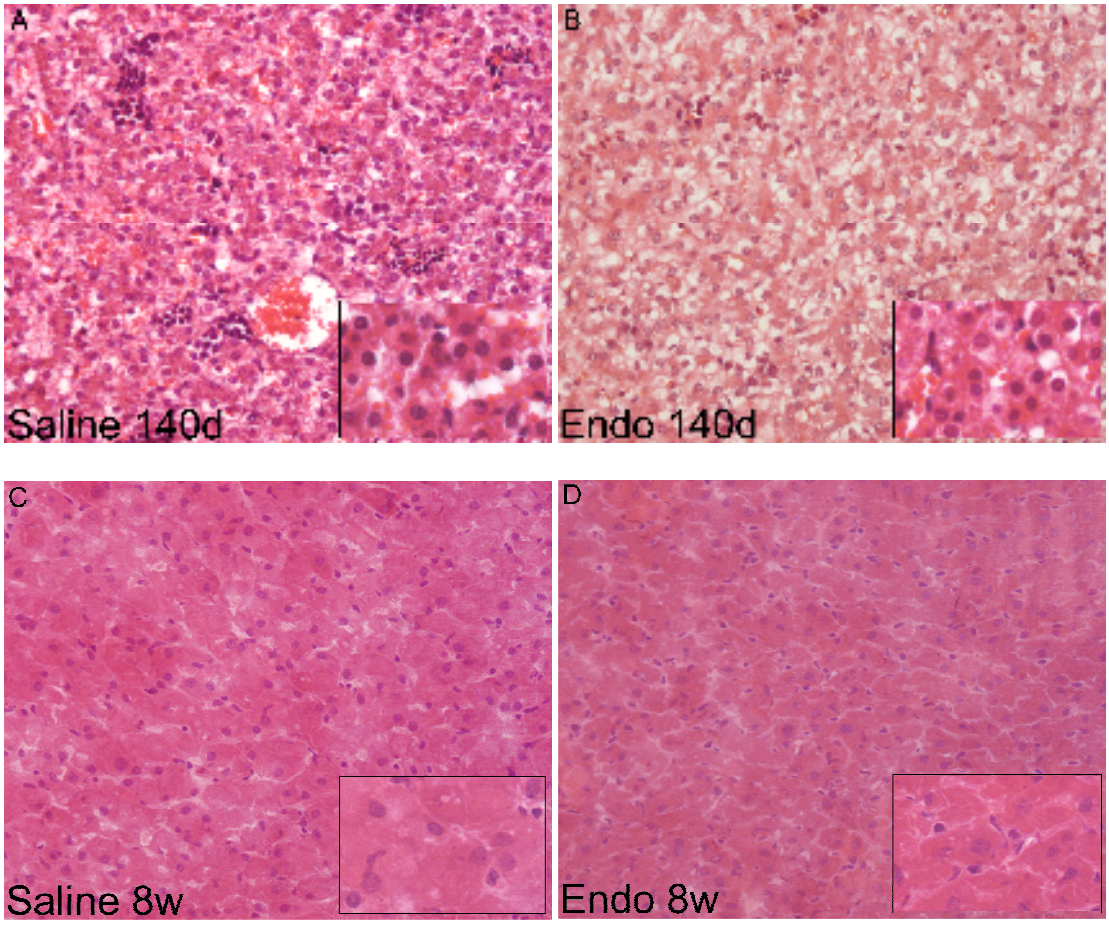
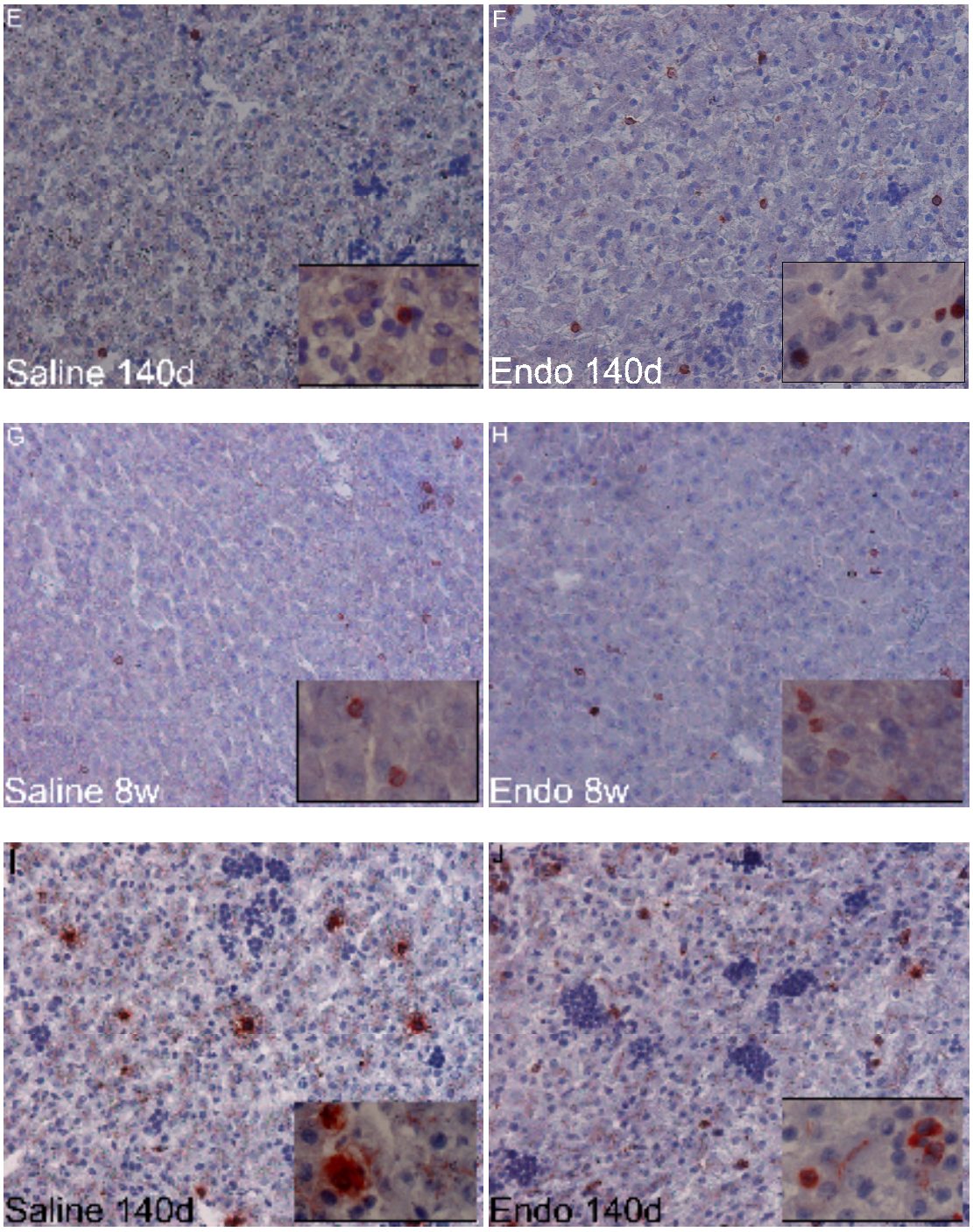


Figure 10. Hepatic inflammation based on liver histology and immunohistochemistry in animals with GA 140 days (140d) and postnatal age eight weeks (8w). (A) Representative inflammatory score of haematoxylin- and eosin- (H/E) stained liver sections from control and endotoxin (endo) exposed animals. **(B+C)** Liver cryosections were stained against T-lymphocytes (CD3) and myeloperoxidase (MPO) respectively. Scale expressed as amount of cells per mm². * Significant different from saline injected group of same GA.

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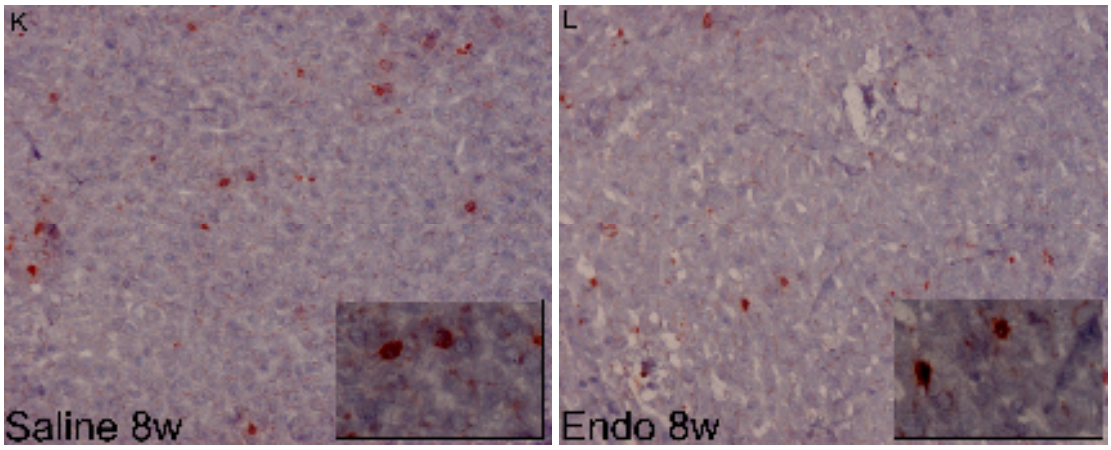


Figure 11. Microscopic pictures of H/E-, CD3- and MPO-stained liver cryosections. (A-D) Representative pictures (200x magnification, inset: 400x magnification) of the H/E staining for the saline and endotoxin (endo) exposed animals with GA 140 days and postnatal age eight weeks respectively. **(E-H)** Representative pictures (200x magnification, inset: 400x magnification) of the CD3 staining for the saline and endotoxin exposed animals with GA 140 days and postnatal age eight weeks respectively. **(I-L)** Representative pictures (200x magnification, inset: 400x magnification) of the MPO staining for the saline and endotoxin exposed animals with GA 140 days and postnatal age eight weeks respectively.

