Aus der Klinik für Allgemeine Pädiatrie und Neonatologie,
Universitätsklinikum des Saarlandes, Homburg/Saar
Direktor: Univ.-Prof. Dr. med. L. Gortner

"VITAMIN A FOR PREVENTION OF BRONCHOPULMONARY DYSPLASIA IN EXTREMELY LOW BIRTH WEIGHT INFANTS – DATA ON A WATER-SOLUBLE, NANO-ENCAPSULED RETINYL PALMITATE MONOPREPARATION IN THE PRETERM LAMB MODEL

Dissertation zur Erlangung des Grades eines Doktors der Medizin der Medizinischen Fakultät der UNIVERSITÄT DES SAARLANDES
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vorgelegt von: Holger Wahl
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Gewidmet den Kindern, denen diese Forschungsarbeit helfen soll.

Content

1 Abbreviations

ADH	Alcohol dehydrogenase
BCO-I	ß,ß-carotene-15,15'–monooxygenase
BHT	Butyl-hydroxytoluene
BP	Blood Pressure
BPD	Bronchopulmonary dysplasia
bpm	Breaths per minute
BW	Birth weight
CRBP	Cellular retinol binding protein
CYP26	Cytochrome P450 family 26
DA	Dalton
DNA	Deoxyribonucleic acid
e.t.	Endotracheal
ETT	Endotracheal tube
F	Here: Frequency (ventilation settings)
$Fi0_2$	Fraction of inspired oxygen
GA	Gestational age
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HPLC	High-performance liquid chromatography
HR	Heart Rate
I:E	Inspiratory to expiratory time
i.m.	Intramuscular

i.v.	Intravenous
IPPV	Intermittend positive pressure ventilation
IVH	Intraventricular haemorrhage
LL	Left lung
LOD	Limit of detection
LOQ	Limit of quantification
LRAT	Lecithin:retinol acyltransferase
NCoA	Co-activator complex
NCoR	Co-repressor complex
p.o.	Per os
PBS	Phosphate-buffered saline
pCO_2	Partial pressure of carbondioxide
PCR	Polymerase Chain reaction
PEEP	Positive endexpiratory pressure
PIP	Positive inspiratory pressure
pO_2	Partial pressure of oxygen
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP	Retinol binding protein
RE	Retinyl ester
REH	Retinyl ester hydrolase
RL	Right lung

RNA	Ribonucleic acid
ROH	Retinol
rRNA	Ribosomal ribonucleic acid
RXR	Retinoid X receptor
SDR	Short-chain dehydrogenase/reductase
sec	seconds
SMRT	Silencing mediator of retinoic acid
STRA6	Thyroid hormone receptor, stimulated by retinoic acid gene 6
THF	Tetrahydrofuran
TTR	Transthyretin
VA	Vitamin A
VAD	Vitamin A deficiency
VARA	Vitamin A-Retinoic Acid Complex
VDR	Vitamin D receptor
VLBW	Very low birth weight
VPT	Very preterm

2 Summary

Vitamin A, a fat soluble vitamin and transcription factor, and its active metabolite, retinoic acid, is essential for human health due to its effects on growth, maintenance and regeneration of many cells and tissues, including the lung tissue.

The availability of vitamin A is crucial for embryonic lung development, maturation and function of the respiratory system including the development of the respiratory epithelium, alveologenesis and septation of alveoli. It is known that pregnant women are often at high risk for vitamin A deficiency, that vitamin A levels in women are directly proportional to cord blood vitamin A levels and that preterm infants suffer from vitamin A deficiency. Vitamin A deficiency is associated with the development of bronchopulmonary dysplasia, a developmental disorder of lung maturation and function, as well as long-term respiratory morbidity in this cohort. Indeed, the pathogenesis of bronchopulmonary dysplasia and morphological changes associated with vitamin A deficiency are similar in nature.

The prevention of bronchopulmonary dysplasia in at-risk infants is an important aspect of early neonatal care since the condition affects a large number of survivors of preterm birth. It is characterized by chronic inflammation, impaired alveolarization and vascularization, which result in chronic lung damage with significant short- and long-term mortality and morbidity.

Additional vitamin A supplementation in preterm neonates, in particular in infants with an extremely low birth weight, has been shown to prevent bronchopulmonary dysplasia and to improve long-term pulmonary outcome. However, the best method of its administration remains unclear.

Currently, vitamin A is supplemented in the neonatal cohort for nutritive reasons using lipid-soluble intravenous multivitamin preparations, lipid-soluble intramuscular monopreparations or lipid-soluble oral solutions. However, when considering additional vitamin A supplementation for the prevention of bronchopulmonary dysplasia, there is still no consensus with regard to the optimal supplementation regime, since the best substrate, method and dose of administration remains to be established.

The aim of my research project was the evaluation of a new substance, a nano-encapsuled, water- and fat-soluble retinyl ester monopreparation, originally used as a commercially available nutritional supplement, for its potential use in the neonatal population.

The substance was applied in a well-established animal model of preterm birth and the feasibility of using the intravenous and endotracheal route of administration was explored.

Vitamin A distribution in serum, lung and liver tissue was measured, and I studied the induction of the mRNA of three retinoid homeostatic genes, STRA6, LRAT and CYP26B1, as markers for changes in expression of early vitamin A homeostatic genes.

Significant increases in retinol and retinyl palmitate levels in serum, lung and liver after intravenous and endotracheal administration were found, and I was able to show changes in the expression of mRNA of the tested retinoid homeostatic genes, demonstrating a very early vitamin A effect through cellular responses on a molecular level.

The data positively supports the feasibility of using the tested nano-encapsuled, water-soluble vitamin A monopreparation for vitamin A supplementation in the preterm neonatal cohort using either the intravenous or endotracheal route. Further studies are warranted to assess practicalities with regard to the mode of application and to explore this substances' kinetics and safety profile, interactions with other medications, short- and long-term effects and its long-term influence on bronchopulmonary dysplasia in the preterm neonatal population.

3 Introduction

3.1 Vitamin A

3.1.1 Substance and biological function

In general linguistic usage, the term vitamin A (VA, ROH, retinol) describes a number of different chemical substances, such as retinol, retinal, retinoic acid or retinyl esters, e.g. retinyl palmitate or retinyl acetate (UNDERWOOD, 1984), whereby retinol is frequently used as a synonym for VA (BIESALSKI, 1989), hereby excluding storage or biologically active forms. In this thesis, the term VA will be used to include all different chemical substances. Where it is important to differentiate between retinol, retinyl ester and retinoic acid, the relevant correct terms will be used.

In humans, VA cannot be synthesized de novo, and it is therefore essential. It can be either ingested directly derived from animal sources (liver, kidney, eggs, and dairy products) or from enriched foods (milk, breakfast cereals) as preformed retinoids (mainly retinol and retinyl esters). Further, it can be synthesised from provitamin A, carotenes (Figure 1), obtained from plant sources (especially dark green leafy and yellow vegetables). In the developed world, 25-75% of total VA intake is provided by preformed retinyl esters or retinol, with the rest being provided by carotenes (HARRISON, 2005).

Any analog of *all-trans* retinol (Figure 2), whether a metabolite of VA compounds or a synthetic derivate, exhibiting the same properties as VA is called retinoid. Retinol, including its isoforms, *all-trans, 11-cis, 13-cis, 9, 13-di-cis, 9-cis, and 11, 13-di-cis* and retinyl esters, VA's storage form, primarily function as precursors for the biosynthesis of endogenous, biologically active retinoids (THEODOSIOU et al., 2010). Endogenous, biologically active retinoids can be generated by intracellular enzymatic conversion from retinol and retinyl esters and include retinoic acid, a small, 300 Da, lipophilic substance, and its isomers, all-trans retinoic acid (Figure 3), 9-cis retinoic acid, 11-cis retinaldehyde, 3,4-didehydro retinoic acid, as well as 14-hydroxy-4, 14-retro retinol, 4-oxo RA, and 4-oxo retinol.

As will be outlined below, retinol's active metabolites are of significant importance for human health due to their effects on embryonic and foetal development as well as growth, maintenance and regeneration of many cells and tissues, not only early in life but also throughout life (MADEN, HIND, 2003).

Figure 1: ß-Carotenes

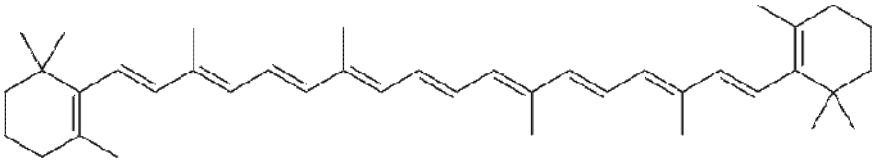


Figure 2: All-trans retinol

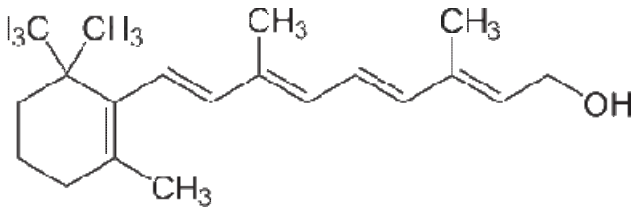
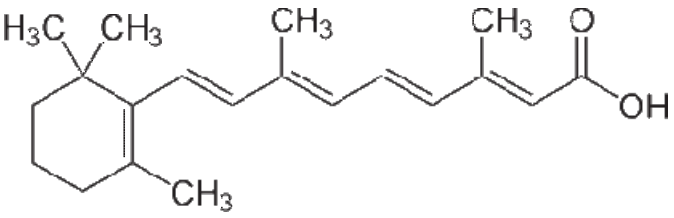


Figure 3: All-trans retinoic acid



3.1.2 Metabolism

Vitamin A metabolism is complex due to the variety of VA homologs involved and due to the potential toxic effect retinoic acid may have on cells. It is known that VA metabolism is subjected to tight homeostatic control (UNDERWOOD et al., 1979), which means that the internal equilibrium is maintained by adjusting physiological processes within the organism. These processes are complicated and remain still only partially understood (SCHREIBER et al., 2012). In particular, our understanding of VA homeostasis and kinetics in newborns remains poorly understood and is a hot topic of current research (TAN et al., 2014).

Principally, in adults, children and term infants dietary VA (ß-Carotenes, retinol, retinyl ester) is absorbed in the small intestine (Figure 4). In young infants, especially in premature infants, ß-Carotenes play only an ancillary role. The digestion of retinyl ester (RE) requires a process of solubilisation that is catalysed by lipases. They are enzymatically converted to retinol by pancreatic lipase and the intestinal brush border enzyme phospholipase B prior to uptake into the enterocyte. Co-ingestion of dietary fat markedly enhances the intestinal absorption of dietary VA. This is due to the stimulation of pancreatic enzyme secretion, secretion of bile salts for micelle production and the provision of other products of lipid digestion such as free fatty acids which themselves can build micelles (HARRISON, 2005). This is an important aspect when contemplating different routes of administration, in particular when considering

oral VA supplementation for infants with an extremely low birth weight (ELBW infants) since they are most often not fed for a prolonged period of time after birth.

Retinol is then taken up by the enterocyte through a saturable carrier-mediated process and non-saturable diffusion-dependent process (QUICK, ONG, 1990). The carrier proteins, however, have not yet been identified or characterized (BLOMHOFF, BLOMHOFF, 2006). The absorption rates of intestinal cells differ between preformed VA from animal sources (retinyl ester absorption rate 70-90%), and provitamin A carotenes from plant sources with absorption rates of 20-50% (depending on VA status and other dietary/non-dietary factors). Transport and storage forms are biologically inactive. The mobilization and homeostatic control of retinol from storage sites, thus serum VA levels, depends on enzymatic activities from retinyl ester hydrolases. The mechanisms of digestion and absorption of dietary VA have been reviewed in detail by Harrison (HARRISON, 2005), and the specific role of retinyl ester hydrolases in vitamin A homeostasis was described in detail by Schreiber et al. (SCHREIBER et al., 2012).

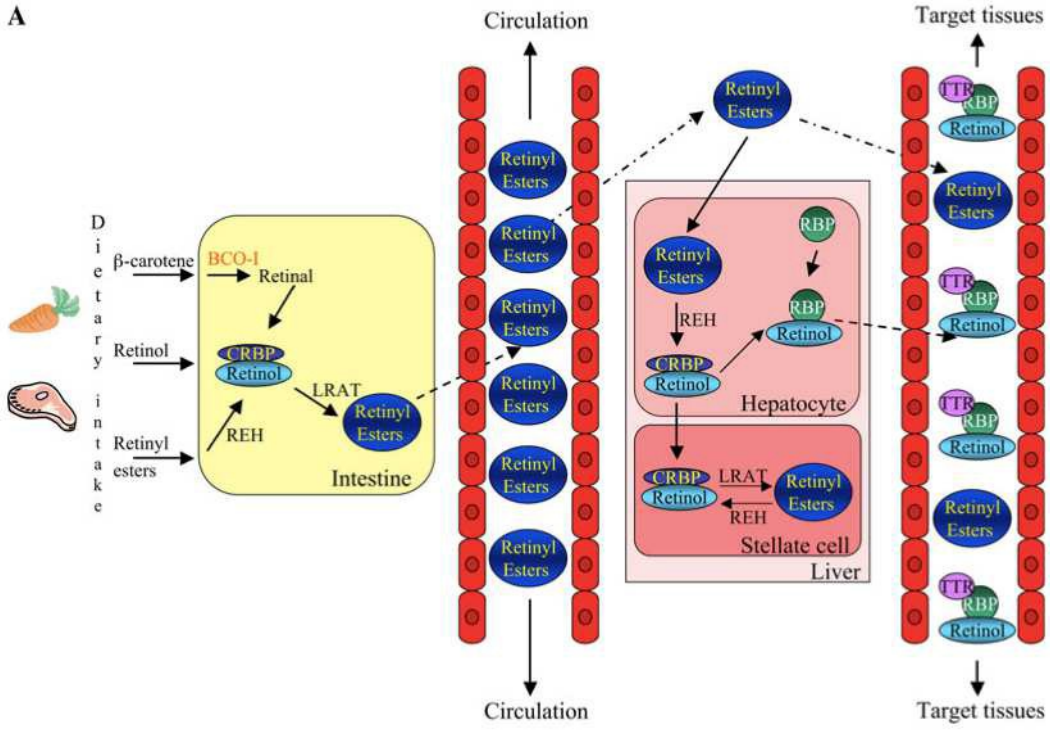
After uptake into the intestinal cells, retinol is bound to cellular retinol binding protein II. Once retinol is bound to cellular retinol binding protein II for the purpose of solubilisation and protection from degradation, it can be delivered to intestinal LRAT for re-esterfication.

Retinyl esters are subsequently secreted as chylomicrones into the lymphatic system, reduced to chylomicron remnants in the general circulation and transported to target tissues or the liver for storage (Figure 4). Extrahepatic uptake of chylomicrone remnants (mammary tissue, bone marrow, adipose tissue, spleen, lungs) has been described (PAIK et al., 2004). This may be an important factor for the immediate delivery of retinyl esters to tissues with high demands and may contribute to the regulation of VA homeostasis (BLOMHOFF, BLOMHOFF, 2006). The question as to whether chylomicrone-associated retinyl ester can act directly as precursors for retinoic acid is currently not known.

The liver forms the organism’s biggest VA storage site (50-80%) (BLOMHOFF et al., 1985; SENOO, 2004), and absorption and hepatic storage of preformed VA is very efficiently organized unless a pathological condition develops. Liver uptake of chylomicron remnants is facilitated by the apolipoprotein E receptor (BIESALSKI, NOHR, 2004). Within the hepatocytes retinyl esters may be stored in hepatic perisinusoidal stellate cells or undergo hydrolysis. The extensive storage of retinyl esters in hepatic stellatae cells and the cell’s ability to control excretion of retinol ensures a steady blood-plasma retinol concentration despite normal fluctuations in VA intake (BLOMHOFF, BLOMHOFF, 2006).

Figure 4: Vitamin A metabolism

From: Theodosiou et al., 2010: From carrot to clinic: an overview of the retinoic acid signaling pathway (THEODOSIOU et al., 2010).



BCO-I: **ß,ß-carotene-15,15'–monooxygenase,** CRBP: **cellular retinol binding protein,** REH: **retinyl ester hydrolase,** LRAT: **lecithin:retinol acetyltransferase** RBP: **retinol binding protein,** TTR: **transthyretin**

Before being excreted from the liver, retinol binds intrahepatically to retinol binding protein (RBP), a 21-kD plasma protein, and is then excreted and transported to the tissue as holo-RBP (retinol-RBP) complex (KANAI et al., 1968) coupled to transthyretin to avoid renal clearance (BIESALSKI, NOHR, 2004). 4-5% of the circulating RBP-retinol complex is not bound to TTR whilst 95 % of the plasma RBP is bound to transthyretin (PETERSON, 1971). The kidneys contribute to recycling of the retinol-RBP complex, contributing about 50% of the total circulating pool (BLOMHOFF et al., 1991; CHRISTENSEN et al., 1999). The holo-RBP complex is under homeostatic control (UNDERWOOD et al., 1979). Only during severe vitamin A deficiency (VAD) as in depleted liver retinyl ester stores is the concentration of the plasma retinol-RBP complex decreased and extrahepatic stores may further contribute to the regulation of VA homeostasis (BLOMHOFF, BLOMHOFF, 2006).

Active retinoids, e.g. retinoic acid, are synthesized within the target cells. A balanced system of synthesis and catabolism is needed to allow control of retinoic acid levels within cells and to prevent toxicity (Figure 5). Several intracellular and membranic enzymes and proteins are key, including retinyl ester hydroxylase, CRABP, STRA6, LRAT, and CYP26, among others (ROSS, 2003; ROSS et al., 2001). These enzymes and proteins are important since they are

variably expressed depending on VA status. They seem to exert control over intracellular VA status (WU, ROSS, 2010).

Retinol uptake from plasma and extracellular fluids into peripheral cells is mediated by STRA6, a multitransmembrane domain protein and a specific membrane receptor for RBP and represents a major physiological mediator of cellular VA endocytosis. It also acts as a cytokine receptor and transduces signalling by holo-RBP, which is able to regulate insulin response. Further, it may not only control influx, but also efflux of retinol from cells, and it therefore acts as a bi-directional transporter, with intracellular retinol concentrations determining the transport direction (KAWAGUCHI et al., 2007).

Intracellular retinol is bound to RBP and either oxidized to retinoic acid or re-esterified to retinyl ester (local in situ storage form) via enzymatic conversion by LRAT. The first step of oxidation is the enzymatic conversion of retinol to retinal by the alcohol-dehydrogenases (ADHs) and to RA by the retinal dehydrogenases (RALDHs) (DUESTER, 2000). Retinoic acid subsequently either exerts its effects via the nuclear cell receptors, transcriptional transregulators, the molecular physiology of which was recently reviewed by Duong and Rochette-Egly (DUONG, ROCHETTE-EGLY, 2011) or is metabolized (detoxified) by the cytochrome P450 systems.

The nuclear retinoic acid receptors **(**RARα, β and γ), which are multi-domain proteins (macromolecular complexes), play a pivotal role in controlling the expression of certain gene subsets hereby maintaining homeostasis through regulating cell proliferation and differentiation (DUONG, ROCHETTE-EGLY, 2011). Not only RAR receptors but also their partners, the RXRs, also play their role in cell homeostasis (IMAI et al., 2001). Furthermore, not only VA appears to be crucial for the regulation of cell proliferation and differentiation but also vitamin D since the vitamin D receptor (VDR) is obligate within the macromolecular complex formed with the retinoid X receptor (RXR) (ZHANG et al., 2011).

