Vitamin A metabolism in the cat

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Summary

The vitamin A metabolism in cats appears to be different, at some levels, from other species. However, there is still much unknown. The main conclusions are:

- Cats depend on retinyl esters in their diet, in contrast to species that can use β-carotene.
- Instead of transporting retinol in blood bound to Retinol Binding Protein, cats transport retinyl esters bound to lipoproteins.
- It appears that the vitamin A level in blood is not homeostatically regulated.
- Cats have a reduced urinary vitamin A excretion compared to dogs.

Deforming cervical spondylosis is often associated with hypervitaminosis A. However, we suggest that there is an interaction between vitamin A and vitamin D. To increase the understanding about this subject, we have reviewed literature. Unfortunately, potential interactions between vitamins A and D have not been examined in cats. Thus, there is still much to be investigated.

The second subject of this study was to determine whether the enzymes CYP24A1, CYP27B1, CYP1A2, CYP26A1, CYP26B1 and LRAT are expressed in cat liver, using quantitative PCR. The enzymes CYP24A1 and CYP27B1 from the vitamin D metabolism were not detected in the cat liver. This is similar to other species. The enzymes of the vitamin A metabolism, CYP1A2 CYP26A1, CYP26B1 and LRAT, are expressed in cat liver. In addition we made a comparison of the quantitative expression between healthy and cirrhotic livers, to see if a shift would occur. The hyperostoses are often seen in older cats, which generally have cirrhotic livers. A shift in expression was significant for CYP26A1. The expression in young livers is very low, while the expression in old livers is relatively high. This is remarkable, because CYP26 is responsible for the breakdown of vitamin A.

The results of this study does not indicate a direct effect of the examined enzymes in the pathophysiology of the metabolic bone disease. We suggest that this disease is most likely multifactorial. More research should be done to understand the pathophysiology of deforming cervical spondylosis.

Content

Introduction

There is still much to be investigated about vitamin A metabolism in cats. Hypervitaminosis A in cats is often associated with the metabolic bone disease ‘deforming cervical spondylosis’. The authors suggest that an interaction with vitamin D also plays a role.

This research is part of a larger study, to increase the understanding of this metabolism. The aim of this series of investigations is to answer the question in which extend the cat is different from other species on the vitamin A metabolism and whether there are interactions with the vitamin D metabolism. Finally we would like to clarify the pathophysiology of the metabolic bone disease that occurs in cats with hypervitaminosis A. In this first part of the study we will review what is already known in literature about this subject.

In addition we will determine whether certain enzymes of the vitamin A and D metabolism are expressed in the liver of cats. A comparison of the quantitative enzyme expression between healthy livers and cirrhotic livers will be made. By determining the difference in enzyme expression among young healthy livers and old cirrhotic livers, we can see if a shift occurs. This is important because hyperostoses are often seen in older cats, which generally have cirrhotic livers.

The questions that are relevant in this study are:

- What is the quantitative expression of CYP24A1 and CYP27B1 from the vitamin D metabolism in cat livers?
- What is the quantitative expression of CYP1A2, CYP26A1, CYP26B1 and LRAT from the vitamin A metabolism in cat livers?
- Does a shift in expression occur between healthy livers and cirrhotic livers?

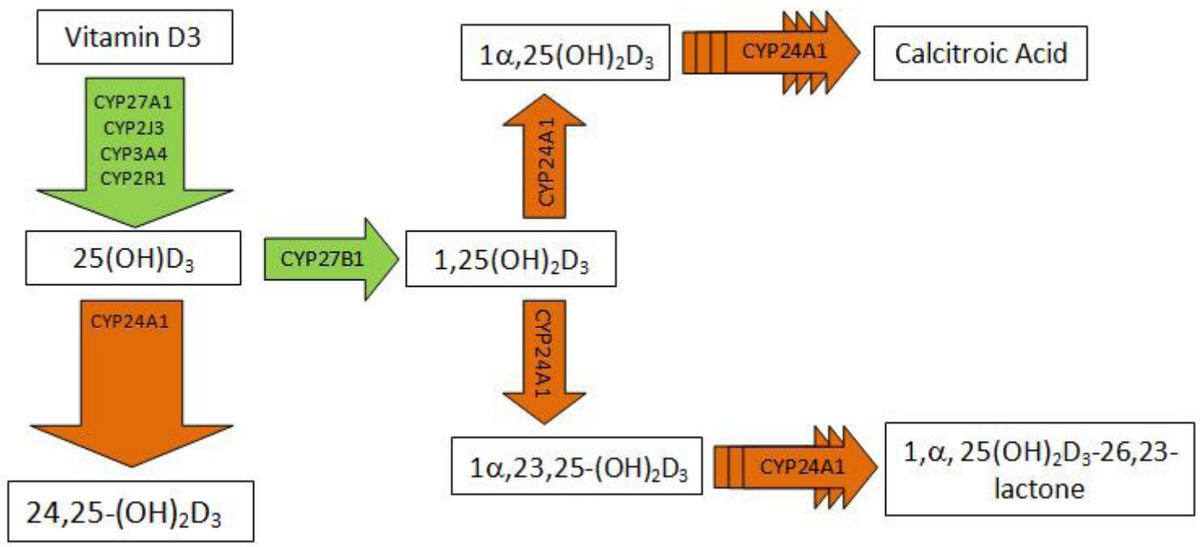


Fig. 1. Overview of the vitamin D metabolism

Vitamin D metabolism

The most important form of vitamin D in animals is cholecalciferol; vitamin D_3 (**Fig. 1**). Most mammals can produce cholecalciferol by activating 7-dehydrocholestrol by the exposure to ultraviolet light in the skin.[Hand et al., 2010; Schuster, 2010; Urushino et al., 2009] However, dogs and cats need dietary vitamin D. The photosynthesis pathway is inefficient because of the higher activity of the enzyme 7-dehydrocholesterol-Δ7 reductase that converts 7-dehydrocholesterol to cholesterol.[How et al., 1994; Morris, 1999]

Cholecalciferol is a hormone precursor and thus lacks intrinsic biological activity. It has to be converted in two subsequent hydroxylation steps to the hormonally active form 1α,25-dihydroxyvitamin D ($1\alpha,25(OH)_2D_3$).[Schuster, 2010]

In the first step of activation, 25-hydroxylation, are four distinct enzymes involved, namely the mitochondrial CYP27A1 and microsomal CYP2R1, CYP2J2 and CYP3A4. A second hydroxylation at the C-1α-site is needed for complete activation to $1\alpha,25(OH)_2D_3$. This step is carried out by the enzyme 25-hydroxyvitamin D-1α-hydroxylase which contains the component CYP27B1. This hydroxylation is performed mainly in the kidneys.[Prosser & Jones, 2004; Schuster, 2010]

Active vitamin D limits its own function via the enzyme CYP24A1. The inactivation pathway is induced by the active $1\alpha,25(OH)_2D_3$. CYP24A1 is the single enzyme responsible for this five-step inactivation. There are two catabolic pathways, leading to the inactive metabolites calcitroic acid and $1\alpha,25(OH)_2D_3$-26,23-lactone.
(Prosser & Jones, 2004; Schuster, 2010)

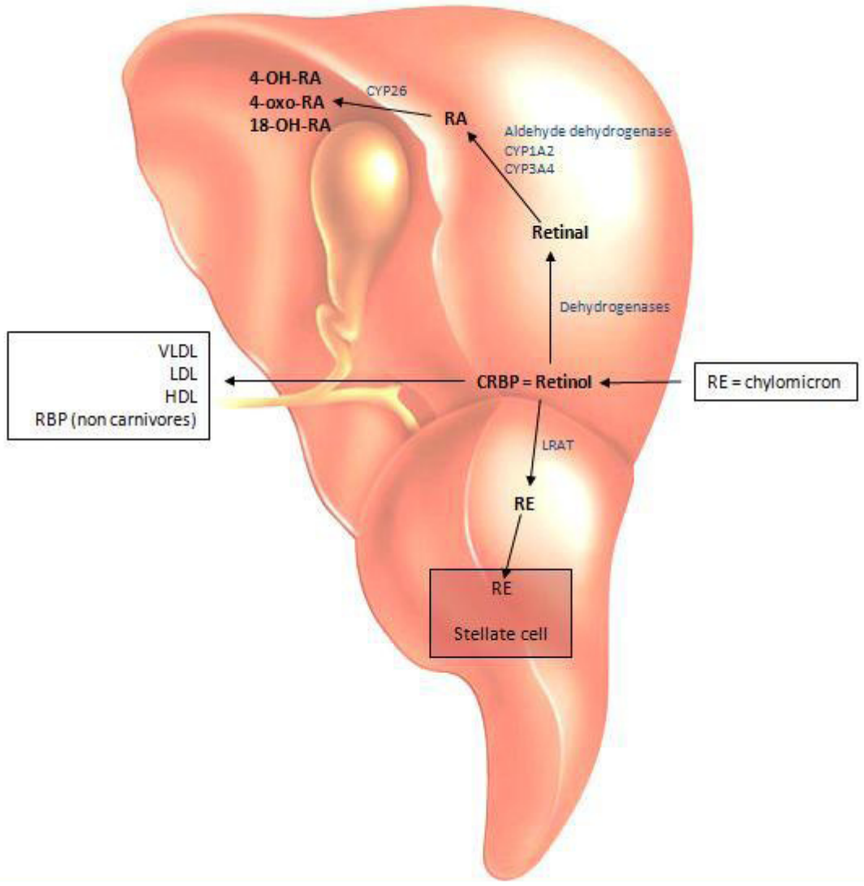


Fig. 2. Overview of the vitamin A metabolism in the liver

Vitamin A metabolism

Most vitamin A in food is in the form of retinyl esters (RE). But also provitamin A carotenoids in the form of β-carotene from plants are common. However, β-carotene can't be used as a source for vitamin A in domestic cats. β-carotene can be absorbed, but cats are unable to convert it into retinol because they lack the enzyme β-carotene 15,15'-dioxigenase.(Chew et al., 2000; Hand et al., 2010; Schweigert, Raila, Wichert et al., 2002)

