Pathophysiologic Aspects of Feline Hepatic Lipidosis

Chiara Valtolina

Ontwerp en grafische begeleiding: Kees van Veenendaal, Heemstede
Illustraties: Joeke van der Meer, Essen (https://joeke.net)
Druk: Drukkerij de Bij, Amsterdam

Pathophysiologic Aspects of Feline Hepatic Lipidosis

Pathofysiologische aspecten van leververvetting bij de kat
(met een samenvatting in het Nederlands)

Aspetti Patofisiologici della Lipidosi Epatica Felina
(con un riassunto in italiano)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op dinsdag 23 april 2019 des ochtends te 10.30 uur

door

Chiara Valtolina

geboren op 14 juni 1974
te Milaan, Italië

Promotor
Prof. dr. J.W. Hesselink

Copromotoren
Dr. J.H. Robben
Dr. R.P. Favier

Dit proefschrift werd (mede) mogelijk gemaakt met financiële steun van:
Royal Canine
MSD Animal Health
Affinity PetCare
IDEXX Laboratories B.V.
Covetrus and Zoetis

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Chapter 1

Scope and aims of the thesis

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Feline hepatic lipidosis (FHL) is considered the most common hepatobiliary disease in cats.[1-6] It is characterized by the excessive accumulation of triglycerides (TGs) in more than 80% of the hepatocytes resulting in secondary impairment of liver function, and intrahepatic cholestasis.[1,2,7-9] Although FHL is mainly reported in middle-aged cats (median age 7 years), cats of any age can be affected. Although no clear breed or gender predilection for this disease has been reported, in some studies female cats seem to be overrepresented. [1,2,7,10] Actual (body condition 4/5) or historical obesity has been mentioned by several investigators as a predisposing factor for the development of FHL.[1,4,7,11]

Feline hepatic lipidosis is classified as primary or secondary hepatic lipidosis (HL). In primary FHL anorexia occurs in a healthy animal secondary to decreased food availability, administration of non-palatable food, or decreased food intake secondary to a stressful event.[8,12] Secondary lipidosis is considered the most common form of lipidosis, occurring in about 95% of the cases. It is the consequence of an underlying disease of which pancreatitis, cholangitis and chronic kidney disease are the most common diseases reported. [1,2,6,13]

Feline hepatic lipidosis presents as an acute critical syndrome that can result in the animal's death due to severe liver dysfunction and failure if therapeutic measures are not taken expeditiously. The development of FHL in an anorectic feline patient during hospitalisation can contribute to a negative outcome.

The pathophysiology of FHL is complex and the variability in reported historical, physical, and clinicopathologic findings in cats with HL suggests that this is a syndrome with different causative factors. [1,2,13] One very important factor is associated with the intrinsic pure carnivore nature of cats that characterises the lipid and protein metabolism. It makes the feline patient dependent on obligatory essential fatty acid (EFAs), amino acids, and vitamins that may become exhausted after a period of prolonged anorexia. These deficiencies are considered important cofounding factors for the development of FHL. [11,13]

Chapter 2 presents a detailed overview of the pathophysiology and clinical presentation of FHL , with emphasis on the specific characteristics and needs of

the cat as an obligate carnivore. Some new insights in the pathophysiology of FHL are discussed, based on the in-depth study of the pathophysiologic mechanisms of HL development in people with non-alcoholic fatty liver disease (NAFLD) and in other obligated carnivores, such as the European polecat (*Mustela putorius*) and the American mink (*Neovison vison*). [14-18]

The primary metabolic abnormalities leading to the accumulation of TGs in hepatocytes leading to FHL are not yet completely understood. Most likely, they consist of disturbances in the pathways that regulate uptake, synthesis, degradation, and secretion of fatty acids (FAs). There is an imbalance between mobilisation of peripheral fat stores and *de novo* synthesis of FAs in the liver or visceral adipose tissue on the one hand, and insufficient increase in the rate of hepatic FAs β-oxidation for energy and insufficient dispersal of hepatic TGs via very-low density lipoprotein (VLDL) on the other hand.[1,2,11,13] The deregulation in these metabolic processes that plays a pivotal role in the accumulation of TGs in the liver and the development of FHL remains unclear. A focused and specific treatment plan could be developed if one pathway is more important than the others in the development of FHL.

The aim of this thesis is an attempt to unravel the characteristics of the different pathways of lipid mobilisation, hepatic lipid metabolism and *de novo* lipid synthesis in healthy cats and cats with FHL.

In the liver FAs are reconstituted into TGs, which are then secreted in the peripheral circulation in VLDLs or are transported over the mitochondrial membrane by L-carnitine to enter the β-oxidation. VLDLs secretion is reported to be enhanced in FHL and although the most recent literature have questioned this finding , it has been suggested that a lower capacity to excrete VLDL, could play an important role in the development of FHL.[2,4,19,20] Secretion of VLDL is dependent on the rate of hepatic phosphatidylcholine (PC) synthesis. [21] Phosphatidylcholine is synthetized in the liver via two pathways, the "Kennedy pathway" and the "phosphatidylethanolamine N-methyltransferase (PEMT) pathway". The Kennedy pathway is the major route for PC synthesis and is present in all mammalian tissues. The synthesis of PC via this pathway depends on the intake of choline.[21]

Cats require higher amounts of several B-vitamins, such as cobalamin, folate, and choline, as compared to other species. Therefore in cats, depletion during periods of anorexia, maldigestion, or malassimilation may have a bigger impact liver function.[11] Suboptimal concentrations of dietary choline has been associated in

the cat with a diminished capacity of the liver to synthesize PC resulting in accumulation of lipids in the liver.[22-24]

The PEMT pathway is liver-specific and is estimated to account for approximately 20–40% of the newly synthesized PC in the liver of humans, mice and rats.[25-27] In humans and mice PEMT expression is known to be upregulated by oestrogens.[26,28,29] Because of its strong oestrogen influence, the majority of premenopausal woman and female rats fed a choline deficient diet, are less likely to develop hepatic lipidosis than the male counterparts, as they have 30-50% more capacity to form choline *de novo* via the PEMT pathway. [28,30] Postmenopausal women and castrated female mice have demonstrated to have lower levels of PEMT activity and therefore to be more susceptible to become choline deficient and develop HL when fed a choline deficient diet. [26,28,31]

Cats that are anorectic will be deficient of choline and the PEMT pathway should be activated to be able to produce sufficient amounts of PC and secondary VLDL. In cats the importance of the PEMT pathway and the hormonal influence on plasmatic and hepatic lipid composition in a healthy feline population has not been evaluated.

In **Chapter 3** the lipid metabolism in healthy cats that are fed a choline-restricted diet is described and the hormonal effect on choline synthesis via the PEMT pathway is evaluated. Furthermore, a detailed description of the difference in lipid composition between male and female cats, pre- and post-gonadectomy is presented in order to gain a better insight into possible predisposing factors in the development of FHL.

The role of lipids in the development and progression of numerous diseases in man, such as diabetes mellitus, obesity, NAFLD, Alzheimer and arteriosclerosis is well recognised [32-34]. The recognition of sex-specific genetic dimorphism in lipid metabolism has helped to identify risk factors and to develop more specific and targeted therapies.[35,36] The essential FAs (EFAs) profile is known to vary considerably between males and females in humans, mice and rats, indicating that sex-lipid dimorphism occurs in different species.[29,37-44] Pre-menopausal women are considered to possess a more favourable (less pro-coagulatory and less pro-atherogenic) plasma lipid profile compared to men and postmenopausal women with lower plasma levels of TGs, total cholesterol (TC), and low density lipoprotein-cholesterol (LDL-C) in combination with higher levels of high density lipoprotein-cholesterol (HDL-C).[45-48]

In human medicine, the study and evaluation of lipid metabolites with the use of lipid profiling or "lipidomics" has become crucial in order to understand the role

of lipids in the pathophysiology of lipid-related diseases.[49,50]

Butterwick *et al.* have reported no important differences in plasma lipid and lipoprotein concentrations between sexually intact females and males or between sexually intact and castrated males cats.[51] However, as sexual dimorphism in lipid metabolism is evident in other species, sexual dimorphism in cats deserves additional exploration.

In **Chapter 4** the lipid profile of intact and spayed/neutered healthy cats fed with a common commercial diet is investigated with the help of lipidomic analysis. Furthermore, the effect of gonadectomy on the liver and plasma lipid profiles is evaluated. Finally, the plasma and liver lipid profile of six cats with HL are examined in detail with the lipidomic technique. By comparing the lipid profiles of healthy cats and cats with HL predisposing factors in the development of FHL may be elucidated.

Another deregulated pathway in lipid metabolism that could contribute to TG accumulation in FHL could be the *de novo* synthesis of FAs. In normal conditions, *de novo* lipogenesis (DNL) converts excess carbohydrate into FAs that are consequently esterified to storage TGs and in a later stage may be used as an energy source via β-oxidation. The main product of DNL is palmitate, but stearate and shorter FAs are also generated.[52-54] The adipogenic transformation of hepatocytes, i.e. the expression of gene profiles by the liver characteristic of healthy adipose tissue, seems to contribute to 26% of FAs that are accumulated in the liver of patients with NAFLD.[52,53,55] Rouvinen-Watt *et al.*, in a recent study evaluating mRNA expression of lipogenic genes in the liver of mink with HL, confirmed that the initial development of steatosis is secondary to increased FAs mobilisation from visceral abdominal fat tissue and the subsequent increase in circulating FAs concentrations, but also partly by the activation of hepatic DNL.[56]

The hypothesis that fat accumulation in the liver could be exacerbated by *de novo* synthesis of FAs (either by the adipose tissue or by the liver itself) was supported by the presence of increased hepatic concentrations of palmitate in cats with FHL.[4]

De novo fatty acid synthesis in the liver is regulated by three important nuclear transcription factors that enhance the expression of several genes involved in lipogenesis: sterol regulatory element-binding protein 1 (SREBP-1c), carbohydrate response element-binding protein (ChREBP) and peroxisome proliferator-activated receptor- y (PPAR-y).[57-59]

In **Chapter 5** we investigate the concept of adipogenic transformation of hepatocytes and de novo lipid synthesis in the liver of cats with FHL and its contribution to lipid load in FHL. Lipogenic gene expression of PPAR-α, PPAR-γ, FAS, SREBF1 and are determined with the use of qRT-PCR on liver of cats affected by HL compared to healthy cats,.

In humans increasing evidence suggests that steatosis should not be considered just an innocent bystander in NAFLD, but is associated with the development of steatohepatitis and fibrosis.[60-62] The primary histological feature of FHL is simple steatosis but, in contrast to NAFLD, progression into steatohepatitis and chronic liver failure has never been documented in cats with HL.[7,63]

Mature liver cells, i.e. hepatocytes and cholangiocytes, have a remarkable capacity to proliferate and restore liver function in response to different types of injury.[64-66] However, in situations of acute severe or chronic ongoing disease the ability of hepatocytes to restore liver mass and function becomes impaired.[66-74] Liver steatosis, in humans and a rodent model of NAFLD, appeared sufficient to inhibit replication of mature hepatocytes and to trigger the activation of a reserve population of cells, known as hepatic stem or progenitor cells (HPCs).[75,76] Once activated, HPCs proliferate and depending on the type of disease, HPCs can differentiate towards hepatocytes and/or cholangiocytes. The balance between renewal, proliferation and differentiation of HPCs is determined by the interaction between the cells and stroma present in the microenvironment, i.e. the "HPC niche".[77-79]

In rodents, humans and dogs, important cellular components in this niche include HPCs, hepatic stellate cells (HSCs), their differentiated counterparts, alpha-smooth muscle actin (α-SMA)-positive myofibroblasts and macrophages/ Kupffer cells with laminin as the main stromal component.[64,65,80-82] In humans and dogs the ductular reaction and the degree of HPC and its niche activation is directly related to the severity of disease.[67,75,80,83] This is indicated by the amount of hepatocyte loss, the amount of inflammation and the extent of fibrosis, and it is seen in diseases like acute hepatitis, chronic hepatitis, biliary disease and hepatic tumours.[67,69-72,74,75,81,84-87]

The remodelling and activation of the HPCs and their niche has recently been described in feline lymphocytic cholangitis (LC).[88] The characteristics of the HPCs and their niche in feline LC share similarities to the HPCs and their niche in sclerosing cholangitis in human medicine. In **Chapter 6** the progenitor liver cells and their interaction in FHL are characterised with the help of immunohistochemistry. The cellular and stromal pattern of the HPC niche

in FHL is described, with emphasis on MAC387-positive Kupffer cells, α-SMA positive myofibroblasts and the important extracellular matrix component laminin. To substantiate the immunohistochemical data double immuno-fluorescence is used to describe co-localisations of the various cell types within the HPC niche in FHL.

In Chapter 7 the results of the study are summarised and discussed. In addition, recommendation and suggestions for further studies are made that could further help in understanding the pathophysiology of FHL, with the goal of improving our current therapeutical opportunities.

Chapter 3

- Analyse the lipid metabolism in healthy cats before and after spaying/neutering
- Evaluation of sex hormone on choline synthesis via the PEMT pathway in healthy cats fed a restricted choline diet

Chapter 4

- Try and highlight predisposing factors in feline hepatic lipidosis studying the lipidomics of healthy cats and cats with hepatic lipidosis
- Perform lipid profiling in intact and spayed/neutered healthy cats fed with a common commercial diet
- Evaluate the effect of gonadectomy on the liver and plasma lipid profiles
- Perform lipid profiling in cats affected by hepatic lipidosis and compare it to healthy cats

Chapter 5

- Investigate the concept of de novo lipid synthesis in the liver of cats with FHL and its contribution to lipid load in FHL
- The expression of the lipogenic gene of PPAR-α, PPAR-y, FAS, SREBF1 are determined with the use of qRT-PCR on liver of cats affected by HL compared to healthy cats

Chapter 6

- To characterised the progenitor liver cells and their interaction in FHL with the help of immunohistochemistry.
- The cellular and stromal pattern of the HPC niche in FHL is described, with emphasis on MAC387-positive Kupffer cells, α-SMA positive myofibroblasts and the important extracellular matrix component laminin.
- To substantiate the immunohistochemical data double immunofluorescence is used to describe co-localisations of the various cell types within the HPC niche in FHL.

Figure 1: Aims of the thesis

References

1. Armstrong PJ, Blanchard G. Hepatic lipidosis in cats. *Vet Clin North Am Small Anim Pract.* 2009;39(3):599-616. doi: 10.1016/j.cvsm.2009.03.003 [doi].

2. Center SA. Feline hepatic lipidosis. *Vet Clin North Am Small Anim Pract.* 2005;35(1): 225-269. doi: S0195-5616(04)00142-1 [pii].

3. Dimski DS. Feline hepatic lipidosis. *Semin Vet Med Surg (Small Anim).* 1997;12(1):28-33.

4. Hall JA, Barstad LA, Connor WE. Lipid composition of hepatic and adipose tissues from normal cats and from cats with idiopathic hepatic lipidosis. *J Vet Intern Med.* 1997;11(4):238-242.

5. Lund EM, Armstrong PJ, Kirk CA, Kolar LM, Klausner JS. Health status and population characteristics of dogs and cats examined at private veterinary practices in the united states. *J Am Vet Med Assoc.* 1999;214(9):1336-1341.

6. Webb CB. Hepatic lipidosis: Clinical review drawn from collective effort. *J Feline Med Surg.* 2018;20(3):217-227. doi: 10.1177/1098612X18758591 [doi].

7. Center SA, Crawford MA, Guida L, Erb HN, King J. A retrospective study of 77 cats with severe hepatic lipidosis: 1975-1990. *J Vet Intern Med.* 1993;7(6):349-359.

8. Biourge VC, Groff JM, Munn RJ, et al. Experimental induction of hepatic lipidosis in cats. *Am J Vet Res.* 1994;55(9):1291-1302.

9. Postic C, Girard J. The role of the lipogenic pathway in the development of hepatic steatosis. *Diabetes Metab.* 2008;34(6 Pt 2):643-648. doi: 10.1016/S1262-3636(08)74599-3 [doi].

10. Brown B, Mauldin GE, Armstrong J, Moroff SD, Mauldin GN. Metabolic and hormonal alterations in cats with hepatic lipidosis. *J Vet Intern Med.* 2000;14(1):20-26.

11. Verbrugghe A, Bakovic M. Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis. *Nutrients.* 2013;5(7):2811-2835. doi: 10.3390/nu5072811 [doi].

12. Biourge VC, Massat B, Groff JM, Morris JG, Rogers QR. Effects of protein, lipid, or carbohydrate supplementation on hepatic lipid accumulation during rapid weight loss in obese cats. *Am J Vet Res.* 1994;55(10):1406-1415.

13. Valtolina C, Favier RP. Feline hepatic lipidosis. *Vet Clin North Am Small Anim Pract.* 2017;47(3):683-702. doi: S0195-5616(16)30160-7 [pii].

14. Mustonen AM, Pyykonen T, Paakkonen T, et al. Adaptations to fasting in the american mink (mustela vison): Carbohydrate and lipid metabolism. *Comp Biochem Physiol A Mol Integr Physiol.* 2005;140(2):195-202. doi: S1095-6433(04)00351-4 [pii].

15. Nieminen P, Rouvinen-Watt K, Collinsb D, Grant J, Mustonen AM. Fatty acid profiles and relative mobilization during fasting in adipose tissue depots of the american marten (martes americana). *Lipids*. 2006;41(3):231-240.

16. Araya J, Rodrigo R, Videla LA, et al. Increase in long-chain polyunsaturated fatty acid n - 6/n - 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin Sci (Lond).* 2004;106(6):635-643. doi: 10.1042/CS20030326 [doi].

17. Videla LA, Rodrigo R, Araya J, Poniachik J. Oxidative stress and depletion of hepatic long-chain polyunsaturated fatty acids may contribute to nonalcoholic fatty liver disease. *Free Radic Biol Med*. 2004;37(9):1499-1507. doi: 10.1016/j.freeradbiomed.2004.06.033 [doi].

18. Rouvinen-Watt K, Mustonen AM, Conway R, et al. Rapid development of fasting-induced hepatic lipidosis in the american mink (neovison vison): Effects of food deprivation and re-alimentation on body fat depots, tissue fatty acid profiles, hematology and endocrinology. *Lipids*. 2010;45(2):111-128. doi: 10.1007/s11745-009-3377-4 [doi].

19. Minamoto T, Walzem RL, Hamilton AJ, et al. Altered lipoprotein profiles in cats with hepatic lipidosis. *J Feline Med Surg.* 2018:1098612X18780060. doi: 10.1177/1098612X18780060 [doi].

20. Pazak HE, Bartges JW, Cornelius LC, Scott MA, Gross K, Huber TL. Characterization of serum lipoprotein profiles of healthy, adult cats and idiopathic feline hepatic lipidosis patients. *J Nutr.* 1998;128(12 Suppl):2747S-2750S.

21. Vance DE. Phospholipid biosynthesis in eukaryotes. In: Vance DE, Vance JE, eds. *Biochemistry of lipids, lipoproteins and membranes*. 5th ed. Amsterdam: Elsevier; 2002:205-232.

22. Da Silva AC, Guerios S MF, Monsao SR. The domestic cat as a laboratory animal for experimental nutrition studies. VI. choline deficiency. *J Nutr.* 1959;67(4):537-547.

23. Anderson PA, Baker DH, Sherry PA, Corbin JE. Choline-methionine interrelationship in feline nutrition. *J Anim Sci.* 1979;49(2):522-527.

24. Schaeffer MC, Rogers QR, Morris JG. Methionine requirement of the growing kitten, in the absence of dietary cystine. *J Nutr.* 1982;112(5):962-971.

25. da Costa KA, Sanders LM, Fischer LM, Zeisel SH. Docosahexaenoic acid in plasma phosphatidylcholine may be a potential marker for in vivo phosphatidylethanolamine N-methyltransferase activity in humans. *Am J Clin Nutr.* 2011;93(5):968-974. doi: 10.3945/ajcn.110.011064 [doi].

26. Noga AA, Vance DE. A gender-specific role for phosphatidylethanolamine N-methyl transferase-derived phosphatidylcholine in the regulation of plasma high density and very low density lipoproteins in mice. *J Biol Chem*. 2003;278(24):21851-21859. doi: 10.1074/jbc.M301982200 [doi].

27. Pani P, Porcu M, Columbano A, Dessi S, Ledda GM, Diaz G. Differential effects of choline administration on liver microsomes of female and male rats. *Exp Mol Pathol.* 1978;28(2):154-162. doi: 0014-4800(78)90048-5 [pii].

28. Resseguie M, Song J, Niculescu MD, da Costa KA, Randall TA, Zeisel SH. Phosphatidylethanolamine N-methyltransferase (PEMT) gene expression is induced by estrogen in human and mouse primary hepatocytes. *FASEB J.* 2007;21(10):2622-2632. doi: fj.07-8227com [pii].

29. Young DL. Estradiol- and testosterone-induced alterations in phosphatidylcholine and triglyceride synthesis in hepatic endoplasmic reticulum. *J Lipid Res.* 1971;12(5):590-595.

30. Fischer LM, daCosta KA, Kwock L, et al. Sex and menopausal status influence human dietary requirements for the nutrient choline. *Am J Clin Nutr.* 2007;85(5):1275-1285. doi: 85/5/1275 [pii].

31. Noga AA, Vance DE. Insights into the requirement of phosphatidylcholine synthesis for liver function in mice. *J Lipid Res.* 2003;44(10):1998-2005. doi: 10.1194/jlr.M300226-JLR200 [doi].

32. Wenk MR. The emerging field of lipidomics. *Nat Rev Drug Discov.* 2005;4(7):594-610. doi: nrd1776 [pii].

33. Watson AD. Thematic review series: Systems biology approaches to metabolic and cardiovascular disorders. lipidomics: A global approach to lipid analysis in biological systems. *J Lipid Res.* 2006;47(10):2101-2111. doi: R600022-JLR200 [pii].

34. Musso G, Gambino R, Cassader M. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Prog Lipid Res.* 2009;48(1):1-26. doi: 10.1016/j.plipres.2008.08.001 [doi].

35. Arnold AP. Promoting the understanding of sex differences to enhance equity and excellence in biomedical science. *Biol Sex Differ.* 2010;1(1):1-6410-1-1. doi: 10.1186/2042-6410-1-1 [doi].

36. Taylor KC, Carty CL, Dumitrescu L, et al. Investigation of gene-by-sex interactions for lipid traits in diverse populations from the population architecture using genomics and epidemiology study. *BMC Genet.* 2013;14:33-2156-14-33. doi: 10.1186/1471-2156-14-33 [doi].

37. Ostwald R, Bouchard P, Miljanich P, Lyman RL. Influence of sex and gonadal hormones on rat-liver and carcass lipids during the development of an essential fatty acid deficiency. *Biochem J.* 1965;97(2):485-499.

38. Lyman RL, Tinoco J, Bouchard P, Sheehan G, Ostwald R, Miljanich P. Sex differences in the metabolism of phosphatidyl cholines in rat liver. *Biochim Biophys Acta.* 1967;137(1):107-114.

39. Lyman RL, Hopkins SM, Sheehan G, Tinoco J. Effects of estradiol and testosterone on the incorporation and distribution of [me-14C]methionine methyl in rat liver lecithins. *Biochim Biophys Acta*. 1968;152(1):197-207.

40. Carroll MD, Lacher DA, Sorlie PD, et al. Trends in serum lipids and lipoproteins of adults, 1960-2002. *JAMA*. 2005;294(14):1773-1781. doi: 294/14/1773 [pii].

41. Mittendorfer B. Sexual dimorphism in human lipid metabolism. *J Nutr.* 2005;135(4): 681-686. doi: 135/4/681 [pii].

42. Burdge GC, Slater-Jefferies JL, Grant RA, et al. Sex, but not maternal protein or folic acid intake, determines the fatty acid composition of hepatic phospholipids, but not of triacylglycerol, in adult rats. *Prostaglandins Leukot Essent Fatty Acids.* 2008;78(1):73-79. doi: S0952-3278(07)00179-2 [pii].

43. Extier A, Langelier B, Perruchot MH, et al. Gender affects liver desaturase expression in a rat model of n-3 fatty acid repletion. *J Nutr Biochem.* 2010;21(3):180-187. doi: 10.1016/j.jnutbio.2008.10.008 [doi].

44. Childs CE, Romeu-Nadal M, Burdge GC, Calder PC. The polyunsaturated fatty acid composition of hepatic and plasma lipids differ by both sex and dietary fat intake in rats. *J Nutr.* 2010;140(2):245-250. doi: 10.3945/jn.109.115691 [doi].

45. Gardner CD, Winkleby MA, Fortmann SP. Population frequency distribution of non-high-density lipoprotein cholesterol (third national health and nutrition examination survey [NHANES III], 1988-1994). *Am J Cardiol.* 2000;86(3):299-304. doi: S0002-9149(00)00918-8 [pii].

46. Gostynski M, Gutzwiller F, Kuulasmaa K, et al. Analysis of the relationship between total cholesterol, age, body mass index among males and females in the WHO MONICA project. *Int J Obes Relat Metab Disord*. 2004;28(8):1082-1090. doi: 10.1038/sj.ijo.0802714 [doi].

47. Regitz-Zagrosek V, Lehmkuhl E, Mahmoodzadeh S. Gender aspects of the role of the metabolic syndrome as a risk factor for cardiovascular disease. *Gend Med.* 2007;4 Suppl B:S162-77. doi: S1550-8579(07)80056-8 [pii].

48. Williams CM. Lipid metabolism in women. *Proc Nutr Soc*. 2004;63(1):153-160. doi: 10.1079/PNS2003314 [doi].

49. Han X, Gross RW. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: A bridge to lipidomics. *J Lipid Res.* 2003;44(6):1071-1079. doi: 10.1194/jlr.R300004-JLR200 [doi].

50. Hu C, van der Heijden R, Wang M, van der Greef J, Hankemeier T, Xu G. Analytical strategies in lipidomics and applications in disease biomarker discovery. *J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877(26):2836-2846.* doi: 10.1016/j.jchromb.2009.01.038 [doi].

51. Butterwick RF, McConnell M, Markwell PJ, Watson TD. Influence of age and sex on plasma lipid and lipoprotein concentrations and associated enzyme activities in cats. *Am J Vet Res.* 2001;62(3):331-336.

52. Lambert JE, Ramos-Roman MA, Browning JD, Parks EJ. Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology.* 2014;146(3):726-735. doi: 10.1053/j.gastro.2013.11.049 [doi].

53. Ameer F, Scandiuzzi L, Hasnain S, Kalbacher H, Zaidi N. De novo lipogenesis in health and disease. *Metabolism.* 2014;63(7):895-902. doi: 10.1016/j.metabol.2014.04.003 [doi].

54. Lee JJ, Lambert JE, Hovhannisyan Y, et al. Palmitoleic acid is elevated in fatty liver disease and reflects hepatic lipogenesis. *Am J Clin Nutr.* 2015;101(1):34-43. doi: 10.3945/ajcn.114.092262 [doi].

55. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest.* 2005;115(5):1343-1351. doi: 10.1172/JCI23621 [doi].

56. Rouvinen-Watt K, Harris L, Dick M, et al. Role of hepatic de novo lipogenesis in the development of fasting-induced fatty liver in the american mink (neovison vison). B*r J Nutr.* 2012;108(8):1360-1370. doi: 10.1017/S0007114511006775 [doi].

57. Reddy JK, Rao MS. Lipid metabolism and liver inflammation. II. fatty liver disease and fatty acid oxidation. *Am J Physiol Gastrointest Liver Physiol.* 2006;290(5):G852-8. doi: 290/5/G852 [pii].

58. Bugianesi E, Leone N, Vanni E, et al. Expanding the natural history of nonalcoholic steatohepatitis: From cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology.* 2002;123(1):134-140. doi: S0016508502000732 [pii].

59. Matsusue K, Haluzik M, Lambert G, et al. Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. *J Clin Invest.* 2003;111(5):737-747. doi: 10.1172/JCI17223 [doi].

60. Day CP, James OF. Hepatic steatosis: Innocent bystander or guilty party? *Hepatology.* 1998;27(6):1463-1466. doi: S0270913998002201 [pii].

61. Persico M, Iolascon A. Steatosis as a co-factor in chronic liver diseases. *World J Gastroenterol.* 2010;16(10):1171-1176.

62. Powell EE, Jonsson JR, Clouston AD. Steatosis: Co-factor in other liver diseases. *Hepatology.* 2005;42(1):5-13. doi: 10.1002/hep.20750 [doi].

63. Center SA, Guida L, Zanelli MJ, Dougherty E, Cummings J, King J. Ultrastructural hepatocellular features associated with severe hepatic lipidosis in cats. *Am J Vet Res.* 1993;54(5):724-731.

64. Roskams T, Yang SQ, Koteish A, et al. Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. *Am J Pathol.* 2003;163(4):1301-1311. doi: S0002-9440(10)63489-X [pii].

65. Fausto N. Liver regeneration and repair: Hepatocytes, progenitor cells, and stem cells. *Hepatology.* 2004;39(6):1477-1487. doi: 10.1002/hep.20214 [doi].

66. Michalopoulos GK. Liver regeneration. *J Cell Physiol.* 2007;213(2):286-300. doi: 10.1002/jcp.21172 [doi].

67. Lowes KN, Brennan BA, Yeoh GC, Olynyk JK. Oval cell numbers in human chronic liver diseases are directly related to disease severity. *Am J Pathol.* 1999;154(2):537-541. doi: S0002-9440(10)65299-6 [pii].

68. Clouston AD, Powell EE, Walsh MJ, Richardson MM, Demetris AJ, Jonsson JR. Fibrosis correlates with a ductular reaction in hepatitis C: Roles of impaired replication, progenitor cells and steatosis. *Hepatology.* 2005;41(4):809-818. doi: 10.1002/hep.20650 [doi].

69. Richardson MM, Jonsson JR, Powell EE, et al. Progressive fibrosis in nonalcoholic steatohepatitis: Association with altered regeneration and a ductular reaction. *Gastroenterology.* 2007;133(1):80-90. doi: S0016-5085(07)00994-8 [pii].

70. Bedossa P, Paradis V. Liver extracellular matrix in health and disease. *J Pathol.* 2003;200(4):504-515. doi: 10.1002/path.1397 [doi].

71. Guyot C, Lepreux S, Combe C, et al. Hepatic fibrosis and cirrhosis: The (myo)fibroblastic cell subpopulations involved. *Int J Biochem Cell Biol.* 2006;38(2):135-151. doi: S1357-2725(05)00272-4 [pii].

72. Morini S, Carotti S, Carpino G, et al. GFAP expression in the liver as an early marker of stellate cells activation. *Ital J Anat Embryol.* 2005;110(4):193-207.

73. Reeves HL, Burt AD, Wood S, Day CP. Hepatic stellate cell activation occurs in the absence of hepatitis in alcoholic liver disease and correlates with the severity of steatosis. *J Hepatol.* 1996;25(5):677-683. doi: S0168-8278(96)80238-8 [pii].

74. Carpino G, Morini S, Ginanni Corradini S, et al. Alpha-SMA expression in hepatic stellate cells and quantitative analysis of hepatic fibrosis in cirrhosis and in recurrent chronic hepatitis after liver transplantation. *Dig Liver Dis.* 2005;37(5):349-356. doi: S1590-8658(05)00018-6 [pii].

75. Roskams TA, Libbrecht L, Desmet VJ. Progenitor cells in diseased human liver. *Semin Liver Dis.* 2003;23(4):385-396. doi: 10.1055/s-2004-815564 [doi].

76. Yang S, Koteish A, Lin H, et al. Oval cells compensate for damage and replicative senescence of mature hepatocytes in mice with fatty liver disease. *Hepatology.* 2004;39(2):403-411. doi: 10.1002/hep.20082 [doi].

77. Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: Stem cells and their niche. *Cell.* 2004;116(6):769-778. doi: S0092867404002557 [pii].

78. Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. *Nature*. 2001;414(6859):98-104. doi: 10.1038/35102160 [doi].

79. Roskams T. Different types of liver progenitor cells and their niches. *J Hepatol*. 2006;45(1):1-4. doi: S0168-8278(06)00242-X [pii].

80. Libbrecht L, Roskams T. Hepatic progenitor cells in human liver diseases. *Semin Cell Dev Biol*. 2002;13(6):389-396. doi: S1084952102001258 [pii].

81. Ijzer J, Schotanus BA, Vander Borght S, et al. Characterisation of the hepatic progenitor cell compartment in normal liver and in hepatitis: An immunohistochemical comparison between dog and man. *Vet J*. 2010;184(3):308-314. doi: 10.1016/j.tvjl.2009.02.016 [doi].

82. Strick-Marchand H, Masse GX, Weiss MC, Di Santo JP. Lymphocytes support oval cell-dependent liver regeneration. *J Immunol*. 2008;181(4):2764-2771. doi: 181/4/2764 [pii].

83. Libbrecht L, Desmet V, Van Damme B, Roskams T. Deep intralobular extension of human hepatic 'progenitor cells' correlates with parenchymal inflammation in chronic viral hepatitis: Can 'progenitor cells' migrate? *J Pathol*. 2000;192(3):373-378. doi: 10.1002/1096-9896(2000)9999:99993.0.CO;2-5 [pii].

84. Katoonizadeh A, Nevens F, Verslype C, Pirenne J, Roskams T. Liver regeneration in acute severe liver impairment: A clinicopathological correlation study. *Liver Int*. 2006;26(10):1225-1233. doi: LIV1377 [pii]

85. Schotanus BA, van den Ingh TS, Penning LC, Rothuizen J, Roskams TA, Spee B. Cross-species immunohistochemical investigation of the activation of the liver progenitor cell niche in different types of liver disease. *Liver Int*. 2009;29(8):1241-1252. doi: 10.1111/j.1478-3231.2009.02024.x [doi].

86. Santoni-Rugiu E, Jelnes P, Thorgeirsson SS, Bisgaard HC. Progenitor cells in liver regeneration: Molecular responses controlling their activation and expansion. *APMIS*. 2005;113(11-12):876-902. doi: APMapm_386 [pii].

87. Spee B, Carpino G, Schotanus BA, et al. Characterisation of the liver progenitor cell niche in liver diseases: Potential involvement of wnt and notch signalling. *Gut*. 2010;59(2):247-257. doi: 10.1136/gut.2009.188367 [doi].

88. Otte CM, Valtolina C, Vreman S, et al. Immunohistochemical evaluation of the activation of hepatic progenitor cells and their niche in feline lymphocytic cholangitis. *J Feline Med Surg*. 2017:1098612X17699723. doi: 10.1177/1098612X17699723 [doi].

Chapter 2

Introduction

Feline hepatic lipidosis

Chiara Valtolina* and Robert P. Favier

Vet Clin Small Anim 47 (2017) 683–702

* *Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 108 (De Uithof) 3584 CM Utrecht, The Netherlands.*
c.valtolina@uu.nl

Synopsis

Feline hepatic lipidosis (FHL) is a common and potentially fatal liver disorder, characterized by an accumulation of triglycerides (TAG) in the liver, leading to an impaired hepatocyte function, cholestasis, and hepatic dysfunction. Although the exact pathophysiologic mechanisms of FHL remain elusive, there is an imbalance between the influx of fatty acids (FA) from peripheral fat stores into the liver, *de novo* liposynthesis, and the rate of hepatic oxidation and dispersal of hepatic TAG via excretion of very-low density lipoproteins. The diagnosis of FHL is based on anamnestic, clinical, and clinicopathologic findings, associated with diagnostic imaging of the liver, and cytology, or histological examination of liver biopsies. Fluid therapy, electrolyte correction and adequate early nutrition are essential components of the therapy for FHL.

Keywords
feline, hepatic lipidosis, cats, liver, triglyceride, obesity, VLDL, TAG

Key point
Pathophysiology of feline hepatic lipidosis; diagnosis of feline hepatic lipidosis; treatment of feline hepatic lipidosis

Introduction

Feline hepatic lipidosis (FHL), the most common hepatobiliary disease in cats, [1-5] is characterized by the accumulation of excessive triglyceride (TGs) in more than 80% of the hepatocytes resulting in a >50% increase in liver weight[2,6,7], secondary impairment of liver function, and intrahepatic cholestasis.[2,6,8,9] A specific geographic distribution of the disease has been suggested based on the available reports of FHL from different countries, including: North America, Great Britain, Japan and Western Europe). The higher prevalence of FHL in these areas might be secondary to feeding habits of cats and a high incidence of obesity in the feline population.[1]

The pathophysiology of FHL is complex. The primary metabolic abnormalities leading to TGs accumulation in the hepatocytes are not yet completely understood, but they could consist of alterations of the pathways of uptake, synthesis, degradation, and secretion of fatty acids (FA). Nonetheless, the variability in reported historical, physical, and clinicopathologic findings in cats with naturally occurring hepatic lipidosis suggests that this is a syndrome with many causative factors.

A negative energy balance, usually caused by anorexia is considered the primary cause for initiating FHL. In an experimental model of FHL, lipidosis occurs within two weeks' of the development of anorexia.[8,10] In a clinical setting, FHL has been seen to develop after a period of anorexia that ranges from 2-14 days.[1,6] Feline hepatic lipidosis is classified as primary or secondary. In primary FHL anorexia occurs in a healthy animal secondary to decreased food availability, administration of non-palatable food[7,8], or decreased food intake secondary to a stressful event. Secondary lipidosis occurs in animals that develop anorexia as a consequence of underlying disease. Secondary lipidosis is the most common form of lipidosis described, occurring in about 95% of cases. The diseases associated with the development of lipidosis are numerous and include: diabetes mellitus, pancreatitis, inflammatory hepatobiliary disease, gastrointestinal disease, renal failure, and neoplasia.[1,2]

Because the cat is a pure a carnivore, its lipid and protein metabolism [11-14] make it dependent on obligatory essential fatty acids (EFA), amino acids, and vitamins

which become deficient following a period of prolonged anorexia. These deficiencies are considered important cofounding factors for the development of FHL.[2,12]

The development of hepatic lipidosis (HL) after a period of anorexia has also been described in other strict carnivores, such as the European Polecat *(Mustela putorius)* and the American Mink *(Neovison vison)*.[15,16] The in-depth study of the pathophysiologic mechanisms behind the development of HL in these other obligated carnivores, could help us to better understand the pathophysiology of FHL in cats.

Pathophysiology

Due to evolutionary pressure, cats have developed unique adaptations of lipid and protein metabolism reflecting a strict carnivorous state[12-14,17-19], which impacts cats' requirements for EFA and essential aminoacids.[2,13,20] Like other mammals cats are unable to synthesize EFA like linoleic acid (18:2*n*-6; LA) and α-linoleic acid (18:3n-3; ALA). In addition to this, unlike other mammals, cats also have a limited capacity to synthesize the long chain polyunsaturated fatty acid (LCPUFA) arachidonic acid (20:4*n*-6; AA) from LA and eicosapentaenoic acid (20:3*n*-3; EPA) and docosahexaenoic acid (22:6*n*-3; DHA) from α-linolenic acid (18:3*n*-3; ALA). The explanation for this peculiarity is that cats have a severely decreased activity of the enzymes Δ5- and Δ6-desaturase, enzymes involved in the formation of LCPUFA from EFA.[21-24] Recently, Trevizan *et al.*, revealed that cats have an active Δ5-desaturase and that they are able to synthetize AA from γ-linolenic acid via bypassing the Δ6-desaturase step but not in an amount that will allow them to store this LCPUFA in condition of anorexia.[11]

Long chain poly-unsaturated fatty acids are involved in numerous processes. Increased levels of LCPUFA are well known to protect against the development of HL via the so-called "fuel partitioning action" of LCPUFA.[25,26] Long chain poly-unsaturated fatty acids, n-3 LCPUFA species (i.e. DHA), rather than the n-6 LCPUFA (i.e. AA), favour FA oxidation over TAG storage and they direct glucose away from FA synthesis by facilitating glycogen synthesis.[27,28] Long chain poly-unsaturated fatty acids downregulate sterol regulatory element binding protein-1 (SREBP-1) expression and impair its processing, resulting in an inhibition of the transcription of lipogenic and glycolytic genes.[28-30] Furthermore, n-3 LCPUFA species act as ligand activators of the peroxisome proliferator-activated receptor-α (PPAR-α) present in liver and adipose tissue upregulating the expression of genes encoding enzymes involved in FA oxidation.[28,31]

Cats possess limited ability to adapt their protein metabolic pathways for conserving nitrogen and they rapidly develop essential amino acid deficiency and protein malnutrition following a period of prolonged anorexia. In both experimentally induced and spontaneous FHL, plasma concentrations of alanine, arginine, citrulline, taurine, and methionine become markedly reduced (>50% reduction from baseline).[2,12,32]

Cats with FHL show changes in carbohydrate metabolism that resembles those seen in critically ill cats. Cats with HL, compared to healthy subjects, have higher circulating concentrations of glucose, lactate, glucagon, non-esterified fatty acid (NEFA), and have lower circulating concentrations of insulin.[33,34]

Although the exact pathophysiologic mechanism of FHL remains elusive, there is clearly an imbalance between the influx of NEFA derived from peripheral fat stores, de novo synthesis of FAs, the rate of hepatic FA oxidation for energy, and the dispersal of hepatic TAG via excretion of very-low density lipoproteins (VLDLs).

Influx of free fatty acid from peripheral fat stores

Feline hepatic lipidosis is considered to be a negative energy balance state and it is characterized by increased circulating concentrations of the counter regulatory hormones (glucagon, growth hormone, cortisol, and catecholamines) that lead to an increased activity of hormone-sensitive lipase (HSL), promoting lipolysis and mobilisation of NEFA from the visceral adipose tissue. Increased levels of counter regulatory hormones and decreased circulating concentrations of insulin lead to increased HSL activity, resulting in decreased lipogenesis, increased peripheral insulin resistance by decreased activity of the glucose transport protein-4 (GLUT-4), and impaired glucose tolerance.35 High concentrations of circulating FFA also contribute to peripheral insulin resistance.[1,2,36,37]

Nonesterified fatty acids are released from the adipose tissue and transported to the liver via the portal circulation. The predominant lipid that accumulates within the hepatocyte is TGs.[4] A higher concentration of palmitate (16:0) was found in liver tissue from cats with FHL compared to control subjects that mirrored the increased concentrations of palmitate in the adipose tissue of the same animals.[4] This finding confirmed the hypothesis that FFAs in livers of cats with FHL are derived from VAT.[2,4,12,33] Besides adipose tissue being the major site for storage of excess energy as TGs during a positive energy balance state, it has also an important endocrine function by secreting multiple adipokines including: adiponectin, leptin, chemokines, and cytokines.[38,39] These adipokines are involved in energy

homeostasis and inflammation and might be responsible for the development of peripheral insulin resistance.[38-40] Common inflammatory cytokines reported to be elevated in obese cats when compared to lean individuals are tumour necrosis factor alpha (TNFα) and interleukin 6 (IL-6).[41,43] Adiponectin exerts a profound insulin-sensitizing effect as well as anti-inflammatory and anti-atherosclerotic effects. Leptin is a regulator of adipose tissue mass and regulates insulin sensitivity.[39] The adipose tissue of FHL cats had markedly increased TNF-α concentrations compared to that of healthy subjects.[42] In cats with FHL both the serum concentrations of adiponectin and leptin were found to be increased compared to healthy subjects, but only leptin was significantly increased in cats with FHL compared to cats with other liver diseases.[39] Obesity predisposes cats to FHL during a period of anorexia, because of the quantity of FA that can be rapidly released from peripheral fat stores and VAT, release of inflammatory adipokines from the adipose tissue, and because of the insulin resistance associated with obesity.[1,2,37,43,44]

De novo lipogenesis

De novo lipid (DNL) synthesis in cats occurs mainly in the adipose tissue, followed by the liver, mammary glands, and muscle. This differs from human and rodents where the liver is the primary site for DNL synthesis.[45-47] While glucose is the precursor for DNL in humans, in cats acetate resulting from incomplete FA oxidation (i.e. ketogenesis), typically increased in FHL, is the substrate for the formation of the FFA palmitate (16:0).[2,36,46,48] Palmitate is found both in the adipose tissue and liver of cats with FHL, whereas it is not in healthy cats.[4] It cannot be ruled out that palmitate accumulation is the result of the *de novo* lipogenesis contributing to HL. Similarly in the mink, an animal that has metabolic similarities with the domestic cat,[19,46] DNL from acetate in HL may be accompanied by the adipogenic transformation of hepatocytes, as is seen in the human NAFLD, where the liver begins to express gene profiles characteristic of healthy adipose tissue.[46,49,50] To date, the concept of adipogenic transformation of hepatocytes has not been evaluated in cats.

