

CONFIDENTIAL*TNO-report***THE EFFECT OF 3 PERFLUORINATED ALKYL
SULPHONATED ON CHOLESTEROL/BILE ACID
METABOLISM IN 15 %-FAT FED E3-LEIDEN
TRANSGENIC MICE *IN VIVO* AND ON FATTY ACID
CONVERSION INTO CHOLESTEROL IN RAT
HEPATOCYTES *IN VITRO*.**

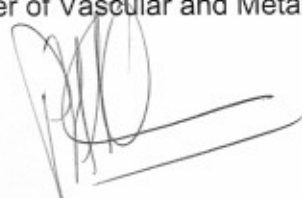
Project / study number: 031.10074 / 3M-02

**TNO Quality of Life
TNO Biosciences**Gaubius laboratory
Zernikedreef 9,
P.O. Box 2215
2301 CE Leiden
The Netherlands
Phone +31 71 518 1209
Fax +31 71 518 1901Authors:
L.H. Cohen
E.J. Pieterman
E. Hoegee-de NobelIn assignment of:
3M, Medical DepartmentStudy monitor:
John L. Butenhoff, Ph.D., CIH, DABT
Corporate Scientist
Medical Department
3M Center, Building 0220-02-E-02
St.Paul, Minnesota 55144-1000
USA
Phone: +1.651.733.1962
Fax: +1.651.733.1773
E-mail: jlbutenhoff@mmm.comStudy director:
Dr. Louis H. CohenStatus and Date:
Final version: 04 December 2006Previous versions:
Draft1: 18 October 2006
Draft2: 30 October 2006
Draft3: 28 November 2006

Number of pages: 35

Authorization by Dr. P.H.A. Quax,
Technology manager of Vascular and Metabolic Diseases:

Signature and date:

 6/12/6

Contents

Testing facility	page 3
Contributors	page 3
1. Introduction	page 3
2. Study design	page 4
3. Results of <i>in vivo</i> experiment (ApoE3Leiden mice)	page 6
3.1. Body weight and food intake measurements	page 6
3.2. Plasma measurements	page 7
3.3. Measurements in metabolic cages	page 13
3.3.1 Food and drink intake	page 13
3.3.2 Moving (X) activity	page 15
3.3.3 O ₂ -consumption, CO ₂ -production and respiratory exchange ratio (RER)	page 15
3.3.4 Energy expenditure	page 17
3.4 Fecal bile acid, sterol, and fatty acid measurements	page 18
3.5 Intestinal cholesterol absorption	page 22
3.6 Perigonadal fat tissue weight	page 23
3.7 Liver weight and hepatic lipid contents	page 23
3.8 Liver enzyme activities: HMG-CoA reductase, Cholesterol 7 α -hydroxylase and sterol 27-hydroxylase	page 25
4. Results of <i>in vitro</i> experiment (rat hepatocytes)	page 30
5. Summary and conclusions	page 32
5.1. Effects of PFBS in ApoE3Leiden mice	page 32
5.2. Effects of PFHS in ApoE3Leiden mice	page 32
5.3. Effects of PFOS in ApoE3Leiden mice	page 33

Testing facility

Business Unit Biosciences

TNO Quality of Life

Gaubius Laboratory

Zernikedreef 9

P.O. Box 2215

2301 CE Leiden

Phone: +31 71 5181209

fax: +31 71 5181901

Contributors

Study director:

Dr. Louis H. Cohen

Phone: +31 71 5181469

E-mail: louis.cohen@tno.nl

Experimental work:

Ing. Christa van Thiel

Ing. Bep Hoegee

Ing. Anita van Nieuwkoop

Ing. Angela Koudijs

Ing. Wim van Duyvenvoorde

Ing. Koen van der Mark

Ing. Ronald van der Sluis

Dr. Peter Voshol

Ing. Elsbet Pieterman

Advisor :

Dr. Hans Princen

Prof. Dr. Louis M. Havekes

1. INTRODUCTION

3M has observed that the compounds of interest have strong cholesterol lowering effects in animal studies. In order to obtain more insight into the possible mechanism of action the effects of the compounds on cholesterol and bile acid synthesis in primary culture of rat hepatocytes and on bile acid uptake in hamster ileum pieces *in vitro* have been investigated in a previously performed study. From this study it was concluded that the compounds possibly increased hepatic bile acid synthesis, however indirect effect on sterol synthesis might be involved as well. In the present study the *in vivo* effect of the test compounds on cholesterol and bile acid metabolism have been investigated in ApoE3Leiden transgenic mice. This animal model shows a humanlike lipoprotein pattern and, unlike wild type mice, is sensitive for lipid lowering drugs like statins. Furthermore, the plasma cholesterol levels can be elevated by adjustment of the diet.

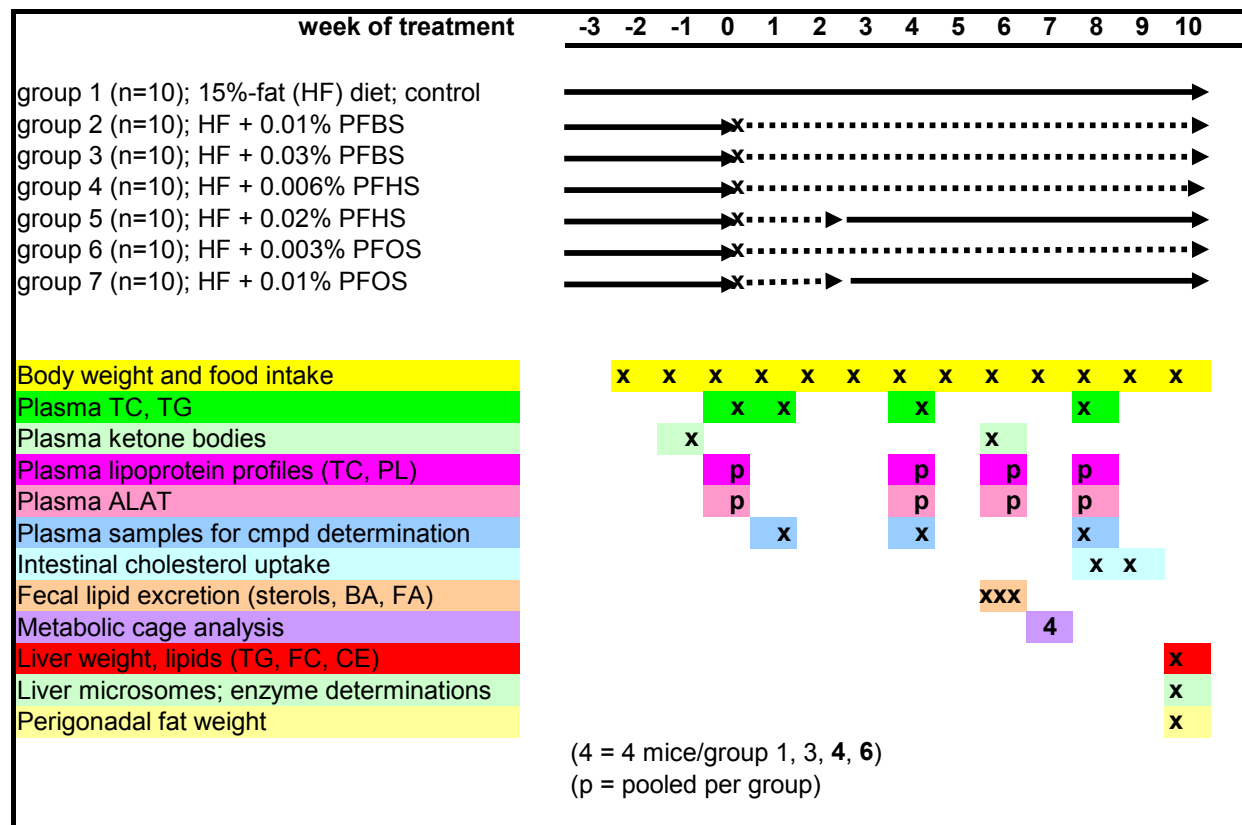
Additionally, in the present project information on the effects of the test compounds on hepatic cholesterol synthesis have been obtained via an *in vitro* study, in which the conversion of long and short chain fatty acids into cholesterol in the presence of the compounds have been investigated.

2. STUDY DESIGN

The design for the performance of this study has been described in the final study protocol of 08-05-2006. Deviations from this protocol have been mentioned below.

Approval for this study by the TNO Animal Experiment Committee has been obtained on 06-06-2006 under number DEC#2125.

The final study design and the concomitant measurement points have been depicted in scheme 1.



Scheme 1. Experimental design of the study

After a 3 weeks run-in period on a semi synthetic high-fat (HF; 14% lard, 1% corn oil and 0.25% cholesterol) diet, 75 male ApoE3Leiden mice (11-12 weeks of age at start of run-in) were randomized on basis of body weight, plasma cholesterol and triglycerides (measured after 4 h fasting). Then, the groups received respectively HF-diet (control group 1) and 0.01%, 0.03% of PFBS (groups 2 and 3), 0.006% PFHS (group 4) and 0.003% PFOS (group 6) in the HF- diet for the following 10 weeks. Groups 5 and 7 received respectively 0.02% PFHS and 0.01% PFOS in the diet, but after 2 weeks of treatment the mice lost on average 3 grams of bodyweight (see Fig. 3.1.1) and food intake has steadily decreased (see Fig. 3.1.2). For this reason it was decided to stop with these treatments and to return to the high fat control diet until the end of the study (this deviation of the protocol has been discussed with the study monitor by E-mail correspondence of 5, 6 and 18 July 2006).

A further deviation concerns the plasma ketone bodies determination. These compounds can only be detected in plasma in a proper way after an overnight fasting period (16 h). For this reason it could not be measured in the same plasma samples of weeks 0 and 8 (taken after 4h fasting) and therefore, it was decided to perform this determination in week -1 and week 6 of treatment (see E-mail correspondence 13 and 30 June 2006).

At weeks 0, 1, 4 and 8 plasma cholesterol and triglyceride levels after 4h-fasting have been measured. In weeks 1 (7 days of treatment), 4 and 8, 15µl-plasma samples have been sent to the 3M company for the determination of the test compound concentrations.

As originally planned, at weeks 0, 4 and 8, lipoprotein profiles were analyzed (at week 0, the average values of three subpools were determined, at week 4 and 8, one pooled sample per group was analyzed). Since the week 4-lipoprotein patterns, especially of groups 5 and 7, were different from expected, extra lipoprotein profiles have been analyzed in treatment-week 6 (see E-mail correspondence with study monitor of 24 July 2006). The latter plasma samples were obtained after overnight fasting (necessary for ketone body determination), which in our experience does not give different results compared to lipoprotein profiles of 4h-fasting samples. In weeks 0, 4, 6 (extra measure points; see E-mail correspondence of 24 July 2006) and 8, plasma ALAT values were determined in pooled samples per group.

In week 5-6 feces has been collected from 3 subgroups per treatment-group during 3 periods of 2-3 days, and fecal bile acids, fatty acids and sterol quantities and composition were determined. Initiated by the E-mail correspondence of 25 July 2006, feces has been collected in week 4 as well. However, up to now, we see no reason yet to investigate it further.

In week 7, the metabolic activity of 4 animals per group from groups 1, 3, 4 and 6 have been analyzed in metabolic cages. The following parameters have been measured: food intake, drink intake, feeding bouts, total physical activity, O₂-consumption and CO₂-production. From these parameters the respiratory exchange ratio (RER) and O₂-consumption-related body energy expenditure have been calculated. Because the food can only be supplied in powdered form in the metabolic cages, during this period (week 7 – 7.5), the other animals in this study also received powdered food. Hereafter all animals received food in chunks again.

In week 8-9 intestinal cholesterol uptake has been determined.

In week 10 the mice were sacrificed, plasma was collected by means of heart puncture, the liver and perigonadal fat were taken out and weighed, two small pieces of the liver were quickly frozen in liquid nitrogen for determination of lipids and eventual RNA analysis, and microsomes and mitochondria have been isolated from the remaining part of the liver. HMG-CoA reductase and cholesterol-7 α -hydroxylase activity have been determined in the microsomal preparations, sterol-27-hydroxylase activity has been measured in the mitochondrial fractions.

3. RESULTS OF *IN VIVO* EXPERIMENT (APOE3LEIDEN MICE)

The resulting individual primary data and subsequent calculated data are provided in a separate appendix to this report. The mean values per group are presented in the figures and tables below. Significance of differences was calculated using the non-parametric Mann-Whitney U test and the resulting p-values are given in the appendix.

In the figures, a colored asterisk at a separate measure point indicate a statistically significant difference ($p < 0.05$) between the value of the indicated group (of the same color) and that of the control group. In the tables, this difference has been indicated by a yellow background.

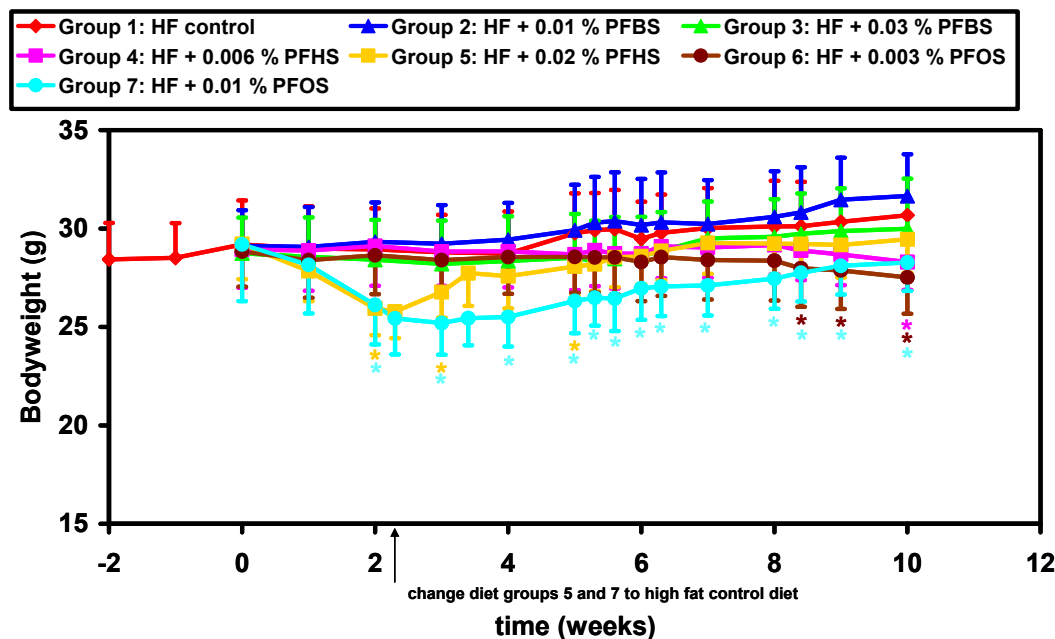
3.1. Body weight and food intake measurements

As shown in Figs. 3.1.1 and 3.1.2, the treatment with the high concentrations of PFHS and PFOS (groups 5 and 7) caused already from the start a remarkable decrease in body weight and food intake. After the change back to the control diet in week 2, these animals return to normal food intake, comparable to all other groups. Subsequently, group 5 animals gained weight again up to the control values, however, the body weight of the group 7 mice, although increasing again, stayed behind during the whole treatment period.

The PFBS treated mice showed the same body weight increase as the controls. The Groups 4 and 6 (low concentrations of PFHS and PFOS) lost weight significantly in the last weeks of the study when compared to control animals. This is in line with the loss of fat tissue as shown below.

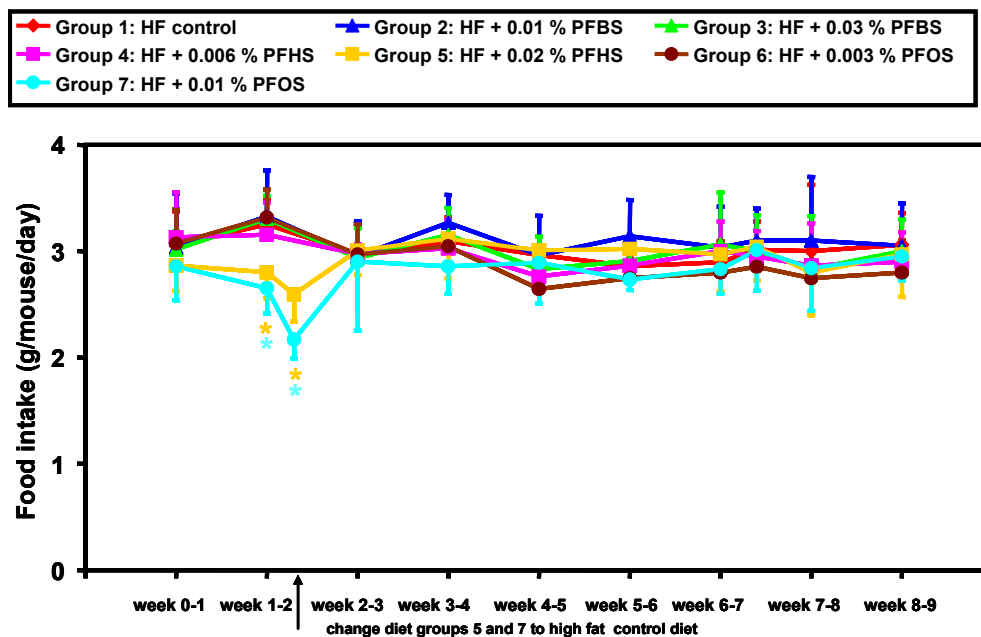
Figure 3.1.1 Body weight

Values are means $\pm$ S.D.