3.2.2 Inflammation based on gene expression profiling

According to the observations in the animals of GA 125 days, where the increase in pro-inflammatory cytokines was resolved 14 days after endotoxin-induced chorioamnionitis, there was no difference in TNF, IL-1β and IL-8 mRNA expression in relation to control animals when the animals were exposed to endotoxin during 30 days (figure 12A-C).

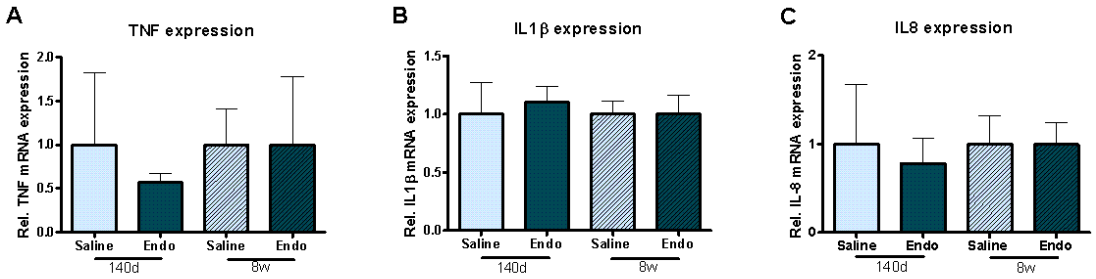
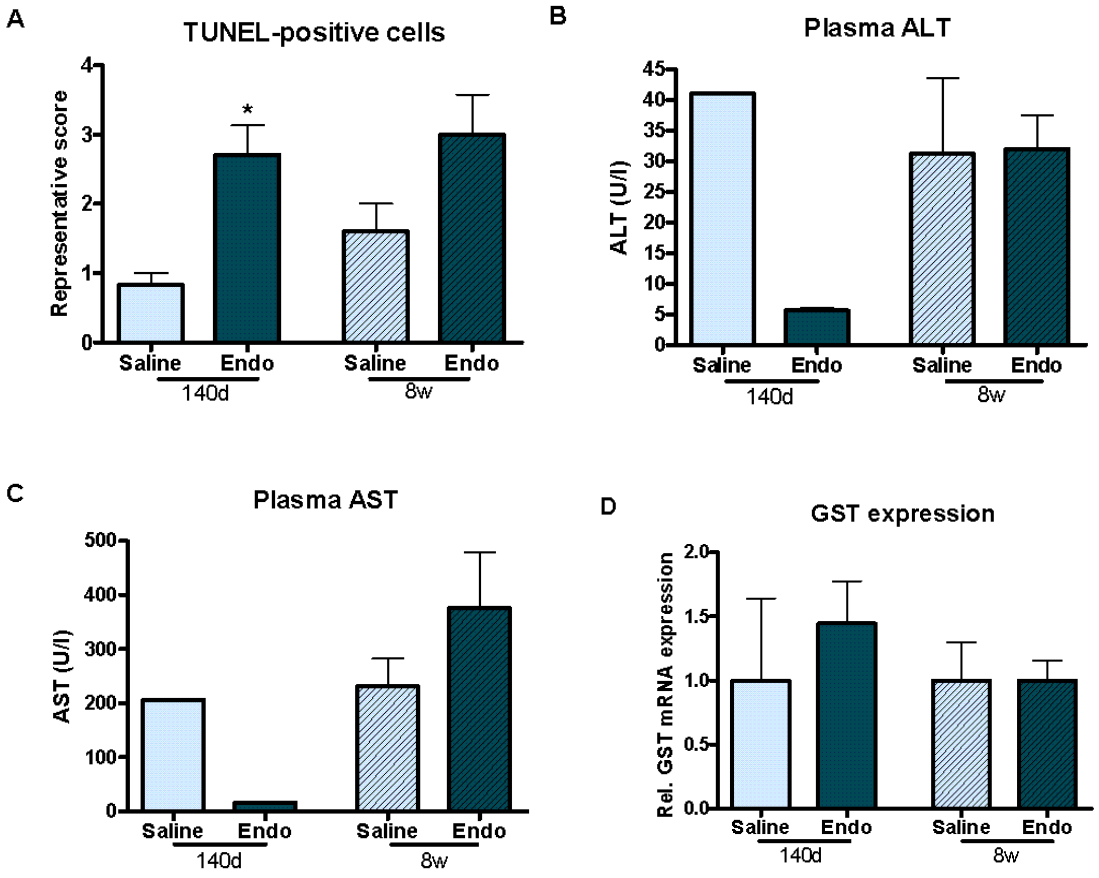


Figure 12. Hepatic gene expression analysis with quantitative reverse-transcription PCR. Cytokine mRNA levels are normalized to cyclophylin A. **(A-C)** Relative gene expression of TNF, IL-1β and IL-8 in the postnatal liver 30 days after endotoxin (endo) compared to saline control.

3.2.3 Induced liver damage after endotoxin-induced chorioamnionitis

To assess the level of hepatocellular damage, the number of apoptotic cells, plasma alanine aminotransferase (ALT), plasma aspartate aminotransferase (AST) levels and mRNA levels of the anti-oxidant glutathione S-transferase (GST) were measured [31].

The amount of apoptotic cells showed a significant increase after endotoxin-induced chorioamnionitis in the animals with GA 140 days (figure 13A, E-F). This is in line with previous observations in the animal group of GA 140 days concerning the increased levels of CD3-positive T-lymphocytes. However, the effect on hepatocellular damage could not be observed based on the markers for liver damage ALT, AST and GST (figure 13B-D). The postnatal livers of animals aged eight weeks showed no indication of liver damage (figure 13A-D, G-H).



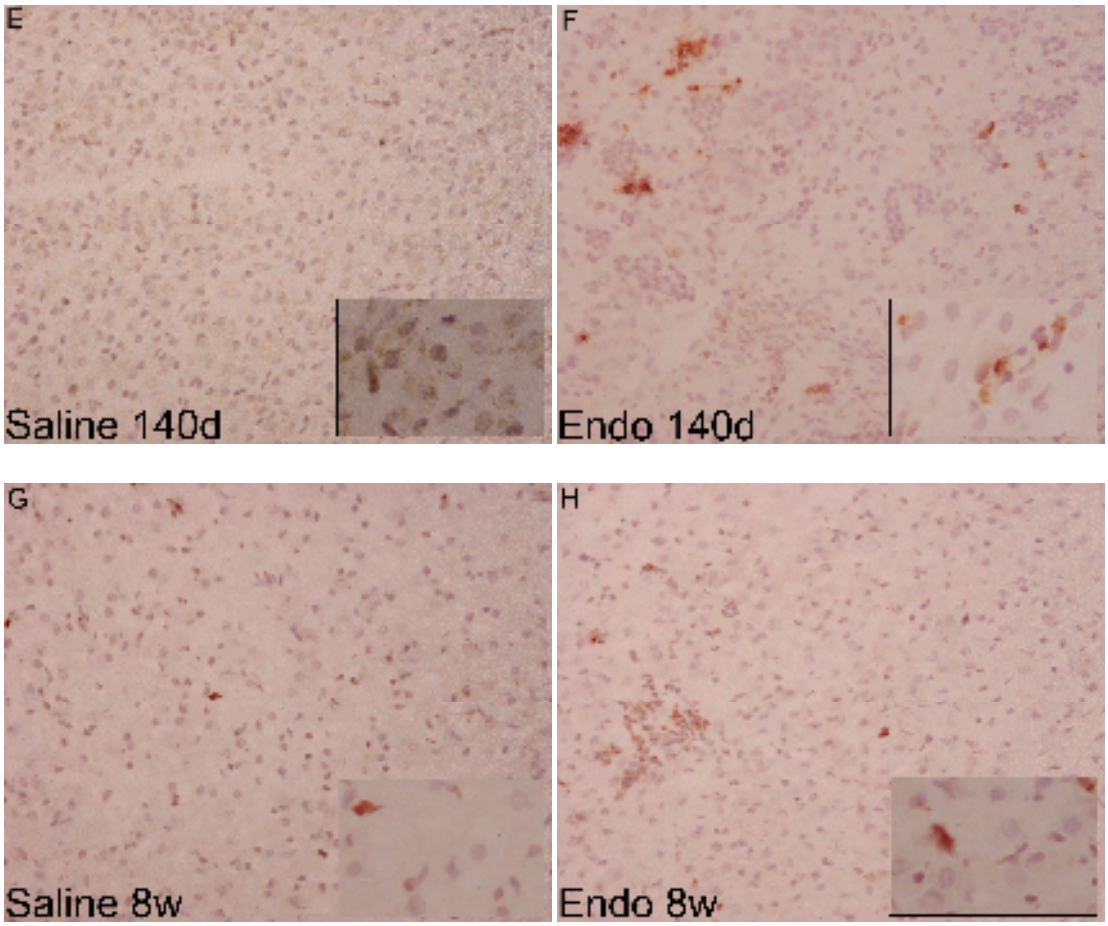


Figure 13. Quantification of hepatocellular damage. (A) Representative apoptotic score of TUNEL-positive cells from control and endotoxin (endo) injected animals. **(B+C)** Plasma levels of alanine aminotransferase activity (ALT) and aspartate aminotransferase activity (AST). **(D)** Relative mRNA expression of glutathione S-transferase (GST) after endotoxin (endo) compared to control animals. **(E-H)** Representative pictures (200x magnification, inset: 400x magnification) of the TUNEL-staining for the saline and endotoxin exposed animals of 140 days (140d) and eight weeks (8w) respectively.* Significant different from saline injected group of same GA.