Once having reached the liver, FFAs can enter two pathways: either they undergo β-oxidation in the mitochondria or they can be esterified to TAG and secreted via the very low-density lipoprotein (VLDL) pathway.

Hepatic beta-oxidation

Mitochondrial β-oxidation is the main oxidative pathway for the disposal of FA under normal physiologic conditions.[51] Short-chain and medium-chain NEFAs freely enter the mitochondria, while the activity of the enzyme carnitine palmitoyl

transferase-1 (CPT-1) regulates the entry of the long-chain FAs. Oxidation of FA produces acetyl-CoA, which can be used to provide energy via the tricarboxylic acid (Krebs) cycle to provide energy and/or to form ketone bodies. L-carnitine is part of the two enzymes that regulate transport of FA from the circulation into the mitochondria and from the hepatic cytosol back into plasma.

Ketone body formation is increased in cats with FHL, suggesting an enhanced rate of β-oxidation of FA.[1,2,32,36,37] However, it is unknown if the rate of β-oxidation is adapted to compensate for the greatly increased FA accumulation in the hepatocytes. The increase of ketone bodies in cases of lipidosis is most likely the result of the more complex catabolic state, increased insulin resistance, and decreased tolerance to glucose that develops in these patients than being the result of an increased rate of β-oxidation.[12,34,36]

In human medicine, abnormal β-oxidation from mitochondrial dysfunction has been suggested and reported as a potential cause for lipid accumulation in hepatocyte during NAFLD.[51] Center et al. reported that in FHL hepatocyte mitochondria were reduced in number and markedly abnormal, suggesting that mitochondrial dysfunction could also occur in FHL.[52]

There is a lot of discussion about the role of carnitine in FHL. Because L-carnitine is essential for the transportation of FA into the mitochondria, L-carnitine deficiency has been proposed as one of the main pathophysiologic mechanisms for the accumulation of fatty acids in the liver.[2,32,53,54] However, measurement of L-carnitine concentrations in different tissues (liver, kidney, blood) from cats affected with FHL failed to support this hypothesis.[53,55,56] On the other hand, there is also evidence that supplementation of L-carnitine in experimental lipidosis dramatically reduces hepatic lipid accumulation[57] and increases the rate of β-oxidation in obese cats.[53,54,58] Furthermore, a protective effect of L-carnitine was demonstrated in fasting cats and in cats with HL where supplementation of L-carnitine reduced the increase of plasma FA concentrations compared to control cats.[53] Therefore it is possible that in a situation of anorexia and an increased catabolic state tissue concentrations of L-carnitine are insufficient to meet demand and supplementation might be beneficial.

Methionine is an essential amino acid fundamental for numerous methylation reactions and an important thiol donor involved in the synthesis of glutathione (GSH). Glutathione is an important oxygen free radical scavenger and is involved in the hepatocellular protection against oxidative injury and its hepatic concentrations are reduced in cats with liver disease.[59] As methionine and its

coenzyme S-adenosyl methionine (SAMe) together are precursors of carnitine, methionine deficiency might contribute to inefficient levels of L-carnitine.[2,12,32,55]

Dispersal of hepatic TAG via VLDL excretion

Once in the hepatocyte, NEFAs can be esterified to TAG. Triglycerides usually accumulate in vacuoles within hepatocytes or can be incorporated into VLDLs to be excreted into the peripheral circulation.

Rapid onset of protein malnutrition and deficiency of essential amino acids are thought to be important pathophysiologic mechanisms for the development of FHL. A lack of apolipoprotein B100, a major component of the VLDL, was proposed as a reason for the diminished ability to excrete TAG from the liver.[3,6]. However, cats with HL are known to have increased levels of TAG in plasma with greatest distribution in the VLDL fraction (~62% vs. 25% in healthy, lean cats)[60] and increased serum concentrations of VLDL, with the VLDL fraction representing approximately 19% of the total lipoprotein mass compared to 2% in healthy lean cats. This suggests that VLDL secretion seems to be enhanced and not deficient in FHL.[20,60-62] However, despite hepatic VLDL secretion being increased in cats with FHL, this increase might not be sufficient to prevent the lipid overload of hepatocytes, in face of a dramatic increase of NEFA transport to the liver.

Arginine and taurine deficiency in cats with FHL could also compromise lipid metabolism and excretion of TAG via the VLDL excretion pathway. Arginine is an important urea cycle substrate and arginine deficiency has been associated with the development of hyperammonemia and hepatic encephalopathy (HE) in cats with FHL.[63,64] Taurine deficiency has been shown to increase lipolysis in peripheral tissues and has been linked to secondary accumulation of NEFAs in the liver.[12,65] Supplementation of taurine in experimental cats during initial weight gain followed by weight loss was associated with decreased hepatic lipid accumulation.[12,62]

As discussed previously, HL in the American Mink and the European polecat, other obligatory carnivores, shares numerous clinical, clinicopathologic, and pathophysiologic characteristics with FHL in domestic cats.[16,46,48,66,67] Both the American mink and the European polecat have been used as animal models to investigate the pathophysiology of NAFLD in people.[16] In the American mink and European polecat, fatty acid data of various adipose tissue depots and liver tissue showed a decrease in the *n*-3 LCPUFA.[16,48,67-69] The depletion of *n*-3 PUFA during food deprivation could be partly due to the mechanisms of selective FA mobilization: the location of the first double bond from the methyl end affects the fractional mobilisation of LCPUFA and n-3 substrates are often preferred over

n-6 ones in FA desaturation reactions and β-oxidation.[70] The decrease in n-3 LCPUFA causes an increase in the *n*-6/*n*-3 PUFA ratio. Non-alcoholic fatty liver disease in human seems to be the result of an unfavourable *n*-6/*n*-3 PUFA ratio, with an increase in the n-6 LCPUFA.[26,27] N-3 LCPUFAs are more potent activators of the PPAR-α receptors than n-6 LCPUFA and a depletion of n-3 PUFA has been proposed to favour FA and TAG synthesis over hydrolysis and FA oxidation and may impair lipid export from the liver by suppressing VLDL secretion.[28,48] An increase in the n-6 LCPUFA concentration in response to food deprivation has been associated with increased inflammation and oxygen free radical formation and n-6 LCPUFA are considered to be a key contributor to the pathophysiology and progression of liver steatosis in NAFLD, in the American mink and in the European polecat.[16,27,28,48] A lower concentration of both total n-6 and total n-3 PUFAs was noted in adipose tissue of cats with FHL compared to controls. The n-6/n-3 PUFA ratio was not statistically assessed, but a study from Hall et al. suggests that a derangement in the *n*-6/*n*-3 PUFA ratio might occur in FHL compared to healthy control cats.[4] Due to the limiting nature of the Δ5- and especially Δ6-desaturase activities, the LCPUFA status, especially the n-3 LCPUFA status, of domestic cats may be severely compromised during food deprivation and/or rapid weight loss contributing to the pathogenesis of FHL.

Recently, research has been performed in experimental animals and in humans on the immunomodulatory role of circulating bile acids and on their role in suppressing the hypothalamic pituitary adrenal axis (HPA axis). In humans the link between critical illness related corticosteroid insufficiency (CIRCI) and high circulating bile acid concentrations has been established.[71,72] High levels of circulating conjugated or unconjugated bile acids in critical illness has been shown to inhibit glucocorticoids metabolising enzyme and to inhibit the release of corticotropin releasing hormone (CHR) and adrenocorticotropic hormone (ACTH) from the hypotalamic-pituitary-adrenal axis (HPA axis).[71-73] The hallmark of CIRCI is hemodynamic instability that manifests itself as refractory hypotension despite fluid resuscitation and levels of corticosteroids that are insufficient for the severity of the underlying illness.[74,75] To evaluate if CIRCI also is present in cats with cholestasis, a pilot study was performed.[76] Basal serum cortisol and delta cortisol (the difference between basal and post-ACTH cortisol) concentrations were evaluated in 20 cats with cholestasis. Cats with refractory hypotension had a lower mean delta cortisol than cats with normal blood pressure, but this was not significant. [76] However we know from human medicine, that delta cortisol is not very specific indicator of the HPA axis and adrenal function as it doesn't take into consideration cortisol breakdown and its availability at the cellular level. [72] Therefore, CIRCI should be suspected in a subpopulation of cats with FHL and refractory hypotension.

Historical and clinical findings

Although FHL is mainly reported in middle-aged cats (median age: 7 years), cats of any age can be affected.[1,2,6] There seems to be no clear breed or sex predilection, although in some studies female cats seem to be overrepresented.[6,33] Actual (body condition 4/5) or historical obesity has been mentioned by several authors as a predisposing factor for the development of FHL.[1,2,4,6,12] Cats affected with HL present with a history of anorexia and weight loss. Other reported clinical signs include: icterus, dehydration, vomiting, nausea and ptyalism, constipation or diarrhoea, and a poor hair coat (Figure 1; Figure.2).[2,6] The mentation of cats with FHL can be severely altered if hypokalaemia and hepatic encephalopathy (HE) are present. In cats with FHL, HE is associated with arginine deficiency and can be worsened by hypokalaemia and decreased liver function, which further impairs the urea cycle.[63,64,77] Cats with HE can present with severe mental depression, ptyalism and severe nausea.[2,6,78] Plasma ammonia concentration should always be measured in a patient with severe alteration of its mental state. Ammonia tolerance testing is not recommended in cats with FHL.[2] Ventroflexion of the neck, often seen in cats with FHL, and severe muscle weakness can be secondary to the concurrent presence of severe hypokalaemia.

Fig. 1. Ptyalism secondary to nausea in a cat with FHL

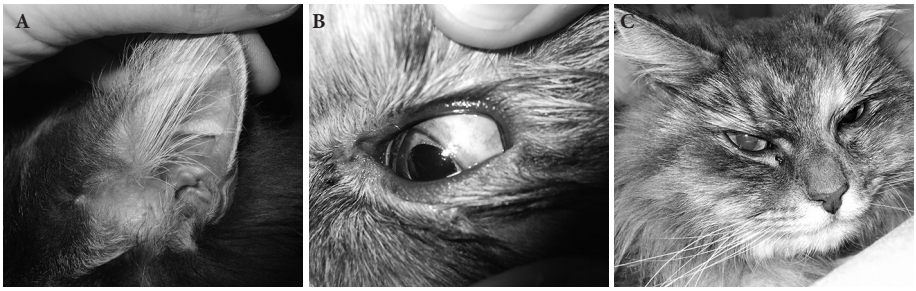

Fig. 2. Cat with FHL. Note the visible icterus of (A) skin and (B) sclera and (C) poor kept hair coat

Serum biochemistry

Feline hepatic lipidosis is characterized by an increased bilirubin concentration and increased serum activities of alkaline phosphatase (ALP) and alanine aminotransferase (ALT), when compared to healthy cats and cats with other liver disease (i.e. cholangitis).[2,6,79] Hyperglycaemia is often present and is due to insulin resistance and an increase in the counter regulatory hormones.[2,34,37] Hypoglycaemia, when present, indicates severely decreased or even end-stage liver function.

Mild hypoalbuminemia is often reported in FHL secondary to anorexia and decreased hepatic function. Blood urea nitrogen was found to be decreased in 51% of cats as consequence of chronic anorexia and/or insufficient urea-cycle function.[1,2,6] Commonly reported electrolyte alterations include: hypokalaemia (30% of cats with FHL), hypomagnesemia (28%) and hypophosphatemia (17%) that can be present at admission or can develop after administration of fluid therapy to correct dehydration.[6] Hypokalaemia and hypophosphataemia have been associated with an increased morbidity and mortality in FHL.[6] Hypokalaemia can increase the encephalopathic effects of ammonia and cause muscle weakness, paralytic ileus, and anorexia; hypophosphatemia can cause severe haemolysis resulting in the need for a blood transfusion.[1,2,6] Cats with HL often have higher serum concentrations of β-hydroxybutyric acid (BHBA) compared to normal cats as reflection of their negative energy balance.[10,36,60] High serum BHBA concentrations are the result of stimulation of the increased lipolysis with mobilisation of NEFA leading to increased hepatic β-oxidation and production of ketone bodies. Clinical signs associated with increased serum BHBA concentrations are usually vague and include anorexia, lethargy, cachexia, and weight loss. Serum BHBA has been suggested as a marker for FHL.[36]

Haematology

The cell blood count (CBC) is often normal in cats with FHL, but in some cases, a mild non-regenerative anaemia and mild leucocytosis might be present. Underlying inflammatory, infectious, or neoplastic disease could result in an inflammatory leukogram. Heinz bodies can be detected on blood smear evaluation both on admission and during recovery.[1,2,6] Thrombocytopenia does not occur frequently in cats with HL, unless they are suffering from DIC.[6,33,80-84]

Tests of coagulation

Coagulation abnormalities and clinical bleeding tendencies are reported to be common (45-73%) in cats with FHL, especially during venepuncture, catheter placement or if invasive procedures, such as oesophageal feeding tube placement or liver biopsy are performed. [2,81-83] Lisciandro et al. reported that prolongation of prothrombin time (PT) was the most common abnormality (found in 77% of cats with FHL), whereas factor VII activity was reduced in 68% and activated partial thromboplastin time was prolonged in 55% in a population of cats affected with liver disease.[83] In a study by Center et al. in cats with HL 75% had increased proteins invoked by vitamin K absence (PIVKA) clotting times, while only a minimal percentage had prolonged prothrombin time or activated partial thromboplastin time (4% and 25%, respectively).[82] More recently, Dirck et al. found that 40% of cats affected with liver disease had a significant prolongation of the activated partial thromboplastin time compared to healthy cats. Protein C was also decreased in 44% of cats with liver disease, while fibrinogen was increased when compared to health controls. No significant difference was found for vitamin K dependant clotting factors (II, VII, IX, X) between healthy controls and cats with liver disease. The most consistent abnormalities in cats with FHL were an increased factor V activity and D-dimer concentrations with 54% of cats having values above the reference range for both parameters.[81] Furthermore, 31% of cats with FHL had a severely decreased factor XIII (fibrin stabilizing factor) activity.[81] Not only decreased production but also activation of haemostasis with secondary increased consumption of coagulation factors could be responsible for the bleeding tendencies of patients with FHL.

Diagnosis of feline hepatic lipidosis

The presumptive diagnosis of FHL is based on the patient history, clinical presentation, clinicopathologic findings, and ultrasonographic appearance of the liver. However, history, clinical and clinicopathologic presentation are not specific for lipidosis and any underlying disease process can confound them.

On ultrasound examination the liver in cats with FHL appears enlarged and diffusely hyperechoic liver when compared to the falciform fat.[78] Using these criteria abdominal ultrasound performed by three board-certified radiologists had an accuracy of 70% for diagnosing lipidosis in cats.[85] Comparable ultrasonographic findings of a diffusely hyperechoic liver can be found in healthy obese cats.[86]

The definitive diagnosis is usually made by cytologic evaluation of a fine needle aspirate (FNA) and in some cases by histologic evaluation of a liver biopsy.[1,2,87-89] Cytology of the liver has been advocated as it is considered to be a safer procedure. It often does not require general anaesthesia and it is associated with few minor complications when compared to liver biopsy.[1,2,88,90] The hallmark of lipidosis on cytology is the presence of steatosis, which can be macro- or microvescicular.[1,2,52] Although vacuolar hepatopathy was the category with the highest percentage of agreement between liver cytology and liver histology, only 51% of feline cases had overall agreement between cytologic and histologic diagnosis of FHL.[87] Furthermore, Willard et al. reported four cases of cats diagnosed with FHL based on cytology where the underlying liver disease (cholangitis or lymphoma) was missed.[89] Cats that are sick tend to accumulate lipid in their liver often mimicking FHL and for this reason a definitive diagnosis of FHL can only be made when >80% of the hepatocytes are affected.[2,6]

Liver biopsy requires general anaesthesia and assessment of the patient's haemostatic system. Thrombocytopenia (<80,000 platelets /μL) and elevated activated partial thromboplastin time (>1.5 times the upper limit of the reference range) were the two reported abnormalities that had the strongest association with severe bleeding.[91] Furthermore, liver biopsy collection using Tru-Cut rapid-firing automatic biopsy needles has been associated with the development of vagotonic shock in cats, characterized by bradycardia and cardiovascular collapse up to 30 min after the biopsy was performed.[90] These are the reasons why the majority of authors do not recommend liver biopsies as the initial tool to diagnose FHL. Cats with FHL present a series of cardiovascular, metabolic, and coagulation derangements that make them unsuitable to undergo general anaesthesia and

liver biopsy. Liver biopsy should be considered in subjects that despite appropriate treatment fail to improve or if they have a history, clinical finding, or clinicopathologic findings suggestive of a possible underlying hepatic disease other than HL.[1,2]

The use of non-contrast computerized tomography (CT) has been advocated in human medicine as a more reliable and repeatable method for the detection fatty hepatic infiltration in patients with non-alcoholic fatty liver disease (NAFLD) when compared to cytological and histological assessment of the liver.[92,93] Fatty infiltration of organs is associated with X-ray attenuation on CT[94] and level of x-ray attenuation of adipose tissue in human medicine is inversely correlated with hepatic fat content.[92,95,96] Computed tomography (CT) has been used to detect visceral adipose tissue deposition in cats and CT evaluation of total body fat seems to correlate well with the body condition score.[97] Two recent studies evaluated the use of CT to detect liver fatty deposition in FHL, but yielded contrasting results. The first experimental study by Nakamura et al. evaluated a colony of adult healthy cats where the mean hepatic fat attenuation was 54 Hounsfield units (HU; range: 43.5-65.9 HU). Hepatic lipidosis was then experimentally induced and when CT images of the liver were evaluated, decreased hepatic X-rays attenuation was observed (<35 HU).[98] In a more recent study, Lamb et al. evaluated x-ray attenuation in the liver and kidneys of a population of client owned cats with suspected FHL. Cats were divided into three different groups based on the risk of suffering from mild, moderate of severe lipidosis. The study however, failed to highlight any differences between groups and the values obtained for x-ray attenuation of the liver were different from the previously published ones. The conclusion of Lamb et al. was that hepatic CT attenuation of the liver might be of limited value in detecting FHL in patients at risk for lipidosis and that values obtained for hepatic x-ray attenuation could vary between CT scanners.[99] Based on the results of these two studies and the need for anaesthesia in a clinically compromised patient, the routine use of CT to diagnose lipidosis cannot yet be recommended and further studies are necessary.

Treatment

Fluid and electrolyte therapy

Cats presented with FHL can suffer from differing degrees of hypoperfusion secondary to vomiting, anorexia, and adipsia. Hypoperfusion in cats is characterized by tachycardia (heart rate >220 bpm) or inappropriate bradycardia (heart rate <140 bpm), pale mucous membranes, prolonged capillary refill time,

often hypothermia, and mild hypotension. Dehydration is a common abnormality seen in cats with FHL. Initial fluid therapy should be directed to correct hypoperfusion if it is present. A balanced isotonic crystalloid infusion (0.9% NaCl, lactated Ringer's solution, or Ringer's acetate solution) is the fluid type of choice. Small volume resuscitation, with a 5-10 mL/kg IV bolus given over 30 min should be instituted in hypovolemic cats while slow rewarming is implemented. Repeated examination of the cardiovascular system will help to decide if further fluid administration is necessary to achieve euvolemia. Fluid therapy to provide for maintenance requirements and correct deficits according to the estimated percentage of dehydration should then be started (Table 1 and Table 2) and the total volume is usually administered over 24 hours. There is a lot of debate on what is the best fluid to administer in patients with FHL. Due to poor hepatic function lactate clearance, might not be appropriate in cats with FHL. Therefore the administration of lactate-based solutions might worsen hyperlactatemia in these patients.[1,2] However, this seems to only be a theoretical concern as lactated Ringer's solution has been used without major complication in these patients.[1] Fluids containing glucose should not be used in cats with FHL to avoid worsening glucose intolerance and concurrent hyperglycemia.[37] The fluid therapy plan should be re-evaluated and adjusted at least once a day based on the cat's new requirements and clinical condition. The newly formulated plan should take into consideration the fluid balance and the body weight of the patient, percentage of dehydration and if ongoing losses (vomiting, diarrhoea) are still occurring.

Estimated dehydration (% of BW)	**Physical examination findings**
<5%	Normal (anamnesis consistent with excessive fluid loss compared to intake)
5-6%	Skin turgor is mildly reduced; mucous membranes are dry
7-8%	Skin turgor is moderately reduced; mucous membranes are dry
8-10%	Skin turgor is severely reduced; mucous membranes are dry; eyes sunken in the orbit
10-12%	Skin turgor is severely reduced; mucous membranes are dry; eyes sunken in the orbit; initial signs of shock (mild tachycardia, pale/ pink mucous membranes, slightly prolonged CRT, weak peripheral pulse)
12-15%	Clinical signs consistent with shock (tachycardia, pale mucous membranes, weak peripheral pulse, prolonged capillary refill time)

Table 1: Clinical estimation of dehydration
Percentage of dehydration is evaluated based on physical evaluation of the turgor, mucous membranes and position of the eyes in the orbit. The estimated percentage will be used to calculate the amount of fluid necessary for the correction of dehydration in the fluid therapy plan (table 2).

Fluid requirements for a hospitalized cat	
Maintenance fluid therapy	40-60 mL/kg/day
Correction of dehydration	Deficit in mL (to be administered in 12-24 hours) = % dehydration x 10 x body weight (kg)
Ongoing fluid losses	Based observed fluid loss: - minimal fluid loss: 2 mL/kg/hour - moderate fluid loss: 4 mL/kg/hour - severe fluid loss: 6 mL/kg/hour

Table 2: Fluid requirements for a hospitalized cat
Based on the cat fluid balance, the fluid therapy plan will take into consideration maintenance fluid therapy plus the correction of dehydration (based on the estimated percentage of dehydration) and the ongoing fluid losses (via vomiting, diarrhea, polyuria).

If enteral or parenteral feeding is implemented, the amount of fluid administered with the nutritional plan should be deducted from the calculated rate of fluid infusion to avoid fluid overload.

Correction of electrolytes abnormalities should take place in the initial phase of hospitalisation and before nutrition is started, as insulin release can cause a further decrease in serum/plasma potassium and phosphate concentrations. Abnormalities of potassium and phosphate should be adequately corrected (Table 3) and these electrolytes should be checked at least twice daily in the beginning of hospitalisation. The rate of potassium administration must not exceed 0.5 mEq/kg/hour. If hypokalaemia is difficult to correct, the serum/plasma magnesium concentration should be also measured and if needed this electrolyte should be supplemented, as hypomagnesemia can worsen renal wasting of potassium.

Nutritional management of feline hepatic lipidosis

The cornerstone of treatment in FHL is early nutrition. Nutrition should be initiated on the day of admission to reverse the negative energy balance and catabolic state typical of FHL. The only reason to delay nutrition is the presence of cardiovascular instability (hypoperfusion, hypotension) and severe electrolyte abnormalities. Nutrition can be provided via the enteral or parenteral routes. Wherever possible enteral feeding is preferred over parenteral nutrition because it helps maintaining intestinal structure function.[100] However, in case of intractable vomiting or because of minimal tolerance to enteral feeding, the parenteral route should be taken into consideration.[100] Partial parenteral nutrition could be easily administered via a peripheral catheter and doesn't require a central venous access, which might initially not be advisable due to coagulation abnormalities.

The ideal diet for FHL should be high in protein (30%–40% of the metabolizable energy), moderate in lipids (about 50% of the metabolizable energy), and relatively poor in carbohydrate (about 20% of the metabolizable energy).[1,2] Glucose should be used as carbohydrate source as it doesn't require digestion and can be used by enterocytes as energy source.[1] In critically ill cats, 6 g of protein/100 kcal (or 25–35% of their total energy requirements) is considered to be enough to support their unique metabolism.[100] In FHL, a diet with a protein content of 25% of the metabolizable energy was shown to attenuate but not to ameliorate HL, while a diet with a higher protein content (35-45% of the metabolizable energy) showed to reverse the catabolic state and improved clinical signs associated with FHL.[7,32]The majority of the veterinary commercial diets formulated for recovery in cats meets these requirements.

The calorie requirements of these patients can be estimated using the formula: RER = 70 x (body weight in kg)$^{0.75}$.

Electrolytes	Formula used	Route of administration	Comments
Potassium	mmol of potassium to administer to correct the deficit up to a potassium level of 4.5 mmol/L = 4.5 - $K^{patient}$ x 0.6 x BW (kg)	IV	The amount in ml of the calculated potassium should be diluted 1:4 with NaCl 0.9% and administered with a dedicated syringe pump IV at a rate not faster than 0.5 mmol/kg/hour. Be careful not to administer the solution containing potassium as a bolus or rapid infusion.
Phosphate	0.01-0.03 mEq/kg/hour for 6 hours 0.12 mEq/kg/hour in severe deficit	IV	Use a dedicated line and syringe pump. Ensure that this line is not flushed. It is important to consider the amount of potassium administered via phosphate correction and subtract it from the potassium correction.
Magnesium sulfate (MgSO4) or Magnesium chloride (MgCl2)	Rapid replacement: 0.75-1 mEq/kg/day for first 24 hours Slow replacement: 0.3-0.5 mEq/kg/day for 2-3 days	IV	Administered as 20% solution diluted in 5% dextrose

Table 3: Treatment of electrolyte abnormalities

The use of an illness factor, typically ranging from 1.0-2.0, by which the RER is multiplied to meet the increased caloric needs of critically ill patients is no longer recommended.[101] The use of the illness factor leads to overfeeding and has been associated with hyperglycaemia, gastrointestinal dysfunction, as well as hepatic dysfunction. The development of hyperglycaemia is especially concerning (particularly during parenteral nutrition) as it is associated with an increased rate of complications and a worse outcome.[102]

Forced enteral feeding should never be considered in a sick cat with FHL because of the risk of the development of food aversion[2] and because it is usually difficult to administer the cat enough food to meet their energy requirements. Feeding tubes (nasoesophageal, esophageal, and gastric) allow clinicians to provide enteral nutrition without excessive stress to the patient (Table 4). The preferred initial feeding tube choice of the authors and others[1,2] for cats with FHL is the nasoesophageal feeding tube. The introduction of a nasoesophageal feeding tube does not require general anaesthesia or heavy sedation and is not invasive (Fig.3). Cats with FHL are often unstable on admission to undergo general anaesthesia and are often coagulopathic. For these reasons the insertion of oesophagostomy and gastrostomy tubes should be considered potentially unsafe in these patients and should be delayed until fluid and coagulation abnormalities have been addressed and the cat is considered stable enough to undergo anaesthesia (Fig 3).

Typically sufficient amounts of a commercial high protein recovery food to provide 1/3 of the calculated RER is fed on the first day followed by a slow incremental increase in the amount over the next 2-3 days until the total RER is being provided. The slow increase of the caloric load should decrease the risk of refeeding syndrome. Refeeding syndrome is characterized by severe hypophosphatemia, hypokalaemia and hyperglycaemia, vomiting, diarrhoea, or shock.[103,104] It is the consequence of a rapid caloric administration to a starved patient in a chronic negative catabolic state, causing rapid insulin release.[103-105]

Cats with FHL are considered feeding volume sensitive and the amount of food they can tolerate per meal might be drastically reduced.[1] The total volume of food required each day should be initially divided into 6-8 portions, or administered as a constant rate infusion (CRI). The authors prefer to administer food as a CRI because it seems to reduce nausea, gastric discomfort, and vomiting associated with gastric distension after intermittent food administration. The food should be always administered lukewarm and if the intermittent administration is chosen, it should be administered over 10-15 minutes. If any signs of discomfort, retching or vomiting are present, the administration of food should be interrupted.

Antiemetics and gastroprotectants should be considered in cats that are nauseated and/or vomit. Metoclopramide (0.2 mg/kg, *IV,* 4 times a day or 1 mg/kg/day *IV* as a CRI), ondansetron (0.1-0.5 mg/kg, *IV*, 2 to 3 times per day) and maropitant[106] (1 mg/kg *SQ* once a day) can be used alone or in combination to decrease vomiting and nausea. Metoclopramide is not a potent antiemetic in cats, but has some prokinetic effect and can facilitate gastric emptying.[107] Omeprazole (1 mg/kg *PO* twice a day) or ranitidine (2.5 mg/kg *IV* twice a day) can be used to prevent reflux esophagitis in animals with frequent emesis.[107] The use of appetite stimulants has been discouraged in cats with FHL.[1,2,100,108]

Vitamin K_1 should be administered in cats with impaired coagulation and some clinicians routinely administer it to all cats with FHL. Because the absorption of Vitamin K_1 from the gastrointestinal tract might be compromised by cholestasis, 0.5–1.5 mg/kg *SQ* at 12-hour intervals for 3-4 doses has been recommended.[2]

Feeding tube for enteral nutrition	Size	Advantages	Disadvantages
Nasoesophageal	7-8 French	No anaesthesia required Easy to place Inexpensive	Short-term solution Only administration of liquid diet
Esophageal	14 French	Easy to place Inexpensive Every diet can be used Suitable for longer duration	Anaesthesia required Possible bleeding Cellulitis as complication
Gastric	14-18 French	Suitable for longer duration Every diet can be used Suitable for longer duration	Anesthesia required Tube displacement can result in peritonitis

Table 4: type and characteristics of available feeding tubes for enteral nutrition in cats

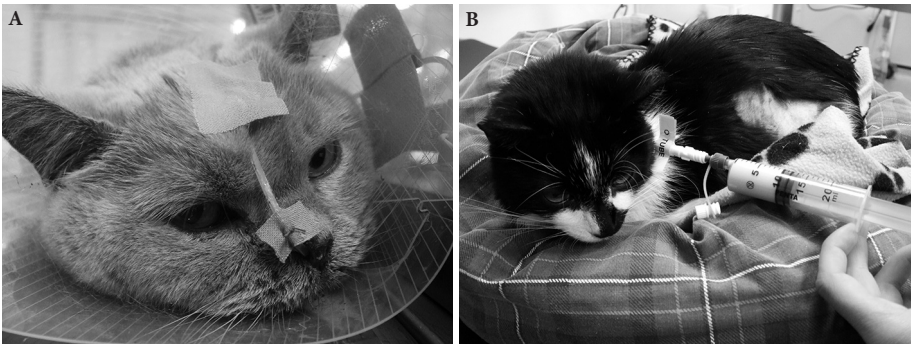

Fig. 3. Cats with (A) nasoesophageal and (B) esophageal feeding tubes.

Other medications and supplements are often suggested for the treatment of FHL (Table 5) but their efficacy has not been adequately demonstrated in order to make definitive recommendations regarding their use.[54,58,59,109,110] L-carnitine supplementation has received more interest than any other food supplements and there are experimental and clinical studies that highlight its benefits. In an experimental study in overweight cats undergoing rapid weight loss, dietary L-carnitine supplementation increased the rate of FFA β-oxidation and decreased TAG accumulation in the liver.[53,54,58,62] Furthermore, clinical observation of improvement of the clinical signs and probability of survival in cats with FHL when supplemented with L-carnitine suggests that carnitine should be considered an important addition to nutrition support.[2] A dose of 250-500 mg/cat/day has been suggested.[1,2]

Medications or nutraceuticals	**Route of administration**	**Dosage**
Vitamin B_{12}	*SQ*	250 µg/injection once weekly for 6 weeks, once every 2 weeks for 6 weeks, and then monthly
Taurine	*PO*	250 mg/ day during the first 7 to 10 days
N-acetylcysteine	*IV, PO*	Initial first dose 140 mg/kg (20% solution diluted 1:4 or greater with saline) *IV* over 30 minutes. 70 mg/kg every 8-12 hours
S-adenosylmethionine (SAMe)	*PO*	20 mg/kg/day

Table 5: Other medications and nutraceuticals suggested by the treatment of FHL

Prognosis

If appropriately and rapidly treated with nutritional support and in the absence of a serious underlying disease, cats with FHL have a reported recovery rate of 80-85%.[2,10] Reported positive prognostic factors were a younger age and a higher median serum potassium concentration and hematocrit.[2,6] A low albumin concentration on admission was associated with a worse prognosis in a population of cats affected with FHL (Valtolina and Favier, unpublished data).

References

1. Armstrong PJ, Blanchard G. Hepatic lipidosis in cats. *Vet Clin North Am Small Anim Pract.* 2009;39(3):599-616. doi: 10.1016/j.cvsm.2009.03.003 [doi].

2. Center SA. Feline hepatic lipidosis. *Vet Clin North Am Small Anim Pract.* 2005;35(1):225-269. doi: S0195-5616(04)00142-1 [pii].

3. Dimski DS. Feline hepatic lipidosis. *Semin Vet Med Surg (Small Anim).* 1997;12(1):28-33.

4. Hall JA, Barstad LA, Connor WE. Lipid composition of hepatic and adipose tissues from normal cats and from cats with idiopathic hepatic lipidosis. *J Vet Intern Med.* 1997;11(4):238-242.

5. Lund EM, Armstrong PJ, Kirk CA, Kolar LM, Klausner JS. Health status and population characteristics of dogs and cats examined at private veterinary practices in the united states. *J Am Vet Med Assoc.* 1999;214(9):1336-1341.

6. Center SA, Crawford MA, Guida L, Erb HN, King J. A retrospective study of 77 cats with severe hepatic lipidosis: 1975-1990. *J Vet Intern Med.* 1993;7(6):349-359.

7. Biourge VC, Massat B, Groff JM, Morris JG, Rogers QR. Effects of protein, lipid, or carbohydrate supplementation on hepatic lipid accumulation during rapid weight loss in obese cats. *Am J Vet Res.* 1994;55(10):1406-1415.

8. Biourge VC, Groff JM, Munn RJ, et al. Experimental induction of hepatic lipidosis in cats. *Am J Vet Res.* 1994;55(9):1291-1302.

9. Postic C, Girard J. The role of the lipogenic pathway in the development of hepatic steatosis. *Diabetes Metab.* 2008;34(6 Pt 2):643-648. doi: 10.1016/S1262-3636(08)74599-3 [doi].

10. Blanchard G, Paragon BM, Serougne C, Ferezou J, Milliat F, Lutton C. Plasma lipids, lipoprotein composition and profile during induction and treatment of hepatic lipidosis in cats and the metabolic effect of one daily meal in healthy cats. *J Anim Physiol Anim Nutr (Berl).* 2004;88(3-4):73-87. doi: 10.1111/j.1439-0396.2003.00462.x [doi].

11. Trevizan L, de Mello Kessler A, Brenna JT, Lawrence P, Waldron MK, Bauer JE. Maintenance of arachidonic acid and evidence of Delta5 desaturation in cats fed gamma-linolenic and linoleic acid enriched diets. *Lipids.* 2012;47(4):413-423. doi: 10.1007/s11745-011-3651-0 [doi].

12. Verbrugghe A, Bakovic M. Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis. *Nutrients.* 2013;5(7):2811-2835. doi: 10.3390/nu5072811 [doi].

13. Zoran DL. The carnivore connection to nutrition in cats. *J Am Vet Med Assoc.* 2002;221(11):1559-1567.

14. MacDonald ML, Rogers QR, Morris JG. Nutrition of the domestic cat, a mammalian carnivore. *Annu Rev Nutr.* 1984;4:521-562. doi: 10.1146/annurev.nu.04.070184.002513 [doi].

15. Mustonen AM, Pyykonen T, Paakkonen T, et al. Adaptations to fasting in the american mink (mustela vison): Carbohydrate and lipid metabolism. *Comp Biochem Physiol A Mol Integr Physiol.* 2005;140(2):195-202. doi: S1095-6433(04)00351-4 [pii].

16. Nieminen P, Mustonen AM, Karja V, Asikainen J, Rouvinen-Watt K. Fatty acid composition and development of hepatic lipidosis during food deprivation--mustelids as a potential animal model for liver steatosis. *Exp Biol Med (Maywood).* 2009; 234(3):278-286. doi: 10.3181/0806-RM-210 [doi].

17. Morris JG. Idiosyncratic nutrient requirements of cats appear to be diet-induced evolutionary adaptations. *Nutr Res Rev.* 2002;15(1):153-168. doi: 10.1079/NRR200238 [doi].

18. Verbrugghe A, Hesta M, Daminet S, Janssens GP. Nutritional modulation of insulin resistance in the true carnivorous cat: A review. *Crit Rev Food Sci Nutr.* 2012;52(2):172-182. doi: 10.1080/10408398.2010.499763 [doi].

19. Eisert R. Hypercarnivory and the brain: Protein requirements of cats reconsidered. *J Comp Physiol B.* 2011;181(1):1-17. doi: 10.1007/s00360-010-0528-0 [doi].

20. Szabo J, Ibrahim WH, Sunvold GD, Bruckner GG. Effect of dietary protein quality and essential fatty acids on fatty acid composition in the liver and adipose tissue after rapid weight loss in overweight cats. *Am J Vet Res.* 2003;64(3):310-315.

21. Hassam AG, Rivers JP, Crawford MA. The failure of the cat to desaturate linoleic acid; its nutritional implications. *Nutr Metab.* 1977;21(5):321-328.

22. Rivers JP, Sinclair AJ, Craqford MA. Inability of the cat to desaturate essential fatty acids. *Nature.* 1975;258(5531):171-173.

23. Rivers JP, Sinclair AJ, Moore DP, Crawford MA. The abnormal metabolism of essential fatty acids in the cat [proceedings. *Proc Nutr Soc.* 1976;35(2):66A-67A.

24. Pawlosky R, Barnes A, Salem N,Jr. Essential fatty acid metabolism in the feline: Relationship between liver and brain production of long-chain polyunsaturated fatty acids. *J Lipid Res.* 1994;35(11):2032-2040.

25. Mahfouz MM, Smith TL, Kummerow FA. Effect of dietary fats on desaturase activities and the biosynthesis of fatty acids in rat-liver microsomes. *Lipids.* 1984;19(3):214-222.

26. Clarke SD. Nonalcoholic steatosis and steatohepatitis. I. molecular mechanism for polyunsaturated fatty acid regulation of gene transcription. *Am J Physiol Gastrointest Liver Physiol*. 2001;281(4):G865-9.

27. Araya J, Rodrigo R, Videla LA, et al. Increase in long-chain polyunsaturated fatty acid n - 6/n - 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin Sci (Lond)*. 2004;106(6):635-643. doi: 10.1042/CS20030326 [doi].

28. Videla LA, Rodrigo R, Araya J, Poniachik J. Oxidative stress and depletion of hepatic long-chain polyunsaturated fatty acids may contribute to nonalcoholic fatty liver disease. *Free Radic Biol Med*. 2004;37(9):1499-1507. doi: 10.1016/j.freeradbiomed.2004.06.033 [doi].

29. Matsuzaka T, Shimano H, Yahagi N, et al. Dual regulation of mouse delta(5)- and delta(6)-desaturase gene expression by SREBP-1 and PPARalpha. *J Lipid Res*. 2002;43(1):107-114.

30. Yoshikawa T, Shimano H, Yahagi N, et al. Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. *J Biol Chem*. 2002;277(3):1705-1711. doi: 10.1074/jbc.M105711200 [doi].

31. Osborne TF. Sterol regulatory element-binding proteins (SREBPs): Key regulators of nutritional homeostasis and insulin action. *J Biol Chem*. 2000;275(42):32379-32382. doi: 10.1074/jbc.R000017200 [doi].

32. Biourge V, Groff JM, Fisher C, Bee D, Morris JG, Rogers QR. Nitrogen balance, plasma free amino acid concentrations and urinary orotic acid excretion during long-term fasting in cats. *J Nutr*. 1994;124(7):1094-1103.

33. Brown B, Mauldin GE, Armstrong J, Moroff SD, Mauldin GN. Metabolic and hormonal alterations in cats with hepatic lipidosis. *J Vet Intern Med*. 2000;14(1):20-26.

34. Chan DL, Freeman LM, Rozanski EA, Rush JE. Alteration in carbohydrate metabolism in critically ill cats. *J Vet Emer Crit Care*. 2006;16(2)(S1):S7-S13.

35. Knieriem M, Otto CM, Macintire D. Hyperglycemia in critically ill patients. *Compend Contin Educ Vet*. 2007;29(6):360-2, 364-72; quiz 372.

36. Aroch I, Shechter-Polak M, Segev G. A retrospective study of serum beta-hydroxybutyric acid in 215 ill cats: Clinical signs, laboratory findings and diagnoses. *Vet J*. 2012;191(2):240-245. doi: 10.1016/j.tvjl.2011.01.010 [doi].

37. Biourge V, Nelson RW, Feldman EC, Willits NH, Morris JG, Rogers QR. Effect of weight gain and subsequent weight loss on glucose tolerance and insulin response in healthy cats. *J Vet Intern Med*. 1997;11(2):86-91.

38. Hoenig M, Thomaseth K, Waldron M, Ferguson DC. Insulin sensitivity, fat distribution, and adipocytokine response to different diets in lean and obese cats before and after weight loss. *Am J Physiol Regul Integr Comp Physiol.* 2007;292(1):R227-34. doi: 00313.2006 [pii].

39. Mazaki-Tovi M, Abood SK, Segev G, Schenck PA. Alterations in adipokines in feline hepatic lipidosis. *J Vet Intern Med.* 2013;27(2):242-249. doi: 10.1111/jvim.12055 [doi].

40. Jung UJ, Choi MS. Obesity and its metabolic complications: The role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. *Int J Mol Sci.* 2014;15(4):6184-6223. doi: 10.3390/ijms15046184 [doi].

41. Hoenig M, McGoldrick JB, deBeer M, Demacker PN, Ferguson DC. Activity and tissue-specific expression of lipases and tumor-necrosis factor alpha in lean and obese cats. *Domest Anim Endocrinol.* 2006;30(4):333-344. doi: S0739-7240(05)00184-0 [pii].

42. Miller C, Bartges J, Cornelius L, Norton N, Barton M. Tumor necrosis factor-alpha levels in adipose tissue of lean and obese cats. *J Nutr.* 1998;128(12 Suppl):2751S-2752S.

43. Hoenig M, Wilkins C, Holson JC, Ferguson DC. Effects of obesity on lipid profiles in neutered male and female cats. *Am J Vet Res.* 2003;64(3):299-303.

44. Hoenig M. The cat as a model for human nutrition and disease. *Curr Opin Clin Nutr Metab Care.* 2006;9(5):584-588. doi: 10.1097/01.mco.0000241668.30761.69 [doi].

45. Richard MJ, Holck JT, Beitz DC. Lipogenesis in liver and adipose tissue of the domestic cat (felis domestica). *Comp Biochem Physiol B.* 1989;93(3):561-564.

46. Rouvinen-Watt K, Harris L, Dick M, et al. Role of hepatic de novo lipogenesis in the development of fasting-induced fatty liver in the american mink (neovison vison). *Br J Nutr.* 2012;108(8):1360-1370. doi: 10.1017/S0007114511006775 [doi].

47. Bergen WG, Mersmann HJ. Comparative aspects of lipid metabolism: Impact on contemporary research and use of animal models. *J Nutr.* 2005;135(11):2499-2502. doi: 135/11/2499 [pii].

48. Rouvinen-Watt K, Mustonen AM, Conway R, et al. Rapid development of fasting-induced hepatic lipidosis in the american mink (neovison vison): Effects of food deprivation and re-alimentation on body fat depots, tissue fatty acid profiles, hematology and endocrinology. *Lipids.* 2010;45(2):111-128. doi: 10.1007/s11745-009-3377-4 [doi].

49. Pardina E, Baena-Fustegueras JA, Llamas R, et al. Lipoprotein lipase expression in livers of morbidly obese patients could be responsible for liver steatosis. *Obes Surg.* 2009;19(5):608-616. doi: 10.1007/s11695-009-9827-5 [doi].

50. Westerbacka J, Kolak M, Kiviluoto T, et al. Genes involved in fatty acid partitioning and binding, lipolysis, monocyte/macrophage recruitment, and inflammation are overexpressed in the human fatty liver of insulin-resistant subjects. *Diabetes*. 2007;56(11):2759-2765. doi: db07-0156 [pii].