Because dietary retinyl esters are fat soluble, bile salts for micelles in the small intestinal lumen are required. In the small intestine, the retinyl esters are hydrolyzed to retinol. This process requires the pancreatic enzyme Pancreatic triglyceride lipase and the brush border enzyme Phospholipase B.(Hand et al., 2010; Schweigert & Raila, 2002) Subsequently retinol can be transported into the enterocytes. The hypothesis is that the uptake of retinol is mediated by lipid transporters. However, no protein has yet been identified.(Hand et al., 2010; Harrison, 2005)

Within the enterocyte retinol is bound by Cellular retinol binding protein II (CRBPII). This complex is a substrate for the enzyme Lecithin:retinol acyltransferase (LRAT), which re-esterifies the retinol. In the absence of CRBP, the enzyme Acyl CoA:retinol acyltransferase (ARAT) can also catalyze the esterification.(Napoli, 1999; Ross, 1982) The retinyl esters then can be incorporated into chylomicrons and then secreted into the lymph, where it is transported with low-density lipoproteins (LDL) to the liver. A small amount of retinol will be oxidized to retinal and then to retinoic acid that will be passed via the portal vene.(Hand et al., 2010; Harrison, 2005; Li & Tso, 2003; Schweigert & Raila, 2002) It is also possible that the secretion into the portal circulation is as free retinol. It is expected that this route is significant in pathologic conditions that affect the secretion of chylomicrons.(Harrison, 2005)

Most of the chylomicron-associated retinyl esters are taken up by the liver. In the hepatocytes the retinyl esters are hydrolyzed and go to different pathways (**Fig. 2**):

[1] Transfer and storage in stellate cells

Under normal conditions, the liver is the main site of vitamin A storage. All-trans-retinol is converted into all-trans-retinyl esters by the enzyme LRAT. This enzyme transfers the sn-1 fatty acid from lecithin to retinol, which is bound to CRBPI or CRBPII. Retinyl palmitate, stearate and oleate are formed and stored in the stellate cells of the liver.(Wolf, 2001; Batten et al., 2004) Most of the vitamin A is present in the form of retinyl palmitate and stearate.(Li & Tso, 2003; Harrison, 2005; Zolfaghari & Ross, 2000)

A study in mouse and rat proposed that the expression of LRAT and retinyl ester storage in the liver is regulated by retinoic acid (RA). Retinol from diet or by recycling from peripheral tissues is stored when the production of RA in the periphery is sufficient. Thus, in this situation LRAT remains in the induced state. When the vitamin A supply is not adequate, the supply of retinol to peripheral tissues will decline. This results in an inadequate feedback of RA to the liver, thus liver LRAT expression and activity would fall. Less retinyl esters will be stored, so that more retinol is available for target tissues **(Fig. 3)**.(Zolfaghari & Ross, 2000)

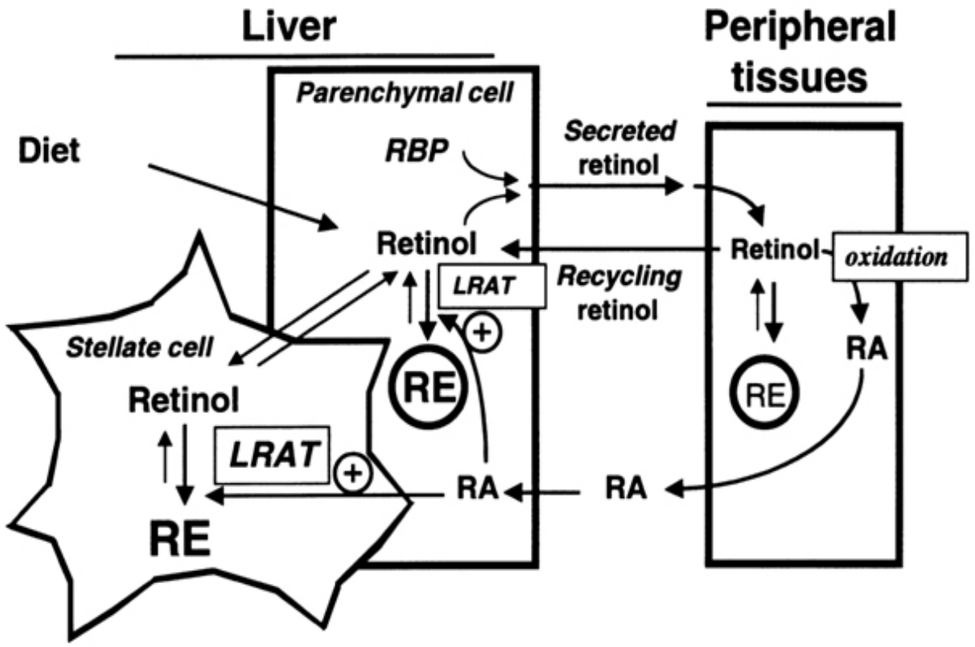


Fig. 3. Regulation of liver LRAT by peripheral RA.(Zolfaghari, 2000)

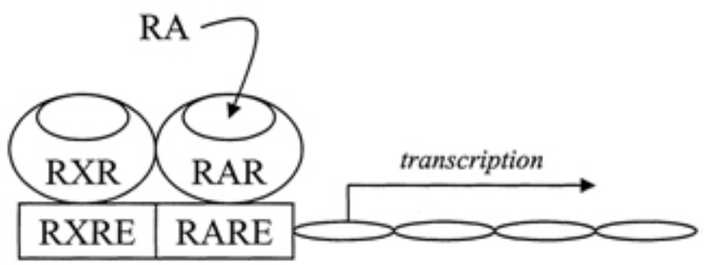


Fig. 4. Mechanisms of retinoid action.(Napoli, 1999)

The amount of storage of vitamin A in the cat liver is disputed. Some groups say that the cat liver contains very little vitamin A.(Futterman & Andrews, 1964) Others reported concentrations of vitamin A in livers of cats that were much higher than those in canines.(Raila et al., 2001) There is also a contrast in findings about whether the liver or the kidneys contain more vitamin A.(Raila et al., 2001; Lowe et al., 1957)

[2] Metabolizing by dehydrogenases and oxidases to generate retinal and retinoic acid

To exert its function, all-trans-retinol is oxidized to retinal, all-trans-retinoic acid and 9-cis-retinoic acid. They can bind in target organs to nuclear receptors that activate the expression of specific target genes.(Chambon, 1996) Retinoic Acid Receptors (RARs) can bind to RA response elements (RARE). They function heterodimeric with Retinoid X Receptors (RXRs). The RXR binds with another responsive element, the RXRE **(Fig. 4)**.(Zolfaghari & Ross, 2000; Napoli, 1999)

All-trans-retinol needs to be converted into all-trans-retinoic acid, which can bind to nuclear retinoic acid receptors. This conversion comprises two subsequent reactions. The first and rate-limiting step is the oxidation of all-trans-retinol into all-trans-retinal. All-trans-retinal is then rapidly oxidized to all-trans-retinoic acid.(Chen et al., 2000; Kim et al., 1992)

Conversion of all-trans-retinol to al-trans-retinal is mainly catalyzed by alcohol dehydrogenase isoenzymes. The subsequent reaction of all-trans-retinal into all-trans-retinoic acid is catalyzed by aldehyde dehydrogenase.(Chen et al., 2000; Napoli, 1999) However, an alternative for the second step is the reaction by various cytochrome P-450 dependent mono-oxygenases. In human adult livers, CYP1A2 and CYP3A4 appeared to be the major CYP enzymes catalyzing the conversion of all-trans-retinol tot all-trans-retinal.(Chen et al., 2000)

[3] Retinol secretion into the plasma

In animals and man with sufficient vitamin A the vitamin A levels in blood are tightly controlled by serum Retinol Binding Protein (RBP). Therefore, the RBP levels in blood do not reflect the dietary intake.(Li & Tso, 2003; Zolfaghari & Ross, 2000; Schweigert et al., 1990) Elevated plasma retinyl esters are only observed under conditions of vitamin A intoxication.(Mallia et al., 1975; Smith & Goodman, 1976) To assess the vitamin A status a biopsy of the liver is needed.(Schweigert et al., 1990)

However, in carnivores, including the cat, most of the vitamin A in blood is not transported as retinol bound to RBP, but as retinyl esters bound to lipoproteins of all fractions (VLDL, LDL, and HDL). The occurrence of retinyl esters in plasma of these species is not associated with any sign of vitamin A intoxication.(Schweigert & Bok, 2000) In contrast to other species, the vitamin A levels in the blood are not homeostatically regulated but it is dependent on the dietary vitamin A intake.(Schweigert et al., 1990; Wilson et al., 1987)

[1] Retinyl ester hydrolysis	
a.	*Lumen*
	Pancreatic triglyceride lipase
b.	*Brush border membrane*
	Phospholipase B
[2] Retinol uptake, transfer and efflux	
	Retinol transporters
	Cellular retinol binding protein I
	Cellular retinol binding protein II
[3] Retinyl ester synthesis	
	Lecithin retinol acyltransferase (LRAT)
	Acyl-CoA retinol acyltransferase (ARAT)
[4] Chylomicron assembly	
	Apolipoprotein B
	Microsomal triglyceride transfer protein

Table 1. Proteins involved in the digestion, absorption and transport of retinol

[4] Breakdown of retinoic acid

RA induces CYP26, and in this way induces its own degradation.(Pavez Lorie et al., 2009) CYP26 is reported as the principal enzyme responsible for the catabolism of all-trans-retinoic acid into 4-OH-RA, 4-oxo-RA and 18-OH-RA.(Lee et al., 2007) 13,cis-RA and 9-cis-RA are not recognized as a substrate by CYP26.(Napoli, 1999; Sonneveld, et al., 1998)

CYP26B1 appears to be essential under physiological conditions, whereas CYP26A1 might play a greater role during retinoic acid excess.(Pavez Lorie et al., 2009)

RA can be metabolized in different reactions. The first reaction is 4-hydroxylation to form 4-OH-RA. The next step is the generation of 4-oxo-RA. 4-oxo-RA in turn undergoes conversion into 13-cis-4-oxo-RA. Another reaction catalyzes 18-hydroxylation to produce 18-OH-RA.(Napoli, 1999)

The major contributor to the 4-hydroxylation in human liver is most likely CYP2C8.(Nadin & Murray, 1999) However, there is also some contribution by CYP3A4 and CYP2C9.(McSorley & Daly, 2000; Nadin & Murray, 1999)

Carnivores excrete substantial amounts of retinol and retinyl esters with the urine. This is a regulated process depending on the concentration of vitamin A in the blood plasma.(Raila et al., 2000; Schweigert & Bok, 2000) When dogs were fed above NRC requirements, they excreted substantial amounts of vitamin A with the urine.(Schweigert & Bok, 2000) This could be a mechanism that protects carnivores from hypervitaminosis A.