3.2.4 Lipid analysis after endotoxin-induced chorioamnionitis

Also in the second experimental design, biochemical analysis was performed to investigate the effect of intra-amniotic endotoxin exposure on lipid levels. After endotoxin exposure, lambs with GA 140 days showed decreased hepatic cholesterol levels compared to control levels. Eight weeks after spontaneous delivery, hepatic cholesterol levels showed no difference compared to basal levels (figure 14A). Hepatic triglyceride levels however, were affected by exposure to endotoxin both in the animals of GA 140 days as in the animals with

postnatal age eight weeks. Remarkably, triglyceride levels decreased compared to control levels in the animals aged 140 days. In contrast, triglyceride levels increased compared to control in the animals aged eight weeks (figure 14B).

Interestingly, both plasma cholesterol and triglyceride levels were not affected after endotoxin-induced chorioamnionitis (figure 14C+D).

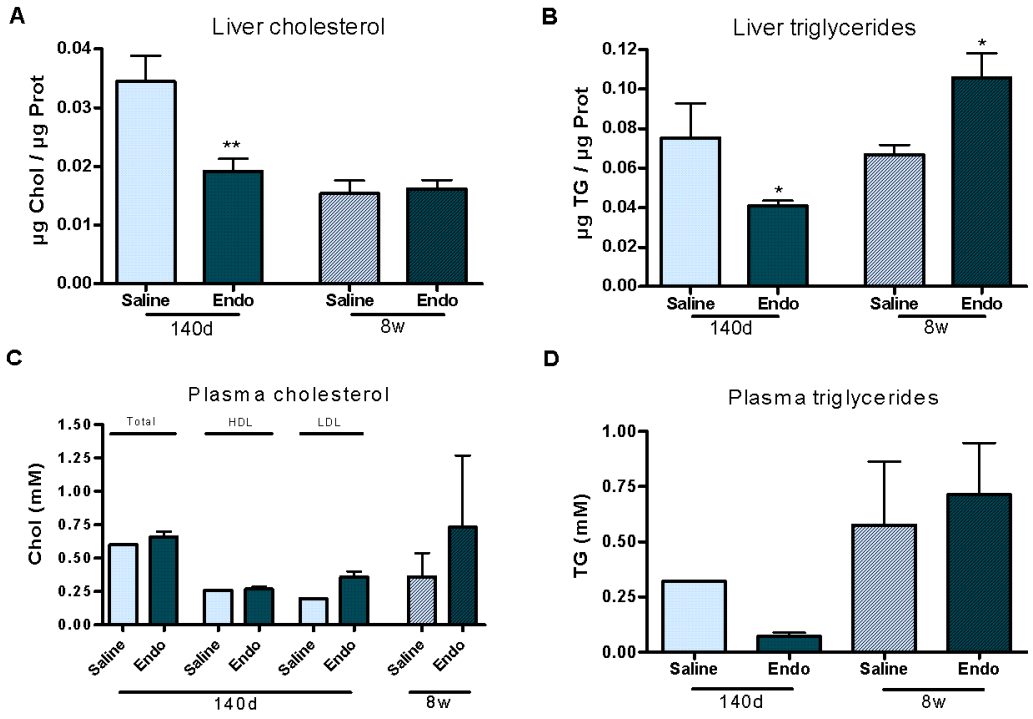


Figure 14. Liver and plasma lipid levels of saline and endotoxin (endo) exposed animals at GA 140d and postnatal age eight weeks. (A-B) Liver cholesterol (Chol) and triglyceride (TG) levels. Hepatic lipid levels are corrected for their protein (Prot) content. **(C)** Plasma total Chol, HDL and LDL levels for animals with GA 140 days. Only total chol levels are represented for animals with postnatal age eight weeks. **(D)** Plasma TG levels. * Significant different from saline injected group of same GA.

4. DISCUSSION

In the current study we have demonstrated for the first time that fetuses exposed to endotoxin-induced chorioamnionitis are sensitized to develop liver damage. These fetuses developed an inflammatory response and increased plasma lipid levels which lead to hepatocellular damage.

Remarkably, in postnatal life the level of hepatic triglycerides was increased, suggesting that the systemic inflammatory response evoked by chorioamnionitis has still harmful effects on the postnatal liver.

Preterm delivery, a major problem in reproductive medicine [1], is associated with chorioamnionitis-induced FIRS [6, 9]. Currently, the mechanisms whereby chorioamnionitis induces FIRS are poorly understood. So far, most studies in the field of FIRS investigate the effects on lungs and brain [11]. Although the liver is a very important organ, no information on the involvement of the fetal liver in chorioamnionitis-induced FIRS is available.

The fetal liver is involved in the response to different injuries and helps to protect the lungs from acute injury during endotoxemia [34]. In addition, it is the major hematopoietic site during fetal life [35]. Although haematopoiesis occurs in the bone marrow instead of in the liver during postnatal life [36], the postnatal liver is still a central organ in an organism's metabolism [37]. The liver has indispensable functions in lipid metabolism [32]. The liver also produces bile to aid in the digestion and uptake of lipids from the intestine. In addition, the liver has central roles in carbohydrate homeostasis, protein metabolism, detoxification, the breakdown of hormones and other substances, as well as synthesizing coagulation factors and several important proteins such as several acute phase proteins [38]. Recent data suggest that the liver plays an early causal role in the systemic inflammatory response evoked by different stimuli [23-26].

Fetuses delivered prematurely that were exposed to chronic chorioamnionitis, are at high risk for postnatal morbidities and mortality, with the most common acute morbidities being brain injury and lung injury [39]. Various animal models using intra-amniotic endotoxin or Ureaplasma injection suggest that antenatal inflammation primes the fetal lung leading to exacerbated postnatal lung inflammation [40, 41]. In addition, it has been suggested that exposure to intrauterine infection might be associated with cognitive limitations [40]. However, the effects of chorioamnionitis on the postnatal liver have never been reported.

4.1 Prenatal hepatic involvement in endotoxin-induced chorioamnionitis

Although the liver is one of the most important organs both in fetal and in adult life, most studies in the field of chorioamnionitis are focused on lungs and brain [11]. In these organs, comparable experiments were performed in order to understand the involvement of lungs and brain in FIRS [18, 42].

While the chorioamnionitis-induced inflammatory response in the brain was the most prominent in the animals that received endotoxin two weeks before the delivery [42], our data demonstrate a hepatic inflammatory response already two days after the endotoxin injection. This early inflammatory response suggests that the liver contributes to the acute systemic inflammation evoked by chorioamnionitis. We have previously shown that also the lungs evoked an inflammatory response two days after the endotoxin injections [18]. However, this observation could be explained by the direct contact of the lungs with the inflamed amniotic fluid, which occurs regardless of the systemic inflammation.

Endotoxin-induced chorioamnionitis provoked an acute hepatic inflammatory response, which started by a rapid and robust elevation of both IL-1β and TNF mRNA expression in the liver. IL-1β and TNF are well-known pro-inflammatory cytokines, involved in the early inflammatory response and in the evolution of the inflammatory events [29]. TNF is one of the major activators of nuclear factor kappa B (NFκB), a transcription factor that plays a crucial role in inflammation and regulates the production of TNF and multiple other cytokines [43]. By promoting migration and adhesion, TNF could contribute to the increased infiltration of inflammatory cells observed two weeks after the endotoxin injections. In addition, both TNF and IL-1β have been shown to play an important role in systemic inflammation [44] and thus the elevation in expression of these cytokines in the liver support an evidence for the involvement of the liver in FIRS development.

Despite the reduction in expression of the pro-inflammatory cytokines two weeks after endotoxin exposure, we have observed an increased inflammatory cell influx on haematoxylin/eosin (H/E) and CD3-T-lymphocyte staining. It seems that the fetal liver sets off a cascade of pro-inflammatory events, leading to the initiation of the inflammatory response with a recruitment of inflammatory cells. In addition, a wide variety of cytokines and chemokines may play roles in the inflammatory response. Factors other than the limited cytokines that we measured may be involved [45].