51. Wei Y, Rector RS, Thyfault JP, Ibdah JA. Nonalcoholic fatty liver disease and mitochondrial dysfunction. *World J Gastroenterol*. 2008;14(2):193-199.

52. Center SA, Guida L, Zanelli MJ, Dougherty E, Cummings J, King J. Ultrastructural hepatocellular features associated with severe hepatic lipidosis in cats. *Am J Vet Res*. 1993;54(5):724-731.

53. Blanchard G, Paragon BM, Milliat F, Lutton C. Dietary L-carnitine supplementation in obese cats alters carnitine metabolism and decreases ketosis during fasting and induced hepatic lipidosis. *J Nutr*. 2002;132(2):204-210.

54. Center SA, Harte J, Watrous D, et al. The clinical and metabolic effects of rapid weight loss in obese pet cats and the influence of supplemental oral L-carnitine. *J Vet Intern Med*. 2000;14(6):598-608.

55. Biourge V, Groff JM, Morris JG, Rogers QR. Long-term voluntary fasting in adult obese cats: Nitrogen balance, plasma amino acid concentrations and urinary orotic acid excretion. *J Nutr*. 1994;124(12 Suppl):2680S-2682S.

56. Jacobs G, Cornelius L, Keene B, Rakich P, Shug A. Comparison of plasma, liver, and skeletal muscle carnitine concentrations in cats with idiopathic hepatic lipidosis and in healthy cats. *Am J Vet Res*. 1990;51(9):1349-1351.

57. Armstrong PJ. Feline hepatic lipidosis. in proceeding of the 7th annual ACVIM forum, san diego, CA, USA, may 1989; pp. 335–337. *Proceeding of the 7th Annual ACVIM Forum, San Diego, CA, USA*. May 1989:335-336,337.

58. Center SA, Warner KL, Randolph JF, Sunvold GD, Vickers JR. Influence of dietary supplementation with (L)-carnitine on metabolic rate, fatty acid oxidation, body condition, and weight loss in overweight cats. *Am J Vet Res*. 2012;73(7):1002-1015. doi: 10.2460/ajvr.73.7.1002 [doi].

59. Center SA, Warner KL, Erb HN. Liver glutathione concentrations in dogs and cats with naturally occurring liver disease. *Am J Vet Res*. 2002;63(8):1187-1197.

60. Pazak HE, Bartges JW, Cornelius LC, Scott MA, Gross K, Huber TL. Characterization of serum lipoprotein profiles of healthy, adult cats and idiopathic feline hepatic lipidosis patients. *J Nutr*. 1998;128(12 Suppl):2747S-2750S.

61. Ibrahim WH, Szabo J, Sunvold GD, Kelleher JK, Bruckner GG. Effect of dietary protein quality and fatty acid composition on plasma lipoprotein concentrations and hepatic triglyceride fatty acid synthesis in obese cats undergoing rapid weight loss. *Am J Vet Res*. 2000;61(5):566-572.

62. Ibrahim WH, Bailey N, Sunvold GD, Bruckner GG. Effects of carnitine and taurine on fatty acid metabolism and lipid accumulation in the liver of cats during weight gain and weight loss. *Am J Vet Res*. 2003;64(10):1265-1277.

63. Lidbury JA, Cook AK, Steiner JM. Hepatic encephalopathy in dogs and cats. *J Vet Emerg Crit Care (San Antonio)*. 2016. doi: 10.1111/vec.12473 [doi].

64. Morris JG, Rogers QR. Ammonia intoxication in the near-adult cat as a result of a dietary deficiency of arginine. *Science*. 1978;199(4327):431-432.

65. Cantafora A, Blotta I, Rossi SS, Hofmann AF, Sturman JA. Dietary taurine content changes liver lipids in cats. *J Nutr*. 1991;121(10):1522-1528.

66. Dick MF, Hurford J, Lei S, Mustonen AM, Nieminen P, Rouvinen-Watt K. High feeding intensity increases the severity of fatty liver in the american mink (neovison vison) with potential ameliorating role for long-chain n-3 polyunsaturated fatty acids. *Acta Vet Scand*. 2014;56:5-0147-56-5. doi: 10.1186/1751-0147-56-5 [doi].

67. Mustonen AM, Puukka M, Rouvinen-Watt K, Aho J, Asikainen J, Nieminen P. Response to fasting in an unnaturally obese carnivore, the captive european polecat mustela putorius. *Exp Biol Med (Maywood)*. 2009;234(11):1287-1295. doi: 10.3181/0904-RM-140 [doi].

68. Nieminen P, Kakela R, Pyykonen T, Mustonen AM. Selective fatty acid mobilization in the american mink (mustela vison) during food deprivation. *Comp Biochem Physiol B Biochem Mol Biol*. 2006;145(1):81-93. doi: S1096-4959(06)00161-8 [pii].

69. Mustonen AM, Kakela R, Nieminen P. Different fatty acid composition in central and peripheral adipose tissues of the american mink (mustela vison). *Comp Biochem Physiol A Mol Integr Physiol*. 2007;147(4):903-910. doi: S1095-6433(07)00871-9 [pii].

70. Nieminen P, Rouvinen-Watt K, Collinsb D, Grant J, Mustonen AM. Fatty acid profiles and relative mobilization during fasting in adipose tissue depots of the american marten (martes americana). *Lipids*. 2006;41(3):231-240.

71. McNeilly AD, Macfarlane DP, O'Flaherty E, et al. Bile acids modulate glucocorticoid metabolism and the hypothalamic-pituitary-adrenal axis in obstructive jaundice. *J Hepatol*. 2010;52(5):705-711. doi: 10.1016/j.jhep.2009.10.037 [doi].

72. Boonen E, Van den Berghe G. Understanding the HPA response to critical illness: Novel insights with clinical implications. *Intensive Care Med*. 2015;41(1):131-133. doi: 10.1007/s00134-014-3545-8 [doi].

73. Quinn M, Ueno Y, Pae HY, et al. Suppression of the HPA axis during extrahepatic biliary obstruction induces cholangiocyte proliferation in the rat. *Am J Physiol Gastrointest Liver Physiol*. 2012;302(1):G182-93. doi: 10.1152/ajpgi.00205.2011 [doi].

74. Fede G, Spadaro L, Tomaselli T, et al. Adrenocortical dysfunction in liver disease: A systematic review. *Hepatology*. 2012;55(4):1282-1291. doi: 10.1002/hep.25573 [doi].

75. Marik PE, Pastores SM, Annane D, et al. Recommendations for the diagnosis and management of corticosteroid insufficiency in critically ill adult patients: Consensus statements from an international task force by the american college of critical care medicine. *Crit Care Med*. 2008;36(6):1937-1949. doi: 10.1097/CCM.0b013e31817603ba [doi].

76. Webster CRL. Metabolic consequences of cholestasis in cats. *Society of Comparative Hepatology 25th Ecvim-Ca Congress*. 2015.

77. Rothuizen J. Important clinical syndromes associated with liver disease. *Vet Clin North Am Small Anim Pract*. 2009;39(3):419-437. doi: 10.1016/j.cvsm.2009.02.007 [doi].

78. Larson MM. Ultrasound imaging of the hepatobiliary system and pancreas. *Vet Clin North Am Small Anim Pract*. 2016;46(3):453-480. doi: 10.1016/j.cvsm.2015.12.004 [doi].

79. Center SA, Baldwin BH, Dillingham S, Erb HN, Tennant BC. Diagnostic value of serum gamma-glutamyl transferase and alkaline phosphatase activities in hepatobiliary disease in the cat. *J Am Vet Med Assoc*. 1986;188(5):507-510.

80. Gagne JM, Armstrong PJ, Weiss DJ, Lund EM, Feeney DA, King VL. Clinical features of inflammatory liver disease in cats: 41 cases (1983-1993). *J Am Vet Med Assoc*. 1999; 214(4):513-516.

81. Dircks B, Nolte I, Mischke R. Haemostatic abnormalities in cats with naturally occurring liver diseases. *Vet J*. 2012;193(1):103-108. doi: 10.1016/j.tvjl.2011.09.026 [doi].

82. Center SA, Warner K, Corbett J, Randolph JF, Erb HN. Proteins invoked by vitamin K absence and clotting times in clinically ill cats. *J Vet Intern Med*. 2000;14(3):292-297.

83. Lisciandro SC, Hohenhaus A, Brooks M. Coagulation abnormalities in 22 cats with naturally occurring liver disease. *J Vet Intern Med*. 1998;12(2):71-75.

84. Kavanagh C, Shaw S, Webster CR. Coagulation in hepatobiliary disease. *J Vet Emerg Crit Care (San Antonio)*. 2011;21(6):589-604. doi: 10.1111/j.1476-4431. 2011.00691.x [doi].

85. Feeney DA, Anderson KL, Ziegler LE, Jessen CR, Daubs BM, Hardy RM. Statistical relevance of ultrasonographic criteria in the assessment of diffuse liver disease in dogs and cats. *Am J Vet Res*. 2008;69(2):212-221. doi: 10.2460/ajvr.69.2.212 [doi].

86. Nicoll RG, O'Brien RT, Jackson MW. Qualitative ultrasonography of the liver in obese cats. *Vet Radiol Ultrasound*. 1998;39(1):47-50.

87. Wang KY, Panciera DL, Al-Rukibat RK, Radi ZA. Accuracy of ultrasound-guided fine-needle aspiration of the liver and cytologic findings in dogs and cats: 97 cases (1990-2000). *J Am Vet Med Assoc.* 2004;224(1):75-78.

88. Weiss DJ, Moritz A. Liver cytology. *Vet Clin North Am Small Anim Pract.* 2002;32(6): 1267-91, vi.

89. Willard MD, Weeks BR, Johnson M. Fine-needle aspirate cytology suggesting hepatic lipidosis in four cats with infiltrative hepatic disease. *J Feline Med Surg.* 1999;1(4):215-220. doi: 10.1053/jfms.1999.0045 [doi].

90. Rothuizen J, Twedt DC. Liver biopsy techniques. *Vet Clin North Am Small Anim Pract.* 2009;39(3):469-480. doi: 10.1016/j.cvsm.2009.02.006 [doi].

91. Bigge LA, Brown DJ, Penninck DG. Correlation between coagulation profile findings and bleeding complications after ultrasound-guided biopsies: 434 cases (1993-1996). *J Am Anim Hosp Assoc.* 2001;37(3):228-233. doi: 10.5326/15473317-37-3-228 [doi].

92. Oliva MR, Mortele KJ, Segatto E, et al. Computed tomography features of nonalcoholic steatohepatitis with histopathologic correlation. *J Comput Assist Tomogr.* 2006;30(1):37-43. doi: 00004728-200601000-00007 [pii].

93. Pickhardt PJ, Park SH, Hahn L, Lee SG, Bae KT, Yu ES. Specificity of unenhanced CT for non-invasive diagnosis of hepatic steatosis: Implications for the investigation of the natural history of incidental steatosis. *Eur Radiol.* 2012;22(5):1075-1082. doi: 10.1007/s00330-011-2349-2 [doi].

94. Roldan-Valadez E, Favila R, Martinez-Lopez M, Uribe M, Mendez-Sanchez N. Imaging techniques for assessing hepatic fat content in nonalcoholic fatty liver disease. *Ann Hepatol.* 2008;7(3):212-220. doi: 867777 [pii].

95. Kodama Y, Ng CS, Wu TT, et al. Comparison of CT methods for determining the fat content of the liver. AJR Am J Roentgenol. 2007;188(5):1307-1312. doi: 188/5/1307 [pii].

96. Ma X, Holalkere NS, Kambadakone RA, Mino-Kenudson M, Hahn PF, Sahani DV. Imaging-based quantification of hepatic fat: Methods and clinical applications. Radiographics. 2009;29(5):1253-1277. doi: 10.1148/rg.295085186 [doi].

97. Lee P, Mori A, Takemitsu H, Yamamoto I, Arai T. Lipogenic gene expression in abdominal adipose and liver tissues of diet-induced overweight cats. Vet J. 2011;190(2):e150-3. doi: 10.1016/j.tvjl.2011.04.003 [doi].

98. Nakamura M, Chen HM, Momoi Y, Iwasaki T. Clinical application of computed tomography for the diagnosis of feline hepatic lipidosis. J Vet Med Sci. 2005;67(11):1163-1165. doi: JST.JSTAGE/jvms/67.1163 [pii].

99. Lam R, Niessen SJ, Lamb CR. X-ray attenuation of the liver and kidney in cats considered at varying risk of hepatic lipidosis. *Vet Radiol Ultrasound*. 2014;55(2):141-146. doi: 10.1111/vru.12113 [doi].

100. Chan DL. The inappetent hospitalised cat: Clinical approach to maximising nutritional support. *J Feline Med Surg*. 2009;11(11):925-933. doi: 10.1016/j.jfms.2009.09.013 [doi].

101. Freeman LM, Chan DL. Total parenteral nutrition. In: DiBartola SP, ed. *Fluid, electrolyte, and acid-base disorders in small animal practice*. 3rd edn ed. St Louis: Elsevier; 2006:584-601.

102. Pyle SC, Marks SL, Kass PH. Evaluation of complications and prognostic factors associated with administration of total parenteral nutrition in cats: 75 cases (1994-2001). *J Am Vet Med Assoc*. 2004;225(2):242-250.

103. Brenner EA, Salazar AM, Zabotina OA, Lubberstedt T. Characterization of european forage maize lines for stover composition and associations with polymorphisms within O-methyltransferase genes. *Plant Sci*. 2012;185-186:281-287. doi: 10.1016/j.plantsci.2011.11.016 [doi].

104. Justin RB, Hohenhaus AE. Hypophosphatemia associated with enteral alimentation in cats. *J Vet Intern Med*. 1995;9(4):228-233.

105. Adams LG, Hardy RM, Weiss DJ, Bartges JW. Hypophosphatemia and hemolytic anemia associated with diabetes mellitus and hepatic lipidosis in cats. *J Vet Intern Med*. 1993;7(5):266-271.

106. Hickman MA, Cox SR, Mahabir S, et al. Safety, pharmacokinetics and use of the novel NK-1 receptor antagonist maropitant (cerenia) for the prevention of emesis and motion sickness in cats. *J Vet Pharmacol Ther*. 2008;31(3):220-229. doi: 10.1111/j.1365-2885.2008.00952.x [doi].

107. Trepanier L. Acute vomiting in cats: Rational treatment selection. *J Feline Med Surg*. 2010;12(3):225-230. doi: 10.1016/j.jfms.2010.01.005 [doi].

108. Delaney SJ. Management of anorexia in dogs and cats. *Vet Clin North Am Small Anim Pract*. 2006;36(6):1243-9, vi. doi: S0195-5616(06)00078-7 [pii].

109. Center SA. Metabolic, antioxidant, nutraceutical, probiotic, and herbal therapies relating to the management of hepatobiliary disorders. *Vet Clin North Am Small Anim Pract*. 2004;34(1):67-172, vi.

110. Webster CR, Cooper J. Therapeutic use of cytoprotective agents in canine and feline hepatobiliary disease. *Vet Clin North Am Small Anim Pract*. 2009;39(3):631-652. doi: 10.1016/j.cvsm.2009.02.002 [doi]

Chapter 3

No up-regulation of the phosphatidylethanolamine N-methyltransferase pathway and choline production by sex hormones in cats

Chiara Valtolina[a] Arie B. Vaandrager[b], Robert P. Favier[a], Joris H. Robben[a], Maidina Tuohetahuntila[b], Anne Kummeling[a], Isabelle Jeusette[c] and Jan Rothuizen[a]

BMC Veterinary Research (2015) 11:280

[a] *Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 108, 3584 CM, Utrecht, The Netherlands*
[b] *Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine and Institute of Biomembranes, Utrecht University, Yalelaan 2, 3584 CM, Utrecht, The Netherlands*
[c] *Research and Development, Affinity Petcare, Pl. Xavier Cugat, 2 Edificio D, 3ª, Planta, 08174 St. Cugat del Vallès, Barcelona, Spain*

Abstract

Background

Feline hepatic lipidosis (FHL) is a common cholestatic disease affecting cats of any breed, age and sex. Both choline deficiency and low hepatic phosphatidylethanolamine N-methyltransferase (PEMT) activity are associated with hepatic lipidosis (HL) in humans, mice and rats. The PEMT expression is known to be upregulated by oestrogens, protecting the females in these species from the development of HL when exposed to choline deficient diets. The aim of the present study was to evaluate the influence of sex hormones on choline synthesis via the PEMT pathway in healthy male and female cats before and after spaying/neutering, when fed a diet with recommended dietary choline content.

Results

From six female and six male cats PEMT activity was assayed directly in liver biopsies taken before and after spaying/neutering, and assessed indirectly by analyses of PEMT–specific hepatic phosphatidylcholine (PC) species and plasma choline levels. Hepatic PEMT activity did not differ between intact female and male cats and no changes upon spaying/neutering were observed. Likewise, no significant differences in liver PC content and PEMT-specific polyunsaturated PC species were found between the sexes and before or after spaying/neutering.

Conclusion

These results suggest that choline synthesis in cats differs from what is observed in humans, mice and rats. The lack of evident influence of sex hormones on the PEMT pathway makes it unlikely that spaying/neutering predisposes cats for HL by causing PC deficiency as suggested in other species.

Keywords

cats, hepatic lipidosis, choline, PEMT, oestrogen

Background

Feline hepatic lipidosis (FHL) is a common cholestatic disease affecting cats. It is considered the consequence of prolonged anorexia and subsequent dramatic lipolysis.[1-3] Feline hepatic lipidosis is characterized by an excessive accumulation of triglycerides (TAG) in hepatocytes. Although the exact mechanisms remain elusive, there is clearly an imbalance between the influx of fatty acids (FA) derived from peripheral fat stores and *de novo* synthesis of FA in the liver on the one hand, and the rate of hepatic FA oxidation and the efflux of hepatic TAGs via very-low density lipoprotein (VLDL) on the other hand.[1,3] In cats with FHL, serum TAG levels significantly increase with the greatest distribution into the VLDL fraction.[4] It has been suggested that a lower capacity to excrete VLDL plays a role in the development of FHL.[1-3]

Secretion of VLDL is dependent on the rate of hepatic phosphatidylcholine (PC) synthesis. Phosphatidylcholine is synthetized in the liver by two pathways, the Kennedy or cytidine diphosphate-choline (CDP-choline) pathway and the phosphatidylethanolamine N-methyltransferase (PEMT) pathway. The Kennedy pathway produces more saturated and mono-unsaturated acyl chain-containing PC, whereas the PEMT pathway synthesizes PC with a long highly unsaturated acyl chain (HUFA) (PC 38:6 and PC 40:6).[5] The Kennedy pathway is the major route for PC synthesis and is present in all mammalian tissues. The synthesis of PC via this pathway depends on the intake of choline.[6] The PEMT pathway is independent of choline intake and converts phosphatidylethanolamine (PE) to PC via the transfer of three methyl-groups from S-adenosylmethionine.[7,8] The PEMT pathway is liver-specific and is estimated to account for approximately 20-40% of the newly synthesized PC in the liver in humans, mice and rats.[9-11] In premenopausal women, intact female mice and rats it has been estimated that a larger fraction of PC is made in the liver via the methylation of PE, than in men, postmenopausal women and sterilised mice and rats.[9-11]

The role of choline in hepatic lipidosis (HL) has been evaluated in humans [12], cats [13-16] and mice.[17] Both choline deficiency and low PEMT activity have been associated with HL.[18-22] Suboptimal concentrations of dietary choline are associated in the cat with a diminished capacity of the liver to synthesize PC resulting in accumulation of lipids in the liver.[13-15]

In humans, mice and rats PEMT expression and activity is known to be upregulated by oestrogens. This explains why premenopausal and intact females are less choline

dependent than males or postmenopausal/spayed females.[9,11,23-25] Pre-menopausal women have a 30-50% higher capacity than men to form PC *de novo* via the PEMT pathway.[23,24] Premenopausal women and female rats on a choline deficient diet are less likely to develop HL than their male counterparts. Postmenopausal women and castrated female mice with less PEMT activity are considered more susceptible to develop PC deficiency and, subsequently, HL.[11,24]

Center et al. have reported that female cats are more affected than male cats, but it is more commonly reported that FHL has no breed, age or sex predisposition.[1,3] An explanation for an occasionally proposed sex predisposition in FHL has never been convincingly described.

As demonstrated in other species, the loss of oestrogen influence could cause spayed female cats to have a lower capacity for PC synthesis than intact females. If so, spaying female cats, a practice widely performed in all western countries, could be a predisposing factor for female cats to develop FHL.

The aim of this study was to analyse the lipid metabolism in healthy cats before and after spaying/neutering and subsequently the influence of sex hormones on PC synthesis via the PEMT pathway.

Methods

This study was approved by the Committee for the Ethical Care of Animals of the Utrecht University.

Cats and study design

Client-owned cats (six intact females, six intact males) admitted to the Department of Clinical Science of Companion Animals of the Faculty of Veterinary Medicine, Utrecht University (DSCA) for spaying/neutering were eligible to enter the study. Informed owner written consent was obtained prior to enrolment of all cats. The cats had to be in good clinical health during the study based on general physical examination performed by one of the authors (CV; veterinary specialist (Diplomate of the American and European College of Veterinary Emergency and Critical Care)), a CBC, and biochemistry test results. Coagulation parameters needed to be within reference range in preparation for the liver biopsy. The health status was checked twice: on admission for the castration and 4 weeks later prior to the second liver biopsy.

Cats enrolled for the study received the same study diet for 8 weeks. Four weeks after the start of the diet, blood samples and liver biopsies were taken. Following confirmation of good health status and sampling, the cats were neutered or spayed. Four weeks after the surgical procedure (8 weeks after the start of the

diet) blood and liver tissue sampling were repeated.
For the results analysis and interpretation, four groups were considered: intact males (before neutering, n=6), intact females (before spaying, n=6), spayed female (after spaying, n=6), neutered male (after neutering, n=6)

Diet

During the study, all the cats were fed a commercially available complete dry diet for adult cat (Affinity Petcare, Barcelona, Spain), provided in 500g bags and as snacks (Table 1). Although originally formulated for adult cats, this diet covered recommended nutrients allowance for growth with a margin of 10% (NRC 2006). The dry diet contained a fixed amount of choline (2,400 ppm as fed, 0.65 g/1,000 kcal), close to the adequate intake of choline (0.64 g/1,000 kcal) as recommended by NRC (2006) [26](Table 1).

Nutrients	Analysis method		Diet	Snack	Diet	Snack		Diet	Snack	Adult cat	Adult cat	800g kitten
		Unit	(as fed)	(as fed)	(DM*)	(DM)	Unit					
Moisture	CE N°152/2009	g/100g	7.2	8.1								
Protein	AOAC 990.03 / NF V18-120	g/100g	28.7	28.2	30.9	30.7	g/1000 kcal	77.8	74.5	40.0	50.0	56.3
Fat	AOAC 954.02	g/100g	11.8	12.7	12.7	13.8	g/1000 kcal	32.1	33.5	ND#	22.5	22.5
Ash	AOAC 942.05	g/100g	7.6	5.8	8.1	6.3	g/1000 kcal	20.5	15.3			
Crude fiber	AOCS Ba6-05	g/100g	2.7	2.0	2.9	2.2	g/1000 kcal	7.3	5.3			
Nitrogen free extract	calculated	g/100g	42.1	43.2	45.3	47.0	g/1000 kcal	114.0	114.2			
Starch	DIR. 72/199/ CEE	g/100g	30.5	ND	32.9	ND	g/1000 kcal	82.7	ND			
Methionine	CE N° 152/2009	g/100g	0.45	0.47	0.48	0.51	g/1000 kcal	1.22	1.24	0.34	0.43	1.10
Cystine	CE N° 152/2009	g/100g	0.64	0.65	0.69	0.71	g/1000 kcal	1.73	1.72			
methionine + cystine	CE N° 152/2009	g/100g	1.09	1.12	1.17	1.22	g/1000 kcal	2.95	2.96	0.68	0.85	2.20
Choline	Ionic Cromato-graphy	g/100g	0.24	0.24	0.26	0.26	g/1000 kcal	0.65	0.63	0.51	0.64	0.64
Taurine	HPLC	g/100g	0.07	0.07	0.08	0.08	g/1000 kcal	0.20	0.18	0.08	0.10	0.10
Betaine			ND	ND	ND	ND		ND	ND			
Folate (Vitamine B9)	HPLC	mg/kg	2.81	1.93	3.03	2.10	mg/1000 kcal	0.76	0.51	0.15	0.19	0.19
Cobalamine (Vitamine B12)	HPLC	µg/kg	40.00	30.00	43.09	32.64	µg/1000 kcal	10.84	7.93	ND	5.60	5.60
added Pyridoxine (Vitamine B6)	AOCS Ba6-05	mg/kg	10.39	8.55	11.19	9.30	mg/1000 kcal	2.82	2.26	0.50	0.63	0.63

Table 1. Nutrient composition of the complete dry diet and snacks as used in this study and the National research Council (NRC) requirements for adult cats and kittens.
* DM = dry matter # ND = not determined

Owners were given a table with the recommended amount of food to administer to their cat based on its weight (100 kcal/kg body weight$^{0.67}$), either 100% delivered with the diet or 95% delivered with the diet and 5% with snacks with similar food composition (Table 1). The energy allowance recommended was the one as published by NRC (2006) for adult cats.[26] Compared to NRC (2006) recommendations for entire growing kittens between 6 and 12 months, it could have been 10 to 13% lower (Table 1). Owners were instructed to report any lack of food intake or palatability to one of the authors (CV).

Blood collection

To determine the health status of the cats, blood (2 mL) was collected by jugular venepuncture and divided into three tubes: 0.5 mL in EDTA (CBC), 0.5 mL in heparin (biochemical analysis), 1 mL in citrate (coagulation).

For hormone and choline analysis, blood (4 mL) was collected by jugular venepuncture and divided into two tubes: 2 mL in heparin (hormone), and 2 mL in heparin (choline). The tubes were centrifuged and the plasma was separated. The plasma for choline analysis was immediately stored at -70°C in cryogenic vials (Corning Inc., NY, USA).

Hormone analysis

Plasma oestradiol-17β concentration was measured by radioimmunoassay (RIA) (Coat-A-Count TKE; Diagnostic Products Corporation, Los Angeles, CA, USA) according to the manufacturer's instructions with modifications as described previously [27] and validated for the dog.[28] The intra-assay and interassay coefficients of variability (CV) were 14.0% and 11.8%, respectively. The lower limit of quantitation was 7 pmol/L.

Plasma testosterone concentration was measured by RIA (Coat-A-Count Total Testosterone; Diagnostic Product Corporation, Los Angeles, CA, USA) according to the manufacturer's protocol with previously described modifications to increase the sensitivity.[29] The intra-assay and interassay CV were 5% and 6%, respectively. The lower limit of quantitation was 51 pmol/L.

Plasma choline analysis

Free choline concentration was determined with a choline detection kit (Biovision, Milpitas, CA, USA) by measuring the absorbance at 570 nm in a 96 well plate according to the manufacturer's instructions. Due to the low free choline concentrations 100 µL instead of 50 µL of cat plasma was used. The choline signal had to be corrected for a linear increase in non-specific signal observed after the endpoint of the assay. The lower limit of quantitation was 1 µM.

Anaesthesia and surgical procedure

Premedication consisted of glycopyrrolate (0.01 mg/kg, intramuscularly (IM)), 30 min later followed by midazolam (0.2 mg/kg IM) and ketamine 5 mg/kg IM. Anaesthesia was induced with alfaxalone (1-2 mg/kg intravenously (IV)) after which the cat was intubated. Anaesthesia was maintained with alfaxalone (4-5 mg/kg/h IV) and inhalation of a mixture of air, oxygen and isoflurane. Castration was performed following a routine procedure. Each animal remained hospitalised until the morning after surgery when it was discharged.

Liver biopsies

Under general anaesthesia, an ultrasound of the liver was performed by a veterinary specialist (Diplomate of the European College of Veterinary Diagnostic Imaging or resident in the same specialty). If no abnormalities were detected in liver structure and echogenicity, two ultrasound guided liver biopsies were taken with a 16 G needle (Super Core Semi-automatic Biopsy Instrument; Angitech, Vancouver, BC, Canada).

One sample was fixated in 4% formalin and another was rinsed in normal saline (NaCl 0.9%), rapidly frozen in liquid nitrogen and stored at -80°C. Ultrasound of the abdomen was repeated 1-3 hours after liver biopsy to evaluate the presence of free abdominal fluid (blood) around the biopsy site.

Histology analysis

Samples fixed in 4% neutral-buffered formalin were embedded in paraffin. Sections (3 μm) were cut for routine staining with haematoxylin and eosin (H&E) staining. The slides were reviewed by a specialist in veterinary pathology.

Liver PEMT activity analysis

For the PEMT activity analyses the frozen liver biopsies were thawed, re-suspended in 350 μL of buffer A (250 mM sucrose, 0.2 mM EDTA, 5 mM DTT, and 10 mM Tris/HCl pH 8.0), and homogenized by mechanical disruption with a pestle tightly fitting to an Eppendorf tube followed by sonication (10 sec, amplitude 10 μm). PEMT activity was determined as previously described.[30] Protein was determined in the homogenates by the BCA method Pierce® BCA protein assay kit (Thermo scientific, Rockford, IL, USA) with BSA as standard.

To 100 μL of homogenized liver biopsy, 100 pmol of di-linolenoyl PC (PC (18:3, 18:3)) was added as internal standard. Subsequently, lipids were extracted and separated in a neutral and phospholipid fraction by fractionation on a silica-G column as described.[31]

The PEMT activity was also assessed indirectly by analyses of PEMT-specific PC species, i.e. HUFA PC species, and the PC/PE ratio.[5] Phosphatydilcholine and PE

species were determined as described by HPLC-mass spectroscopy.[32] In short, 150 µL of the homogenized liver biopsy was centrifuged for 5 min at 7,000 x g and 115 µL of the supernatant was incubated for 1 h at 37°C with 0.4 mM 16:0-dimethyl PE (PEDM; Avanti Polar Lipids, Alabaster, AL, USA), 50 µM S-(5'-Adenosyl)-L-methionine (SAM; Sigma-Aldrich, St. Louis, MO, USA) and 0.34 µCi ^{3}H-labeled SAM (Perkin Elmer, Waltham, MA, USA) in a final volume of 150 µL. After the incubation the lipids were extracted and separated with thin layer chromatography (TLC), and the PC spot was scraped and counted.

Statistical analysis

As no previous studies in cats have been performed, the results of the study by DaCosta K.A et al in humans were used to perform a power analysis to determine the minimal number of cats to be used in this study.[9] Based on the power calculations, the minimal number of female cats needed to demonstrate the effect of spaying on phosphatidylcholine-docosahexaenoic acid (PC-DHA) was six, based on the following data: mean PC—DHA before spay: 100 ± 25 nmol/ml; mean PC—DHA after spay: 59 ± 25 nmol/ml; alpha 0.05; power 0.88; paired t-test testing).

Data analysis was performed using IBM SPSS 22 statistic software (IBM Corporation Armonk, NY, United States of America).

The outcome variables plasma choline, PEMT, total liver PC content and PC:PE ratio were analysed using a linear mixed model with cat ID as random effect to take repeated observations within the subject into account. Variables time (before/after spaying/neutering) and gender (male/female) and the interaction between time and gender were used as explanatory variables. The outcome variables were log transformed to meet the model assumptions normality and the constance of variance. The akaike information criterion (AIC) was used to select the best model. Residuals plots were used to assess the validity of the model.

For outcome variables PC38:6 and PC40:6 the difference between both time moments (before/after spaying/neutering) and gender (male/female) were calculated as the model assumptions could not be met using the linear mixed effect model. A nonparametric Mann Whitney U test was applied on the differences to assess the difference in means between both genders.

The P-value <0.05 was used to assess statistical significance. Results are expressed as mean and standard deviation, if not indicated otherwise.

Results

Cats

Six intact females with a median age of 20.5 months (range: 6.0 – 84.0) and 6 intact males with a median age of 7.0 months (range: 6.0 – 9.0) were enrolled. Median body weight of the female cats was 3.0 kg (range: 2.6 – 3.9); median body weight of the male cats was 4.0 kg (range: 3.5 – 4.5).

All cats accepted the diet without any problems. Results of the complete blood count (CBC), biochemistry and coagulation profile of all twelve cats, analysed following standard chemical analyses, were within reference intervals (results not shown). The surgical procedures and ultrasound guided liver biopsies were without incident in all cats. The cats recovered uneventfully from anaesthesia and were discharged the day after.

Histological analysis

Histological evaluation of the liver biopsies performed in each subject (before and after spaying/neutering) at 4 weeks and at 8 weeks from the start of the diet revealed no histological changes compatible with hepatic lipidosis or other histological changes.

Hormone analysis

The results of the sex hormones measurements in the blood of the male and female cats pre and post spaying/neutering are reported in table 2. Oestrogen levels dropped significantly after spaying ($P = 0.041$), indicating successful spay in all female cats. In one female cat, both before and after spaying, the oestrogen levels were below the detection limit of the test.

The testosterone level dropped significantly ($P = 0.001$) in male cats, indicating successful neutering.

	Group 1 (male intact) **(n = 6)**	Group 2 (male neutered) **(n = 6)**	Group 3 (female intact) **(n = 6)**	Group 4 (female spayed) **(n = 6)**
Testosterone* (pmol/L)	824.3 ± 929.4	36.6 ± 17.0		
Oestrogen# (pmol/L)			9.6 ± 6.4	3.0 ± 1.0

Table 2. Plasma oestrogen and testosterone levels (mean ± SD) before and after spaying/neutering.

* $P < 0.00193$ male intact versus neutered

# $P < 0.041$ female intact versus spayed

Plasma choline and liver PEMT activity analysis

Free plasma choline levels were not statistically different between intact female cats compared to intact males (P = 0.76) (Table 3). After spaying the choline levels increased in female cats by 35% to levels similar to those in male cats pre and post neutering (Table 3), but the increase was not significant.

Activity of PEMT varied considerably between samples and did not differ significantly between male and female cats before or after spaying/neutering, and no effects of spaying/neutering were found in either group (Table 3). No correlation between the individual plasma choline levels and PEMT activity was observed (results not shown).

As presented in table 3, no significant differences were observed in total liver PC and the PEMT-specific PC species *i.e.* long chain highly unsaturated (HUFA) PC species, PC 38:6 (PC 16:0, 22:6) and PC 40:6 (PC 18:0, 22:6), and the PC/PE ratio between male and female cats before or after spaying/neutering.

	Group 1 (male intact) (n = 6)	Group 2 (male neutered) (n = 6)	Group 3 (female intact) (n = 6)	Group 4 (female spayed) (n = 6)
plasma choline (μM)	1.9 ± 0.8	1.8 ± 0.7	1.5 ± 0.3	2.0 ± 0.5
liver PEMT* activity (nmol/mg protein/h)	0.24 ± 0.18	0.19 ± 0.11	0.15 ± 0.09	0.21 ± 0.16
liver PC content (nmol/mg protein)	24.2 ± 3.8	24.1 ± 3.2	27.5 ± 3.1	24.1 ± 2.3
liver PC 38:6# (% of total PC)	3.3 ± 1.1	3.7 ± 1.6	4.2 ± 0.4	3.8 ± 0.5
liver PC 40:6# (% of total PC)	3.3 ± 1.3	4.2 ± 2.3	4.1 ± 0.6	3.7 ± 0.6
liver PC/PE§ ratio	3.4 ± 0.4	3.1 ± 0.3	4.0 ± 1.4	4.0 ± 1.5

Table 3. Plasma choline levels and hepatic indicators of phosphatidylcholine (PC) metabolism (mean ± SD) in male and female cats before and after spaying/neutering.

* PEMT = phosphatidylethanolamine N-methyltransferase
# PC species implicated in PEMT activity i.e. PC containing long highly unsaturated acyl chains
§ PE: phosphatidylethanolamine

Discussion

Choline deficiency and low hepatic PEMT activity have been associated with HL in humans, mice and rats. Pre-menopausal and intact females in these species appear better protected from the development of lipidosis when exposed to a

choline deficient diet. The up-regulation by oestrogens increases their capacity to synthesise PC via the PEMT pathway, reducing the dependency on choline intake.[11,24,25] Also in cats, the importance of choline in the development of HL has been substantiated in supplementation and deficiency studies.[13-16] This study could not demonstrate an effect of a reduced exposure to oestrogens on the PEMT pathway and the level of PEMT-specific PC species in cats. Therefore, this study does not support the hypothesis that a down-regulated PEMT pathway, as result of spaying/neutering, is a predisposing factor for the development of HL when cats are fed at recommended choline levels. [26]

The choline levels in the plasma (range 1 – 3.2 μM) were low compared to what has been established in humans (range 7 – 20 μM) [12] and the earlier reported choline levels in cat plasma (3 – 7 μM).[33] However, the latter range has been established with a different assay method.[12,33] The lower choline levels in the plasma may have been influenced by assay interference of substances in cat plasma.

The choline levels in female intact cats tended to be lower compared to the levels in spayed female cats and male cats. Nevertheless, this finding should be interpreted with caution based on the overall low choline levels close to the lower limit of quantitation. We have no clear explanation for the rise in plasma free choline levels after spaying in female cats, but it may suggest an effect of oestrogen on either choline uptake from the diet or on the conversion of choline to other metabolites like acetylcholine, betaine or phosphatidylcholine.

In contrast to what has been reported in humans, mice and rats, the PEMT activity tended to be lower in intact female cats than in male cats and spayed female cats. Unexpectedly, there was no higher PEMT activity in intact females before spaying, nor a decrease in PEMT related parameters after spaying. This is in contradiction to what is anticipated when oestrogens would up-regulate the PEMT pathway. The low PEMT activity in intact female cats may not have reached significance because of the large variation in activity between samples. This may be partly due to the relatively low signals in the respective assays, making them more susceptible to variation in background noise.

To further elucidate the activity of the PEMT pathway, PC 38:6 and PC 40:6 and the PC/PE ratio were evaluated. Phosphatidylcholine 38:6 and PC 40:6 are products of the PEMT pathway and therefore reflect indirectly the activity of this pathway.[5,9] The PC/PE ratio is also considered a proxy for the PEMT pathway although it may also be influenced by the rate of the PC and PE synthesis and breakdown by other pathways.[8] It is suggested that the higher the PC/PE ratio, the higher the PEMT activity should be, as more PE would be converted to PC in the hepatocytes via three sequential steps of methylation.[5,7,9] Also, in these indirect parameters, there

were no significant differences between the groups that could suggest an effect of oestrogen levels on activity of the PEMT pathway.

As the study was performed in client-owned cats, a diet with recommended choline content and not a choline restricted diet was used. With sufficient choline in the diet to allow PC to be formed via the Kennedy pathway, the expression and activity of the PEMT pathway may not have been fully stimulated. However, as it has been demonstrated in humans, mice and rats, the PEMT pathway is also active with diets that contain sufficient choline for the specific species, contributing to 20-40% of the PC produced.[9-11]

This study was performed in a relatively small series of animals. A power analysis indicated that 6 cats per group would have been sufficient to evaluate the hormonal effect on the PEMT pathway. The power analysis was based on the assumption that changes needed to be large to be considered clinically relevant. Nevertheless, it cannot be excluded that significant differences would be found with larger numbers.

It is possible that the difference of prevalence of lipidosis in female cats compared to male cats might be associated to a specific female lipid profile. Further evaluation of lipid profiling of liver and plasma of healthy female and male cats before and after spaying/neutering may help to clarify potential sex difference in cats.

Conclusion

Phosphatidylcholine synthesis in cats, when fed a recommended amount of choline in the diet, seems to differ from what has been observed in humans, mice and rats, with no evidence of influence of sex hormones on the PEMT pathway. Current recommendations for diet choline levels are adequate for spayed female cats and it is unlikely that castration predisposes cats for HL by causing PC deficiency as suggested in other species when recommended dietary choline level are provided.

Abbreviations

FHL: feline hepatic lipidosis; HL: hepatic lipidosis; cytidine diphosphate-choline: CPD-choline; PEMT: phosphatidylethanolamine N-methyltransferase; VLDL: very low density lipoprotein; PC: phosphatidylcholine TAG: triglycerides; DHA: docosahexaenoic acid; HUFAs: highly unsaturated acyl chains; PE: phosphatidylethanolamine

Competing Interest

The author Isabelle Jeusette declares to have competing interest with the manuscript by being currently employed by the study sponsor.

Funding

This research was financially supported by Affinity Petcare, Barcelona, Spain. Affinity Petcare also supplied the study of the diet that was fed to the cats in this study.

Contributions

CV, JR, RPF, JHR, ABV, IJ contributed to the development and writing of the paper. RPF, JR performed the liver biopsies. ABV and MT performed the lipidomic and PEMT analysis and the statistics. AK performed the surgeries. All authors read and approved the final manuscript.

Acknowledgment

We thank Dr. Louis C. Penning for critically reading this manuscript.
We thank Dr Hans J.C.M Vernooij for assistance with the statistics.
We thank Affinity Petcare, Barcelona, Spain, for funding this research.

References

1. Armstrong PJ, Blanchard G. Hepatic lipidosis in cats. *Vet Clin North Am Small Anim Pract*. 2009;39(3):599-616. doi: 10.1016/j.cvsm.2009.03.003 [doi].

2. Center SA, Crawford MA, Guida L, Erb HN, King J. A retrospective study of 77 cats with severe hepatic lipidosis: 1975-1990. *J Vet Intern Med*. 1993;7(6):349-359.

3. Center SA. Feline hepatic lipidosis. *Vet Clin North Am Small Anim Pract*. 2005;35(1):225-269. doi: S0195-5616(04)00142-1 [pii].

4. Pazak HE, Bartges JW, Cornelius LC, Scott MA, Gross K, Huber TL. Characterization of serum lipoprotein profiles of healthy, adult cats and idiopathic feline hepatic lipidosis patients. *J Nutr*. 1998;128(12 Suppl):2747S-2750S. doi: 10.1093/jn/128.12.2747S [doi].

5. DeLong CJ, Shen YJ, Thomas MJ, Cui Z. Molecular distinction of phosphatidylcholine synthesis between the CDP-choline pathway and phosphatidylethanolamine methylation pathway. *J Biol Chem*. 1999;274(42):29683-29688.

6. Vance DE. Phospholipid biosynthesis in eukaryotes. In: Vance DE, Vance JE, eds. *Biochemistry of lipids, lipoproteins and membranes.* 5th ed. Amsterdam: Elsevier; 2002:205-232.

7. Vance DE, Ridgway ND. The methylation of phosphatidylethanolamine. *Prog Lipid Res*. 1988;49:1187-1194.

8. Li Z, Vance DE. Phosphatidylcholine and choline homeostasis. *J Lipid Res*. 2008;49(6): 1187-1194.

9. da Costa KA, Sanders LM, Fischer LM, Zeisel SH. Docosahexaenoic acid in plasma phosphatidylcholine may be a potential marker for in vivo phosphatidylethanolamine N-methyltransferase activity in humans. *Am J Clin Nutr*. 2011;93(5):968-974. doi: 10.3945/ajcn.110.011064 [doi].

10. Pani P, Porcu M, Columbano A, Dessi S, Ledda GM, Diaz G. Differential effects of choline administration on liver microsomes of female and male rats. *Exp Mol Pathol*. 1978;28(2):154-162. doi: 0014-4800(78)90048-5 [pii].

11. Noga AA, Vance DE. A gender-specific role for phosphatidylethanolamine N-methyltransferase-derived phosphatidylcholine in the regulation of plasma high density and very low density lipoproteins in mice. *J Biol Chem*. 2003;278(24):21851-21859. doi: 10.1074/jbc.M301982200 [doi].

12. Zeisel SH. Choline: An essential nutrient for humans. *Nutrition*. 2000;16(7-8):669-671. doi: S0899-9007(00)00349-X [pii].

13. Da Silva AC, Guerios S MF, Monsao SR. The domestic cat as a laboratory animal for experimental nutrition studies. VI. choline deficiency. *J Nutr*. 1959;67(4):537-547.

14. Anderson PA, Baker DH, Sherry PA, Corbin JE. Choline-methionine interrelationship in feline nutrition. *J Anim Sci*. 1979;49(2):522-527.