In cats, urinary concentrations of vitamin A metabolites were found which were up to 100-fold lower than those observed in canine species. A reduced urinary vitamin A excretion could be an explanation for the sensitivity to vitamin A intoxication in cats. On the other hand it could be a protection mechanism against hypovitaminosis A. β-carotene can't be used as a source for vitamin A in domestic cats, therefore they must spare vitamin A by reducing the excretion in urine.(Raila et al., 2000; Raila et al., 2001) Control feeding experiments are necessary to investigate this.

In dogs, western blot analysis excludes RBP as the carrier for retinol or retinyl esters in the urine. This process is associated with the Tamm-Horsfall like glycoprotein that is produced and actively secreted by the epithelial cells of the distal tubules in the kidney.(Schweigert, Raila, & Haebel, 2002; Raila et al., 2000; Raila et al., 2002)

Hypervitaminosis A

Cats are occasionally fed with a diet that mainly consists of liver, which contains high levels of vitamin A. But also commercial cat food formulations can contain high concentrations of vitamin A as a result of the use of animal liver as an ingredient. Furthermore, vitamin A is added to commercial diets at the end of the production line to prevent low vitamin A levels in the end products, because vitamin A is susceptible to high temperatures in the extrusion process. Because determination of vitamin A is expensive, the amount of vitamin A stated on the package of commercial pet foods mostly is only the amount added, so the total amount of vitamin A is underestimated.(Freytag et al., 2003; Heanes, 1990) In these cases the cats are susceptible to vitamin A toxicity.(Hayes, 1982) The long-term effects of vitamin A excess are established after months or years. In the most cases adult cats are involved ranging in age from 2 to 9 years.(Seawright et al., 1970)

The condition following vitamin A excess is called deforming cervical spondylosis.(Seawright & English, 1964; Seawright & English, 1967) This disease is characterized by the formation of extensive osteophytes and exostoses. The most common affected sites include the occipital bone and the cervical and thoracic vertebrae. Less common are the extra spinal sites; shoulder, elbow, sternum, thoracic cage and pelvis.(Hayes, 1982; Seawright & English, 1964; Seawright & English, 1967; Polizopoulou et al., 2005)

The cats clinically develop stiffness and soreness of muscle and cervicothoracal skeleton, anorexia, weight loss, exophthalmia, alopecia, and dermatitis.(Hayes, 1982; Seawright & English, 1967)

Besides the deforming cervical spondylosis, chronic ingestion of high levels of vitamin A may result in teratogenic effects. Freytag et al. showed in their study that there is an increase of kitten malformations including palatoschisis, mandible shortening, tongue protrusion, pelvic hypoplasia, stenotic colon, enlarged heart and agenesis of spinal cord and small intestine.(Freytag et al., 2003)

Interactions of vitamin A and D

Skeletal problems are often associated with hypervitaminosis A. However, we suggest that there is an interaction between vitamin A and vitamin D.

Potential interactions between vitamins A and D have not been examined in cats. However, excessive vitamin A intake (606.000 RE/kg diet) in cats, did not cause deforming cervical spondylosis after three years. In this study an interaction between vitamin A and D was not specifically addressed and requires further study.(Freytag et al., 2003)

Other species

A study in humans found that a high level of vitamin A antagonizes the serum calcium response to vitamin D. The group suggests that the antagonism may be exerted at the level of intestinal calcium absorption. (Johansson & Melhus, 2001)

In rats it is also established that that there is an antagonistic interaction between vitamin A and D at the physiological level. Furthermore, the effect of vitamin D on the maintenance of serum calcium was eliminated by retinyl acetate. Both vitamin A and D require the RXR. When large amounts of vitamin A are given, 9-cis-RA can dominate the RXRs that are available. The action of vitamin D might then be compromised. However, other mechanisms cannot be ruled out. Vitamin A could interfere with the absorption of vitamin D, effect transport proteins of vitamin D, or effect the conversion of vitamin D to its active form.(Rohde et al., 1999) Later investigations concluded retinol does not affect the metabolism of calciferol or its metabolites through the CYP24 degradation pathway.(Rohde & DeLuca, 2005)

Turkey poults that had a deficient intake of both vitamin A and D developed severe lameness, growth depression, mortality and lesions consistent with rickets. When fed with the estimated required level of vitamin D and a high level of vitamin A the same symptoms were observed. Poults fed with high vitamin D and the estimated required level of vitamin A developed hypervitaminosis D with growth depression and renal tubular mineralization. When the diet consisted of high levels of both vitamin A and D, no symptoms occurred. The growth rate and bone mineral content were similar to the control group. This study concludes that there is an antagonism between vitamin A and D (**Table 2**).(Metz et al., 1985)

In chickens, high concentrations of dietary vitamin A interfered with the utilization of vitamin D, increased the incidence of rickets and neutralized the toxic effect of excess vitamin D.(Aburto et al., 1998)

Vitamin A	Vitamin D	Conclusion
Estimated required level	Estimated required level	No symptoms, normal growth rate and mineral content
Deficient	Deficient	Severe lameness, growth depression, mortality, rickets
High	Estimated required level	Severe lameness, growth depression, mortality, rickets
Estimated required level	High	Hypervitaminosis D: growth depression, renal tubular mineralization
High	High	No symptoms, normal growth rate and mineral content

Table 2. The interaction between vitamin A and D in Turkey poults.

Receptor level

In human, the mouse and rat many research is done on the interaction between vitamin A and D at the receptor level. Many of these study's are done in vitro.

The effect of RA and calcitriol are mediated by the nuclear RARs and vitamin D receptor (VDR), which are ligand-inducible transcription factors.(Jimenez-Lara & Aranda, 2000; Kindmark et al., 1995) Induction of RAR and VDR responsive elements in target genes requires a cofactor the RXR, with its ligand 9-cis-RA.(Kindmark et al., 1995) The RXR forms a heterodimer with the VDR to activate genes that are regulated by 1,25(OH)2D3.(Reinhardt et al., 1999) $1,25(OH)_2D_3$ binds to the VDR, which regulates gene expression in target cells by binding to vitamin D response elements (VDREs). The $1,25(OH)_2D_3$ binding to the VDR changes the conformation of the VDR, which promotes heterodimerization with the RXR. The VDR-RXR heterodimer forms a complex with the VDRE.(Reinhardt et al., 1999)

$1,25(OH)_2D_3$ and 9-cis-RA can be inhibitory, additive or synergistically to each other.

Inhibitory

9-cis-RA destabilizes the VDR-RXR heterodimer by promoting the formation of RXR homodimers. The equilibrium shifts toward RXR homodimers and a reduce in VDR-RXR dimers. This reduced VDR-RXR binding to the VDRE presumably reduces the $1,25(OH)_2D_3$ mediated gene expression.(Reinhardt et al., 1999; Cheskis & Freedman, 1994; Kindmark et al., 1995)

Synergistically

In the rat, 9-cis-RA and $1,25(OH)_2D_3$ can act synergistically in the regulation of 24-hydroxylase in vivo. Co dosing of 9-cis-RA and $1,25(OH)_2D_3$ results in an increased activity of 24-hydroxylase, above dosing only $1,25(OH)_2D_3$. Furthermore, $1,25(OH)_2D_3$ had an effect on the RA metabolism. Administering both 9-cis-RA and $1,25(OH)_2D_3$ resulted in higher circulating concentrations of 9-cis-RA than when 9-cis-RA was singly dosed.(Reinhardt et al., 1999)

$1,25(OH)_2D_3$ and retinoids can inhibit the growth and/or induce the differentiation of various cell types. Blutt et al. showed that the combination of 1,25-dihydroxyvitamin D_3 and 9-cis retinoic acid synergistically inhibits the growth of LNCaP prostate cancer cells, by accumulating the cell cycle in the G1 phase.(Blutt et al., 1997)

Additive

$1,24(OH)_2D_3$ and retinoids exert additive effects for the 24-hydroxylase expression. In this model, RAR/RXR and RXR/VDR heterodimers, not RXR/RXR homodimers, mediate the effect.(Zou et al., 1997)

Enzyme expression

Enzyme expression in other species than the domestic cat

In this study, we will also determine whether certain enzymes of the vitamin A and D metabolism are expressed in the liver of cats. The enzymes that are going to be investigated are CYP24A1 and CYP27B1 from the vitamin D metabolism, and CYP1A2, CYP26A1, CYP26B1 and LRAT from the vitamin A metabolism.

To investigate in which extend the cat is different from other species, it is relevant to know how these enzymes are expressed in other animals and humans.