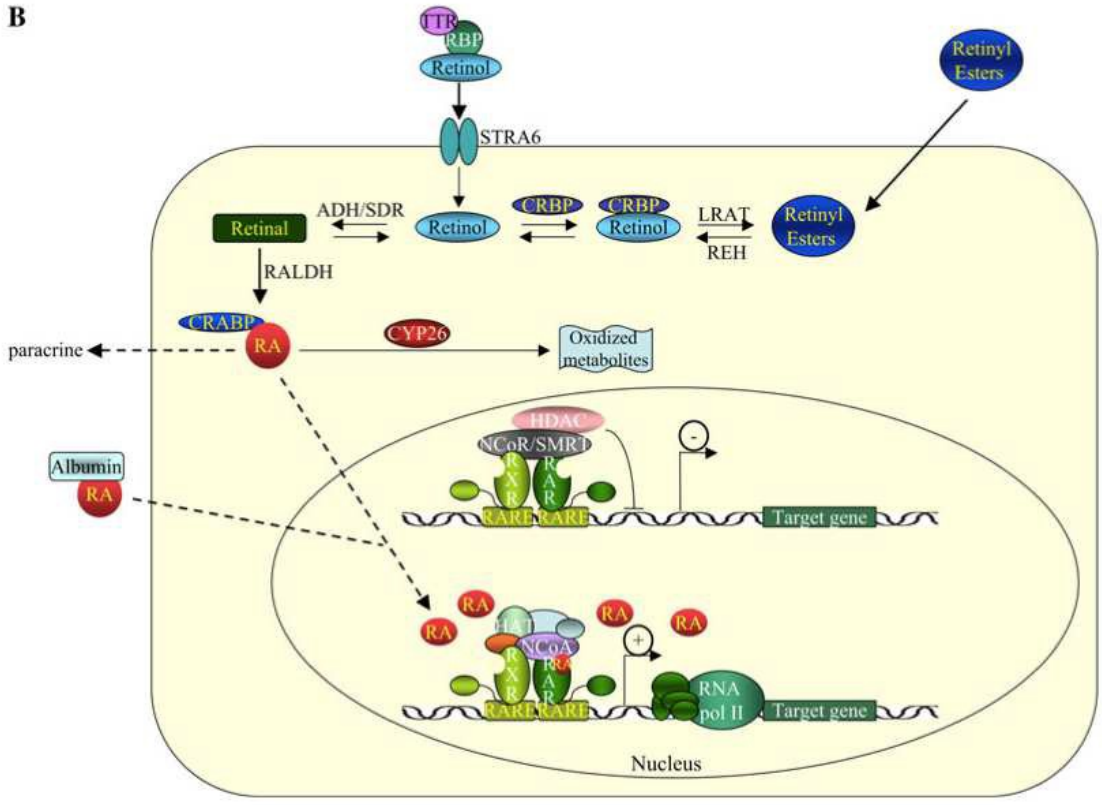
The cytochrome P450 systems (P450RAI) are responsible for oxidation of retinol to retinoic acid and protect against toxic exposure to retinoic acid. Different enzymes are known, such as CYP26B1 and CYP26A1. CYP26B1, identified shortly after CYP26A1, metabolizes all-trans retinoic acid to the polar metabolites 4-oxo retinoic acid, 4-hydroxy retinoic acid, and 18-hydroxy-all-trans retinoic acid (WHITE et al., 2000). The oxidative processes are thought to be initiated by hydroxylation of the C4 or C18 position of the beta-ionone ring of retinoic acid. CYP26 oxidizes all-trans retinoic acid but does not appreciably metabolize the 9-cis-or 13-cis-RA isomers, which suggests that it plays a unique role in retinoid biologic factors

(PETKOVICH, 2001). Enzymes that catalyse the intracellular reactions for retinoic acid conversion may be of particular importance since those may act as regulators of tissue retinoic acid levels and hence protect the cells from toxic influences.

Further, it should be mentioned that retinol can be conjugated with glucoronic acid, which leads to retinoyl- and retinyl glucoronidase and can subsequently be eliminated in faeces and urine. This has been studied by analysing urinary, biliary, and faecal metabolites of radiolabeled retinol and retinoic acid. Several polar metabolites have been detected. It has been suggested that catabolism seems to involve pathways involving retinol as well as retinoic acid, but the relative contribution of these as well as the intermediates and enzymes involved are not well understood (BLOMHOFF, BLOMHOFF, 2006).

Figure 5: Transcriptional activation

From: Theodosiou et al., 2010: From carrot to clinic: an overview of the retinoic acid signaling pathway (THEODOSIOU et al., 2010).



TTR: transthyretin, **RBP**: retinol binding protein, **STRA6:** thyroid hormone receptor, stimulated by retinoic acid gene 6, **ADH:** alcohol dehydrogenase, **SDR:** short-chain dehydrogenase/reductase, **CRBP**: cellular retinol binding protein, **REH:** retinyl ester hydrolase, **LRAT:** lecithin:retinol acetyltransferase, **CYP26:** cytochrome P450 family 26, **RA:** retinoic acid, **RAR:** retinoic acid receptor, **RXR:** retinoid X receptor, **RARE:** retinoic acid response element, **NCoR:** co-repressor complex, **NCoA:** co-activator complex, **SMRT:** silencing mediator of retinoic acid, **HAT:** histone acetyltransferase, **HDAC:** histone deacetylase.

3.2 The importance of vitamin A for lung development

Retinoic acid is elementary for the adequate formation of the embryonic axis and limbs due to its control of the expression of key developmental target genes (CLAGETT-DAME, DELUCA, 2002; DUONG, ROCHETTE-EGLY, 2011). It has the greatest endogenous activity as a ligand for retinoid acid nuclear receptors (RAR-α, RAR-β, and RAR-γ) (BASTIEN, ROCHETTE-EGLY, 2004), through which it influences development and cell differentiation. It is necessary for the orderly development of the central nervous system, vision, hematopoietic system, immune system and reproductive system, and also for the embryonic and postnatal development of lung tissue (BIESALSKI, 2011; MADEN, HIND, 2004; MALPEL et al., 2000).

Alveologenesis, septation of alveoli and surfactant production are all dependent on the influence of VA (MASSARO, MASSARO, 2002; METZLER, SNYDER, 1993; ROTH-KLEINER, POST, 2005).

Geevarghese and Chytil demonstrated that during the alveolar stage of lung development major accumulation and utilization of retinyl ester occur (GEEVARGHESE, CHYTIL, 1994; SHENAI, CHYTIL, 1990). Retinoic acid peaks precede alveolar septation, which is accompanied by a simultaneous fall in retinol, suggesting utilization of retinoids during this process, (HIND et al., 2002b). Already in 1995, McGowan et al. showed that retinoid acid receptors are expressed during alveolar formation (MCGOWAN et al., 1995). The close temporal and spatial expressions of the RAR isoforms α1, β2, β4 and γ2 during alveolar development have subsequently been characterized in the mouse model (HIND et al., 2002a). Knockout mice for RARβ gene have smaller, more numerous alveoli, suggesting that RARβ may function as a negative regulator of alveolar function (MASSARO et al., 2000) and null mutants for the RARγ gene have fewer, larger alveoli, suggesting that RARγ is required for alveolar formation (MCGOWAN et al., 2000).

Changes occur not only on receptor level but also on enzymatic and protein levels. Retinoic acid synthesising enzymes (RALDH1 [alveolar parenchyma]; RALDH2 [lung periphery]) have been demonstrated to show a close spatial distribution with patterns of alveolar proliferation postnatally (HIND et al., 2002b; MADEN, HIND, 2004) and significant changes of the levels *of* RBP (CRBP1 and CRABP1) in both rat (MCGOWAN et al., 1995; ONG, CHYTIL, 1976) and mouse (HIND et al., 2002a) are observed during the time of alveolar development.

There is further strong evidence that retinoic acid is required not only at life's beginning but also throughout life for the maintenance of lung alveoli integrity and elastin formation (BLAND et al., 2003; MADEN, HIND, 2004) and may indeed also exhibit regenerative properties. It has been proposed that regeneration uses the same pathways as organ or

tissue development (MUNEOKA, BRYANT, 1982), and retinoic is known to induce limb regeneration and super-regeneration in animals (NIAZI, SAXENA, 1978). With regard to lung development, Maden and Hind suggested that RA re-stimulates the same RA-responsive gene pathways that are used during normal alveologenesis (MADEN, HIND, 2003). Retinoic acid-induced alveolar regeneration was first described by Massaro and Massaro in 1996 (MASSARO, MASSARO, 1996), and in 1997 they showed that pathological features of experimentally induced emphysema can be reversed in the adult rat model (MASSARO, MASSARO, 1997). Other studies were able to show similar data (BELLONI et al., 2000; TEPPER et al., 2000).

A lack of vitamin A, in particular during embryogenesis, has therefore significant health implications. It has been shown that severe VAD can result in early embryonic death (CLAGETT-DAME, DELUCA, 2002), and less severe VAD induces foetal developmental malformations (SPIEGLER et al., 2012). Inhibition of retinoic acid synthesis leads to disruption of alveolar formation and the development of large air spaces (MADEN, HIND, 2003). In the animal model, Baybutt et al. showed in 2000 that VAD induced inflammation and emphysema and reduced surfactant synthesis in the lungs of rodents. The observed histopathological changes are similar to those found in bronchopulmonary dysplasia (BPD) and adult emphysema (BAYBUTT et al., 2000).

3.3 Vitamin A toxicity

As with any other substance that may be over-consumed, VA shows unwanted and toxic effects if taken in large quantities. As it is impossible to describe any universal mode of action of VA derivates, it is equally impossible to generalize their toxic effects. When administered, VA is nearly completely absorbed and stored to a certain extent by the organism. Prolonged and excessive overdosing will give rise to symptoms of intoxication (BIESALSKI, 1989). In addition, teratogenic effects are known.

According to Poisindex®, any VA ingestion of more than 1 million IU in adults and more than 300,000 IU in children has caused acute toxicity. With regard to chronic toxicity, more than 10 times the RDA for weeks or months, or more than 50,000 IU/d in adults and more than 25,000 IU/d in children, are associated with toxic effects.

In children, headaches, fever and gastrointestinal disturbances have been reported following therapeutic ingestion of 100,000 IU VA during clinical trials (FLORENTINO et al., 1990). Lam et al. reported the casuistic of three children < 6 years who ingested 200,000 to 300,000 IU and remained asymptomatic, although retinol plasma levels were elevated and remained so for months (LAM et al., 2006). With regard to premature infants, data is lacking.

Clinical adverse effects associated with acute VA overdoses are various and include gastrointestinal effects (such as nausea, vomiting and hepatotoxicity), skin disorders (exanthema, dry skin), effects on bone (bone and joint pain), lipid metabolism, and neurological disorders (such as headaches, intracranial hypertension, papillaedema, bulging fontanelle in infants, and seizures) (BAQUI et al., 1995; DE FRANCISCO, BAQUI, 1996; DE FRANCISCO et al., 1993). Humphrey et al. were able to demonstrate that VA-induced bulging fontanelle is not associated with developmental delay or adverse growth (HUMPHREY et al., 1998).

Chronic ingestion, so called hypervitaminosis A, may also involve fatigue, anorexia, hair loss, weight loss, oedema, anaemia, low-grade fever, polyuria, hypercalcaemia, hepatosplenomegaly, muscle pain, bone pain and diplopia. Liver damage or bone hyperostosis (early closure of the epiphysis of long bones) in children may be possible. Furthermore, hypercalciuria, haemolytic anaemia and renal failure may be seen. Typical changes may be present on X-rays (skull, skeleton) (MÜHLENDAHL et al., 2007).

It is known that hepatotoxicity is one of the most severe outcomes of chronic high-dose VA intake (BAUERNFEIND, 1980; GEUBEL et al., 1991; KOWALSKI et al., 1994). It is assumed that the mechanisms of hepatic adverse effects are linked to the overloaded hepatic storage capacity for VA (EUROPEAN FOOD SAFETY AUTHORITY (EFSA), 2006). Several suggestions for underlying reasons for hepatic injury have been made: cell membrane damage and lysosomal rupture (ELLIS et al., 1986), obstructed blood flow through VA-loaded hepatic stellate cells within the sinusoidal space with the consequence of portal hypertension (HRUBAN et al., 1974; RUSSELL et al., 1974) or a dysfunction of the excretory function of the hepatic stellate cells (production of collagen type III) as responsible for the pathophysiology of hepatic cirrhosis (SVEGLIATI-BARONI et al., 2001). Some data on animal experiments exist (LEO et al., 1982; SHINTAKU et al., 1998b, a), although, overall the data is limited. In most cases toxicity appeared after prolonged ingestion of high doses of VA. Hepatotoxicity was very frequently associated with elevated retinol and retinyl ester in serum (EUROPEAN FOOD SAFETY AUTHORITY (EFSA), 2006). However, none of the clinical studies in premature infants that have been considered in Darlow and Graham's Cochrane review (DARLOW, GRAHAM, 2007) have reported any evidence of hepatic or biochemical toxicity, which may be explained by their already deficient body stores.

On a different note, care should be taken when assessing neonatal VA supplementation with regard to immunological effects. It has previously been shown that the risk for atopy was increased (AAGE et al., 2015). Clinical studies must continue to observe any unwanted long-term effects, including effects on the developing immune system.

3.4 Vitamin A deficiency

Vitamin A deficiency is a global health problem. Around 190 million pre-school children and 19 million pregnant women are affected by VAD worldwide (WHO, 2009). Maternal health cannot be separated from newborn health, and it is known that if VA supply to the pregnant woman is impaired, negative consequences for the offspring are expected (ROTONDI, KHOBZI, 2010). Associations between maternal multiple-micronutrient deficiencies, which are commonly exacerbated during pregnancy, and infant health, especially in small for gestational age and low birth weight babies, has been shown and extensively investigated, (HAIDER, BHUTTA, 2012). It was found that premature infants are particularly susceptible to the sequelae of their micronutrient deficiencies due to their fragile health and poor reserves (HANSON et al., 2012). Pregnancy, post partum complications and death are known to be increased in chronic VAD (GORSTEIN et al., 2003; WEST et al., 1999) and, as pointed out above, there are clear indications that VAD is associated with the development of BPD.

Evidence exists that maternal repletion with VA at recommended dietary levels, before, during and after pregnancy improves lung function in the offspring (CHECKLEY et al., 2010), and VA supplementation in late pregnancy for the prevention of BPD remains a topic of current research (BABU, SHARMILA, 2010).

3.5 Vitamin A deficiency and prematurity

Premature infants, who are known to have low VA plasma concentrations, low RBP, and low hepatic stores (BRANDT et al., 1978; INDER et al., 1998; KOSITAMONGKOL et al., 2011; SHENAI et al., 1981; SHENAI et al., 1985a; WEINMAN et al., 2007), are considered to suffer from, and are particularly prone to the consequences of VAD, proving this to be a major public health concern.

Rates of preterm birth vary between countries. Rates between 12-13% in the US (IAMS et al., 2008; SLATTERY, MORRISON, 2002) and other developed countries, including European countries (FIELD et al., 2009), are given. In Germany, data suggests a rate of 13.9 VPT/1000 births (excluding termination of pregnancies) (FIELD et al., 2009). Causes of preterm labour are multifactorial and include infections, inflammations, uteroplacental compromises (ischaemia, haemorrhage, overdistension of uterus) and immunologically mediated processes (ROMERO et al., 2006). Consequences of prematurity are significant. Complications affect various organ systems such as the brain (e.g. intraventricular haemorrhages [IVH], periventricular leukomalacia), the eye (e.g. retinopathy of prematurity), the musculoskeletal system (e.g. osteopenia of prematurity), the gut (e.g. necrotizing enterocolitis, feeding difficulties) and the lungs (e.g. respiratory distress syndrome, BPD), among others.

Associations have been made between VAD in premature infants and the occurrence of BPD and long-term respiratory morbidity (SHENAI et al., 1985b; SPEARS et al., 2004). It has been postulated for some time that VAD predisposes premature infants to BPD (BRANDT et al., 1978; HUSMANN et al., 1992; INDER et al., 1998; SPEARS et al., 2004).

3.6 Prematurity and bronchopulmonary dysplasia

Bronchopulmonary dysplasia, a developmental disorder of lung maturation and function, (COALSON, 2003) constitutes a major complication of prematurity and affects around 17% of very preterm babies (VPT, GA 24+0 - 31+6) surviving up to 36 weeks GA, affecting even more amongst the population of <28 weeks GA, (28-56%) (GORTNER et al., 2010) and the extremely low birth weight (birth weight < 1000g) population (up to 35%) (SMITH et al., 2005).

Pathogenesis of the lung injury in BPD reflects an extremely immature lung with impaired alveolar and capillary growth caused by developmental arrest as a result of pre- (e.g. inflammation, infection) and postnatal (e.g. oxygen toxicity, mechanical ventilation) contributing factors and abnormal reparative processes (COALSON, 2003; HAYES et al., 2010). Those processes give rise to the hallmarks of BPD of chronic inflammation, impaired alveolarization and vascularization resulting in chronic lung damage with significant short- and long-term mortality and morbidity (KINSELLA et al., 2006).

It has been known for some time that BPD constitutes a severe clinical problem for premature infants (SPEARS et al., 2004; SPEER, SILVERMAN, 1998) since those who suffer from BPD are at significant short-term risk, including a heightened risk for IVH, and long-term risk for increased morbidity such as increased hospital admissions for respiratory infections (SMITH et al., 2004), poorer lung function in adolescence (DOYLE et al., 2006), risk of emphysema (WONG et al., 2008), and developmental delays (SHORT et al., 2007) including a heightened risk for cerebral palsy (VAN MARTER et al., 2010). The prevention of BPD in at-risk infants is one of the most challenging aspects in early neonatal care.

Treatment options for BPD are limited, and finding measures for the prevention of the disease is therefore highly significant.

3.7 Vitamin A supplementation for the prevention of BPD

Vitamin A supplementation as a potential measure for the prevention of BPD in the premature population has been extensively researched with a significant number of relevant clinical studies (AMBALAVANAN et al., 2003; BENTAL et al., 1999; KENNEDY et al., 1997; PEARSON et al., 1992; SHENAI et al., 1987; SHENAI et al., 1990; TYSON et al., 1999;

WARDLE et al., 2001; WERKMAN et al., 1994) and a large number of scientific reviews (BAYBUTT, MOLTENI, 2007; BIESALSKI, 2011; BIESALSKI, NOHR, 2003; BIESALSKI, SEELERT, 1989; CHYTIL, 1985, 1992, 1996, 1999; GUIMARAES et al., 2012; MACTIER, WEAVER, 2005; MASSARO, MASSARO, 2001, 2003, 2010) indicating the considerable research interest in this area.

Darlow and Graham concluded in their Cochrane Review that *"Supplementing very low birth weight infants with vitamin A is associated with a reduction in death or oxygen requirement at one month of age and oxygen requirement among survivors at 36 weeks postmenstrual age, with this latter outcome being confined to infants with birth weight less than 1000 g."* (DARLOW, GRAHAM, 2007).

In addition, several translational trials have shown that VA supplementation can positively influence the process of reducing lung injury and damage of the respiratory system (COUROUCLI et al., 2006; JAMES et al., 2010; VENESS-MEEHAN et al., 2002). In 2003 Maden and Hind showed that dexamethasone-treated mice in a model of alveolar loss show complete regeneration after daily retinoic acid injection (MADEN, HIND, 2003). Bland et al. created a lamb model for BPD and showed benefits when treating the animals with VA (BLAND et al., 2003). In a 2010 study using the lamb model, Albertine et al showed that VA treatment partially improved lung development in chronically ventilated preterm neonates (ALBERTINE et al., 2010).

Thus, we conclude that due to the fact that VA is essential for the growth, development and maturation of lung tissue and because it controls in its active form the regulation and differentiation of cells including those of the respiratory tract, it is an important micronutrient for the preterm neonate who is known to be VA depleted. Early improvement of VA supply in the ELBW population is considered to be an important factor for positively influencing the progression of BPD, a disease with a high morbidity and (co-) mortality rate. Questions remain about the most meaningful way of achieving VA sufficiency in ELBW infants.

3.8 Problem statement

3.8.1 Current recommendations and pitfalls

No general recommendation for the standard use of VA for BPD prevention in the premature neonatal population has been made, and current supplementation regimes still do not seem to sufficiently correct VAD in the ELBW cohort, whereby the optimal dose as well the most appropriate route of administration have yet to be identified (MACTIER, 2013).

It is of even more crucial importance to extensively explore all options of postnatal VA supplementation in the infant because, despite initial promising results (CHECKLEY et al., 2010), recent Cochrane reviews did suggest that there is no role for antenatal VA supplementation to reduce perinatal mortality (MCCAULEY et al., 2015) and no evidence that VA supplementation in postpartum women reduces infant mortality or morbidity (OLIVEIRA et al., 2016). However, data on antenatal VA supplementation and peri- or postnatal morbidity such as BPD is unfortunately unavailable. Ongoing research in this area is still warranted.

Current regimes for postnatal VA supplementation in ELBW infants use either the oral, intravenous or intramuscular route (DARLOW, GRAHAM, 2011; MEYER et al., 2014).

Oral solutions are available (e.g. Vitadral® drops), and there is currently a multicentre trial underway to clarify whether additional early postnatal high-dose oral vitamin A supplementation in the form of a lipid-soluble retinyl ester preparation is able to prevent bronchopulmonary dysplasia or death in extremely low birth weight infants (MEYER et al., 2014). However, often due to delayed feeding regimes in this cohort, the necessary co-ingestion of dietary fat that is needed for enhanced absorption, is reduced. Additionally, intestinal brush border enzymes, the synthesis of RE hydrolases, pancreatic enzyme secretion, the production of bile salts and carrier mediates processes may all be immature in very premature infants which further impede intestinal absorption of orally administered dietary or supplementary retinol or retinyl ester. Therefore, intravenous, intramuscular, or endotracheal application may be more reliable.