The presence of inflammation was also seen on the MPO-stained sections. Myeloperoxidase (MPO) is an important enzyme that is stored in azurophilic granules of polymorphonuclear neutrophils and is therefore used as a neutrophil marker [46]. Hepatic infiltration of polymorphonuclear neutrophils is an early response to systemic inflammation [47] and was already seen two days after endotoxin exposure. In line with this observation, it has been suggested that MPO is involved in acute inflammation [46]. However, the increase in MPO-positive cells was not observed by the H/E staining. A possible explanation could be attributed to the substantial amount of haematopoietic cells present in the fetal liver. Consequently, it is more difficult to make a distinction between the haematopoietic cells and the inflammatory cells on the H/E staining.

The elevation in MPO-positive cells was also seen two weeks after endotoxin-induced chorioamnionitis. This observation is in line with an increased infiltration of the CD3-positive T-lymphocytes. Although MPO is especially involved in the acute inflammatory response [46], MPO has also been found in monocytes and certain macrophages [46], indicating a chronic role of MPO. In addition, MPO catalyzes the conversion of chloride and hydrogen peroxide to hypochlorite [46]. These toxic agents can be released from the cell during inflammatory conditions, where they may attack normal tissue and thus contribute to acute liver injury and hepatic fibrogenesis [28, 48]. The resulting liver damage promotes expression and secretion of CXC chemokines, which attract additional MPO-positive cells [47] creating a vicious circle. It is known that MPO plays a role in oxidative stress as such the liver may sustain the condition of FIRS.

Oxidative damage to tissue proteins has been implicated in the pathogenesis of liver disease [28] and has been shown to be present two days as well as two weeks after endotoxin-induced chorioamnionitis by the immunohistochemical staining against MPO. Another indication for the increased oxidative stress in the livers of these fetuses was the decreased expression of glutathione S-transferase two days after the endotoxin injections. Glutathione S-transferase is an enzymatic anti-oxidant that is released by neutrophils and macrophages at sites of inflammation and its activity protects cells against oxidative stress [49, 50]. The level of this anti-oxidant might provide a clear indication on the extent of cytotoxic damage that occurs in the liver [51]. The early downregulation in the expression of glutathione S-transferase suggests that the fetus has difficulties in responding to oxidative stress. Indeed, defense mechanisms against reactive oxygen species in the fetus are significantly different from these in adults: levels of anti-oxidants are lower in the fetal liver than in the adult liver. Consequently,

preterm infants are particularly susceptible to the damage caused by reactive oxygen species since the anti-oxidant system has yet to mature. Oxidative stress can overwhelm the anti-oxidant defense mechanisms and perturb the structure and functions of the fetal liver [49, 50].
Hepatic dysfunction is followed by the elevated levels of serum enzymes indicating cellular leakage and loss of functional integrity of hepatic membranes [51]. Indeed, the decrease in anti-oxidant capacity in the liver of these fetuses was associated with liver damage. Both plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were increased two weeks after fetal exposure to endotoxin, indicating the presence of liver injury in these fetal sheep. Changes in activities of these enzymes are considered as a tool to study liver damage, varying cell viability and cell membrane permeability [51]. However, no excessive liver necrosis was seen on the H/E stained liver sections, indicating a protective role of the haematopoietic cells in the fetal liver [52].

Another interesting factor associated with hepatic inflammation is an increase in plasma cholesterol [53, 54]. Indeed, a similar peak in cholesterol levels and inflammatory response could be observed two days after exposure to endotoxin. It is not clear why the rise in cholesterol levels did not persist two weeks after the endotoxin injections, since cholesterol has been associated with both acute and chronic inflammation [55]. Therefore, the observed effect could be attributed to the capability of the fetus to modulate and downregulate the increase in cholesterol levels [14, 15]. At present, the exact mechanisms by which cholesterol evokes liver inflammation are largely unknown.

4.2 Postnatal hepatic involvement in endotoxin-induced chorioamnionitis

Based on the adverse effects of endotoxin-induced chorioamnionitis on the liver during prenatal life, we hypothesized that endotoxin-induced chorioamnionitis can also affect liver function during postnatal life.

Although the prenatal liver was significantly inflamed by endotoxin-induced chorioamnionitis, the only hepatic postnatal effect on inflammation could be seen on the CD3-T-lymphocyte-staining in the animals that were sacrificed near term. As these animals received endotoxin 30 days before the delivery, this might be an indication for chronic inflammation. However, other markers for inflammation such as the mRNA expression of pro-inflammatory cytokines were not increased in this animal group. This is in line with the observations in the prenatal study where an elevation of IL-1β and TNF mRNA expression

could also not be observed two weeks after endotoxin-induced chorioamnionitis. A plausible explanation could be that these cytokines are not playing an important role in chronic inflammation since they mediate particularly the early inflammatory response [29].

In addition, also no effect on inflammation could be observed concerning the H/E- and MPO-staining. Although it has been suggested that MPO is involved in acute inflammation [46], MPO has also been reported to have a chronic role [46]. This chronic role of MPO was seen in the gut, where a massive influx of MPO-positive cells has been observed 30 days after intra-amniotic endotoxin injection [56]. In addition, antenatal exposure to endotoxin caused also increased postnatal lung MPO levels [18, 40]. Since MPO plays a postnatal role in other tissues after endotoxin-induced chorioamnionitis, it is likely that the postnatal liver can mitigate the inflammatory response.

In line with the observations about the MPO-staining, no effect on mRNA glutathione S-transferase levels was seen in the postnatal livers. Consequently, no liver injury [28, 48] could be observed, neither by liver necrosis on the H/E stained liver sections nor by plasma ALT and AST levels.

The mechanisms involved in this postnatal inflammatory process may include alterations of the innate immune system [40]. It has been shown that the inflammatory response fully develops during the first weeks of postnatal life [57, 58, 59]. Based on these findings, it is likely that during prenatal life the hepatic inflammatory response was not developed yet to prevent the liver damage. However, in the postnatal liver the immune system is maturing so that the liver can handle with the pro-inflammatory stimuli to clear the inflammation.

In line with these observations, there was an increase in apoptotic cells after endotoxin-induced chorioamnionitis in the 140 days old animals. The increase in apoptotic cell death can be an explanation for the absence of necrosis and cell damage. After the initiation of inflammation by chorioamnionitis, neutrophils are recruited into the liver from the blood circulation. Subsequently, neutrophils undergo apoptotic cell death. The apoptotic vesicles are readily phagocytosed by neighbouring cells, monocytes and macrophages. If apoptotic vesicles are not taken up in a specific time range, they become necrotic. So the vesicles will burst open and spill their contents, which is a pro-inflammatory stimulus on his own leading to the reinitiation of the inflammatory cascade [19].

In the present study we did not study the systemic inflammatory changes as measured by serum cytokine and white blood cell counts. This could give additional information about the involvement of the liver in the response to chorioamnionitis. Further studies looking at

additional parameters of liver development and maturation will also be necessary to address the potential synergy between inflammation and maturation in the perinatal period.

Irrespective of the resolved inflammation in the postnatal liver, the most interesting observation was the increase in hepatic triglycerides eight weeks after delivery. Several studies already indicated that pro-inflammatory cytokines such as TNF, IL-12 and IL-18 induce hepatic steatosis [60, 61]. Consequently, it is likely that the prenatal inflammation is the trigger to develop steatosis during postnatal life. This observation needs further investigation since hepatic lipid accumulation can result in the development of hepatic inflammation and insulin resistance [62, 63, 27].

5. SUMMARY

In summary, the present study demonstrates for the first time that both the prenatal and postnatal liver are involved in the response to chorioamnionitis.

Already two days after the endotoxin injection, the number of hepatic myeloperoxidase (MPO)-positive cells was increased and prenatal hepatic mRNA levels of pro-inflammatory genes interleukin (IL)-1ß and tumor necrosis factor (TNF) were significantly higher compared to controls. The inflammatory response was sustained two weeks after the endotoxin injections and the levels of markers for liver damage (ALT and AST) were significantly elevated. As the hepatic damage appears to be resolved near term delivery, it seems that the neonate can recover from the initial hit by the absence of further aggravation of the existing inflammation. However, there is a significant increase in hepatic triglycerides at postnatal age after endotoxin-induced chorioamnionitis. These results indicate that the prenatal hepatic damage caused by the inflammatory response to chorioamnionitis, also has his consequences in postnatal life.

At present, preterm birth is associated with neonatal morbidity and mortality and therefore, it should be essential to monitor hepatic function and lipid levels in preterm babies.

Future research is needed to clearly explore the mechanisms by which chorioamnionitis induces liver damage in preterm delivered babies. Additional studies can further define factors in the immunologic response that mitigate host colonization, and to broaden the evaluation of other inflammatory mediators/processes. The details of the modulation of liver injury and maturation by pro-inflammation have not yet been identified, and support the need for further research in this area.