Except for the deviations in food intake behavior of groups 5 and 7 (see above), all other animals ate about the same throughout the treatment period (Fig 3.1.2).

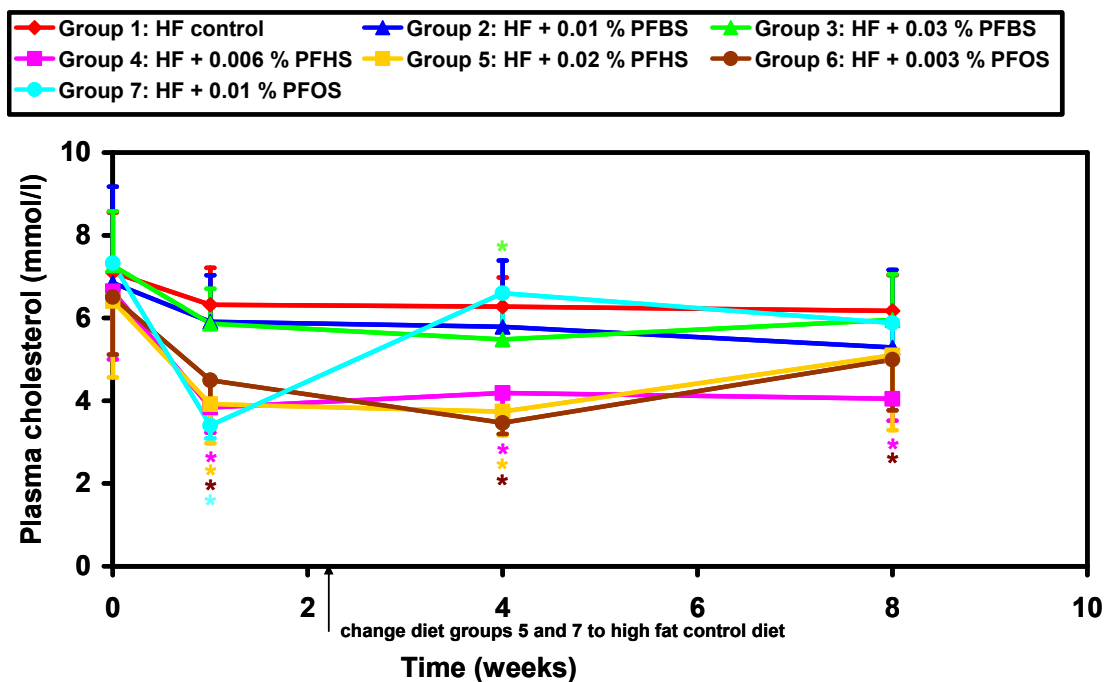
Figure 3.1.2 Food intake
Values are means $\pm$ S.D.



3.2. Plasma measurements

The following parameters have been determined in plasma in the course of the study: cholesterol (Fig. 3.2.1), triglycerides (Fig. 3.2.2), lipoprotein profiles (Fig 3.2.3 – 3.2.5), ALAT (Fig. 3.2.6) and ketone bodies (Fig. 3.2.7).

Figure 3.2.1 Plasma cholesterol
Values are means $\pm$ S.D.



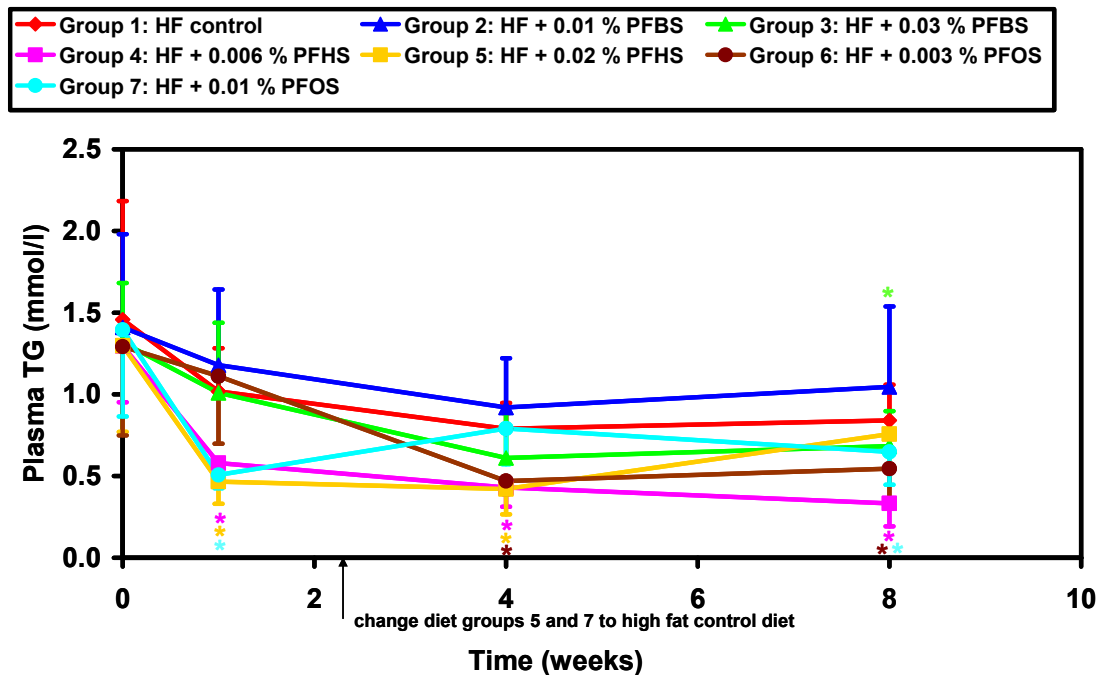
As seen in other animal studies, PFHS and PFOS strongly decreased plasma cholesterol. The reversal to control diet in the groups 5 and 7 resulted in a return to control values for

the PFOS group (7), however this return was delayed until week 8 for the PFHS group (5). The PFBS treatment did not decrease significantly the cholesterol levels.

More or less the same results have been obtained for the plasma triglyceride (TG) levels.

Figure 3.2.2 Plasma triglyceride

Values are means $\pm$ S.D.



PFHS and PFOS decreased TG levels, but also the highest concentration of PFBS (group 3) after 8 weeks of treatment; the lowest concentration of PFBS, group 2, was not different from control. TG levels rose again in the group 7 after return to the control diet, however it remained lower than the control TG level in week 8. Group 5 TG-levels turn back to control levels after the shift to control diet.

The differentiation into lipoproteins showed more differences than observed with the lipid levels alone. The patterns have been determined by measuring the cholesterol contents of the different column fractions, which indicate all lipoprotein fractions (Figs 3.2.3a, 3.2.4a, 3.2.5a), and by measuring the phospholipid content, which is major a constituent of the HDL fractions (Figs 3.2.3b, 3.2.4b, 3.2.5b).

The PFBS treatment resulted in only minor changes in the lipoprotein fractions in time. VLDL seems to be increased a little, but IDL/LDL and HDL are somewhat lower than these fractions in the control patterns.

The low concentration of PFHS (group 4) showed the appearance of a lipoprotein fraction which is located before the HDL fraction and half overlapping with the IDL/LDL fraction. Since phospholipids are a major constituent, this fraction has been designated as large, less-dense HDL. It might arise from an impaired clearance of smaller HDL by the liver. This fraction appeared to increase upon longer treatment with PFHS. Concomitantly, VLDL and IDL are strongly decreased, which might indicate a decrease in production/secretion of apoB-containing lipoproteins by the liver.

The 4-week pattern of the group 5 animals (high conc. PFHS; after 2 weeks on control diet again) resembled very much the group 4 pattern with a major increase in the large HDL. However, after 6 and 8 weeks, the lipoprotein profiles went back to the control patterns with again more VLDL, IDL and small HDL.

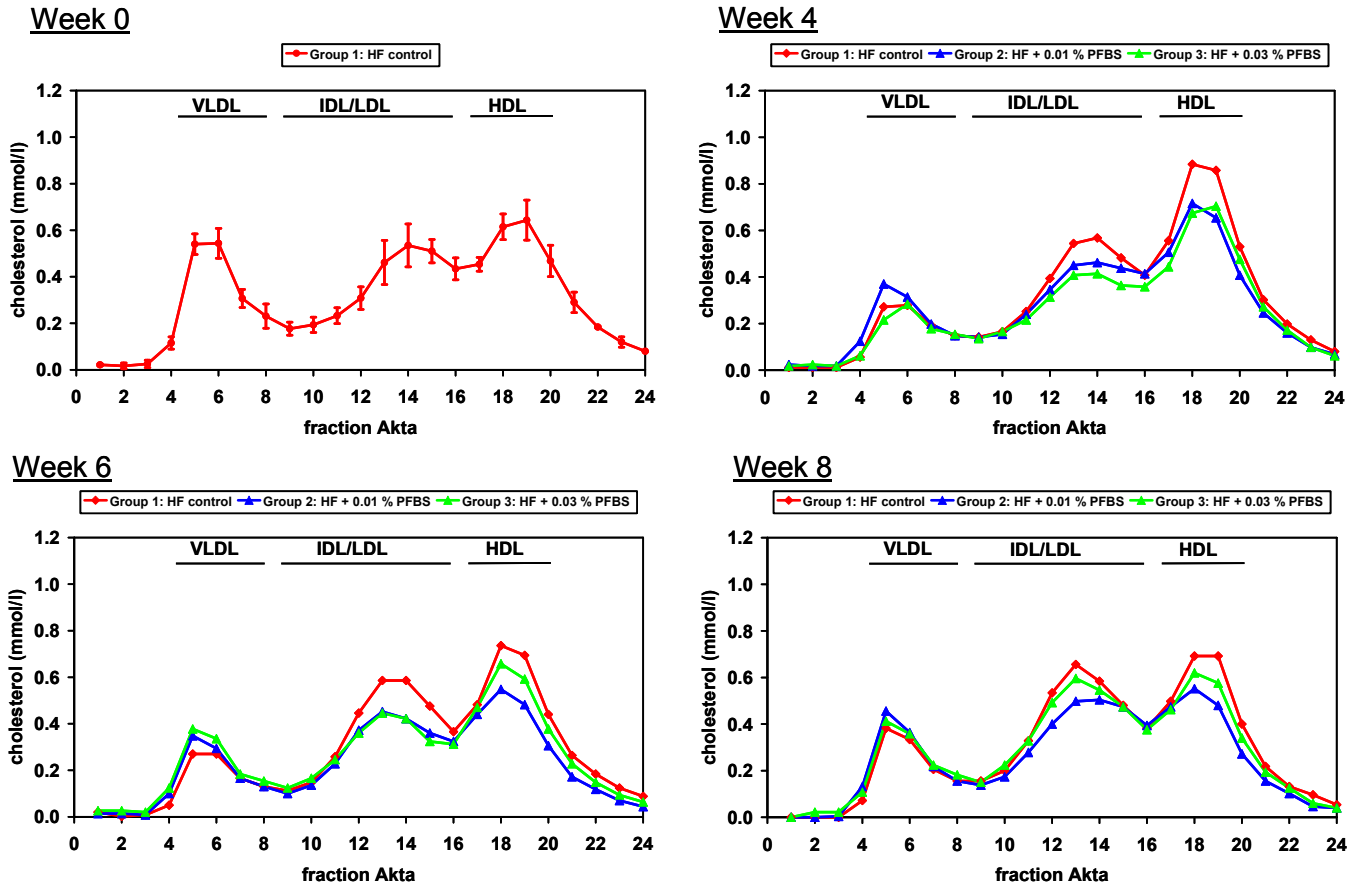
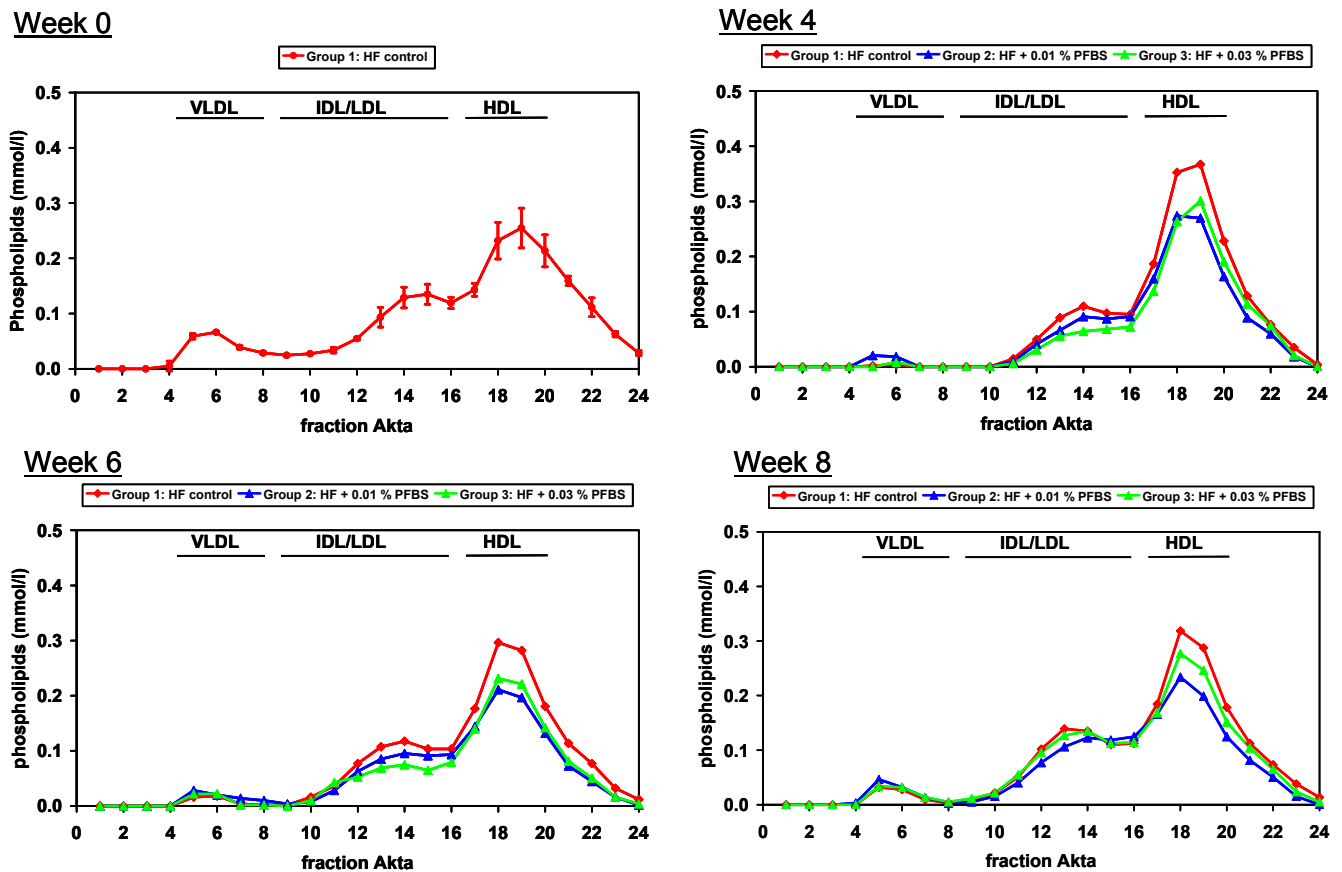
Figure 3.2.3a Pooled plasma lipoprotein (cholesterol) profiles ; PFBS treatment**Figure 3.2.3b Pooled plasma lipoprotein (phospholipid) profiles ; PFBS treatment**