For intravenous supplementation only multivitamin preparations are at present licensed for use. These do not allow for separate dose adjustment and are lipid-soluble substances, which are not easily dissolved in aqueous solutions. In 1994 Werkman et al. studied an additional high dose intravenous VA (retinyl palmitate monopreparation, Aquasol A) supplementation regime added to a commercial lipid emulsion in a cohort of low birth weight infants and found that supplemented infants had significantly higher plasma retinol concentrations in the first month than infants receiving routine supplementation (WERKMAN et al., 1994). Unfortunately, the product used subsequently needed to be withdrawn from intravenous use due to anaphylaxis concerns (personal conversation). There is currently no VA monopreparation licensed for additional intravenous use.

Although additional intramuscular VA supplementation in preterm babies has been shown to prevent BPD and to improve long-term pulmonary outcome (DARLOW, GRAHAM, 2011), it has yet to gain wide acceptance clinically, mainly due to perceived pain in the infant (KAPLAN et al., 2010).

The optimal dose for ELBW infants has been extensively argued and debated in the past, (AMBALAVANAN et al., 2005; KENNEDY et al., 1997; MACTIER, 2013; MACTIER et al., 2005; MACTIER et al., 2011; MACTIER, WEAVER, 2005; WEST, CUMMINGS, 2005). Tyson et al. supplemented 5000 IU of VA intramuscularly three times per week for four weeks in extremely low birth weight infants and found a reduction of biochemical evidence of VAD and a slightly decreased risk of CLD (TYSON et al., 1999). Wardle et al. administered 5000 IU/kg of oral VA to 77 premature infants. The incidence of potential side effects, seizures and persistent vomiting did not differ between the groups (WARDLE et al., 2001). The previously mentioned multicentre trial currently underway is investigating the oral application of a dose of 5000 IU VA/kg/day versus placebo in ELBW infants (MEYER et al., 2014). Ambalavanan et al. administered doses up to 15.000 IU intramuscularly three times a week. They reported a case of transient fullness of fontanelle in one infant (AMBALAVANAN et al., 2003). There was no evidence of any other significant side effects. However, they did not find an advantage of this regime over a dose of 5000 IU three times a week for this patient cohort.

In animal trials, Albertine et al. used 5000 IU/kg daily intramuscular injections in the sheep model to investigate effects on lung tissue (alveolar septation, thickness of airspace walls and capillary growth) (ALBERTINE et al., 2010).

The European Food Safety Authority asserts that *"the upper level of 3000 µg RE/days is appropriate for men, and for infants and children after correction for differences in metabolic rate, because it is 2.5-fold lower than the lowest daily intake that has been associated with hepatotoxicity during chronic intake"* (EUROPEAN FOOD SAFETY AUTHORITY (EFSA), 2006). As a tolerable upper intake level for preformed vitamin A (retinol and retinyl ester) in the vitamin A-sufficient age group of 1-3 years the authors suggest 800 µg RE/days (2,640 IU). This is considered to be 2.5-fold lower than any assumed hepatotoxic dose. From their statement, one can conclude that 6.600 IU (2.5 times 2,640) may be considered the lowest dose at which toxicity, in their view, may be expected.

Because of the above outlined reasons, a dose of 5000 IU/kg body weight as a supplementation dose was chosen in this study.

3.8.2 A nano-encapsuled, water-soluble vitamin A monopreparation

The availability of a commercially-sold, nano-encapsuled, water-soluble vitamin A monopreparation intended for use as nutritional supplement (NovaSOL® A) in humans but, due to its composition, principally usable as parenteral substrate, led us to explore the idea of investigating this substance, the dose of which can be easily weight-targeted, in a well-established animal model using preterm lambs (SEEHASE et al., 2012). Using a nano-

encapsuled, water-soluble mono-substance would potentially have advantages over a lipid-soluble substance with regard to pharmacological properties, easier handling, administration and dose adjustment.

3.8.3 The animal model

The pre-clinical lamb model allows the use of neonatal equipment in realistic conditions and has been shown to be an adequate model for translation of experimental findings into clinical practice (KRAMER, 2008b). Preterm lambs have been used as large animal models for investigating BPD in the past (ALBERTINE et al., 1999). At a gestational age of between 128-133 days (Term: ~ 150 days) lung maturation resembles about 30 weeks of gestation in humans (KRAMER, 2008a) (SEEHASE et al., 2012). Preterm lambs have also previously already served in VA translational trials when VA was administered intramuscularly (ALBERTINE et al., 2010; KRAMER et al., 2008; WILLET et al., 2000). Applying VA as retinyl palmitate intravenously and endotracheally in the preterm lamb model is novel, and a comparison of those two application modes in this model has not been done before.

In contrast, piglets have before served in studies investigating the combined endotracheal application of VA as retinyl acetate and surfactant. Importantly, no negative influence was found on therapeutic surfactant effects, and a combination of both therapies appears feasible (SINGH et al., 2010).

3.8.4 The genetic markers

Vitamin A in its active form, RA, regulates several genes. A number of them, including STRA6, a transmembrane receptor for RBP, which mediates retinol uptake from plasma and extracellular fluid into cells (KAWAGUCHI et al., 2007), as well as LRAT and the CYP26 family, play an important role in VA homeostasis (ROSS et al., 2001). Those enzymes and proteins are variably expressed with VA status and seem to exert tight control over intracellular VA status (ROSS, 2003). It is known that if retinoic acid is available, which is an indication for a sufficient VA status, the expression of LRAT is maintained (ROSS, 2003). Conversely, LRAT is down-regulated during VAD (RANDOLPH, ROSS, 1991). A similar situation applies to the expression of CYP26B1. It also increases with a rise in the concentration of RA (PETKOVICH, 2001) and is maintained at very low levels in VAD (WANG et al., 2002; YAMAMOTO et al., 2000).

These findings were confirmed in a recent translational trial in rodents, a model that can only cautiously be used for translation of research findings for human premature neonatal application, where it has been shown that when treating neonatal rats orally using a combination of VA and different acidic retinoids, lung RE increased and retinol uptake and

esterification during the period of absorption correlated with increased expression of both STRA6 and LRAT after 6 hours. This working group was also able to show a strong induction of levels of CYP26B1 following retinoid treatment and concluded that CYP26B1 may therefore play a major role in metabolizing RA in neonatal lungs (WU, ROSS, 2010).

From the available evidence we concluded that it was useful to determine and analyse the early genetic expression of mRNA of VA homeostatic genes (STRA6, LRAT and CYP26B1) in order to comment not only on distribution pattern after different modes of VA application but also on potential early metabolic effects in our translational model. Further, using two different routes of application, any possible difference among the expression pattern may also influence any future experimental set-up when investigating VA short- and long-term effects as well as dose and mode of delivery.

3.9 Aim of the study

Therefore, the aims of this study were three-fold: firstly, to test the feasibility and practicability of intravenous and endotracheal application of a new substance, namely a nano-encapsuled, water-soluble retinyl ester (retinyl palmitate) monopreparation in an appropriate animal model, secondly, to obtain data on VA distribution and VA kinetics in this model comparing two different methods of application and thirdly, to examine the effects on early cellular responses on a molecular level. For reasons of completion, potential histopathological changes that would indicate early hyper-acute systemic toxic effects following VA application on different organ systems were investigated.

This study is meant to inform future research activities with the aim of providing a new substrate for VA supplementation in the ELBW neonatal cohort for the prevention of BPD. This substrate was never tested in this form before.

In conclusion, the objectives of this study were to:

1. Test the feasibility and practicability of substrate administration.
2. Evaluate VA distribution in serum, urine, lung and liver tissue using intravenous and endotracheal application methods.
3. Determine the expression of mRNA of retinoid homeostatic genes related to VA uptake (STRA6), esterification (LRAT), and catabolism (CYP26B1).

4 Material and Methods

This research project was initiated and conceptualized by myself under supervision of Prof. Gortner, Department of Paediatrics and Neonatology, Saarland University Medical Centre, Homburg/Saar, Germany, in collaboration with the University of Maastricht, Department of Paediatrics, Maastricht University Medical Centre, Faculty of Health, Medicine and Life Sciences, School for Oncology and Developmental Biology, Maastricht, Netherlands, the Department of Biology, Chemistry and Nutrition, University of Hohenheim, Stuttgart, Germany and with the Institute of Anatomy and Cell Biology, Saarland University, Homburg/Saar, Germany,

The animal experiments and tissue sampling took place at Maastricht University. Retinol and retinyl palmitate analysis of serum and tissue samples were performed at Hohenheim University. Histological analysis of tissue samples was performed at the Institute of Anatomy and Cell Biology in Homburg. Molecular analysis of mRNA of lung tissue and overall data analysis was performed at the Department of Paediatrics and Neonatology in Homburg.

I organized and coordinated the different working groups, visited all locations and was introduced to all research techniques. I performed the molecular analysis of the lung tissue samples as well as overall data analysis and interpretation under supervision of Prof. L. Gortner.

4.1 Ethical approval

The experimental protocol and study design were in line with the institutional guides for animal experiments and were approved by the *Institutional Animal Ethics Research Committee* of Maastricht University, Netherlands.

4.2 Animals and experimental design

Medication, chemicals and equipment tables can be found at the end of each chapter.

4.2.1 Animals

Time-mated Texel ewes were kept in a welfare-oriented animal husbandry and were fed species-appropriate fodder containing grass, hay and fermented grain. Ten lambs were delivered by caesarean section (modified EXIT procedure) at a gestational age of 128-133 days. Intramuscular Betamethasone (12mg) was given to the ewe one day prior to the experiment in order to induce foetal lung maturation (JOBE et al., 2007). After operative delivery, the lambs were weighed, underwent oral intubation and ventilation (ventilator: Babylog 8000, mode of ventilation: IPPV, settings of ventilation: FiO2=1, PEEP 8 cm H2O,

PIP 30 cm H2O, F: 60/min, I:E 1:2) and arterial and venous catheters were placed in the umbilical vessels for BP and HR monitoring as well as blood gas analysis for ventilation control and the application of medication (Table 1 and Table 2). The lambs were further continuously sedated with intravenous midazolam and ketamine and kept on an open, heated incubator maintaining the physiologic body temperature of 38 °C. Parenteral nutrition was provided and body temperature was monitored using a rectal temperature probe.

4.2.2 Retinyl ester dose preparation

A 10% vitamin-A-palmitate solution (NovaSOL® A) was received from AQUANOVA AG, Darmstadt, Germany. NovaSOL® A contains polysorbate 80 (E433, polyoxyethylen-sorbitant-monooleat), CAS-Nr. 9005-65-6, ascorbic acid (E 300), CAS-Nr. 50-81-7 and mixed tocopherol (E306). NovaSOL® A is a solubilisate. This means that a liposoluble product was made water-soluble using micelle technology (nano-encapsulation into a micelle structure). The micellar structure has a size of less than 10 nm and a molar mass distributed between 2e+5 and 6e+5 g/mol. The substance contains at least 170.000 IU/g VA. Prior to application, one millilitre of NovaSOL® A 10% was solved in 33 ml of normal saline hereby preparing 5000 IU/ml solution. This solution was subsequently protected from light, warmed to body temperature (37°C) and finally applied to the animal.

4.2.3 Retinyl ester delivery

At half an hour of life, the animals randomly received 5000 IU/kg body weight of retinyl palmitate diluted in saline (5000 IU/mL) either via the umbilical venous catheter (“i.v.”) or via a gastric tube placed within the endotracheal tube (“e.t.”). The vitamin A solution was given directly after an endotracheal surfactant bolus. Animals that were not treated served as the control group.

4.2.4 Sample collection, storage and preparation

During the animal experiment, arterial blood samples of 1 ml for serum ROH and RP analysis were taken immediately prior to treatment (pre- treatment value) and at 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h and at the end of the experiment. Due to technical reasons there was no serum available for data analysis in one of the three control animals. Blood was taken in 1.1 ml small containers (microtubes), protected from light through wrapping in aluminium foil and placed on ice. After the experiment, those samples were placed in a centrifuge and spun for 10 minutes at 1300 xg to obtain serum. The serum probe was then promptly frozen at -80 degrees Celsius. Subsequently, the samples were sent to the laboratory on dry ice in a Styrofoam box and stored at -80°C prior to analysis. Light protection was maintained throughout.

After the experiment, lambs were euthanized with an overdose of pentobarbital after 3.5 +/- 0.5 h. After necropsy, solid organs (lung, liver, kidney) and urine were sampled and immediately snap frozen. All specimens were shielded from light and preparation steps were performed on ice and under yellow light. Subsequently the specimens were placed in light-tight Styrofoam boxes and sent for analysis.

4.2.5 Medication and equipment tables

Table 1: Medications used during experimental design

Fodder	Common grass and fodder by HAVENS Graanhandel NV, Vierlingsbeek, Netherlands
Betamethasone	Celestone®, Schering-Plough, North Ryde, New South Wales, Australia
Midazolame	Midazolam Actavis®, Hafnafjordur, Iceland
Ketamine	Nimtek, Eurovet®, Bladel, Netherlands
Surfactant	Poractant alfa 100 mg/kg, Chiesi Pharmaceuticals, Parma, Italy
Phenobarbital	Euthasol 20%, AST Beheer BV, Oudewater, Nederlands

Table 2: Equipment used during experimental design

Endotracheal-Tube (ETT)	Microcuff® 4.5 mm, Kimberly-Clarke, Zaventem, Belgium
Gastric tube	6 Fr., Vygon, Ecouen, France
Umbilical arterial, venous catheters	Argyle®, 3.5 Fr., Covidien, Tullamore, Ireland
Scale	Sartorius balance type ICI6000S
Heated Incubator	IW930 Series CosyCot™ Infant Warmer, Fisher & Paykel Healthcare, Auckland, New Zealand

Infant Ventilator	Babylog 8000, Draeger, Luebeck, Germany
Microtubes	Serum Gel with Clotting Activator, Saarstedt, Nümbrecht, Germany

4.3 Retinol and retinyl palmitate analysis

4.3.1 High-performance liquid chromatography

All sample preparation steps were performed cooled on ice and under amber light for degradation protection. Vitamin A analysis was performed using high-performance liquid chromatography (HPLC) (Table 4).

For tissue samples, BHT was used as an antioxidant. 100 ppm BHT was added to n-hexane and 10 ppm BHT was added to ethanol. Samples of between 100 mg to 500 mg were measured on an analytical balance in a small tube (Falcon®). 2 ml of PBS (Table 3) and 2 ml of ethanol were added, vortexed and subsequently homogenised 20 seconds using an Ultra-Turrax® disperser on position three of the regulator. Afterwards, the disperser was disassembled and thoroughly cleaned using water and ethanol. For extraction, 5 ml n-hexane was added; the sample was vortexed and rocked for 10 minutes on a nutator before centrifuging the sample at 10°C with 4500 g. The hexane supernatant was removed and placed in a 50 ml flask. The hexane extraction was performed three times. The pooled hexane phases were evaporated using a rotary evaporator (SpeedVac). The water quench temperature was < 25°C.

The liver samples were solved in 2.5 ml HPLC buffer and lung samples in 1 ml HPLC buffer. The HPLC mixture was 80% acetonitrile and 20% THF. Two aliquots of each probe were filled into HPLC vials. One aliquot was used for HPLC measurement; the other aliquot was used as a retained sample and frozen at -80°C.

Serum and urine samples were tempered at 20°C. 200 µl of ethanol were added to 200 µl in a micro-reaction container (Eppi or E-cup) and vortexed. Afterwards, 500 µl N-hexane were added, and the sample was vortexed again before it was centrifuged for 1 minute at 16000 g. The supernatant (hexane phase) was pipetted and placed into a micro-reaction container. The pooled hexane phases were evaporated using a rotary evaporator (SpeedVac), subsequently re-suspended in 200 µl HPLC buffer and centrifuged for 1 minute at 16,000 g. The supernatant was transferred to an HPLC vial.

In order to determine Vitamin A concentrations in tissue and blood samples by means of HPLC, a Prominence Modular HPLC with 2 pumps equipped with a Nucleosil 5C18 (300mm x 4mm, 3µm) column, an autosampler and a photo-diode array detector (SPD-M20A), wavelength set at 325nm were used.

HPLC results were individually checked for plausibility and confirmed by repeated measurements when found to be outside the linear calibration slope. For tissue samples the *Limit of Detection* (LOD) for ROH was at 13 ng/ml and for RP at 47 ng/ml (*Limit of Quantification* [LOQ] for ROH 42 ng/ml and 158 ng/ml for RP). For liquid samples the LOD for ROH was at 44 mM and for RP at 87 nM (LOQ for ROH 145 nM and 291 nM for RP). Due to occasional significant variation between measurements and where there was doubt with regard to the accuracy of measurements these were repeated and individually checked for plausibility. Afterwards, mean values of the repeated measurements were calculated to ensure high levels of data quality.

4.3.2 HPLC chemicals and equipment table

Table 3: HPLC chemicals

HPLC buffer	Acetonitrile (80%), THF (12%), methanol solution (0.1M ammonium acetate in methanol, 8%).
PBS buffer	NaCL (8.0 g/L), KCL (0.2 g/L), Na_2HPO_4 (1.42 g/L), KH_2PO_4 (0.27 g/L), pH 7.5
Acetonitrile	Carl Roth, Karlsruhe, Germany Purity: HPLC Gradient
Methanol	Carl Roth, Karlsruhe, Germany Purity: HPLC Gradient
THF	VWR, Darmstadt, Germany
Standards (retinol, retinolpalmitate)	Sigma Aldrich, Steinheim, Germany Purity: HPLC (>98%)

Table 4: HPLC equipment

Prominence Modular HPLC (Shimadzu Prominence Gradient System) with 2 pumps (LC20AT)	Shimadzu, Kyoto, Japan
HPLC Column: Nucleosil 5C18 (300mm x 4mm, 3µm)	Trentec, Gerlingen, Germany
Autosampler (Sil-20AC)	Shimadzu, Kyoto, Japan
Photo-diode Array detector (SPD-M20A), wavelength set at 325nm	Shimadzu, Kyoto, Japan

4.4 Molecular analysis of lung tissue samples

For expression analysis the MIQE guidelines (BUSTIN et al., 2009) were applied.

4.4.1 RNA isolation

RNA isolation was performed using the NucleoSpin© RNA II kit according to the manufacturer's instructions (Table 8).

For RNA isolation, small tissue samples were obtained from frozen lung tissue using a scalpel and forceps, ensuring a frozen environment throughout for RNA protection. Lysis of cells was performed by using 350 µl of buffer RA1 and 3.5 µl β-mercaptoethanol added to small tissue samples and homogenized using a small mortar. The lysate was then filtrated through a NucleoSpin© filter by centrifuging with 11,000 x g for 1 minute. The filter was discarded, and RNA binding conditions were adjusted using 350 µl 70 % ethanol. In order to bind RNA, the lysate was loaded to the NucleoSpin© RNA Column, which was placed in a collection tube. This was centrifuged for 30 seconds at 11,000 x g, and the column was placed in a new collection tube. Membrane desalting buffer (350 µl) was added, and the sample was once again centrifuged at 11,000 x g for 1 minute to dry the membrane.

DNA digestion as part of the RNA isolation procedure: DNase reaction mixture was prepared in a sterile 1.5 ml microcentrifuge tube and for each isolation 10 µl reconstituted rDNase was added to 90 µl reaction buffer for rDNase. The sample was mixed by flicking the tube. DNase reaction mixture (95 µl) was applied directly onto the centre of the silica membrane of the column and incubated at room temperature for 15 minutes.

Afterwards the silica membrane was washed three times using 200 µl, 600 µl and 250 µl of ready-prepared solutions from the NucleoSpin© RNA II kit and dried by centrifuging at 11,000 x g at set time intervals (twice at 30 seconds, once at 2 minutes). Ribonucleic acid was eluted with 60 µl RNase free water. On some samples this step was performed twice using the originally produced volume in the second step in order to try to yield higher RNA concentrations.

After elution, photometric measurement was performed on the samples to check nucleic acid concentrations for ongoing analysis.

4.4.2 Photometric measurement of nucleic acids

Nucleic acids concentrations can be measured using a photometer (Table 9). Absorbance of light is a natural property of DNA and RNA, and maximum absorption of nucleic acids in a solution occurs at 260 nm. Proteins absorb naturally at 280 nm. The determination of the concentrations follows the Beer-Lambert law.

$$T = \frac{I}{I_0} = e^{-\Sigma \ell} = e^{-\varepsilon \ell c}$$

T = *Transmission of light through a substance*

I_0 *and* I *= Intensity (power per unit area)*

ε = absorptivity of the attenuator

Σ = attenuation coefficient of the substance

ℓ =distance the light travels through the material (path length)

c = concentration of the attenuating species in the material

Varius other molecules besides DNA and RNA absorb light at 260 nmA, and differentiation between RNA and DNA is not possible; this may lead to potentially highly inaccurate measurements. However, detection of contamination of an RNA/DNA solution with proteins is possible. Aromatic amino acids have an absorption maximum at 280 nm, and using the absorbance ratio of 260 nm/ 280 nm, the purity of an RNA/DNA solution can be determined. The ratio should be between 1.8 and 2.0; results below this ratio indicate contamination.