Most data is found in literature. However, for human gene expression a web-based atlas exists. *Amazonia!* is a freely accessible tool that contains a collection of representative microarray data covering a wide range of normal and malignant human samples. In this way it is easy to obtain an expression profile, shown in bar plots, for each gene in humans.[(Le Carrour et al., 2010)]

Enzyme	Species	Expression in
CYP27B1	Human	Kidney, leucocytes, gastrointestinal tract, pancreas, epidermis, parathyroids, brain, endothelial cells, adipose tissue, placenta
		Spermatids, mature spermatozoa, vesicles within the caput epididymis, and glandular epithelium of cauda epididymis, seminal vesicle, prostate
	Rat	Kidney
CYP24A1	Human	Major levels: oöcytes, kidney Moderate levels: trachea, bronchus, tonsils Lower levels: fibroblasts, brain, thyroid, heart, monocytes, endometrium, placenta
		Spermatids, mature spermatozoa, vesicles within the caput epididymis, and glandular epithelium of cauda epididymis, seminal vesicle, prostate
	Rat	Kidney

Table 3. Enzyme expression: vitamin D metabolism

Expression of CYP24A1 and CYP27B1

CYP24A1 is located in the inner mitochondrial membrane. The enzyme is, in human, detected in many different organs at low basic levels but can be induced in response to active vitamin D in almost all of its target cells. The highest expression was detected in oöcytes and the proximal tubule of the kidney. Moderate levels were found in trachea, bronchus, and tonsils. Low levels were present in various tissues including fibroblasts, brain, thyroid, heart, monocytes, endometrium, and placenta. However, CYP24A1 has not been detected in the liver.(Schuster, 2010; Le Carrour et al., 2010)

The primary site of expression of CYP27B1 in human is in the proximal tubule of the kidney. In many other tissues the enzyme is expressed at low levels. Important extra renal sites of CYP27B1 expression include leucocytes(Adams et al., 1983), the gastrointestinal tract, pancreas, epidermis, parathyroids, parts of the brain, endothelial cells, adipose tissue and placenta. No expression was detected in the liver, heart and adrenal cortex.(Schuster, 2010; Hewison et al., 2000; Townsend et al., 2005)

Blomberg Jensen et al. performed a study of the expression of, among others, CYP27B1 and CYP24A1 enzymes in the testis, epididymis, seminal vesicle, prostate and spermatozoa of man. They detected expression of CYP24A1 and CYP27B1 in round and elongated spermatids, vesicles within the caput epididymis, and glandular epithelium of cauda epididymis, seminal vesicle and prostate. There was also expression observed in the neck and midpiece of a subpopulation of mature spermatozoa.(Blomberg Jensen et al., 2010)

CYP24A1 and CYP27B1 were also identified in rat kidney. Both enzymes are regulated by PTH, calcium and calcitonin. The expression appeared to be regulated too by these factors. mRNA levels of CYP27B1 were significantly higher in animals fed low calcium concentration then in animals fed higher calcium concentrations.(Anderson et al., 2003) The expression of CYP24 was significantly higher in animals fed a high calcium concentration compared to animals fed a lower calcium concentration. This is probably due to a decrease in PTH level.(Zierold et al., 2001; Anderson et al., 2003) The mRNA expression of CYP27B1 is in vivo up regulated by PTH in hypocalcaemic rats. Under normocalcaemic conditions, calcitonin is probably the major regulator of CYP27B1 transcription. However, a direct effect of calcium is still possible.(Anderson et al., 2003; Shinki et al., 1999)

An overview of the enzyme expression of the vitamin D metabolism is depicted in **Table 3**.

Enzyme	Species	Expression in
CYP1A2	Human	Liver
	Pig	Liver
CYP26A1	Rat	Liver, testis, embryo
CYP26B1	Human	Parts of the brain, skin, adipose tissue, fibroblasts, joints, synovial membrane
	Rat	Liver, testis, embryo
LRAT	Rat	Liver, small intestine, testis
	Mouse	Liver, small intestine, testis

Table 4. Enzyme expression: vitamin A metabolism

Enzyme expression: Vitamin A metabolism

Expression of CYP1A2

The conversion of all-trans-retinol into all-trans-retinal is mainly located in the liver. In human, CYP1A2 is mainly expressed in the liver.(Chen et al., 2000; Le Carrour et al., 2010) In other tissues, the enzyme is expressed at very low levels.(Le Carrour et al., 2010)

In pigs, the enzyme is also detected in the liver. The constitutive expression seems to be down regulated by androgens.(Kojima et al., 2010)

Expression of CYP26A1 and CYP26B1

The degrading enzymes CYP26A1 and CYP26B1 are found in the rat liver,(Zolfaghari et al., 2007; Zhang et al., 2010) testis and embryo. Expression in the kidney of lung was not detected.(Napoli, 1999)

In human, CYP26B1 is found in many tissues including parts of the brain, skin, adipose tissue, fibroblasts, joints and synovial membrane. However, detection in the liver is absent.(Le Carrour et al., 2010)

Expression of LRAT

LRAT is detected in the liver of mouse and rat. In the liver, the enzyme is regulated by vitamin A. In other organs, like the small intestine and testis, LRAT is constitutively expressed.(Zolfaghari & Ross, 2000)

An overview of the enzyme expression of the vitamin A metabolism is depicted in **Table 4**.

Hypothesis

In the practical part of this study, we will determine whether certain enzymes of the vitamin A and D metabolism are expressed in the liver of cats. To support my hypothesis, I examined the enzyme expression in other species.

The expression of the vitamin D enzymes CYP24A1 and CYP27B1 is investigated in humans and rats. Although the expression is detected in many different tissues, neither of the enzymes is detected in the liver. In both human and rat, the highest expression seems to be in de kidney. Although a difference in species may occur, my hypothesis is that we will not detect any enzyme in the cat liver.

Anderson et al. suggests that the expression and activity of CYP24A1 and CYP27B1 are regulated by PTH, calcium and calcitonin. In this study, there is no information about the status of these factors. Thus, we cannot make a statement about the quantitative expression of those enzymes. We can only say whether or not the enzyme is expressed in the cat liver. Control feeding experiments are necessary to investigate this.

The expression of the vitamin A enzymes CYP1A2, CYP26A1, CYP26B1 and LRAT is investigated in humans, rats, pigs and mice. All enzymes, except CYP26B1 in human, are expressed in the liver. I expect that we will detect those enzymes in cat liver also.

In addition I will determine whether there is a difference in enzyme expression among young healthy livers and old cirrhotic livers. This is important because deforming cervical spondylosis is often seen in older cats, which generally have cirrhotic livers.

When we assume that hypervitaminosis A plays a role in this disease, it would be likely that CYP26 is decreased in affected animals because this enzyme induces retinoid degradation. On the other hand, CYP1A2 is active in catalyzing retinol to the active form. It would be possible that this enzyme will be more active in this syndrome. When storage of retinol by LRAT fails, there can be more active vitamin A in the animal. This can also contribute to a hypervitaminosis A. However, in this experiment we are dealing with cirrhotic livers, which are not necessarily of diseased animals. The mechanisms of hypervitaminosis are probably multifactorial and thus further studies are needed.

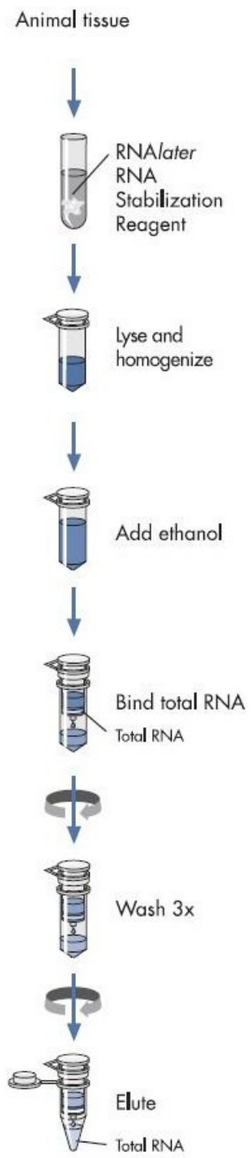


Fig. 5. RNA purification using RNeasy technology

Tissue collection and storage

The material was obtained from euthanized cats, referred for pathological investigation at the Pathology Department of the Veterinary Faculty in Utrecht. No experimental animals were used for the sole purpose of this study. For this study we collected livers from 4 kittens, 8 weeks old, and 4 adult cats ranging in age from 12 to 17 years.

To prevent unwanted changes in the gene expression profile, the samples are immediately immersed in RNA*later* RNA stabilization reagent, stored at -20°C for 1 hour, then the reagent is being removed and the sample tissue is subsequently stored at -70°C until RNA isolation. To ensure rapid and reliable stabilization of RNA even in the inner parts of solid tissues, the sample must be cut into slices less than 0.5 cm thick. For the protocol from the RNeasy® Mini Handbook see Attachment 1.

Primer design and testing

No primers were available for the enzymes CYP1A2, CYP26A1, CYP26B1 and LRAT. So primer sets had to be developed. Those were based on known cat sequences that are published on Ensembl (www.ensembl.org) and GenBank (www.ncbi.nlm.nih.gov). Primer design was performed with Perl primer v.1.1.19 software (http://perlprimer.sourceforge.net/download.html). Primers were placed in different exons to prevent amplifying traces of genomic DNA. Uniqueness and specificity of each primer were verified using the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast). To determine whether secondary structures were formed, Mfold was used (www.bioinfo.rpi.edu/applications/mfold). The primers were tested using quantitative PCR on liver and kidney tissue from one cat. Optimal T_m was determined using a temperature gradient.

RNA isolation

RNeasy technology was used to extract RNA from the liver samples (**Fig. 5**). This technology is based on the selective binding properties of a silica-based membrane and microspin technology.
The samples are first lysed and homogenized in a buffer that inactivates RNases to prevent RNA degradation. Ethanol is added to provide appropriate binding conditions. The sample is applied to an RNeasy Mini spin column, where the RNA binds to the membrane. To prevent DNA contamination, a DNase is added. Subsequently, contaminants are washed away in three washing steps. The RNA is then eluted in RNA free water. For the protocol from the RNeasy® Mini Handbook see Attachment 2.

Sample	Concentration of harvested RNA (ng/µl)	RNA (µl)	RNA (µg)
Kitten 1	0,044	12	0,528
Kitten 2	0,276	2,0	0,552
Kitten 3	0,277	2,0	0,554
Kitten 4	0,287	2,0	0,574
Cat 5	0,213	2,5	0,532
Cat 6	0,33	1,5	0,495
Cat 7	0,406	1,0	0,406

Table 5. Calculation of volume RNA

cDNA synthesis

The iScript® cDNA Synthesis Kit was used to synthesize cDNA out of RNA. This kit is based on a two-step RT-PCR. For the protocol of the iScript® cDNA Synthesis Kit see Attachment 3.