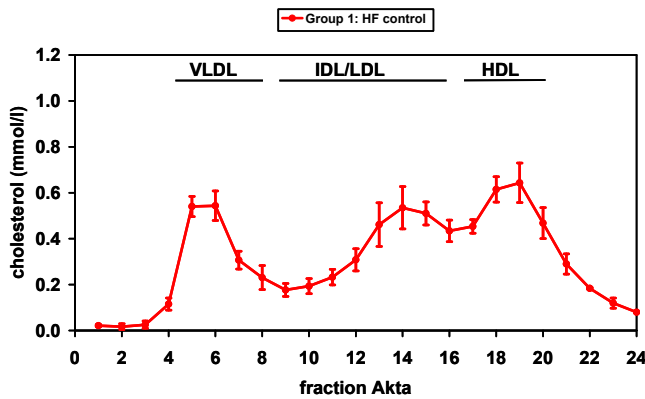
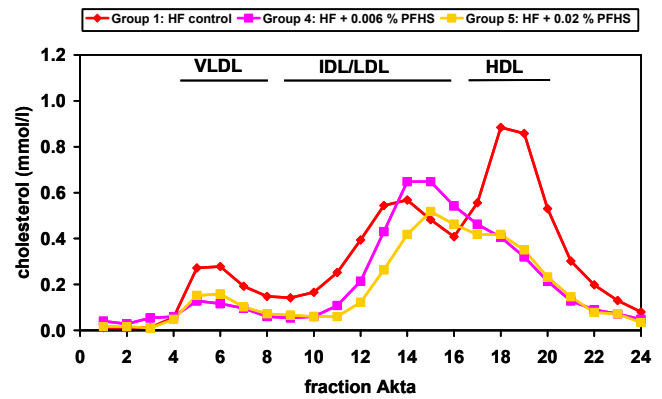
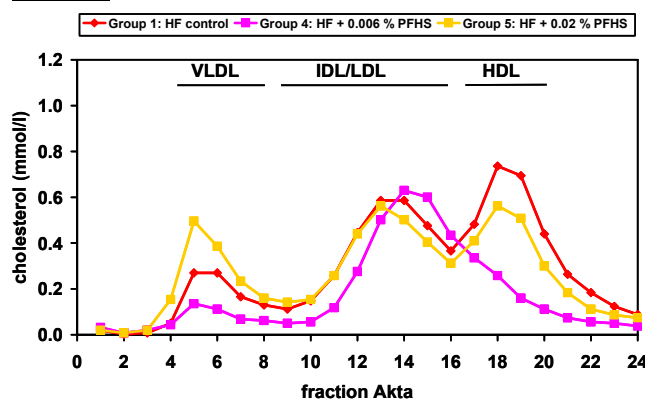
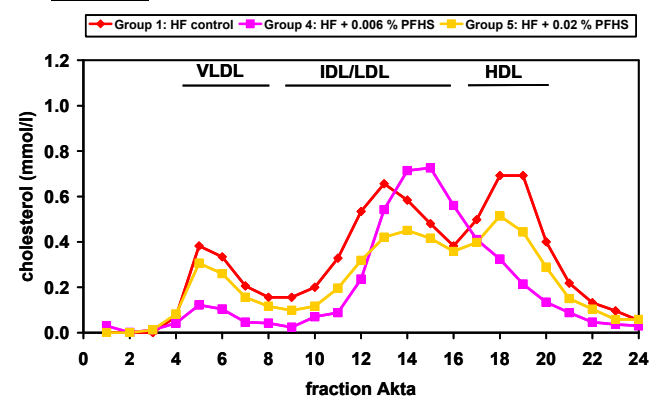
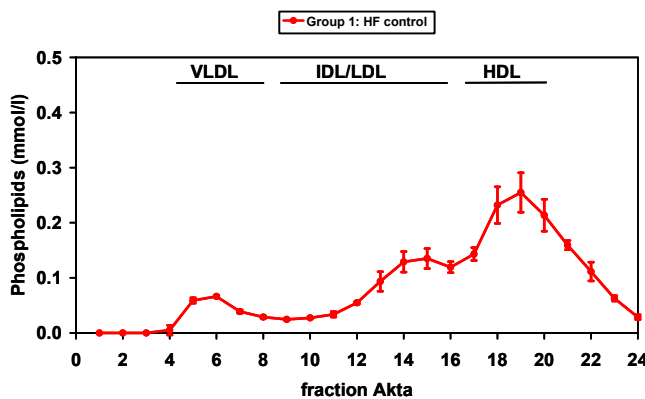
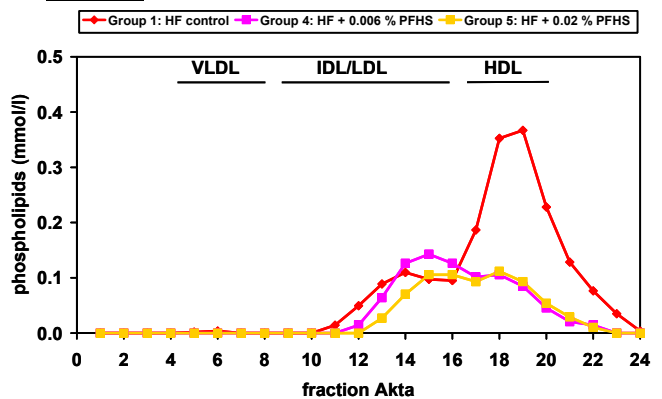
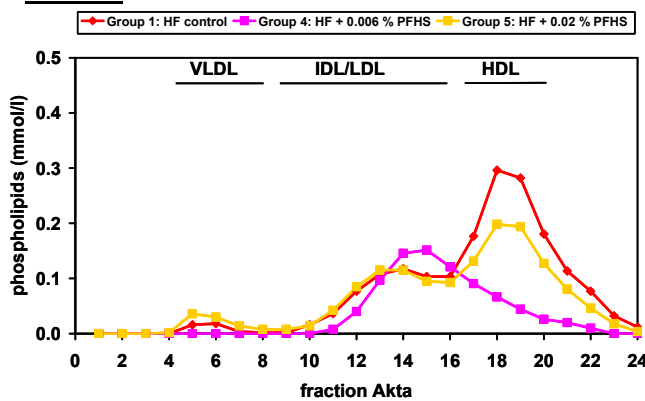
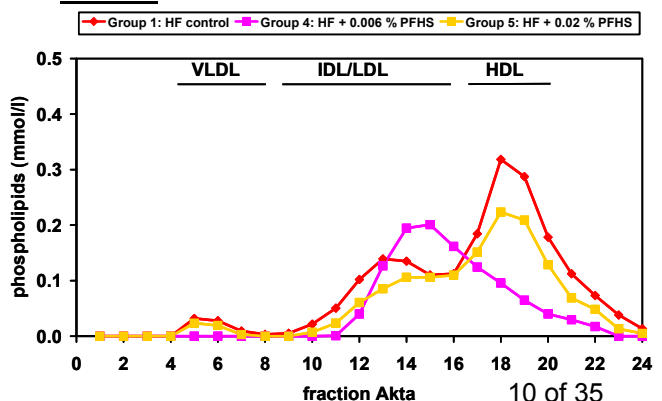
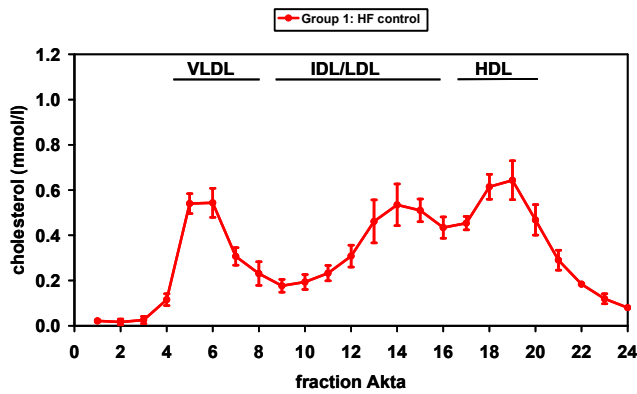
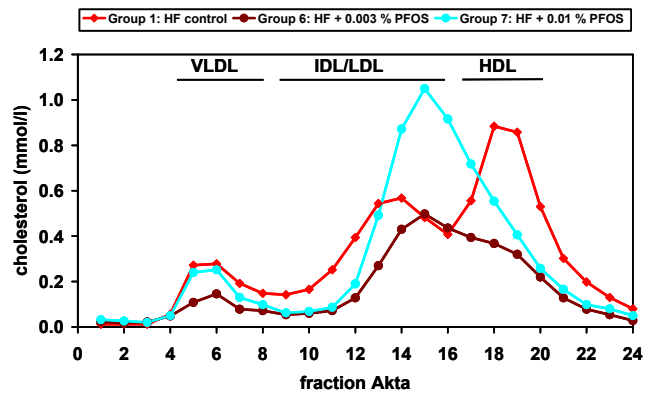
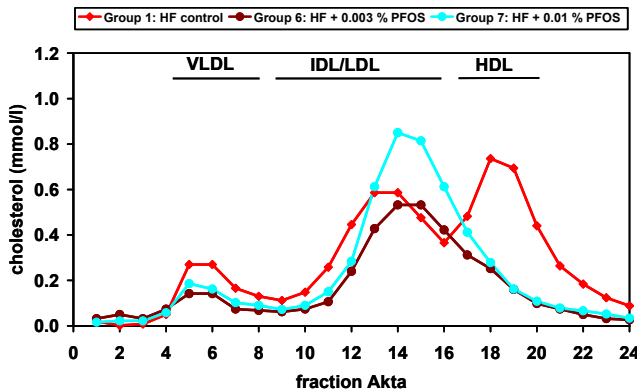
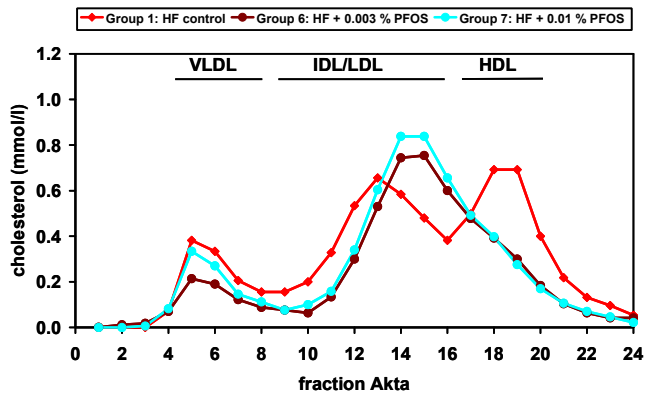
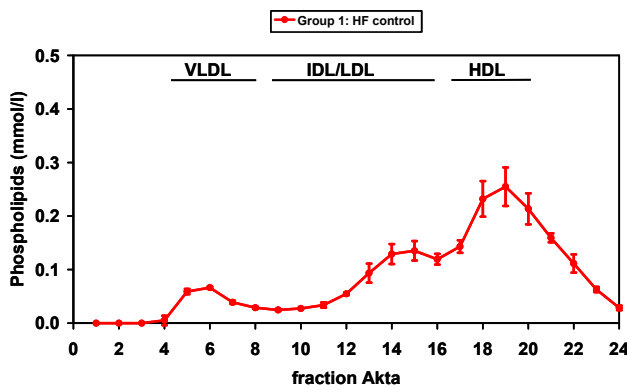
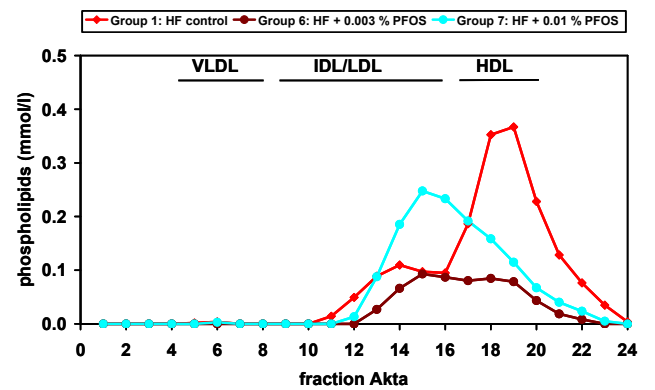
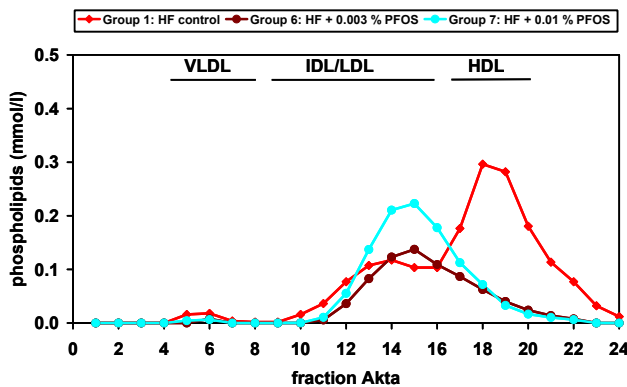
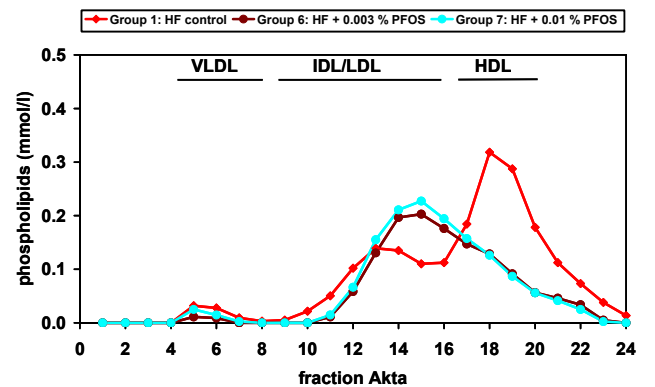
Figure 3.2.4a Pooled plasma lipoprotein (cholesterol) profiles ; PFHS treatmentWeek 0Week 4Week 6Week 8**Figure 3.2.4b Pooled plasma lipoprotein (phospholipid) profiles ; PFHS treatment**Week 0Week 4Week 6Week 8

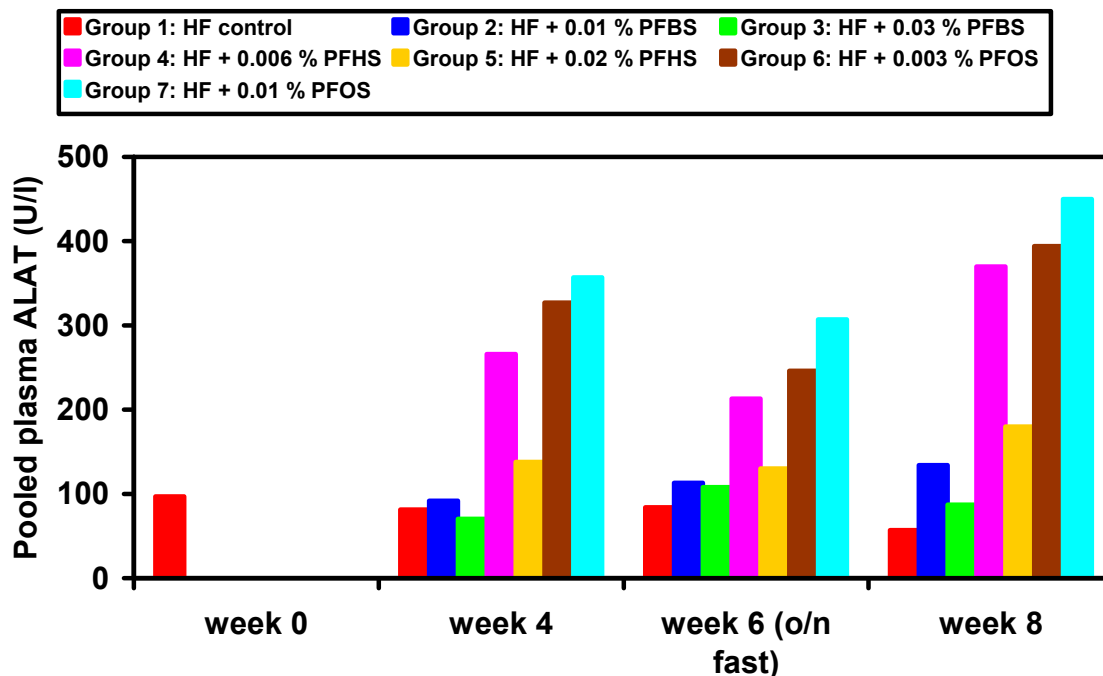
Figure 3.2.5a Pooled plasma lipoprotein (cholesterol) profiles ; PFOS treatment**Week 0****Week 4****Week 6****Week 8****Figure 3.2.5b Pooled plasma lipoprotein (phospholipid) profiles ; PFOS treatment****Week 0****Week 4****Week 6****Week 8**

The lipoprotein profiles of the PFOS treated animals are remarkable. The low concentration (group 6) showed major the large-HDL fraction which is steadily increasing during the treatment. ApoB-containing lipoproteins have been decreased. Similar as with PFHS, VLDL and IDL fractions have been decreased.