For the photometric measurement of our samples 148 µl of RNA free water was used, and 2 µl of eluted RNA was added. Measurements were performed using the BioPhotometer®.

4.4.3 Agarose-Gel-Electrophoresis

Agarose-Gel electrophoresis was performed on eluted RNA samples to check the quality and integrity of RNA. Nucleic acids are charged negatively due to their sugar-phosphate groups and therefore move towards the anode in the electric field. Small fragments move quicker through a gel matrix than large fragments and therefore separation due to the length of fragments is possible. With non-degraded RNA the 28s and 18s rRNA of cells from eukaryotic organisms are visible as distinct bands with a ratio of 2:1 (28s rRNA : 18s rRNA) in the gel (see Figure **6**).

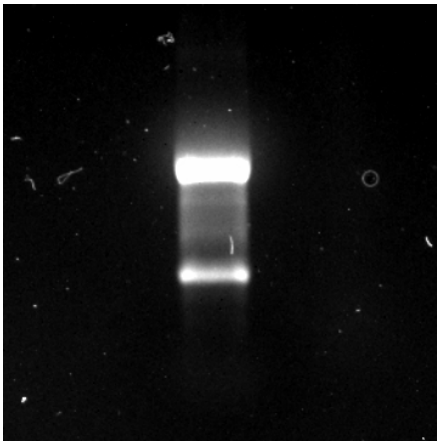
A ratio of 2:1 means that the 28s RNA band is twice as intense (brighter and thicker) as the 18s RNA. If a sample is contaminated with RNase and degradation has occurred, the separation of bands will be washed-out, not sharply defined and less intense.

The gel for the agarose-gel electrophoresis was prepared using agarose, distilled water and MOPS, which were added together in an Erlenmeyer flask, mixed and heated in a microwave until the agarose was dissolved. After cooling the solution down to approximately 45 °C, Formaldehyde 10% was added, and the product was emptied into a gel chamber (with crest).

For the gel-electrophoresis chamber, 500 ml “running buffer” was prepared by diluting 10 x MOPS (Table 8) to 1 x MOPS in RNase free distilled water and added to the electrophoresis chamber.

Sample preparation involved adding 4 µl of RNA to 2 µl “RNA loading buffer” and incubating the samples at 50°C for 20 minutes in a Thermomixer. After incubation, the mix of “RNA loading buffer” and RNA samples was applied to a pocket in the gel. A current of 80 V was applied for approximately 1 hour to separate the bands. The gel was analysed using Imaging System Molecular Imager®, Gel Doc™ XR+.

Figure 6: Gel electrophoresis of an exemplary RNA sample



Two distinct bands are visible; one band appears brighter and thicker (28s RNA band), and the bands are sharply defined.

4.4.4 DNA Digestion

No RNA isolation method can extract RNA that is completely free from DNA contamination. However, it is of crucial importance that, prior to transcribing the available RNA into cDNA, no DNA contamination is present in the samples. If traces of DNA were detectable (see section 5.3.6.8 PCR, Non-RT-PCR) despite DNase digestion during RNA isolation, the sample underwent a DNase digestion procedure using the TURBO DNA-free™ kit according to the user manual. “Rigorous DNase treatment”, which is used for high amounts of nucleic acids, was chosen. Recommended reaction size according to the user manual is a volume of 10-100 µL. 10 x TURBO DNase Buffer, which was added to one-tenth of the reaction volume, and 2-3 µL TURBO™ DNase were gently mixed together. The mixture was incubated for 30 minutes at a temperature of 37°C using the Thermomixer, and 0.2 volumes of DNase inactivation reagent (at least 2 µL) were added to the solution. The reaction was continuously mixed for 5 minutes whilst incubating at room temperature. Afterwards, the samples were centrifuged for 1.5 minutes at 10,000 rpm. The supernatant was pipetted, thereby avoiding introduction of the DNase Inactivation Reagent into the RNA solution. The samples were subsequently moved to a newly-labelled sample container.

4.4.5 QubitTM Assay (broad range) - Determination of RNA Concentrations

Qubit™ fluorometer uses specific fluorescent dyes to determine the concentration of nucleic acids in a sample. Each dye is specific for one type of molecule, e.g. DNA, RNA or protein. It is therefore more precise than those with UV absorbance methods (photometric measurement), which are non-selective. This difference is relevant for calculations preparing for quantitative PCR. The Qubit™ fluorometer uses RNA standards to derive the relationship

between RNA concentration and fluorescence. This relationship is then used to calculate the concentration of the sample.

For the measurement of the RNA concentrations the samples were prepared according to Qubit™ Assay user manual using the Qubit™ reagent, Qubit™ buffer and Qubit™ standards. Measurements were taken using the Qubit® 2.0 Fluormeter.

4.4.6 cDNA synthesis from total RNA (RT-Reaction)

The cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (without RNase inhibitor) according to the manufacturer's instructions. During this procedure random primers are used for initiating cDNA synthesis. This ensures an efficient first-strand synthesis with all RNA molecules (mRNA and rRNA) present in the sample. To commence the procedure 2X reverse transcription (RT) master mix was prepared according to the manufacturer's instructions. Contrary to the manufacturer's instructions, nuclease-free water was not added during the procedure in order to increase the total amount of RNA in the reaction. The solution was incubated using a thermal cycler (Mastercycler gradient) with the specified thermal cycling conditions (Table 5). Afterwards, the samples were appropriately diluted in order to use 10 ng for the PCR reaction.

Table 5: Thermal cycling conditions

Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	∞

4.4.7 Primerdesign

Determination of the primer sequences for gene analysis was performed using the *Applied Biosystems*® database. Firstly, the database was searched for commercially-available expression assays for the genes of interest in cattle. The gene transcripts that had been used for the primer design of these assays were used for a standard nucleotide blast at NCBI. The homologue sequences found for sheep were then used with the Custom TaqMan® Assay Design Tool (Applied Biosystems) to design primers and probes for the genes of interest for sheep expression assays. An alignment was done for sheep sequences (homologue sequences). Ultimately, *Applied Biosystems*® provided the final sequences that were used for PCR analysis in the following four genes: β-Actin, STRA 6, CYP26 and LRAT. β-Actin was used as the endogenous control ("housekeeping" gene).

The following sequences were used for the primer design:

Ovis aries clone ACTB_SLIP_1 beta-actin variant 2 (ACTB) mRNA, complete cds, GenBank: HM067830.1

Ovis aries cytochrome P450 26B1-like (LOC101110922), mRNA, NCBI Reference Sequence: XM_004006067.1

Ovis aries lecithin retinol acyltransferase-like (LOC101121235), mRNA

Ovis aries stimulated by retinoic acid 6 homolog (mouse) (STRA6), mRNA, NCBI Reference Sequence: XM_004018101.1

Primer and probe (-head) combinations were as follows:

- **OVIS_STRA6_F:** 5'-GCTGCTAGTGGGTGTGGTA-3' (forward), 5'-GGAGACGTCCGTGGTGATC-3' (reverse), 5'-CCGCCCTCACCTTC-3' (probe)
- **OVIS_LRAT_F:** 5'-TCAAGAAGAAGGCACTGCTCAA-3' (forward), 5'-GTGCCCAGCAGCTTCTCT-3' (reverse), 5'-CTGTGCCACCTCTTCG-3' (probe)
- **OVIS_CYP26B1_F**: 5'-CGCAGGGCAAGGACTACT-3' (forward), 5'-CTCCTTGCTGCTCTCGATGAG-3' (reverse), 5'-CAGACGCGCTGGACAT-3' (probe)
- **OVIS_ACTB_F**: 5'-CTTCCTTCCTGGGCATGGA-3' (forward), 5'-ACGTCACACTTCATGATGGAATTGA-3' (reverse), 5'-CTGCGGCATTCACG-3' (probe)

4.4.8 Polymerase Chain reaction (PCR)

PCR is used to exponentially amplify a small amount of specific regions of a DNA strand to generate a huge number of copies of DNA of a defined sequence that is present in a particular sample. Thermal cycling and enzymatic replication, which uses short, single-stranded DNA fragments (oligonucleotides) as primers, are crucial for the amplification process. A heat-stable DNA polymerase (e.g. Taq [from a thermophilic bacterium called *Thermus aquaticus*]) is used for enzymatic replication during defined cycles of alternate heating and cooling (thermal cycling).

In this study, PCR was used for three reasons:

1. Non-RT (reverse transcriptase) PCR

   A qualitative check of the RNA samples for DNA contamination was done using Non-RT PCR. If DNA contamination was shown, a further DNase digestion step was performed. RNA without reverse transcription was used as a template that should not yield any PCR product except in case of DNA contamination.

2. Performance of Dynamic range PCR

This is the range over which an increase in starting material concentration results in a corresponding increase in amplification product. Ideally, the dynamic range for real-time PCR should be 7-8 orders of magnitude for plasmid DNA and at least a 3–4 log range for cDNA or genomic DNA. Standard curve dynamic range validation determines what template concentrations are acceptable in a given assay (Reference: Real-time PCR handbook).

3. Quantitative real-time PCR

Quantitative real-time PCR for β-Actin, STRA6, CYP26B and LRAT was performed with the cDNA lung tissue samples. For quality assurance the quantitative real-time PCR was performed in triplicates. For each assay a negative control containing no cDNA template was performed.

For data analysis, comparative quantification, not absolute quantification, was used. In comparative quantification the level of expression for the gene of interest is assayed for up- or down-regulation in a normal (calibrator) sample and one or more experimental samples. Precise copy number determination is not necessary. The technique focuses on fold change compared to the calibrator sample. As a marker of stable gene expression in a given sample, which is independent of the experimental intervention, a housekeeping gene is usually used. In this study, β-Actin served as the housekeeping gene (normaliser sample). Untreated samples serve as calibrator samples. Comparative quantification (Ct) is obtained for expression of the gene of interest from both a test and a calibrator sample. The difference between them is the ΔCt. The fold difference is then simply 2 to the power of ΔCt.

Fold difference = $2^{\Delta Ct}$

In addition, the ΔΔCt method compares results from experimental samples with both a calibrator and a normaliser.

Real-time PCR is different from standard PCR in that the amplified DNA is detected and therefore can be quantified during each cycle in “real-time”, as the reaction continues. In standard PCR, the product of the reaction is only detected at the end of the reaction and only end-point analysis occurs. Real-time PCR uses fluorescent substances for detection of DNA. Real-time PCR is time-saving due to the fact that annealing and elongation occur during the same reaction step at 60°C.

In this study, PCR was performed in triplicates with 10 ng cDNA per 15 µl reaction using Custom TaqMan® Gene Expression Assays from Applied Biosystems on an Applied Biosystems 7500 Fast Real-Time PCR system. Beta Actin served as an endogenous control.

Table 6: PCR reaction mix

Mastermix	7.5 µl
Assay	0.75 µl
RNase free water	4.75 µl
cDNA	2 µl (= 10ng)
Total Volume	15 µl

TaqMan® Fast Advanced Master Mix, Applied Biosystems™ was used to prepare the samples for the PCR reaction.

The following PCR-Program was used:

Table 7: PCR program

7500 Applied Biosystems Real-Time PCR System	Thermal-cycling profile			
	Parameter:	Polymerase Activation	PCR (40 cycles)	
		Hold	Denature	Anneal/extend
	Temp. (C°)	95	95	60
	Time (mm:ss)	00:20	00:03	00:30

4.4.9 Data analysis of PCR results

The Comparative CT Method (ΔΔCT) using Data Assist™ Software v3.0 was used for data analysis. Student t-test was used for comparative assessment.

4.4.10 Chemical and equipment tables

Table 8: Chemicals used for molecular analysis of lung tissue

NucleoSpin® RNA II kit	MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany
Agarose Gel for electrophoresis	0.7 g Agarose, 63 ml Aqua dest., 7 ml 10 x MOPS (3-[N- Morpholino] Propane Sulfonic Acid) mixed and boiled until all ingredients solved, subsequently 3 ml Formaldehyde 10% added and casted into the appropriate gel chamber
Running buffer for electrophoresis	1 x MOPS (3-[N- Morpholino] Propane Sulfonic Acid), see also 10 x MOPS
RNA loading buffer	72 µl Formamide, 16 µl 10 x MOPS, 44 µl DEPC-H_2O (see below), 10 µl Midori-Green direct, 18 µl Glycerol, small amount of "bromphenol blue", (protected from light in 4° refrigerator)
DEPC (RNase free) Water	1 ml of DEPC (diethyldicarbonate) in 1 l of aqua dest.
10 x MOPS	0.2 M MOPS, 0.05 M NaAc (sodium acetate), 0.01 M EDTA, pH 5.5 – 7.0, incubation overnight, autoclaving, protected from light
TURBO DNA-*free*™ Kit	Ambion®, Life Technologies™, Thermo Fisher Scientific Inc., Waltham, MA, USA
Qubit™ Assay	Life Technologies™, Thermo Fisher Scientific Inc., Waltham, MA, USA
High-Capacity cDNA Reverse Transcription Kits for 200 and 1000 reactions	Applied Biosystems™, Life Technologies™, Thermo Fisher Scientific Inc., Waltham, MA, USA

TaqMan® Fast Advanced Master Mix,	Applied Biosystems™, Life Technologies™, Thermo Fisher Scientific Inc., Waltham, MA, USA AmpliTaq® Fast DNA Polymerase, uracil-N glycosylase (UNG), dNTPs with dUTP, ROX™ dye (passive reference), optimized buffer components. Supplied at a 2X concentration.

Table 9: Equipment used for molecular analysis of lung tissue

Fisher Scientific, Power 300, Electrophoresis power supply	Fisher Scientific UK Ltd, Loughborough, UK
BioPhotometer	Eppendorf AG, Hamburg, Germany
Qubit® 2.0 Fluorometer	Invitrogen™, life technologies™
Centrifuge 5415R	Eppendorf AG, Hamburg, Germany
Thermomixer comfort	Eppendorf AG, Hamburg, Germany
7500 Fast Real-Time PCR System	Applied Biosystems, life technologies™
Mastercycler gradient	Eppendorf AG, Hamburg, Germany
Molecular Imager®, Gel Doc™ XR+	Bio-Rad Laboratories GmbH, München, Germany

4.5 Histology

4.5.1 Histological analysis of tissue samples

Post-mortem samples were cut and immediately fixed in formalin by immersion. In the laboratory, samples were separated from water and imbedded in paraffin, and at least 4 histological cuts per organ were performed. Haematoxylin and eosin (H&E) staining was performed on paraffin sections using routine procedures.

Microphotographs were generated using a Zeiss Axiophot microscope with an Olympus camera and software. Light microscopic studies of the anatomy of cells of liver, kidney and brain were performed by two independent examiners. Results were reported descriptively.

Table 10: Chemicals used for histological analysis

Formalin	3.7 % formaldehyde in phosphate buffered saline
Haematoxylin and eosin (H&E) stainings	
Paraffin	

Table 11: Equipment used for histological analysis

Zeiss Axiophot microscope	Carl Zeiss, Jena
Olympus camera (ColorView II™) and software (CellSens Standard 1.8.1)	Olympus, Hamburg, Germany

4.6 Statistical analysis

HPLC data was prepared using Excel™ and analysed using SPSS statistical software™, version 20 (IBM, Armink, NY). In order to compare animal characteristics, retinyl palmitate and retinol tissue levels, the non-parametric Kruskal-Wallis H test was used. When comparing the various groups of interest, the Mann-Whitney U test was performed. The Friedman test was utilised to compare data from retinol and retinyl palmitate serum level measurements. The Student t-test was used for comparative assessment of PCR data. At a p-value of less than 0.05, statistical significance was accepted. GraphPad Prism v5.0 (GraphPad Software, San Diego, CA) was used to draw graphs.

5 Results

5.1 Animal Characteristics

Ten subjects were assigned to three different groups: control (no treatment - three animals), intravenous (i.v.) treatment group (three animals) and endotracheal (e.t.) treatment group (four animals). Animal characteristics, organ weight and physiological parameters did not show significant differences (Table 12 - Table 14). In particular, there were no statistically significant differences (Kruskal-Wallis-H test) with regard to gestational age ($p = 0.624$), birth weight ($p = 0.943$) and organ weight (right lung $p = 0.962$, left lung $p = 0.741$, liver $p = 0.405$, left kidney $p = 0.882$) among the subjects. However, in the e.t. group there was a combined acidosis during the whole ventilation period. This can primarily be attributed to a single animal with pneumothorax and lung bleeding seen in necropsy. This animal showed intrauterine growth restriction (1.8 kg), and the animal needed the highest PIP (40cmH_2O), compared to a median maximum PIP of 23 cmH_2O in all other animals.

Table 12: Animal characteristics I: Age and weight data

Animal characteristics	control (n=3)	i.v. (n=3)	e.t. (n=4)	p
GA (days)	131 ± 1.5	131 ± 1	130 ± 1	0.62
BW (kg)	2.6 ± 0.5	2.3 ± 0.3	2.4 ± 0.3	0.94
LL weight (g)	32.1 ± 9.5	27.3 ± 6.9	33.9 ± 5.6	0.74
RL weight (g)	21.7 ± 6.5	21.7 ± 2.3	23.3 ± 1.7	0.96
Liver weight (g)	39.6 ± 7.1	59.3 ± 15.1	68.1 ± 19.6	0.41
Left kidney (g)	7.7 ± 1.7	7.5 ± 1.4	8.5 ± 1.1	0.88

Data given as mean ± SEM; GA: gestational age; BW: birth weight; LL: Left lung; RL: Right lung

Table 13: Animal characteristics II: Physiological data prior to treatment

Animal characteristics	control (n=3)	i.v. (n=3)	e.t. (n=4)	p
pH	7.12 ± 0.10	7.25 ± 0.05	7.00 ± 0.08	0.08
pCO_2 (mmHg)	68 ± 26	38 ± 6	86 ± 18	0.08
pO_2 (mmHg)	136 ± 101	95 ± 49	125 ± 61	0.82

Data given as mean ± SEM; pCO_2: partial pressure of carbon dioxide; pO_2: partial pressure of oxygen

Table 14: Animal characteristics: Physiological data at the end of treatment

Animal characteristics	control (n=3)	i.v. (n=3)	e.t. (n=4)	p
pH	7.26 ± 0.02	7.18 ± 0.04	7.09 ± 0.08	0.29
pCO_2 (mmHg)	54 ± 2	57 ± 8	64 ± 9	0.76
pO_2 (mmHg)	29 ± 1	54 ± 15	57 ± 6	0.10

Data given as mean ± SEM; pCO_2: partial pressure of carbon dioxide; pO_2: partial pressure of oxygen

5.2 Retinol and retinyl palmitate analysis of serum and tissue samples

5.2.1 Serum ROH and RP analysis

Serum ROH and RP analysis in untreated animals

Serum ROH and RE levels during all selected time-points (pre- treatment and at 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h and at the end of the experiment) in untreated controls were narrowly distributed between 99 nM (minimum) and 284 nM (maximum). Serum retinylpalmitate values were below the detection limit, which was set at 87 nM, in all untreated animals (Table 15 and Table 16). Pre-treatment ROH and RP levels in animals treated with VA intravenously or endotracheally were similar to those measured in untreated controls (Kruskal-Wallis-test, p=0.61 and p=1.00).

Serum ROH and RP values peak immediately between 0.5 and 1.5 hours after intravenous application

Measured serum retinol and retinyl palmitate levels are at the highest between 0.5 and 1.5 hours after intravenous application. Retinol falls sharply shortly afterwards; retinyl palmitate concentrations remain raised without a significant drop (Figure 7A and B). Overall, levels of serum retinol and serum retinyl palmitate after treatment increased significantly in the intravenous group (Friedman-Test, $p < 0.017$) when compared to controls.

Serum ROH and RP values show a steady but significant incline after endotracheal application

Similarly, both serum ROH and RP levels are significantly increased after endotracheal application (Friedman-Test, $p < 0.018$) when compared to controls but demonstrate, in comparison to the i.v. application, a slower, but steady incline without a sudden peak and subsequent sharp drop (Figure 7A and B).