In the reaction set-up we need around 0,5 µg RNA template. To calculate how much volume of RNA we need, we must look at the RNA concentration of the harvested RNA. This is depicted in **Table 5**.

Quantitative PCR

The Bio-RAD My-IQ detection system in combination with the DNA-binding dye SYBR green was used for quantitative PCR. To ensure an internal standard, the following reference genes were used: hypoxanthine phosphoribosyltransferase (HPRT), glyceraldehyde-3-phosphatedehydrogenase (GAPDH), ribosomal protein S19 (RPS19) and ribosomal protein L17 (RPL17).(Penning et al., 2007)

Reactions started with 3 minutes at 95°C followed by 45 cycles of 10 seconds at 95°C and 45 cycles of 30 seconds at T_m. This reaction was continued by 24 cycles of 30 seconds at 72°C.

Calculations to estimate expression stability and the pair wise variation were performed using geNorm (http://medgen.ugent.be/~jvdesomp/genorm)

Results

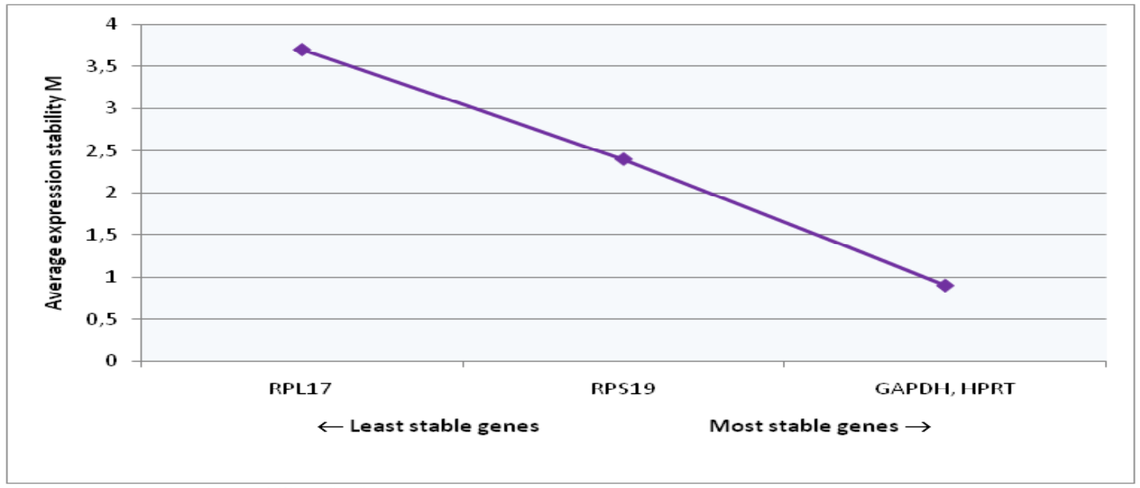
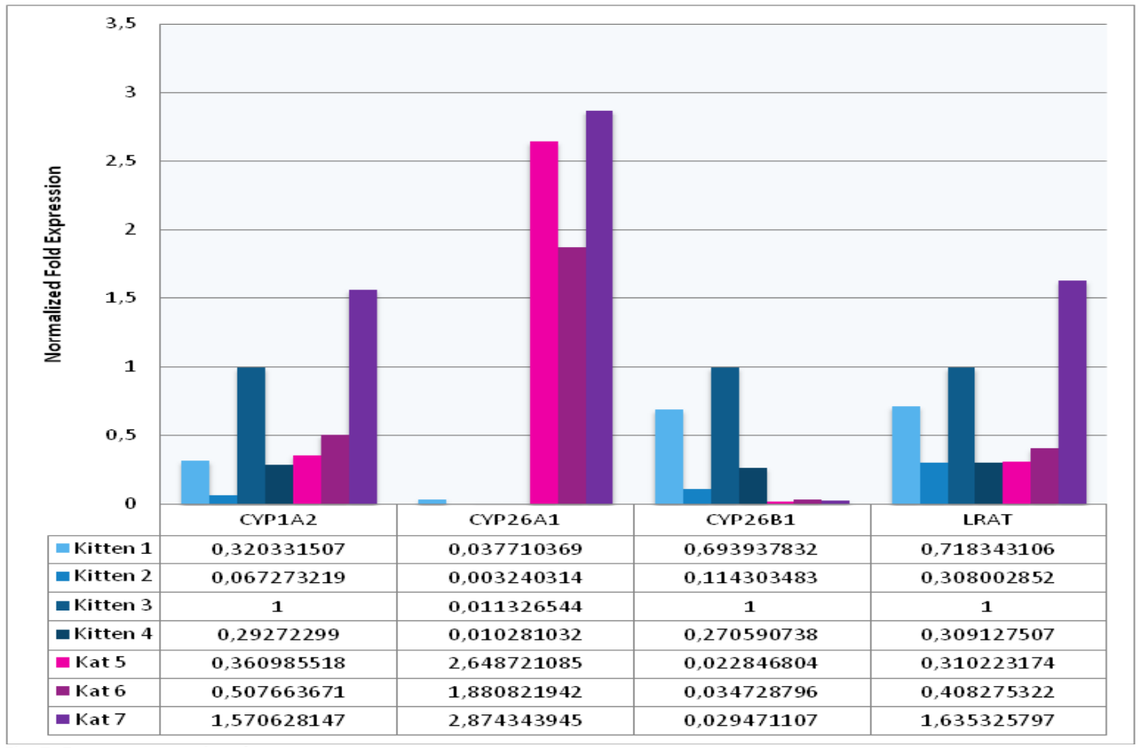


Fig. 6. Avarage expression stability of reference genes



	CYP1A2	CYP26A1	CYP26B1	LRAT
Kitten 1	0,320331507	0,037710369	0,693937832	0,718343106
Kitten 2	0,067273219	0,003240314	0,114303483	0,308002852
Kitten 3	1	0,011326544	1	1
Kitten 4	0,29272299	0,010281032	0,270590738	0,309127507
Kat 5	0,360985518	2,648721085	0,022846804	0,310223174
Kat 6	0,507663671	1,880821942	0,034728796	0,408275322
Kat 7	1,570628147	2,874343945	0,029471107	1,635325797

Fig. 7. Enzyme expression data.

Primers

Primers were developed for the enzymes CYP1A2, CYP26A1, CYP26B1 and LRAT. Accession numbers, primer sequences and optimal T_m values are depicted in **Table 6**.

The primers were tested using qPCR on liver and kidney tissue from one cat. All standard curve efficiencies were between 95% and 105%, indicating the primers are able to duplicate the amplicons each cycle. The melting curve of all tested primers showed a single peak, thus one specific product was formed.

Gene	Accession Number	Forward Primer 5' → 3'	Reverse Primer 5' → 3'	T_m
CYP1A2	NM_001048013	CCTCACGAGAAGATTGTCAG	ACTGTGTCCAGCTCCTC	62,5 °C
CYP26A1	ENSFCAG00000007248	CAAGAGTAAGGGTTTACTTTGC	TGTAGATAACATTCCAGCCC	62,5 °C
CYP26B1	ENSFCAG00000004358	CCACCTCTTTGAAGTCTACC	AGTAGTCCTTGCCCTGG	62,5 °C
LRAT	ENSFCAG00000008647	GAAGAACCCAATGCTGGA	ATCTCATAGAAACTGTTCCTCG	60,5 °C

Table 6. Details of primers

Enzyme expression

Relative gene expression by quantitative measurements of mRNA levels can be normalized using an internal standards such as reference genes. The expression variation and stability of those reference genes for cat liver tissue has to be determined. The program geNorm calculates the gene expression stability (*M*). A high *M* value indicates a gene with less stable expression, thus for a less optimal reference gene. **Fig. 6.** shows that GAPDH and HPRT are the most suitable reference genes for this study. With these control genes we normalized the data for the genes of interest.

CYP24A1 and CYP27B1

The enzymes CYP24A1 and CYP27B1 from the vitamin D metabolism were not detected in the livers of the 7 cats.

CYP1A2, CYP26A1, CYP26B1 and LRAT

All other enzymes investigated, the vitamin A enzymes CYP1A2, CYP26A1, CYP26B1 and LRAT, are expressed in cat liver. The quantitative expression is shown in **Fig. 7**.

Does a shift occur?

To compare the enzyme expression in the group of young livers with the group of old livers, we used the statistical program SPSS. Data were analyzed using the T-test. A p-value of <0,05 was considered significant.

A shift in expression between healthy and cirrhotic livers does not significantly occur in the enzymes CYP1A2 (P=0.427), CYP26B1 (P=0.095) and LRAT (p=0.695). This is not the case for the expression of CYP26A1 (P<0,01). The expression of CYP26A1 in kittens is very low. In contrast, the expression in older cats is relatively high for CYP26A1.

Discussion & Conclusion

Review of literature

In the first part of this study we reviewed what is already known in literature about the vitamin A metabolism in general and in which extend the cat is different from other species. Below, the investigated differences are listed up.

- Cats lack the enzyme to convert β-carotene, which thus cannot be used as a source for vitamin A. Thus, cats depend of retinyl esters in their diet.
- In contrast to other species, cats do not transport retinol in blood bound to RBP, but as retinyl esters bound to lipoproteins. Thus, retinyl esters in plasma are not associated with vitamin A intoxication.
- Vitamin A levels in blood are not homeostatically regulated, but depend on the dietary vitamin A intake.
- Cats have a reduced urinary vitamin A excretion compared to dogs. This could be an explanation for the sensitivity to vitamin A intoxication in cats or just a protection mechanism against hypovitaminosis A. However, a reduced urinary vitamin A excretion could also be an explanation for the sensitivity to vitamin A intoxication in cats.

However, literature is not consistent on some topics. Those are listed below.

- There is a contradiction in studies about the amount of storage of vitamin A in the cat liver. Some groups claim that it contains very little vitamin A while others say that cat liver contains high concentrations of Vitamin A, even compared to other species like the dog.
- It is unsure whether the liver or the kidneys contain most Vitamin A.

Hypervitaminosis A alone?