Group 7, which showed a recovery of plasma TC/TG levels after 2 weeks control diet, has VLDL levels similar to the control group again, but showed low IDL and a very large "large"-HDL peak, which remained the same over the rest of the treatment period. A possible explanation could be that liver VLDL production has recovered (or is enhanced), but lipoprotein clearance is still impaired. It all might be the result of the long half-life of PFOS in the body. This explains also the gradually increase of the "large"-HDL peak in time in the low concentration PFOS group 6, which in week 8 has become as large as this peak in the group 7 plasma.

Since the liver seems to be strongly involved in the mechanism of action of PFHS and PFOS, the liver enzyme alanine aminotransferase (ALAT) has been determined a few times more than originally planned (Fig. 3.2.6).

Figure 3.2.6 Pooled plasma ALAT activity



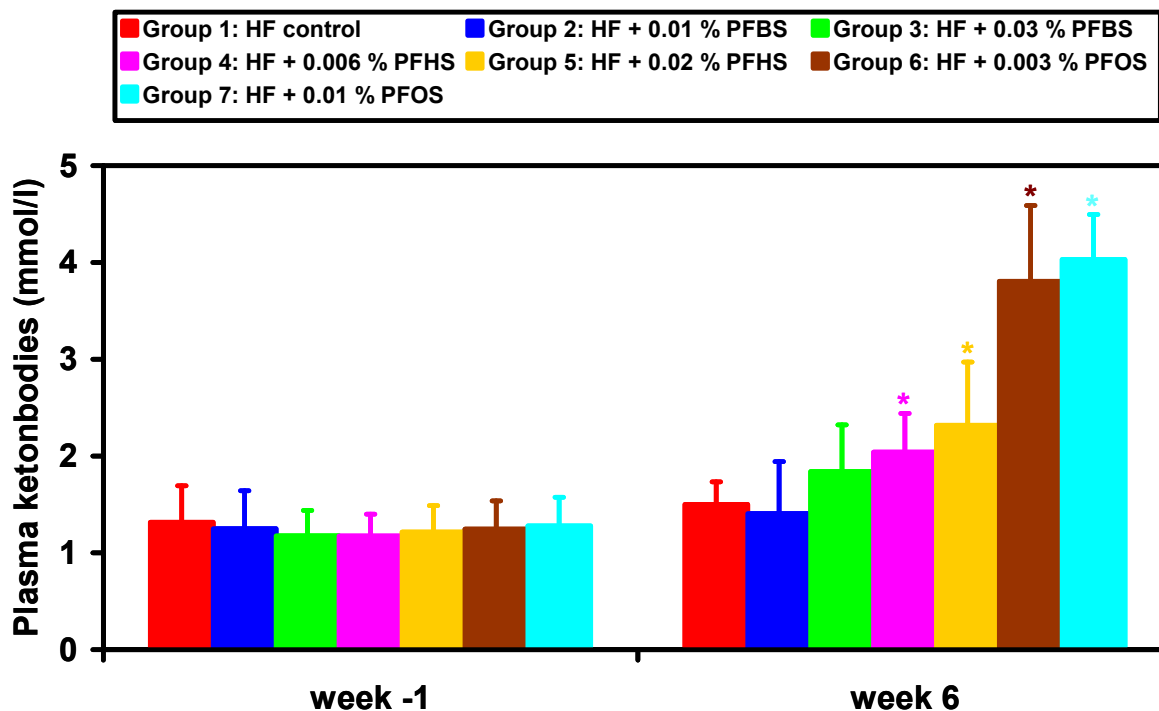
At all measure points ALAT has been increased in the PFOS-treated groups (groups 6 and 7), although from week 2 group 7 received the control diet again. PFHS increased ALAT as well (group 4), but here the return to control diet (group 5) caused a (partial) recovery of this activity. Although plasma ALAT activity is often used as a marker for liver damage, the increase in the plasma can be the result as well of an induction of the expression of the liver enzyme by the treatment, but can also result from the enlargement of the liver as seen in this study (see Fig. 3.7.1).

PFBS had no or only a minor effect on plasma ALAT levels.

Since it was expected that the test compounds might have an effect on fatty acid metabolism, plasma ketone bodies have been determined before and after 6 weeks of treatment. As shown in Fig. 3.2.7, PFHS and, even more so, PFOS increased significantly the plasma ketone body levels. The two weeks of treatment with the highest dose, followed by 4 weeks of control diet (groups 5 and 7) still resulted in increased levels in week 6. PFBS did not significantly increase the ketone bodies.

Figure 3.2.7 Plasma ketone bodies

Values are means $\pm$ S.D.



So, PFHS and PFOS induced fatty acid oxidation, which is supported by the results of the analysis in metabolic cages performed in week 7 of treatment.

3.3. Measurements in metabolic cages

After 7 weeks of treatment, 4 mice of the different treatment groups, the HF control group (group 1), the 0.03% PFBS group (group 3), the 0.006% PFHS group (group 4) and the 0.003% PFOS group (group 6) have been analyzed individually for 3 days in metabolic cages (after 1 day of acclimatization in the same cage). The following parameters have been measured: food and drink intake (Figs. 3.3.1.1 and 3.3.1.3), feeding size bouts (Fig. 3.3.1.2), moving activity (X activity; Fig. 3.3.2), O₂-consumption and CO₂-production (Figs. 3.3.3.1 and 3.3.3.2). From the latter parameters the respiratory exchange ratio (RER; Fig. 3.3.3.3), which gives information on the substrate that is used for energy generation, and additionally, the O₂-consumption-related energy expenditure (Fig. 3.3.4) have been calculated.

3.3.1 Food and drink intake

No significant differences have been observed between the groups in food intake, feeding bout size and drink intake, although the PFHS treated mice might have eaten somewhat more.

Figure 3.3.1.1 Food intake

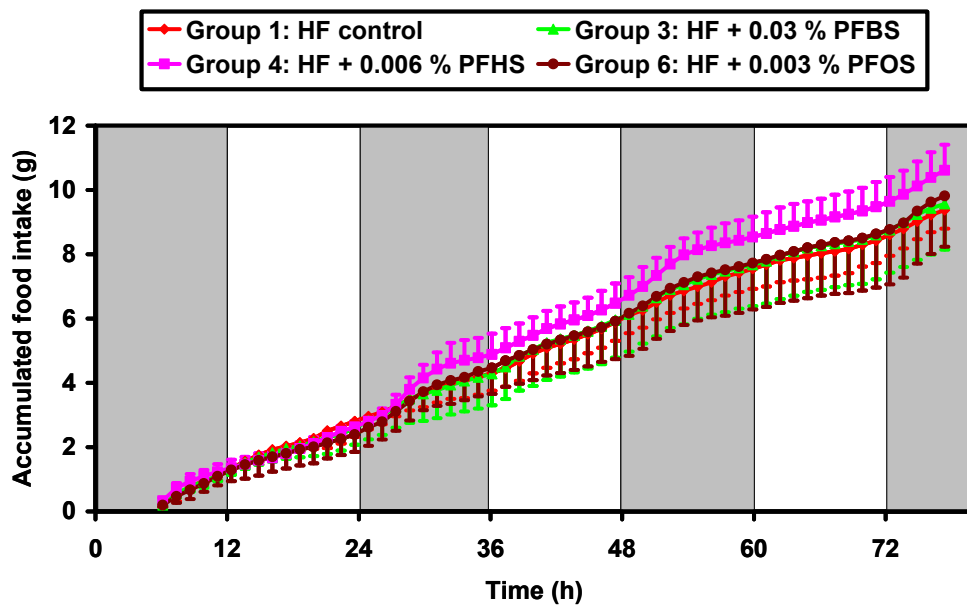


Figure 3.3.1.2 Feeding bout size

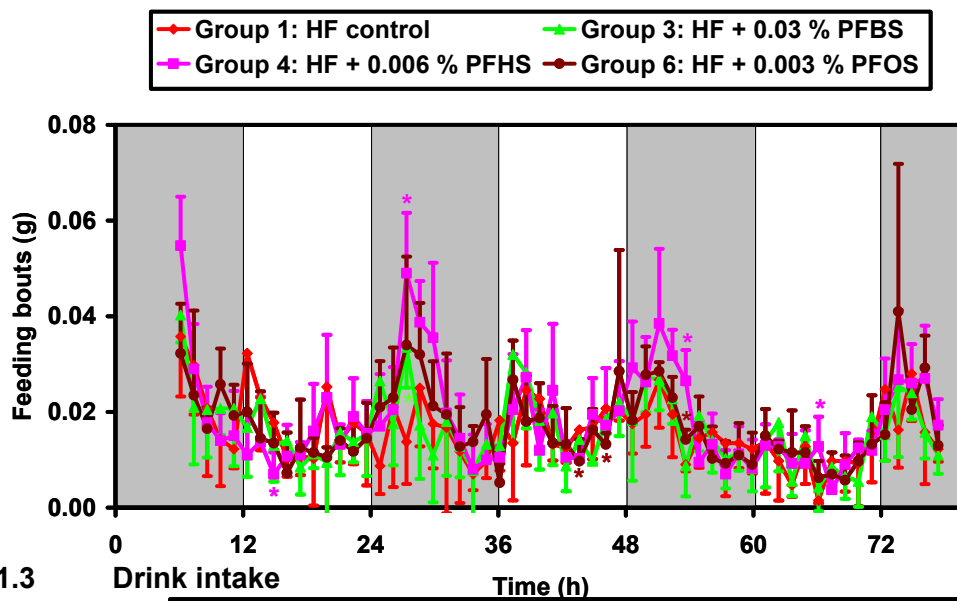
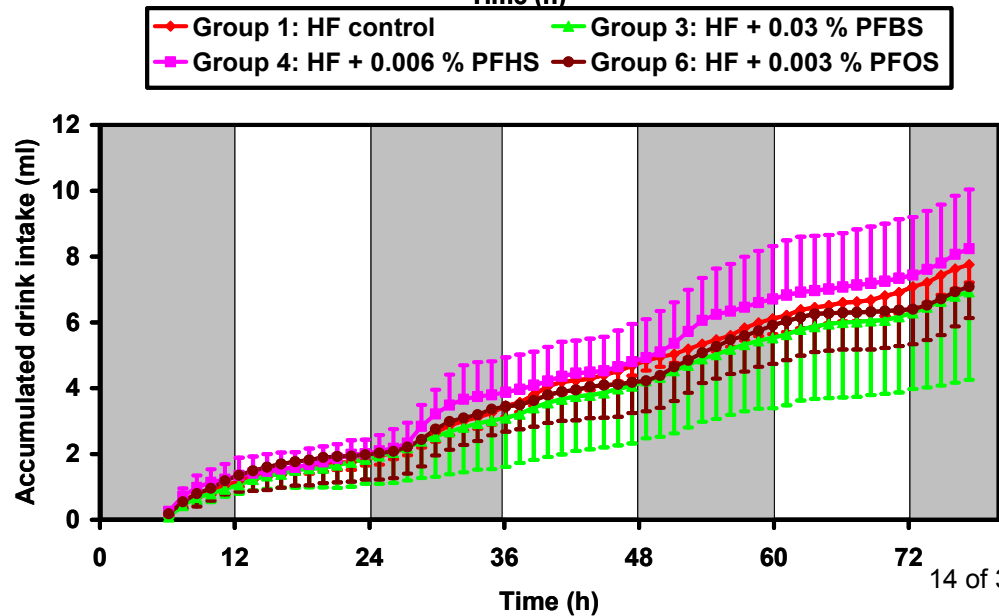


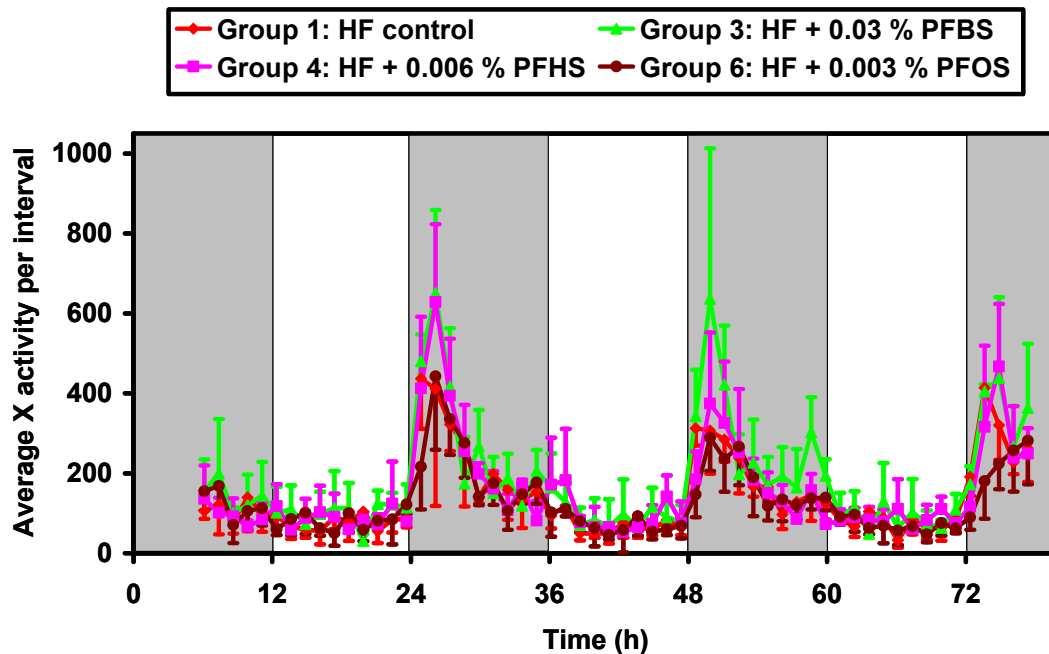
Figure 3.3.1.3 Drink intake



3.3.2 Moving (X) activity

No significant differences have been observed between the groups in moving activity.

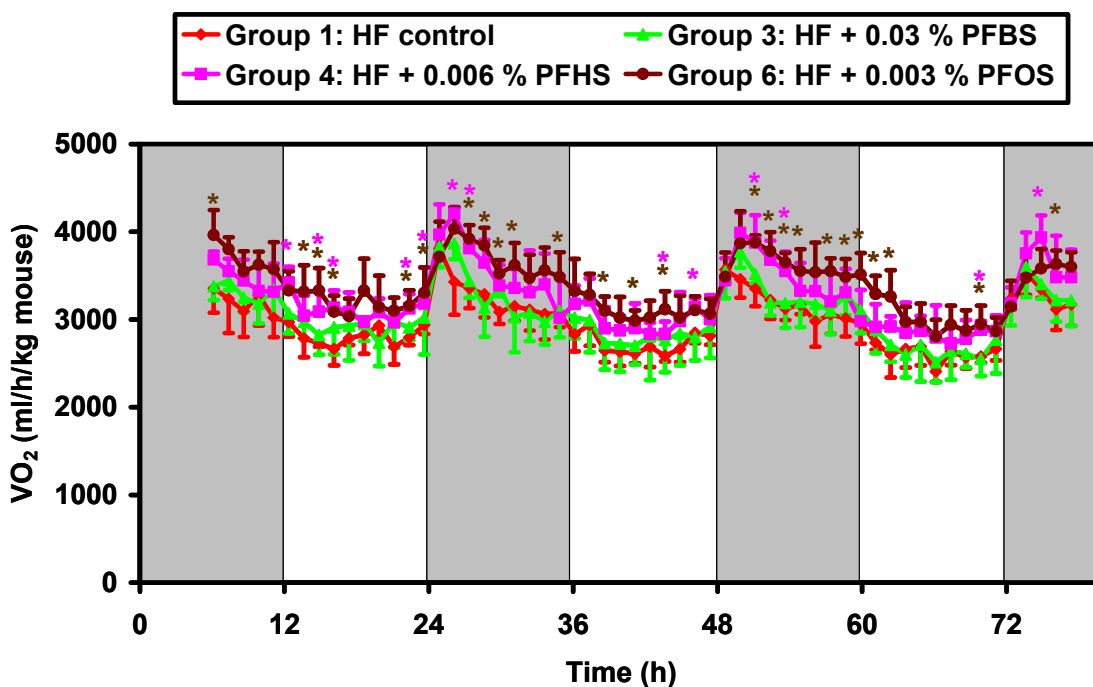
Figure 3.3.2 X Activity



3.3.3 O₂-consumption, CO₂-production and respiratory exchange ratio (RER)

It is remarkable to notice that differences in these parameters have been observed between the groups, while using rather small groups. O₂-consumption of the PFHS and PFOS treated animals was significantly increased, while no difference between control and PFBS-treated mice have been found.