Table 15: Serum ROH values during selected time-points

Group		Mean	SD	Minimum	Maximum	25th	Median	75th
controls	ROH_pre	207	86	146	268	110	207	201
	ROH_05	215	98	146	284	110	215	213
	ROH_10	209	95	141	276	106	209	207
	ROH_15	175	54	137	213	103	175	160
	ROH_20	195	81	137	252	103	195	189
	ROH_final	152	74	99	204	74	152	153
intravenous	ROH_pre	286	135	150	420	150	287	420
	ROH_05	11934	9022	1961	19528	1961	14314	19528
	ROH_10	8527	5995	2669	14650	2669	8262	14650
	ROH_15	3834	1785	2436	5845	2436	3220	5845
	ROH_20	2647	2503	684	5465	684	1791	5465
	ROH_25	1887	23	1871	1903	1403	1887	2794
	ROH_final	1610	1092	657	2801	657	1372	2801
endotracheal	ROH_pre	249	104	170	403	179	212	357
	ROH_05	357	58	302	438	310	344	417
	ROH_10	434	87	336	513	349	444	510
	ROH_15	434	74	380	542	384	407	512
	ROH_20	525	110	362	595	410	572	594
	ROH_25	515	90	459	619	459	468	619
	ROH_final	525	65	436	591	460	536	579

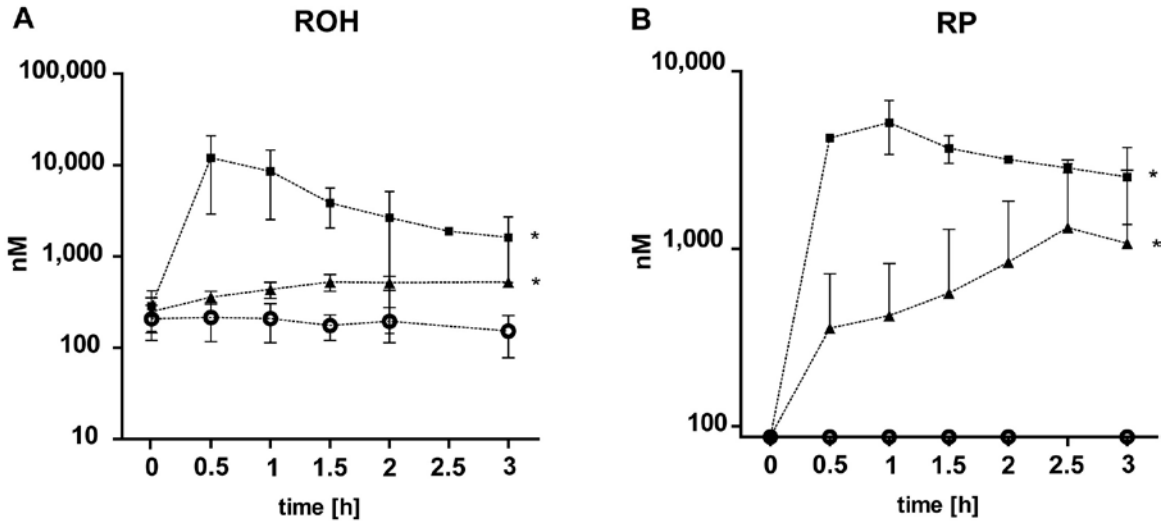
Data given in nM; SD: Standard deviation; 25th percentile; 75 percentile

Table 16: Serum RP values during selected time-points

Group		Mean	SD	Minimum	Maximum	25th	Median	75th
controls	RP_pre	87	0	87	87	65	87	65
	RP_05	87	0	87	87	65	87	65
	RP_10	87	0	87	87	65	87	65
	RP_15	87	0	87	87	65	87	65
	RP_20	87	0	87	87	65	87	65
	RP_final	87	0	87	87	65	87	65
intravenous	RP_pre	87	0	87	87	87	87	87
	RP_05	4214	146	4107	4381	4107	4155	4381
	RP_10	5141	1727	3859	7104	3859	4459	7104
	RP_15	3692	660	3925	4454	3295	3328	4454
	RP_20	3188	146	3020	3289	3020	3255	3289
	RP_25	2858	169	2738	2977	2054	2858	3055
	RP_final	2545	1171	1295	3616	1295	2724	3616
endotracheal	RP_pre	87	0	87	87	87	87	87
	RP_05	357	368	87	866	87	238	747
	RP_10	420	408	87	920	87	336	836
	RP_15	562	728	87	1624	87	268	1330
	RP_20	839	1020	98	2307	123	475	1918
	RP_25	1318	1855	87	3451	87	415	3451
	RP_final	1073	1704	109	3626	143	279	2798

Data given in nM; SD: Standard deviation; 25th percentile; 75 percentile

Figure 7A and 7B: Serum ROH and RP levels before and after treatment



Pre-treatment (A) serum ROH and (B) serum RP in i.v. (square) and e.t. (triangle) treated animals were similar to those measured in untreated controls (circle) and increased significantly in both groups after treatment (*p < 0.05 compared to control).

5.2.2 Tissue ROH and RP analysis

Retinol and retinyl palmitate values are equally distributed in lungs and liver

Retinol and retinyl palmitate analysis in control animals showed retinol and retinyl palmitate levels just at or below the detection limit mean (±SD) 17.3 ng/g±13.0. When comparing the retinol and retinyl palmitate values for different lung and liver areas, no statistically significant difference could be found between retinol and retinyl palmitate distribution in lung apex, lung base and lung hilus (p=0.484 and p=0.428 respectively), nor in liver hilus and periphery (p= 1.0 and p=0.428 respectively) in any of the three study groups.

Retinol and retinyl palmitate concentrations are increased in lungs and liver after intravenous application

After intravenous application of the study substance, levels of ROH and RP increased at a statistically significant rate in lung [ROH (p=0.001); RP (p<0.001)] and liver [ROH (p=0.05); RP (p<0.05)] tissue when compared to the control group (Figure 8 and Figure 9).

No significant changes of concentrations of retinol or retinyl palmitate have been found in the kidney after intravenous administration.

Retinol and retinyl palmitate levels are increased in lungs but not in liver after endotracheal application

After endotracheal application, ROH ($p<0.001$) and RP ($p<0.001$) levels increased significantly in lungs but missed an increase with statistical significance in the liver (Mann-Whitney-Test; $p=0.08$ [ROH] and $p=0.14$ [RP] when compared to controls (Figure 8 and Figure 9).

No significant changes could be shown for kidney retinol or retinyl palmitate levels.

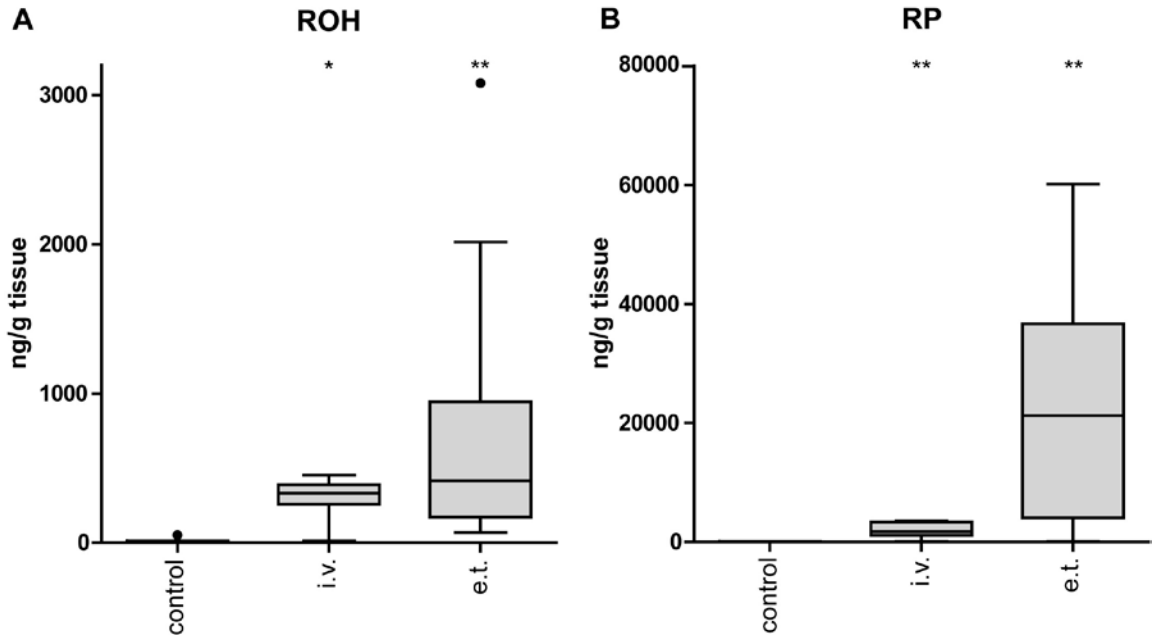
Retinyl palmitate concentrations are increased in lungs and decreased in liver when comparison is made between endotracheal and intravenous mode of application

Retinyl palmitate levels were significantly higher in lungs after endotracheal application than after intravenous application ($p<0.05$), but there was no significant statistical difference in retinol levels ($p=0.931$) between endotracheally or intravenously treated animals.

Liver concentrations of retinol ($p=0.017$) and retinyl palmitate ($p=0.004$) in endotracheally-treated animals were significantly lower than of those animals that received intravenous treatment.

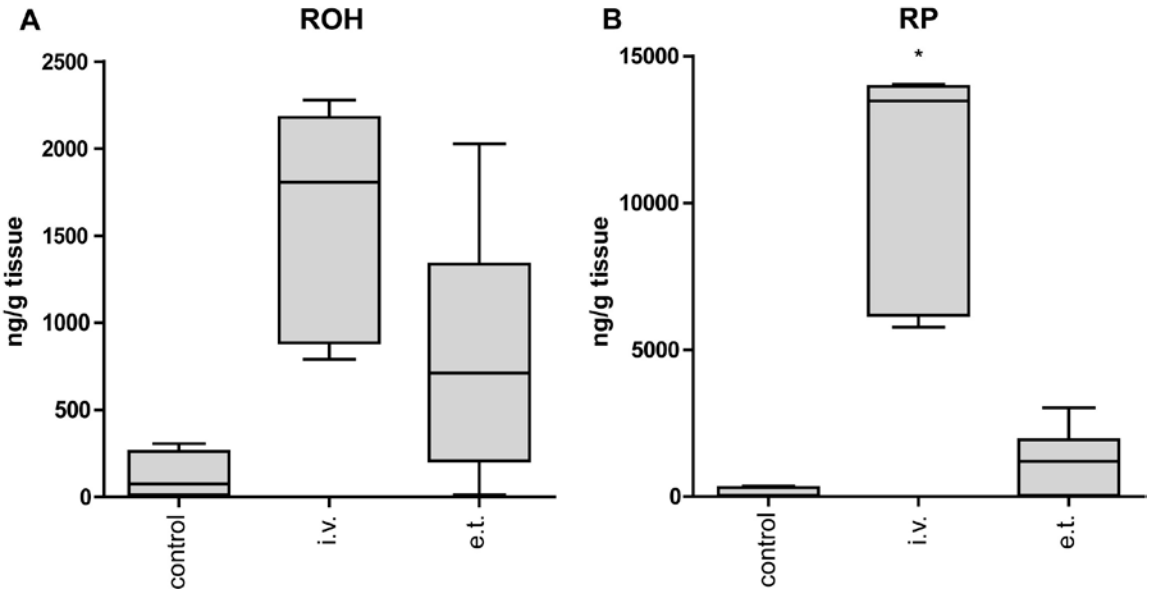
No difference could be shown for ROH or RP levels in the kidneys.

Figure 8A and B: Lung tissue concentration of ROH and RP



In control animals, (A) ROH and (B) RP levels were found to be just at or below the detection limit. Both i.v. and e.t. treated animals showed statistically significant increase of lung tissue concentrations of ROH and RP at autopsy (*p=0.001 and **p<0.001 vs. control).

Figure 9A and B: Liver tissue concentration of ROH and RP



In the liver, (A) ROH and (B) RP levels increased after intravenous application (p=0.05 [ROH] and *p<0.05 [RP]). However, vitamin A increase in the liver after e.t. administration missed statistical significance (p=0.08 [ROH] and p=0.14 [RP].

5.3 Molecular analysis

Intravenously-administered retinylpalmitate induces the expression of CYP26B1

After intravenous application of VA, I was able to show an increased expression of CYP26B1 (p=0.029) in lung tissue when compared to controls after four hours post-treatment (Table 17 and Figure 10). There was no such difference between the endotracheal group and the non-treatment group. No changes of expressions could be shown for LRAT or STRA6 when compared to control animals.

Endotrachealy-administered retinylpalmitate is associated with a reduced expression of LRAT

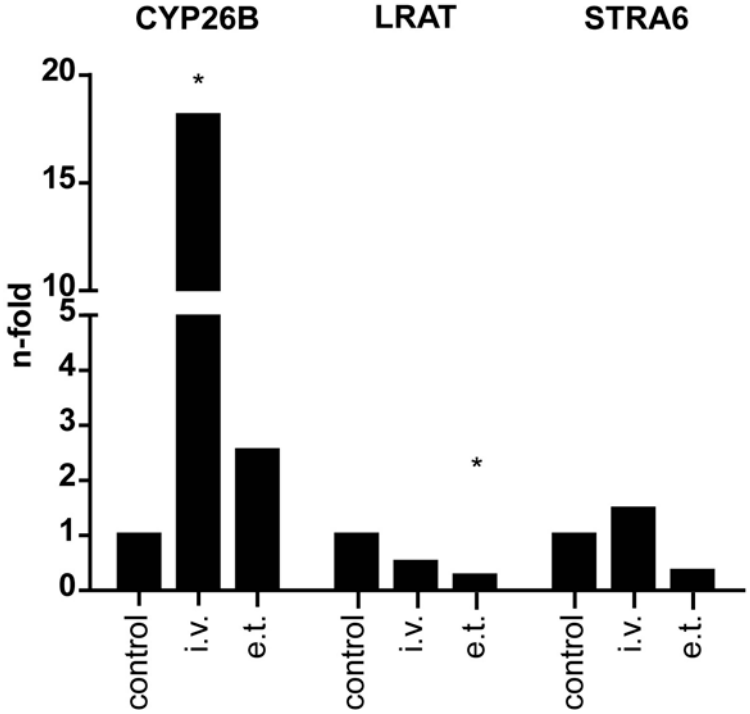
After endotracheal application of RP, LRAT showed a significantly reduced expression when compared to the non-treatment group (p=0.0399). Neither CYP26B1 nor STRA6 showed a change in expression following e.t. treatment (Table 17 and Figure 10).

Table 17: P-values of mRNA analysis in different groups

Assay	Type	e.t.	i.v.	no VA
B-Actin	Selected Control			
CYP26	Target	0.1819	**0.0292***	1
LRAT	Target	**0.0399***	0.218	1
STRA6	Target	0.1462	0.5297	1

P-Values for examined genes after i.v. and e.t. treatment when compared to controls (no VA)

Figure 10: Results of molecular analysis



Molecular analysis of enzymes of vitamin A metabolism showed a significant increase in CYP26 mRNA levels in the i.v. group and a significant decrease in LRAT mRNA levels in the e.t. group (*p<0.05 vs. control).

5.4 Histological analysis

Light microscopic studies performed by two independent examiners on the anatomy of cells of liver, kidney, spleen, large and small intestines and brain did not reveal any difference in the appearance between the treatment and non-treatment groups. A photo documentation of each sectioning was performed in both a wide and detailed view. A collection of photos with representations of each group is included in the appendix.

6 Discussion

6.1 Serum and tissue retinol and retinyl ester levels

The results of this study, which was undertaken in an adequate translational model of preterm birth (KRAMER, 2008b), show that both intravenous and endotracheal supplementation using 5000 IU/kg body weight of a nano-encapsulated, water-soluble retinyl ester (retinyl palmitate) monopreparation increase retinol and retinylpalmitate levels in serum and target organs (liver and lungs) in preterm lambs significantly, though differently, depending on the method of application. From the available data we conclude that it is feasible to use the investigated product as a source for vitamin A supplementation and the modes of delivery as potential routes for vitamin A application in future translational trials.

This is important for clinical reasons since currently-used supplementation regimes do not sufficiently correct vitamin A deficiency, which is assumed to be an important contributing factor to bronchopulmonary dysplasia in the extremely low birth weight infant cohort. There is an urgent need to explore alternative preparations and alternative ways of administration in this group of patients (MACTIER et al., 2011). Traditionally, vitamin A is supplemented in extremely low birth weight infants for nutritional reasons as a lipid-soluble intravenous multivitamin preparation directly after birth. Due to strong evidence that additional supplementation may be helpful for the prevention of bronchopulmonary dysplasia (DARLOW, GRAHAM, 2007), currently available VA multivitamin preparations for intravenous use are neither sufficient nor suitable for increasing dosages. A previously available retinyl ester monopreparation for additional intravenous use, which was studied in a clinical trial (WERKMAN et al., 1994), is no longer available.

Additional intramuscular supplementation, which circumvents the oral and gut route, proved to be promising (ALBERTINE et al., 2010; DARLOW, GRAHAM, 2007); however, due to the painful nature of the procedure, regular intramuscular administrations are not widely accepted in clinical practice (KAPLAN et al., 2010).

Additional vitamin A administration using the oral route has been studied in clinical trials in the past (WARDLE et al., 2001) and is currently the subject of an ongoing multicentre trial (MEYER et al., 2014). Unfortunately, no clinical studies have thus far been shown to have any beneficial treatment effects using this route. The failure of oral supplementation to show any significant treatment benefit in the extremely low birth weight cohort might be the result of poor absorption, primarily due to multiple, either circumstantial factors such as delayed feeding (co-ingestion of dietary fat markedly enhances the intestinal absorption of dietary vitamin A) or other factors related to the infants' gut immaturity that do not allow for the

necessary high postabsorbative retinyl ester concentrations to achieve sufficient uptake in the lungs.

Impaired release of retinol from (already depleted) liver stores which occur in protein-energy malnutrition due to insufficient synthesis of retinol binding protein (BIESALSKI et al., 1999; RAHMATHULLAH et al., 1991) may further contribute to the problem of insufficient vitamin A supply to target organs such as the lungs. The problem of organism maturity is supported by recent findings in a rodent model, where it has been shown that kinetics and distribution of orally supplemented vitamin A greatly differs in neonatal, compared to adult rats (TAN et al., 2015). Extrahepatic vitamin A stores may therefore play a pivotal role (BLOMHOFF, BLOMHOFF, 2006) in sufficient vitamin A supply for crucial target organs such as the lungs.

Alternatively, it has been claimed that inhalative application may circumvent problems of oral administration (BIESALSKI, 1996). Biesalski et al. investigated endotracheally administered vitamin A in rodents with promising results (BIESALSKI, 1996) and subsequently showed in a placebo-controlled trial that vitamin A supplementation by inhalation of retinyl palmitate successfully improved VA status in preschool children (BIESALSKI et al., 1999). Schaffer et al. treated guinea pigs with short-term inhalation comparing a formulation of all-trans retinoic acid and placebo and found a rise of all-trans retinoic acid levels in lung but not liver or plasma (SCHAFFER et al., 2010).

Subsequent clinical studies using aerolized VA preparations further demonstrated those clinical benefits (BIESALSKI et al., 1999; KOHLHAUFL et al., 2002). However, to the best of our knowledge, no such trials have thus far been conducted in the extremely low birth weight neonatal population, presumably due to the lack of appropriate preparations and due to the high ethical standards that would be required for any clinical study in the neonatal population.

The data shows that both methods, endotracheal and intravenous supplementation of a retinyl palmitate preparation, increased serum levels of retinol and retinyl ester at a statistically significant rate when compared to controls. Considering animals that were treated using the e.t. route, this means that the substrate was not only deposited locally within the respiratory tree or the extracellular space but must have also been taken up by the respiratory endothelium and distributed within the organism hereby bypassing the liver's first pass effect.

This is important since it has been postulated that the cells of target organs such as the lungs, kidneys and intestine may store retinyl esters in lipid droplets (NAGY et al., 1997), and extrahepatic storage of retinyl esters may have an important function for local supply of vitamin A to organs with high demand (BLOMHOFF, BLOMHOFF, 2006). Those extracellular

stores may further contribute to the synthesis of active retinoid metabolites (BLOMHOFF, BLOMHOFF, 2006). From our results we conclude that not only intravenous delivery but also endotracheal delivery therefore has the potential to increase extrapulmonary and extrahepatic stores. This has important relevance for the premature organism since not only the lungs are affected by vitamin A deficiency but also other organs, such as the eyes, where earlier studies have shown a trend in reduction of the incidence of retinopathy of prematurity (DARLOW, GRAHAM, 2011; SHENAI et al., 1987). In our sample, however, we have not been able to show any vitamin A deposition within the kidneys. This remains a surprising result, not only in view of renal clearance but also in view of the fact that the kidneys are assumed to have their own storage capacity. Future trials should address the issue of substance distribution, not least because we used a water-soluble, nano-encapsulated product, and the whereabouts of the substance is of crucial importance, mainly but not solely out of toxicological concerns.