15. Schaeffer MC, Rogers QR, Morris JG. Methionine requirement of the growing kitten, in the absence of dietary cystine. *J Nutr*. 1982;112(5):962-971.

16. Verbrugghe A, Bakovic M. Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis. *Nutrients*. 2013;5(7):2811-2835. doi: 10.3390/nu5072811 [doi].

17. Li Z, Agellon LB, Vance DE. Phosphatidylcholine homeostasis and liver failure. *J Biol Chem*. 2005;280(45):37798-37802. doi: M508575200 [pii].

18. Vance JE, Vance DE. The role of phosphatidylcholine biosynthesis in the secretion of lipoproteins from hepatocytes. *Can J Biochem Cell Biol*. 1985;63(8):870-881. doi: 10.1139/o85-108 [doi].

19. Yao ZM, Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J Biol Chem*. 1988;263(6):2998-3004.

20. Yao ZM, Vance DE. Head group specificity in the requirement of phosphatidylcholine biosynthesis for very low density lipoprotein secretion from cultured hepatocytes. *J Biol Chem*. 1989;264(19):11373-11380.

21. Yao ZM, Vance DE. Reduction in VLDL, but not HDL, in plasma of rats deficient in choline. *Biochem Cell Biol*. 1990;68(2):552-558.

22. Buchman AL, Dubin MD, Moukarzel AA, et al. Choline deficiency: A cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation. *Hepatology*. 1995;22(5):1399-1403. doi: S0270913995003879 [pii].

23. Fischer LM, daCosta KA, Kwock L, et al. Sex and menopausal status influence human dietary requirements for the nutrient choline. *Am J Clin Nutr*. 2007;85(5):1275-1285. doi: 85/5/1275 [pii].

24. Resseguie M, Song J, Niculescu MD, da Costa KA, Randall TA, Zeisel SH. Phosphatidylethanolamine N-methyltransferase (PEMT) gene expression is induced by estrogen in human and mouse primary hepatocytes. *FASEB J*. 2007;21(10):2622-2632. doi: fj.07-8227com [pii].

25. Young DL. Estradiol- and testosterone-induced alterations in phosphatidylcholine and triglyceride synthesis in hepatic endoplasmic reticulum. *J Lipid Res*. 1971;12(5):590-595.

26. National Research Council (NRC). Nutrient requirements of dogs and cats. . 2006:81-110.

27. Dieleman SJ, Bevers MM. Effects of monoclonal antibody against PMSG administered shortly after the preovulatory LH surge on time and number of ovulations in PMSG/PG-treated cows. *J Reprod Fertil*. 1987;81(2):533-542.

28. van Haaften B, Bevers MM, van den Brom WE, et al. Increasing sensitivity of the pituitary to GnRH from early to late anoestrus in the beagle bitch. *J Reprod Fertil*. 1994;101(1):221-225.

29. Buijtels JJ, Beijerink NJ, Kooistra HS, Dieleman SJ, Okkens AC. Effects of gonadotrophin releasing hormone administration on the pituitary-ovarian axis in anoestrous vs ovariectomized bitches. *Reprod Domest Anim*. 2006;41(6):555-561. doi: RDA714 [pii].

30. Ridgway ND, Vance DE. Phosphatidylethanolamine N-methyltransferase from rat liver. *Methods Enzymol*. 1992;209:366-374. doi: 0076-6879(92)09045-5 [pii].

31. Testerink N, Ajat M, Houweling M, et al. Replacement of retinyl esters by polyunsaturated triacylglycerol species in lipid droplets of hepatic stellate cells during activation. *PLoS One*. 2012;7(4):e34945. doi: 10.1371/journal.pone.0034945 [doi].

32. Bleijerveld OB, Brouwers JF, Vaandrager AB, Helms JB, Houweling M. The CDP-ethanolamine pathway and phosphatidylserine decarboxylation generate different phosphatidylethanolamine molecular species. *J Biol Chem*. 2007;282(39):28362-28372. doi: M703786200 [pii].

33. Gardiner JE, Paton WD. The control of the plasma choline concentration in the cat. *J Physiol*. 1972;227(1):71-86.

Chapter 4

Sex specific differences in hepatic and plasma lipid profiles in healthy cats pre and post spaying and neutering: relationship with feline hepatic lipidosis

Chiara Valtolina[a] Arie B. Vaandrager[b], Robert P. Favier[a], Maidina Tuohetahuntila[b], Anne Kummeling[a], Isabelle Jeusette[c], Jan Rothuizen[a], and Joris H. Robben[a]

BMC Veterinary Research (2017) 13:231

[a] *Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 108, 3584 CM, Utrecht, The Netherlands*

[b] *Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine and Institute of Biomembranes, Utrecht University, Yalelaan 2, 3584 CM, Utrecht, The Netherlands*

[c] *Research and Development, Affinity Petcare, Pl. Xavier Cugat, 2 Edificio D, 3ª, Planta, 08174 St. Cugat del Vallès, Barcelona, Spain*

Abstract

Background

A link between lipid metabolism and disease has been recognized in cats. Since hepatic lipidosis is a frequent disorder in cats, the aim of the current study was to evaluate liver and plasma lipid dimorphism in healthy cats and the effects of gonadectomy on lipid profiling. From six female and six male cats plasma and liver lipid profiles before and after spaying/neutering were assessed and compared to five cats (three neutered male and two spayed female) diagnosed with hepatic lipidosis**.**

Results

Intact female cats had a significantly lower level of plasma triacylglycerides (TAG) and a higher liver level of the long chain polyunsaturated fatty acid arachidonic acid (AA) compared to their neutered state. Both male and female cats with lipidosis had a higher liver, but not plasma TAG level and an increased level of plasma and liver sphingomyelin compared to the healthy cats.

Conclusion: Although lipid dimorphism in healthy cats resembles that of other species, intact female cats show differences in metabolic configuration that could predispose them to develop hepatic lipidosis. The increased sphingomyelin levels in cats with lipidosis could suggest a potential role in the pathogenesis of hepatic lipidosis in cats.

Key words

Lipid dimorphism, cats, feline hepatic lipidosis, sex hormones, sphingomyelin.

Background

In human medicine, the study and evaluation of lipid metabolites with the use of lipid profiling or "lipidomics" has become crucial in order to understand the role of lipids in the pathophysiology of lipid-related diseases.[1,2] The role of lipids in the development and progression of numerous diseases in man, such as diabetes mellitus, obesity, non-alcoholic fatty liver disease (NAFLD), Alzheimer and arteriosclerosis is well recognised.[3-5] The recognition of sex specific genetic dimorphism in lipid metabolism has helped to identify risk factors and to develop more specific and targeted therapies.[6,7]

Pre-menopausal women are considered to possess a more favourable (less pro-coagulatory and less pro-atherogenic) plasma lipid profile compared to men and post-menopausal women, with lower plasma levels of triacylglyceride (TAG), total cholesterol (TC), and low density lipoprotein-cholesterol (LDL-C), and higher levels of high density lipoprotein-cholesterol (HDL-C).[8-11]

Parallels in lipid profiles between species have been demonstrated. The essential fatty acid (FA) profile is known to vary considerably between males and females in humans, mice and rats, indicating that sex lipid dimorphism occurs in different species.[12-20] When compared to their male counterpart, premenopausal women possess a higher percentage of the long chain polyunsaturated fatty acids (LCPUFA) arachidonic acid (AA 20:4 n-6) and docosahexaenoic acid (DHA, 22:6 n-3) in plasma and liver.[20-22] Also in rats, LCPUFA levels in plasma and liver are higher in intact female rats compared to males, while this difference disappears after gonadectomy.[18,20]

In man, mice and rats LCPUFA are synthesised from the essential FAs linoleic acid (LA 18:2 n-6) and α-linolenic acid (ALA, 18:3 n-3) by the sequential activities of desaturases (Δ5, Δ6) and elongases.[23,24] Oestrogen has been shown to stimulate the FA desaturase activities [20,25-29] and is also known to increase the activity of the hormone sensitive lipases, resulting in an increased level of circulating FA.[28,29] Furthermore, oestrogen appears to be involved in lowering the plasma concentration of TAG and very low density lipoprotein (VLDL) triglycerides in premenopausal females.[17,30,31]

In cats, the link between lipid metabolism and specific diseases has also been acknowledged. Conditions like Niemann-Pick in Siamese cats, obesity, diabetes mellitus, and hepatic lipidosis (HL) have been associated with an altered lipid

metabolism in cats.[32,33] Hepatic lipidosis is considered one of the most common liver diseases in cats and is characterised by severe accumulation of triglycerides in the liver, resulting in an impaired liver function.[34,35] The pathogenesis of HL in cats is still not completely elucidated. The strict carnivorous diet and the unique protein and lipid metabolism of cats have been indicated as important predisposing factors in cats.[36] Cats, as other strict carnivores, possess a unique lipid metabolism, characterised by their limited ability, unlike other mammals, to synthesise AA from LA and eicosapentaenoic acid (EPA) and DHA from ALA due to limited desaturases Δ5 and Δ6 activities.[37,38]

It has been reported that feline HL has no breed, age or sex predisposition [34,35], although in one study female cats have been reported to be more affected than their male counterparts.[39] This seems to be in contrast with what is known in other species, where females, especially the premenopausal woman and intact female rats and mice, seem to be more protected from lipid related disease than the postmenopausal/spayed female and their male counterparts. If however, as has been suggested before, female cats are more susceptible to the development of HL compared to males, the answer could also be found in a unique sex related lipid profiling.

The aim of the current study is to perform lipid profiling in intact and spayed/neutered healthy cats fed with a common commercial diet and to evaluate the effect of gonadectomy on the liver and plasma lipid profiles. Additionally, we compare lipid profiles from healthy cats to those in cats affected by HL in an attempt to elucidate possible predisposing factors in the development of HL.

Materials and methods

This study was approved by the Committee for the Ethical Care of Animals of the Utrecht University.

Healthy cats

The healthy cats and the study design, diet, blood collection, hormone measurements, anaesthesia and surgical procedure, and liver biopsy taking have been described previously.[40] Plasma and pre and post spaying/neutering liver biopsies obtained in this study [40] were also used for analysis.

In summary, six intact females and six intact males client-owned cats admitted to the Department of Clinical Science of Companion Animals of the Faculty of Veterinary Medicine, Utrecht University (DCSCA) for spaying/neutering were considered for the study. Following confirmation of good health cats were neutered and spayed and a liver biopsy taken. The health status as well as blood parameters were evaluated again 4 weeks later during the second admission to the hospital

and before the additional liver biopsy was performed. A study diet was fed to all healthy cats recruited in the study for a total of 8 weeks, starting four weeks before the first admission to the hospital and terminating after the second blood and liver tissue sampling 4 weeks after the spaying/neutering surgery.
For analysis and interpretation of the results, healthy cats were divided in four groups: intact males (before neutering, n=6), intact females (before spaying, n=6), spayed females (after spaying, n=6), neutered males (after neutering, n=6).

Cats with hepatic lipidosis

Cats admitted from January 2013 to January 2014 to the DCSCA with suspected HL based on clinical symptoms (anorexia, vomiting and icterus) were considered for this study. In all cats ultrasonography showed diffuse echodensity of the liver compatible with HL. Hepatic lipidosis was confirmed based on cytological evaluation of fine needle aspirates of the liver. Only cats that were euthanized due to deterioration despite intense treatment and/or financial constraints of the owner were enrolled in the study. Owners' consent was asked to be signed prior to consideration of the cats for the study.
In contrast to the healthy cats, cats with HL did not receive any special diet.
Plasma that remained from heparinised blood collected during hospitalisation for diagnostic and clinical monitoring was freshly stored at -70°C in cryogenic vials (Corning Inc., Corning NY, USA) for lipidomics analysis.
In cats with HL two wedge liver biopsies were immediately collected post-mortem via laparotomy. As reported before (Valtolina et al) one sample was fixated in 4% neutral-buffered formalin and the other liver biopsy was rinsed in normal saline (NaCl 0.9%), rapidly frozen in liquid nitrogen and then stored at -80°C. The latter was used for the lipidomics analysis.

Lipidomics analysis

For the lipidomic analyses the liver biopsies of the healthy cats pre- and post spaying/neutering and of the cats with HL were re-suspended in 350 µL of buffer A (250 mM sucrose 0.2 mM EDTA, 5 mM DTT, and 10 mM Tris/HCl pH 8.0) and homogenized by mechanical disruption with a pestle tightly fitting to an Eppendorf tube followed by sonication (10 sec, amplitude 10 µm). Protein was determined in the homogenates by the BCA method Pierce® BCA protein assay kit (Thermo scientific, Rockford, IL, USA) with BSA as standard.
To 100 µl of homogenized liver biopsy or 100 µL of plasma, 200 pmol of tri-penta-decanoylglycerol (TAG 45:0) and 100 pmol of di-linolenoyl phosphatidylcholine (PC (18:3, 18:3)) were added as internal standards. Subsequently, lipids were extracted and separated in a neutral and phospholipid fraction by fractionation on a silica-G column as described [41]. In short, lipid extracts were dissolved in

methanol/chloroform (1/9, v/v) and loaded on top of the silica column. Neutral lipids were eluted with two volumes acetone, dried under nitrogen gas and stored at -20°C. In the neutral fraction, TAG species, cholesterol, cholesterol esters (CE), and retinyl esters (RE) were determined by HPLC-MS as described.[41] Typically, just before HPLC-MS analysis, the neutral lipid fraction was reconstituted in methanol/chloroform (1/1, v/v) and separated on a Lichrospher RP18-e column (5 µm, 250 x 4.6 mm; Merck, Darmstadt, Germany). A gradient was generated from acetonitrile to acetone/chloroform 85/15, v/v, at a constant flow rate of 1 ml/min. Mass spectrometry of lipids was performed using Atmospheric Pressure Chemical Ionization (APCI) on a Biosystems API-4000 Q-trap (MDS Sciex, Concord, Canada). The system was controlled by Analyst version 1.4.2 software (MDS Sciex, Concord, ON, Canada) and operated in positive ion mode. Full scan runs spectra were obtained from m/z 250-1100. Total TAG was determined by quantitating all ions between m/z 530 and 1050 with a retention time of TAG species and corrected for the presence of second and third isotope peaks. The percentage of TAG 56:6/7 was determined by quantitation of the ions with a m/z 905 and 907. TAG unsat/sat ratio was calculated after quantitiation of the ions with a m/z of 853 (TAG52:5), 879 (TAG54:6), 859 (TAG 52:2) and 885 (TAG54:3).

In the phospholipid fraction, sphingomyelin (SM) and PC species were determined as described.[42] The phospholipid fraction was dissolved in chloroform/methanol (1:1, v/v), and PC and SM molecular species were separated LiChrospher 100 RP18-e column (Merck), with a mobile phase of acetonitrile/methanol/triethylamine (25:24:1, v/v/v). Identification of molecular species was performed by on-line tandem mass spectrometry in the positive-ion mode on an API 4000 Q Trap mass spectrometer fitted with an electrospray ionization source (Sciex) by precursor scans of 184 m/z (choline head group).

Data analysis was performed using Analyst 1.4.2 software (MDS Sciex, Concord, ON, Canada) and calibration curves of all lipid classes were established under similar conditions as the samples.

Statistical analysis

Healthy cats, pre versus post spaying/neutering

The outcome variables TAG, TAG unsat/sat, TAG56:6/7, cholesterol, cholesterol ester, total PC, PC species (% of total PC) and SM were respectively analysed using a linear mixed model with the cat identification as random effect to take repeated observations within the subject into account. "Time" (before/after spaying/neutering) and "gender" (male/female) and the interaction between time and gender were used as explanatory variables. The Akaike Information Criterion (AIC) was used to select the best model. Residuals plots were used to assess the validity of the model by visual inspection of the QQ-plot of residuals for normality

and scatterplots of residuals versus predicted values for constant variance.
Some outcome variables (liver TAG sat/unsat; plasma PC 34:1) were log transformed to meet the model assumptions normality and constant variance. In this model we also could take the correlation between observations into account.
If data were not normally distributed, neither after log transformation (liver TAG 56:6/7; liver PC 38:6; plasma TAG sat/unsat), a nonparametric Kruskall and Wallis test was applied to assess the difference in means between groups of combined gender and time (preMale, preFemale, postMale en postFemale respectively) and a non-parametric Wilcoxon test was applied to assess the difference within genders between both times. Unfortunately this model was not able to take the correlation between observations into account.
In order to identify factors involved in the variation in liver TAG levels in the healthy cats a Pearson correlation analysis was performed.

Healthy cats versus cats with hepatic lipidosis

Comparison between the mean of the respective outcomes of the healthy cats after spaying/neutering and cats with HL were calculated with the independent Student's *T*-test.
P-value <0.05 was used to assess statistical significance. Results are expressed as mean and standard deviation (SD), if not stated otherwise.
Data analysis was performed using IBM SPSS 22 statistic software (IBM Corporation Armonk, NY, USA).

Results

Animal's characterization

A description of the healthy cats has been previously reported.[40] In summary, six healthy females (20.5 (6.0 – 84.0 months); 3.0 (2.6 – 3.9) kg; (median (range)) and six healthy males (7.0 (6.0 – 9.0) months; 4.0 (3.5 – 4.5) kg; (median (range)) were enrolled in this study Results of the CBC, biochemistry and coagulation profile of all 12 cats were within reference intervals. Both oestrogen in the female group (*P*= 0.041) and testosterone in the male group (P= 0.001) dropped significantly confirming successful gonadectomy.[40]
Histological evaluation of the liver biopsies performed in the healthy cats (pre and post spaying/neutering) at 4 weeks and at 8 weeks after the beginning of administration of the diet, revealed no histological changes compatible with HL or any other pathological change in any of the healthy cats.[40]
Two spayed females (50.0 and 52.0 months; 3.5 kg and 4.5 kg, respectively), and three neutered males (38.0, 40.0, and 48.0 months; 3.0, 4.0, and 4.3 kg, respectively)

with HL were enrolled in this study. During a period of one month to three days prior to admittance cats showed clinical signs consisting of, but not limited to, lethargy, anorexia, vomiting, and weight loss. Diagnosis of HL during life was confirmed with cytological examination of liver fine needle aspirates performed by a diplomate in European College of Veterinary Clinical Pathology.

Lipid profiles

Healthy cats, pre- and post-spaying/neutering

Plasma level of TAG was almost one-and-a-half-fold lower ($P = 0.017$) in intact female cats compared to intact male cats (Table 1). There was also a significant difference of the plasma level of TAG between males pre and post neutering, where neutered male cats had higher plasma levels of TAG compared to the intact males ($P = 0.033$).

In contrast, intact female cats had two-fold higher TAG levels in the liver compared to intact males (Table 1). Due to a large variation, this difference did not reach statistical significance ($P = 0.17$). After castration the TAG liver levels in the female cats dropped, but were still higher (but not significant), compared to the TAG levels in castrated males (Table 1).

	Liver				Plasma			
	Group 1 Male intact (n = 6)	Group 2 Male neutered (n = 6)	Group 3 Female intact (n = 6)	Group 4 Female spayed (n = 6)	Group 1 Male intact (n = 6)	Group 2 Male neutered (n = 6)	Group 3 Female intact (n = 6)	Group 4 Female spayed (n = 6)
Total TAG[1] (nmol/mg prot) (mmol/l)	51 ± 35	58 ± 45	99 ± 70	78 ± 52	0.22 ± 0.05[abc]	0.30 ± 0.10[abc]	0.15 ± 0.03[ac]	0.14 ± 0.05[ac]
TAG 56:6/7[2] (%)	0.6 ± 0.2	0.8 ± 0.4	0.7 ± 0.1[b]	0.5 ± 0.1[b]	0.7 ± 0.3	0.8 ± 0.3	0.8 ± 0.2	0.7 ± 0.2
TAG unsat/sat[3]	1.0 ± 0.3	1.1 ± 0.4	1.1 ± 0.4	0.8 ± 0.3	1.8 ± 0.4	1.8 ± 0.2	2.0 ± 0.6	1.8 ± 0.4
Cholesterol (rel. units)	13.1 ± 1.7	14.2 ± 1.3	15.3 ± 1.6	13.6 ± 2.0	8.9 ± 0.7	9.9 ± 1.2	9.3 ± 1.1	9.0 ± 0.6
Cholesterol ester (rel. units)	7.8 ± 2.9	7.6 ± 2.6	10.3 ± 4.6	9.1 ± 3.0	61 ± 8	62 ± 10	56 ± 6	59 ± 3
Total PC[4] (nmol/mg prot) (mmol/l)	24.2 ± 3.8	24.1 ± 3.2	27.5 ± 3.1	24.1 ± 2.3	0.16 ± 0.02	0.17 ± 0.03	0.15 ± 0.03	0.15 ± 0.03
SM[5] 16:0 (% of PC)	3.8 ± 0.8	3.8 ± 0.5	3.4 ± 0.9	3.9 ± 0.8	7.4 ± 0.7	6.9 ± 0.5	7.4 ± 0.7	7.5 ± 1.1

Table 1. Effect of sex hormones and spaying/neutering on liver and plasma lipids of healthy cats (means ± SD).

1. TAG = triacylglycerol
2. TAG 56:6/7 = TAG containing relative long unsatured acyl chains
3. TAG unsat/sat = the ratio between TAG with 5 or 6 double bonds versus 2 or 3 double bonds
4. PC = phosphatidylcholine
5. SM = sphingomyelin (SM)

a P < 0.05 male vs female (gender)
b = P < 0.05 intact vs spayed/neutered (time)
c = P < 0.05 interaction between gender and time

The Pearson correlation analysis demonstrated the highest ranked correlations for liver TAG levels with PC 36:4 in both the liver and plasma (r = +0.62; P = 0.001 for liver PC36:4, and r = +0.540; P = 0.006 for plasma PC36:4). A significant positive correlation (r) of liver TAG levels with liver cholesterol esters was also observed (r = +0.59; P = 0.003), but no significant correlation was found between plasma and liver TAG levels (r = - 0.19; P = 0.39).

The total levels of PC in plasma and liver did not differ significantly between male and female cats, both before and after spaying /neutering (Table 1). The overall species profile of PC in plasma was similar to that in liver tissue (Fig. 1). The most abundant PC species were PC34:2 and PC36:2, both predominantly containing linoleic acid (18:2) in combination with palmitic acid (16:0) (PC34:2) or stearic acid (18:0) (PC36:2). There were differences in PC species profiles between the male and female cats before and after spaying/neutering (Fig. 1). The most significant differences in PC species between female and male cats were with those with an AA (20:4). The sum of the percentage of PC containing LCPUFA AA, PC36:4 and PC38:4, in intact female cats were significantly (P < 0.01 and P < 0.002, respectively) higher in both plasma (21.8 ± 1.6 %) and liver (22.2 ± 2.3 %), compared to male (plasma: 18.2 ± 1.7 %; liver: 17.1 ± 1.4 %) (Fig. 1 and 2).

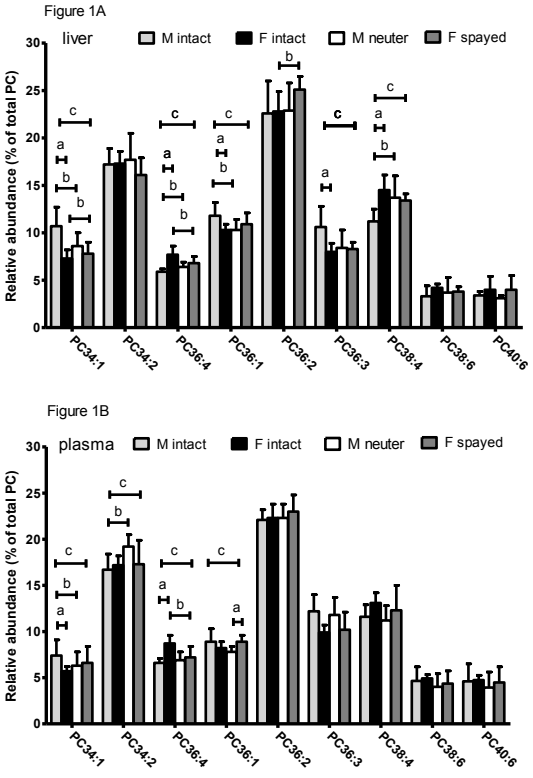

Figure 1. Effect of sex (gender), spaying/neutering (time) and their interaction (gender x time) on species distribution of phosphatidylcholine (means ± SD)

The major phosphatidylcholine (PC) species were determined in liver biopsies (Fig 1A) and plasma (Fig 1B).

PC36:4 and PC38:4 contain arachidonic acid (20:4)
PC38:6 and PC40:6 contain docosahexaenoic acid (22:6).

a = P < 0.05 male vs female (gender),
b = P < 0.05 intact vs spayed/neutered (time)
c = P < 0.05 interaction between gender and time

These percentages were also higher in both plasma and liver of intact female cats were compared to spayed female cats (plasma:19.5 ± 2.7 %; 20.2± 1.2 % liver), however the differences were not statistically significant ($P < 0.08$ and $P < 0.1$ respectively) (Fig. 1 and 2). The lower levels of AA-containing PC species in intact male cats coincided with higher levels of PC34:1 and PC36:3, predominantly containing non-PUFAs (16:0, 18:1, and 18:2). Phosphatidylcholine species with another abundant LCPUFA, i.e. DHA (22:6 n-3), PC38:6 and PC40:6, did not differ between intact males and females and castrated males and females. After spaying/neutering, the levels of PC36:4 dropped significantly in female cats and increased significantly in male cats (Fig. 1 and 2), resulting in similar levels of the AA-containing PCs in male and female cats after spaying/neutering.

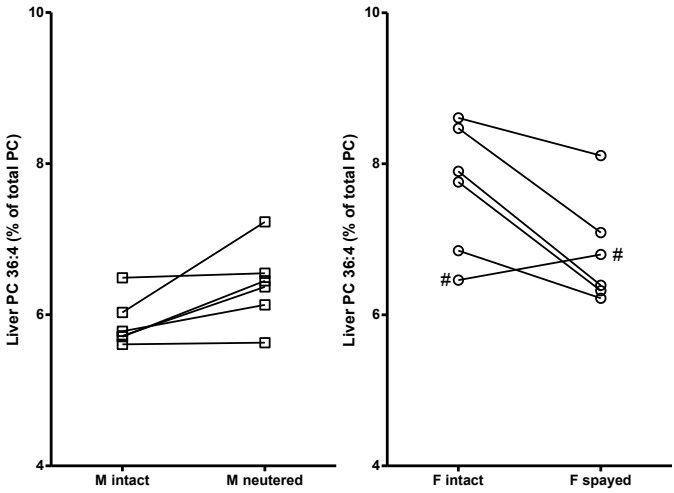

Figure 2. Effect of sex and castration on palmitoyl-arachidonoyl-phosphatidylcholine (PC36:4). The phosphatidylcholine (16:0, 20:4) species (PC36:4) were determined in liver biopsies from 6 cats per group. Significant differences were present in liver PC 36:4 between male and female cats (gender) and between male cats pre and post neutering and female cats pre and post spaying. Notice how in the male cats, neutering increases the PC 36:4 liver values. In female cats, spaying decreases the PC 36:4 liver values. # intact cat had low estrogen level.

Healthy cats versus cats with hepatic lipidosis

The cats with HL had the same level of plasma TAG as the healthy spayed/neutered cats, but an almost ten-fold higher level of TAG in the liver ($P = 0.004$) (Table 2). The levels of AA-containing PC species (36:4; 38:4), which were found to correlate positively with the liver TAG levels in healthy cats, were not higher in the HL cats and were even lower for PC 38:4 in the liver (see Table 2). Sphingomyelin (SM) (16:0) as a percentage of total PC, was significantly higher (two-fold) both in plasma and in liver when compared to the healthy cats.

	Liver		Plasma	
	Cats spayed/neutered (n=12)	HL cats (n=5)	Cats spayed/neutered (n=12)	HL cats (n=5)
Total TAG[1] (nmol/mg prot) (mmol/l)	68 ± 48	584 ± 277[a]	0.22 ± 0.11	0.40 ± 0.25
%TAG 56:6/7[2]	0.7 ± 0.3	1.0 ± 0.3[a]	0.8 ± 0.3	1.8 ± 1.4
TAG unsat/sat[3]	1.0 ± 0.4	1.0 ± 0.7	1.8 ± 0.3	1.0 ± 0.7
Total PC[4] (nmol/mg prot) (mmol/l)	24.1 ± 2.7	10.2 ± 5.0 [a]	0.16 ± 0.03	0.16 ± 0.04
% PC[4] 34:2	16.9 ± 2.4	20.7 ± 4.0	18.3 ± 2.2	19.3 ± 1.9
% PC 34:1	8.2 ± 1.3	12.5 ± 4.3	6.4 ± 1.6	12.8 ± 7.0
% PC 36:4	6.6 ± 0.6	5.3 ± 1.5	7.1 ± 1.0	5.8 ± 2.0
% PC 36:3	8.4 ± 1.4	6.4 ± 2.5	11.0 ± 2.0	8.4 ± 2.3[a]
% PC 36:2	24.0 ± 2.5	24.0 ± 6.2	22.6 ± 1.6	22.8 ± 3.0
% PC 36:1	10.6 ± 1.1	11.6 ± 3.6	8.4 ± 0.9	10.2 ± 2.7
% PC 38:4	13.6 ± 1.7	8.8 ± 3.9 [a]	11.8 ± 2.2	10.0 ± 3.2
% PC 38:6	3.8 ± 1.1	3.7 ± 1.7	4.2 ± 1.4	4.0 ± 1.5
% PC 40:6	4.0 ± 1.6	3.7 ± 2.0	4.2 ± 1.6	3.7 ± 2.0
SM 16:0[5] (% of PC)	3.8 ± 0.6	8.0 ± 3.5 [a]	7.2 ± 0.9	14.2 ± 6.4[a]

Table 2. Comparison of liver and plasma lipids between healthy and cats with hepatic lipidosis (HL)

1. TAG = triacylglycerol
2. TAG 56:6/7 = TAG containing relative long unsatured acyl chains
3. TAG unsat/sat = the ratio between TAG with 5 or 6 double bonds versus 2 or 3 double bonds
4. PC = phosphatidylcholine
5. SM = sphingomyelin (SM)

a = $P < 0.005$ HL vs healthy cats

Discussion

Butterwick et al. has reported no important differences in plasma lipid and lipoprotein concentrations between sexually intact females and males or between sexually intact and castrated males have been demonstrated.[43] However, as sexual dimorphism in the lipid metabolism has been demonstrated so clearly in different species, we wanted to explore this further in cats. In a recent study we were unable to demonstrate significant differences in liver PC levels between male and female cats pre- and post- spaying/neutering and a lack of evident influence of sex hor-

mones on the PC synthesis by the phosphatidylethanolamine N-methyltransferase (PEMT) pathway.[40] However, other lipid factors might be involved if a potential sexual dimorphism in cats exists. To evaluate if a sexual dimorphism could still be established and if differences could be related to HL, we evaluated in this study the lipid profile in cats in more detail.

Intact female cats have significantly lower TAG levels in plasma compared to their male counterparts, similarly to premenopausal women, intact female rats and mice.[12-14,18,19] However, this difference was not reversed by spaying but rather enhanced due to a rise in the plasma TAG level in male cats after neutering.

Although not significant possibly due to large variation, the liver TAG levels were lower in male cats compared to female cats. The significantly higher plasma TAG levels in male cats may be related to a higher rate of apolipoprotein B and VLDL synthesis by hepatocytes in male cats [30,31], as the TAG levels in plasma most likely reflect the VLDL fraction[44] . Also a reduced plasma clearance of VLDL triglyceride in male cats compared to their female counterparts cannot be excluded. Interestingly, in premenopausal women a lower concentration of VLDL-TAG and LDL particles compared to men seems to be associated with accelerated (rather than reduced) VLDL-TAG and LDL production and increased plasma clearance.[30,31,45-48] Our observations warrant further studies into gender specific VLDL secretion/breakdown in cats.

The results of the lipidomic analysis in the cats with HL in this study confirm findings in previous studies analysing lipids in liver and in plasma.[49,50] We also observed that cats with HL have significantly higher TAG levels in their liver. However, in our study cats with HL had no significantly different plasma TAG levels compared to healthy cats, whereas in other studies high plasma TAG levels in cats with HL have been described.[39,49,50]

The secretion of VLDL in HL may prevent lipid accumulation in the liver by exporting the surplus TAG and it can be stimulated by higher levels of TAG in the liver of cats with lipidosis.[44] However, the results in the cats with HL suggest there may be a maximum capacity of the liver in cats to secrete VLDL, so that no further increase in plasma VLDL is observed in association with high liver TAG levels.[51]

Intact female cats have a higher content of LCPUFA AA (20:4 n-6) in PC in both plasma and liver tissue, when compared to spayed female and the male counterparts. This finding is similar to what has been reported in premenopausal women, intact female rats and mice.[12-14,18,19] However, in contrast to what has been reported in these species, PC levels containing LCPUFA DHA (22:6 n-3) were not elevated in intact female cats.[18,21,52,53] Despite this difference, there seems an influence of sex hormones on the profile of PC species in cats likely as the differences between the sexes disappeared after spaying/neutering.

In humans, the increased LCPUFA levels appear to be related to an oestrogen effect on FA desaturase activity via increased expression of Δ5 and Δ6 desaturase.[18,25,26] Although cats were believed to have low Δ5 and Δ6 desaturase activity [38,54], in a more recent study cats were able to synthetize AA, but not DHA, from the substrate γ-linolenic acid (18:3 n−6) via Δ5 desaturase, bypassing the Δ6 desaturase step.[37] This is supported by findings in this study as the primary increase in AA but not DHA levels in the liver of intact female cats compared to the male and spayed female counterparts might also suggest a possible role of Δ5 desaturases.

In humans and mice the higher LCPUFA levels may protect against the development of HL via the so-called "fuel partitioning action" of LCPUFA.[55-57] Long chain polyunsaturated fatty acids favour FA oxidation over TAG storage and they direct glucose away from FA synthesis by facilitating glycogen synthesis. However, the n-3 LCPUFA species (*i.e.* DHA), rather than the n-6 LCPUFA (*i.e.* AA), are mainly responsible for these effects via the activation of the peroxisome proliferating receptors (PPARs) in liver and adipose tissue. [57-60] The low liver levels of n-3 LCPUFA in the intact female cats may suggest less resistance to HL compared to females of other species.

In contrast to what it would be expected, in the cats with HL in this study, we did not find direct evidence for an increase in the n-6/n-3 ratio as indeed, the AA-containing PC 38:4 in cats with HL was even significantly lower compared to healthy cats and there was no difference in n-3 LCPUFA between HL and healthy cats. In cats with HL a lower percentages of the LCPUFA (i.e. AA) in the liver have been demonstrated before.[49,50]

Sphingomyelin, a membrane lipid primarily present in the membranous myelin sheath that surrounds nerve cell axons, and its relation in the development of steatosis has been observed in different animal models of obesity and fatty liver.[61,62] The increased synthesis of SM in livers affected by NAFLD promotes hepatic insulin resistance, hepatocyte apoptosis, an increased release of inflammatory mediators, and ceramide accumulation in peripheral tissues.[63] The increased SM concentrations, both in liver and in plasma, in our cats with HL warrants further study in order to understand the potential role of SM in feline HL.

Conclusions

Although some differences are found, the sexual dimorphism in the hepatic and plasma lipid profile of healthy cats resembles observations in other species with lower plasma TAG levels and increased plasma and liver AA in intact females

compared to males. However, the higher plasma and liver levels of AA but not DHA could predispose intact female cats for HL. Also spaying of female cats may not increase the risk for HL.

In contrast to previous studies, the higher liver but not plasma TAG levels in cats with HL compared to healthy cats could suggest a maximum capacity for liver excretion of VLDL. The increased liver and plasma SM concentration in cats with HL compared to healthy cats could indicate a novel mechanism in the development of HL in cats.

Abbreviations

NAFLD: non-alcoholic fatty liver disease; TAG: triglyceride; TC: total cholesterol; LDL: low density lipoprotein; HDL: high density lipoprotein; FA: fatty acid; AA: arachidonic acid; LA: linoleic acid; LCPUFA: long chain polyunsaturated fatty acid; DHA: docosahexaenoic acid; VLDL: very low density lipoprotein; HL; hepatic lipidosis; Δ: desaturase; PC: phosphatidylcholine; PEMT: phosphatidylethanolamine N-methyltransferase; SM: sphingomyelin;

Declarations

Ethics approval and consent to participate

The project was approved by the responsible ethical committees for the use of client owned animals according to Dutch legislation. Informed owner consent was obtained prior to enrolment of all cats

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing Interest

The author Isabelle Jeusette declares to have competing interest with the manuscript by being currently employed by the study sponsor.

Funding

This research was financially supported by Affinity Petcare, Barcelona, Spain. Affinity Petcare also supplied the study of the diet that was fed to the cats in this study.

Contributions

CV, RHJ, RPF, JHR, ABV, IJ contributed to the development and writing of the paper. RPF, JR performed the liver biopsies. ABV and MT performed the lipidomics and the statistics. AK performed the surgeries.

Acknowledgment

We thank Dr Hans J.C.M. Vernooij for assistance with the statistics.

References

1. Han X, Gross RW. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: A bridge to lipidomics. *J Lipid Res*. 2003;44(6):1071-1079. doi: 10.1194/jlr.R300004-JLR200 [doi].

2. Hu C, van der Heijden R, Wang M, van der Greef J, Hankemeier T, Xu G. Analytical strategies in lipidomics and applications in disease biomarker discovery. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009;877(26):2836-2846. doi: 10.1016/j.jchromb.2009.01.038 [doi].

3. Wenk MR. The emerging field of lipidomics. *Nat Rev Drug Discov*. 2005;4(7):594-610. doi: nrd1776 [pii].

4. Musso G, Gambino R, Cassader M. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Prog Lipid Res*. 2009;48(1):1-26. doi: 10.1016/j.plipres.2008.08.001 [doi].

5. Watson AD. Thematic review series: Systems biology approaches to metabolic and cardiovascular disorders. lipidomics: A global approach to lipid analysis in biological systems. *J Lipid Res*. 2006;47(10):2101-2111. doi: R600022-JLR200 [pii].

6. Arnold AP. Promoting the understanding of sex differences to enhance equity and excellence in biomedical science. *Biol Sex Differ*. 2010;1(1):1-6410-1-1. doi: 10.1186/2042-6410-1-1 [doi].

7. Taylor KC, Carty CL, Dumitrescu L, et al. Investigation of gene-by-sex interactions for lipid traits in diverse populations from the population architecture using genomics and epidemiology study. *BMC Genet*. 2013;14:33-2156-14-33. doi: 10.1186/1471-2156-14-33 [doi].

8. Gardner CD, Winkleby MA, Fortmann SP. Population frequency distribution of non-high-density lipoprotein cholesterol (third national health and nutrition examination survey [NHANES III], 1988-1994). *Am J Cardiol*. 2000;86(3):299-304. doi: S0002-9149(00)00918-8 [pii].

9. Gostynski M, Gutzwiller F, Kuulasmaa K, et al. Analysis of the relationship between total cholesterol, age, body mass index among males and females in the WHO MONICA project. *Int J Obes Relat Metab Disord*. 2004;28(8):1082-1090. doi: 10.1038/sj.ijo.0802714 [doi].

10. Regitz-Zagrosek V, Lehmkuhl E, Mahmoodzadeh S. Gender aspects of the role of the metabolic syndrome as a risk factor for cardiovascular disease. *Gend Med*. 2007;4 Suppl B:S162-77. doi: S1550-8579(07)80056-8 [pii].

11. Williams CM. Lipid metabolism in women. *Proc Nutr Soc*. 2004;63(1):153-160. doi: 10.1079/PNS2003314 [doi].

12. Ostwald R, Bouchard P, Miljanich P, Lyman RL. Influence of sex and gonadal hormones on rat-liver and carcass lipids during the development of an essential fatty acid deficiency. *Biochem J*. 1965;97(2):485-499.

13. Lyman RL, Tinoco J, Bouchard P, Sheehan G, Ostwald R, Miljanich P. Sex differences in the metabolism of phosphatidyl cholines in rat liver. *Biochim Biophys Acta*. 1967;137(1):107-114.

14. Lyman RL, Hopkins SM, Sheehan G, Tinoco J. Effects of estradiol and testosterone on the incorporation and distribution of [me-14C]methionine methyl in rat liver lecithins. *Biochim Biophys Acta*. 1968;152(1):197-207.

15. Young DL. Estradiol- and testosterone-induced alterations in phosphatidylcholine and triglyceride synthesis in hepatic endoplasmic reticulum. *J Lipid Res*. 1971;12(5):590-595.

16. Carroll MD, Lacher DA, Sorlie PD, et al. Trends in serum lipids and lipoproteins of adults, 1960-2002. *JAMA*. 2005;294(14):1773-1781. doi: 294/14/1773 [pii].

17. Mittendorfer B. Sexual dimorphism in human lipid metabolism. *J Nutr*. 2005;135(4):681-686. doi: 135/4/681 [pii].

18. Burdge GC, Slater-Jefferies JL, Grant RA, et al. Sex, but not maternal protein or folic acid intake, determines the fatty acid composition of hepatic phospholipids, but not of triacylglycerol, in adult rats. *Prostaglandins Leukot Essent Fatty Acids*. 2008;78(1):73-79. doi: S0952-3278(07)00179-2 [pii].

19. Extier A, Langelier B, Perruchot MH, et al. Gender affects liver desaturase expression in a rat model of n-3 fatty acid repletion. *J Nutr Biochem*. 2010;21(3):180-187. doi: 10.1016/j.jnutbio.2008.10.008 [doi].

20. Childs CE, Romeu-Nadal M, Burdge GC, Calder PC. The polyunsaturated fatty acid composition of hepatic and plasma lipids differ by both sex and dietary fat intake in rats. *J Nutr*. 2010;140(2):245-250. doi: 10.3945/jn.109.115691 [doi].

21. Bakewell L, Burdge GC, Calder PC. Polyunsaturated fatty acid concentrations in young men and women consuming their habitual diets. *Br J Nutr*. 2006;96(1):93-99. doi: S0007114506001826 [pii].

22. Giltay EJ, Gooren LJ, Toorians AW, Katan MB, Zock PL. Docosahexaenoic acid concentrations are higher in women than in men because of estrogenic effects. *Am J Clin Nutr*. 2004;80(5):1167-1174. doi: 80/5/1167 [pii].

23. Sprecher H. The roles of anabolic and catabolic reactions in the synthesis and recycling of polyunsaturated fatty acids. *Prostaglandins Leukot Essent Fatty Acids*. 2002;67(2-3):79-83. doi: S0952327802904023 [pii].

24. Brenner RR. Hormonal modulation of delta6 and delta5 desaturases: Case of diabetes. *Prostaglandins Leukot Essent Fatty Acids*. 2003;68(2):151-162. doi: S095232780200265X [pii].

25. Burdge GC, Calder PC. Dietary alpha-linolenic acid and health-related outcomes: A metabolic perspective. *Nutr Res Rev*. 2006;19(1):26-52. doi: 10.1079/NRR2005113 [doi].

26. Burdge GC, Wootton SA. Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. *Br J Nutr*. 2002;88(4):411-420. doi: 10.1079/BJN2002689 [doi].

27. Pawlosky R, Hibbeln J, Lin Y, Salem N,Jr. N-3 fatty acid metabolism in women. *Br J Nutr*. 2003;90(5):993-4; discussion 994-5. doi: S0007114503002125 [pii].

28. Irwin RW, Yao J, Hamilton RT, Cadenas E, Brinton RD, Nilsen J. Progesterone and estrogen regulate oxidative metabolism in brain mitochondria. *Endocrinology*. 2008;149(6):3167-3175. doi: 10.1210/en.2007-1227 [doi].

29. Oztekin E, Tiftik AM, Baltaci AK, Mogulkoc R. Lipid peroxidation in liver tissue of ovariectomized and pinealectomized rats: Effect of estradiol and progesterone supplementation. *Cell Biochem Funct*. 2007;25(4):401-405. doi: 10.1002/cbf.1313 [doi].

30. Wang X, Magkos F, Mittendorfer B. Sex differences in lipid and lipoprotein metabolism: It's not just about sex hormones. *J Clin Endocrinol Metab*. 2011;96(4):885-893. doi: 10.1210/jc.2010-2061 [doi].