Figure 3.3.3.1 O₂-consumption



CO₂-production of the PFHS and PFOS groups was found to be increased as well, although in much less time points compared to the increase in O₂-consumption. For this reason the RER for these groups are lower (significantly for PFOS, but not yet statistically significantly for PFHS) than the RER of the control and PFBS-treated group. This is an indication that fat is preferentially used for energy generation over the use of carbohydrates. This is in agreement with an increase in fatty acid oxidation as reflected by an increased plasma ketone body level in PFHS- and PFOS-treated mice and also for the decrease in fat tissue as found later in this study.

Figure 3.3.3.2 CO₂-production

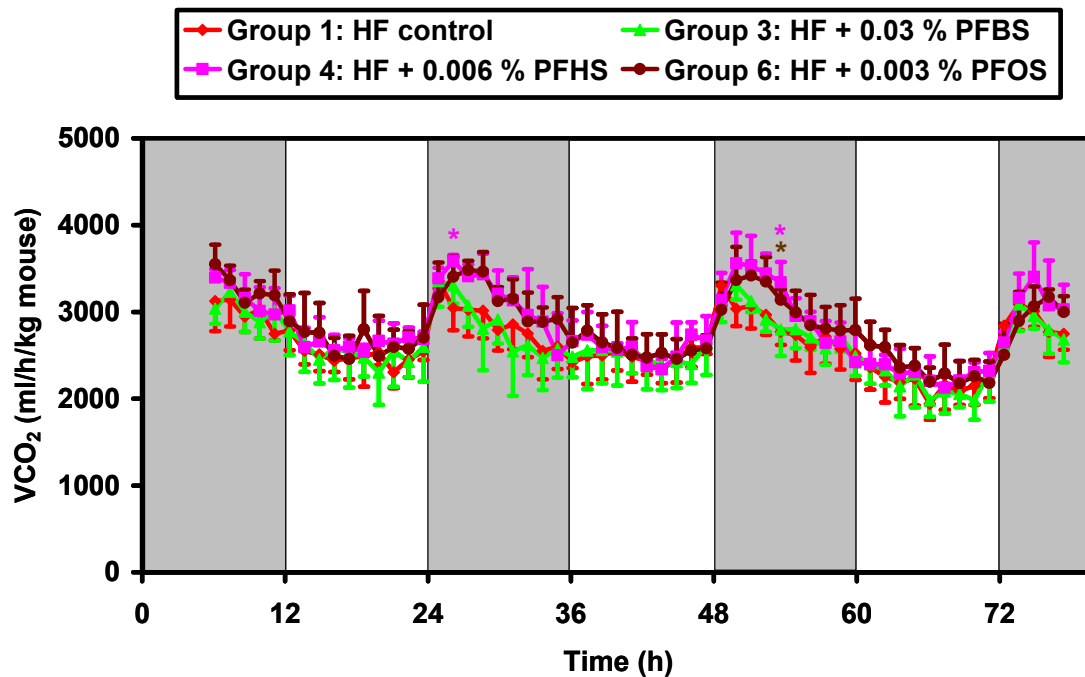
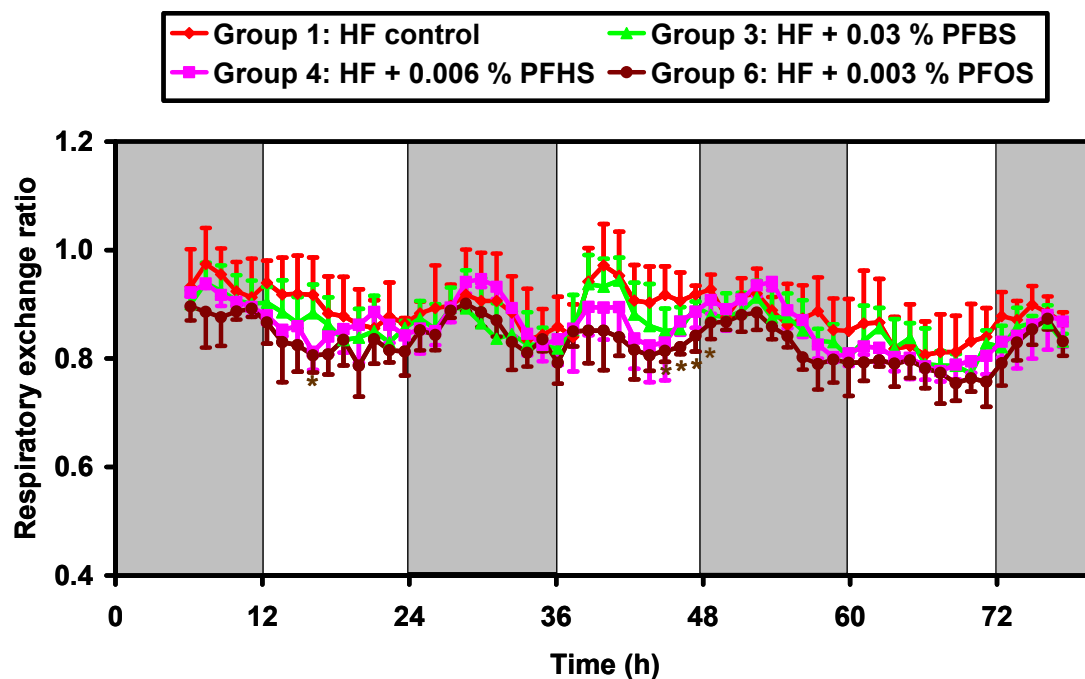


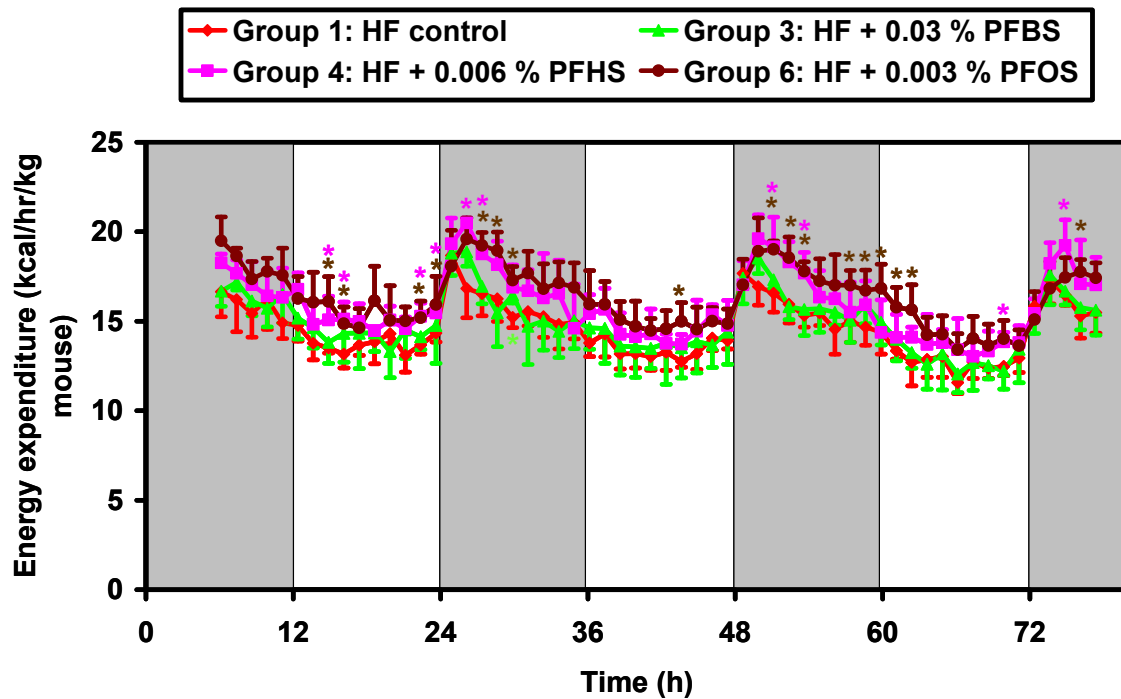
Figure 3.3.3.3 Respiratory exchange ratio (RER)



3.3.4 Energy expenditure

Congruent with these observations it has been calculated that the O₂-consumption-dependent energy expenditure has been increased as well by the PFHS and PFOS treatment.

Figure 3.3.4 Energy expenditure



The differences observed in the metabolic cage analysis might be more pronounced by using larger groups in eventual future studies (preferable n = 8).

3.4. Fecal bile acid, sterol and fatty acid measurements

In week 6 of treatment, feces have been collected and its lipid and bile acid contents determined.

Fecal bile acids have been decreased by PFHS (0.006%; group 4) and even more by PFOS (0.003%). Although groups 5 and 7 were back on control diet for 4 weeks, both these compounds have still kept the bile acid excretion below the control values.

PFBS-treatment seems to have increased the fecal bile acid excretion, which was even significantly different from the control group for group 2.

Figure 3.4.1 Bile acid excretion

Values are means $\pm$ S.D.

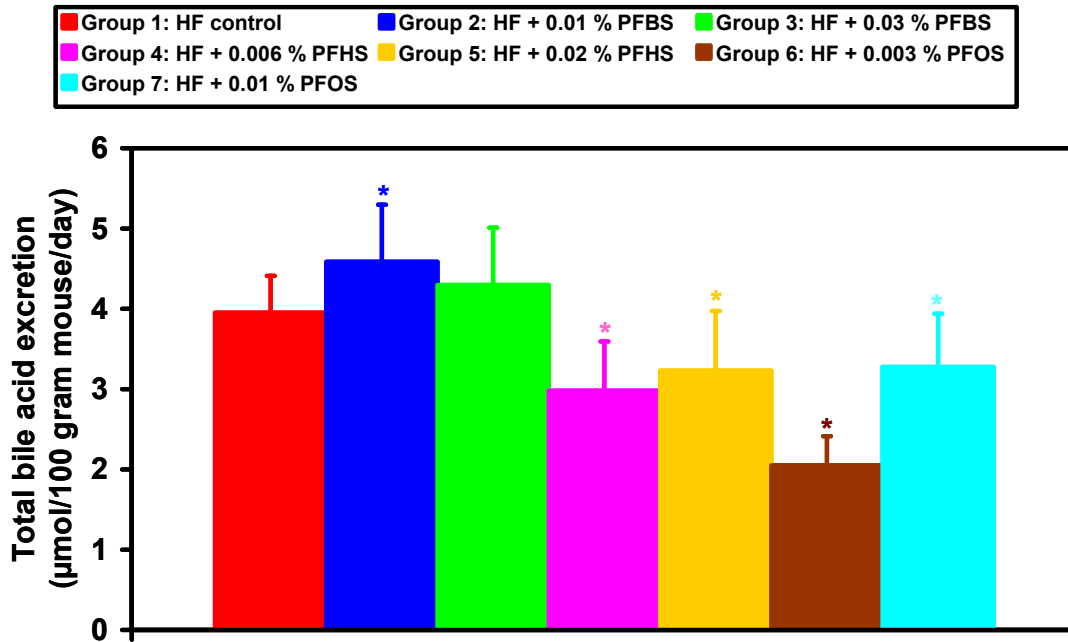


Table 3.4.1 Fecal bile acid composition

group	% of bile acids						
	α-muricholate	deoxycholate	cholate	lithocholate	β-muricholate	ω-muricholate	hyodeo/urso
Group 1: HF control	6.1 $\pm$ 2.8	14.7 $\pm$ 5.7	7.9 $\pm$ 2.7	9.6 $\pm$ 4.1	20.4 $\pm$ 5.0	39.8 $\pm$ 8.8	1.5 $\pm$ 0.8
Group 2: HF + 0.01 % PFBS	4.8 $\pm$ 1.2	12.4 $\pm$ 2.1	8.4 $\pm$ 1.2	10.7 $\pm$ 2.4	19.6 $\pm$ 7.2	42.2 $\pm$ 4.4	1.9 $\pm$ 1.0
Group 3: HF + 0.03 % PFBS	4.2 $\pm$ 1.3	12.8 $\pm$ 1.2	7.6 $\pm$ 2.3	13.1 $\pm$ 4.5	19.9 $\pm$ 4.1	40.9 $\pm$ 3.4	1.4 $\pm$ 0.9
Group 4: HF + 0.006 % PFHS	7.1 $\pm$ 2.3	11.3 $\pm$ 1.8	10.2 $\pm$ 4.2	10.9 $\pm$ 3.3	17.2 $\pm$ 4.5	40.9 $\pm$ 3.8	2.3 $\pm$ 2.2
Group 5: HF + 0.02 % PFHS	5.3 $\pm$ 0.8	13.9 $\pm$ 2.4	8.5 $\pm$ 3.7	16.0 $\pm$ 5.2	16.8 $\pm$ 5.7	36.6 $\pm$ 7.0	2.8 $\pm$ 1.7
Group 6: HF + 0.003 % PFOS	6.8 $\pm$ 2.0	15.9 $\pm$ 5.5	14.3 $\pm$ 4.0	11.3 $\pm$ 3.2	12.5 $\pm$ 3.5	37.0 $\pm$ 5.7	2.3 $\pm$ 1.4
Group 7: HF + 0.01 % PFOS	10.8 $\pm$ 2.7	22.1 $\pm$ 5.5	16.4 $\pm$ 4.9	6.1 $\pm$ 2.4	16.8 $\pm$ 4.0	24.2 $\pm$ 4.0	3.5 $\pm$ 3.5

yellow indicates a significant difference from control ($p < 0.05$).

The latter increase with PFBS did not change the fecal bile acid composition. Concomitantly with the decrease by PFHS and PFOS, a change in composition of excreted bile acids has been observed as well, which can result from a change in bile acid synthesis pathways in the liver, but also from a change in intestinal bile acid conversion. The latter might also happen in the liver after reabsorption and return to the liver.

Only in group 4 (PFHS) was fecal neutral sterol excretion significantly increased. Fecal neutral sterols, which are primarily composed of cholesterol, are determined by food intake, intestinal absorption and liver production. Since the diet used contained 0.25% cholesterol, food intake plays a major role. This is more or less the same in all groups. Since intestinal cholesterol absorption is also the same or increased (see Fig.3.5.1), most probably the significantly increased fecal sterol excretion in group 4 (PFHS) is the result of an increased hepatic cholesterol synthesis. This is in agreement with the increased HMG-CoA reductase activity observed (see Fig. 3.8.2).

Although no differences have been seen in the quantity of excreted plant sterols, which originate from the diet, the composition was slightly changed in the PFHS and PFOS groups. It might be the result of a change in intestinal absorption/resecretion.

Figure 3.4.2 Sterol excretion

Values are means $\pm$ S.D.