In this study, vitamin A was administered in combination with surfactant in the preterm lamb model with induced lung injury (KRAMER et al., 2008). With the exception of one animal that has shown a pneumothorax and lung bleeding in necropsy, no further adverse effects were seen during the clinical phase of the intervention. Nevertheless, future studies need to consider the results by Bronshtein et al. who showed 40% lower surface activity when vitamin A was combined with surfactant compared to surfactant alone (BRONSHTEIN et al., 2009).

The results of this study have confirmed that the studied substance has properties that are thought to be advantageous over currently available substances. As a water-soluble retinyl ester monopreparation, it allowed for exact weight-adjusted dosing and targeted delivery. Preparation and intravenous as well as endotracheal administration of the substance was easy and comparable to other medications in the clinical environment. Due to its property of water solubility, administration via the endotracheal route when combined with surfactant was uncomplicated. It is perceivable that the substance could also be used for nebulisation, as was previously done in a clinical trial with a different retinyl ester preparation by Biesalski et al. (BIESALSKI et al., 1999).

Nonetheless, it is important to consider the various principle options for delivering vitamin A, which are either delivery by its storage form as retinyl ester preparation as in this trial, among others (BIESALSKI, 1996), or in its active form as retinoic acid (SCHAFFER et al., 2010), or as a combination of both (WU, ROSS, 2010). Retinyl ester preparations may be more advantageous over other supplementation forms such as retinoic acid due to their physiological properties: it is known that retinyl esters act as storage forms, and their local

accumulation and utilization prior to birth is of crucial importance for lung development, in particular during the stages of alveolar septation and for surfactant production (GEEVARGHESE, CHYTIL, 1994; GEORGE, SNYDER, 1997; HIND et al., 2002b; MCGOWAN et al., 2000; MCGOWAN et al., 1995; ROTH-KLEINER, POST, 2005; SHENAI, CHYTIL, 1990; THEBAUD et al., 2001). It is known that in-utero alveolarization starts around the 36th gestational week (GALAMBOS, DEMELLO, 2008), and retinyl esters accumulate in the lungs in the 3rd trimenon. Those stores are being depleted immediately prior to term birth (GEEVARGHESE, CHYTIL, 1994; MASUYAMA et al., 1995) in order to provide retinoic acid that in turn induces lung maturation (HIND et al., 2002b). Preterm birth, by definition, occurs at an earlier stage highlighting the need for early provision of sufficient vitamin A stores. Vitamin deficiencies, including an insufficient vitamin A status at earlier gestational age have been associated with adverse outcomes in skeletal and pulmonary health (DOKOS et al., 2013). Thus the ongoing and sufficient repletion of those vitamin A storing cells in the developing lung is of crucial importance since this is seen as an important factor for long-term lung health (BIESALSKI, 2011; DIRAMI et al., 2004; SHENAI, CHYTIL, 1990).

In contrast, any direct supplementation with vitamin A active metabolite, retinoic acid, may only provide short-term effects and may present the additional problem of up-regulation of retinoic acid inhibiting enzymes such as CRABP or CYP26 system (LUU et al., 2001; MCGOWAN et al., 1995; SCHAFFER et al., 2010) and therefore may subsequently result in diminished retinoic acid effect. An alternative approach, as suggested by James et al., may be the supplementation of a combination of retinyl ester and retinoic acid together (VARA). This has proven to have synergistic effects on lung retinyl ester concentrations and has attenuated the hypoxia-induced lung injury in the newborn mouse model (JAMES et al., 2010). This may be a promising approach, and future studies should carefully consider whether to supplement vitamin A as retinyl ester or retinoic acid preparations alone or, in order to possibly enhance their effects, in combination.

Our results demonstrated that the topical application of retinyl esters using the endotracheal route lead to significant rises of retinol and retinyl palmitate concentrations in the lungs. Our results are in line with the results of previous translational and clinical studies that have demonstrated a sufficient VA supply when administering retinyl esters directly to the sensitive target tissue. All studies demonstrate an increase of intracellular stores of retinol as well as an improvement of overall vitamin A status (BIESALSKI et al., 1999; BIESALSKI, 1996; KOHLHAUFL et al., 2002). This is important since a pre-existing vitamin A deficiency in extremely low birth weight infants (BRANDT et al., 1978; SHENAI et al., 1981; SHENAI et al., 1985a) and its contribution to the development of bronchopulmonary dysplasia is well documented (BRANDT et al., 1978; HUSMANN et al., 1992; INDER et al., 1998; SPEARS et

al., 2004) It has been shown that vitamin A must be significantly stored in the lungs in late gestation before alveolarization and surfactant synthesis can commence (FRASLON, BOURBON, 1994), and supplementation with vitamin A reduces the mortality associated with bronchopulmonary dysplasia (SHENAI et al., 1987; SHENAI et al., 1992). In particular, surfactant synthesis is crucially important for adequate lung function in premature infants. If local stores within the lungs are depleted, as is the case in vitamin A deficiency, histopathological changes, including keratinizing metaplasia, that are similar to those of bronchopulmonary dysplasia are seen (CHYTIL, 1996). It has been postulated that retinyl ester deposits in the respiratory tract act as local stores from which retinol and subsequently retinoic acid can be derived following hydrolysis of retinyl ester (BIESALSKI, 1990; BIESALSKI et al., 1990; ZACHMAN et al., 1992). Furthermore, previous studies have even shown that epithelial cells can directly take up retinyl esters without prior hydrolysis (BIESALSKI, NOHR, 2004), and sufficient local vitamin A stores help to induce a normal phenotype in cases of damaged epithelial cells (BIESALSKI, NOHR, 2004; SOBECK et al., 2003). Within the cell, retinyl esters can be hydrolysed to retinol and subsequently converted to retinoic acid. However, thus far a process whereby extracellular retinyl ester can be utilized for retinoic acid formation is not known. Therefore it is prudent to show in future studies that the deposited retinyl esters are indeed taken up intracellularly and are not only deposited within the extracelluar space.

Further, our data shows not only an increase of retinol and retinyl palmitate levels in lung tissue for endotracheal application but also for i.v. application. Following i.v. application liver retinol and retinyl palmitate levels likewise increased, but those missed statistical significance for e.t. administration.

This result requires careful considerations: although, on the one hand, vitamin A supplementation in the neonatal cohort is principally considered to be nontoxic at recommended dose (AGOSTONI et al., 2010; EUROPEAN FOOD SAFETY AUTHORITY (EFSA), 2006; INSTITUTE OF MEDICINE. FOOD AND NUTRITION BOARD, 2001; KOLETZKO et al., 2005; MIRTALLO et al., 2004; ULBRICHT et al., 2012; VAN EYKEN et al., 2012), toxic effects of long-term use of high doses of vitamin A can result in retinyl ester accumulation within the liver and later liver dysfunction and potential cirrhosis since a risk for vitamin A toxicity remains when given in high amounts (PENNISTON, TANUMIHARDJO, 2006). It seems perceivable that particularly premature neonates are vulnerable since the metabolic capacity of the liver is still immature and may be further compounded by a number of other drugs used in this population such as antibiotics and antifungal, among others. Therefore, an effective application mode, such as inhalation or nebulisation, that circumvents

the liver metabolism but still results in the desired clinical effect within the main target organ may be more advantageous by preventing potential systemic side effects.

On the other hand, however, it is known that vitamin A stores of extremely low birth weight infants, including those of the liver are highly depleted. The liver serves as the main vitamin A storage organ, mainly in hepatic stellatae cells with an ability to control excretion of retinol which ensures a steady blood-plasma retinol concentration despite normal fluctuations in vitamin A intake (BLOMHOFF, BLOMHOFF, 2006). Increasing those stores may provide long-term advantages as long as no toxic effects are observed. So far, and in favour of this argument, unphysiological formation of retinoic acid after retinyl ester supplementation does not seem possible due to the strictly controlled metabolism (BIESALSKI, 2011).

Interestingly, although we tested a water-soluble vitamin A preparation, no significant levels of retinol and retinyl palmitate were shown in the urine or the kidneys of treated animals. This favours the conclusion that, during the study period, no significant losses occurred via glomerulofiltration or diuresis, and the substance was maintained within the organism. It is known that retinol under physiological conditions is bound to retinol binding protein (KANAI et al., 1968) and coupled to transthyretin to avoid renal clearance (BIESALSKI, NOHR, 2004). Future studies need to determine whether this mechanism remains intact or where exactly the substance was deposited and whether, in view of its micellar structure, indeed any other risks exist.

6.2 The genetic markers

Following on from the data on distribution of retinol and retinyl palmitate, we continued to explore, in line with several other working groups and current thought, any possible traceable early biological effects through detection of mRNA levels of certain genes.

Retinoic acid exerts its influence on cells through binding with nuclear retinoid receptors (RAR). These receptors build heterodimers with RXR proteins and influence target and subsequently downstream genes (BASTIEN, ROCHETTE-EGLY, 2004), of which STRA6, LRAT and CYP26 are considered major genes for regulating vitamin A metabolism, among others (PETKOVICH, 2001; ROSS, 2003; WU, ROSS, 2010). In line with those previous studies we decided to explore the mRNA levels of STRA6, LRAT and CYP26B1 (SCHAFFER et al., 2010; WU, ROSS, 2010).

In this study, we did not find increased levels in lung tissue of STRA6, a widely expressed multitransmembrane protein in vitamin A dependent tissue that mediates the bi-directional cellular uptake of retinol through retinol binding protein from plasma and extracellular fluids into cells (KAWAGUCHI et al., 2007). Our results, though obtained through a different route

of administration and in a different animal model, are in keeping with the findings by Wu and Ross, who showed that when vitamin A was given orally and not in combination with retinoic acid, there was also no increase in expression of STRA6 mRNA (WU, ROSS, 2010) in lung tissue. Besides the obvious concern that the study timeline may have been too short to see an effect, it is also perceivable that the administered retinyl esters were directly absorbed into cells, hereby bypassing the common mechanism of cellular uptake via retinol binding protein. Supportive of this argument is a previous study by Gerlach et al. that demonstrated direct uptake of retinyl ester into different tissues (GERLACH et al., 1989). Wu and Ross have, however, demonstrated 3-4 times higher expression of STRA6 when vitamin A was given with retinoic acid. They claim that the elevated level of STRA6 mRNA at 6 hours points towards an increase in retinol uptake from the extracellular RBP-retinol complex into the neonatal lungs via the STRA6 receptor. In their view, this could be a mechanism for the rapid response of the neonatal lungs to acidic retinoids (WU, ROSS, 2010). It remains unclear in which direction the flow of retinol moves, and future studies that include the administration of a combination of vitamin A and retinoic acid may consider measuring transthyretin, which is needed for the RBP-Retinol complex to function in direction of cellular uptake. If transthyretin were also to be elevated this might suggest a directional flow towards the cell, implying that the lung cells are in need of more active retinoids.

We found, however, a statistically significant reduced expression of mRNA levels of LRAT in lung tissue of animals that were treated endotracheally when compared to untreated controls. Animals who were treated intravenously did not show the same statistically significant effect of LRAT expression in lung tissues. LRAT is responsible for catalysing the esterification of retinol into retinyl esters and is variably expressed with vitamin A status. It has been shown that lung LRAT mRNA levels closely correlate with LRAT enzymatic activities (MATSUURA et al., 1997; ZOLFAGHARI, ROSS, 2000). Our results are in contrast to results by Wu et al. who demonstrated that both lung retinyl ester concentrations and LRAT expression increased significantly after 6 hours following oral supplementation in the rodent model (WU, ROSS, 2010). It is perceivable that the cellular response is clearly different depending on the application mode. As in our case, we speculate that by decreased expression of LRAT the esterification pathway is turned off, indicating intracellular saturation or excessive supply of the VA storage forms within the cells. This could further support the notion of carrier-independent uptake of retinyl esters into the cells and be indicative of a cellular saturation with retinyl esters. The interpretation of our results is challenging since both methods of application, i.v. and e.t., increased retinol and retinyl esters levels in lung tissue but only supplementation via the e.t. route showed a statistically significant change in LRAT mRNA levels. Overall, absolute levels in ng/g tissue of retinol and retinyl palmitate, although not statistically significant, appear to be higher in the e.t. group than in the i.v.

group. It is unclear whether this is a sufficient explanation for the observed differences. It would allow for the speculation that cellular response may depend primarily upon the level of substrate and secondarily on the route of administration. Cellular responses may therefore be noticeably different when using different application methods. Nevertheless, despite the short time-period of the trial intervention, any statistically significant change of expression of mRNA may point towards an early cellular response to a build-up of vitamin A stores within the cells.

As a third genetic marker we explored the mRNA expression of CYP26B1, an enzyme system that catalyses the oxidation of retinoic acid to non-toxic metabolites. It is assumed that it plays a major role in vitamin A catabolism and detoxification and serves as a cell protector (WU, ROSS, 2010). CYP26B1 increases with a rise in the concentration of retinoic acid (PETKOVICH, 2001) and is thought to be the cell's response for detoxification of retinoic acid (DAS et al., 2014). CYP26B1 is maintained at very low levels in vitamin A deficiency (WANG et al., 2002; YAMAMOTO et al., 2000).

The results of this study have shown an early increase of mRNA levels of CYP26B1 in lung tissue in the i.v. group but not in the e.t. group. The assumption is that an early expression of CYP26B1 may be an indication of a high influx of potentially toxic retinoic acid. However, this interpretation may not be as straightforward since it is not perceivable why potentially more toxic substance should have built up when, in comparison, relatively less substrate was found in lung tissue of i.v. treated animals when compared to e.t. treated animals.

From the results it may be only possible to draw the conclusion that differences in the route of application may play an important role with regard to the safety profile. Nevertheless, the possible increased expression of CYP26 after intravenous application of retinyl esters may be concerning since it may signify possible intracellular toxicity. Those concerns should be investigated in future studies.

6.3 Limitations

This study was conceptualized as a feasibility study and is limited by the small sample size and the short duration of the study protocol. Specifically, the short duration of the study protocol does not allow for any conclusions with regard to long-term or disease-modifying aspects of the intervention.

Further, no conclusion can be drawn from this study regarding safety aspects of the investigated substance. Although the risk to human health is thought to be minor, little is known about the adverse effects on overall health of nano-encapsulated medication.

With regard to specific organ effects in this study, we observed a pulmonary complication in one animal in the e.t. group. This animal was unusually small for gestational age and required higher ventilation settings in contrast to all other animals. Therefore, individual risk factors may have played a role, although it cannot be ruled out, that the intervention may have been a contributing factor for the complication.

There may be less physiological active intracellular or extracellular vitamin A lung content in this study than presumed from the data. Overestimation of active substance may occur since the likelihood of contamination by deposits within the tracheal tree is high. To overcome this concern future research may consider obtaining additional data on effect such as genetic expression of further relevant genes or proteins in order to show the metabolic effects of vitamin A in more detail.

6.4 Futures studies

Future studies should focus on both the intervention and/or on pharmacokinetics and the disease-modifying aspect of the substrate.

Initial data on this novel supplement is promising and warrants ongoing pharmacokinetic studies to determine exact kinetic and safety data. Future pharmacokinetic studies may not only consider whether to apply this substance using the inhalative or nebulised route but also consider whether to use retinyl ester preparations alone or in combination with retinoic acid. With regard to inhalation one should be aware that there is a risk that inhalation of pharmacological agents may also result in poor delivery and distribution within the respiratory tract (LIPWORTH, 1996) and therefore well-conceptualised trials addressing those concerns are warranted. As outlined above, it has been postulated that a combination (VARA) of vitamin A in the form of retinyl esters and its active form, retinoic acid, may be more beneficial than vitamin A alone (WU, ROSS, 2010). This approach may therefore be considered when planning future studies.

Because extremely low birth weight infants regularly receive multiple different medications, any interaction between those and a potential vitamin A supplementation must be considered. Singh et al. states that in newborn neonates who require surfactant replacement therapy, providing vitamin A with surfactant may serve as a less-invasive mode of delivery than intramuscular delivery (SINGH et al., 2010). However, when considering applying vitamin A via the tracheal route, either by topical application or by inhalation, any interaction with surfactant should be examined, and it may be more suitable to administer vitamin A and surfactant separately in view of their pharmacological interactions (BRONSHTEIN et al., 2009). Reassuringly, no influence on the effect of therapeutic surfactant application was

demonstrated in an earlier study after endotracheal vitamin A application in lavaged piglets (SINGH et al., 2010).

Further, it is particularly important to consider any interaction between vitamin A and vitamin D since both build a common receptor complex (VAD-RXR). Zhang et al. were able to show that there is extensive allosteric communication throughout this VAD-RXR complex. They suggest that the ligand itself may influence the DNA binding properties of this macromolecular complex, and they hypothesize that different classes of ligands may differentially alter the DNA binding domains (ZHANG et al., 2011). This opens up a wide range of molecular research opportunities in this field and the interaction between vitamin A and vitamin D, and their influence on cell proliferation and differentiation should be investigated in future studies.

It will continue to be important, when exploring this substance further, to look for both any disease-modifying aspects as well as toxicological effects or unwanted storage sides. Future studies may therefore be conceptualised not only to address the influence of the therapeutic intervention on the primary target organs such as lung and liver but also to consider obtaining data on vitamin A distribution in organs such as the brain or the visual system. So far, simple histological data did not reveal any obvious detrimental effect on brain tissue samples and may therefore be reassuring with regard to the safety profile. Nevertheless, more studies are needed to address those issues. Further, the immune system may play a pivotal role as well (AAGE et al., 2015) and therefore potential clinical studies must observe any unwanted long-term effects, including effects on the developing immune system.

6.5 Conclusion

The purpose of this study was to investigate a new substance for its potential use as a substrate for vitamin A supplementation in the extremely low birth weight infant cohort using the preterm lamb model. I conclude that the newly tested substrate is feasible for both intravenous and endotracheal weight-targeted vitamin A supplementation and should continue to be investigated further in future trials in order to assess practicalities with regard to the mode of application and to explore this substances' kinetics and safety profile as well as interactions with other medication such as surfactant among others. The study contributes to ongoing research activities in the field of vitamin A supplementation for the prevention of bronchopulmonary dysplasia in the extremely low birth weight cohort by adding data about the differences in vitamin A distribution, namely different rises of systemic and tissue bond vitamin A levels, and about differences in metabolic effects, namely different changes in the expression of mRNA of retinoid homeostatic genes after intravenous and endotracheal application. The results support the notion that vitamin A metabolism can be influenced through timely and appropriately-placed supplementation of retinyl ester.

Although this study has a number of limitations impacting on its generalisation, in my view, impulses are given to continue to investigate the substance's direct application to the target organ, the lungs. Results from this and previous trials are promising and bear the potential to encourage future researchers to explore in particular the endotracheal or less invasive topical application routes, such as nebulisation, further.

Finally, clinical trials and safety studies using appropriate substrates are warranted to translate the findings of animal studies like this one into human benefit with regard to biological and long-term effects for the benefit of a very vulnerable patient population such as very low birth weight infants.

7 References

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8 Publications

The results of this study were presented at the GNPI scientific meeting in Frankfurt on the 3rd of June 2016 by myself during an oral presentation with the title “Endotracheale vs. intravenöse Gabe eines wasserlöslichen, nano-verkapselten Vitamin A Präparates – Pilotstudie in frühgeborenen Lämmern“.

The study, titled “Short term effects of endotracheally vs. intravenously administered nano-encapsuled, water-soluble vitamin A in preterm lambs”, is currently under review for publication at the Journal of Nutrition.

9 Acknowledgements

I would like to thank foremost my wife, Donna Alley-Wahl, for all of her support and patience while conducting this research study, for looking after our children when my time was short and for her invaluable advice, support and editing skills whilst drafting my dissertation script. My children also deserve a specially thank you for their patience and understanding while I was sitting at my desk for hours with my headphones on.

I would further like to thank Prof. L. Gortner for giving me the opportunity to conduct this study under his supervision and for his time, expertise and support during this research project. I am grateful for an excellent learning experience for my own professional and personal development.

Further I would like to thank my colleagues at Nottingham University Hospitals, in particular Dr. Craig Smith, Dr. Bernard Schoonakker, Prof. J. Dorling and Prof. H. Budge for their invaluable support when giving me the opportunity to take on a research post as part of my ongoing training (OOPR).

Our research projects concerning vitamin A in preterm neonates would have not been possible without the support of the DFG (Deutsche Forschungsgemeinschaft; ME 3827/1-1), through which I was originally appointed as study coordinator of a clinical multicentre study: NeoVitaA EudraCT No. 2013- 001998-24. This project enabled me to network and motivated me to collaborate with other colleagues, ultimately leading to the inspiration to conduct a research project with a new substance in an animal model.