Studies of the effects of chorioamnionitis on lipid metabolism are important future goals. An interesting follow-up study would be to study the livers at later postnatal time points to investigate the effects on lipid metabolism in more detail. Moreover, reoccurrence of an inflammatory response, resulting in liver damage can not be excluded on the long run since the rise in triglyceride levels can be a pro-inflammatory trigger [25, 26]. Here, the focus should be on the synthesis and degradation of lipid genes that are important in lipid metabolism. Also, the impact on mitochondrial DNA can be studied since the mitochondria are important modulators in energy conversion. Several studies already showed that prematurity influences the functionality of the mitochondria [7, 64], indicating a plausible role of the mitochondria in the disturbed lipid metabolism.

6. REFERENCES

1. Behrman, R. & Butler, A. Preterm birth: causes, consequences, and prevention (The National Academies Press, Washington, D.C., 2007).
2. Faye-Petersen, O. M. The placenta in preterm birth. J Clin Pathol 61, 1261-75 (2008).
3. Kramer, B. W. et al. Decreased expression of angiogenic factors in placentas with chorioamnionitis after preterm birth. Pediatr Res 58, 607-12 (2005).
4. Romero, R., Gotsch, F., Pineles, B. & Kusanovic, J. P. Inflammation in pregnancy: its roles in reproductive physiology, obstetrical complications, and fetal injury. Nutr Rev 65, S194-202 (2007).
5. Bibby, E. & Stewart, A. The epidemiology of preterm birth. Neuro Endocrinol Lett 25 Suppl 1, 43-7 (2004).
6. Goldenberg, R. L., Hauth, J. C. & Andrews, W. W. Intrauterine infection and preterm delivery. N Engl J Med 342, 1500-7 (2000).
7. Velez, D. R. et al. Mitochondrial DNA variant A4917G, smoking and spontaneous preterm birth. Mitochondrion 8, 130-5 (2008).
8. Steel, J. H. et al. Bacteria and inflammatory cells in fetal membranes do not always cause preterm labor. Pediatr Res 57, 404-11 (2005).
9. Romero, R., Espinoza, J., Chaiworapongsa, T. & Kalache, K. Infection and prematurity and the role of preventive strategies. Semin Neonatol 7, 259-74 (2002).
10. Fahey, J. O. Clinical management of intra-amniotic infection and chorioamnionitis: a review of the literature. J Midwifery Womens Health 53, 227-35 (2008).
11. Gotsch, F. et al. The fetal inflammatory response syndrome. Clin Obstet Gynecol 50, 652-83 (2007).
12. Romero, R. et al. The role of inflammation and infection in preterm birth. Semin Reprod Med 25, 21-39 (2007).
13. Romero, R. et al. Inflammation in preterm and term labour and delivery. Semin Fetal Neonatal Med 11, 317-26 (2006).
14. Duncan, J. R. et al. White matter injury after repeated endotoxin exposure in the preterm ovine fetus. Pediatr Res 52, 941-9 (2002).
15. Kramer, B. W. & Jobe, A. H. The clever fetus: responding to inflammation to minimize lung injury. Biol Neonate 88, 202-7 (2005).
16. Elovitz, M. A. & Mrinalini, C. Animal models of preterm birth. Trends Endocrinol Metab 15, 479-87 (2004).
17. Pringle, K. C. Human fetal lung development and related animal models. Clin Obstet Gynecol 29, 502-13 (1986).
18. Kramer, B. W. et al. Dose and time response after intraamniotic endotoxin in preterm lambs. Am J Respir Crit Care Med 164, 982-8 (2001).
19. Newnham, J. P. et al. Betamethasone effects on chorioamnionitis induced by intra-amniotic endotoxin in sheep. Am J Obstet Gynecol 189, 1458-66 (2003).
20. Kramer, B. W. in Unpublished data 7-131 (Kindergeneeskunde, Maastricht, The Netherlands, 2007).
21. Li, X. Y. et al. Maternally administered lipopolysaccharide (LPS) upregulates the expression of heme oxygenase-1 in fetal liver: The role of reactive oxygen species. Toxicol Lett 176, 169-77 (2008).

22. Garnier, Y. et al. Proliferative responses in the placenta after endotoxin exposure in preterm fetal sheep. Eur J Obstet Gynecol Reprod Biol (2007).
23. Kleemann, R. & Kooistra, T. HMG-CoA reductase inhibitors: effects on chronic subacute inflammation and onset of atherosclerosis induced by dietary cholesterol. Curr Drug Targets Cardiovasc Haematol Disord 5, 441-53 (2005).
24. Kmiec, Z. Cooperation of liver cells in health and disease. Adv Anat Embryol Cell Biol 161, III-XIII, 1-151 (2001).
25. Matsuzawa, N. et al. Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. Hepatology 46, 1392-403 (2007).
26. Hotamisligil, G. S. Inflammation and metabolic disorders. Nature 444, 860-7 (2006).
27. Shiri-Sverdlov, R. et al. Early diet-induced non-alcoholic steatohepatitis in APOE2 knock-in mice and its prevention by fibrates. J Hepatol 44, 732-41 (2006).
28. Klebanoff, S. J. Myeloperoxidase: friend and foe. J Leukoc Biol 77, 598-625 (2005).
29. Caille, V., Bossi, P., Grimaldi, D. & Vieillard-Baro, A. [Physiopathology of severe sepsis]. Presse Med 33, 256-61; discussion 269 (2004).
30. Kramer, B. W., Kramer, S., Ikegami, M. & Jobe, A. H. Injury, inflammation, and remodeling in fetal sheep lung after intra-amniotic endotoxin. Am J Physiol Lung Cell Mol Physiol 283, L452-9 (2002).
31. Ozer, J., Ratner, M., Shaw, M., Bailey, W. & Schomaker, S. The current state of serum biomarkers of hepatotoxicity. Toxicology 245, 194-205 (2008).
32. Neville, M. C. Adaptation of maternal lipid flux to pregnancy: research needs. Eur J Clin Nutr 53 Suppl 1, S120-3 (1999).
33. Tilg, H. & Hotamisligil, G. S. Nonalcoholic fatty liver disease: Cytokine-adipokine interplay and regulation of insulin resistance. Gastroenterology 131, 934-45 (2006).
34. Sell, S. Heterogeneity and plasticity of hepatocyte lineage cells. Hepatology 33, 738-50 (2001).
35. Bendon, R. W. & Coventry, S. Non-iatrogenic pathology of the preterm infant. Semin Neonatol 9, 281-7 (2004).
36. Huang, X., Cho, S. & Spangrude, G. J. Hematopoietic stem cells: generation and self-renewal. Cell Death Differ 14, 1851-9 (2007).
37. Spear, B. T., Jin, L., Ramasamy, S. & Dobierzewska, A. Transcriptional control in the mammalian liver: liver development, perinatal repression, and zonal gene regulation. Cell Mol Life Sci 63, 2922-38 (2006).
38. Stevens, A. & Lowe, J. Histologie van de mens (Bohn Stafleu Van Loghum, Houten/Diegem, 1997).
39. Jobe, A. H. & Ikegami, M. Antenatal infection/inflammation and postnatal lung maturation and injury. Respir Res 2, 27-32 (2001).
40. Normann, E. et al. A novel mouse model of Ureaplasma-induced perinatal inflammation: effects on lung and brain injury. Pediatr Res 65, 430-6 (2009).
41. Yoder, B. A. et al. Effects of antenatal colonization with ureaplasma urealyticum on pulmonary disease in the immature baboon. Pediatr Res 54, 797-807 (2003).
42. Gavilanes, A. W. et al. Chorioamnionitis induced by intraamniotic lipopolysaccharide resulted in an interval-dependent increase in central nervous system injury in the fetal sheep. Am J Obstet Gynecol (2009).
43. Tacke, F., Luedde, T. & Trautwein, C. Inflammatory pathways in liver homeostasis and liver injury. Clin Rev Allergy Immunol 36, 4-12 (2009).