31. Magkos F, Patterson BW, Mohammed BS, Klein S, Mittendorfer B. Women produce fewer but triglyceride-richer very low-density lipoproteins than men. *J Clin Endocrinol Metab*. 2007;92(4):1311-1318. doi: jc.2006-2215 [pii].

32. Snyder SP, Kingston RS, Wenger DA. Niemann-pick disease. sphingomyelinosis of siamese cats. *Am J Pathol*. 1982;108(2):252-254.

33. Watson TDG, Barrie J. Lipoprotein metabolism and hyperlipidemia in the dog and cat: A review. *Journal of Small Animal Practice*. 1993;34:479-487.

34. Armstrong PJ, Blanchard G. Hepatic lipidosis in cats. *Vet Clin North Am Small Anim Pract*. 2009;39(3):599-616. doi: 10.1016/j.cvsm.2009.03.003 [doi].

35. Center SA. Feline hepatic lipidosis. *Vet Clin North Am Small Anim Pract*. 2005;35(1):225-269. doi: S0195-5616(04)00142-1 [pii].

36. Verbrugghe A, Bakovic M. Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis. *Nutrients*. 2013;5(7):2811-2835. doi: 10.3390/nu5072811 [doi].

37. Trevizan L, de Mello Kessler A, Brenna JT, Lawrence P, Waldron MK, Bauer JE. Maintenance of arachidonic acid and evidence of Delta5 desaturation in cats fed gamma-linolenic and linoleic acid enriched diets. *Lipids*. 2012;47(4):413-423. doi: 10.1007/s11745-011-3651-0 [doi].

38. Pawlosky R, Barnes A, Salem N,Jr. Essential fatty acid metabolism in the feline: Relationship between liver and brain production of long-chain polyunsaturated fatty acids. *J Lipid Res*. 1994;35(11):2032-2040.

39. Center SA, Crawford MA, Guida L, Erb HN, King J. A retrospective study of 77 cats with severe hepatic lipidosis: 1975-1990. *J Vet Intern Med*. 1993;7(6):349-359.

40. Valtolina C, Vaandrager AB, Favier RP, et al. No up-regulation of the phosphatidylethanolamine N-methyltransferase pathway and choline production by sex hormones in cats. *BMC Vet Res*. 2015;11:280-015-0591-6. doi: 10.1186/s12917-015-0591-6 [doi].

41. Testerink N, Ajat M, Houweling M, et al. Replacement of retinyl esters by polyunsaturated triacylglycerol species in lipid droplets of hepatic stellate cells during activation. *PLoS One*. 2012;7(4):e34945. doi: 10.1371/journal.pone.0034945 [doi].

42. Bleijerveld OB, Brouwers JF, Vaandrager AB, Helms JB, Houweling M. The CDP-ethanolamine pathway and phosphatidylserine decarboxylation generate different phosphatidylethanolamine molecular species. *J Biol Chem*. 2007;282(39):28362-28372. doi: M703786200 [pii].

43. Butterwick RF, McConnell M, Markwell PJ, Watson TD. Influence of age and sex on plasma lipid and lipoprotein concentrations and associated enzyme activities in cats. *Am J Vet Res*. 2001;62(3):331-336.

44. Pazak HE, Bartges JW, Cornelius LC, Scott MA, Gross K, Huber TL. Characterization of serum lipoprotein profiles of healthy, adult cats and idiopathic feline hepatic lipidosis patients. *J Nutr*. 1998;128(12 Suppl):2747S-2750S. doi: 10.1093/jn/128.12.2747S [doi].

45. Matthan NR, Jalbert SM, Barrett PH, Dolnikowski GG, Schaefer EJ, Lichtenstein AH. Gender-specific differences in the kinetics of nonfasting TRL, IDL, and LDL apolipoprotein B-100 in men and premenopausal women. *Arterioscler Thromb Vasc Biol*. 2008;28(10):1838-1843. doi: 10.1161/ATVBAHA.108.163931 [doi].

46. Nikkila EA, Kekki M. Polymorphism of plasma triglyceride kinetics in normal human adult subjects. *Acta Med Scand*. 1971;190(1-2):49-59.

47. Olefsky J, Farquhar JW, Reaven GM. Sex difference in the kinetics of triglyceride metabolism in normal and hypertriglyceridaemic human subjects. *Eur J Clin Invest*. 1974;4(2):121-127.

48. Watts GF, Moroz P, Barrett PH. Kinetics of very-low-density lipoprotein apolipoprotein B-100 in normolipidemic subjects: Pooled analysis of stable-isotope studies. *Metabolism*. 2000;49(9):1204-1210. doi: S0026-0495(00)82057-0 [pii].

49. Hall JA, Barstad LA, Connor WE. Lipid composition of hepatic and adipose tissues from normal cats and from cats with idiopathic hepatic lipidosis. *J Vet Intern Med*. 1997;11(4):238-242.

50. Brown B, Mauldin GE, Armstrong J, Moroff SD, Mauldin GN. Metabolic and hormonal alterations in cats with hepatic lipidosis. *J Vet Intern Med*. 2000;14(1):20-26.

51. Fabbrini E, Mohammed BS, Magkos F, Korenblat KM, Patterson BW, Klein S. Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. *Gastroenterology*. 2008;134(2):424-431. doi: 10.1053/j.gastro.2007.11.038 [doi].

52. Giltay EJ, Duschek EJ, Katan MB, Zock PL, Neele SJ, Netelenbos JC. Raloxifene and hormone replacement therapy increase arachidonic acid and docosahexaenoic acid levels in postmenopausal women. *J Endocrinol*. 2004;182(3):399-408.

53. Hoene M, Li J, Haring HU, Weigert C, Xu G, Lehmann R. The lipid profile of brown adipose tissue is sex-specific in mice. *Biochim Biophys Acta*. 2014;1842(10):1563-1570. doi: 10.1016/j.bbalip.2014.08.003 [doi].

54. Rivers JP, Sinclair AJ, Craqford MA. Inability of the cat to desaturate essential fatty acids. *Nature*. 1975;258(5531):171-173.

55. Mahfouz MM, Smith TL, Kummerow FA. Effect of dietary fats on desaturase activities and the biosynthesis of fatty acids in rat-liver microsomes. *Lipids*. 1984;19(3):214-222.

56. Reddy JK. Nonalcoholic steatosis and steatohepatitis. III. peroxisomal beta-oxidation, PPAR alpha, and steatohepatitis. *Am J Physiol Gastrointest Liver Physiol*. 2001;281(6):G1333-9.

57. Clarke SD. Polyunsaturated fatty acid regulation of gene transcription: A molecular mechanism to improve the metabolic syndrome. *J Nutr*. 2001;131(4):1129-1132.

58. Videla LA, Rodrigo R, Araya J, Poniachik J. Oxidative stress and depletion of hepatic long-chain polyunsaturated fatty acids may contribute to nonalcoholic fatty liver disease. *Free Radic Biol Med*. 2004;37(9):1499-1507. doi: 10.1016/j.freeradbiomed.2004.06.033 [doi].

59. Araya J, Rodrigo R, Videla LA, et al. Increase in long-chain polyunsaturated fatty acid n - 6/n - 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin Sci (Lond)*. 2004;106(6):635-643. doi: 10.1042/CS20030326 [doi].

60. Nieminen P, Mustonen AM, Karja V, Asikainen J, Rouvinen-Watt K. Fatty acid composition and development of hepatic lipidosis during food deprivation--mustelids as a potential animal model for liver steatosis. *Exp Biol Med (Maywood)*. 2009;234(3):278-286. doi: 10.3181/0806-RM-210 [doi].

61. Li Y, Dong J, Ding T, et al. Sphingomyelin synthase 2 activity and liver steatosis: An effect of ceramide-mediated peroxisome proliferator-activated receptor gamma2 suppression. *Arterioscler Thromb Vasc Biol*. 2013;33(7):1513-1520. doi: 10.1161/ATVBAHA.113.301498 [doi].

62. Pagadala M, Kasumov T, McCullough AJ, Zein NN, Kirwan JP. Role of ceramides in nonalcoholic fatty liver disease. *Trends Endocrinol Metab*. 2012;23(8):365-371. doi: 10.1016/j.tem.2012.04.005 [doi].

63. Bikman BT, Summers SA. Sphingolipids and hepatic steatosis. *Adv Exp Med Biol*. 2011;721:87-97. doi: 10.1007/978-1-4614-0650-1_6 [doi].

Chapter 5

The role of hepatic *de novo* lipogenesis in the development of feline hepatic lipidosis

Chiara Valtolina[1], Joris H. Robben[1], Monique E. van Wolferen[1], Hedwig S. Kruitwagen[1], Ronald J. Corbee[1], Robert P. Favier[2], Louis C. Penning[1].

Submitted to the Journal Feline medicine and Surgery

[1]*Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 108, 3584 CM, Utrecht, the Netherlands.*
[2] *Evidensia Netherlands, Wilhelminalaan 6, 3451 HJ Vleuten, The Netherlands*

Abstract

Objectives

To evaluate if *de novo* hepatic lipid synthesis contributes to fatty acids overload in the liver of cats with feline hepatic lipidosis (FHL) .

Methods

Lipogenic gene expression of peroxisome proliferator-activated receptor-α (*PPAR-α)*, peroxisome proliferator-activated receptor-*γ* (*PPAR-γ*), fatty acid synthetase (FAS), and sterol regulatory element-binding factor (*SREBF)*, were evaluated using qRT-PCR in liver tissue of six cats with feline hepatic lipidosis and compared to liver tissue of eight healthy cats.

Results

In liver tissue the *PPAR-α*, *PPAR-γ*, and *FAS* mRNA expression levels were not significantly different ($p > 0.12$; $p > 0.89$; $p > 0.5$, respectively) in the FHL group compared to the control group. *SREBF* gene expression was around 10-times down regulated in the FHL group compared to the control group ($p = 0.039$)
Conclusions and relevance: The result of our study with the downregulation of *SREBF* in liver tissue of cats with FHL, does not support the hypothesis that *de novo* lipogenesis in the liver is an important pathway of fatty acids accumulation in feline hepatic lipidosis.

Keywords

Feline hepatic lipidosis, de novo lipogenesis, SREBP-1c, qRT-PCR

Introduction

Feline hepatic lipidosis (FHL) is considered the most common hepatobiliary disease in cats and is characterized by the accumulation of excessive triglycerides (TGs) in more than 80% of the hepatocytes, resulting in liver dysfunction and cholestasis.[1-4] The pathophysiology of FHL is complex and the recent literature has highlighted how FHL results from multiple alterations in the pathways involved in the hepatic uptake of fatty acid (FA), lipoprotein and glucose, TGs degradation and FA oxidation, and lipoprotein secretion in the form of very low density lipoproteins (VLDL).[1,3-5] *De novo* TGs synthesis in the liver could cause part of the hepatic FAs load and may further exacerbate liver fat accumulation.[4-6]

De novo lipogenesis (DNL) is a complex and highly regulated metabolic pathway.[7,8] In normal conditions DNL converts excess carbohydrate into FAs, that are then esterified to storage triacylglycerols (TGs). The main product of DNL is palmitic acid (C16:0). Palmitic acid can be desaturated by stearoyl-CoA desaturase-1 (SCD-1) to produce palmitoleic acid or it can be elongated to yield stearic acid (C18:0) and other shorter fatty acids.[8-10]
In healthy humans, DNL is active in both liver and adipose tissue, whereas in healthy cats the adipose tissue is primarily involved, while the liver plays a secondary role. Furthermore, while glucose is the precursor for DNL in humans, in cats the substrate to form the FA palmitate is acetate.[3,11-13] *De novo* lipogenesis is considered to be a minor contributor to lipid formation and lipid homeostasis in healthy individuals.[14-17]

However in man, hepatic *DNL* has been demonstrated to contribute to liver steatosis in different metabolic diseases that cause deregulation of the lipogenic pathways, like obesity, diabetes type II and non-alcoholic fatty liver disease (NAFLD).[18-22]
The adipogenic transformation of hepatocytes, with the expression of gene profiles characteristic of healthy adipose tissue, contributes to liver overload with excess free FAs (FFAs).[8,23,24] *De novo* lipogenesis has been demonstrated to contribute up to 26% of FFAs that accumulate in the liver of patients with NAFLD.[8,10,25]

The role of the molecular mediators of lipogenesis in NAFLD has recently been reviewed.[26] Sterol regulatory element-binding protein-1c has been defined as the

pivotal controller of lipid biosynthesis and main regulator of the expression of several genes involved in lipogenesis. [27-30] In NAFLD and obese patients, elevated *de novo* hepatic FAs synthesis and secondary TGs accumulation in the liver has been reported being secondary to an increased mRNA expression in the hepatic *SREBP-1c*. Increased *SREBP-1c* by inducing lipogenic genes expression such as fatty acid synthetase (FAS) and peroxisome proliferator-activated receptor-*γ* (PPAR-γ) and suppressing the mRNA expression of peroxisome proliferator-activated receptor-*α* (PPAR-α), favours hepatic lipogenesis over FA oxidation and lead to steatosis. [28-33] In patients with NAFLD, the increased expression of *SREBP*-1c in NAFLD suppresses PPAR-α, further inhibiting carnitine-palmitoyl transferase 1 (*CPT-1*) and FAs β-oxidation.[31]

The role of *DNL* in the development of HL, has been evaluated in the American Mink (*Neovison vison*), an obligated carnivore often used as an experimental animal model for NAFLD. [12,13] These animals share physiological similarities with the domestic cat and they can easily develop HL after a short period of anorexia.[4,5,13,34] In a recent study evaluating mRNA expression of lipogenic genes in the liver of Mink with HL, Rouvinen-Watt *et al.* confirmed that liver steatosis is partially secondary to the activation of the hepatic *DNL*.[13]

The concept that hepatic *DNL* could contribute to liver FAs overload in cats with hepatic lipidosis (HL) was for the first time introduced by Hall et al. in their study on the lipid composition of hepatic and adipose tissue in cats with FHL.[35] The fatty acid composition of visceral adipose tissue and of the liver of cats with HL revealed greater percentages of palmitate and monounsaturated fatty acids (MUFA) compared to healthy controls. The increased hepatic concentrations of palmitate in cats with HL compared to healthy cats supported the hypothesis that some *de novo* synthesis of fatty acids occurs both in the liver and adipose tissue of cats with HL.[35] Furthermore in FHL, acetate, like other ketone bodies, is increased as a result of a more complex catabolic state that includes increased insulin resistance, and decreased tolerance to glucose. [5,11,36] The increased acetate in liver of cats with FHL constitutes a potential excess substrate for hepatic *DNL*, contributing and worsening hepatic FAs overload.

To date, the concept of adipogenic transformation of hepatocytes and its contribution to hepatic FA accumulation has not been adequately evaluated in cats.

The aim of the present study was to evaluate adipogenic transformation of hepatocytes and *de novo* lipid synthesis by determination of lipogenic gene expression of *PPAR-α*, *PPAR-γ*, *FAS*, and sterol regulatory element-binding factor

((*SREBF)* equivalent in function to SREBP-1c) using qRT-PCR in liver tissue of cats with FHL.

Materials And Methods

This study was approved by the Committee for the Ethical Care of Animals of the Utrecht University. Written, informed consent was obtained from all cat owners prior to study enrolment of the cats.

Animals

Cats admitted from January 2014 to January 2015 to the Department of Clinical Science of Companion Animals of the Faculty of Veterinary Medicine, Utrecht University (DCSCA) diagnosed with HL based on clinical symptoms (anorexia, vomiting and icterus), ultrasonography finding (diffuse echodensity of the liver) and cytological evaluation of fine needle aspirates of the liver consistent with FHL, were considered for the study. Only cats that were euthanized due to deterioration of their clinical condition despite intense treatment and/or because of financial constraints, were enrolled in the study.

In cats with FHL wedge liver biopsies were immediately collected post-mortem via laparotomy. The biopsies were rinsed in normal saline (NaCl 0.9%), rapidly frozen in liquid nitrogen and then stored at -80°C. The latter has been used to perform qRT-PCR.

Aged matched liver biopsies of eight healthy cats were used as controls. These liver biopsies were retrieved from surplus material from a study on the effect of administration of Vitamin A on skeletal and hepatic tissue as part of the University's 3R-policy.[37] Good health in these cats was based on a normal physical examination and haematological, biochemical and coagulation parameters within the reference ranges. In this study, percutaneous ultrasound-guided liver biopsies were taken with a 16 gauge, 20 cm long biopsy needle (Pro*Mag Biopsy Needle; Argon Medical Devices Inc., Athens, Texas, United States of America) under general anaesthesia. The liver biopsies were snap frozen in liquid nitrogen and then stored at -80°C.

RNA isolation, reverse transcription and quantitative reverse transcriptase PCR

Total RNA was isolated from liver tissue using RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's instructions. An on-column DNase-I (QIAFEB, RNase-free DNase kit) treatment was included to avoid contamination with residual genomic DNA. RNA concentrations and quality were

measured spectrophotometrically using the Nanodrop ND-1000 (Isogen Life Science BV, IJsselstein, The Netherlands). RNA integrity was checked on a Bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). The RNA integrity number of all samples exceeded seven. Per sample at least 3 µg of RNA was used for further processing. From all RNA samples cDNA was synthesized with the iScriptTM cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands) containing both oligo-dT and random hexamer primers to ensure that the cDNA levels were reliably representing the mRNA levels. Around 600 ng of RNA was incubated with iScript reaction mix, iScript reverse transcriptase and nuclease free water at 42°C for 30 min, in a 60 µl reaction volume.

As required under MIQE-precise expression stability of the reference genes was evaluated and a combination of ribosomal protein S5 (RPS5), β -glucuronidase (GUSB), and tyrosine 3-monooxygenase/tryptophan 5-momooxygenase activation protein zeta isoform (YWHAZ) was optimal.[38] Primer sequences for specific sequence-confirmed amplicons and optimal annealing temperature are described in Table 1. The qRT-PCRs were performed in duplicate using the Bio-Rad detection system.

name	primer 5'-->3'	annealing Temp (^{0}C)	amplicon size (bp)
FAS	F: GAAATCGGCAAATTCGACCT	65	115
	R: CTGTTCCCACCTTCATCCA		
PPARγ	F: TGTGACCTTAACTGTCGTATCC	66-67	134
	R: CTTCTCTTTCTCCGCCTGTG		
SREBF	F: CGTTTCTTCGTGGATGGG R:ACAAATTCAGTGCTGCTC	63	140
PPARα	F: GACAAATGTGACCGTAGCTG	60	109
	R: AAACGAATTGCGTTATGGGA		
RPS5	F: CAGGTCTTGGTGAATGCG	58	129
	R: CCAGATGGCCTGATTCAC		
GUSB	F: TGACATCACCATCAGCACCAGC	67	114
	R: GCCTTCCTCATCCAGAAGACGC		
YWHAZ	F:GAAGAGTCCTACAAAGACAGCACGC	65	115
	R: AATTTTCCCCTCCTTCTCCTGC		

Table 1: Primers for *FAS, PPAR-γ, SREBF and PPAR-α*. Sequences based on Kruitwagen et al. and Penning et al.[50,51]
FAS: fatty acid synthetase; *PPAR-γ*: peroxisome proliferator-activated receptor-*γ, SREBF:* sterol regulatory element-binding factor; *PPAR-α:* peroxisome proliferator-activated receptor-*α*; *RPS5*: ribosomal protein S5; *GUSB*: β -glucuronidase; *YWHAZ*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

Amplifications were carried out in a volume of 25 µl containing 12.5 µl of 2xSYBR green supermix (BioRad), 0.4 µM of forward and reverse primer and 1 µl cDNA and milliQ water. Cycling conditions included: denaturation at 95 °C for 3 minutes, followed by 45 cycles of denaturation (95 °C for 10 s), and amplicon-specific annealing temperature for 30 s. A melt curve analysis was performed for every reaction to verify amplicon specificity. IQ5 Real-Time PCR detection system software (BioRad) was used for data analysis. A no template control was also run in duplicate with each plate as a negative control. Expression levels were calculated taken the PCR efficiency into account, the PCR efficiency was always between 96-106%.

Data are reported as median (range). The Mann–Whitney U-Test was used to determine significance for qRT-PCR results between groups with statistical significance being set at $p < 0.05$.

Results

Animals

The FHL group consisted of six client-owned cats (four male castrated, one male intact and one female sterilised) that fitted the inclusion criteria for the study. Three cats were European short hair, two Main Coon, one Norwegian forest cat and one Turkish Angora. Median age was 4 years and 4 months (range 2– 8.5 years) and median body weight was 4 kg (range 3-6.7 kg). Anorexia, lethargy and icterus were the major problems for which 5/6 cats were referred to the DCSCA. One cat was admitted for dyspnoea secondary to chronic traumatic diaphragmatic rupture and developed HL while in hospital.
The control group consisted of 6 castrated males and 2 intact males cats. They all were European short air cats. Median age was 4 years and 3 months (range 1.5– 7 years) and median body weight was 4.6 kg (range 3-6.3 kg).

Lipogenic gene expression

In liver tissue the *PPAR-α*, *PPAR-γ*, and *FAS* mRNA expression levels were not significantly different ($p > 0.12$; $p > 0.89$; $p > 0.5$, respectively) in the FHL group compared to the control group (Figure 1). *SREBF* gene expression was around 10-times down regulated in the FHL group compared to the control group ($p = 0.039$)(Figure 1).

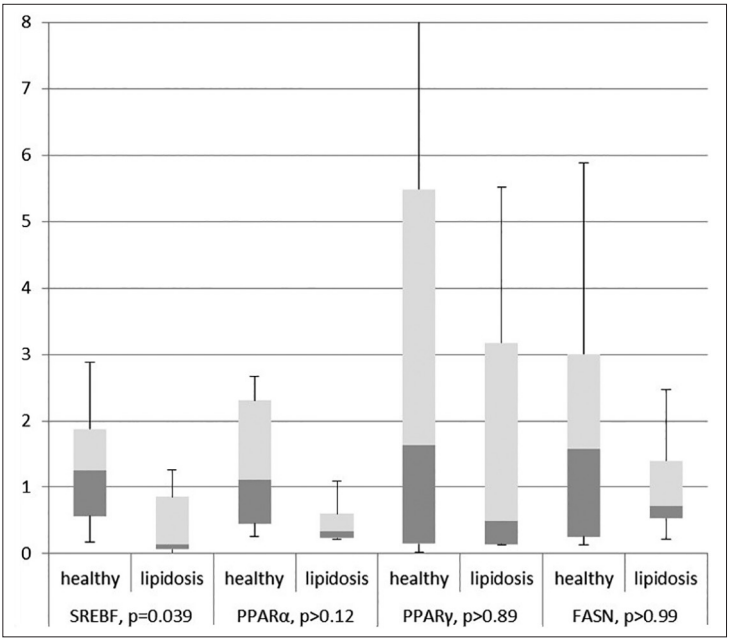

Figure 1: Presented are median, first quartile (box) and range (whiskers). The expression per gene is related to the average of the healthy control group which is set at "1". Expression corrected for three reference genes *RPS5*, *GUSB*, and *YWHAZ*. * Only the expression of SREBF is significantly downregulated in FHL samples compared to heathy controls.

FAS: fatty acid synthetase; *PPAR-γ*: peroxisome proliferator-activated receptor-*γ, SREBF:* sterol regulatory element-binding factor; *PPAR-α:* peroxisome proliferator-activated receptor-*α*; *RPS5*: ribosomal protein S5; *GUSB*: β -glucuronidase; *YWHAZ*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

Discussion

In humans in situations of metabolic dysregulation, *DNL* has been suggested to be abnormally increased and contributes to the pathogenesis of liver steatosis in diseases like obesity and NAFLD. [10,20-22] The increased *DNL* is characterised by the presence of FA palmitate, the primary product of *de novo* FA synthesis, and the MUFA palmitoleic acid synthesized from palmitate by the action of the enzyme Stearoyl-CoA desaturase-1 (*SCD-1*).[9,10]

In cats with FHL a higher concentration of palmitate and MUFA were found both in the visceral adipose tissue and liver, when compared to healthy cats.[35] This finding was suggestive for an increased *DNL* in visceral adipose tissue and most likely also in the liver as part of the pathophysiologic mechanism of liver steatosis, contributing and worsening FAs liver overload.[35]

In this study the decrease in liver *SREBF* gene expression and no expression change in other *DNL*-related enzymes suggest that *DNL* does not occur in the liver of cats with FHL. This is in contrast with what has been reported in humans with NAFLD and in other obligated carnivores.[13,18-21] Furthermore, the expression of *PPAR-α* was similar to its expression in healthy cats. This suggests that CPT-1a expression and the following degree of β-oxidation in the liver of cats with HL is not affected by a downregulation of *PPAR-α*.

De novo fatty acid synthesis in the liver is regulated by three important nuclear transcription factors: *SREBP-1c*, carbohydrate response element-binding protein (*ChREBP*)[7,39], and *PPAR-γ*.[7,40-42] Sterol regulatory element-binding transcription factor 1 regulates and induces the expression of several genes involved in lipogenesis and lipid biosynthesis such as *FAS*, *SCD-1* and acetyl-CoA carboxylase (*ACC*).[8,28,30,43,44] Fatty acid synthase is involved in conversion of malonyl-CoA into 16-carbon saturated FA palmitate.[26] Peroxisome proliferator-activated receptor-*γ* is required for normal adipocyte differentiation and is expressed at very low levels in the liver of healthy individuals. Overexpression of PPAR-*γ* in the liver leads to hepatic steatosis, also by enhancing the expression of other adipogenic genes in the liver.[32,42,45,46]

The physiological significance of *DNL* in NAFLD has been recently reviewed by Hodson and Frayn. The authors argued that the most important role of *DNL* might not be its quantitative contribution to hepatic FA overload, but its part in the suppression of FA oxidation.[27] Malonyl-CoA, a key a metabolic intermediate in *de novo* fatty acid biosynthesis in lipogenic tissues, is in fact a potent inhibitor of the mitochondrial outer membrane enzyme *CPT1*.[47] Carnitine-palmitoyl transferase 1 is responsible for the transport of fatty acyl-CoA into the mitochondrion, a fundamental step in FA oxidation. [48] The increased expression of *SREBP*-1c in NAFLD suppresses PPAR-α, further inhibiting *CPT-1* and FA β-oxidation.[31]

Using labelled FAs Donnelly et al. (2005) concluded that 59% of TGs in the livers of patients with NAFLD were from increased lipolysis in the adipose tissue and increased FA flux to the liver, 26% from hepatic *DNL* and 15% from the diet.[25] Hepatic *DNL* contributes significantly to FA accumulation in the liver of subject with NAFLD.

The development of *HL* has been investigated in the American Mink, as model of NAFLD.[12,13] In the American Mink food deprivation for 3 days was adequate to induce HL, with liver fat percentage increasing twice in the fasted group compared to the control group. The liver steatosis in the initial days of fasting, was secondary to the flux of FAs from extensive peripheral lipolysis from the visceral adipose tissue.[12,13] In the following days, the percentage of liver lipid accumulation

increased dramatically to the end of the 7-day fasting period. Significant increases were observed in the levels of mRNA encoding for *ACC-1* and *FAS* after 5–7 days of fasting compared to non-fasted mink. It was concluded that hepatic *DNL* could have further exacerbated liver fat accumulation in fasted Minks and that 5–7 days of fasting appeared to be the critical time period for this to develop. Although *SREBP-1c* was not measured in this study, the higher expression of *FAS* and *ACC,* and the higher hepatic palmitate and MUFAs concentrations suggested that the gene expression of the key enzymes for *DNL* was increased. Feeding the minks for 28 days after the end of the fasting period, allowed the relative expression of *ACC* and *FAS* to return to pre-fasting levels.[13]

In a model of diet-induced overweight in cats lipogenic gene expression (*SREBP-1c, FAS* and *ATP citrate lyase* (*ACL*)) in lipid sensitive tissue, *i.e.* liver, subcutaneous and abdominal adipose tissue, has been evaluated and compared to lean animals.[49] As already demonstrated in man, a positive energy imbalance due to over-nutrition and obesity can activate *SREBP-1c* and other secondary lipogenic gene expressions, enhancing lipogenesis in adipose and liver tissues contributing to liver steatosis.[18,19,21] In the overweight cats, the mRNA expression of *SREBP-1c* and *ACL* were markedly decreased in liver tissue whereas *FAS* expression remained similar to that of control cats. Abdominal *SREBP-1c* mRNA expression was markedly lower in abdominal subcutaneous and higher in omental adipose tissues of overweight cats as compared to control cats. The conclusion of this study was that also in an overweight cat model *DNL* does not occur in the liver but it occurs in the visceral adipose tissue.[49] The conclusion of the study supports the finding of Hall et al. that the origin of the enhanced hepatic TGs in cats with FHL is the visceral adipose tissue and not the liver.

Our study has some limitations. Although the collection process of healthy liver tissue, i.e. needle biopsies, and of FHL livers, i.e. post-mortem wedge material, were different, there are no indications that the biopsy technique itself could have caused differences in relative mRNA expression levels.

In order to validate the qRT-PCR data, the reduced mRNA level need to be confirmed by quantitative measurement at the protein level. This confirmation step is currently hampered by the very low hepatic expression of *SREBF-1* together with the lack of validated antibodies for *SREBF-1* in feline tissues. Interestingly, the *SREBF-1* downregulation as observed here *in vivo* is similar to the downregulation as observed in 3D feline liver organoids cultured *in vitro* under FA-overload, lending credence to robustness of the qRT-PCR measurements. [50]
In conclusion, the result of our study with the downregulation of *SREBF* in liver

tissue of cats with FHL does not support the hypothesis that *DNL* in the liver is an important pathway of FAs accumulation in FHL. As cats with HL have an increased concentration of palmitate and MUFAs compared to healthy cats in their liver and visceral adipose tissue, *DNL* in FHL most likely occurs in the abdominal adipose tissue. Subsequently, the *de novo* synthetized MUFAs are transported from the adipose tissue to the liver, where they contribute to fat overload in FHL.

Acknowledgements
CV and LCP wrote the body of the paper, JHR and RPF critically reviewed the manuscript.

Conflict of interest
The authors declared no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

Funding
LCP received a grant from The Netherlands Organization for Health Research and Development (NWO Zon/MW number 16004121) for liver progenitor research.

References

1. Armstrong PJ, Blanchard G. Hepatic lipidosis in cats. *Vet Clin North Am Small Anim Pract*. 2009;39(3):599-616. doi: 10.1016/j.cvsm.2009.03.003 [doi].

2. Center SA, Guida L, Zanelli MJ, Dougherty E, Cummings J, King J. Ultrastructural hepatocellular features associated with severe hepatic lipidosis in cats. *Am J Vet Res*. 1993;54(5):724-731.

3. Center SA. Feline hepatic lipidosis. *Vet Clin North Am Small Anim Pract*. 2005;35(1):225-269. doi: S0195-5616(04)00142-1 [pii].

4. Valtolina C, Favier RP. Feline hepatic lipidosis. *Vet Clin North Am Small Anim Pract*. 2017;47(3):683-702. doi: S0195-5616(16)30160-7 [pii].

5. Verbrugghe A, Bakovic M. Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis. *Nutrients*. 2013;5(7):2811-2835. doi: 10.3390/nu5072811 [doi].

6. Jiang ZG, Robson SC, Yao Z. Lipoprotein metabolism in nonalcoholic fatty liver disease. *J Biomed Res*. 2013;27(1):1-13. doi: 10.7555/JBR.27.20120077 [doi].

7. Reddy JK, Rao MS. Lipid metabolism and liver inflammation. II. fatty liver disease and fatty acid oxidation. *Am J Physiol Gastrointest Liver Physiol*. 2006;290(5):G852-8. doi: 290/5/G852 [pii].

8. Ameer F, Scandiuzzi L, Hasnain S, Kalbacher H, Zaidi N. De novo lipogenesis in health and disease. *Metabolism*. 2014;63(7):895-902. doi: 10.1016/j.metabol.2014.04.003 [doi].

9. Lee JJ, Lambert JE, Hovhannisyan Y, et al. Palmitoleic acid is elevated in fatty liver disease and reflects hepatic lipogenesis. *Am J Clin Nutr*. 2015;101(1):34-43. doi: 10.3945/ajcn.114.092262 [doi].
10. Lambert JE, Ramos-Roman MA, Browning JD, Parks EJ. Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology*. 2014;146(3):726-735. doi: 10.1053/j.gastro.2013.11.049 [doi].

11. Aroch I, Shechter-Polak M, Segev G. A retrospective study of serum beta-hydroxybutyric acid in 215 ill cats: Clinical signs, laboratory findings and diagnoses. *Vet J*. 2012;191(2):240-245. doi: 10.1016/j.tvjl.2011.01.010 [doi].

12. Rouvinen-Watt K, Mustonen AM, Conway R, et al. Rapid development of fasting-induced hepatic lipidosis in the american mink (neovison vison): Effects of food deprivation and re-alimentation on body fat depots, tissue fatty acid profiles, hematology and endocrinology. *Lipids*. 2010;45(2):111-128. doi: 10.1007/s11745-009-3377-4 [doi].

13. Rouvinen-Watt K, Harris L, Dick M, et al. Role of hepatic de novo lipogenesis in the development of fasting-induced fatty liver in the american mink (neovison vison). *Br J Nutr*. 2012;108(8):1360-1370. doi: 10.1017/S0007114511006775 [doi].

14. Richard MJ, Holck JT, Beitz DC. Lipogenesis in liver and adipose tissue of the domestic cat (felis domestica). *Comp Biochem Physiol B*. 1989;93(3):561-564.

15. Campos CF, Duarte MS, Guimaraes SE, et al. Review: Animal model and the current understanding of molecule dynamics of adipogenesis. *Animal*. 2016;10(6):927-932. doi: 10.1017/S1751731115002992 [doi].

16. Bergen WG, Mersmann HJ. Comparative aspects of lipid metabolism: Impact on contemporary research and use of animal models. *J Nutr*. 2005;135(11):2499-2502. doi: 135/11/2499 [pii].

17. Bjorntorp P, Sjostrom L. Carbohydrate storage in man: Speculations and some quantitative considerations. *Metabolism*. 1978;27(12 Suppl 2):1853-1865. doi: 0026-0495(78)90242-1 [pii].

18. Strable MS, Ntambi JM. Genetic control of de novo lipogenesis: Role in diet-induced obesity. *Crit Rev Biochem Mol Biol*. 2010;45(3):199-214. doi: 10.3109/10409231003667500 [doi].

19. Byrne CD. Fatty liver: Role of inflammation and fatty acid nutrition. *Prostaglandins Leukot Essent Fatty Acids*. 2010;82(4-6):265-271. doi: 10.1016/j.plefa.2010.02.012 [doi].

20. Choudhury J, Sanyal AJ. Insulin resistance and the pathogenesis of nonalcoholic fatty liver disease. *Clin Liver Dis*. 2004;8(3):575-94, ix. doi: 10.1016/j.cld.2004.04.006 [doi].

21. Diraison F, Dusserre E, Vidal H, Sothier M, Beylot M. Increased hepatic lipogenesis but decreased expression of lipogenic gene in adipose tissue in human obesity. *Am J Physiol Endocrinol Metab*. 2002;282(1):E46-51. doi: 10.1152/ajpendo.2002.282.1.E46 [doi].

22. Rector RS, Thyfault JP, Wei Y, Ibdah JA. Non-alcoholic fatty liver disease and the metabolic syndrome: An update. *World J Gastroenterol*. 2008;14(2):185-192.
23. Pardina E, Baena-Fustegueras JA, Llamas R, et al. Lipoprotein lipase expression in livers of morbidly obese patients could be responsible for liver steatosis. *Obes Surg*. 2009;19(5):608-616. doi: 10.1007/s11695-009-9827-5 [doi].

24. Westerbacka J, Kolak M, Kiviluoto T, et al. Genes involved in fatty acid partitioning and binding, lipolysis, monocyte/macrophage recruitment, and inflammation are overexpressed in the human fatty liver of insulin-resistant subjects. *Diabetes*. 2007;56(11):2759-2765. doi: db07-0156 [pii].

25. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest*. 2005;115(5):1343-1351. doi: 10.1172/JCI23621 [doi].

26. Browning JD, Horton JD. Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest*. 2004;114(2):147-152. doi: 10.1172/JCI22422 [doi].

27. Hodson L, Frayn KN. Hepatic fatty acid partitioning. *Curr Opin Lipidol*. 2011;22(3):216-224. doi: 10.1097/MOL.0b013e3283462e16 [doi].

28. Horton JD, Goldstein JL, Brown MS. SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest*. 2002;109(9):1125-1131. doi: 10.1172/JCI15593 [doi].

29. Horton JD, Goldstein JL, Brown MS. SREBPs: Transcriptional mediators of lipid homeostasis. *Cold Spring Harb Symp Quant Biol*. 2002;67:491-498.

30. Horton JD. Sterol regulatory element-binding proteins: Transcriptional activators of lipid synthesis. *Biochem Soc Trans*. 2002;30(Pt 6):1091-1095. doi: 10.1042/ [doi].

31. Pettinelli P, Del Pozo T, Araya J, et al. Enhancement in liver SREBP-1c/PPAR-alpha ratio and steatosis in obese patients: Correlations with insulin resistance and n-3 long-chain polyunsaturated fatty acid depletion. *Biochim Biophys Acta*. 2009;1792(11):1080-1086. doi: 10.1016/j.bbadis.2009.08.015 [doi].

32. Pettinelli P, Videla LA. Up-regulation of PPAR-gamma mRNA expression in the liver of obese patients: An additional reinforcing lipogenic mechanism to SREBP-1c induction. *J Clin Endocrinol Metab*. 2011;96(5):1424-1430. doi: 10.1210/jc.2010-2129 [doi].

33. Yang ZX, Shen W, Sun H. Effects of nuclear receptor FXR on the regulation of liver lipid metabolism in patients with non-alcoholic fatty liver disease. *Hepatol Int*. 2010;4(4):741-748. doi: 10.1007/s12072-010-9202-6 [doi].

34. Eisert R. Hypercarnivory and the brain: Protein requirements of cats reconsidered. *J Comp Physiol B*. 2011;181(1):1-17. doi: 10.1007/s00360-010-0528-0 [doi].

35. Hall JA, Barstad LA, Connor WE. Lipid composition of hepatic and adipose tissues from normal cats and from cats with idiopathic hepatic lipidosis. *J Vet Intern Med*. 1997;11(4):238-242.

36. Chan DL, Freeman LM. Nutrition in critical illness. *Vet Clin North Am Small Anim Pract*. 2006;36(6):1225-41, v-vi. doi: S0195-5616(06)00100-8 [pii].

37. Corbee RJ, Tryfonidou MA, Grinwis GC, et al. Skeletal and hepatic changes induced by chronic vitamin A supplementation in cats. *Vet J*. 2014;202(3):503-509. doi: 10.1016/j.tvjl.2014.09.029 [doi].

38. Bustin S, Penning LC. Improving the analysis of quantitative PCR data in veterinary research. *Vet J*. 2012;191(3):279-281. doi: 10.1016/j.tvjl.2011.06.044 [doi].

39. Hashimoto T, Cook WS, Qi C, Yeldandi AV, Reddy JK, Rao MS. Defect in peroxisome proliferator-activated receptor alpha-inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting. *J Biol Chem*. 2000;275(37):28918-28928. doi: 10.1074/jbc.M910350199 [doi].

40. Bugianesi E, Leone N, Vanni E, et al. Expanding the natural history of nonalcoholic steatohepatitis: From cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology*. 2002;123(1):134-140. doi: S0016508502000732 [pii].

41. Evans RM, Barish GD, Wang YX. PPARs and the complex journey to obesity. *Nat Med*. 2004;10(4):355-361. doi: 10.1038/nm1025 [doi].

42. Matsusue K, Haluzik M, Lambert G, et al. Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. *J Clin Invest*. 2003;111(5):737-747. doi: 10.1172/JCI17223 [doi].

43. Li X, Li Y, Yang W, et al. SREBP-1c overexpression induces triglycerides accumulation through increasing lipid synthesis and decreasing lipid oxidation and VLDL assembly in bovine hepatocytes. *J Steroid Biochem Mol Biol*. 2014;143:174-182. doi: 10.1016/j.jsbmb.2014.02.009 [doi].

44. Xiong X, Wang X, Lu Y, et al. Hepatic steatosis exacerbated by endoplasmic reticulum stress-mediated downregulation of FXR in aging mice. *J Hepatol*. 2014;60(4):847-854. doi: 10.1016/j.jhep.2013.12.003 [doi].

45. Yu S, Matsusue K, Kashireddy P, et al. Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor gamma1 (PPARgamma1) overexpression. *J Biol Chem*. 2003;278(1):498-505. doi: 10.1074/jbc.M210062200 [doi].

46. Boelsterli UA, Bedoucha M. Toxicological consequences of altered peroxisome proliferator-activated receptor gamma (PPARgamma) expression in the liver: Insights from models of obesity and type 2 diabetes. *Biochem Pharmacol*. 2002;63(1):1-10. doi: S0006295201008176 [pii].

47. Saggerson D. Malonyl-CoA, a key signaling molecule in mammalian cells. *Annu Rev Nutr*. 2008;28:253-272. doi: 10.1146/annurev.nutr.28.061807.155434 [doi].

48. Reddy JK, Hashimoto T. Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: An adaptive metabolic system. *Annu Rev Nutr*. 2001;21:193-230. doi: 10.1146/annurev.nutr.21.1.193 [doi].

49. Lee P, Mori A, Takemitsu H, Yamamoto I, Arai T. Lipogenic gene expression in abdominal adipose and liver tissues of diet-induced overweight cats. *Vet J*. 2011;190(2):e150-3. doi: 10.1016/j.tvjl.2011.04.003 [doi].

50. Kruitwagen HS, Oosterhoff LA, Vernooij IGWH, et al. Long-term adult feline liver organoid cultures for disease modeling of hepatic steatosis. *Stem Cell Reports*. 2017;8(4):822-830. doi: S2213-6711(17)30082-6 [pii].

51. Penning LC, Vrieling HE, Brinkhof B, et al. A validation of 10 feline reference genes for gene expression measurements in snap-frozen tissues. *Vet Immunol Immunopathol*. 2007;120(3-4):212-222. doi: S0165-2427(07)00272-3 [pii].

Chapter 6

An immunohistochemical characterisation of the hepatic stem cell niche in feline hepatic lipidosis: a preliminary morphological study

C. Valtolina[a], J.H. Robben[a], R.P. Favier[a,c], J. Rothuizen[a], G.C.M. Grinwis[b], B.A. Schotanus[a,d], L.C. Penning[a]

J Feline Med Surg 2019 Feb;21(2)165-172

[a.] *Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 108, 3584 CM, Utrecht, the Netherlands*

[b.] *Department of Pathobiology, Faculty of Veterinary Medicine and Veterinary Pathology Diagnostic Centre, Utrecht University, Yalelaan 1, 3584 CM, Utrecht, the Netherlands*

[c.] *Present address: Evidensia Dierenziekenhuis Nunspeet, Nunspeet, the Netherlands*

[d.] *Present address: Intercept Pharmaceuticals, Gouda, the Netherlands*

Abstract

Objective

To describe the cellular and stromal components of the hepatic progenitor cell niche in feline hepatic lipidosis (FHL).

Methods

Immunohistochemical staining for the progenitor/bile duct marker (K19), activated Kupffer cells (MAC387), myofibroblasts (α-SMA), and the extracellular matrix component laminin were used on 7 liver biopsies of cats with FHL and 3 healthy cats. Double immunofluorescence stainings were performed to investigate colocalization of different cell types in the hepatic progenitor cells (HPC) niche.

Results

HPCs, Kupffer cells, myofibroblasts and laminin deposition were observed in the liver samples of FHL although with variability in the expression and positivity of the different immunostainings between different samples. When compared to the unaffected cats where K19 positivity and minimal aSMA and laminin positivity was seen mainly in the portal area, in the majority of FHL samples K19 and aSMA positive cells and laminin positivity were seen also in the periportal and parenchymatous area. MAC387 positive cells were present throught the parenchyma.