I am very grateful for the support of Prof. Kramer and his team, who conducted the translational trials in the preterm lamb model and for the support of Prof. Biesalski and Prof. Nohr and their team for conceptual advice and the vitamin A analysis. A special thank you also to Prof. Tschernig for his advice and expertise as well as his investigation of the

histological samples. The tested vitamin A preparation (NovaSOL®A 10%) was a gift by AquaNova ®, Darmstadt, Germany and free of charge. Here I would like to thank Dariush Behnam for his generous support. The surfactant (Curosurf 80 mg/mL®) was gifted by Chiesi Farmaceutici, Parma, Italy, and also free of charge.

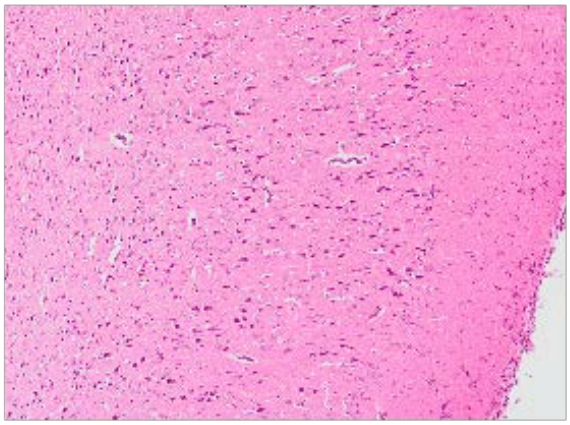
Throughout this project I worked very closely with Domink Monz, Erol Tutdibi and Matthias Hütten. Their help and advice was invaluable while I was conducting my own laboratory work, performing the data collection, and analyzing and interpreting my data. I would further like to thank A. Koza, E. Maurer, and I. Lang for excellent technical assistance and support. Last but not least I would like to thank Nadine Stahl for her patiently conducted organisational support during the project and her assistance in formatting and reviewing the script.

10 Figures

11 Tables

12 Appendices

Figure 11: 80R - no VA



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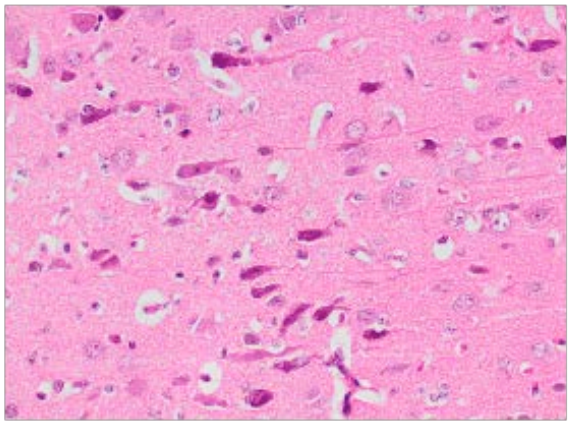

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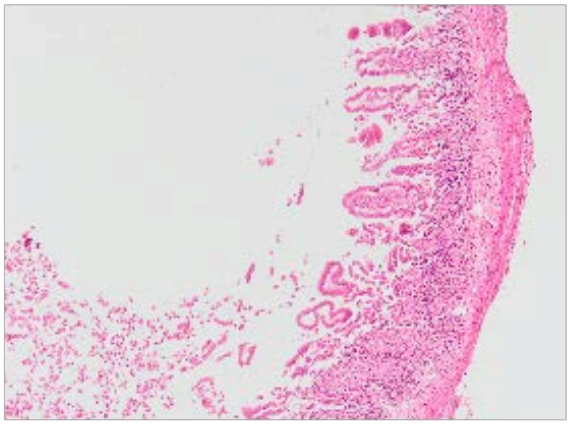

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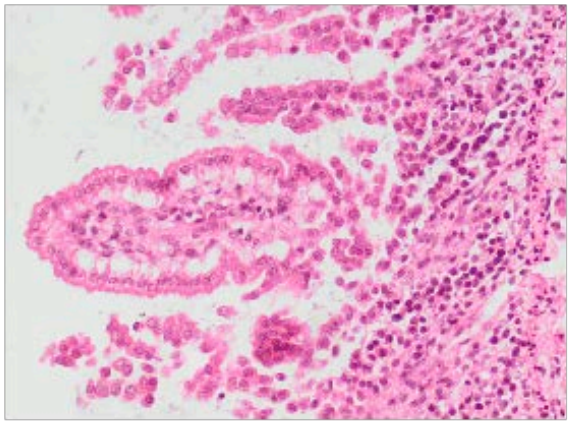

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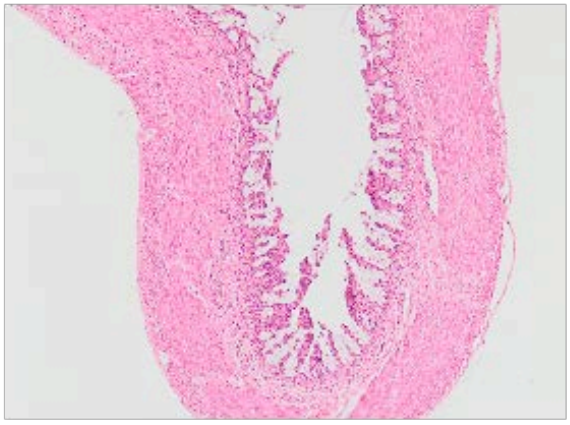

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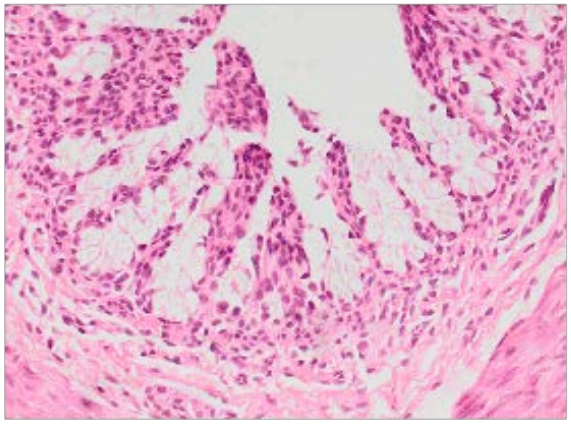

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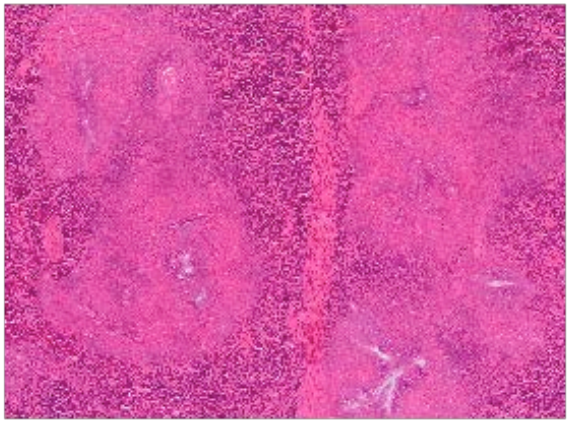

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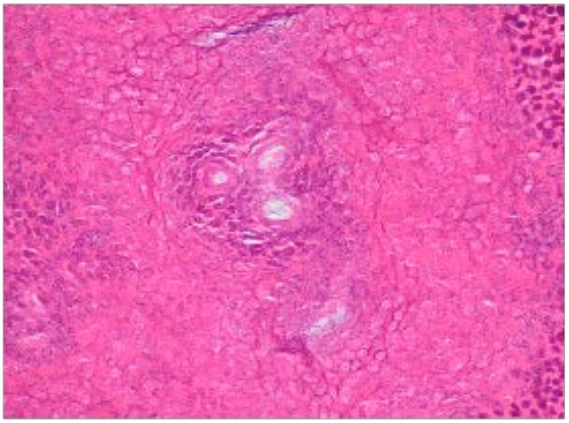

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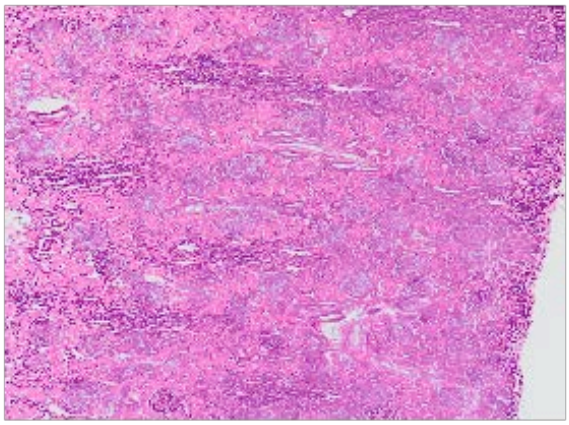

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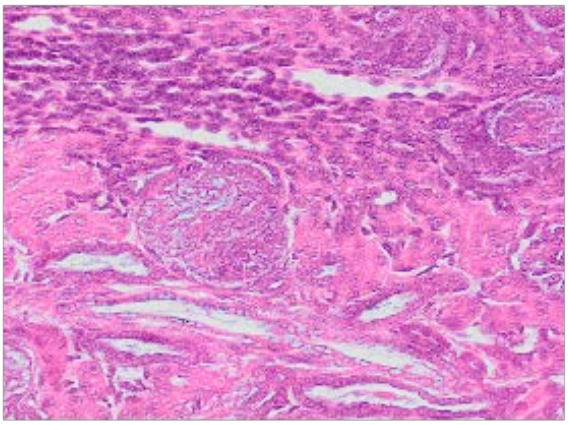

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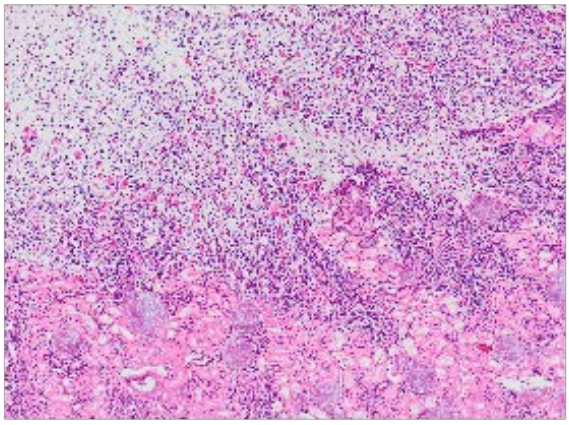

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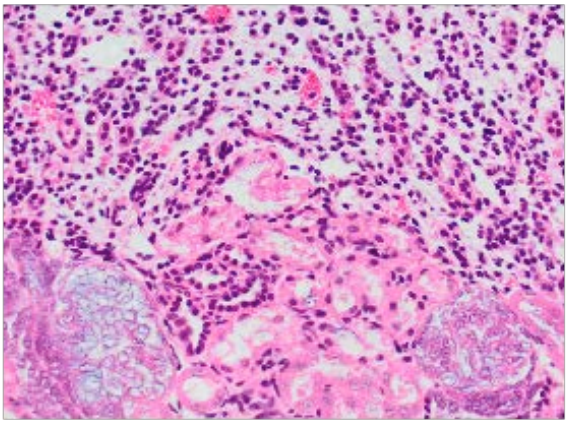

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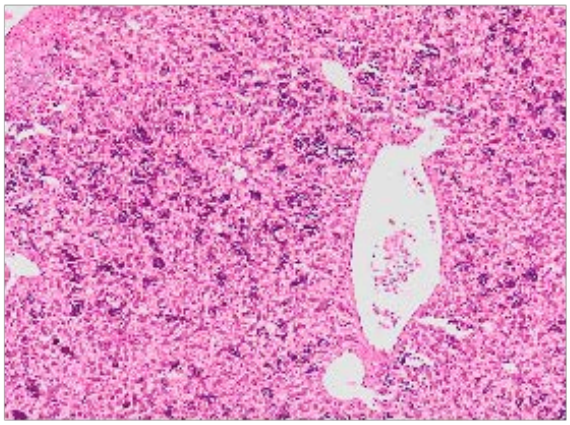

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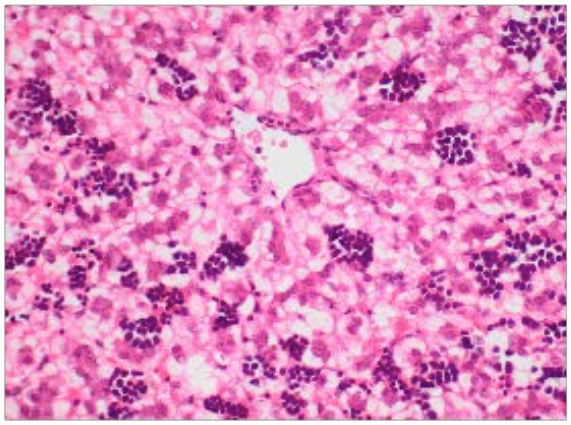

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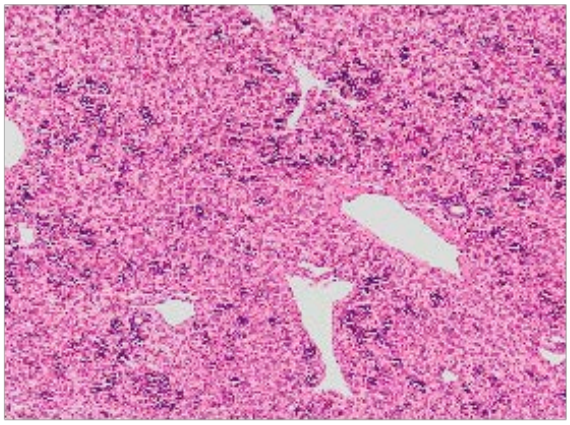

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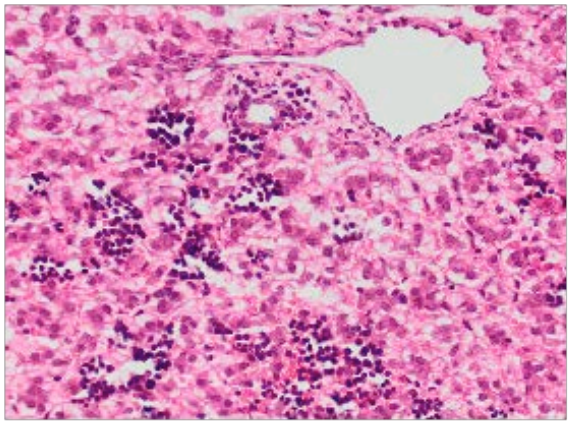

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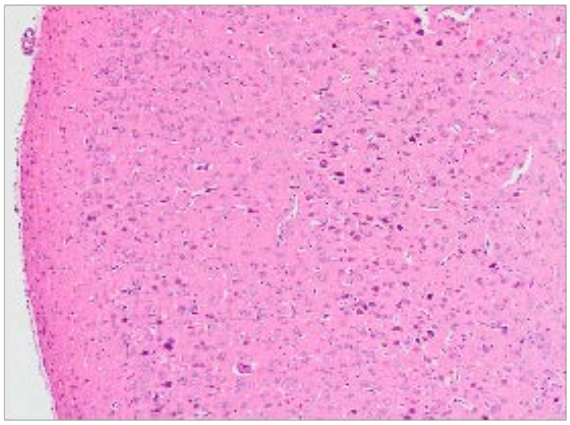
Figure 14: 229R2 - VA i.v.



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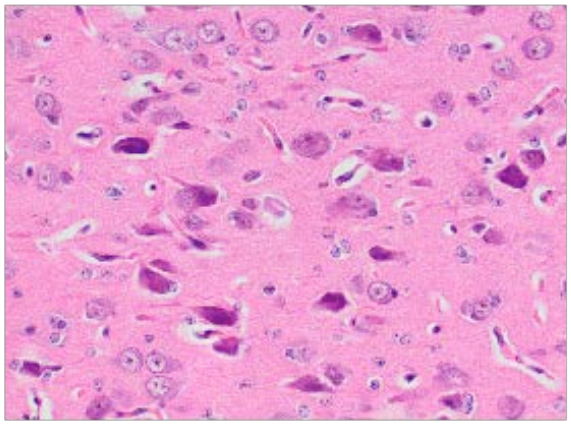

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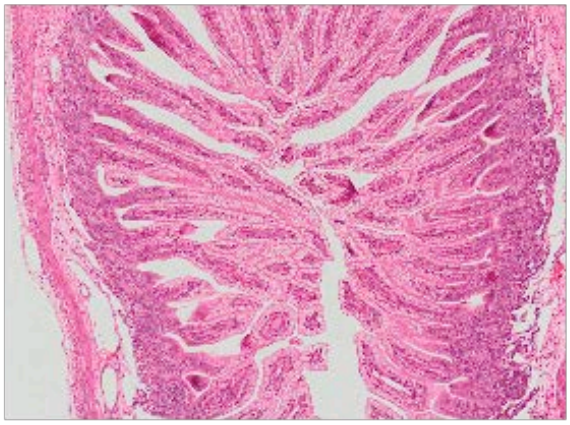

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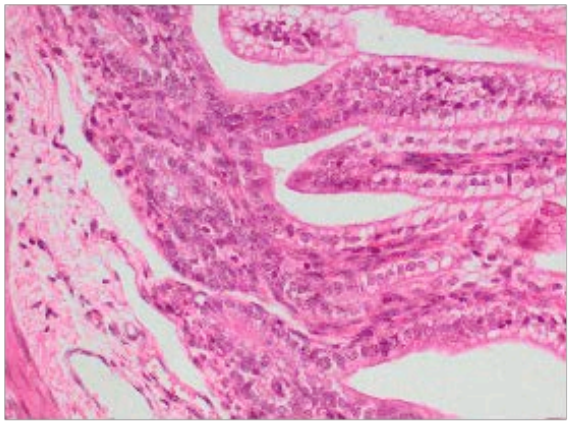

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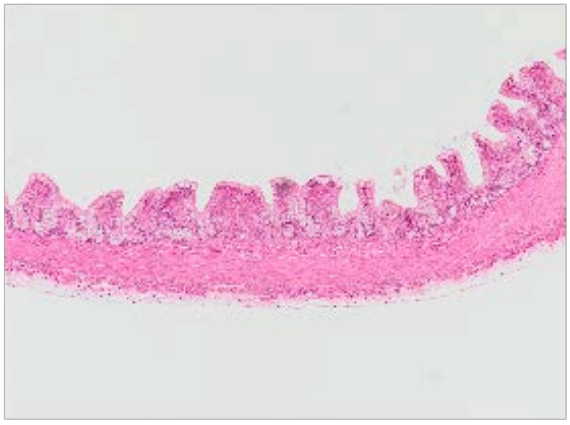

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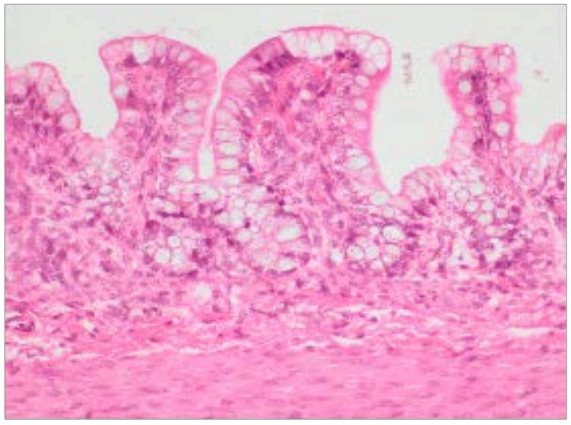

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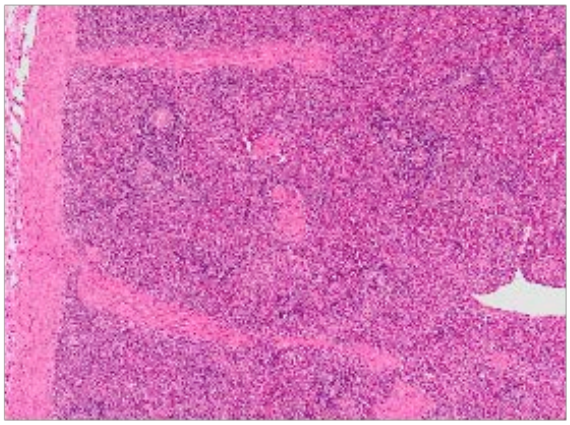

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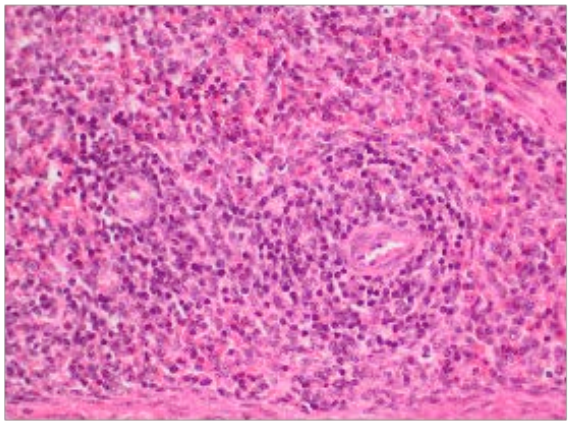