Skeletal problems like deforming cervical spondylosis are often associated with hypervitaminosis A. Cats that are fed a diet that mainly consists of liver, which contains high levels of vitamin A, are susceptible to a vitamin A toxicity. However, we suggest that in this disease there is an interaction between vitamin A and vitamin D.

Some studies showing that excess of vitamin A is toxic, used livers as a source of vitamin A in the diet.[(Seawright & English, 1967)] Others based their investigations on a case report where the cat(s) did have a high amount of liver in their diet.[(Hayes, 1982, Franch, 2000, Polizopoulou et al., 2005)] However, liver also contains high levels of vitamin D.

Unfortunately, potential interactions between vitamins A and D have not been examined in cats. However, excessive (606.000 RE/kg diet) intake of vitamin A alone did not cause deforming cervical spondylosis after three years. In this study it was not specifically addressed and thus requires further study.[(Freytag et al., 2003)]

Enzyme expression

In the second part of the study, I determined whether the enzymes CYP24A1, CYP27B1, CYP1A2, CYP26A1, CYP26B1 and LRAT are expressed in cat liver. In addition I made a comparison of the quantitative expression between healthy and cirrhotic livers, to see if shift would occur.

- The enzymes CYP24A1 and CYP27B1 from the vitamin D metabolism were not detected in cat liver. Those enzymes are probably not expressed in cat liver. Another possibility is that the enzyme expression is below the detection limit.
- The expression and activity of CYP24A1 and CYP27B1 appears to be regulated by PTH, calcium and calcitonin. In this study, there is no information about the status of these factors. Thus, we cannot make a statement about the quantitative expression of those enzymes. Control feeding experiments are necessary to investigate this.
- The enzymes of the vitamin A metabolism, CYP1A2, CYP26A1, CYP26B1 and LRAT, are expressed in cat liver.
- A shift in expression of CYP26A1 occurs between kittens and old cats. The expression in young livers is very low, while the expression in old livers is relatively high. However, this is not consistent with my hypothesis. When we assume that hypervitaminosis A plays a role in the metabolic bone disease 'deforming cervical spondylosis' , it would be likely that CYP26 is decreased because this enzyme is responsible for the degradation of vitamin A. However, CYP26A1 might play a greater role during RA excess, while CYP26B1 appear to be essential under physiological conditions. If this is true, it is more likely that CYP26A1 is indeed more expressed in older livers, to degradate the excess.
- There is no significantly shift in expression for the enzymes CYP1A2, CYP26B1 and LRAT. However, the groups were very small. Thus, we should be cautious with conclusions. Larger groups will make the results more reliable. This is particularly true for CYP26B1, since the p-value was only 0,095.

The results of this study does not indicate a direct effect of the examined enzymes in the pathophysiology of the metabolic bone disease. This disease is most likely multifactorial. More research should be done to understand the pathophysiology of 'deforming cervical spondylosis'!

References

Adams, J.S., O.P. Sharma, M.A. Gacad et al. (1983), Metabolism of 25-hydroxyvitamin D3 by cultured pulmonary alveolar macrophages in sarcoidosis. The Journal of clinical investigation 72(5), pp.1856-1860.

Anderson, P.H., P.D. O'Loughlin, B.K. May et al. (2003), Quantification of mRNA for the vitamin D metabolizing enzymes CYP27B1 and CYP24 and vitamin D receptor in kidney using real-time reverse transcriptase- polymerase chain reaction. Journal of Molecular Endocrinology 31(1), pp.123-132.

Batten, M.L., Y. Imanishi, T. Maeda et al. (2004), Lecithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver. The Journal of biological chemistry 279(11), pp.10422-10432.

Blomberg Jensen, M., J.E. Nielsen, A. Jorgensen et al. (2010), Vitamin D receptor and vitamin D metabolizing enzymes are expressed in the human male reproductive tract. Human reproduction (Oxford, England) 25(5), pp.1303-1311.

Blutt, S.E., E.A. Allegretto, J.W. Pike et al. (1997), 1,25-dihydroxyvitamin D3 and 9-cis-retinoic acid act synergistically to inhibit the growth of LNCaP prostate cells and cause accumulation of cells in G1. Endocrinology 138(4), pp.1491-1497.

Chambon, P. (1996), A decade of molecular biology of retinoic acid receptors. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology 10(9), pp.940-954.

Chen, H., W.N. Howald & M.R. Juchau. (2000), Biosynthesis of all-trans-retinoic acid from all-trans-retinol: catalysis of all-trans-retinol oxidation by human P-450 cytochromes. Drug metabolism and disposition: the biological fate of chemicals 28(3), pp.315-322.

Cheskis, B. & L.P. Freedman. (1994), Ligand modulates the conversion of DNA-bound vitamin D3 receptor (VDR) homodimers into VDR-retinoid X receptor heterodimers. Molecular and cellular biology 14(5), pp.3329-3338.

Chew, B.P., J.S. Park, B.C. Weng et al. (2000), Dietary beta-carotene absorption by blood plasma and leukocytes in domestic cats. The Journal of nutrition 130(9), pp.2322-2325.

Franch, J., J. Pastor, B. Franch et al. (2000), Back-scattered electron imaging of a non-vertebral case of hypervitaminosis A in a cat. Journal of feline medicine and surgery 2(1), pp.49-56.

Freytag, T.L., S.M. Liu, Q.R. Rogers et al. (2003), Teratogenic effects of chronic ingestion of high levels of vitamin A in cats. Journal of Animal Physiology and Animal Nutrition 87(1-2), pp.42-51.

Futterman, S. & J.S. Andrews. (1964), The Composition of Liver Vitamin a Ester and the Synthesis of Vitamin a Ester by Liver Microsomes. The Journal of biological chemistry 239, pp.4077-4080.

Hand, M.S., C.D. Thatcher, R.L. Remillard et al. (2010). Small animal clinical nutrition. Small Animal Clinical Nutrition5th ed., pp. 126-129. Kansas: Mark Morris Intitute.

Harrison, E.H. (2005), Mechanisms of digestion and absorption of dietary vitamin A. Annual Review of Nutrition 25, pp.87-103.

Hayes, K.C. (1982), Nutritional problems in cats: taurine deficiency and vitamin A excess. The Canadian veterinary journal.La revue veterinaire canadienne 23(1), pp.2-5.

Heanes, D.L. (1990), Vitamin A concentrations in commercial foods for dogs and cats. Australian Veterinary Journal 67(8), pp.291-294.

Hewison, M., D. Zehnder, R. Bland et al. (2000), 1alpha-Hydroxylase and the action of vitamin D. Journal of Molecular Endocrinology 25(2), pp.141-148.

How, K.L., H.A. Hazewinkel & J.A. Mol. (1994), Dietary vitamin D dependence of cat and dog due to inadequate cutaneous synthesis of vitamin D. General and comparative endocrinology 96(1), pp.12-18.

Jimenez-Lara, A.M. & A. Aranda. (2000), Interaction of vitamin D and retinoid receptors on regulation of gene expression. Hormone research 54(5-6), pp.301-305.

Johansson, S. & H. Melhus. (2001), Vitamin A antagonizes calcium response to vitamin D in man. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 16(10), pp.1899-1905.

Kim, C.I., M.A. Leo & C.S. Lieber. (1992), Retinol forms retinoic acid via retinal. Archives of Biochemistry and Biophysics 294(2), pp.388-393.

Kindmark, A., H. Melhus, S. Ljunghall et al. (1995), Inhibitory effects of 9-cis and all-trans retinoic acid on 1,25(OH)2 vitamin D3-induced bone resorption. Calcified tissue international 57(3), pp.242-244.

Kojima, M., M. Sekimoto & M. Degawa. (2010), Androgen-mediated down-regulation of CYP1A subfamily genes in the pig liver. The Journal of endocrinology 207(2), pp.203-211.

Le Carrour, T., S. Assou, S. Tondeur et al. (2010), Amazonia! An Online Resource to Google and Visualize Public Human whole Genome Expression Data. The Open Bioinformatics Journal 4, pp.5-10.

Lee, S.J., L. Perera, S.J. Coulter et al. (2007), The discovery of new coding alleles of human CYP26A1 that are potentially defective in the metabolism of all-trans retinoic acid and their assessment in a recombinant cDNA expression system. Pharmacogenetics and genomics 17(3), pp.169-180.

Li, E. & P. Tso. (2003), Vitamin A uptake from foods. Current opinion in lipidology 14(3), pp.241-247.

Lowe, J.S., R.A. Morton & J. Vernon. (1957), Unsaponifiable constituents of kidney in various species. The Biochemical journal 67(2), pp.228-234.

Mallia, A.K., J.E. Smith & D.W. Goodman. (1975), Metabolism of retinol-binding protein and vitamin A during hypervitaminosis A in the rat. Journal of lipid research 16(3), pp.180-188.

McSorley, L.C. & A.K. Daly. (2000), Identification of human cytochrome P450 isoforms that contribute to all-trans-retinoic acid 4-hydroxylation. Biochemical pharmacology 60(4), pp.517-526.

Metz, A.L., M.M. Walser & W.G. Olson. (1985), The interaction of dietary vitamin A and vitamin D related to skeletal development in the turkey poult. The Journal of nutrition 115(7), pp.929-935.

Morris, J.G. (1999), Ineffective vitamin D synthesis in cats is reversed by an inhibitor of 7-dehydrocholestrol-delta7-reductase. The Journal of nutrition 129(4), pp.903-908.

Nadin, L. & M. Murray. (1999), Participation of CYP2C8 in retinoic acid 4-hydroxylation in human hepatic microsomes. Biochemical pharmacology 58(7), pp.1201-1208.

Napoli, J.L. (1999), Interactions of retinoid binding proteins and enzymes in retinoid metabolism. Biochimica et biophysica acta 1440(2-3), pp.139-162.

Pavez Lorie, E., H. Li, A. Vahlquist et al. (2009), The involvement of cytochrome p450 (CYP) 26 in the retinoic acid metabolism of human epidermal keratinocytes. Biochimica et biophysica acta 1791(3), pp.220-228.