Conclusions and relevance

This is a preliminary morphological study to describe the activation and colocalization of components of the HPC niche in FHL. Although the HPC niche in FHL resembles that described in hepatopathies in dogs and in feline lymphocytic cholangitis, the expression of K19, α-SMA, MAC and lamin is more variable in FHL and a common pattern of activation could not be established. Nevertheless, when HPC were activated, a spatial association between HPCs and its niche could be demonstrated.

Keywords

Feline, feline hepatic lipidosis, hepatic stem cell niche, immunohistochemistry, immunofluorescence

Introduction

Feline hepatic lipidosis (FHL) is considered the most common hepatobiliary disease in cats. [1-3] Clinical signs include lethargy, anorexia, weight loss, icterus and often vomiting and constipation. Histologically it is characterized by the excessive accumulation of triglycerides in more than 60% of the hepatocytes resulting in secondary impairment of liver function and intrahepatic cholestasis.[2,4] The histological feature of FHL is that of simple steatosis and current literature has suggested that the disease lacks necroinflammatory lesions and fibrosis.[4,5] Feline hepatic lipidosis often presents as an acute critical syndrome that can result in the animal's death if therapeutic measures are not rapidly implemented. Its progression into steatohepatitis and chronic liver failure has not been documented in cats.[1,2]
Mature liver cells, *i.e.* hepatocytes and cholangiocytes, have a remarkable capacity to proliferate and restore liver function in homeostasis and in response to injury.[6-8] Three decades ago "the streaming liver hypothesis" was proposed based on pulse-chace experiments: periportally derived hepatocytes migrate along the central vein to the hepatic vein axis.[9] Recent cell-tracing experiments demonstrated a population of proliferating and self-renewing cells adjacent to the central vein in the liver lobule. These cells seem to be able to replace all hepatocytes in the liver lobule during homeostatic renewa.l[10] These experiments helped the understanding of liver regeneration and the importance of specific hepatic stem- or progenitor cells (HPCs) as recently reviewed.[11,12]

Irrespective of the origin of hepatic progenitor cells, once the ability of hepatocytes to restore liver mass and function is exhausted or impaired, HPCs become activated as described for several species.[8,13-18] These quiescent HPCs reside in the smallest branches of the biliary tract, also known as the canal of Hering, and are recognized by their morphological appearance, portal location, and expression of specific markers, such as cytokeratin 7 (K7) and K19.[6,14,19-21] Once activated, HPCs proliferate and their expansion results in the formation of histological structures known as ductular reactions, also known as bile duct proliferation[14]. There are few descriptions of the HPCs in cats. [15,22,23] The first study that reported on progenitors cells in healthy cats was the study from IJzer et al. in 2009.[15] Those progenitor cells were described as small periportally located cells positive for K7, with a morphology and location identical to resident HPCs located in the canal of Hering as described in humans and dogs.[14,15,24] The same study identified the presence of ductular

structures (ductular reaction) in different types of feline liver disease (acute hepatitis, neutrophilic and lymphocityc cholangitis and lipidosis) and characterised their histological and phenotypical features similarly to what has been described in humans and dogs[8,13,15-18,23-28]. However, the simple description of the ductular reaction does not further implicate the activation and co-localization of the HPCs and their niche.[29]

The balance between HPC self-renewal, proliferation and differentiation is determined by the interaction between the cells and stroma present in the microenvironment, *i.e.* the "HPC niche".[30-32] In rodent models, men, and dog important cellular components in this niche include HPCs, hepatic stellate cells (HSCs), their differentiated counterparts, αSMA positive myofibroblasts, and macrophages/Kupffer cells with laminin as the main stromal component.[6,7,19,24,33] Laminin is important for HPC proliferation and maintenance of the progenitor phenotype. Once the HPCs have left the laminin-enveloped niche, they can differentiate into a hepatocyte phenotype.[34]

In humans and dogs the ductular reaction and the degree of HPC and its niche activation is directly related to the severity of disease[17,35,36] indicated by the amount of hepatocyte loss, the amount of inflammation, and the extend of fibrosis and it is seen in disease like acute hepatitis, chronic hepatitis, bliary disease and hepatic tumour.[13,17,24,27,28,36-41]
In a recent publication, the remodeling and activation of the HPC and its niche was described in feline lymphocytic cholangitis.[42] The characteristics of the HPC and its niche shares similarity to the HPC and its niche in sclerosing cholangitis in human medicine.

In human and rodent models of non-alcoholic fatty liver disease (NAFLD) steatosis is sufficient to inhibit replication of mature hepatocytes and to trigger the activation of HPCs.[17,18] There is growing evidence that steatosis per se is associated with the development of steatohepatitis and fibrosis and should not be considered just an innocent bystander in liver disease.[43-45]
To our knowledge there is no literature available for cats on the changes of the HPC and its niche in FHL. Therefore, the aim of this study was to describe the cellular and stromal pattern of the HPC niche in FHL, with emphasis on MAC-positive Kupffer cells, αSMA-positive myofibroblasts and the important extracellular matrix component laminin. To substantiate the immunohistochemical data we further used double immunofluorescence to investigate possible co-localizations of the various cell types within the HPC niche.

Materials and methods

Samples of diseased livers were obtained from cats submitted for routine *post-mortem* investigation. Normal liver from healthy cats were obtained from fresh cadavers of non-liver related studies as required under the University 3R-policy. Projects have been approved by the Animal Welfare Committee on Experimental Animal Use, as required by Dutch legislation.

Seven liver specimens (formalin-fixed and paraffin embedded) with a histological diagnosis of FHL were selected from the archives of the Department of Pathobiology of the Faculty of Veterinary Medicine, Utrecht University, the Netherlands. The seven FHL samples demonstrated a severe degree of lipidosis (> 70% of hepatocytes involved), based on histological evaluation performed by a board-certified veterinary pathologist (GCMG). In one sample extensive lipidosis coincided with a histological diagnosis of per-acute hepatitis. No other liver co-morbidities were present in the six other FHL samples. Diagnoses were based on criteria as reported previously by the World Small Animal Veterinary Association (WSAVA).[46]

Tissue samples from three healthy cats were taken *post-mortem* and processed similar to the FHL samples. Sections (3 µm) of all samples were cut for haematoxylin and eosin (H&E) and immunohistochemical and immunofluorescence staining. The samples were confirmed histologically normal by the same board-certified veterinary pathologist (GCMG).

Immunohistochemistry

All FHL and control samples were examined immunohistochemically for the expression of K19 (keratin-19; marker of bile duct and progenitor cells)[27], MAC387 (myelomonocytic antigen; marker of infiltrated and resident macrophages)[22,47], αSMA (smooth muscle actin antibody; marker of activated hepatic stellate cells/myofibroblasts)[27,34], laminin (extracellular matrix component and part of the HPC niche)[34], and Ki67 (proliferation marker)[23] to characterise the HPC niche in cats (Table 1).

The immunohistochemical procedure was performed as described before.[23,48] Briefly, sections were deparaffinised and rehydrated in xylene and a graded alcohol series, respectively. After antigen retrieval, endogenous peroxidase was inhibited with a ready-to-use peroxidase block (Dako, Glostrup, Denmark). Sections were blocked with 10% normal goat serum and the primary antibody was diluted in antibody diluent (Dako,) and incubated one hour at room temperature or overnight at 4°C depending on the marker (Table 1). EnVision HRP-labelled secondary antibodies (anti-rabbit or anti-mouse; Dako) were used and 3,3'-diaminobenzidine was used as the substrate for visualization. Sections were counterstained with haematoxylin.

Marker*	Source	IgG-Type	Clone	Antigen Retrieval	Incubation	Company	Product Code	Dilution
K19	Mouse	IgG1	b170	Prot. K	O/N 4°C	Novocastra	NCL-CK19	1:100
MAC387	Mouse	IgG1	MAC387	Prot. K	O/N 4°C	Abcam	ab22506	1:1000
αSMA	Mouse	IgG2a	1A4	None	60 min RT	BioGenex	MU128-UC	1:200
LAM	Rabbit	Ig		Prot. K	O/N 4°C	Abcam	ab11575	1:100
Ki67	Mouse	IgG1	MIB1	Citrate pH 6.0	O/N 4°C	Dako		1:30
panCK	Rabbit	Polyclonal		P.K/citr.	O/N 4°C	Dako	Z0622	1:400

Table 1. Antibodies and processing characteristics as applied in the immunohistochemistry and double immunofluorescence of liver samples of cats with hepatic lipidosis.
*K19: keratin-19; MAC387: anti-macrophage antibody 387; αSMA: alpha-smooth muscle actin; LAM: laminin; Ki67: proliferation marker; panCK: pancytokeratin, widespectrum cytokeratin marker. Mouse derived antobdoies are monoclonal, whereas the rabbit is polyclonal.

As a negative control, the primary antibody was omitted and the IgG1 isotypes served as internal non-specific controls for each other. For washing steps PBS/Tween 0.1% was used.

The stainings for K19, Ki67, αSMA and laminin were scored by one operator at high (40x) magnification. Five random fields were observed for positively stained hepatocytes and myofibroblasts and the number of positive cells/fields were reported. Only the cells which displayed nuclei on the section were considered. A distinction was made within initial scoring for portal, periportal and parenchymal cell positivity for K19 and αSMA and laminin. To perform a cell count for MAC387, ImageJ was used, a java-based image processing program from the National Institutes of Health. Images of representative slide were made using an Olympus ColorviewIIIu (Olympus) digital camera and an Olympus BX41 microscope. Cells that stained positive to MAC387 were reported as total cells per total area analyzed (mm^2) for each sample.

Double immunofluorescence staining

To evaluate the spatial relationship between macrophages, activated HSCs, laminin, and HPCs double immunofluorescence staining was performed on 3 samples of cats with FHL showing varying K19-positivity. The decision not to include double immunofluorescence on healthy samples was related to the fact that the positivity to α-SMA and laminin in healthy sample was almost negligible and mainly seen around the portal area (bile ducts/blood vessel), also as described in previously performed studies.[15,29]

Staining was performed using the aforementioned markers for macrophages (MAC387), activated stellate cells (αSMA) and laminin. To detect HPCs, PanCK, a wide spectrum cytokeratin and well validated HPC marker, was used (Table 1).[49] Immunofluorescence staining was performed as described for the immunohistochemical procedure. Sections were incubated with the primary antibodies in a parallel approach overnight at 4°C. The secondary antibodies (goat-anti-mouse AlexaFluor-488 and goat-anti-rabbit AlexaFluor 568; Invitrogen, Etten-Leur, the Netherlands) were applied 1:100, diluted in antibody diluent (Dako). Sections were counterstained using 4',6-diamidino-2-phenylindole (DAPI) 1:2000 for 10 min. at room temperature and mounted using Fluorsave. In the negative controls the first antibody was omitted and replaced by an aspecific isotype control. Slides were analysed with a Leica DMRE fluorescent microscope with Photometrics Coolsnap CCD digital photo camera and CellB software (AnalySIS, Olympus, Zoeterwoude, the Netherlands).

Results

Immunohistochemistry

The results of the immunohistochemical staining for normal liver and FHL samples are summarized in table 2, 3 and figure 1 depicts examples of each staining. Negative controls remained unstained, indicating a specific signal for each antibody (data not shown). Samples were considered for the study when at least five portal areas were evaluated.

K19

In unaffected livers, K19, indicating bile duct and/or progenitor cells, was present in bile ducts and in a variable number of small epithelial cells located in the periportal area (Table 2 and Figure 1) confirming the validity of the antiK19 antibody. No ductular reactions were observed. Of the 7 cats with lipidosis 4 showed an increase in the number of K19-positive cells in the (peri)portal area and clusters of cells in the parenchymal area (Table 2 and Figure 1). In three of seven cases an increased number of cells positive to K19 staining were seen in the (peri)portal areas and a positive staining for parenchymal ductular reactions was observed in one case.

αSMA

Alpha-Smooth Muscle Actin positive staining, indicating the presence of HSCs and differentiated smooth muscle cells. In healthy livers very few cells stained positive and mainly in the portal area, around bile ducts and in smooth muscle of

Sample		Portal	Periportal	Parenchyma	Relative Size (mm2)
F1	K19	1	2	2	0.04479
	aSMA	2	2	3	
F2	K19	1	2	2	0.04576
	aSMA	3	2	2	
F3	K19	1	1	2	0.05568
	aSMA	2	4	4	
F4	K19	2	3	3	0.05557
	aSMA	1	3	4	
F5	K19	2	2	1	0.05521
	aSMA	2	2	2	
F6	K19	4	3	4	0.05336
	aSMA	4	4	4	
F7	K19	2	2	1	0.05562
	aSMA	2	4	4	
C1	K19	3	1	1	0.03165
	aSMA	1	0	0	
C2	K19	2	0	1	0.02451
	aSMA	1	0	0	
C3	K19	1	1	0	0.02779
	aSMA	1	1	0	

Table 2: K19 and aSMA positive cells and their distribution per portal area in FHL and in unaffected liver samples . Cells were evaluated at high magnification (x40) and their number are reported as cells/fields. # K19: keratin-19 (marker of bile duct and progenitor cells); αSMA (alpha-smooth muscle actin; marker of hepatic stellate cells/myofibroblasts)

blood vessels (Table 2, Figure 1). In four of seven FHL samples, αSMA expression was strongly increased in both the periportal area and throughout the parenchyma. In three of seven FHL samples, αSMA positive cells were increased both in the portal and periportal area, when compared to the unaffected samples.

MAC387

In healthy livers variable amounts of MAC387 positive cells were found spread throughout the parenchyma as single cells with no clear spatial relationship with the portal areas (Table 3, Figure 1). All FHL samples demonstrated also variable amounts of MAC387 positive cells . In these samples, the MAC387 positive cells formed clusters varying in number from 2-3 to 4-6 cells per cluster, mostly located in close proximity to the portal area.

Laminin

A weak laminin staining was observed in and around the portal areas in healthy livers (Table 3, Figure 1). Four out of five FHL samples showed an increase in laminin positivity in the periportal area, ceasing towards the central vein. The

central areas in all samples were negative for laminin. One FHL sample remained negative for the laminin staining.

Ki67

Ki67 positivity was minimal in the healthy liver. Three FHL samples showed a distinctive increase in Ki67 positve cells, whereas four remained negative (Table 3, Figure 1).

Sample	K167	MAC387	Laminin
F1	7	4063	Positive in the portal and periportal area
F2	5	3125	Positive in the portal area
F3	3	4022	Strong positivity in the portal area and mild periportal positivity
F4	15	2556	np
F5	1	3170	Mild positivity in the portal area
F6	32	42856	Strong positivity in the portal area and moderate parenchymal positivity
F7	3	5969	np
C1	3	10867	Minimal positivity in portal area
C2	5	4797	Minimal positivity in the portal area
C3	5	2986	Minimal positivity in portal area

Table 3: K167, MAC 387 positive cells and laminin positive staining in FHL and in unaffected liver samples. For Ki67 cells were evaluated at high magnification (x40) and their number are reported as cells/fields; MAC387 were evaluated with ImageJ and reported as number of total cells per total area (mm^2)
Ki67: proliferation marker MAC: anti-macrophage antibody 387 (marker of macrophages); LAM: laminin.
n.p.= not performed

Double immunofluorescence (IF) staining

Double immunofluorescence showed that αSMA and laminin expression were increased and in close proximity to the pancytokeratin/K19 (PanCK/K19)-positive cells. Laminin strictly co-localized with HPCs, while αSMA was also located at other histological sites. MAC387-positive clusters of cells were found adjacent to K19-positive cells. (Figure 2).

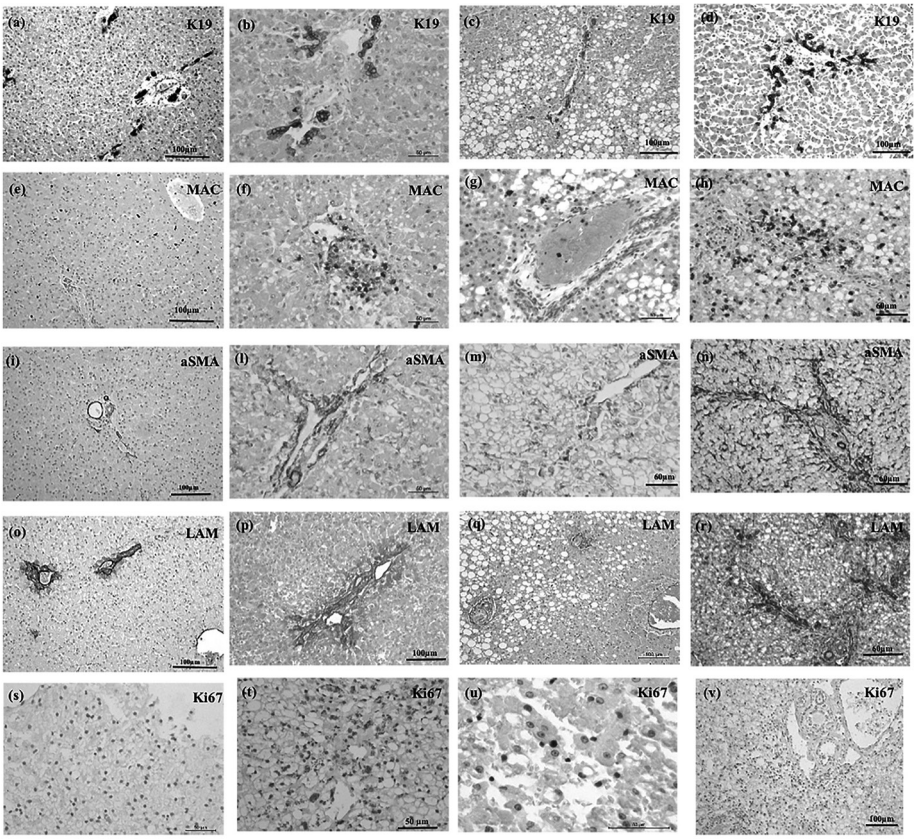

Figure 1. The activated feline HPC niche immunohistochemically stained for K19 (HPCs), MAC387 (macrophages), aSMA (stellate cells), LAM (laminin) and Ki67 (hepatocyte regeneration). The figure shows one unaffected cat C2 (a,e,i,o,s) and selected FHL cases with different marker expression F1(b,f,l,p,t); F5 (c,g,m,q,u); F6 (d,h,n,r,v) .

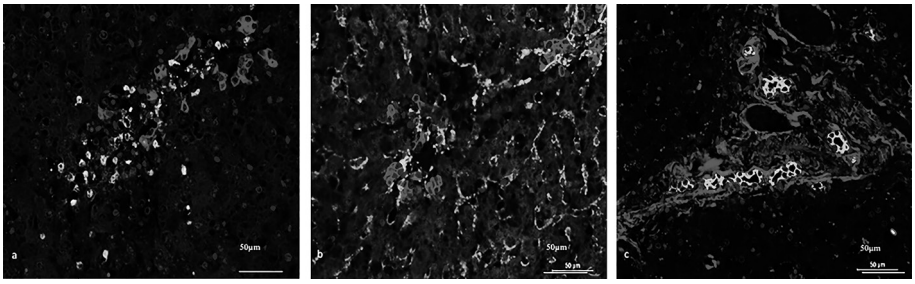

Figure 2. HPCs co-localized with macrophages, stellate cells and laminin in examples of feline hepatic lipidosis. Double immunofluorescent stained with PanCK (HPCs) and MAC (macrophages), aSMA (stellate cells) and LAM (laminin), respectively.

a) anCK/MAC: increase in macrophages and clustering of these cells in close proximity to the HPCs

b) PanCK/SMA: increase in aSMA positivity in parenchyma and around the HPCs

c) PanCK/Laminin: the laminin can be seen as sheets around the HPCs

Discussion

This study characterised the feline HPC niche by immunohistochemical and immunofluorescence staining in a common feline hepatic disease, FHL. The results of our study demonstrate that ductular reactions observed in the feline HPC niche in FHL contain Kupffer cells, activated HSCs (myofibroblasts) and the extracellular matrix component laminin. This is comparable with other hepatopathies in man and dogs, such as acute and chronic hepatitis, hepatobilary diseases and hepatic carcinoma.[13,15,16,24-27,32,48] It is also in line with the HPC activation in feline lymphocytic cholangitis, a chronic feline hepatobiliary disease.[42]

In human, rat and murine models of impaired hepatocyte replication in NAFLD, simple steatosis is enough to inhibit replication of mature hepatocytes and to trigger the activation of HPCs.[17,18] Due to elevated levels of serum-free fatty acids and insulin resistance, lipotoxicity is promoted, reactive oxygen species are formed and hepatic inflammation and hepatocyte apoptosis are induced.[18,43,44] This results in an inhibition of the replication capability of the hepatocytes and stimulation of the activation of the HPCs. Liver steatosis seems to affect the gravity and progression of more chronic liver disease.[43-45] Although a liver with steatosis is less protected against inflammation and fibrosis, only a small population of people affected by liver steatosis will further develop steatohepatitis and fibrosis.[43-45]

However, we were unable to demonstrate a definite pattern between the histopathological presence of lipidosis and the activation of the HPCs or the upregulation of the niche components in all cats with hepatic lipidosis. Different degrees of HPC activation in FHL were suggested by the variability in the expression and positivity of the different immunostainings in the different samples. Despite the presence of severe steatosis (> 70 % of hepatocytes affected), this might be explained by the fact that hepatic lipidosis in cats often has an acute onset and progression due to a negative energy balance resulting from a heterogeneous group of underlying diseases, which may have different secondary effects on the liver. In the cases with only minimal number of positive cells to the staining, the damage to the hepatocytes could have been minimal or not severe enough to induce hepatocytes apoptosis/death and secondary activation of HPCs.

Nevertheless, when HPC were activated, a spatial association between HPCs and its niche could be demonstrated. The result of the double IF in FHL cases with an enhanced number of progenitor cells, suggested that a relationship was present between the HPCs, Kupffer cells/macrophages and HSCs/myofibroblasts

Stellate cells/myofibroblasts are the main producers of extracellular matrix components in the liver and could be responsible for the enhanced laminin deposition.[34] Recently, it has been suggested that the activated HPCs produce their own laminin sheet to perpetuate their proliferation and maintain their undifferentiated phenotype.[34,49-51] The close anatomical relationship between HPCs and laminin suggests that this may also be true in cats. *In situ hybridization* for laminin mRNA can resolve this issue.

This is the first study to characterise the feline HPC niche, HPC activation, and their relation with FHL. The low number of FHL cases and the lack of longitudinal samples limits the power of the study. Staining differences may have been affected by variations in fixation and/or storage time. However, the quality control with omission of the first antibody resulted in negative staining for all antibodies that were used. Furthermore, the staining patterns were not identical despite the use similar IgG isotypes and the same secondary antibody in the stainings for K19, MAC387, and Ki67. This strongly argues against a-specific staining patterns.

We did not use liver samples collected during life, because histological biopsies are not routinely taken in cats suspected to have hepatic lipidosis.[1-3] High quality biopsies of sufficient size are therefore difficult to obtain.[52] Furthermore, liver diseases in cats are often associated with coagulation disturbances and hemorrhage is a potential complication.[1,3,5252] Especially serial histological liver biopsies for the evaluation of disease progression are difficult to obtain due to reservations made by veterinarians and the understandable reluctance by pet-owners. In addition to this, histological liver biopsies are also not necessary for a diagnosis of FHL.[3] Because of the absence of sequential sampling and the descriptive nature of the immunohistochemistry, this study does not provide dynamic information and limits conclusions on cause-effect relationships. Despite these restrictions, we were able to highlight some interesting aspects on the HPCs and their niche in FHL.

Conclusion

Based on this study the composition of the feline HPC niche in FHL and its observed cellular and stromal interactions resemble that of normal and diseased livers in other companion animals like dogs and in cats with lymphocutic cholangitis. However, the feline HPC niche in FHL shows variability between cats regarding the expression of K19, α-SMA, MAC and lamin and a common pattern of activation could not be established. Nevertheless, when HPC were activated, a colocalization between HPCs and its niche could be demonstrated. It remains speculation whether this variation is caused by the duration of lipidosis, variations

in the underlying disease, or the effect of the therapeutic measures initiated by the referring clinician.

Abbreviations

HPCs: hepatic progenitor cells; HSCs: hepatic stellate cells; K19: keratin-19; MAC387: anti-macrophage antibody 387; αSMA: alpha-Smooth Muscle Actin; NAFLD: non-alcoholic fatty liver disease.

Competing Interest

None of the authors have competing interests.

Funding

LCP receives grants form the Netherlands Organisation for Health Research and Development (NWO ZON/MW numbers 92003538 and 16004121) for liver progenitor cell research.

Contributions

CV, BAS and LCP wrote the body of the paper, JHR and RPF criticaly reviewed the manuscript. CV, JHR, RPF and JR collected the samples. GCMG performed histological evaluation of the samples. BAS performed the immunostaining.

References

1. Armstrong PJ, Blanchard G. Hepatic lipidosis in cats. *Vet Clin North Am Small Anim Pract*. 2009;39(3):599-616. doi: 10.1016/j.cvsm.2009.03.003 [doi].

2. Center SA. Feline hepatic lipidosis. *Vet Clin North Am Small Anim Pract*. 2005;35(1):225-269. doi: S0195-5616(04)00142-1 [pii].

3. Valtolina C, Favier RP. Feline hepatic lipidosis. *Vet Clin North Am Small Anim Pract*. 2017;47(3):683-702. doi: S0195-5616(16)30160-7 [pii].

4. Center SA, Crawford MA, Guida L, Erb HN, King J. A retrospective study of 77 cats with severe hepatic lipidosis: 1975-1990. *J Vet Intern Med*. 1993;7(6):349-359.
5. Center SA, Guida L, Zanelli MJ, Dougherty E, Cummings J, King J. Ultrastructural hepatocellular features associated with severe hepatic lipidosis in cats. *Am J Vet Res*. 1993;54(5):724-731.

6. Roskams T, Yang SQ, Koteish A, et al. Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. *Am J Pathol*. 2003; 163(4):1301-1311. doi: S0002-9440(10)63489-X [pii].

7. Fausto N. Liver regeneration and repair: Hepatocytes, progenitor cells, and stem cells. *Hepatology*. 2004;39(6):1477-1487. doi: 10.1002/hep.20214 [doi].

8. Michalopoulos GK. Liver regeneration. *J Cell Physiol*. 2007;213(2):286-300. doi: 10.1002/jcp.21172 [doi].

9. Zajicek G. Do livers "stream"? *Am J Pathol*. 1995;146(3):772-776.

10. Wang B, Zhao L, Fish M, Logan CY, Nusse R. Self-renewing diploid Axin2(+) cells fuel homeostatic renewal of the liver. *Nature*. 2015;524(7564):180-185. doi: 10.1038/nature14863 [doi].

11. Alison MR, Lin WR. Diverse routes to liver regeneration. *J Pathol*. 2016;238(3):371-374. doi: 10.1002/path.4667 [doi].

12. Huch M. Regenerative biology: The versatile and plastic liver. *Nature*. 2015;517(7533):155-156. doi: 10.1038/517155a [doi].

13. Katoonizadeh A, Nevens F, Verslype C, Pirenne J, Roskams T. Liver regeneration in acute severe liver impairment: A clinicopathological correlation study. *Liver Int*. 2006;26(10):1225-1233. doi: LIV1377 [pii].

14. Roskams TA, Theise ND, Balabaud C, et al. Nomenclature of the finer branches of the biliary tree: Canals, ductules, and ductular reactions in human livers. *Hepatology*. 2004;39(6):1739-1745. doi: 10.1002/hep.20130 [doi].

15. IJzer J, Kisjes JR, Penning LC, Rothuizen J, van den Ingh TS. The progenitor cell compartment in the feline liver: An (immuno)histochemical investigation. *Vet Pathol*. 2009;46(4):614-621. doi: 10.1354/vp.07-VP-0097-I-FL [doi].

16. Roskams T, Cassiman D, De Vos R, Libbrecht L. Neuroregulation of the neuroendocrine compartment of the liver. *Anat Rec A Discov Mol Cell Evol Biol*. 2004;280(1):910-923. doi: 10.1002/ar.a.20096 [doi].

17. Roskams TA, Libbrecht L, Desmet VJ. Progenitor cells in diseased human liver. *Semin Liver Dis*. 2003;23(4):385-396. doi: 10.1055/s-2004-815564 [doi].

18. Yang S, Koteish A, Lin H, et al. Oval cells compensate for damage and replicative senescence of mature hepatocytes in mice with fatty liver disease. *Hepatology*. 2004;39(2):403-411. doi: 10.1002/hep.20082 [doi].

19. Libbrecht L, Roskams T. Hepatic progenitor cells in human liver diseases. *Semin Cell Dev Biol*. 2002;13(6):389-396. doi: S1084952102001258 [pii].

20. Sell S. Comparison of liver progenitor cells in human atypical ductular reactions with those seen in experimental models of liver injury. *Hepatology*. 1998;27(2):317-331. doi: S0270913998000494 [pii].

21. Yin L, Lynch D, Ilic Z, Sell S. Proliferation and differentiation of ductular progenitor cells and littoral cells during the regeneration of the rat liver to CCl4/2-AAF injury. *Histol Histopathol*. 2002;17(1):65-81.

22. Warren A, Center S, McDonough S, et al. Histopathologic features, immunophenotyping, clonality, and eubacterial fluorescence in situ hybridization in cats with lymphocytic cholangitis/cholangiohepatitis. *Vet Pathol*. 2011;48(3):627-641. doi: 10.1177/0300985810384409 [doi].

23. Otte CM, Rothuizen J, Favier RP, Penning LC, Vreman S. A morphological and immunohistochemical study of the effects of prednisolone or ursodeoxycholic acid on liver histology in feline lymphocytic cholangitis. *J Feline Med Surg*. 2014;16(10):796-804. doi: 10.1177/1098612X14520811 [doi].

24. IJzer J, Schotanus BA, Vander Borght S, et al. Characterisation of the hepatic progenitor cell compartment in normal liver and in hepatitis: An immunohistochemical comparison between dog and man. *Vet J*. 2010;184(3):308-314. doi: 10.1016/j.tvjl.2009.02.016 [doi].

25. Hixson DC, Chapman L, McBride A, Faris R, Yang L. Antigenic phenotypes common to rat oval cells, primary hepatocellular carcinomas and developing bile ducts. *Carcinogenesis*. 1997;18(6):1169-1175.

26. Tan J, Hytiroglou P, Wieczorek R, et al. Immunohistochemical evidence for hepatic progenitor cells in liver diseases. *Liver*. 2002;22(5):365-373. doi: 1622 [pii].

27. Schotanus BA, van den Ingh TS, Penning LC, Rothuizen J, Roskams TA, Spee B. Cross-species immunohistochemical investigation of the activation of the liver progenitor cell niche in different types of liver disease. *Liver Int*. 2009;29(8):1241-1252. doi: 10.1111/j.1478-3231.2009.02024.x [doi].

28. Santoni-Rugiu E, Jelnes P, Thorgeirsson SS, Bisgaard HC. Progenitor cells in liver regeneration: Molecular responses controlling their activation and expansion. *APMIS*. 2005;113(11-12):876-902. doi: APMapm_386 [pii].

29. Kruitwagen HS, Spee B, Schotanus BA. Hepatic progenitor cells in canine and feline medicine: Potential for regenerative strategies. *BMC Vet Res*. 2014;10:137-6148-10-137. doi: 10.1186/1746-6148-10-137 [doi].

30. Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: Stem cells and their niche. *Cell*. 2004;116(6):769-778. doi: S0092867404002557 [pii].

31. Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. *Nature*. 2001;414(6859):98-104. doi: 10.1038/35102160 [doi].

32. Roskams T. Different types of liver progenitor cells and their niches. *J Hepatol*. 2006;45(1):1-4. doi: S0168-8278(06)00242-X [pii].

33. Strick-Marchand H, Masse GX, Weiss MC, Di Santo JP. Lymphocytes support oval cell-dependent liver regeneration. *J Immunol*. 2008;181(4):2764-2771. doi: 181/4/2764 [pii].

34. Lorenzini S, Bird TG, Boulter L, et al. Characterisation of a stereotypical cellular and extracellular adult liver progenitor cell niche in rodents and diseased human liver. *Gut*. 2010;59(5):645-654. doi: 10.1136/gut.2009.182345 [doi].

35. Libbrecht L, Desmet V, Van Damme B, Roskams T. Deep intralobular extension of human hepatic 'progenitor cells' correlates with parenchymal inflammation in chronic viral hepatitis: Can 'progenitor cells' migrate? *J Pathol*. 2000;192(3):373-378. doi: 10.1002/1096-9896(2000)9999:99993.0.CO;2-5 [pii].

36. Lowes KN, Brennan BA, Yeoh GC, Olynyk JK. Oval cell numbers in human chronic liver diseases are directly related to disease severity. *Am J Pathol*. 1999;154(2):537-541. doi: S0002-9440(10)65299-6 [pii].

37. Richardson MM, Jonsson JR, Powell EE, et al. Progressive fibrosis in nonalcoholic steatohepatitis: Association with altered regeneration and a ductular reaction. *Gastroenterology*. 2007;133(1):80-90. doi: S0016-5085(07)00994-8 [pii].

38. Bedossa P, Paradis V. Liver extracellular matrix in health and disease. *J Pathol*. 2003; 200(4):504-515. doi: 10.1002/path.1397 [doi].

39. Carpino G, Morini S, Ginanni Corradini S, et al. Alpha-SMA expression in hepatic stellate cells and quantitative analysis of hepatic fibrosis in cirrhosis and in recurrent chronic hepatitis after liver transplantation. *Dig Liver Dis*. 2005;37(5):349-356. doi: S1590-8658(05)00018-6 [pii].

40. Morini S, Carotti S, Carpino G, et al. GFAP expression in the liver as an early marker of stellate cells activation. *Ital J Anat Embryol*. 2005;110(4):193-207.

41. Guyot C, Lepreux S, Combe C, et al. Hepatic fibrosis and cirrhosis: The (myo)fibroblastic cell subpopulations involved. *Int J Biochem Cell Biol*. 2006;38(2):135-151. doi: S1357-2725(05)00272-4 [pii].

42. Otte CM, Valtolina C, Vreman S, et al. Immunohistochemical evaluation of the activation of hepatic progenitor cells and their niche in feline lymphocytic cholangitis. *J Feline Med Surg*. 2017:1098612X17699723. doi: 10.1177/1098612X17699723 [doi].

43. Day CP, James OF. Hepatic steatosis: Innocent bystander or guilty party? *Hepatology*. 1998;27(6):1463-1466. doi: S0270913998002201 [pii].

44. Powell EE, Jonsson JR, Clouston AD. Steatosis: Co-factor in other liver diseases. *Hepatology*. 2005;42(1):5-13. doi: 10.1002/hep.20750 [doi].

45. Persico M, Iolascon A. Steatosis as a co-factor in chronic liver diseases. *World J Gastroenterol*. 2010;16(10):1171-1176.

46. van den Ingh TSGAM, Cullen JM, Twedt DC, Van Winkle T, Desmet VJ, Rothuizen J. Morphological classification of biliary disorders of the canine and feline liver. *WSAVA Standards for Clinical and Histological Diagnosis of Canine and Feline Liver Diseases*. 2006.

47. Pilling D, Fan T, Huang D, Kaul B, Gomer RH. Identification of markers that distinguish monocyte-derived fibrocytes from monocytes, macrophages, and fibroblasts. *PLoS One*. 2009;4(10):e7475. doi: 10.1371/journal.pone.0007475 [doi].

48. Kruitwagen HS, Spee B, Viebahn CS, et al. The canine hepatic progenitor cell niche: Molecular characterisation in health and disease. *Vet J*. 2014;201(3):345-352. doi: 10.1016/j.tvjl.2014.05.024 [doi].

49. Kallis YN, Robson AJ, Fallowfield JA, et al. Remodelling of extracellular matrix is a requirement for the hepatic progenitor cell response. *Gut*. 2011;60(4):525-533. doi: 10.1136/gut.2010.224436 [doi].

50. Van Hul NK, Abarca-Quinones J, Sempoux C, Horsmans Y, Leclercq IA. Relation between liver progenitor cell expansion and extracellular matrix deposition in a CDE-induced murine model of chronic liver injury. *Hepatology*. 2009;49(5):1625-1635. doi: 10.1002/hep.22820 [doi].

51. Schotanus BA, van Steenbek FG, Viebahn CS, et al. *Molecular pathways of massive hepatic progenitor cell niche activation in canine dissecting hepatitis*. [PhD thesis]. Faculty of Veterinary Medicine,Utrecht University, The Netherlands; 2011.

52. Proot SJ, Rothuizen J. High complication rate of an automatic tru-cut biopsy gun device for liver biopsy in cats. *J Vet Intern Med*. 2006;20(6):1327-1333.

Chapter 7

Summarising discussion

Feline hepatic lipidosis (FHL) is considered the most common form of acute acquired potentially lethal liver disease in the cat. It is believed to be a syndrome with multiple causative factors, observed in anorexic animals.[1-3] Cats have a propensity for hepatocellular lipid vacuolisation secondary to illness, that likely reflects unique aspects of feline metabolism. Their obligate carnivore status comes with several physiological and metabolic peculiarities in protein, carbon and fatty acid metabolism that make them different from other non-strictly carnivores, like dogs, rats, mice, and humans.[1,3,4]

Anorexia is the trigger for FHL, however individual predisposition to develop FHL cannot be excluded as not every anorexic cat will develop hepatic lipidosis (LH) and not every sick cat with lipidosis will succumb to the disease. A specific geographic distribution of the disease has been suggested based on the available reports of FHL from different areas, including North America, Great Britain, Japan, and Western Europe. The higher prevalence of FHL in these areas might be secondary to feeding habits and a high incidence of reported obesity in the feline population.[1,2] The prognosis does not seem to be related to the severity of the FHL, but more to the underlying disease and the early timing of treatment.

The knowledge gap in relation to FHL is highlighted by the many uncertainties and questions there still remain. To get answers, we need a better understanding of the lipid metabolism in healthy cats. Furthermore, we need to better comprehend the pathways of lipid metabolism in cats with FHL to understand the interaction between triglyceride (TG) accumulation in the liver and the alterations in the pathways for uptake, synthesis, degradation, and secretion of fatty acids (FAs).

In **Chapter 1** the scope and aims of this thesis were presented. They were designed to give a better insight into the pathophysiologic mechanisms that play a role in FHL in an attempt to unravel the characteristics of the different pathways of lipid mobilisation, hepatic lipid metabolism specifically, and *de novo* lipid synthesis in healthy cats and cats with FHL.
In this chapter (**Chapter 7**) the main findings of all the studies together with future perspectives are summarised and discussed. This summary is visually illustrated in figure 1.

Chapter 2 starts with an introduction on FHL: clinical signs, current diagnostics and treatment possibilities are discussed. The pathophysiology of FHL is discussed based on the available literature in relation to HL in cats, other obligate carnivores such as the European polecat (*Mustela putorius*) and the American mink (*Neovison vison*), and humans with non-alcoholic fatty liver disease (NAFLD).[5-8]

Although the exact pathophysiologic mechanism of FHL remains elusive, we know that FHL is the result of an imbalance between the influx of FAs into the liver derived from peripheral fat stores, the excretion of hepatic TGs via very low-density lipoproteins (VLDLs), *de novo* synthesis of FAs, and the rate of hepatic FAs oxidation for energy.

Once in the hepatocyte, FAs can be esterified to TGs. TGs usually accumulate in vacuoles within hepatocytes or can be incorporated in VLDLs to be excreted into the peripheral circulation. Cats with HL have always been considered to have increased levels of TGs in plasma, with the largest distribution in the VLDL fraction and increased plasma concentrations of VLDL.[9] This suggests that VLDL secretion is enhanced and not deficient in FHL.[9-12] However, while hepatic VLDL secretion appears increased in cats with FHL, this increase may not be sufficient to prevent the lipid overload of hepatocytes by the dramatic increase of FAs uptake into the liver. Secretion of VLDL is dependent on the rate of hepatic phosphatidylcholine (PC) synthesis.[13] If choline is not absorbed via the gastrointestinal tract during a period of anorexia, PC cannot be synthesised via the "Kennedy pathway". Consequently, the alternative "phosphatidylethanolamine N-methyltransferase (PEMT) pathway" should become the most important source of newly synthesised PC. Choline deficiency and low hepatic PEMT activity have been associated with HL in humans, mice, and rats.[14-16] Premenopausal and intact females in these species appear better protected from the development of lipidosis when exposed to a choline deficient diet. The up-regulation by oestrogens increases their capacity to synthesise PC via the PEMT pathway, reducing the dependency on choline intake.[17-19]

In **Chapter 3** we evaluated the lipid metabolism in healthy cats fed a choline restricted diet and studied the effect of hormones on choline synthesis via the PEMT pathway. We hypothesised that the loss of oestrogen influence could cause spayed female cats to have a lower capacity for PC synthesis than intact females. If this would be the case, the widely performed practice in all western countries of spaying female cats could be a predisposing factor to develop FHL.
Our study demonstrates that plasma choline levels were not statistically different between intact female and intact male cats. The choline levels increased in female

cats by 35% after spaying to levels similar to those in male cats pre- and post-neutering, but the increase was not significant. Activity of PEMT varied widely with no significant difference between female and male cats before or after spaying/neutering. Phosphatidylcholine 38:6 and PC 40:6 as products of the PEMT pathway reflect indirectly the activity of this pathway.[20,21] And the PC/phosphatidylethanolamine (PE) ratio is also considered a proxy for the PEMT pathway.[22] There were no significant differences between the groups in these indirect parameters. These results confirm that there was no oestrogen effect on activity of the PEMT pathway. Unexpectedly, there was no higher PEMT activity in intact females before spaying, nor a decrease in PEMT related parameters after spaying. In contrast to what has been reported in humans, mice, and rats, the PEMT activity tended to be lower in intact female cats than in male cats and spayed female cats.

The increase in plasma free choline levels after spaying in female cats may suggest an effect of oestrogen on either choline uptake from the diet or on the conversion of choline to other metabolites like acetylcholine, betaine or phosphatidylcholine. Furthermore, the amount of choline in the "restricted" but not deficient diet may still have been enough to allow PC to be formed via the Kennedy pathway, with a blunted stimulation of PEMT pathway expression and activity as a result. However, even with a diet sufficiently supplemented with choline some contribution to the PC synthesis from the PEMT pathway could have been expected, as this has been demonstrated in humans, mice, and rats. In these species, the PEMT pathway still contributes 20–40 % to the PC normally produced with diets that contain sufficient choline for the specific species.[17,21,23,24]

We could conclude from this study that the current recommendations for dietary choline levels are adequate for spayed female cats and it is unlikely that castration predisposes cats for HL by causing PC deficiency, as suggested in other species, if recommended dietary choline level are provided.