44. Hildebrand, F., Pape, H. C. & Krettek, C. [The importance of cytokines in the posttraumatic inflammatory reaction]. Unfallchirurg 108, 793-4, 796-803 (2005).
45. Yoon, B. H. et al. Clinical implications of detection of Ureaplasma urealyticum in the amniotic cavity with the polymerase chain reaction. Am J Obstet Gynecol 183, 1130-7 (2000).
46. Loria, V., Dato, I., Graziani, F. & Biasucci, L. M. Myeloperoxidase: a new biomarker of inflammation in ischemic heart disease and acute coronary syndromes. Mediators Inflamm 2008, 135625 (2008).
47. Jaeschke, H. & Hasegawa, T. Role of neutrophils in acute inflammatory liver injury. Liver Int 26, 912-9 (2006).
48. Brown, K. E., Brunt, E. M. & Heinecke, J. W. Immunohistochemical detection of myeloperoxidase and its oxidation products in Kupffer cells of human liver. Am J Pathol 159, 2081-8 (2001).
49. Saugstad, O. D. Oxidative stress in the newborn--a 30-year perspective. Biol Neonate 88, 228-36 (2005).
50. Sato, E. F. et al. Oxidative stress promotes the regression of fetal liver hemopoiesis. Biochemistry (Mosc) 69, 18-22, 1 p following 74 (2004).
51. Pari, L. & Prasath, A. Efficacy of caffeic acid in preventing nickel induced oxidative damage in liver of rats. Chem Biol Interact 173, 77-83 (2008).
52. Kunisaki, S. M. et al. Fetal hepatic haematopoiesis is modulated by arterial blood flow to the liver. Br J Haematol 134, 330-2 (2006).
53. Wouters, K. et al. Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. Hepatology 48, 474-86 (2008).
54. Mari, M. et al. Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. Cell Metab 4, 185-98 (2006).
55. Chait, A., Han, C. Y., Oram, J. F. & Heinecke, J. W. Thematic review series: The immune system and atherogenesis. Lipoprotein-associated inflammatory proteins: markers or mediators of cardiovascular disease? J Lipid Res 46, 389-403 (2005).
56. Wolfs, T. G. et al. Endotoxin induced chorioamnionitis prevents intestinal development during gestation in fetal sheep. PLoS One 4, e5837 (2009).
57. Schwarzenberg, S. J., Potter, C. J. & Berry, S. A. Developmental regulation of the hepatic acute phase response. Am J Physiol 261, C461-6 (1991).
58. Patricolo, M. et al. [Hepatic resection in the fetal rabbit. Histologic comparison of tissue regeneration in the fetus versus the adult]. Minerva Chir 51, 971-7 (1996).
59. Patricolo, M. et al. In utero partial liver resection in the rabbit model: a study on fetal tissue regeneration. Fetal Diagn Ther 12, 232-5 (1997).
60. Mundt, B. et al. Tumour necrosis factor related apoptosis inducing ligand (TRAIL) induces hepatic steatosis in viral hepatitis and after alcohol intake. Gut 54, 1590-6 (2005).
61. Chikano, S. et al. IL-18 and IL-12 induce intestinal inflammation and fatty liver in mice in an IFN-gamma dependent manner. Gut 47, 779-86 (2000).
62. Ruvolo, P. P. Intracellular signal transduction pathways activated by ceramide and its metabolites. Pharmacol Res 47, 383-92 (2003).
63. Browning, J. D. & Horton, J. D. Molecular mediators of hepatic steatosis and liver injury. J Clin Invest 114, 147-52 (2004).
64. Wenchich, L. et al. Mitochondrial energy metabolism in very premature neonates. Biol Neonate 81, 229-35 (2002).

APPENDIX 1

1.1 Protocol: CD3-staining on paraffin sections

A. Deparaffineren

1. Xylol 2 x 3 min
2. EtOH 100% 2 x 3 min
3. EtOH 96% 2 x 3 min
4. EtOH 70% 1 x 3 min
5. Spoelen met water

B. Blokkeren endogene peroxidase-activiteit

1. Plaats de glaasjes gedurende 20 min. in 0,3% H_2O_2 in 1x PBS (2 ml H_2O_2 30% in 198 ml 1x PBS).
2. Was 2x 5 min. met 1x PBS.

C. Antigen Retrieval

1. Vul een Coplin jar met Citraat buffer pH 6.0 (DAKO Real Target Retrieval Solution 10x S2031, 180 ml Milli-Q + 20 ml retrieval solution).
2. Breng de vloeistof aan de kook in de magnetron (2,5 minuut stand III).
3. Plaats de coupes in de Coplin jar en laat gedurende 10 minuten uit de magnetron staan.
4. Kook de Coplin jar weer 5 minuten in de magnetron (stand II) → Zonder de coupes!.
5. Laat de coupes 20 min. afkoelen in de citraat buffer.
6. Was 3x 3 min. met 1x PBS.

D. Blokkeren aspecifieke binding

1. Omcirkel de coupes met de DAKO pen (DAKO S 200230-2: om te zorgen dat de vloeistoffen op de coupe blijven zitten).
2. Incubeer de objectglaasjes gedurende 30 minuten in 5% BSA/1x PBS bij kamertemperatuur in een vochtige doos (200-400 µl per coupe)
3. Laat de vloeistof van de objectglaasjes afdruipen.

E. Binding 1^{e} antilichaam

1. Verdun het primaire antilichaam (polyclonal rabbit anti-human CD3, DAKO A0452) 1:200 in 0,1%BSA/PBS. Denk aan de negatieve controle (alleen 0,1%BSA/PBS)!
2. Breng 200 µl op het weefselfragment.

3. Plaats de objectglaasjes horizontaal in een afgesloten plastic bak met vochtig tissue en incubeer overnacht bij 4°C.
4. Was de preparaten 3 x 5 min met 1x PBS.

F. Binding 2e antilichaam

1. Verdun het secundaire antilichaam (polyclonal swine anti-rabbit immunoglobulins*Biotin, DAKO E0353) 1:200 in 0,1%BSA/PBS, wederom 200 µl per coupe (nu inclusief negatieve controles).
2. Incubeer gedurende 1 uur in een afgesloten plastic back met vochtig tissue in een donkere ruimte op kamertemperatuur. (Maak ondertussen AB-oplossing)
3. Was de preparaten 3 x 5 min met 1x PBS.

G. ABC-complex

1. Gebruik StrepABComplex/HRP (DAKO K0377)
2. Meng A bij 0,1% BSA/PBS (1:500) en vortex.
3. Meng B bij bovenstaande oplossing (1:500) en vortex.
4. Maak de oplossing 30 minuten voor gebruik!
5. Breng 200 µl per coupe aan en incubeer 30 min. bij kamertemperatuur.
6. Was 3x 5 min. met 1x PBS.

H. Kleuren met AEC en tegenkleuring

1. Kleur gedurende 15 min. met AEC: 2% buffer/ 3% AEC/ 2% H_2O_2 in demi water
2. Was 3x5 min. met 1xPBS.
3. Wassen met kraanwater.
4. Tegenkleuren met Haematoxylin (10 sec.).
5. Wassen onder stromend water gedurende 5 min.
6. Wassen met demi water.
7. Plak een dekglaasje op de coupes met Faramount

1.2 Protocol: CD3-staining on frozen sections

Dry coupes at least overnight in a dry box at room temperature before staining!!!!!!

1) Fixate coupes for 10' in 'dry' Acetone.
2) Dry slides under a ventilator for 15'.
3) Incubate slides for 5' in 1xPBS.
4) Block with H_2O_2 (V1:1000 in 1xPBS) for 5'.
5) Wash 2 times with 1xPBS.
6) Encircle samples with DAKO-pen.
7) Incubate for 30' in 5%BSA/ 1xPBS + **Avidin D Block solution V1:5**.
8) Incubate for 60' in 1%BSA/ 1xPBS + 1st antibody (polyclonal rabbit anti-human CD3, DAKO A0452) 1:200 in 0,1%BSA/PBS + **Biotin Block solution V1:5**.
9) Wash 3 times with 1xPBS.
10) Incubate for 60' in 1%BSA/1xPBS + 2nd antibody (polyclonal swine anti-rabbit immunoglobulins*Biotin, DAKO E0353) 1:200 in 0,1%BSA/PBS
    (Incubate for 5' before adding to the section!)
    (This step is to block aspecific binding of the secondary AB to mouse fragments.)
    (Meanwhile: 1xPBS + 1:50 Avidin D solution + 1:50 Biotin solution should be made)
11) Wash 3 times with 1xPBS.
12) **Incubate for 30' in 1xPBS + 1:50 Avidin D solution + 1:50 Biotin solution.**
13) **Wash 3 times with 1xPBS.**
14) Stain for 15' with AEC: 2% buffer/ 3% AEC/ 2% H_2O_2 in demi water.
15) Wash with 1xPBS.
16) Wash with tab water.
17) Stain a few seconds with Haematoxylin.
18) Rinse with running tab water for 5'.
19) Wash with demi water.
20) Mount the slide with Faramount.

APPENDIX 2

2.1 Protocol: MPO-staining on paraffin sections

1. Deparaffination – old xylol 5 min, fresh xylol 5 min	
2. Rehydration - fresh 100% alcohol 3 min, old 100% alcohol 3 min, 96% alcohol 3 min, 96% alcohol 3 min, 70% alcohol 3 min, demi-water 3 min	
3. Block endogenous peroxidases – 0,6% H_2O_2 in methanol	30 min RT
4. Rinse with demi water	5 min RT
5. Rinse with 1x PBS	3 x 3 min
6. Dry slide and encircle section with DAKO-Cytomation pen	
7. Pre-incubation with normal swine serum **1:10**	10 min RT
8. Incubate with anti-MPO **1:500**	60 min RT
9. Rinse with 1x PBS	3 x 3 min
10. Incubate with Swine-anti-Rabbit Biotin **1:500**	60 min RT
11. MEANWHILE prepare Strep-AB/HRP complex	
✓ Take 1 ml of PBS	
✓ Add 9 µl of reagent A	
✓ Add 9 µl of reagent B	
✓ Mix well by vortexing; **leave for at least 30 min** at 4°C	
12. Rinse with PBS	3 x 3 min
13. Incubate with Strep-AB/HRP complex	60 min RT
14. Rinse with PBS	3 x 3 min
15. Incubate with AEC	~10 min
✓ 2% buffer/	
✓ 3% AEC	
✓ 2% H_2O_2	
✓ demi water	
16. Rinse with 1x PBS	3 x 5 min
17. Haematoxylin staining; 10 sec (liver) to 1 min (fat)	
18. Rinse with **tap water**	
19. Seal with Faramount aqueous mounting medium	

2.2 Protocol: MPO-staining on frozen sections

Dry coupes at least overnight in a dry box at room temperature before staining!!!!!!