Penning, L.C., H.E. Vrieling, B. Brinkhof et al. (2007), A validation of 10 feline reference genes for gene expression measurements in snap-frozen tissues. Veterinary immunology and immunopathology 120(3-4), pp.212-222.

Polizopoulou, Z.S., G. Kazakos, M.N. Patsikas et al. (2005), Hypervitaminosis A in the cat: a case report and review of the literature. Journal of feline medicine and surgery 7(6), pp.363-368.

Prosser, D.E. & G. Jones. (2004), Enzymes involved in the activation and inactivation of vitamin D. Trends in biochemical sciences 29(12), pp.664-673.

Raila, J., I. Buchholz, H. Aupperle et al. (2000), The distribution of vitamin A and retinol-binding protein in the blood plasma, urine, liver and kidneys of carnivores. Veterinary research 31(6), pp.541-551.

Raila, J., U. Mathews & F.J. Schweigert. (2001), Plasma transport and tissue distribution of beta-carotene, vitamin A and retinol-binding protein in domestic cats. Comparative biochemistry and physiology.Part A, Molecular & integrative physiology 130(4), pp.849-856.

Raila, J., R. Radon, A. Trupschuch et al. (2002), Retinol and retinyl ester responses in the blood plasma and urine of dogs after a single oral dose of vitamin A. The Journal of nutrition 132(6 Suppl 2), pp.1673S-5S.

Reinhardt, T.A., N.J. Koszewski, J. Omdahl et al. (1999), 1,25-Dihydroxyvitamin D(3) and 9-cis-retinoids are synergistic regulators of 24-hydroxylase activity in the rat and 1, 25-dihydroxyvitamin D(3) alters retinoic acid metabolism in vivo. Archives of Biochemistry and Biophysics 368(2), pp.244-248.

Rohde, C.M. & H.F. DeLuca. (2005), All-trans retinoic acid antagonizes the action of calciferol and its active metabolite, 1,25-dihydroxycholecalciferol, in rats. The Journal of nutrition 135(7), pp.1647-1652.

Rohde, C.M., M. Manatt, M. Clagett-Dame et al. (1999), Vitamin A antagonizes the action of vitamin D in rats. The Journal of nutrition 129(12), pp.2246-2250.

Ross, A.C. (1982), Retinol esterification by rat liver microsomes. Evidence for a fatty acyl coenzyme A: retinol acyltransferase. The Journal of biological chemistry 257(5), pp.2453-2459.

Schuster, I. (2010), Cytochromes P450 are essential players in the vitamin D signaling system. Biochimica et biophysica acta.

Schweigert, F.J. & V. Bok. (2000), Vitamin A in blood plasma and urine of dogs is affected by the dietary level of vitamin A. International journal for vitamin and nutrition research.Internationale Zeitschrift fur Vitamin- und Ernahrungsforschung.Journal international de vitaminologie et de nutrition 70(3), pp.84-91.

Schweigert, F.J. & J. Raila. (2002), Mechanisms involved in the intestinal digestion and absorption of dietary vitamin A. The Journal of nutrition 132(2), pp.324-325.

Schweigert, F.J., J. Raila & S. Haebel. (2002), Vitamin A excreted in the urine of canines is associated with a Tamm-Horsfall like protein. Veterinary research 33(3), pp.299-311.

Schweigert, F.J., J. Raila, B. Wichert et al. (2002), Cats absorb beta-carotene, but it is not converted to vitamin A. The Journal of nutrition 132(6 Suppl 2), pp.1610S-2S.

Schweigert, F.J., O.A. Ryder, W.A. Rambeck et al. (1990), The majority of vitamin A is transported as retinyl esters in the blood of most carnivores. Comparative biochemistry and physiology.A, Comparative physiology 95(4), pp.573-578.

Seawright, A.A. & P.B. English. (1964), Deforming Cervical Spondylosis in the Cat. The Journal of pathology and bacteriology 88, pp.503-509.

Seawright, A.A. & P.B. English. (1967), Hypervitaminosis A and deforming cervical spondylosis of the cat. Journal of comparative pathology 77(1), pp.29-39.

Seawright, A.A., P.B. English & R.J. Gartner. (1970), Hypervitaminosis A of the cat. Advances in Veterinary Science and Comparative Medicine 14, pp.1-27.

Shinki, T., Y. Ueno, H.F. DeLuca et al. (1999), Calcitonin is a major regulator for the expression of renal 25-hydroxyvitamin D3-1-alpha-hydroxylase gene in normocalcemic rats. Proceedings of the National Academy of Sciences of the United States of America 96(14), pp.8253-8258.

Smith, F.R. & D.S. Goodman. (1976), Vitamin A transport in human vitamin A toxicity. The New England journal of medicine 294(15), pp.805-808.

Sonneveld, E., C.E. van den Brink, B.M. van der Leede et al. (1998), Human retinoic acid (RA) 4-hydroxylase (CYP26) is highly specific for all-trans-RA and can be induced through RA receptors in human breast and colon carcinoma cells. Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research 9(8), pp.629-637.

Townsend, K., K.N. Evans, M.J. Campbell et al. (2005), Biological actions of extra-renal 25-hydroxyvitamin D-1alpha-hydroxylase and implications for chemoprevention and treatment. The Journal of steroid biochemistry and molecular biology 97(1-2), pp.103-109.

Urushino, N., K. Yasuda, S. Ikushiro et al. (2009), Metabolism of 1alpha,25-dihydroxyvitamin D2 by human CYP24A1. Biochemical and biophysical research communications 384(2), pp.144-148.

Wilson, D.E., J. Hejazi, N.L. Elstad et al. (1987), Novel aspects of vitamin A metabolism in the dog: distribution of lipoprotein retinyl esters in vitamin A-deprived and cholesterol-fed animals. Biochimica et biophysica acta 922(3), pp.247-258.

Wolf, G. (2001), Retinoic acid homeostasis: retinoic acid regulates liver retinol esterification as well as its own catabolic oxidation in liver. Nutrition reviews 59(12), pp.391-394.

Zhang, Y., R. Zolfaghari & A.C. Ross. (2010), Multiple retinoic acid response elements cooperate to enhance the inducibility of CYP26A1 gene expression in liver. Gene 464(1-2), pp.32-43.

Zierold, C., J.A. Mings & H.F. DeLuca. (2001), Parathyroid hormone regulates 25-hydroxyvitamin D(3)-24-hydroxylase mRNA by altering its stability. Proceedings of the National Academy of Sciences of the United States of America 98(24), pp.13572-13576.

Zolfaghari, R., C.J. Cifelli, S.O. Lieu et al. (2007), Lipopolysaccharide opposes the induction of CYP26A1 and CYP26B1 gene expression by retinoic acid in the rat liver in vivo. American journal of physiology.Gastrointestinal and liver physiology 292(4), pp.G1029-36.

Zolfaghari, R. & A.C. Ross. (2000), Lecithin:retinol acyltransferase from mouse and rat liver. CDNA cloning and liver-specific regulation by dietary vitamin a and retinoic acid. Journal of lipid research 41(12), pp.2024-2034.

Zou, A., M.G. Elgort & E.A. Allegretto. (1997), Retinoid X receptor (RXR) ligands activate the human 25-hydroxyvitamin D3-24-hydroxylase promoter via RXR heterodimer binding to two vitamin D-responsive elements and elicit additive effects with 1,25-dihydroxyvitamin D3. The Journal of biological chemistry 272(30), pp.19027-19034.

Attachment 1

Protocol: Stabilisation of RNA in Harvested Animal Tissues
RNeasy® Mini Handbook

This protocol describes how to stabilize and store human and animal tissues in RNAlater RNA Stabilization Reagent, included in the RNeasy Protect Mini Kit.

Important notes about RNAlater RNA Stabilization Reagent
RNA in harvested animal tissue is not protected until the tissue is completely submerged in a sufficient volume of RNA*later* RNA Stabilization Reagent. After harvesting, the tissue should be immediately placed in at least 10 volumes of the reagent (or approximately 10 µl reagent per 1 mg tissue). Larger volumes can be used if necessary or desired. Smaller volumes may lead to RNA degradation during storage. Storage containers should be wide enough so that the reagent covers the entire tissue. Storage containers or tubes with large diameters may require more reagent to completely cover the tissue. The procedures for tissue harvesting and RNA stabilization should be carried out as quickly as possible.

Tissue size is critical for successful RNA stabilization with RNA*later* RNA Stabilization Reagent. Immediately upon contact, the reagent diffuses into the surface layer and outer portions of solid tissues. To ensure rapid and reliable stabilization of RNA even in the inner parts of solid tissues, the sample must be cut into slices less than 0.5 cm thick. The slices can be any convenient size, provided one dimension of the sample is <0.5 cm. If the slices are thicker than 0.5 cm, the reagent will diffuse too slowly into the interior of the sample and RNA degradation will occur.

Procedure

1. Before excising the tissue sample, estimate the volume (or weight) of the sample to be stabilized in RNA*later* RNA Stabilization Reagent.

2. Determine the appropriate volume of RNA*later* RNA Stabilization Reagent for preserving the tissue. At least 10 volumes of the reagent (or approximately 10 µl reagent per 1 mg of tissue) is required. Pipet the correct amount of reagent into an appropriate collection vessel.

Note: Be sure to completely submerge the tissue in RNAlater RNA Stabilization Reagent.

3. Excise the tissue sample from the animal and, if necessary, cut it into slices less than 0.5 cm thick. Perform this step as quickly as possible and proceed immediately to step 4.

4. Completely submerge the tissue piece(s) in the collection vessel containing RNAlater RNA Stabilization Regent from step 2.

Note: The tissue sample must be immediately submerged in RNAlater RNA Stabilization Reagent to protect the RNA.

5. Store the tissue submerged in RNAlater RNA Stabilization Reagent for up to 4 weeks at 2–8°C, up to 7 days at 15–25°C, or up to 1 day at 37°C. For archival storage at –20°C, first incubate the tissue overnight in the reagent at 2–8°C. Then transfer the tissue, in the reagent, to –20°C for storage. For archival storage at –80°C, first incubate the tissue overnight in the reagent at 2–8°C. Then remove the tissue from the reagent, and transfer it to –80°C for storage.