The essential FAs profile is known to vary considerably between males and females in humans, mice and rats, indicating that sex lipid dimorphism occurs in different species.[19,25-32] We evaluated the lipid profile in healthy cats and in cats with HL with the use of lipidomics to evaluate if sexual lipid dimorphism could be established in cats, and if these differences could be related to HL (**Chapter 4**). The results of this study indicate that intact female cats have significantly lower TGs levels in plasma compared to their male counterparts, similarly to premenopausal women, intact female rats and mice.[19,25-27,30,32] This difference was not reversed after spaying/neutering, but rather enhanced due to a significant rise in the plasma TGs level in male cats. The higher plasma TGs levels in male cats may be related to a higher rate of apolipoprotein B and VLDL synthesis by

hepatocytes in male cats as the TGs levels in plasma most likely reflect the VLDL fraction.[9,33,34] Also a reduced plasma clearance of VLDL triglyceride in male cats compared to their female counterparts cannot be excluded. Interestingly, in premenopausal women a lower concentration of VLDL-TGs and LDL particles compared to men appears to be associated with accelerated (rather than reduced) VLDL-TGs and LDL production and increased plasma clearance.[33-38]

The results of the lipidomic analysis in the cats with HL in this study confirm findings in previous studies analysing lipids in liver and in plasma.[39,40] We confirmed that cats with HL have significantly higher TGs levels in their liver compared to the healthy cats. However in our study, cats with HL had no significantly different plasma TGs levels compared to healthy cats, whereas in other studies high plasma TGs levels in cats with HL have previously been described.[39-41]

Triglycerides in plasma from fasted animals most likely reflect the VLDL lipoprotein fraction.[9] VLDL is relative enriched in TGs compared to low density lipoprotein (LDL) and high density lipoprotein (HDL). The relationship between FHL and VLDL is however not straightforward because of conflicting results of both increased and decreased plasma TGs or VLDL concentrations from different studies.[9,42,43] A recent study on altered lipoprotein profiles in cats with HL corroborates our findings that cats with FHL have a low concentration of TGs in the plasma. Knowing that VLDLs are hydrolysed to VLDL remnants in peripheral tissues and further metabolized by hepatic lipase to become LDLs, the authors of that study speculated that the increased amounts of LDLs found in cats with FHL might indicate that VLDL secretion had been elevated earlier on in the disease process.[44,45] Due to the shorter half-life of VLDLs versus LDLs, VLDL secretion and circulating TGs concentrations may increase early in feline HL and then decrease as the disease progresses. Low VLDL levels in cats with HL suggest there may be a maximum capacity of the feline liver to secrete VLDL. Therefore, no further increase in plasma VLDL is observed in association with high liver TGs levels.[45]

Like other mammals, cats are unable to synthesize essential fatty acids (EFAs), like linoleic acid (18:2n-6) and a-linoleic acid (18:3n-3). Unlike other mammals, cats have a limited capacity to synthesize the long-chain polyunsaturated FA (LCPUFA) arachidonic acid (AA) (20:4n-6) from linoleic acid, and eicosapentaenoic acid (20:3n-3) and docosahexaenoic acid (DHA) (22:6n-3) from a-linoleic acid (18:3n-3). The severely decreased activity of the enzymes Δ 5-desaturase and Δ 6-desaturase, enzymes involved in the formation of LCPUFA from EFA, explains this peculiarity in cats.[46-49]

Intact female cats in our study, have a higher content of LCPUFA AA (20:4 n-6)

in PC in both plasma and liver tissue, when compared to spayed female and the male counterparts. This finding is similar to what has been reported in premenopausal women, intact female rats and mice.[19,25-27,32] However, in contrast to what has been reported in these species, PC levels containing LCPUFA DHA (22:6 n-3) were not elevated in intact female cats.[30,50,51] Despite this interspecies difference, there appears an influence of sex hormones on the profile of PC species in cats as the differences between the sexes disappeared after spaying/neutering. In a recent study on liver lipid metabolism in mice and its relation to sex hormones, it has been demonstrated that circulating oestrogens are able to suppress the enzyme Cyp4a12a, which normally is responsible for degrading the LCPUFA AA in liver and plasma. [52] This could be an explanation in our study for the elevated level of AA in intact female cats compared to the spayed cats and male counterparts. Furthermore, as demonstrated by Trevisan et al. cats are able to synthetize AA, but not DHA, from the substrate γ-linolenic acid (18:3 n – 6) via Δ5 desaturase, bypassing the Δ6 desaturase step.[53] As desaturases can be stimulated by oestrogen, this could explain the primary increase in AA but not DHA levels in the liver of intact female cats compared to the male and spayed female counterparts.

Although our observations clearly suggest gender specific differences in lipid metabolism, current knowledge is still incomplete and contradictory. For this reason, presenting a final hypothesis on the role of gender in the development of HL in cats is premature.

The increased liver and plasma concentration of sphingomyelin (SM) is an interesting and new finding from the lipidomics analysis in cats with HL. Sphingomyelin is a type of sphingolipid primarily present in the cell membrane, especially in the membranous myelin sheath that surrounds nerve cell axons. Sphingomyelin consists of a phosphocholine head group, a sphingosine, and a fatty acid. The sphingosine and fatty acid together form a ceramide. Sphingomyelin *de novo* biosynthesis starts in the endoplasmic reticulum with the condensation of serine and palmitoyl-CoA catalysed by serine palmitoyl transferase (SPT). [54] The availability of palmitic acid (C16:0) is a rate-limiting step for ceramide and SM synthesis. Liver represents a central site for ceramide production and generally contains a far higher quantity of sphingolipids, especially ceramide and SM, than all other tissues.[55-58] The increased synthesis of SM in livers affected by NAFLD promotes hepatic insulin resistance, hepatocyte apoptosis and increased release of inflammatory mediators. [56,57] The role of SM and ceramide in the development of steatosis has also been observed in different animal models of obesity and fatty liver disease.[56,57,59] In obese subjects, ceramide and SM concentrations are higher in the liver compared to subcutaneous and intra-abdominal adipose tissue, and

the presence of hepatic steatosis in humans coincides with increased ceramide concentrations.[58]
The presence of SM and ceramide in the liver has been indicated in NAFLD as one of the "second hit" that is responsible for the progression from simple steatosis to steatohepatitis.[56,57] The traditional "two-hit" pathophysiological approach to NAFLD recognises simple steatosis as the first hit and the prerequisite for hepatocyte injury to develop, whereas cytokines, adipokines, bacterial endotoxin, mitochondrial dysfunction and/or endoplasmic reticulum stress represent the second hit for the progression to non-alcoholic steatohepatitis (NASH). [60]

The increased SM concentrations, both in liver and in plasma, in cats with HL warrants further study in order to understand the potential role of SM in feline HL and its correlation with cytokines, insulin resistance and severity of the disease. Experimental studies targeting the enzymes involved in the pathway of ceramide synthesis seem to impact and decrease the development of steatosis in a mouse model of obesity and NAFLD, and remain subject of research in human medicine.[56,61,62]

In **Chapter 5** *de novo* hepatic lipogenesis (*DNL*) as a possible contributing pathophysiologic pathway to liver steatosis in FHL has been evaluated. In human medicine *DNL* has been suggested to be abnormally increased and to contribute to the pathogenesis of liver steatosis in diseases like obesity and to 26% to the liver lipid accumulation in NAFLD. [63-66]

Sterol regulatory element-binding factor (*SREBF1*), which is the equivalent in function to SREBP-1c, has been defined as the pivotal controller of lipid biosynthesis and main regulator of the expression of several genes involved in lipogenesis.[67-70] This study demonstrates that *SREBF* gene expression was around 10-times downregulated in cats with FHL. Furthermore, in liver tissue the mRNA expression of *PPAR-α*, *PPAR-γ*, and *FASN*, indicators of increased *DNL*, was not significantly different in cats with FHL compared to healthy cats. In a cat model with a diet induced overweight, lipogenic gene expression (*SREBP-1c, FAS* and *ATP citrate lyase* (*ACL*)) in lipid sensitive tissues confirmed our findings that the liver of cats does not foster *DNL*.[71] The downregulation of *SREBF/SREBP-1c* in liver of cats with FHL does not support the hypothesis that *DNL* in the liver is an important pathway of FAs accumulation in FHL in contrast with what has been reported in other obligated carnivores and humans with NAFLD.[64,72-74]

As cats with HL have an increased concentration of palmitate and MUFA compared to healthy cats in their liver and visceral adipose tissue, *DNL* in FHL

most likely occurs in the visceral adipose tissue. Subsequently, the *de novo* synthetized MUFAs are transported from the adipose tissue to the liver, where they contribute to fat overload in FHL. The evaluation of lipogenic gene expression in visceral adipose tissue in cats affected with HL and the comparison between lipogenic genes expression in visceral adipose tissue and liver might bring more understanding on *DNL* in cats.

The importance of the visceral adipose tissue deposition in the development of NAFLD is well known in human and veterinary medicine. Besides being the major site for storage of excess energy as TGs during a positive energy balance state, visceral adipose tissue has also an important endocrine function by secreting multiple adipokines (adiponectin, leptin), chemokines, and cytokines involved in the development of insulin resistance, and increased lipogenesis. [75-77]. Matsuzawa et al. proposed the pathophysiological importance of visceral fat deposition in the development of metabolic dysfunctions and hepatic steatosis and introduced the term "visceral fat syndrome.[77] The adipose tissue of FHL cats has markedly increased tumour necrosis factor, leptin and adiponectin concentrations compared with that of healthy subjects and it contributes greatly to FAs released in a state of negative energy balance like in FHL.[78]

The traditional "two-hit" pathophysiological approach to NAFLD does not seem to apply in cats with FHL. Feline hepatic lipidosis has always been described in the literature as an "acute disease that lacks a necro-inflammatory component". The liver contains parenchymal (hepatocytes, the main liver cells) and non-parenchymal cells, including Kupffer cells (liver resident macrophages), hepatic stellate cells (HSCs, involved in vitamin A metabolism), and biliary epithelial cells (forming bile ducts), which contribute to maintaining liver homeostasis. All of these cell types are targets of the hepatic lipotoxicity that in NAFLD often signs the progression from steatosis to NASH.[79-81] When liver damage is severe enough to inhibit the replication capability of the hepatocytes, a quiescent population of pluripotent cell is activated: the hepatic progenitor cells (HPCs). The few descriptions in the literature of the ductular reaction in cats do not clearly implicate the activation and co-localisation of the HPCs and their niche. [82-84] In **Chapter 6** the feline HPCs and its niche was investigated in cats with FHL and compared to healthy cats by immunohistochemical and double immuno-fluorescence staining. Immunohistochemical staining for the progenitor/bile duct marker (K19), activated Kupffer cells (MAC387), myofibroblasts (alpha-smooth muscle actin [α-SMA]) and the extracellular matrix component laminin were used. The study demonstrated that ductular reactions observed in the feline HPC niche in FHL contain Kupffer cells, activated myofibroblasts and the extracellular matrix component laminin. In the majority of FHL samples HPCs,

myofibroblast, and laminin positivity were seen also in the periportal and parenchymatous area. Macrophages were present throughout the parenchyma. This is comparable with other hepatopathies in humans and dogs, such as acute and chronic hepatitis, hepatobiliary diseases and hepatic carcinoma.[82,83,85-89] These findings are also in line with the HPC activation in feline lymphocytic cholangitis, a chronic feline hepatobiliary disease.[90]

We were, however, unable to establish a clear and definite pattern between the histopathological presence of lipidosis and the activation of the HPCs or the upregulation of the niche components in all cats with HL. Different degrees of HPC activation in FHL were suggested by the variability in the expression and positivity of the different immunostainings. This is different feline lymphocytic cholangitis (LC) in which immunohistochemical markers applied in liver tissue were expressed in more cells, or more intensely, than in the liver tissue of unaffected cats.[90] In human, rat and murine models of impaired hepatocyte replication in NAFLD, simple steatosis is enough to inhibit replication of mature hepatocytes and to trigger the activation of HPCs, however this does not seem the case in FHL. [91,92]

Despite the presence of severe steatosis in all cats affected with FHL, the lack of a pattern of activation of the HPCs might be explained by different factors. Feline hepatic lipidosis is classified as an acute disease in which the clinical condition of the cat improves and lipidosis resolves quickly or the cat dies or is euthanised. Lymphocytic cholangitis is considered a more chronic disease, characterised by portal fibrosis and bile duct proliferation.[84,93,94] In human and animal models the degree of HPC and its niche activation is directly related to the severity of disease, indicated by the amount of hepatocyte loss, the amount of inflammation, and the extent of fibrosis.[88,91,95-103] The acute nature of FHL may have limited the development of a clear relationship between HPCs activation and hepatic lipidosis. Furthermore, the different results in HPC activation in FHL could also be caused by variations in the underlying disease that caused the anorexia and secondary lipidosis or the effect of the therapeutic measures initiated by the clinician. To determine the cause of the variability from this study, a follow up study is needed in which the expression of the markers for HPC and its niche are evaluated in relation to the clinical and histopathological severity of lipidosis in a larger number of cases. However, the available literature on FHL has already demonstrated a variable clinical outcome with no association with histopathological results.

In conclusion, our studies have contributed to a better understanding of the lipid physiology in healthy cats and some particular aspects of the pathophysiology of

FHL. Although these results do not bring forth a clear picture on the pathophysiology of FHL, they do strongly suggest the cat is a species on its own with physiological and metabolic peculiarities in fatty acid and lipid metabolism that makes it different from other non-strictly carnivores animals, like dogs, rats, mice and humans. Therefore, feline hepatic lipidosis should be considered a syndrome with multiple pathophysiologic factors that originate from the cat's unique lipid, one-carbon and protein metabolism, presence of insulin resistance, obesity and maybe some individual predispositions.

Considering the results from our studies and data from other experimental studies in overweight cats undergoing rapid weight loss to induce HL, we argue that both decreased VLDL excretion and increased *de novo* hepatic lipid synthesis are unlikely to be the most important causes of hepatic FAs accumulation in FHL.[10,11] The most probable pathophysiologic pathway seems to be an insufficient increase in the rate of fatty-acid oxidation in the face of increased peripheral FA mobilization. This seems to be in agreement with the clinical experience that exogenous L-carnitine supplementation can improve FA oxidation in cats with FHL. Also in an organoid model for steatosis supplementation of L-carnitine to feline liver organoids attenuated lipid accumulation in high-fat conditions and improved cellular viability.[82,104] Individual characteristics, environmental influences and eventually pre-existing obesity could complete the picture of FHL as predisposing or complicating factors.

Finally, we suggest that although cats with FHL share some metabolic similarities with human NAFLD patients, the FHL syndrome appears very different from NAFLD and NASH. Feline hepatic lipidosis represents an acute severe disease associated with compromised hepatic function, but there is no evidence that hepatic steatosis progresses to a NASH-like syndrome in the cat.

Chapter 3

- There is no evidence of influence of sex hormones on the PEMT pathway in cats, when fed a recommended amount of choline in the diet
- The current recommendations for dietary choline levels are adequate for spayed female cats and it is unlikely that castration predisposes cats for HL by causing PC deficiency

Chapter 4

- In healthy cats sexual dimorphism in the hepatic and plasma lipid profile resembles observations in other species with lower plasma TGs levels and increased plasma and liver AA in intact females compared to males.
- Intact female cats with higher plasma and liver levels of AA but not DHA could be predisposed for HL. Also spaying of female cats may not increase the risk for HL.
- In contrast to previous studies, the higher liver but not plasma TGs levels in cats with HL could suggest a maximum capacity for liver excretion of VLDL.
- The increased liver and plasma SM concentration in cats with HL compared to healthy cats could indicate a novel mechanism in the development of HL in cats

Chapter 5

- In this study the decrease in liver *SREBF* gene expression and no expression change in other *DNL*-related enzymes suggest that *DNL* does not occur in the liver of cats with FHL
- In cats with FHL *DNL* most likely occurs in the abdominal adipose tissue. Subsequently, the de novo synthetized MUFAs are transported from the adipose tissue to the liver, where they contribute to fat overload in FHL.

Chapter 6

- The study demonstrated that ductular reactions observed in the feline HPC niche in FHL contain Kupffer cells, activated myofibroblasts and the extracellular matrix component laminin.
- There is no a clear and definite pattern between the histopathological presence of lipidosis and the activation of the HPCs or the upregulation of the niche components in all cats with HL

Figure 1: Conclusions of the thesis

References

1. Armstrong PJ, Blanchard G. Hepatic lipidosis in cats. *Vet Clin North Am Small Anim Pract*. 2009;39(3):599-616. doi: 10.1016/j.cvsm.2009.03.003 [doi].

2. Center SA. Feline hepatic lipidosis. *Vet Clin North Am Small Anim Pract*. 2005;35(1):225-269. doi: S0195-5616(04)00142-1 [pii].

3. Verbrugghe A, Bakovic M. Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis. *Nutrients*. 2013;5(7):2811-2835. doi: 10.3390/nu5072811 [doi].

4. Valtolina C, Favier RP. Feline hepatic lipidosis. *Vet Clin North Am Small Anim Pract*. 2017;47(3):683-702. doi: S0195-5616(16)30160-7 [pii].

5. Mustonen AM, Pyykonen T, Paakkonen T, et al. Adaptations to fasting in the american mink (mustela vison): Carbohydrate and lipid metabolism. *Comp Biochem Physiol A Mol Integr Physiol*. 2005;140(2):195-202. doi: S1095-6433(04)00351-4 [pii].

6. Araya J, Rodrigo R, Videla LA, et al. Increase in long-chain polyunsaturated fatty acid n - 6/n - 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin Sci (Lond)*. 2004;106(6):635-643. doi: 10.1042/CS20030326 [doi].

7. Nieminen P, Mustonen AM, Karja V, Asikainen J, Rouvinen-Watt K. Fatty acid composition and development of hepatic lipidosis during food deprivation--mustelids as a potential animal model for liver steatosis. *Exp Biol Med (Maywood)*. 2009; 234(3):278-286. doi: 10.3181/0806-RM-210 [doi].

8. Videla LA, Rodrigo R, Araya J, Poniachik J. Oxidative stress and depletion of hepatic long-chain polyunsaturated fatty acids may contribute to nonalcoholic fatty liver disease. *Free Radic Biol Med*. 2004;37(9):1499-1507. doi: 10.1016/j.freeradbiomed.2004.06.033 [doi].

9. Pazak HE, Bartges JW, Cornelius LC, Scott MA, Gross K, Huber TL. Characterization of serum lipoprotein profiles of healthy, adult cats and idiopathic feline hepatic lipidosis patients. *J Nutr*. 1998;128(12 Suppl):2747S-2750S.

10. Ibrahim WH, Bailey N, Sunvold GD, Bruckner GG. Effects of carnitine and taurine on fatty acid metabolism and lipid accumulation in the liver of cats during weight gain and weight loss. *Am J Vet Res*. 2003;64(10):1265-1277.

11. Ibrahim WH, Szabo J, Sunvold GD, Kelleher JK, Bruckner GG. Effect of dietary protein quality and fatty acid composition on plasma lipoprotein concentrations and hepatic triglyceride fatty acid synthesis in obese cats undergoing rapid weight loss. *Am J Vet Res*. 2000;61(5):566-572.

12. Szabo J, Ibrahim WH, Sunvold GD, Bruckner GG. Effect of dietary protein quality and essential fatty acids on fatty acid composition in the liver and adipose tissue after rapid weight loss in overweight cats. *Am J Vet Res*. 2003;64(3):310-315.

13. Vance DE. Phospholipid biosynthesis in eukaryotes. In: Vance DE, Vance JE, eds. *Biochemistry of lipids, lipoproteins and membranes*. 5th ed. Amsterdam: Elsevier; 2002:205-232.

14. Yao ZM, Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J Biol Chem*. 1988;263(6):2998-3004.

15. Yao ZM, Vance DE. Head group specificity in the requirement of phosphatidylcholine biosynthesis for very low density lipoprotein secretion from cultured hepatocytes. *J Biol Chem*. 1989;264(19):11373-11380.

16. Vance JE, Vance DE. The role of phosphatidylcholine biosynthesis in the secretion of lipoproteins from hepatocytes. *Can J Biochem Cell Biol*. 1985;63(8):870-881. doi: 10.1139/o85-108 [doi].

17. Noga AA, Vance DE. A gender-specific role for phosphatidylethanolamine N-methyltransferase-derived phosphatidylcholine in the regulation of plasma high density and very low density lipoproteins in mice. *J Biol Chem*. 2003;278(24):21851-21859. doi: 10.1074/jbc.M301982200 [doi].

18. Resseguie M, Song J, Niculescu MD, da Costa KA, Randall TA, Zeisel SH. Phosphatidylethanolamine N-methyltransferase (PEMT) gene expression is induced by estrogen in human and mouse primary hepatocytes. *FASEB J*. 2007;21(10):2622-2632. doi: fj.07-8227com [pii].

19. Young DL. Estradiol- and testosterone-induced alterations in phosphatidylcholine and triglyceride synthesis in hepatic endoplasmic reticulum. *J Lipid Res*. 1971;12(5):590-595.

20. DeLong CJ, Shen YJ, Thomas MJ, Cui Z. Molecular distinction of phosphatidylcholine synthesis between the CDP-choline pathway and phosphatidylethanolamine methylation pathway. *J Biol Chem*. 1999;274(42):29683-29688.

21. da Costa KA, Sanders LM, Fischer LM, Zeisel SH. Docosahexaenoic acid in plasma phosphatidylcholine may be a potential marker for in vivo phosphatidylethanolamine N-methyltransferase activity in humans. *Am J Clin Nutr*. 2011;93(5):968-974. doi: 10.3945/ajcn.110.011064 [doi].

22. Li Z, Vance DE. Phosphatidylcholine and choline homeostasis. *J Lipid Res*. 2008;49(6):1187-1194.

23. da Costa KA, Gaffney CE, Fischer LM, Zeisel SH. Choline deficiency in mice and humans is associated with increased plasma homocysteine concentration after a methionine load. *Am J Clin Nutr*. 2005;81(2):440-444. doi: 81/2/440 [pii].

24. Pani P, Porcu M, Columbano A, Dessi S, Ledda GM, Diaz G. Differential effects of choline administration on liver microsomes of female and male rats. *Exp Mol Pathol*. 1978;28(2):154-162. doi: 0014-4800(78)90048-5 [pii].

25. Ostwald R, Bouchard P, Miljanich P, Lyman RL. Influence of sex and gonadal hormones on rat-liver and carcass lipids during the development of an essential fatty acid deficiency. *Biochem J*. 1965;97(2):485-499.

26. Lyman RL, Tinoco J, Bouchard P, Sheehan G, Ostwald R, Miljanich P. Sex differences in the metabolism of phosphatidyl cholines in rat liver. *Biochim Biophys Acta*. 1967;137(1):107-114.

27. Lyman RL, Hopkins SM, Sheehan G, Tinoco J. Effects of estradiol and testosterone on the incorporation and distribution of [me-14C]methionine methyl in rat liver lecithins. *Biochim Biophys Acta*. 1968;152(1):197-207.

28. Carroll MD, Lacher DA, Sorlie PD, et al. Trends in serum lipids and lipoproteins of adults, 1960-2002. *JAMA*. 2005;294(14):1773-1781. doi: 294/14/1773 [pii].

29. Mittendorfer B. Sexual dimorphism in human lipid metabolism. *J Nutr*. 2005;135(4):681-686. doi: 135/4/681 [pii].

30. Burdge GC, Slater-Jefferies JL, Grant RA, et al. Sex, but not maternal protein or folic acid intake, determines the fatty acid composition of hepatic phospholipids, but not of triacylglycerol, in adult rats. *Prostaglandins Leukot Essent Fatty Acids*. 2008;78(1):73-79. doi: S0952-3278(07)00179-2 [pii].

31. Childs CE, Romeu-Nadal M, Burdge GC, Calder PC. The polyunsaturated fatty acid composition of hepatic and plasma lipids differ by both sex and dietary fat intake in rats. *J Nutr*. 2010;140(2):245-250. doi: 10.3945/jn.109.115691 [doi].

32. Extier A, Langelier B, Perruchot MH, et al. Gender affects liver desaturase expression in a rat model of n-3 fatty acid repletion. *J Nutr Biochem*. 2010;21(3):180-187. doi: 10.1016/j.jnutbio.2008.10.008 [doi].

33. Magkos F, Patterson BW, Mohammed BS, Klein S, Mittendorfer B. Women produce fewer but triglyceride-richer very low-density lipoproteins than men. *J Clin Endocrinol Metab*. 2007;92(4):1311-1318. doi: jc.2006-2215 [pii].

34. Wang X, Magkos F, Mittendorfer B. Sex differences in lipid and lipoprotein metabolism: It's not just about sex hormones. *J Clin Endocrinol Metab*. 2011;96(4):885-893. doi: 10.1210/jc.2010-2061 [doi].

35. Matthan NR, Jalbert SM, Barrett PH, Dolnikowski GG, Schaefer EJ, Lichtenstein AH. Gender-specific differences in the kinetics of nonfasting TRL, IDL, and LDL apolipoprotein B-100 in men and premenopausal women. *Arterioscler Thromb Vasc Biol*. 2008;28(10):1838-1843. doi: 10.1161/ATVBAHA.108.163931 [doi].

36. Nikkila EA, Kekki M. Polymorphism of plasma triglyceride kinetics in normal human adult subjects. *Acta Med Scand*. 1971;190(1-2):49-59.

37. Olefsky J, Farquhar JW, Reaven GM. Sex difference in the kinetics of triglyceride metabolism in normal and hypertriglyceridaemic human subjects. *Eur J Clin Invest*. 1974;4(2):121-127.

38. Watts GF, Moroz P, Barrett PH. Kinetics of very-low-density lipoprotein apolipoprotein B-100 in normolipidemic subjects: Pooled analysis of stable-isotope studies. *Metabolism*. 2000;49(9):1204-1210. doi: S0026-0495(00)82057-0 [pii].

39. Hall JA, Barstad LA, Connor WE. Lipid composition of hepatic and adipose tissues from normal cats and from cats with idiopathic hepatic lipidosis. *J Vet Intern Med*. 1997;11(4):238-242.

40. Brown B, Mauldin GE, Armstrong J, Moroff SD, Mauldin GN. Metabolic and hormonal alterations in cats with hepatic lipidosis. *J Vet Intern Med*. 2000;14(1):20-26.

41. Center SA, Crawford MA, Guida L, Erb HN, King J. A retrospective study of 77 cats with severe hepatic lipidosis: 1975-1990. *J Vet Intern Med*. 1993;7(6):349-359.

42. Blanchard G, Paragon BM, Serougne C, Ferezou J, Milliat F, Lutton C. Plasma lipids, lipoprotein composition and profile during induction and treatment of hepatic lipidosis in cats and the metabolic effect of one daily meal in healthy cats. *J Anim Physiol Anim Nutr (Berl)*. 2004;88(3-4):73-87. doi: 10.1111/j.1439-0396.2003.00462.x [doi].

43. Kuzi S, Segev G, Kedar S, Yas E, Aroch I. Prognostic markers in feline hepatic lipidosis: A retrospective study of 71 cats. *Vet Rec*. 2017;181(19):512. doi: 10.1136/vr.104252 [doi].

44. Minamoto T, Walzem RL, Hamilton AJ, et al. Altered lipoprotein profiles in cats with hepatic lipidosis. *J Feline Med Surg*. 2018:1098612X18780060. doi: 10.1177/1098612X18780060 [doi].

45. Fabbrini E, Mohammed BS, Magkos F, Korenblat KM, Patterson BW, Klein S. Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. *Gastroenterology*. 2008;134(2):424-431. doi: 10.1053/j.gastro.2007.11.038 [doi].

46. Hassam AG, Rivers JP, Crawford MA. The failure of the cat to desaturate linoleic acid; its nutritional implications. *Nutr Metab*. 1977;21(5):321-328.

47. Rivers JP, Sinclair AJ, Craqford MA. Inability of the cat to desaturate essential fatty acids. *Nature*. 1975;258(5531):171-173.

48. Rivers JP, Sinclair AJ, Moore DP, Crawford MA. The abnormal metabolism of essential fatty acids in the cat [proceedings. *Proc Nutr Soc*. 1976;35(2):66A-67A.

49. Pawlosky R, Barnes A, Salem N,Jr. Essential fatty acid metabolism in the feline: Relationship between liver and brain production of long-chain polyunsaturated fatty acids. *J Lipid Res*. 1994;35(11):2032-2040.

50. Bakewell L, Burdge GC, Calder PC. Polyunsaturated fatty acid concentrations in young men and women consuming their habitual diets. *Br J Nutr*. 2006;96(1):93-99. doi: S0007114506001826 [pii].

51. Hoene M, Li J, Haring HU, Weigert C, Xu G, Lehmann R. The lipid profile of brown adipose tissue is sex-specific in mice. *Biochim Biophys Acta*. 2014;1842(10):1563-1570. doi: 10.1016/j.bbalip.2014.08.003 [doi].

52. Vehmas AP, Adam M, Laajala TD, et al. Liver lipid metabolism is altered by increased circulating estrogen to androgen ratio in male mouse. *J Proteomics*. 2016;133:66-75. doi: S1874-3919(15)30208-6 [pii].

53. Trevizan L, de Mello Kessler A, Brenna JT, Lawrence P, Waldron MK, Bauer JE. Maintenance of arachidonic acid and evidence of Delta5 desaturation in cats fed gamma-linolenic and linoleic acid enriched diets. *Lipids*. 2012;47(4):413-423. doi: 10.1007/s11745-011-3651-0 [doi].

54. Holland WL, Summers SA. Sphingolipids, insulin resistance, and metabolic disease: New insights from in vivo manipulation of sphingolipid metabolism. *Endocr Rev*. 2008;29(4):381-402. doi: 10.1210/er.2007-0025 [doi].

55. Luukkonen PK, Zhou Y, Sadevirta S, et al. Hepatic ceramides dissociate steatosis and insulin resistance in patients with non-alcoholic fatty liver disease. *J Hepatol*. 2016;64(5):1167-1175. doi: S0168-8278(16)00005-2 [pii].

56. Bikman BT, Summers SA. Sphingolipids and hepatic steatosis. *Adv Exp Med Biol*. 2011;721:87-97. doi: 10.1007/978-1-4614-0650-1_6 [doi].

57. Pagadala M, Kasumov T, McCullough AJ, Zein NN, Kirwan JP. Role of ceramides in nonalcoholic fatty liver disease. *Trends Endocrinol Metab*. 2012;23(8):365-371. doi: 10.1016/j.tem.2012.04.005 [doi].

58. Kotronen A, Seppanen-Laakso T, Westerbacka J, et al. Comparison of lipid and fatty acid composition of the liver, subcutaneous and intra-abdominal adipose tissue, and serum. *Obesity (Silver Spring)*. 2010;18(5):937-944. doi: 10.1038/oby.2009.326 [doi].

59. Li Y, Dong J, Ding T, et al. Sphingomyelin synthase 2 activity and liver steatosis: An effect of ceramide-mediated peroxisome proliferator-activated receptor gamma2 suppression. *Arterioscler Thromb Vasc Biol*. 2013;33(7):1513-1520. doi: 10.1161/ATVBAHA.113.301498 [doi].

60. Levene AP, Goldin RD. The epidemiology, pathogenesis and histopathology of fatty liver disease. *Histopathology*. 2012;61(2):141-152. doi: 10.1111/j.1365-2559.2011.04145.x [doi].

61. Zhao H, Przybylska M, Wu IH, et al. Inhibiting glycosphingolipid synthesis ameliorates hepatic steatosis in obese mice. *Hepatology*. 2009;50(1):85-93. doi: 10.1002/hep.22970 [doi].

62. Bijl N, Sokolovic M, Vrins C, et al. Modulation of glycosphingolipid metabolism significantly improves hepatic insulin sensitivity and reverses hepatic steatosis in mice. *Hepatology*. 2009;50(5):1431-1441. doi: 10.1002/hep.23175 [doi].

63. Lambert JE, Ramos-Roman MA, Browning JD, Parks EJ. Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology*. 2014;146(3):726-735. doi: 10.1053/j.gastro.2013.11.049 [doi].

64. Choudhury J, Sanyal AJ. Insulin resistance and the pathogenesis of nonalcoholic fatty liver disease. *Clin Liver Dis*. 2004;8(3):575-94, ix. doi: 10.1016/j.cld.2004.04.006 [doi].

65. Diraison F, Dusserre E, Vidal H, Sothier M, Beylot M. Increased hepatic lipogenesis but decreased expression of lipogenic gene in adipose tissue in human obesity. *Am J Physiol Endocrinol Metab*. 2002;282(1):E46-51. doi: 10.1152/ajpendo.2002.282.1.E46 [doi].

66. Rector RS, Thyfault JP, Wei Y, Ibdah JA. Non-alcoholic fatty liver disease and the metabolic syndrome: An update. *World J Gastroenterol*. 2008;14(2):185-192.

67. Hodson L, Frayn KN. Hepatic fatty acid partitioning. *Curr Opin Lipidol*. 2011;22(3):216-224. doi: 10.1097/MOL.0b013e3283462e16 [doi].

68. Horton JD, Goldstein JL, Brown MS. SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest*. 2002;109(9):1125-1131. doi: 10.1172/JCI15593 [doi].

69. Horton JD, Goldstein JL, Brown MS. SREBPs: Transcriptional mediators of lipid homeostasis. *Cold Spring Harb Symp Quant Biol*. 2002;67:491-498.

70. Horton JD. Sterol regulatory element-binding proteins: Transcriptional activators of lipid synthesis. *Biochem Soc Trans*. 2002;30(Pt 6):1091-1095. doi: 10.1042/ [doi].

71. Lee P, Mori A, Takemitsu H, Yamamoto I, Arai T. Lipogenic gene expression in abdominal adipose and liver tissues of diet-induced overweight cats. *Vet J*. 2011;190(2):e150-3. doi: 10.1016/j.tvjl.2011.04.003 [doi].

72. Strable MS, Ntambi JM. Genetic control of de novo lipogenesis: Role in diet-induced obesity. *Crit Rev Biochem Mol Biol*. 2010;45(3):199-214. doi: 10.3109/10409231003667500 [doi].

73. Rouvinen-Watt K, Mustonen AM, Conway R, et al. Rapid development of fasting-induced hepatic lipidosis in the american mink (neovison vison): Effects of food deprivation and re-alimentation on body fat depots, tissue fatty acid profiles, hematology and endocrinology. *Lipids*. 2010;45(2):111-128. doi: 10.1007/s11745-009-3377-4 [doi].

74. Byrne CD. Fatty liver: Role of inflammation and fatty acid nutrition. *Prostaglandins Leukot Essent Fatty Acids*. 2010;82(4-6):265-271. doi: 10.1016/j.plefa.2010.02.012 [doi].

75. Hoenig M, Thomaseth K, Waldron M, Ferguson DC. Insulin sensitivity, fat distribution, and adipocytokine response to different diets in lean and obese cats before and after weight loss. *Am J Physiol Regul Integr Comp Physiol*. 2007;292(1):R227-34. doi: 00313.2006 [pii].

76. Mazaki-Tovi M, Abood SK, Segev G, Schenck PA. Alterations in adipokines in feline hepatic lipidosis. *J Vet Intern Med*. 2013;27(2):242-249. doi: 10.1111/jvim.12055 [doi].

77. Matsuzawa Y, Funahashi T, Nakamura T. Molecular mechanism of metabolic syndrome X: Contribution of adipocytokines adipocyte-derived bioactive substances. *Ann N Y Acad Sci*. 1999;892:146-154.

78. Miller C, Bartges J, Cornelius L, Norton N, Barton M. Tumor necrosis factor-alpha levels in adipose tissue of lean and obese cats. *J Nutr*. 1998;128(12 Suppl):2751S-2752S.

79. Lewis JR, Mohanty SR. Nonalcoholic fatty liver disease: A review and update. *Dig Dis Sci*. 2010;55(3):560-578. doi: 10.1007/s10620-009-1081-0 [doi].

80. Petta S, Muratore C, Craxi A. Non-alcoholic fatty liver disease pathogenesis: The present and the future. *Dig Liver Dis*. 2009;41(9):615-625. doi: 10.1016/j.dld.2009.01.004 [doi].

81. Musso G, Gambino R, Cassader M. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Prog Lipid Res*. 2009;48(1):1-26. doi: 10.1016/j.plipres.2008.08.001 [doi].

82. Kruitwagen HS, Spee B, Schotanus BA. Hepatic progenitor cells in canine and feline medicine: Potential for regenerative strategies. *BMC Vet Res*. 2014;10:137-6148-10-137. doi: 10.1186/1746-6148-10-137 [doi].

83. IJzer J, Kisjes JR, Penning LC, Rothuizen J, van den Ingh TS. The progenitor cell compartment in the feline liver: An (immuno)histochemical investigation. *Vet Pathol*. 2009;46(4):614-621. doi: 10.1354/vp.07-VP-0097-I-FL [doi].

84. Warren A, Center S, McDonough S, et al. Histopathologic features, immunophenotyping, clonality, and eubacterial fluorescence in situ hybridization in cats with lymphocytic cholangitis/cholangiohepatitis. *Vet Pathol*. 2011;48(3):627-641. doi: 10.1177/0300985810384409 [doi].

85. Katoonizadeh A, Nevens F, Verslype C, Pirenne J, Roskams T. Liver regeneration in acute severe liver impairment: A clinicopathological correlation study. *Liver Int*. 2006;26(10):1225-1233. doi: LIV1377 [pii].

86. Roskams T, Cassiman D, De Vos R, Libbrecht L. Neuroregulation of the neuroendocrine compartment of the liver. *Anat Rec A Discov Mol Cell Evol Biol*. 2004;280(1):910-923. doi: 10.1002/ar.a.20096 [doi].

87. IJzer J, Schotanus BA, Vander Borght S, et al. Characterisation of the hepatic progenitor cell compartment in normal liver and in hepatitis: An immunohistochemical comparison between dog and man. *Vet J*. 2010;184(3):308-314. doi: 10.1016/j.tvjl.2009.02.016 [doi].

88. Schotanus BA, van den Ingh TS, Penning LC, Rothuizen J, Roskams TA, Spee B. Cross-species immunohistochemical investigation of the activation of the liver progenitor cell niche in different types of liver disease. *Liver Int*. 2009;29(8):1241-1252. doi: 10.1111/j.1478-3231.2009.02024.x [doi].

89. Guyot C, Lepreux S, Combe C, et al. Hepatic fibrosis and cirrhosis: The (myo) fibroblastic cell subpopulations involved. *Int J Biochem Cell Biol*. 2006;38(2):135-151. doi: S1357-2725(05)00272-4 [pii].

90. Otte CM, Valtolina C, Vreman S, et al. Immunohistochemical evaluation of the activation of hepatic progenitor cells and their niche in feline lymphocytic cholangitis. *J Feline Med Surg*. 2017:1098612X17699723. doi: 10.1177/1098612X17699723 [doi].

91. Roskams TA, Libbrecht L, Desmet VJ. Progenitor cells in diseased human liver. *Semin Liver Dis*. 2003;23(4):385-396. doi: 10.1055/s-2004-815564 [doi].

92. Yang S, Koteish A, Lin H, et al. Oval cells compensate for damage and replicative senescence of mature hepatocytes in mice with fatty liver disease. *Hepatology*. 2004;39(2):403-411. doi: 10.1002/hep.20082 [doi].

93. Twedt DC, Armstrong PJ. Feline inflammatory liver disease. In: Bonagura JD, Twedt DC, eds. *Kirk's current veteriary therapy XIV*. Vol 14. 14 edition ed. Saunders Elsevier, Philadelphia.; 2008:576.

94. Gagne JM, Weiss DJ, Armstrong PJ. Histopathologic evaluation of feline inflammatory liver disease. *Vet Pathol*. 1996;33(5):521-526.

95. Santoni-Rugiu E, Jelnes P, Thorgeirsson SS, Bisgaard HC. Progenitor cells in liver regeneration: Molecular responses controlling their activation and expansion. *APMIS*. 2005;113(11-12):876-902. doi: APMapm_386 [pii].

96. Libbrecht L, Desmet V, Van Damme B, Roskams T. Deep intralobular extension of human hepatic 'progenitor cells' correlates with parenchymal inflammation in chronic viral hepatitis: Can 'progenitor cells' migrate? *J Pathol*. 2000;192(3):373-378. doi: 10.1002/1096-9896(2000)9999:99993.0.CO;2-5 [pii].

97. Lowes KN, Brennan BA, Yeoh GC, Olynyk JK. Oval cell numbers in human chronic liver diseases are directly related to disease severity. *Am J Pathol*. 1999;154(2):537-541. doi: S0002-9440(10)65299-6 [pii].

98. Roskams T. Different types of liver progenitor cells and their niches. *J Hepatol*. 2006;45(1):1-4. doi: S0168-8278(06)00242-X [pii].

99. Richardson MM, Jonsson JR, Powell EE, et al. Progressive fibrosis in nonalcoholic steatohepatitis: Association with altered regeneration and a ductular reaction. *Gastroenterology*. 2007;133(1):80-90. doi: S0016-5085(07)00994-8 [pii].

100. Spee B, Carpino G, Schotanus BA, et al. Characterisation of the liver progenitor cell niche in liver diseases: Potential involvement of wnt and notch signalling. *Gut*. 2010;59(2):247-257. doi: 10.1136/gut.2009.188367 [doi].

101. Carpino G, Renzi A, Onori P, Gaudio E. Role of hepatic progenitor cells in nonalcoholic fatty liver disease development: Cellular cross-talks and molecular networks. *Int J Mol Sci*. 2013;14(10):20112-20130. doi: 10.3390/ijms141020112 [doi].

102. Bedossa P, Paradis V. Liver extracellular matrix in health and disease. *J Pathol*. 2003;200(4):504-515. doi: 10.1002/path.1397 [doi].

103. Morini S, Carotti S, Carpino G, et al. GFAP expression in the liver as an early marker of stellate cells activation. *Ital J Anat Embryol*. 2005;110(4):193-207.

104. Center SA, Warner KL, Randolph JF, Sunvold GD, Vickers JR. Influence of dietary supplementation with (L)-carnitine on metabolic rate, fatty acid oxidation, body condition, and weight loss in overweight cats. *Am J Vet Res*. 2012;73(7):1002-1015. doi: 10.2460/ajvr.73.7.1002 [doi].

Chapter 8

Samenvatting in het Nederlands

Riassunto della tesi in italiano

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Acknowledgments

Samenvatting in het Nederlands

De lever is een orgaan met diverse belangrijke functies: het maakt eiwitten, speelt een rol bij de stofwisseling van glucose, ontgift het bloed, produceert gal die een rol speelt bij de vertering van vet en de absorptie van vitaminen uit het maagdarmkanaal, maakt stollingsfactoren die een belangrijke rol spelen bij de bloedstolling en reguleert de stofwisseling van vet. Leververvetting of leverlipidose is de meest voorkomende leverziekte bij de kat en treedt op bij katten die gedurende een aantal dagen niet hebben gegeten. De ziekte wordt gekenmerkt door de stapeling van vet in de lever (steatose), voornamelijk triglyceriden, waardoor de lever groter wordt en niet meer normaal kan functioneren. Dit leidt tot verstoringen in de vele leverfuncties en uiteindelijk tot het overlijden van de patiënt.
Hoewel leververvetting vooral voorkomt bij katten van middelbare leeftijd (rond de leeftijd van 7 jaar), kunnen katten van elke leeftijd het krijgen. Er is geen duidelijke voorkeur wat betreft ras of geslacht (mannelijk versus vrouwelijk) voor deze ziekte, maar in sommige studies lijken poezen meer te zijn vertegenwoordigd. Leververvetting wordt onderverdeeld in een primaire en secundaire vorm. Primaire leververvetting treedt op bij gezonde katten die stoppen met eten door een sterk verminderd aanbod van voer, het geven van voer dat ze niet lekker vinden of verminderde inname als gevolg van stressvolle omstandigheden. Secundaire leververvetting is de meest voorkomende vorm, die in ongeveer 95% van de gevallen optreedt. De oorzaak hierbij is een onderliggende ziekte die de kat zo ziek maakt dat ze stopt met eten.

Het mechanisme, de pathofysiologie, waardoor leververvetting ontstaat bij de kat is complex en heeft zeer waarschijnlijk meerdere oorzaken. Katten zijn bovendien 'obligate vleeseters' op basis van hun voedingsbehoefte en zijn afhankelijk van hun eten voor de beschikbaarheid van een aantal belangrijke essentiële vetten en vitaminen. Zodra ze stoppen met eten zouden voedingsdeficiënties dus de leverziekte kunnen veroorzaken of verergeren.

In **hoofdstuk 2** wordt een gedetailleerd overzicht gegeven over de pathofysiologie, de klinische verschijnselen en presentatie van leververvetting bij de kat. Het accent ligt hierbij op de karakteristieken en behoeften van de kat als een obligate vleeseter.

In een gezonde kat worden vetzuren die vrijkomen uit de vetopslag in de buik of het onderhuids weefsel naar de lever getransporteerd, waar zij worden omgezet in triglyceriden. Vervolgens worden de triglyceriden in de bloedsomloop gebracht door middel van specifieke eiwitten, zogeheten 'very-low-density-lipoproteïnen' (VLDLs). Vetzuren kunnen ook worden gebruikt om energie te produceren via een proces genaamd 'bèta-oxidatie' of ze kunnen worden gebruikt om nieuwe (*de novo*) vetten te maken. Ook al is de exacte pathofysiologie van leververvetting bij de kat nog niet bekend, het is in ieder geval een gevolg van een disbalans tussen de hoeveelheid vetzuren die door de lever worden opgenomen aan de ene kant en de verwijdering van triglyceriden uit de lever via VLDLs, de vorming van *de novo* vetten en de snelheid van de bèta-oxidatie aan de andere kant.