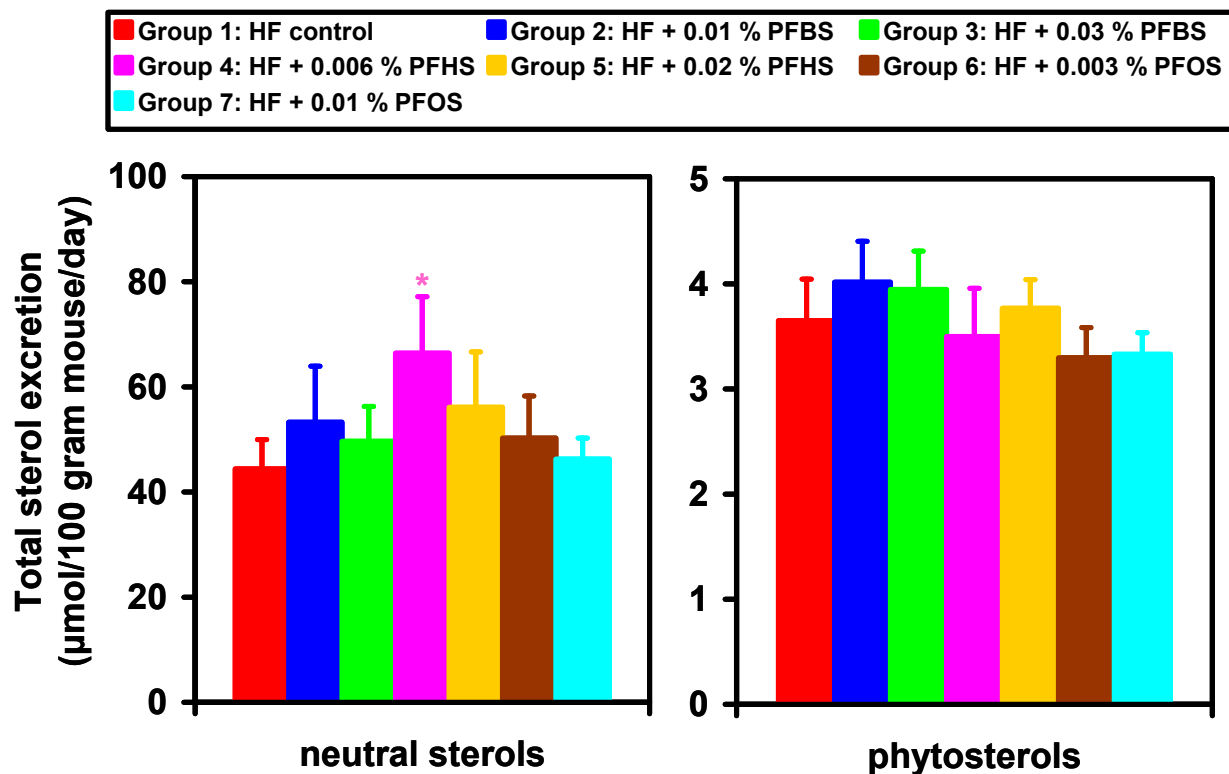
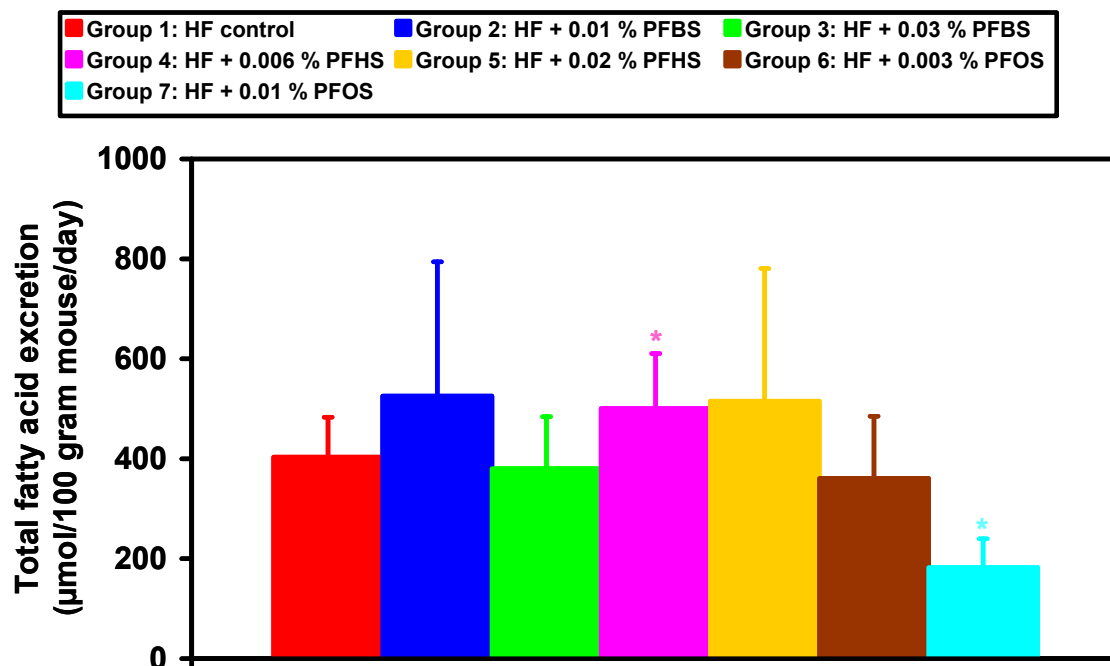


Table 3.4.2a Fecal neutral sterol composition

group	% of neutral sterols				
	coprostanol	coprostanon	cholesterol	cholestanol	lathosterol
Group 1: HF control	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	94.4 $\pm$ 0.4	2.7 $\pm$ 0.2	2.9 $\pm$ 0.3
Group 2: HF + 0.01 % PFBS	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	95.0 $\pm$ 0.6	2.3 $\pm$ 0.8	2.7 $\pm$ 0.3
Group 3: HF + 0.03 % PFBS	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	94.6 $\pm$ 0.7	2.7 $\pm$ 0.4	2.7 $\pm$ 0.6
Group 4: HF + 0.006 % PFHS	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	95.5 $\pm$ 0.3	2.4 $\pm$ 0.3	2.1 $\pm$ 0.1
Group 5: HF + 0.02 % PFHS	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	94.4 $\pm$ 1.4	3.1 $\pm$ 1.5	2.5 $\pm$ 0.2
Group 6: HF + 0.003 % PFOS	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	94.3 $\pm$ 0.7	3.4 $\pm$ 0.6	2.4 $\pm$ 0.2
Group 7: HF + 0.01 % PFOS	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	93.7 $\pm$ 0.7	4.6 $\pm$ 0.6	1.7 $\pm$ 0.1

Table 3.4.2b Fecal phytosterol composition

group	% of phytosterols			
	campesterol	stigmasterol	β -sitosterol	β -sitostanol
Group 1: HF control	26.2 $\pm$ 0.8	8.2 $\pm$ 1.1	65.6 $\pm$ 1.1	0.0 $\pm$ 0.0
Group 2: HF + 0.01 % PFBS	26.2 $\pm$ 1.0	8.1 $\pm$ 1.7	65.7 $\pm$ 1.5	0.0 $\pm$ 0.0
Group 3: HF + 0.03 % PFBS	26.5 $\pm$ 0.7	7.7 $\pm$ 1.9	65.9 $\pm$ 1.7	0.0 $\pm$ 0.0
Group 4: HF + 0.006 % PFHS	27.9 $\pm$ 1.1	4.3 $\pm$ 2.0	67.7 $\pm$ 1.3	0.0 $\pm$ 0.0
Group 5: HF + 0.02 % PFHS	26.6 $\pm$ 1.1	6.5 $\pm$ 2.7	66.9 $\pm$ 2.1	0.0 $\pm$ 0.0
Group 6: HF + 0.003 % PFOS	27.6 $\pm$ 0.8	3.6 $\pm$ 0.5	68.8 $\pm$ 0.8	0.0 $\pm$ 0.0
Group 7: HF + 0.01 % PFOS	27.9 $\pm$ 1.0	5.0 $\pm$ 3.5	67.1 $\pm$ 2.9	0.0 $\pm$ 0.0

Figure 3.4.3 Fatty acid excretionValues are means $\pm$ S.D.**Table 3.4.3 Fecal fatty acid composition**

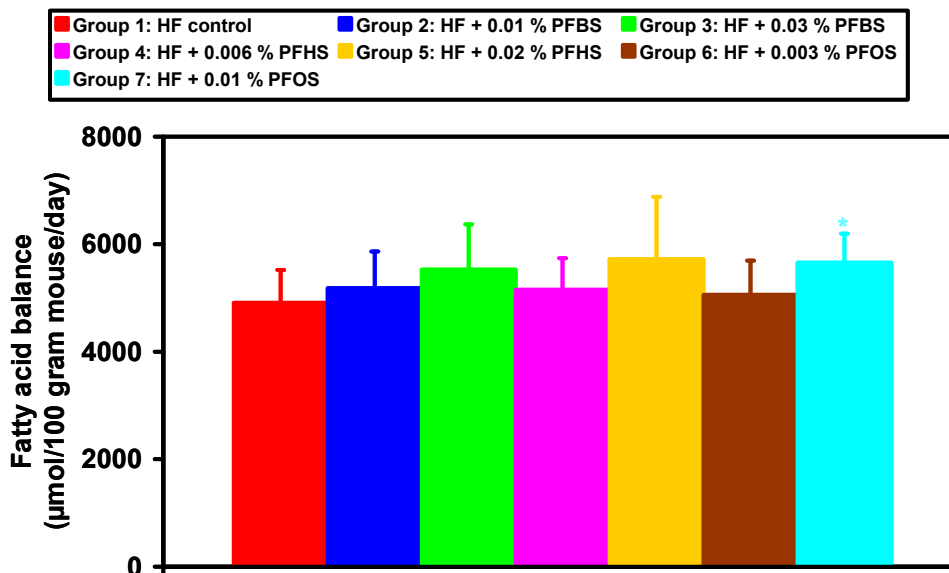
group	% of fatty acids						
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Group 1: HF control	2.0 $\pm$ 0.4	46.0 $\pm$ 3.9	0.38 $\pm$ 0.04	46.0 $\pm$ 3.7	4.8 $\pm$ 0.5	0.30 $\pm$ 0.04	0.55 $\pm$ 0.21
Group 2: HF + 0.01 % PFBS	1.9 $\pm$ 0.5	44.1 $\pm$ 2.7	0.35 $\pm$ 0.13	47.5 $\pm$ 2.9	5.2 $\pm$ 0.6	0.30 $\pm$ 0.05	0.62 $\pm$ 0.10
Group 3: HF + 0.03 % PFBS	1.8 $\pm$ 0.3	45.1 $\pm$ 2.3	0.39 $\pm$ 0.04	47.0 $\pm$ 2.4	4.8 $\pm$ 0.3	0.31 $\pm$ 0.02	0.58 $\pm$ 0.08
Group 4: HF + 0.006 % PFHS	1.7 $\pm$ 0.3	43.6 $\pm$ 1.9	0.35 $\pm$ 0.02	48.6 $\pm$ 1.9	4.8 $\pm$ 0.4	0.31 $\pm$ 0.03	0.60 $\pm$ 0.07
Group 5: HF + 0.02 % PFHS	2.0 $\pm$ 0.5	45.5 $\pm$ 3.2	0.39 $\pm$ 0.04	46.2 $\pm$ 3.2	5.1 $\pm$ 0.7	0.29 $\pm$ 0.03	0.57 $\pm$ 0.10
Group 6: HF + 0.003 % PFOS	1.8 $\pm$ 0.4	45.6 $\pm$ 2.9	0.34 $\pm$ 0.03	46.6 $\pm$ 3.0	4.8 $\pm$ 0.3	0.29 $\pm$ 0.03	0.56 $\pm$ 0.09
Group 7: HF + 0.01 % PFOS	2.2 $\pm$ 0.4	48.2 $\pm$ 2.4	0.35 $\pm$ 0.03	43.5 $\pm$ 2.7	4.9 $\pm$ 0.5	0.38 $\pm$ 0.00	0.46 $\pm$ 0.06

Fecal fatty acids are derived from the fat from food intake (TG) and lipids produced by the liver (PL). Most of the groups (some have large SD) showed no significant difference from control. Group 4 (PFHS) had significantly increased fecal fatty acids, whereas group 7 (2 weeks PFOS and 4 weeks control) showed a strong decrease, with a concomitant small change in fatty acid composition.

From the difference between the total fatty acid (TG) intake per subgroup and total fatty acid excreted in the feces, the fatty acid balance has been calculated. The values are expressed as the difference between total fatty acid input and output in μmol fatty acid per 100 gram mouse per day. As shown in Fig. 3.4.5, all groups have more or less the same fat intake, except that in group 7 this is significantly higher. This is of interest in the light of the loss of fat tissue in the same group (see Fig. 3.6.1).

Figure 3.4.5 Fatty acid balance

Values are means $\pm$ S.D.

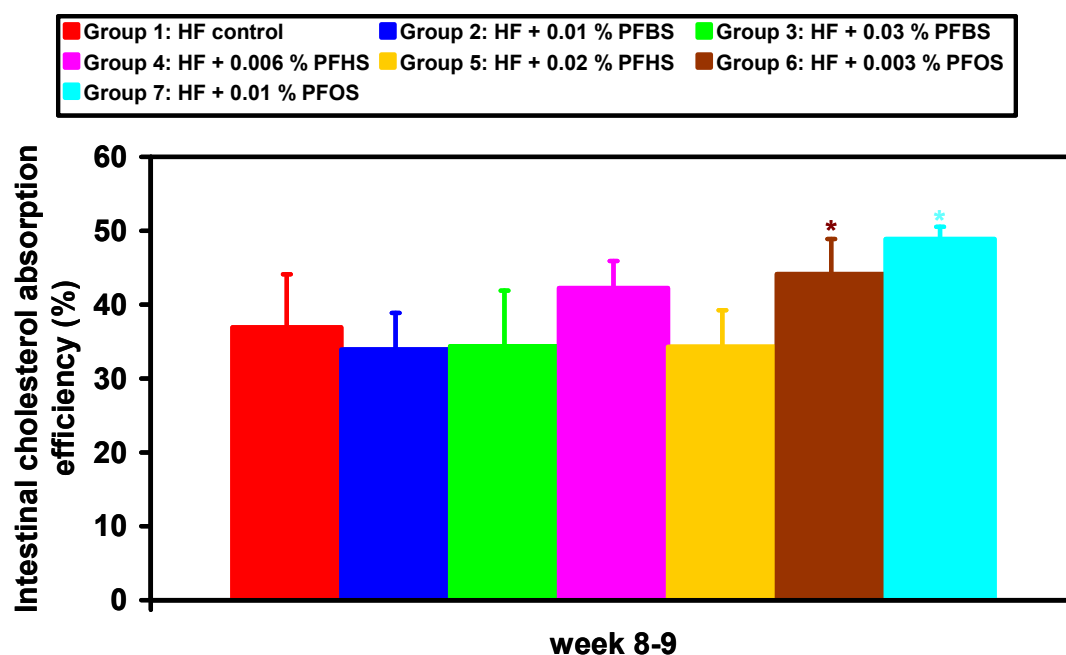


3.5. Intestinal cholesterol absorption

The intestinal cholesterol absorption was assessed by the fecal dual-isotope method. In week 8 all mice were individually housed and received an oral dose of 200 µl of olive oil, containing 1 µCi [¹⁴C]-cholesterol and 1 µCi [³H]-sitostanol, per mouse. Bodyweight and food intake were monitored, and feces were collected for the following 4 days. Feces were homogenized, pooled per mouse over the 4-day period, and dissolved in ethanolic potassium (3mol/l, 60% ethanol). Radioactivity was determined in the fecal samples to assess the ratio of [¹⁴C]-labeled cholesterol and [³H]-labeled sitostanol. Sitostanol was used as the reference compound since it is known to be poorly absorbed (< 3 %) in mice. The formula used to calculate cholesterol absorption was: % cholesterol absorption = ($[\text{C}^{14}]/[\text{H}^3]\text{-dosing mixture} - [\text{C}^{14}]/[\text{H}^3]\text{-feces}$) / $[\text{C}^{14}]/[\text{H}^3]\text{-dosing mixture} \times 100$.

Figure 3.5.1 Intestinal cholesterol absorption

Values are means ± S.D.

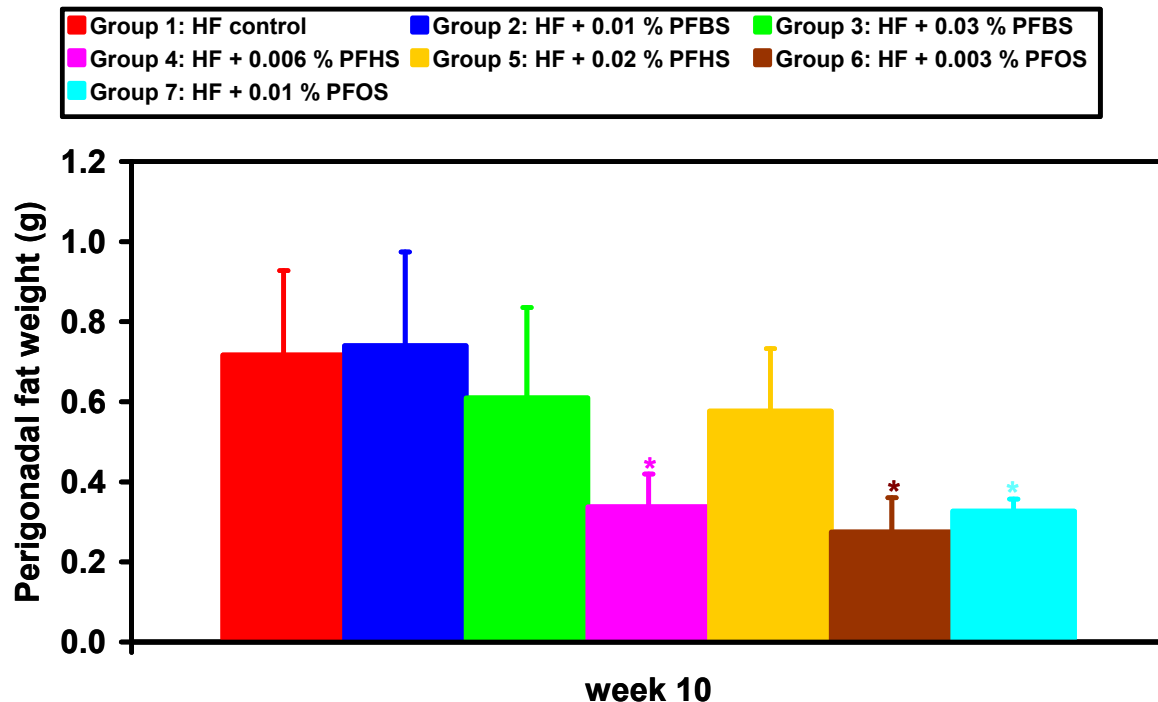


Both PFOS-treated groups 6 and 7 have increased intestinal cholesterol absorption. Since all groups have about the same cholesterol intake via the food and the same fecal cholesterol output (except group 4), this increase must be caused by an increased hepatic cholesterol synthesis. Indeed, the increased HMG-CoA reductase activity in these groups (see Fig. 3.8.2b) suggests an increased hepatic cholesterol synthesis. Although not significantly, the intestinal cholesterol absorption of group 4 (PFHS) seems to be slightly increased as well, which, beside additionally an increased fecal excretion, might be explained as well by an increased hepatic cholesterol synthesis.