Note: Lower temperatures are recommended for longer storage (e.g., 2–8°C for up to 4 weeks instead of 37°C or room temperature; –20°C or –80°C for longer storage). Tissues stored in RNAlater RNA Stabilization Reagent at –20°C may not freeze. The low temperature may cause the formation of crystals or a precipitate in the reagent. This will not affect subsequent RNA purification. There is no need to redissolve the precipitate. RNAlater stabilized tissues stored at –20°C or –80°C can be thawed at room temperature and frozen again for up to 20 freeze–thaw cycles without affecting RNA quality or yield.

If transporting tissue samples in RNAlater RNA Stabilization Reagent, ensure that the tissues always remain submerged in the reagent. Either keep the tubes upright during transport or fill the tubes completely with RNAlater RNA Stabilization Reagent.

Attachment 2

Protocol: Purification of Total RNA from Animal Tissues

RNeasy® Mini Handbook

This protocol requires the RNeasy Mini Kit or RNeasy Protect Mini Kit.

Determining the correct amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. A maximum amount of 30 mg fresh or frozen tissue or 15–20 mg RNAlater stabilized tissue (which is partially dehydrated) can generally be processed. For most tissues, the RNA binding capacity of the RNeasy spin column and the lysing capacity of Buffer RLT will not be exceeded by these amounts.

Some tissues, such as spleen, parts of brain, lung, and thymus are more difficult to lyse or tend to form precipitates during RNA purification. The volume of Buffer RLT may need to be increased to facilitate complete homogenization and to avoid significantly reduced RNA yields, DNA contamination, or clogging of the RNeasy spin column. See the procedure below for details.

Do not overload the RNeasy spin column, as this will significantly reduce RNA yield and quality.

Weighing tissue is the most accurate way to quantitate the amount of starting material. As a guide, a 3 mm cube (27 mm3) of most animal tissues weighs 30–35 mg.

Important points before starting

- Fresh, frozen, or RNAlater stabilized tissues can be used. Tissues can be stored at –70°C for several months. Flash-freeze tissues in liquid nitrogen, and immediately transfer to –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates from step 4 can also be stored at –70°C for several months. Incubate frozen lysates at 37°C in a water bath until completely thawed and salts are dissolved before continuing with step 5. Avoid prolonged incubation, which may compromise RNA integrity.
- If desired, more than 30 mg tissue can be disrupted and homogenized at the start of the procedure (increase the volume of Buffer RLT proportionately). Use a portion of the homogenate corresponding to no more than 30 mg tissue for RNA purification, and store the rest at –80°C.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- Perform all steps of the procedure at room temperature. During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT containing β-ME can be stored at room temperature for up to 1 month.
- Alternatively, add 20 µl of 2 M dithiothreitol (DTT) per 1 ml Buffer RLT. The stock solution of 2 M DTT in water should be prepared fresh or frozen in single-use aliquots. Buffer RLT containing DTT can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- If performing optional on-column DNase digestion, prepare DNase I stock solution as described in Appendix D (page 69).

Procedure

1. Excise the tissue sample from the animal or remove it from storage. Remove RNAlater stabilized tissues from the reagent using forceps. Determine the amount of tissue. Do not use more than 30 mg.

Weighing tissue is the most accurate way to determine the amount.

Note: If the tissues were stored in RNAlater Reagent at –20°C, be sure to remove any crystals that may have formed.

2. For RNAlater stabilized tissues: If using the entire tissue, place it directly into a suitably sized vessel for disruption and homogenization, and proceed to step 3. If using only a portion of the tissue, cut it on a clean surface. Weigh the piece to be used, and place it into a suitably sized vessel for disruption and homogenization. Proceed to step 3.

RNA in RNAlater stabilized tissues is protected during cutting and weighing of tissues at ambient temperature (15–25°C). It is not necessary to cut the tissues on ice or dry ice or in a refrigerated room. Remaining tissues can be stored in RNAlater RNA Stabilization Reagent. Previously stabilized tissues can be stored at –80°C without the reagent.

3. Disrupt the tissue and homogenize the lysate in Buffer RLT (do not use more than 30 mg tissue) according to step 3a, 3b, 3c, or 3d.

Note: Ensure that β-ME is added to Buffer RLT before use (see “Things to do before starting”).

After storage in RNAlater RNA Stabilization Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization using standard methods is usually not a problem. For easier disruption and homogenization, we recommend using 600 µl Buffer RLT.

Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with the TissueLyser and rotor–stator homogenizers generally results in higher RNA yields than with other methods.

Amount of starting material (mg)	Volume of Buffer RLT (µl)
<20	350 or 600*
20-30	600

* Use 600 µl Buffer RLT for tissues stabilized in RNAlater RNA Stabilization Reagent or for difficult-to-lyse tissues.

Table. Volumes of Buffer RLT for Tissue disruption and Homogenization

a. Disruption and homogenization using a rotor–stator homogenizer:
Place the weighed (fresh, frozen, or RNAlater stabilized) tissue in a suitably sized vessel. Add the appropriate volume of Buffer RLT (see Table 8). Immediately disrupt and homogenize the tissue using a conventional rotor–stator homogenizer until it is uniformly homogeneous (usually 20–40 s). Proceed to step 4.

b. Disruption using a mortar and pestle followed by homogenization using a QIAshredder homogenizer:
Immediately place the weighed (fresh, frozen, or RNAlater stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase- free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of Buffer RLT (see Table 8). Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Proceed to step 4.

c. Disruption using a mortar and pestle followed by homogenization using a needle and syringe:
Immediately place the weighed (fresh, frozen, or RNAlater stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of Buffer RLT (see Table 8), and homogenize by passing the lysate at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe. Proceed to step 4.

d. Disruption and homogenization using the TissueLyser:
See the TissueLyser Handbook. Then proceed to step 4.

4. Centrifuge the lysate for 3 min at full speed. Carefully remove the supernatant by pipetting, and transfer it to a new microcentrifuge tube (not supplied). Use only this supernatant (lysate) in subsequent steps.

In some preparations, very small amounts of insoluble material will be present after the 3 min centrifugation, making the pellet invisible.

5. Add 1 volume of 70% ethanol* to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.

Note: The volume of lysate may be less than 350 µl or 600 µl due to loss during homogenization and centrifugation in steps 3 and 4.

Note: Precipitates may be visible after addition of ethanol. This does affect the procedure.

6. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Reuse the collection tube in step 7.

If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.

Optional: If performing optional on-column DNase digestion, follow steps D1–D4 after performing this step.

Optional On-Column DNase Digestion

with the RNase-Free DNase Set

The RNase-Free DNase Set provides efficient on-column digestion of DNA during RNA purification. The DNase is efficiently removed in subsequent wash steps.

Note: Standard DNase buffers are not compatible with on-column DNase digestion. Use of other buffers may affect the binding of RNA to the RNeasy membrane, reducing RNA yield and integrity.

Lysis and homogenization of the sample and binding of RNA to the RNeasy membrane are performed according to the standard protocols. After washing with a reduced volume of Buffer RW1, the RNA is treated with DNase I while bound to the RNeasy membrane. The DNase I is removed by a second wash with Buffer RW1. Washing with Buffer RPE and elution of RNA are then performed according to the standard protocols.

Important points before starting

- Generally, DNase digestion is not required since RNeasy technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). DNA can also be removed by a DNase digestion following RNA purification.
- Do not vortex the reconstituted DNase I. DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.

Things to do before starting

- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNasefree water provided. To avoid loss of DNase I, do not open the vial. Inject RNasefree water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex.
- For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

Procedure

Prepare and load samples onto the RNeasy spin column as indicated in the individual protocols. Instead of performing the first wash step, follow steps D1–D4 below.

D1. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step D4.

D2. Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube. Buffer RDD is supplied with the RNase-Free DNase Set.

Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

D3. Add the DNase I incubation mix (80 µl) directly to the RNeasy spin column membrane, and place on the benchtop (20–30°C) for 15 min.

Note: Be sure to add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

D4. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.Continue with the first Buffer RPE wash step in the relevant protocol.

Note: In most of the protocols, the immediately following Buffer RW1 wash step is skipped (as indicated in the protocol). Continue with the first Buffer RPE wash step.

7. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 9.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see “Things to do before starting”).

8. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane.

The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere withdownstream reactions.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

9. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.

Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

10. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.

11. If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl RNasefree water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 11.

If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

Attachment 3

Protocol: iScript™cDNA Synthesis Kit

iScript cDNA Synthesis kit provides a sensitive and easy-to-use solution for two-step RT-PCR. This kit includes just three tubes - comprehensive of the reagents required for successful RT-PCR.

The iScript reverse transcriptase is RNAse H+, resulting in greater sensitivity than RNAse H- enzymes. iScript is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided pre-blended with RNAse inhibitor. The unique blend of oligo (dT) and random hexamer primers in the iScript Reaction Mix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets < 1kb in length.

iScript cDNA Synthesis Kit produces excellent results in both real-time and conventional RT-PCR.

Storage and Stability

Store the iScript™ cDNA Synthesis Kit at -20 °C in a constant temperature freezer. When stored under these conditions the kit components are stable for a minimum of one year after ship date. Nuclease-free water can be stored at room temperature.

Reaction Set Up

Component	Volume per reaction
5x iScript Reaction Mix	4 µl
iScript Reverse Transcriptase	1 µl
Nuclease-free water	x µl
RNA template (0,5µg)	x µl
Total Volume	*20 µl*

Reaction Protocol

Incubate complete reaction mix: 5 minutes at 25ºC, 30 minutes at 42ºC, 5 minutes at 85ºC, Hold at 4ºC (optional)

Recommendations for optimal results using the iScript cDNA Synthesis Kit:
The maximum amount of the cDNA reaction that is recommended for downstream PCR is one-tenth of the reaction volume, typically 2µl. When using larger amounts of input RNA (>1µg) the reaction should be scaled up e.g. 40µl reaction for 2µg, 100µl reaction for 5µg to ensure optimum synthesis efficiency.