Het doel van dit promotieonderzoek is enkele karakteristieken van de diverse routes voor vetmobilisatie, de vetstofwisseling in de lever en de *de novo* vetvorming bij gezonde katten en katten met leververvetting te ontrafelen. In verschillende hoofdstukken in dit promotieonderzoek wordt als vergelijking de situatie beschreven bij de mens, diersoorten die kunnen worden vergeleken met de kat als obligate vleeseter, de nerts. Niet-alcoholische vetleverziekte ('non-alcoholic fatty liver disease'; NAFLD) is een aandoening bij de mens waarbij door overgewicht of een onderliggende ziekte, zoals suikerziekte, enorme hoeveelheden vetzuren zich stapelen in de lever.

In **hoofdstuk 3** wordt de rol van het transporteiwit VLDL onderzocht in gezonde katten en het effect van geslachtshormonen op de vorming van dit eiwit. Uit studies bij de mens en bij andere diersoorten is bekend dat de productie van VLDLs afhankelijk is van de aanwezigheid van fosfatidylcholine, dat door de lever wordt gemaakt van choline. Choline is één van de B-vitamines en de kat is voor de beschikbaarheid van vitamine B afhankelijk van voeding. Als katten stoppen met eten en de inname van choline sterk afneemt, vermindert de beschikbaarheid van fosfatidylcholine en kan ook een afname optreden in de hoeveelheid VLDLs. De lever zou hierdoor geen triglyceriden kunnen uitscheiden en stapelen zij zich op in de lever. Het beeld van leververvetting of steatose kan zich ontwikkelen.

Vrouwen vóór de menopauze zijn in staat fosfatidylcholine te produceren onder de invloed van het geslachtshormoon oestrogeen, waarbij een alternatieve route, de zogeheten 'PEMT route', wordt gebruikt als zij op een dieet met een laag choline gehalte staan. Als dit fenomeen ook optreedt bij de kat, zouden intacte poezen beter beschermd kunnen zijn tegen leververvetting, terwijl daarentegen

katers en gecastreerde poezen een predispositie zouden kunnen hebben als zij stoppen met eten. Om deze hypothese te toetsen zijn fosfatidylcholine en andere vetten, die voornamelijk via de PEMT route worden geproduceerd, gemeten bij poezen en katers voor- en nadat ze werden gecastreerd. De katten kregen een dieet met een laag, maar niet deficiënt, gehalte aan choline. Het resultaat van de studie toont aan dat er geen invloed van geslachtshormonen op de fosfatidylcholine synthese en op de PEMT route is. De huidige aanbevelingen voor de diëtaire behoefte aan choline zijn voldoende voor gecastreerde poezen. En het is dus onwaarschijnlijk dat castratie, door het veroorzaken van een fosfatidylcholine deficiëntie, katten predisponeert voor leververvetting zoals dat wordt gevonden bij andere soorten. Tenminste, als het aanbevolen gehalte aan choline in het dieet aanwezig is.

De rol van vetten in het ontstaan en de ontwikkeling van diverse ziekten bij de mens, zoals suikerziekte, vetzucht, NAFLD, Alzheimer en arteriosclerose, is bekend. De erkenning van geslacht-specifieke genetische verschillen in de vetsamenstelling en de vetstofwisseling tussen mannen en vrouwen heeft bijgedragen aan het identificeren van risicofactoren voor deze aandoeningen en specifieke, gerichte behandelingen te ontwikkelen. In de humane geneeskunde wordt de vetstofwisseling bestudeerd en geëvalueerd door het vetprofiel te analyseren met behulp van 'lipidomics'. Deze analysetechniek is zeer belangrijk geworden in het ophelderen van de rol van vetten in de pathofysiologie van vet-gerelateerde ziekten. Geslachtsverschillen tussen poezen en katers en hun 'geslachtsprofiel', i.e. intact versus gecastreerd, verdient meer aandacht.

In **hoofdstuk 4** is het vetprofiel van intacte en gecastreerde gezonde katten, die werden gevoed met een standaard, commercieel beschikbaar dieet, onderzocht met behulp van lipidomics. Bovendien werd het effect van castratie op het plasma en lever vetprofiel geëvalueerd. Tenslotte werd ook het plasma en lever vetprofiel van katten met leververvetting in detail onderzocht met de lipidomics techniek.

Het resultaat van deze studie liet zien dat geslachtsverschillen in het vetprofiel ook bij de kat bestaan. Intacte poezen hebben significant lagere triglyceriden gehalten in het plasma in vergelijking tot katers. Dit is vergelijkbaar met de situatie bij vrouwen vóór de menopauze en bij intacte vrouwelijke ratten en muizen. Dit verschil verdween echter niet na een verlaging van het plasma oestrogeen gehalte door middel van castratie. In tegendeel, het verschil nam toe door een toename in het plasma triglyceriden gehalte bij katers na castratie. Intacte poezen hadden ook een hogere hoeveelheid van een specifiek vetmolecuul, arachidonzuur, in zowel plasma als lever in vergelijking tot gecastreerde poezen

en katers. Arachidonzuur wordt geassocieerd met de ontwikkeling van ontstekingen. Castratie vermindert het arachidonzuur gehalte bij poezen en vermindert ook de negatieve effecten die dit molecuul kan hebben op de lever.
Zoals ook vastgesteld in andere studies, was bij katten met leververvetting het triglyceride gehalte in hun lever verhoogd in vergelijking tot de situatie bij gezonde katten. Het verhoogde gehalte van sfingomyeline bij katten met leververvetting was een interessante, nieuwe bevinding van deze studie. Sfingomyeline is een specifiek soort vet dat vooral voorkomt in de celmembraan. De beschikbaarheid van palmitinezuur, dat is verhoogd in de lever en vetweefsel bij katten met leververvetting, is een snelheidsbepalende factor in de vorming van sfingomyeline. Bij de mens veroorzaakt de toegenomen vorming van sfingomyeline in de lever, die is aangetast door NAFLD, een toename in de ontsteking van de lever en in het afsterven van levercellen. Sfingomyeline is dus geassocieerd met een progressie van de leverziekte. De toename in sfingomyeline gehaltes, zowel in het plasma als de lever, bij katten met leververvetting vraagt om aanvullend onderzoek om beter te begrijpen wat de potentiële rol van sfingomyeline is bij de kat met leververvetting.

In **hoofdstuk 5** wordt de mogelijke bijdrage van *de novo* synthese van vetten in de lever (*de novo* lipogenese) aan de vetophoping in de lever geëvalueerd. Bij de mens is gesuggereerd dat *de novo* lipogenese abnormaal is toegenomen en een belangrijke bijdrage levert aan de pathogenese van steatose in ziekten zoals NAFLD. Het is een belangrijke, afwijkende route in de ontwikkeling van lipidose. Het belangrijkste product van *de novo* lipogenese is het vetzuur palmitinezuur. In eerdere studies werd palmitinezuur bij katten met leververvetting in zowel de lever als vetweefsel in de buik gevonden. Dit suggereert dat de vetzuren in de lever zowel afkomstig kunnen zijn van buikvet, als ook nieuw kunnen worden gemaakt in de lever van zieke katten.

Er zijn enkele genen die belangrijk zijn in de regulatie van *de novo* lipogenese. 'Sterol regulatory element-binding factor' (SRBF) is de centrale controleur van de vet biosynthese en belangrijkste regulator van de expressie van diverse genen, die zijn betrokken bij nieuwe vetsynthese. Met de hulp van een speciale techniek, 'polymerase chain reaction' (PCR), kon de activatie van genen worden geëvalueerd in de lever en het bloed van gezonde katten en katten met leververvetting. Bij katten met leververvetting was niet alleen de SRBF expressie tien keer minder in vergelijking met de situatie bij gezonde katten, maar was ook de expressie van andere genen betrokken bij de lipogenese niet toegenomen. Dit suggereert dat bij de kat met leververvetting *de novo* lipogenese niet optreedt in de lever zoals bij de mens en de nerts, maar eerder afkomstig is uit het vetweefsel van de buik.

Dus, *de novo* lipogenese in de lever is niet een belangrijke route in de ontwikkeling van leververvetting bij de kat.
Vetophoping in de lever moet, volgens toenemend bewijs uit de humane geneeskunde, niet worden beschouwd als een 'onschuldige toeschouwer' bij NAFLD. Het is gelinkt aan de ontwikkeling van ontsteking (steatohepatitis) en de progressie van de leverziekte. Als leververvetting bij de kat wordt geëvalueerd op basis van histologisch onderzoek, i.e. het onderzoek van de betrokken celtypen en de verandering in het leverweefsel als gevolg van de ziekte, leek het centrale kenmerk simpele steatose. In tegenstelling tot de situatie bij NAFLD bij de mens is de aanwezigheid van ontsteking en de ontwikkeling van steatohepatitis en chronisch leverfalen bij de kat nog niet eerder gedocumenteerd.

Het weefsel van de lever, i.e. het parenchym, bestaat uit hepatocyten, de meest voorkomende levercellen, en andere niet-parenchymale cellen, zoals Kupffer cellen (in de lever voorkomende macrofagen), stellaatcellen, die betrokken zijn bij de stofwisseling van vitamine A, en epitheelcellen van de galgangen. Zij dragen allemaal bij aan de homeostase van de lever.

Deze cellen kunnen allemaal het doelwit zijn van de toxiciteit van vet en bij NAFLD is schade aan cellen vaak een aanwijzing voor de overgang van steatose naar steatohepatitis en chronische leverziekte. Een 'slapende' populatie stamcellen, de lever progenitor cellen die het vermogen hebben om zich te differentiëren in diverse celtypen, wordt geactiveerd als de leverschade ernstig genoeg is om de delingscapaciteit van hepatocyten te remmen, zoals bij de ophoping van vet in de lever. Belangrijke cellulaire componenten van de 'lever progenitor cel niche' zijn stellaatcellen, en de Kupffer cel/levermacrofaag. Laminine is de meest voorkomende component in het steunweefsel, het stroma. Het aantal lever-progenitor cellen en de activatie van hun niche zijn bij de mens en de hond gerelateerd aan de ernst van een ziekte. Eerder onderzoek heeft aangetoond dat de lever van katten met leververvetting progenitor cellen bevat, maar de manier waarop zij worden geactiveerd en de wijze waarop zij op elkaar inwerken is nooit verder onderzocht.

In **hoofdstuk 6** is de lever progenitor cel bij de kat en haar niche onderzocht bij katten met leververvetting. Deze bevindingen zijn vergeleken met de situatie bij gezonde controle katten. Hiervoor is er gebruik gemaakt van specifieke immunokleuringen die in staat zijn om de verschillende celtypen, die de lever progenitor cel niche vormen, te kleuren en hun interactie te herkennen. De resultaten van de studie toonden aan dat bij katten met leververvetting de lever

progenitor cel niche bestaat uit Kupffer cellen, geactiveerde stellaatcellen, i.e. myofibroblasten, en de extracellulaire matrix component laminine. Dubbele immunofluorescentie toonde bovendien aan dat als lever progenitor cellen worden geactiveerd, een ruimtelijke relatie tussen de progenitor cellen en hun niche kon worden aangetoond. Er was echter geen duidelijk omschreven verband tussen de histopathologische mate van vervetting en de mate van de activatie van de lever progenitor cellen of de celcomponenten van de niche bij de katten met leververvetting.

Het gebrek aan een duidelijk activatiepatroon van lever progenitor cellen ondanks de aanwezigheid van ernstige steatose bij de katten met leververvetting, zoals dat gezien wordt bij de mens en hond, kan door verschillende factoren worden verklaard. Allereerst is leververvetting bij de kat een acute aandoening. Leververvetting ontwikkelt zicht bij de kat meestal vrij snel, ook al kan het soms dagen tot weken duren voordat het ontstaat; als de kat weer eten binnenkrijgt, verbetert de klinische conditie van de kat waarbij de vervetting verdwijnt, of de kat verbetert niet en sterft of wordt geëuthanaseerd nog voordat veranderingen in lever progenitor cel niche zich kunnen ontwikkelen. Ten tweede kunnen de wisselende resultaten in de lever progenitor cel activatie ook zijn veroorzaakt door de effecten op de lever van de diverse onderliggende ziekten, die voor de kat de directe aanleiding waren om te stoppen met eten, of de therapeutische maatregelen die de behandelend dierenarts heeft genomen. Al deze verklaringen zijn op dit moment nog erg speculatief.

Samenvattend kan worden gezegd dat er meer inzicht is verkregen in de vetstofwisseling bij de gezonde kat en enkele specifieke aspecten van de pathofysiologie van leververvetting bij de kat. Eén aspect is in ieder geval opvallend in deze studies: de kat is een uniek dier met fysiologische en metabole eigenaardigheden in het vet- en vetzuurmetabolisme, waardoor de kat zich onderscheidt van ander niet-obligate vleeseters, zoals de hond, rat, muis en mens. Als alle resultaten worden overwogen en worden gecombineerd met experimentele studies bij katten met overgewicht die extreem gewichtsverlies ondergingen en daarbij leververvetting ontwikkelden, kan er worden beargumenteerd dat het onwaarschijnlijk is dat afgenomen VDLD excretie door de lever en toegenomen *de novo* vetsynthese in de lever een zeer belangrijke rol spelen in de ophoping van vet in de lever bij leverlipidose bij de kat. De meest waarschijnlijke pathofysiologische route voor de ontwikkeling van leververvetting bij de kat lijkt een insufficiënte toename in de snelheid van de vetzuuroxidatie in de lever met tegelijkertijd een sterk toegenomen perifere vetmobilisatie.

Riassunto della tesi in italiano

Il fegato è un organo importante che normalmente ricopre molteplici funzioni: produce nuove proteine, aiuta la regolazione del glucosio, disintossica il sangue dalle sostanze prodotte dall'organismo, produce la bile che è importante per la digestione dei grassi e l'assorbimento di vitamine, produce i fattori della coagulazione importanti per la coagulazione del sangue e regola il metabolismo dei lipidi.

La lipidosi epatica felina è considerata la malattia epatica più comune nei gatti e si verifica nei gatti che non mangiano da alcuni giorni. È caratterizzata dall'accumulo nel fegato di molecole di grasso (*steatosi epatica*), principalmente trigliceridi, che fanno diventare il fegato più grosso e malfunzionante. Sebbene la lipidosi epatica sia principalmente riportata nei gatti di mezza età (età media 7 anni), possono soffrirne gatti di qualsiasi età. Anche se non è stata segnalata una predisposizione di razza o di genere (maschio vs femmina) per questa malattia, in alcuni studi le gatte femmine sembrano essere più rappresentate.

La lipidosi epatica felina è classificata come lipidosi epatica primaria o secondaria. La lipidosi epatica felina primaria si verifica in gatti sani che smettono di mangiare a causa della somministrazione di cibo non appetitoso, o per la riduzione dell'assunzione di cibo a seguito di un evento che provoca stress eccessivo. Invece, la lipidosi secondaria, che si verifica in circa il 95% dei casi, è considerata la forma più comune di lipidosi, e. si sviluppa quando un gatto smette di mangiare inseguito a molteplici cause, soprattutto per una malattia stottostante.
In generale, il meccanismo (fisiopatologia) alla base della lipidosi epatica felina è complesso. Inoltre, poiche' i gatti essendo 'carnivori obbligati' dipendono dall'assunzione con il loro cibo di importanti vitamine e acidi grassi essenziali, quando smettono di mangiare, queste carenze potrebbero innescare e peggiorare la lipidosi epatica.

Il **Capitolo 2** fornisce una riassunto dettagliato di ciò che è noto sulla fisiopatologia, sui segni clinici e sulla presentazione della lipidosi epatica felina, con particolare attenzione alle caratteristiche e ai bisogni specifici del gatto come carnivoro.

In un gatto sano, gli acid grassi che vengono rilasciati dai depositi di grasso nell'addome o nel tessuto sottocutaneo, raggiungono il fegato dove possono: essere trasformati in trigliceridi ed esportati nella circolazione attraverso delle proteine specifiche, chiamate lipoproteine a bassissima densità ("very low density lipoprotein": VLDL); essere utilizzati per produrre energia attraverso un processo chiamato beta ossidazione; oppure essere usati per produrre nuovi lipidi (*de novo* lipogenesi). Sebbene l'esatto meccanismo patofisiologico della lipidosi epatica felina non sia del tutto compreso, sappiamo che la lipidosi epatica felina è il risultato di uno squilibrio tra la quantità di acidi grassi che raggiunge il fegato, l'eliminazione dal fegato dei trigliceridi, la formazione di nuovi acidi grassi ed l'utilizzo degli acidi grassi per la formazione di energia.

Lo scopo di questa tesi è un di ricercare e descrivere le diverse vie patofisiologiche della lipidosi epatica felina: la mobilizzazione dei lipidi, il metabolismo dei lipidi a livello epatico e la sintesi *de novo* lipidica in gatti sani ed in gatti con lipidosi epatica. Nei diversi articoli di questa tesi abbiamo spesso utilizzato, come confronto, ciò che è noto nella medicina umana e in un altro animale che ha molte similitudini con il gatto, anch'esso un carnivoro obbligato, il visone. Per quanto riguarda l'uomo, a causa dell'obesità o della presenza di altre malattie come il diabete, gli acid grassi si possono accumulare nel fegato dando origine ad una malattia chiamata steatosi epatica non alcolica (NAFLD).

Nel **Capitolo 3** abbiamo studiato il ruolo della proteina di trasporto VLDL nei gatti sani e l'effetto degli ormoni sessuali sulla sintesi di questa proteina. Sappiamo dalla medicina umana e da studi su altri animali da ricerca, che la produzione di VLDL dipende dalla presenza di fosfatidilcolina, che a sua volta è costituita dalla molecola colina. La colina è considerata una vitamina del gruppo B e sappiamo che i gatti dipendono dall'assunzione di vitamine del gruppo B con il loro cibo. Se smettono di mangiare, l'assunzione di colina può essere molto bassa, diminuendo la disponibilità di fosfatidilcolina e causando un ulteriore diminuzione delle VLDL. Se le VLDL non sono disponibili, non è possibile esportare i trigliceridi dal fegato che si accumuleranno dando origine alla steatosi epatica.

Tuttavia, dalla medicina umana sappiamo che gli ormoni estrogeni, elevati nella donna in pre-menopausa, sono in grado, a fronte di una dieta a basso contenuto di colina, di stimolare la sintesi di fosfatidilcolina attarverso un via metabolica alternativa chiamata. Se questo si verificasse anche nei gatti, allora la gatta femmina non sterilizzata sarà protetta dalla lipidosi, mentre i maschi e le gatte sterilizzate potrebbero essere predisposti allo sviluppo di lipidosi per carenza di

fosfatidilcolina, una volta smesso di mangiare. Per valutare la nostra ipotesi abbiamo misurato la fosfatidilcolina, la colina e altri lipidi che si formano principalmente attraverso la via PEMT, nel fegato e nel sangue di gatti sani (sia maschi che femmine) prima e dopo la loro sterilizzazione/castrazione. I gatti avevano ricevuto una dieta che era bassa, ma non priva, di colina. I risultati del nostro studio mostrano tuttavia che non vi è alcuna influenza degli ormoni sessuali estrogeni sulla sintesi della fosfatidilcolina e sulla via PEMT nel gatto. Da questo studio possiamo concludere che le attuali raccomandazioni per i livelli di colina nella dieta sono adeguate per i gatti ed è improbabile che la sterilizzazione predisponga le gatte femmine alla lipidosi epatica causando una carenza di fosfatidilcolina.

Il ruolo dei lipidi nello sviluppo e nella progressione di alcune malattie negli uomini, come il diabete mellito, l'obesità, la NAFLD l'Alzheimer e l'arteriosclerosi è ben riconosciuto. In medicina umana, il riconoscimento delle differenze genetiche nel metabolismo lipidico specifiche tra maschio e femmina ha aiutato a sviluppare terapie più specifiche e mirate per queste patologie. In medicina umana, il profilo lipidico, chiamato anche "lipidomics", è diventato fondamentale per comprendere il ruolo dei lipidi nella patofisiologia di alcune malattie che coinvolgono alterazioni dei grassi. Le differenze lipidiche tra maschio e femmina e tra il loro profilo sessuale (intatto o sterilizzato / castrato) nei gatti merita ulteriore attenzione.

Nel **Capitolo 4** il profilo lipidico di gatti sani intatti nutriti con una dieta commerciale comune, viene studiato con l'aiuto dell'analisi lipidomica. Inoltre, viene valutato l'effetto della sterilizzazione/castrazione sul profilo lipidico nei gatti sani. Infine, il profilo lipidico dei gatti con lipidosi epatica viene esaminato in dettaglio.

I risultati di questo studio indicano che esistono differenze sessuali nel profilo dei lipidi nei gatti. Infatti, le femmine intatte hanno livelli di trigliceridi significativamente più bassi nel plasma rispetto ai gatti maschi, analogamente a quello che viene riportato nelle donne in premenopausa, ratti e topi femmina intatti. Tuttavia, questa differenza non cambia con la diminuzione degli ormoni estrogeni dopo sterilizzazione nelle gatte femmine, ma piuttosto aumenta a causa di un incremento del livello di trigliceridi nel plasma dei gatti maschi dopo la castrazione. Le gatte femmine non sterilizzate hanno anche un contenuto più elevato di una specifica molecola lipidica, acido arachidonico, sia nel plasma che nel tessuto epatico, rispetto alla femmina sterilizzata e ai gatti maschili. L'acido arachidonico è stato associato allo sviluppo di un processo infiammatorio.

La sterilizzazione nelle gatte femmine, riducendo la concentrazione di acido arachidonico, riduce anche i possibili effetti negativi che questa molecola potrebbe avere sul fegato.

Nei gatti affetti da lipidosi epatica abbiamo confermato i risultati degli studi precedenti, che hanno rilevato un'elevata concentrazione di trigliceridi nel fegato di questi animali, rispetto a gatti sani. L'aumento della concentrazione epatica e plasmatica di sfingomielina è una scoperta interessante e nuova nei gatti con lipidosi epatica. La sfingomielina è un tipo specifico di lipide principalmente presente nella membrana cellulare. La disponibilità di palmitato, che è elevata nel fegato e nel tessuto adiposo in gatti con lipidosi epatica, viene considerata il prerequisito fondamentale per la sintesi di sfingomielina. L'aumentata sintesi di sfingomielina nel fegato delle persone affette da NAFLD, promuove la morte delle cellule epatiche, aumenta l'infiammazione del fegato ed è stata associata alla progressione della malattia epatica. Le maggiori concentrazioni di sfingomielina, sia nel fegato che nel plasma dei gatti con lipidosi epatica, richiedono ulteriori studi al fine di comprendere il potenziale ruolo di questa molecola nella patofisiologia della lipidosi epatica felina.

Nel **Capitolo 5** viene valutata la nuova (*de novo*) sintesi epatica dei lipidi (*de novo* lipogenesi) come possibile via patofisiologica in grado di contribuire all'accumulo del grasso nel fegato nella lipidosi epatica felina. In medicina umana è stato riportato che la *de novo* lipogenesi aumenta in modo anormale in malattie come la NAFLD contribuendo in modo significativo alla sua patogenesi. Il prodotto principale della *de novo* lipogenesi è l'acido palmitico. Studi precedenti hanno evidenziato che nei gatti con lipidosi epatica, tale acido grasso e' presente in grandi quantità sia a livello epatico che nel grasso addominale. Questa scoperta suggerisce che la fonte primaria dei grassi che si accumulano nel fegato nei soggetti con lipidosi, è il tessuto adiposo addominale , ma non esclude una *de novo* lipogenesi a livello epatico.

Sappiamo che ci sono alcuni geni che sono importanti per la regolazione della *de novo* lipogenesi. Il gene chiamato SRBF, è considerato il gene più importante per la regolazione della sintesi dei grassi ed è il regolatore principale dell'espressione di altri geni coinvolti nella nuova sintesi lipidica. Con l'aiuto di una speciale tecnica chiamata "reazione a catena della polimerasi"(PCR) , la presenza di questo gene è stata valutata nel fegato e nel sangue di gatti sani e gatti con lipidosi. Tuttavia, nei gatti con lipidosi, l'espressione di SRBF nel fegato era 10 volte inferiore rispetto ai gatti sani, e l'espressione epatica degli altri geni coinvolti nella

nuova sintesi di lipidi, non era aumentata. Ciò suggerisce che nella lipidosi epatica felina, la sintesi *de novo* degli acidi grassi non si verifica nel fegato, come nelle persone e in altri carnivori obbligati, ma molto probabilmente principalmente nel tessuto grasso addominale. Quindi la *de novo* sintesi epatica dei grassi, non è una via patogenetica importante nello sviluppo della lipidosi nei gatti.

In medicina umana nei pazienti NAFLD, l'accumulo di lipidi nel fegato non viene considerato un processo benigno o di poca importanza, ma è stato associato allo sviluppo di infiammazione (steatoepatite), e alla progressione della malattia epatica. Quando la lipidosi epatica felina è valutata da un punto di vista istologico, la sua caratteristica principale sembra essere quella di semplice steatosi. A differenza della NAFLD, nella lipidosi epatica felina non sono mai state documentate la presenza di infiammazione, la sua la progressione a steatoepatite e l'insufficienza epatica cronica.

Il fegato contiene nel suo tessuto (parenchima) epatociti, le principali cellule del fegato, e altre cellule non parenchimali, come le cellule di Kupffer (macrofagi del fegato), cellule stellate epatiche (coinvolte nel metabolismo della vitamina A) e le cellule epiteliali biliari (costituiscono i dotti biliari), che contribuiscono a mantenere l'omeostasi del fegato. Tutte queste cellule sono bersagli della tossicità dei lipidi che nella NAFLD spesso indica la progressione dalla semplice steatosi alla steatoepatite e alla malattia epatica cronica. Quando il danno al fegato è sufficiente ad inibire la capacità replicativa degli epatociti, una popolazione di cellule a riposo, con la capacità di differenziarsi in diversi tipi di cellule, viene attivata: le cellule progenitrici epatiche. Nei roditori, nell'uomo e nei cani, queste cellule interagiscono tra loro ed un altro componente importante chiamato "laminina" e formano una nicchia, la nicchia delle cellule progenitrici epatiche. Le componenti cellulari importanti di questa nicchia includono le cellule progenitrici epatiche, le cellule epatiche stellate, le cellule di Kupffer e la laminina. Nell'uomo e nel cane il grado di attivazione delle cellule progenitrici epatiche e della nicchia sono direttamente correlate alla gravità della malattia epatica stessa. Studi precedenti hanno evidenziato la presenza delle cellule progenitrici epatiche nel gatto con lipidosi epatica, ma la loro attivazione ed interazione non è mai stato valutata.

Nel **Capitolo 6** le cellule progenitrici epatiche e la loro nicchia viene analizzata nei gatti con lipidosi epatica. Sono state utilizzate tecniche immunologiche di colorazione specifica, per evidenziare le diverse cellule che costituiscono le cellule progenitrici epatiche e la nicchia e per evidenziare la loro interazione. I risultati

dello studio dimostrano che nel gatto con lipidosi epatica le cellule progenitrici e la loro nicchia includono le cellule di Kupffer, le cellule epatiche stellate, e la componente extracellulare della matrice, la laminina. L'utilizzo della tecnica di colorazione con la doppia immunofluorescenza ha dimostrato che quando le cellule progenitrici sono attivate, esiste un'associazione spaziale tra le cellule stesse e la loro nicchia. Tuttavia, non siamo riusciti a trovare un pattern definito tra la presenza istopatologica della lipidosi e l'attivazione delle cellule progenitrici o delle componenti della nicchia in tutti i gatti con lipidosi epatica. Nonostante la presenza di grave steatosi in tutti i gatti con lipidosi, la mancanza di un pattern di attivazione comune della cellule progenitrici, come si vede in altri animali, potrebbe essere spiegato da diversi fattori.

Innanzitutto, la lipidosi epatica è classificata come malattia acuta. Anche se a volte richiede tempo per svilupparsi (giorni-settimane), la sua progressione è in genere piuttosto rapida; o la lipidosi si risolve rapidamente o il gatto spesso muore o viene addormentato. Questo potrebbe accadere prima che possano svilupparsi cambiamenti nella nicchia e nelle cellule progenitrici epatiche. In secondo luogo, le diverse modalità terapeutiche che vengono instaurate dal medico veterinario, potrebbero influire sull'attivazione delle cellule progenitrici. Tutte queste spiegazioni rimangono in questo momento speculative.

In conclusione, grazie a questi studi abbiamo fatto progressi nella comprensione della fisiologia del metabolismo dei lipidi nei gatti sani e in alcuni aspetti specifici della fisiopatologia della lipidosi epatica del gatto. Una cosa che è apparsa chiara da tutti gli studi è che il gatto è sicuramente un animale unico, con peculiarità fisiologiche e metaboliche nel metabolismo dei lipidi e degli acidi grassi che lo rende diverso dagli altri animali non strettamente-carnivori, come i cani, ratti, topi e gli uomini . Considerando i risultati dei nostri studi e i dati derivati da altri studi sperimentali in gatti in sovrappeso sottoposti ad una rapida perdita di peso con conseguente sviluppo di lipidosi epatica, possiamo concludere che una diminuzione dell'escrezione dei trigliceridi per una carenza delle VLDL e aumento della sintesi *de novo* lipidica a livello epatico non sono le cause più importanti di accumulo di grasso a livello epatico. La via fisiopatologica più probabile sembra quindi essere un insufficiente tasso di ossidazione a livello epatico degli acidi grassi a fronte di una maggiore mobilitazione periferica dei lipidi.

About the author

Chiara Valtolina, DVM, Research Doctorate, Dipl. ACVECC, Dipl. ECVECC

Chiara Valtolina was born on the 14th of June 1974 in Milan, Italy.

She started her veterinary medicine study in 1994 and she graduated with cum laude in 2000 at the Faculty of Veterinary Medicine, University of Milan, Italy. She worked for 4 years at the Surgery Department of Small Animal of the same faculty as a research doctorate.
In December 2004, at the end of her doctorate, after receiving the title of doctor in veterinary medicine, Chiara began an externship program at the Intensive Care Unit (IZA) of the Department of Clinical Science of Small Animals at the Faculty of Veterinary Medicine, Utrecht University, The Netherlands.
In June 2006 Chiara started her residency in Emergency and Critical Care at the Royal Veterinary College in London.
She terminated her residency in June 2009 and in September 2009 she became a diplomate of the American College of Emergency and Critical Care.
In 2014 she obtain the title of diplomate of the European College of Emergency and Critical Care.

She is currently working, since November 2009 as a staff clinician and lecturer in the Intensive Care Unit (IZA) of the Department of Clinical Science of Small Animals at the Faculty of Veterinary Medicine, Utrecht University in the Netherlands.

Chiara is also the vice-president of the European Emergency and Critical Care Society (EVECCS) and she is invited speaker in numerous national and international ECC congresses.

List of publications

Acocella F, Brizzola S, Valtolina C, Scanziani E, Marchesi F, Mantero S, Garreau H and Vert M. Prefabricated tracheal prosthesis with partial biodegradable materials: a surgical and tissue engineering evaluation in vivo. J. Biomater. Sci. Polymer Edn, Vol. 18, No. 5, pp. 579–594 (2007)

Valtolina C and Adamantos S. Evaluation of Small-Bore Wire-Guided Chest Drains For Management Of Pleural Space Disease. Journal of Small animal Practice, 50(6); 290-297.

Valtolina C, Robben J.H, Uilenreef J , Murrell J.C, Aspegrén J, McKusick B, Hellebrekers L.J. Clinical evaluation of the efficacy and safety of a constant rate infusion of dexmedetomidine for postoperative pain management in dogs. Vet Anaesth Analg. 2009 Jul;36(4):369-83.

Valtolina C, Vaandrager A.B, Favier P, Robben J.H Tuohetahuntila M, Kummeling A, Jeusette I and Rothuizen J. No up-regulation of the phosphatidylethanolamine N-methyltransferase pathway and choline production by sex hormones in cats. BMC Veterinary Research 2015; 11:280 .DOI: 10.1186/s12917-015-0591-6

Valtolina C, Vaandrager AB, Favier RP, Tuohetahuntila M, Kummeling A, Jeusette I, Rothuizen J, Robben JH. Sex specific differences in hepatic and plasma lipid profiles in healthy cats pre and post spaying and neutering: relationship with feline hepatic lipidosis. BMC Vet Res. 2017 Aug 8;13(1):231.

Valtolina C, Robben JH, Favier RP, Rothuizen J, Grinwis GC, Schotanus BA, Penning LC. Immunohistochemical characterisation of the hepatic stem cell niche in feline hepatic lipidosis: a preliminary morphological study. J Feline Med Surg. 2019 Volume: 21 issue: 2, page(s): 165-172

Valtolina C and Favier R.P. Feline Hepatic Lipidosis. Vet Clin Small Anim 47 (2017) 683–702

Otte CM, Valtolina C, Vreman S, Hubers S, van Wolferen ME, Favier RP, Rothuizen J, Penning LC. Immunohistochemical evaluation of the activation of hepatic progenitor cells and their niche in feline lymphocytic cholangitis. J Feline Med Surg. 2018 Jan;20(1):30-37.

den Toom M.L, Dobak T.P, Broens E.M and Valtolina C. Interstitial pneumonia and pulmonary hypertension associated with suspected ehrlichiosis in a dog Acta Vet Scand. 2016; 58: 46.

Valtolina C. and Goggs R. Pain Recognition and Management. Advanced Monitoring and Procedures for Small Animal Emergency and Critical Care, edited by Jamie Burkitt Creedon and Harold Davis. Section 7, chapter 42.

Acknowledgments

"Alone we can do so little. Together we can do so much" Helen Keller

The people that have been close to me during these last six years of my life know how much I often struggled with the "stupid" PhD.. I just wanted to be on clinics with the patients, where my heart is.. It has not always been a smooth road and often my motivation and my usual positive attitude were not always at their best during a "PhD week"..
However looking back, it is unfair to say that I did not enjoy at all the process and working on a PhD thesis allowed me to develop new skills that will remain with me for the rest of my carrier: from writing and developing a research project, analysing data, writing scientific papers and developing a taste for research.

"Success is a journey, not a destination. The doing is often more important than the outcome" A. Ashe

This journey also allowed me to work with great people that have helped me growing as a person and as a researcher and arriving at the finishing line, either with their collaboration, mental and physical support. I would not have been here today defending this PhD if it was not for all of you, that patiently listened to me, helped me, supported me during those years.

Unfortunately it is impossible to mention everyone in this chapter but for sure there are some persons that deserve my special appreciation.

My promotor Prof. Jan Willem Hesselink who took over few years ago this important role for my PhD. Thank you for your moral support and for your practical help with all the forms and other obscure things that needed to be done first on Horaest and then on MyPhD…but mostly for believing that my work was worth becoming a PhD thesis when I was still in my pessimistic sceptical mood.

My co-promotor Dr. Joris H. Robben. My greatest thanks is for you Joris, co-promotor during this PhD, mentor in my daily work, invaluable and dearest friend in my daily life. Thanks for everything, I would not have been the person and veterinarian I am without your important support and guidance in all these

years. Long (long) time ago you took a little Italian veterinarian under your shield and by restoring my motivation and enthusiasm for veterinary medicine you allowed me to spread my wings and obtain first my diplomate status and then this PhD. You are everyday an inspiration for me. I know that looking at you sitting (probably more nervous than me) with the committee, will give me the strength to stand up and defend this PhD and the conviction that everything at the end will be all right. Today my achievement will also be yours.

My co-promotor Dr. Robert Favier. Dear Robert I still remember when we met the first time back in 2005 during my externship period in ICU. I was for the majority of people just another visitor, but you were one of the few person to greet me every day and to ask how I was. You made me feel less lonely and invisible. I respect and admire your positive attitude in life, your enthusiasm for education and your work. Your contribution to this Phd, your mental support, nice words and texts have been irreplaceable during these long years and really helped me going through the finish line.

My "unofficial/official" co-promotor Dr. Louis Penning. Dear Louis, without you and your precious help my last two articles would have never been published. I respect your endless passion for research, your great mind that produces bright (although sometimes to cryptic for me to fully grasp) ideas. I am very grateful that you came on board of team "PhD Chiara" because your help and support has been really irreplaceable .

My two paranymphs and dearest friends Claudia and Karen. My dear Claudia, I told you once that you have been (and are) my rock, my best friend, my invaluable support through all these years. Together we shared secrets, holidays, laughs, tears, good moments and bad moment. We shared life in all her forms and we are still here and I could not imagine my life without you in it, at my side. Lieve Karen, my dearest friend, with the time passing by our friendship became stronger and stronger and you became for me an irreplaceable part of my life. You are one of the bravest, smartest person I know and the courage, strength you use in your life are for me an inspiration. Thanks for being here for me.

Dr. Bas Vandraager. Dear Bas, thank you for your help, important scientific contribution and support during the writing of my first two articles. I was lost in the world of biochemistry and lipids but you managed to make things clear and more understandable.

Dr. Hans Vernooj, I would like to thank you for attempting to teach me some

statistics and to show me that I could also (well..more or less) do it on my own.. but without your guidance it would have never been possible.

Dr Boukje Schotanus, Dr Guy Grinwijs, Dr Maidina Tuohetahuntila, Dr Anne Kummeling, Monique E. van Wolferen, Dr Hedwig S. Kruitwagen and all the other people that have participated and helped with the practical work in realising the different studies. Thanks you very much because without your help those studies would have never progressed as they did. Dr. Corma Otte, dear Corma, we share the same passion for cats, for liver disease and for veterinary medicine. It has been a pleasure for me to meet you and I still remember your supporting words when we met at the Caringvets meeting. They really helped me to look at my PhD in a more positive way!

The anaesthesia department and team and the UVDL team for helping me with my research studies, from sedating the cats for the surgeries and liver biopsies to analysing all the blood collected. Thanks for your great service!

Prof dr. Jan Rothuizen for giving me the possibility to do this PhD and for your support and help in the beginning of the initial studies. You were always very supportive and your incredible knowledge of veterinary hepatology very helpful during the writing of the papers.

Kees van Veneendaal for helping me making this book possible, for your professionalism, your great work. Joeke van der Meer for accepting to drawn the cover of my thesis and for your beautiful artwork. Your drawing made my thesis unique and beautiful.

"Be true to your work, your words and your friends" Henry David Thoreau

There are friends, there is family, and then there are friends that become family …and I am so lucky to have an "extended family" that I love dearly and that has always been there to support me in this PhD journey and at my side through these past years. I don't know what I would have done without you. My life is better with all of you in it.

Dear Susanne the first person that comes to my mind when I think of my extended family is you. Since the beginning when we met back in 2004, you have been always very kind to me. You open your arms, your house and your heart to me and you always treated me as a member of your family.. for me this feeling is priceless. You, Joris, Sam and Tim are for me my Dutch family. Your true

friendship and love supported me through all these years and I am so happy to share this day with you at my side.

Dear Delia, dear Tess! Our friendship goes back in time, a long time ago. I still remember the first day I met you at the reception desk of the surgery department of the university of Milan. That was the beginning of our friendship that in the years grew to something beautiful and the bond that we have is really strong. You are for me a sister and even if we are far away from each other we are never far from the heart.

Dear friend Annemarie. I always have to tell the story on how we have met in ICU, with me blessing everyone and you thinking I was your saving angel… I truly bless those first days in ICU back in 2009 because they allowed me to meet you and to get to know you. You are one of my dearest friend, a caring and loving person, always true to yourself and to the others, always available for your friends, even if that meant waking up very early in Australia to skype.

Lieve Mare, my dear friend Mare. We have known each other quite some time and been there with each other (for each other) in happy and difficult moments of our lives. Your courage, determination to change bad situations life confronted you with, into something good and positive, your self-compassion in accepting the person you became without looking back at what was before, is for me a great inspiration and I love you dearly.

Lieve Barbara. I still remember when I heard that a new young buurvrouw was coming to live above my house, I didn't know what to expect.. I definitely didn't expect to meet an amazing strong person, true animal lover and an invaluable great friend. We share the same beliefs and ethical life choices, the same frustrations on mankind, the love for vegan food and I love that we are trying to make this world a more compassionate place. Meeting you and your friendship made me a better person.

Anneleen and Sara my two dear Belgium friends with whom I shared dinner, drinks, birthdays, holidays, happy moment and personal lives. You were there for me also when I most needed you. You are very important to me and I truly hope, but I am sure of it, that our friendship will survive time and physical distance.

Dear Lars, we also know each other for many years and we share, in between lot of other things, our love for veterinary medicine and cats. I value our true

friendship very much and our dinner and conversations about life and personal matters are so precious to me. You can see easily through me and I always know that your words of reassurance and wisdom will always be there for me.

Dear Mark, we agree that being true friends doesn't mean calling each other every day but it means making the most of every time we see each other, being true to ourselves and open to the other. I am so grateful for every time we spent together and even if you are now a bit further away, nothing in between our friendship will change.

Steve, gekke en lieve Steve. I don't remember anymore how from that guy that works in the UVDL and makes jokes on the phone when you call about a blood results, you became one of my dearest friend. In between playing squash, dinners we became good friends. You are one of the best person I know and I am happy you can be here on this day.

Bas, Stefanie and Maurice, Hugo, Meredith, Christine and Rosalie, Sara Galac and Nicole. I met you all at work, where I didn't expect to find per se some good friends and as always, life surprises me. You are all more than just colleagues to me and I truly values all the time we spent together sharing our stories, frustration, hopes for the future and life matters. I love you all very much.

Dear Hans, a special acknowledgment goes to you for your great support in clinics and with my PhD and especially for trying to let me see the beauty in research and that research can be fun and exciting.

ICU (and not) technicians and friends: Maarten, Ilse, Dominique, Wout, Monique, Nick, Jose, Marie-Jose', Renske, Yvonne, Egbert, Karin Koelewijn. For keeping up with me in ICU and for always being supportive and an invaluable help during my daily work and during the data collection for my PhD.

Ruud, Kim, Tessa, Lisanne and Sanne. Colleagues from the SGMN, Sanne our resident in ECC, we worked together in ICU and in between a patient discussion and some emergencies, we managed to get to know each other better. Thanks for your support and good work.

Dear Tera, Marja, Lindsay, Alma, Rick, Tijn, Floor, Tjarda, Judith, Iris and everyone else that rotated with me in ICU. Thanks for your enthusiasm and great work. Working with all of you is always a pleasure.

My dear Italian friends Chiara, Nadia, Valentina, Anna, Paola. We know each other for so long, with some of you for more than 20 years, and even if we do not see each other very often you are for me a safe haven, a place that I can always come back to and that it is filled with love and warmth. The friendship and love that we share doesn't know distance or time passing by.

My mum, Rosanna. There is not a day that I don't think of you and wish you could be here with me. I miss your smile, your positive energy, your endless care, your love... every single day. You have always been my biggest supporter and you thought me that no matter how hard life is, it is always worth living it with a smile and a positive attitude.. you probably didn't realise how hard it is to be happy and positive in a life without you in it. I know that wherever you are now, you are probably smiling, very proud of me today for my achievement and for the person that I became.

My father Giuseppe and my brother Andrea, my family that I love very much, no matter what. Papino caro, spero davvero che tu possa essere con me in questo giorno importante perché non sarebbe completo senza averti al mio fianco. Grazie per il supporto e l'amore che mi hai dato in questi anni. Fratellino caro, Andrea, mi dispiace che tu non possa fare parte di questa giornata, dividere con me un momento felice e per una volta vedere il mondo in cui vivo. Anche se mi mancherai, so che ci sarai con la mente e con il cuore. Marina, sei diventata parte della mia famiglia ed in questi 15 anni abbiamo imparato a conoscerci e a volerci bene. Sono felice di averti vicina a me in questo giorno importante

My favourite aunt Lia. Dear Lia you are of my best friend, a second mum for me and I love you dearly. Without you in it, my life, this day would have not felt complete. In every moment in every steps of my life, I feel your love and support.

The "Gallo family" just because you all exist and you are there... I can always feel the love even if from a WhatsApp conversation.

Morpheo and Ophelia, my two crazy beautiful and lovely cats... for making me smile with your quirky personalities and behaviours and for bringing a spark of joy and love into my house and in my heart.

And now onwards to another challenge ..

"If you find a path with no obstacles, it probably doesn't lead anywhere" F.A. Clark

"You are never too old to set a new goal or to dream a new dream" C. S. Lewis

ISBN 978-90-819817-3-6