1) Fixate coupes for 10' in 'dry' Acetone.
2) Dry slides under a ventilator for 15'.
3) Incubate slides for 5' in 1xPBS.
4) Block with H_2O_2 (V1:1000 in 1xPBS) for 5'.
5) Wash 2 times with 1xPBS.
6) Encircle samples with DAKO-pen.
7) Incubate for 30' in 0,1%BSA/ 1xPBS + V1:10 NSS **+ Avidin D Block solution V1:5**.
8) Incubate for 60' in 0,1%BSA/ 1xPBS + 1st antibody (anti-MPO **1:500) + Biotin Block solution V1:5**.
9) Wash 3 times with 1xPBS.
10) Incubate for 60' in 1%BSA/1xPBS + 2nd antibody (polyclonal swine anti-rabbit immunoglobulins*Biotin, DAKO E0353) 1:500 in 0,1%BSA/PBS
    (Incubate for 5' before adding to the section!)
    (This step is to block aspecific binding of the secondary AB to mouse fragments.)
    (Meanwhile: 1xPBS + 1:50 Avidin D solution + 1:50 Biotin solution should be made)
11) Wash 3 times with 1xPBS.
12) **Incubate for 30' in 1xPBS + 1:50 Avidin D solution + 1:50 Biotin solution.**
13) **Wash 3 times with 1xPBS.**
14) Stain for 10' with AEC: 2% buffer/ 3% AEC/ 2% H_2O_2 in demi water.
15) Wash with 1xPBS.
16) Wash with tab water.
17) Stain a few seconds with Haematoxylin.
18) Rinse with running tab water for 5'.
19) Wash with demi water.
20) Mount the slide with Faramount.

APPENDIX 3

3.1 Protocol: Haematoxylin/Eosin-staining on paraffin sections

Deparaffinate:

1. 2 x 5min. in xylol
2. 2 x 3min. in 100% ethanol
3. 1 x 2min. in 96% ethanol
4. 1 x 2min. in 70% ethanol
5. 1 x 2min. in 50% ethanol
6. Rinse in demiwater

Staining:

1. 5min. in haematoxylin solution
2. Rinse in tap water (blue)
3. 3min. in eosin solution
4. Rinse in tap water (blue)

Dehydrate:

1. Dip 3 times in 70% ethanol
2. Dip 3 times in 96% ethanol
3. Dip 3 times in 96% ethanol
4. Dip 3 times in 100% ethanol
5. Dip 3 times in 100% ethanol
6. 2 x 2min. in xylol

Include:

1. Coverslip using Entellan

3.2 Protocol: Haematoxylin/Eosin-staining on frozen sections

1. Airdry

2. Staining:
    1. 2 min. in haematoxylin solution
    2. Rinse in tap water (blue) for 10 minutes
    3. 10-30 sec. in eosin solution
    4. Rinse in tap water (blue)
    5. Wash in demi water

3. Dehydrate:
    1. Dip 3 times in 50% ethanol
    2. Dip 3 times in 70% ethanol
    3. Dip 3 times in 96% ethanol
    4. Dip 3 times in 100% ethanol

4. Include: Coverslip using Entellan

APPENDIX 4

Protocol: Oil Red O staining

- 1h fixation of air dried thawed cryo-sections using 3.7% formaldehyde solution in deionised water.
- 3 rinses in deionised water for 30s to remove excess of formaldehyde.
- Sections for 30 minutes in working solution oil red O.
- Washing with deionised water 3 times 30s.
- If necessary counterstaining with Mayer's haematoxylin for 60s.
- Washing with running tab water for 10 minutes and covered with coverslip using 10% glycerol in PBS.

Stock solution Oil red O:

- 500mg Oil red O dissolved in 100ml 60% triethyl-phosphate.

Working solution oil red O:

- Fresh prepared before staining: 12 ml stock solution plus 8 ml deionised water and filtered to remove crystallized Oil red O.

APPENDIX 5

Protocol: TUNEL staining

Department of Molecular Genetics — Maastricht University

In Situ Cell Death Detection Kit, POD (TUNEL technology)

Material:

- *In Situ* Cell Death Detection Kit (Roche)
- Fixation solution–parafix
- 1xPBS
- Blocking solution: 3% H_2O_2 in methanol
- Permeabilisation solution: 0.1% Triton X-100, 0.1% sodium citrate at 4°C (freshly prepared)
- DNase I (1000 U/ml: Fermentas)
- *Ethanol (100%)*
- *Acetone*
- Coverlips
- Humidified chamber (with lid) at 37°C
- Vector *NovaRED* Substrate Kit for Peroxidase (Vector Laboratories: catalog nr: SK-4800)
- 95% ethanol (freshly prepared)
- 100% ethanol (fresh)
- Xylene

Method:

Pretreatment of cryopersrved tissue

1. Take slides out of the -20°C and let them dry for 20 minutes under the ventilator.
2. Fix tissue sections with **fixation solution** for 20 minutes at RT. (Fix the slides lying with plenty of fixation solution; after 10 minutes of fixation add some additional fixation solution).
3. Rinse slides shortly in PBS to get rid of the fixation solution.
4. Wash the slides for 30 minutes in new PBS.
5. Incubate with **blocking solution** for 10 minutes at RT.
6. Rinse slides for 1 minute with PBS.
7. Incubate slides in Permeabilisation solution for 2 minutes on ice.
8. Rinse slides twice for 1 minute with PBS (Let slides in PBS while you perform a DNase treatment of 2 slides to take along as a positive and negative control).

A negative and positive control should be included in each experimental set up.

9. For a **positive** control and a **negative control**: incubate fixed and permeabilized cells with DNase I (3U/ml in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mg/ml BSA) for 15 minutes at RT to induce DNA strand breaks.
10. Wash slides twice for 1 minute in PBS (NOT in the same slide holder as where the untreated slides are in).

Labeling protocol

The TUNEL reaction mixture should be prepared immediately before use and should not be stored. For the **negative control**; incubate fixed, permeabilized and DNase treated cells in 50µl/slide **TUNEL-Label** solution (without terminal transferase) instead of TUNEL reaction mixture.

Note:

Keep TUNEL reaction mixture on ice until use.

11. Dilute TUNEL Enzyme 3x in TUNEL Dilution Buffer (5 µl TUNEL Enzyme + 10 µl TUNEL Dilution Buffer).
    Add for 1 test: 5 µl of **TUNEL Enzyme** solution to 45 µl **TUNEL Label** solution to obtain 50 µl TUNEL reaction mixture. Mix well to equilibrate components.

TUNEL POD zonder aceton fixatie — - 1 - — Last modified 02-03-2009 Name

12. Take slides one by one out of the PBS and treat them as follows.
13. Dry area around samples. (Do not let the slide dry out).
14. Add 50 µl **TUNEL reaction mixture** on each slide. *Note:* For the negative control add 50 µl Label solution.
    To ensure a homogeneous spread of TUNEL reaction mixture across the slide and to avoid evaporative loss, samples should be covered with a coverslip during incubation. (In case you have a large amount of slides, split them up in 2 or 3 parts; if you have handled about 8 slides (takes about 15 minutes) put them in the humidified chamber for incubation and then go on with the next 8 slides etc).
15. Add lid and incubate 60 minutes at 37°C in a humidified chamber in the dark.
16. Rinse slides 3 times for 1 minute in PBS. For the first washing step: place slide together with the coverslip in the PBS. The coverslip will detach from the slide in the PBS (check this).

Samples can be analysed in a drop of PBS under a fluorescence microscope at this state. Use an excitation wavelength in the range of 450-500 nm and detection in the range of 515-565 nm (green).

Signal conversion (Optional)
Using TUNEL POD the fluorescent label may be converted into a colorimetric signal. Thus, the samples may be analyzed by light microscopy. If preparations will be analyzed by light microscopy using TUNEL POD as secondary detection system any precipitating substrate suitable for immunohistochemistry may be used.

17. Dry area around samples.
18. Add 50 µl **TUNEL POD** on each slide.
    Note: To ensure a homogeneous spread of Converter POD across the slide and to avoid evaporative loss, samples should be covered with a coverslip during incubation.
19. Incubate slides in a humidified chamber for 30 minutes at 37°C.
20. Rinse slides 3 times for 1 minute in PBS. For the first washing step: place slide together with the coverslip in the PBS. The coverslip will detach from the slide in the PBS (check this).

Immediately before use on tissue sections, prepare the substrate solution as follows:

- To 5 ml mQ add 5 drops of Reagent 1 and mix well.
- Add 2 drops of Reagent 2 and mix well.
- Add 2 drops of Reagent 3 and mix well.
- Add 2 drops of the Hydrogen Peroxide Solution and mix well.