3.6. Perigonadal fat tissue weight

Figure 3.6.1 Perigonadal fat weight

Values are means $\pm$ S.D.



PFHS (group 4) and PFOS (groups 6 and 7) strong reduced perigonadal fat tissue. Although not measured, this might reflect a decrease in total body fat tissue. This notion is supported by the decrease in body weight. Plasma leptin levels might be measured, which should be found to be decreased as a reflection of decreased fat tissue. The reduction in perigonadal fat tissue was not seen in the PFBS-treated groups and the PFHS group 5.

3.7. Liver weight and hepatic lipid contents

Livers are strongly increased by PFOS (groups 6 and 7) and PFHS (group 4). The return to control diet of the group 5 PFHS-treated animals caused a return to only slightly but still significantly enlarged livers. When expressed as % of total body weight (Fig. 3.7.1b), the PFBS-treated group 3 also had slightly but significantly enlarged livers when compared to controls.

As shown in Fig. 3.7.2, the lipids, triglycerides and cholesterylesters, have been increased in the livers of PFOS-treated groups 6 and 7. In the other livers these contents were comparable with that of the control mice.

Free cholesterol contents have not been changed by any of the compounds.

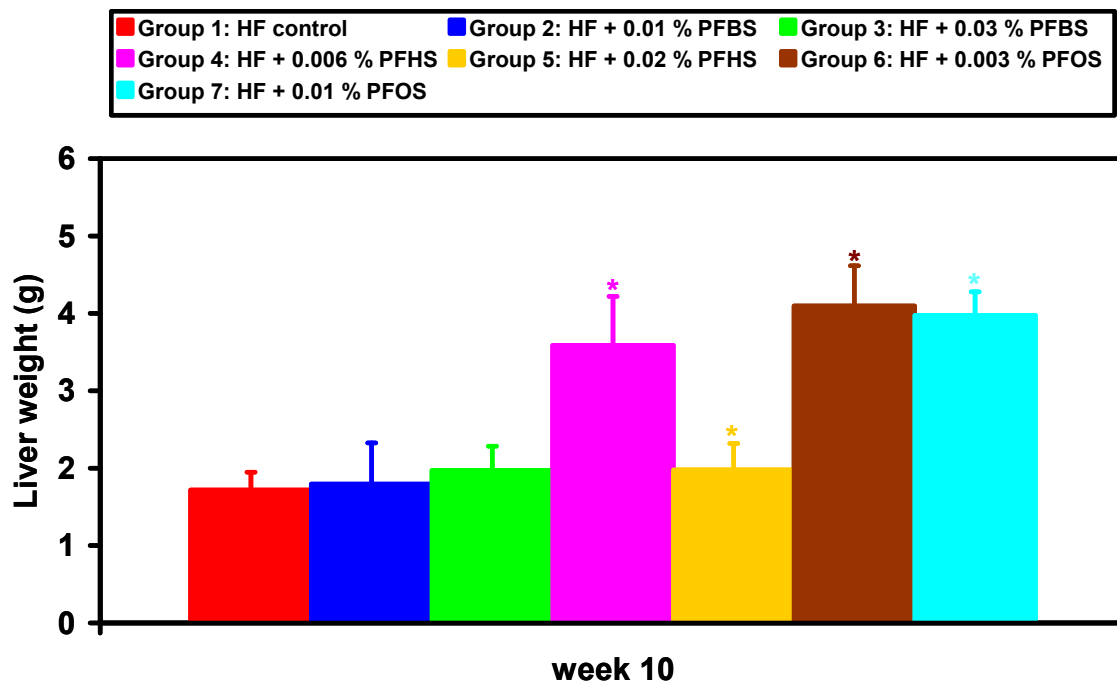
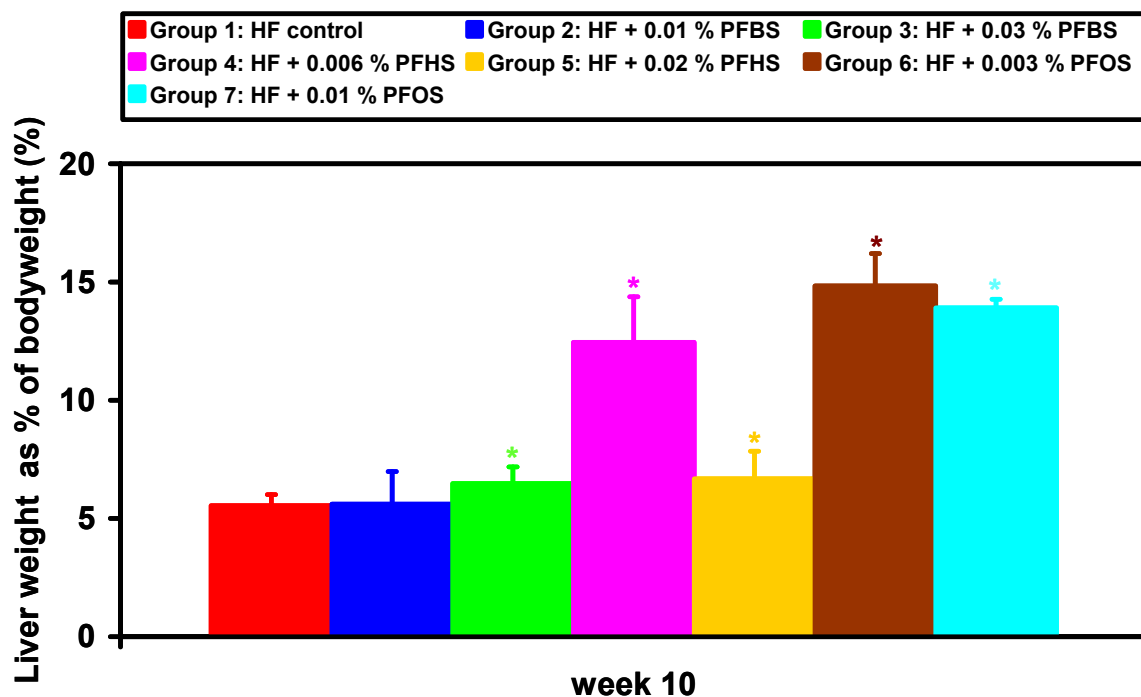
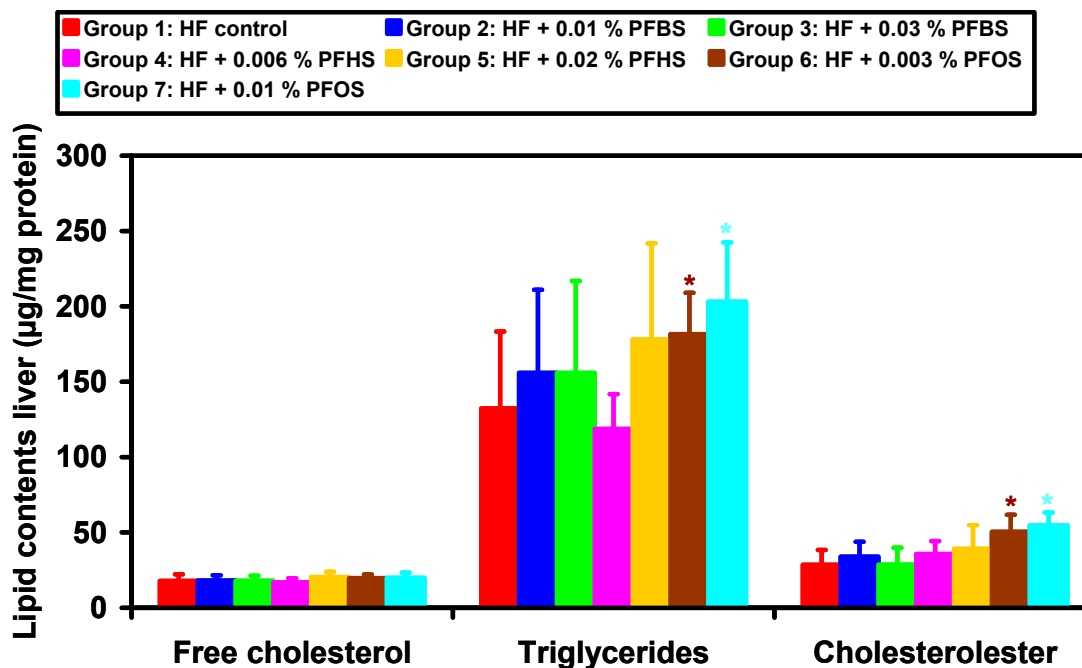
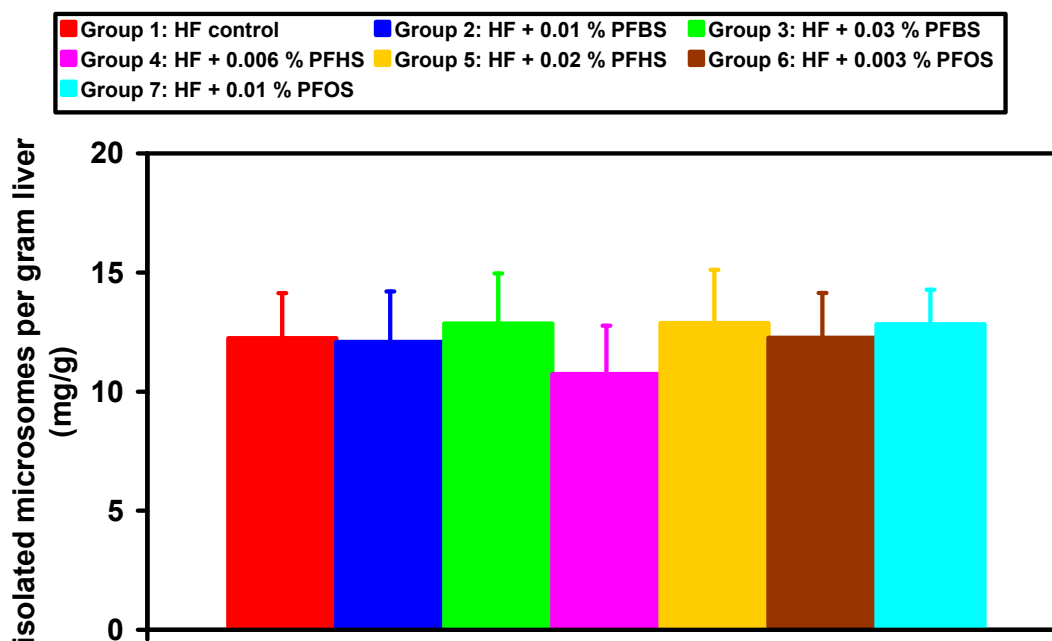
Figure 3.7.1a Liver weightValues are means $\pm$ S.D.**Figure 3.7.1b Liver weight as % of body weight**Values are means $\pm$ S.D.

Figure 3.7.2 Lipid contents liverValues are means $\pm$ S.D.**3.8. Liver enzyme activities: HMG-CoA reductase, cholesterol 7 α -hydroxylase and sterol 27-hydroxylase.**

Immediately after sacrifice, livers have been removed and microsomes and mitochondria have been isolated from the liver tissue. Thereafter, HMG-CoA reductase and cholesterol-7 α -hydroxylase activity have been determined in the microsomal preparations and sterol-27-hydroxylase activity in the mitochondria.

The yield of microsomes per g liver is about the same in all groups (Fig. 3.8.1)

Fig. 3.8.1 Microsomal preparations

Although HMG-CoA reductase activity was rather low in all preparations and therefore SD's are rather large, PFHS and PFOS seem to have increased this activity. This is significantly different from control for PFHS group 4. Since the yield of the microsomes per g liver is more or less the same and large differences between the liver weights have been observed, the activity has been recalculated and expressed as total activity per liver (Fig. 3.8.2b). This might indicate that large differences in total hepatic cholesterol synthesis between the groups exist. Total HMG-CoA reductase activity per liver has been increased significantly in groups 4 (PFHS), 6 (PFOS) and 7 (PFOS).

Figure 3.8.2a HMG-CoA reductase activity in liver microsomes

Values are means $\pm$ S.D.

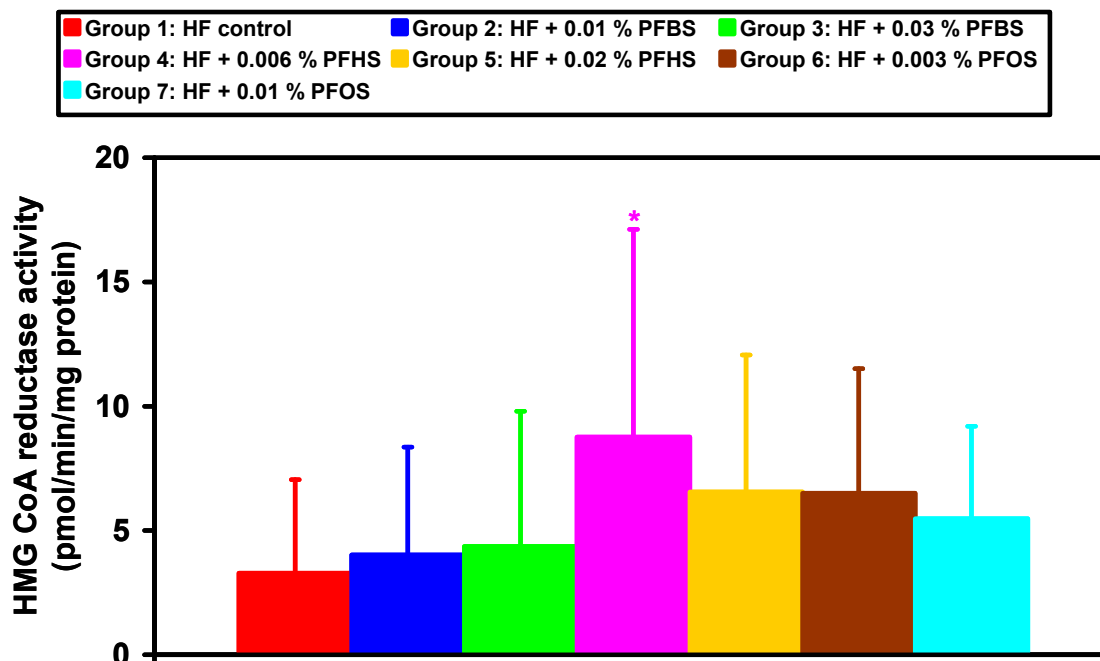


Figure 3.8.2b Total HMG-CoA reductase activity per liver

Values are means $\pm$ S.D.

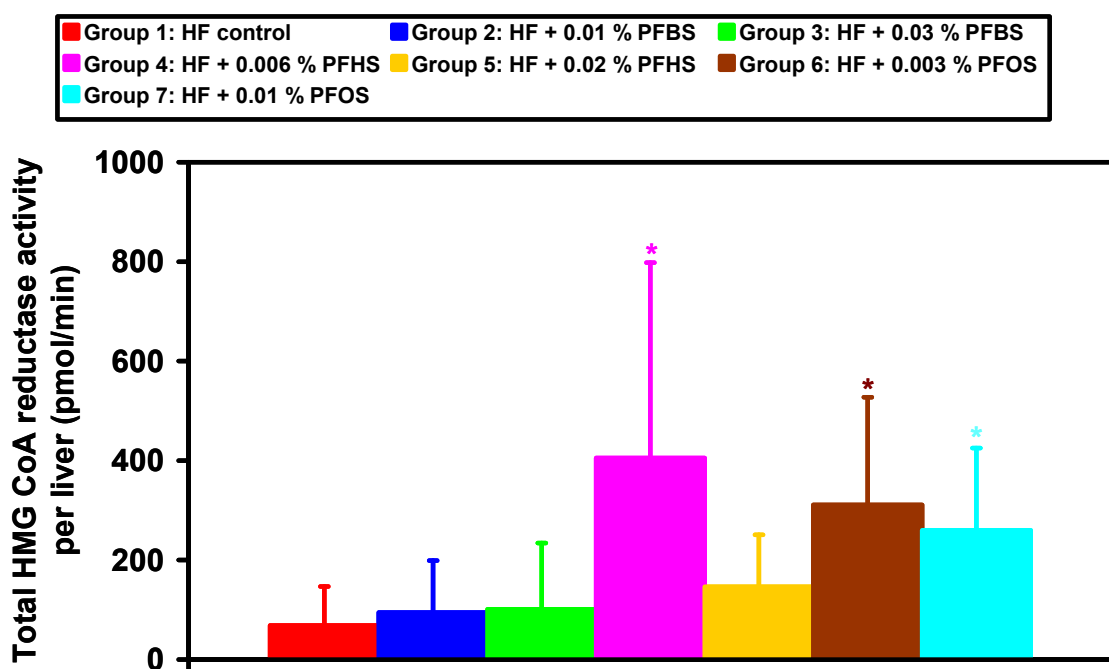


Figure 3.8.3a Cholesterol 7 α -hydroxylase activity in liver microsomes
Values are means $\pm$ S.D.