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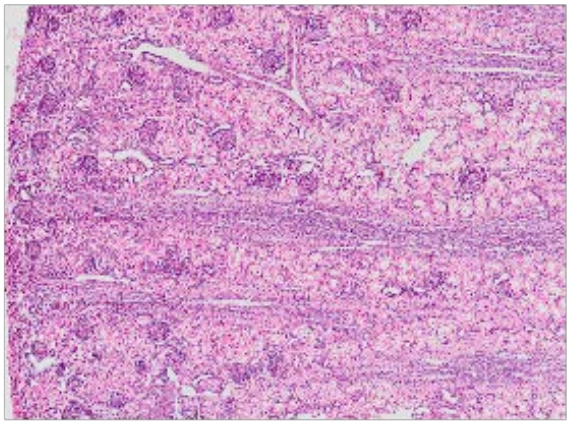

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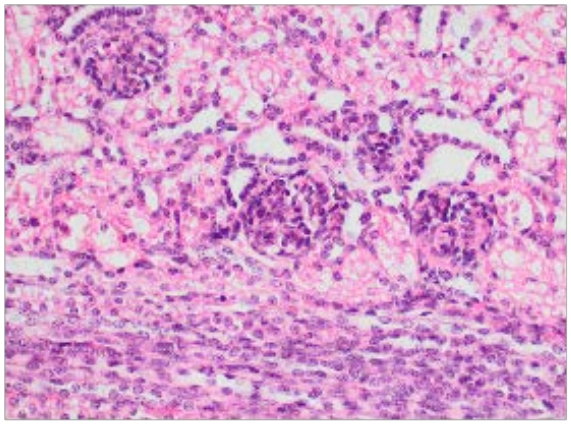

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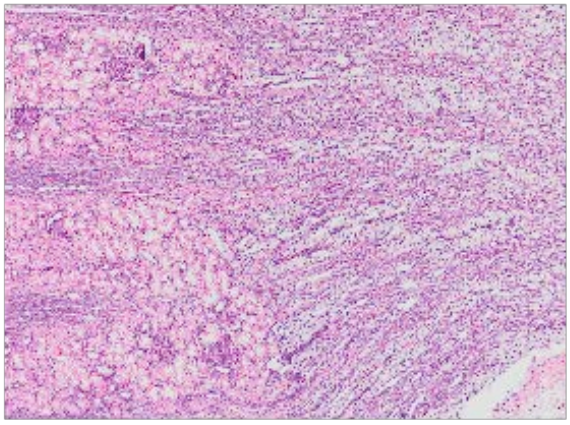

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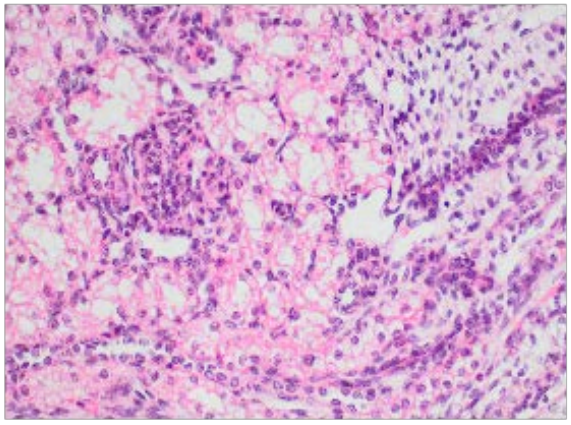

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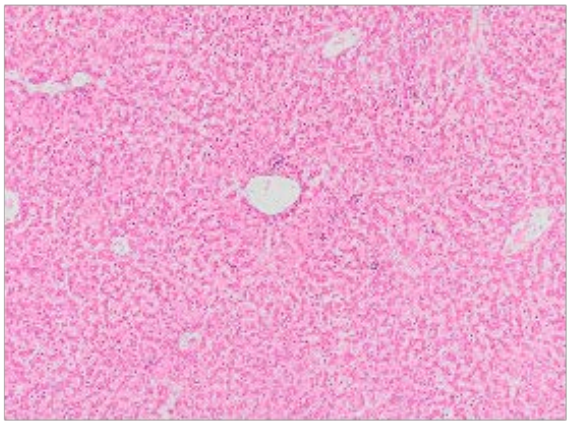

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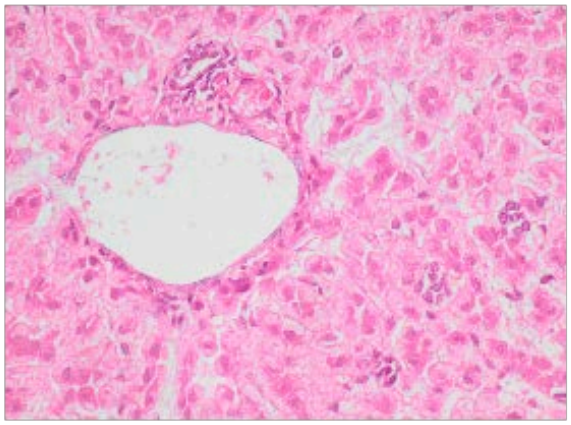

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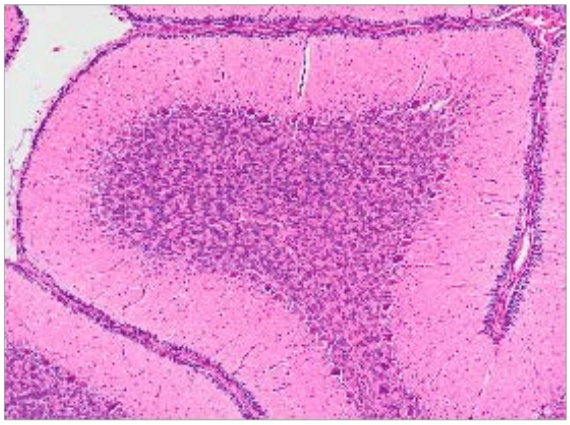

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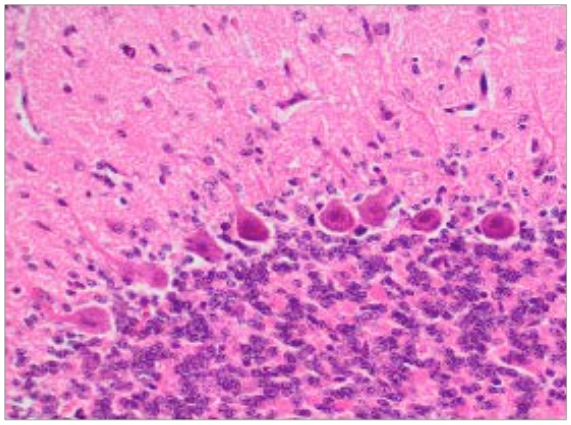

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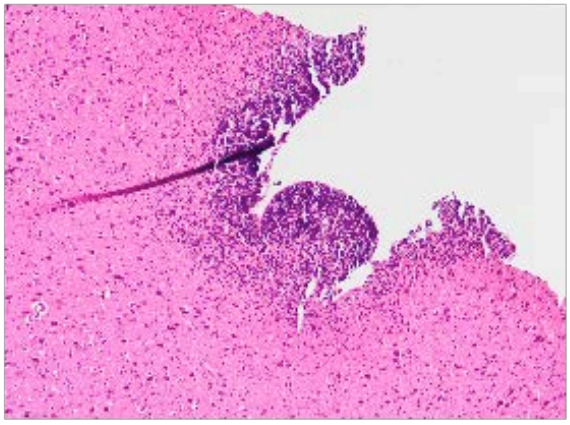

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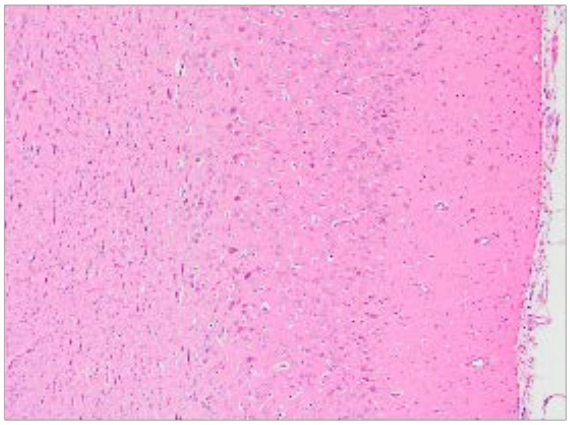

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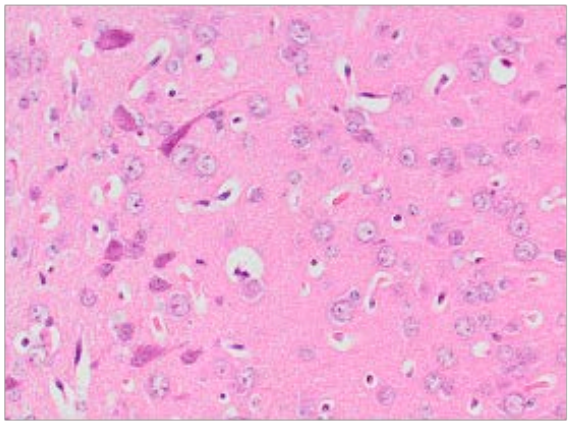

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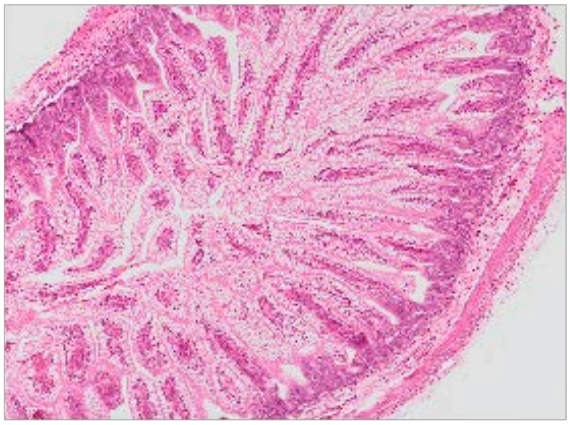

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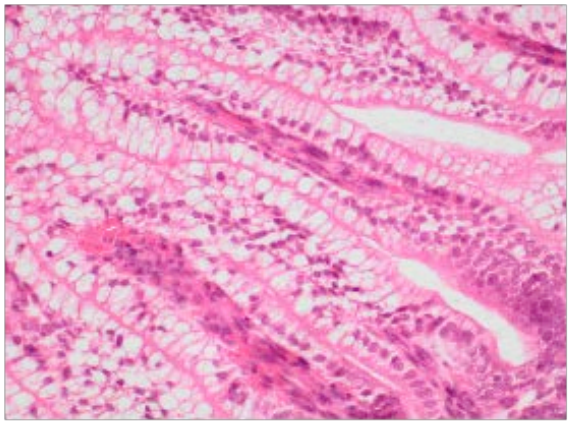

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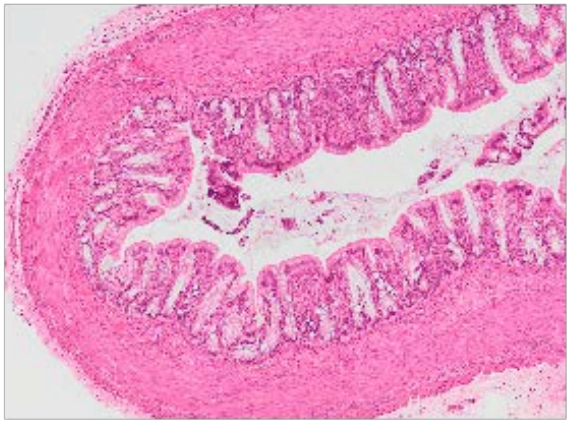

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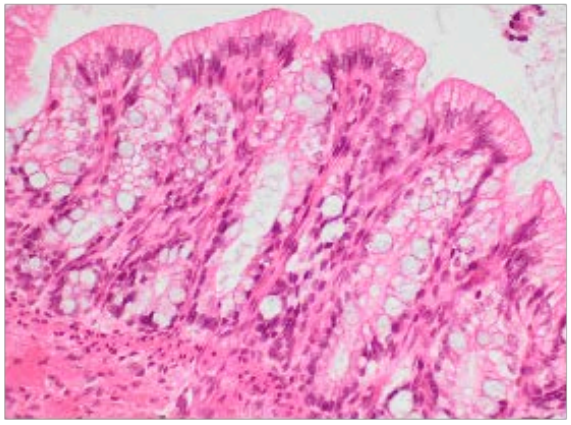

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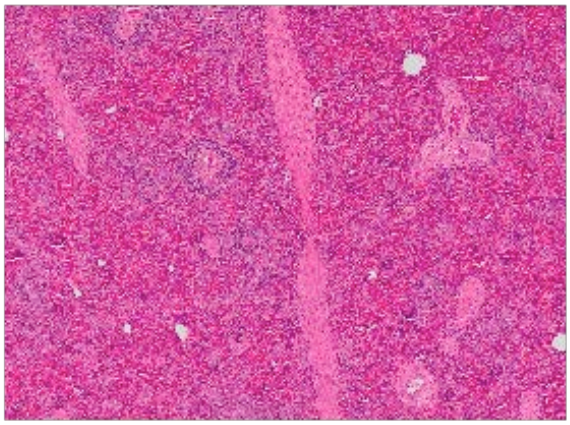

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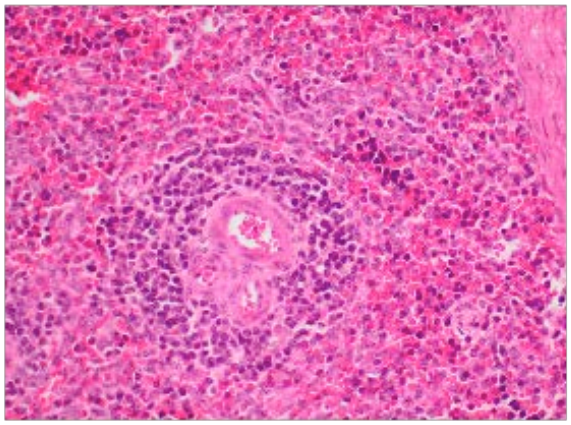

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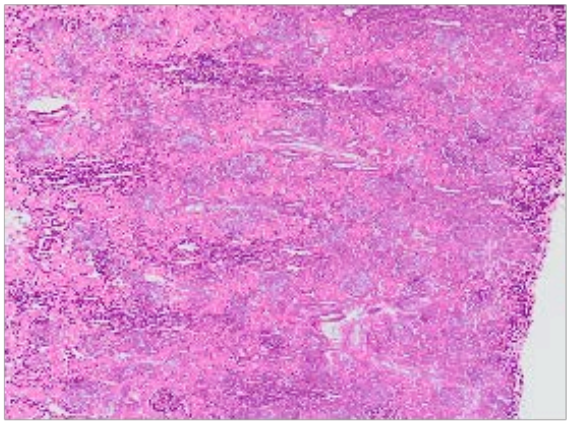

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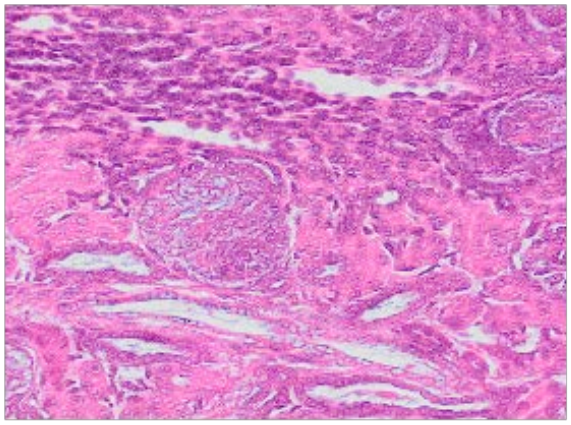

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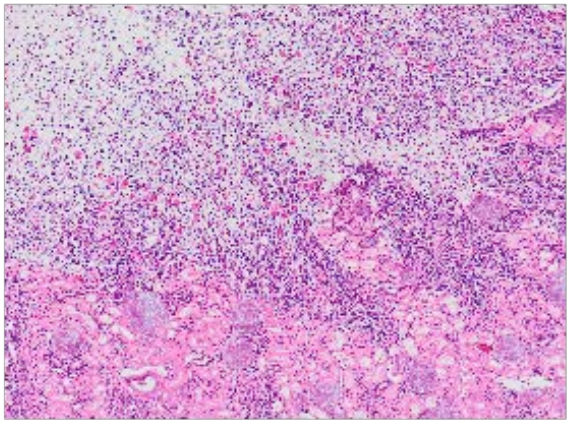

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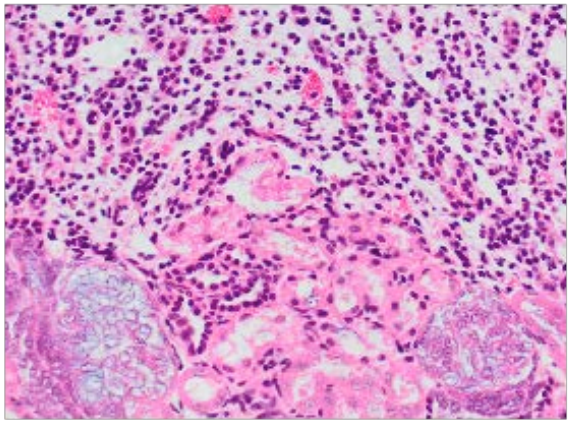

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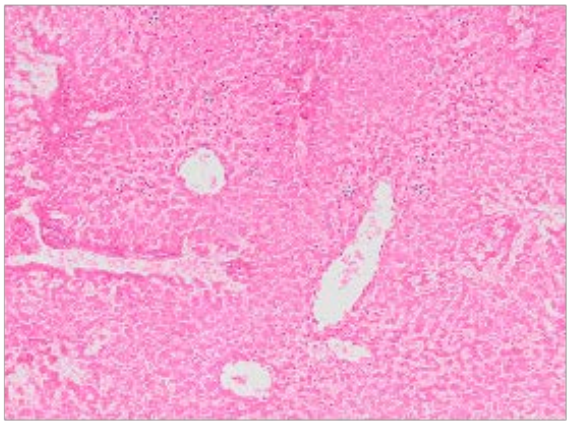

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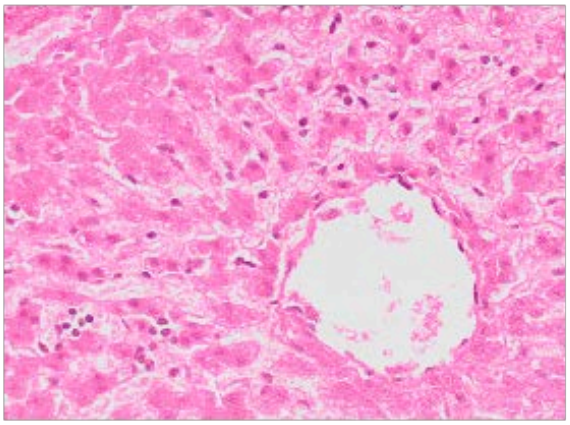

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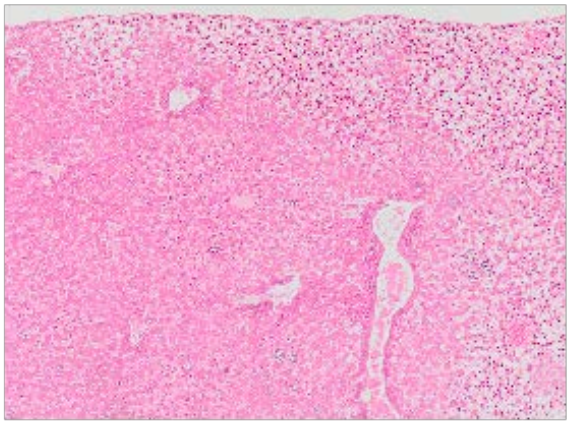

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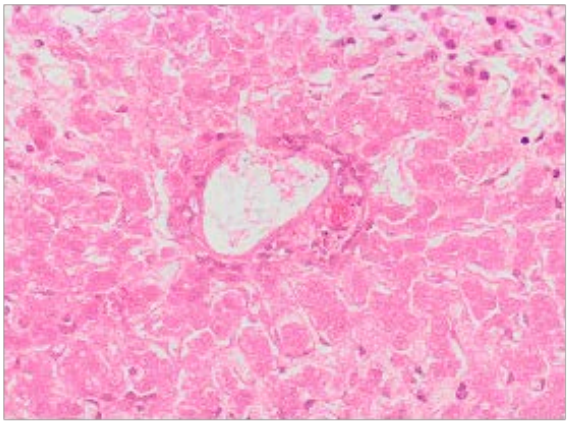

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