21. Add 250 µl **Substrate solution** on each slide.
22. Incubate slides for 5-15 minutes at RT. Development times should be determined by the investigator. Longer incubations may increase sensitivity.
23. Wash slides for 5 minutes in demineralized H_2O.
24. Incubate slides for 3 seconds in hematoxilin (diluted 1:5 in Demi H_2O).
25. Wash slides with tab H_2O till there is no colour coming of the slides and then wash slides for an additional 2-3 minutes under running tab H_2O.
26. Wash slides for 1 minute in demineralized H_2O.
27. Place the slides into 2 changes of 85% ethanol for 2 minutes each.
28. Place the slides directly into 2 changes of 100% ethanol for 2 minutes each.
29. Place slides into 2 changes of xylene for about 2 minutes each
30. Enclose the slides in entellan using a coverslip

Solutions

Parafix: 4% paraformaldehyde in PBS, pH 7.4, freshly prepared

Per 100 ml parafix:

Add 4 grams of paraformaldehyde to 80 ml mQ. Warm to 40-50°C and stir. Add approximately 10 drops of 1N NaOH and stir. Add 10 ml 10xPBS and 10 ml 1M HEPES (out of the tissue culture room). Filter the solution over filtrapaper and cool on ice. The pH should be around 7.4. Aliquot the parafix in tubes of 10 ml and put in the -20°C (freezer on the right of the -80°C).

Blocking solution: 3% H_2O_2 in methanol

Add 10 ml H_2O_2 (30%) to 90 ml methanol

Permeabilisation solution: 0.1% Triton X-100, 0.1% sodium citrate, freshly prepared

Add 100 mg sodium citrate and 100 µl Triton and miliQ to a final volume of 100 ml. Stir well to dissolve the Triton and place on ice.

DNase reaction buffer: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mg/ml BSA, 3 U/ml DNaseI

For 1 ml add together:

100 µl 0.5M Tris-HCl, pH 7.4
200 µl 50 mM $MgCl_2$
100 µl BSA (9.8 mg/ml)
3 µl DNase (1 U/µl)
597 µl mQ

APPENDIX 6

Protocol: RNA isolation

Department of Molecular Genetics — Maastricht University

RNA Isolation from tissue, monolayer cells or suspension cells
(by using Tri-Reagent of Sigma)

Material

- Tri Reagent (Sigma)
- Cultured cells
- Chloroform
- Isopropanol
- 70 % Ethanol
- Formamide (preferred: 3x crystallized) or DEPC treated sterilized water
- RNAse Away

Note: Make sure that your materials are Rnase free !! Clean surfaces with RNAseAway and rinse with 70 % EtOH

Method

Sample Preparation

Tissue:
Homogenize tissue sample in Tri Reagent in the minibeadbeater.
- Fill the tube with max 0.8 cm of glass beads.
- Add 1.0 ml Tri Reagent and place the tube on ice.
- Add the frozen tissue (50-100mg per ml Tri Reagent).
- Put the tube in the minibeatbeater and shake for 30 sec. at 5000 rpm.
- Place the tube on ice (repeat previous steps for other samples, before going on).

Monolayer cells:
- Remove the cell medium and wash the cells with 1 ml prewarmed Optimem (37°C).
- Remove the Optimem.
- Lyse cells directly on the culture dish by adding 0.5 ml Tri Reagent per well (3.8 cm^2) of a 12 or 24 wells plate (or 1 ml Tri Reagent per 10 cm^2 of glass culture plate surface area).
- To form a homogeneous lysate pass the cell lysate several times through a pipette.
- Transfer the lysate to a fresh tube.

Suspension cells:
- Isolate the cells by centrifugation (5 minutes; 1100 rpm at RT). Remove the medium.
- Wash the cells with 1 ml prewarmed Optimem (37°C)
- Isolate the cells by centrifugation (5 minutes; 1100 rpm at RT). Remove the Optimem.
- Lyse the cells in Tri Reagent by repeated pipetting. One ml of the reagent is sufficient to lyse 5-10x 10^6 cells.
- Transfer the lysate to a fresh tube.

RNA Isolation
If samples have a high content of fat, protein, polysaccharides or extracellular material such as muscle, fat tissue and tuberous parts of plants an additional step may be needed. After homogenization, centrifuge the homogenate at 12000 *g* for 10 minutes at 4 °C to remove the insoluble material

(extracellular membranes, polysaccharides, and high molecular weight DNA). The supernatant contains RNA and protein. If the sample had a high fat content there will be a layer of fatty material on the surface of the aqueous phase that should be removed. Transfer the clear supernatant to a fresh tube.

- To ensure complete dissociation of nucleoprotein complexes, allow samples to stand for 5 minutes at room temperature in the Tri Reagent.
- Add 200 µl of chloroform per 1 ml Tri Reagent used.
- Shake vigorously for 15 seconds (i.e. do NOT vortex) and allow to stand for 2-15 minutes at room temperature.
- Centrifuge at 12000 x g for 15 minutes at 4 °C.
- Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA) and a colorless upper aqueous phase (containing RNA).

Note: The chloroform used for phase separation should not contain isoamyl alcohol or other additives.

Note: RNAses are inactive in Tri Reagent. When the Tri Reagent is removed keep the RNA samples on ice.

- Transfer the aqueous phase to a fresh tube and keep it on ice.
- Add 0.5 ml isopropanol per ml Tri Reagent used in sample preparation and mix vigorously (do NOT vortex).
- Place on ice for 10 minutes.
- Centrifuge at 12000 x g for 10 minutes at 4 °C.
- The RNA precipitate will form a pellet on the side and bottom of the tube.
- Remove the supernatant and wash the RNA pellet by adding 0.5 ml 70% ethanol (1 ml/ml Tri-Reagent).
- Centrifuge at 12000 x g for 5 minutes at 4 °C.
- Remove the supernatant completely and let it dry to the air for a short period.
  Add an appropriate volume of formamide or DEPC treated sterile H_2O.
- Dissolve the pellet, in 50 µl DEPC treated milliQ, by pipetting or vortexing.
- Store tubes at -80°C

Note: Final preparation of RNA is free of DNA and proteins. It should have an OD 260/280 ratio of 2.1.

Note: Typical yields from tissues (µg RNA/mg tissue): liver, spleen, 6-10 µg; 3-4 µg; skeletal muscle, brain, 1-1.5 µg; placenta, 1-4 µg.

Note: Typical yields from cultured cells (µg RNA/10^6 cells): epithelial cells, 5-15 µg; fibroblasts, 5-7 µg.

APPENDIX 7

Protocol: Hepatic lipid analysis

Lipid quantification liver/muscle and adipose tissue

- Homogenize (mini bead beater and glass beads, 30 sec/50rpm) frozen tissue in 1 ml SET buffer (0.5 l)
  - Sucrose 250mM : 42.79g
  - EDTA 2mM : 0.37g
  - Tris 10mM : 0.61 g
- Let sample undergo one or **two** freeze-thaw cycles for cell destruction
- Further cell destruction is possible by suction up and down with a very fine needle (orange needle)
- Transfer homogenate into:
  - New eppendorf tube (1.5ml)
  - PCR plate for assays (easy to transform to 'measure plate')
- Let sample undergo another freeze-thaw cycle

- **Dilutions** for TG and protein in LDLR -/- mice (EXAMPLE, dependent on size of organ slice):
  - Protein dilution = 20x
    - 2.5 µl sample (always make double for repeating the assay) = 60 µl
    - Use TG dilution (5x) and dilute 4x
    - 1:4 – 1 µl TG dilution and 3 µl PBS
    - 15 µl TG dilution and 45 µl PBS = 60 µl
  - TG dilution = 5x
    - 7.5 µl sample (always make double for repeating assay) 20 µl
    - Extra for protein samples = 20 µl + 15 µl = 35 µl

- **Standards** for TG/CHOL :
  - In duplo (20 µl)
  - S7 = 400 mg/ml – 15 µl stock
  - S6 = 200 mg/ml = 7.5 µl stock
  - S5 = 100 mg/ml – 10 µl S6 + 10 µl PBS
  - S4 = 50 mg/ml = 10 µl S5 + 10 µl PBS
  - S3 = 25 mg/ml = 10 µl S4 + 10 µl PBS
  - S2 = 12.5 mg/ml = 10 µl S3 + 10 µl PBS
  - S1 = 6.25 mg/ml – 10 µl S2 + 10 µl PBS
  - S0 = 0 mg/ml = 7.5 µl PBS

- **Standards** for protein :
  - In duplo (60 µl)
  - S5 = 2 µg/µl – 25 µl stock
  - S4 = 1 µg/µl = 30 µl S5 + 30 µl PBS
  - S3 = 0.5 µg/µl = 30 µl S4 + 30 µl PBS
  - S2 = 0.25 µg/µl = 30 µl S3 + 30 µl PBS
  - S1 = 0.125 µg/µl = 30 µl S2 + 30 µl PBS
  - S0 = 0 µg/µl = 25 µl PBS

- **Measure protein content** of sample (BCA kit, Pearce)
  - add 25 µl (of the diluted) sample in microplate (flat bottom!)
  - add 200 µl reagens (50 parts A, 1 part B)
  - mix
  - 30 minutes at 37° 595
  - read absorbance at 560 nm

- **Measure Triglyceride and/or cholesterol content** (Roche GPO-PAP kit)
  - add 7.5 µl sample (diluted for TG and undiluted for CHOL) in microplate (flat bottom!)
  - add 200µl reagens
  - mix
  - 45 minutes at room temperature
  - read absorbance at 490 nm

* Concentration of TG or chol can be set relative to the protein content of the sample