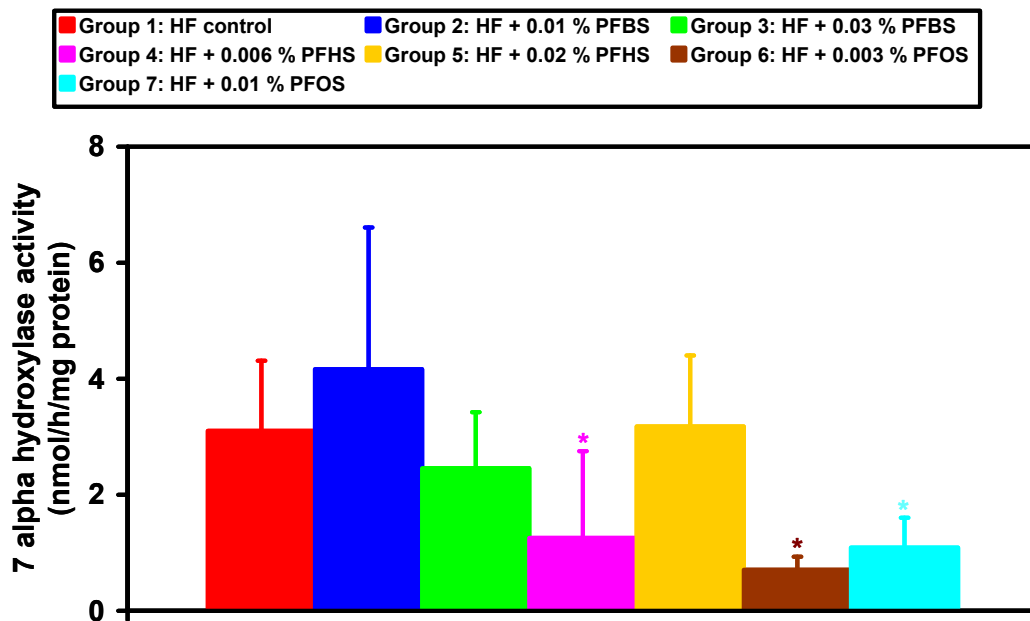
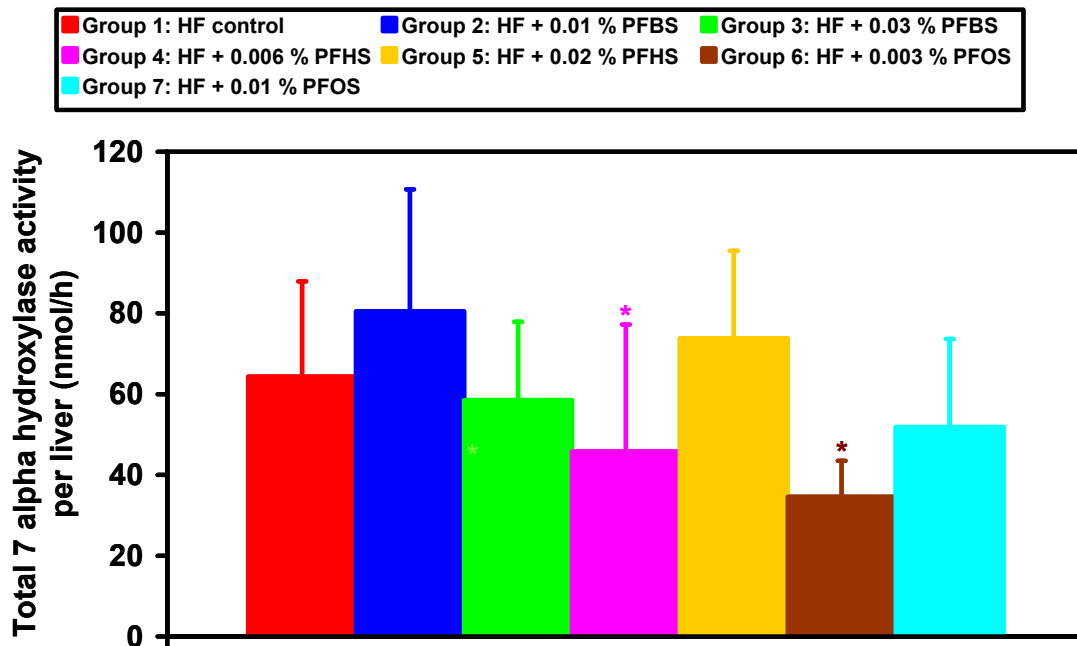


Figure 3.8.3b Total cholesterol 7 α -hydroxylase activity per liver
Values are means $\pm$ S.D.

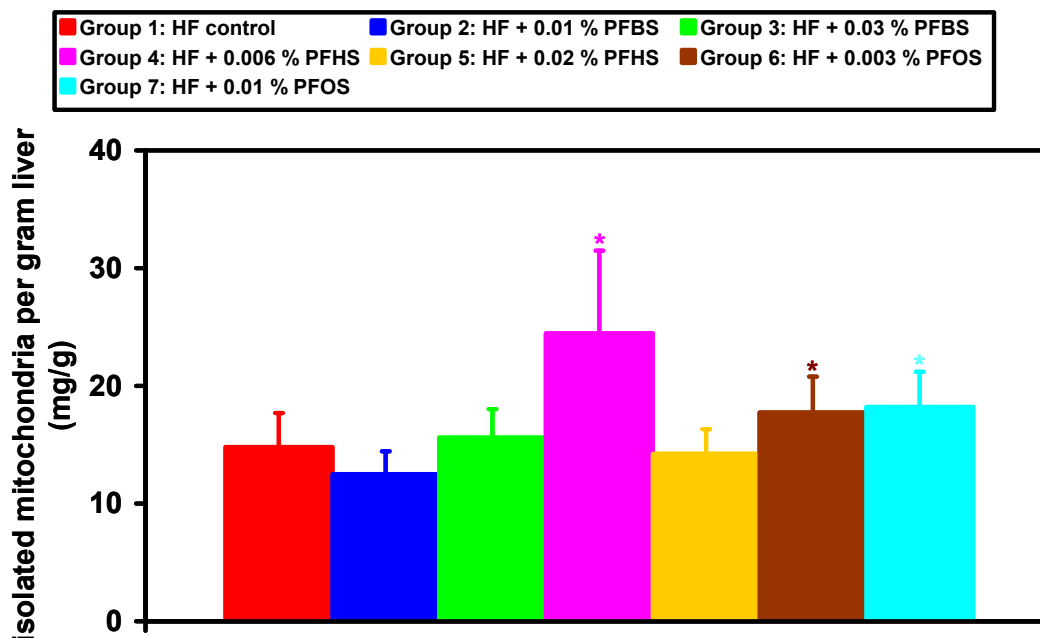


One of the rate limiting enzymes of bile acid synthesis, the microsomal enzyme cholesterol 7 α -hydroxylase, has found to be decreased by PFHS (group 4) and by PFOS (groups 6 and 7). The activity in the other groups did not differ significantly from the control value (although that of group 2 seems to be somewhat enhanced). If the activity is expressed as total activity per liver a significant decrease by PFHS (group 4) and PFOS (group 6) remained, suggesting that total hepatic bile acid production might be reduced as well.

The other regulatory enzyme of bile acid synthesis, sterol 27- hydroxylase was measured in the mitochondrial preparations. It was remarkable that the yield of mitochondria (mg/g liver; Fig. 3.8.4) seemed to be increased in the PFHS-treated and PFOS-treated groups, suggesting that these compounds increased the number of mitochondria. This might be related with the observed increase of fatty acid oxidation.

Figure 3.8.4 Liver mitochondrial preparations

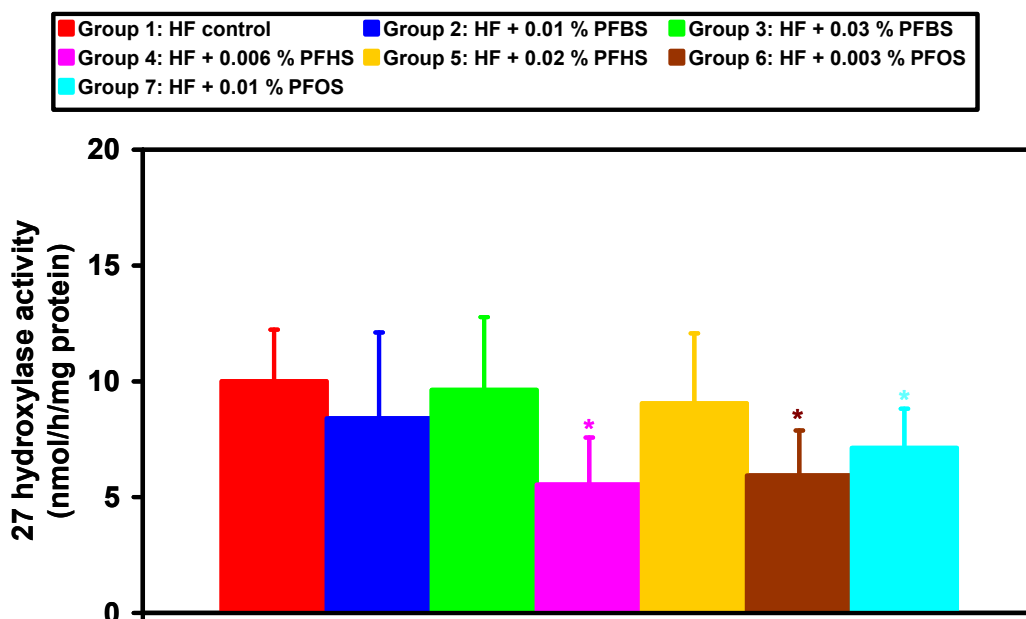
Values are means $\pm$ S.D.



As expresses per mg of mitochondrial protein sterol 27-hydroxylase was found to be decreased by PFHS (group 4) and PFOS (groups 6 and 7) as well.

Figure 3.8.5a sterol 27-hydroxylase activity in liver mitochondria

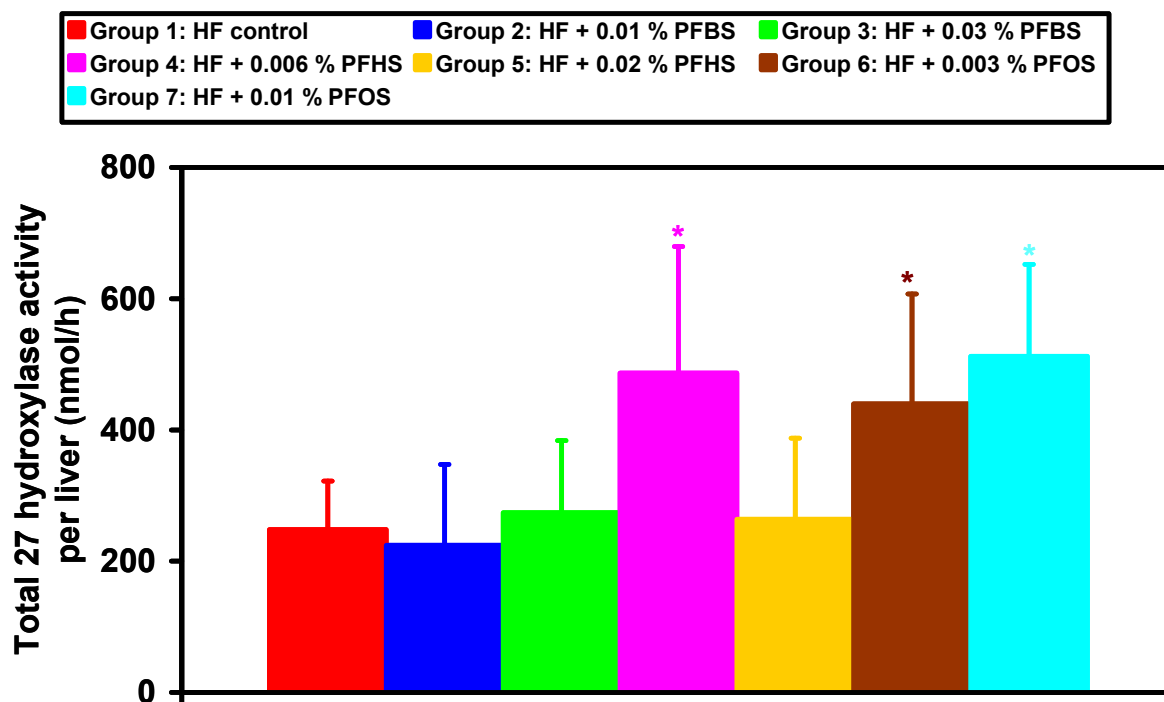
Values are means $\pm$ S.D.



However, taking into account of the differences in mitochondrial yields and in liver weights and expressing the activity per liver (Fig. 3.8.5b), the total sterol 27-hydroxylase activity was found to be increased in the same groups.

Figure 3.8.5b Total sterol 27-hydroxylase activity per liver

Values are means $\pm$ S.D.



4. RESULTS *IN VITRO* EXPERIMENT (RAT HEPATOCYTES)

From the first *in vitro* experiments performed in project 3M-1, it was suggested that the PFAS compounds might influence the internal acetate pool, which is the substrate for hepatic cholesterol synthesis. Since fatty acid catabolism is a source for acetate, the effect of the test compounds on the incorporation of different sized radiolabelled fatty acids into cholesterol has been investigated in the present project. As a positive control, a strong and specific inhibitor of cholesterol synthesis, rosuvastatin has been taken along in each separate experiment. Identical experiments have been performed 3 – 4 times (as mentioned at the figures)

Figure 4.1 Incorporation of [14 C]-acetate into cholesterol in rat hepatocytes *in vitro*
Values are means $\pm$ S.D. (n = 4).

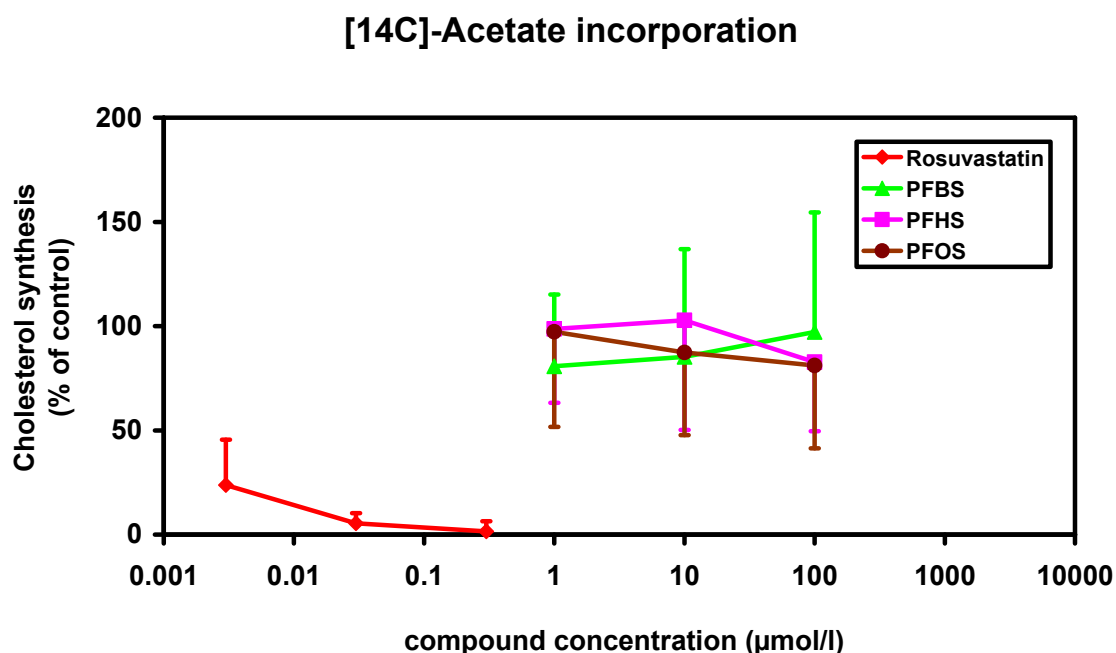
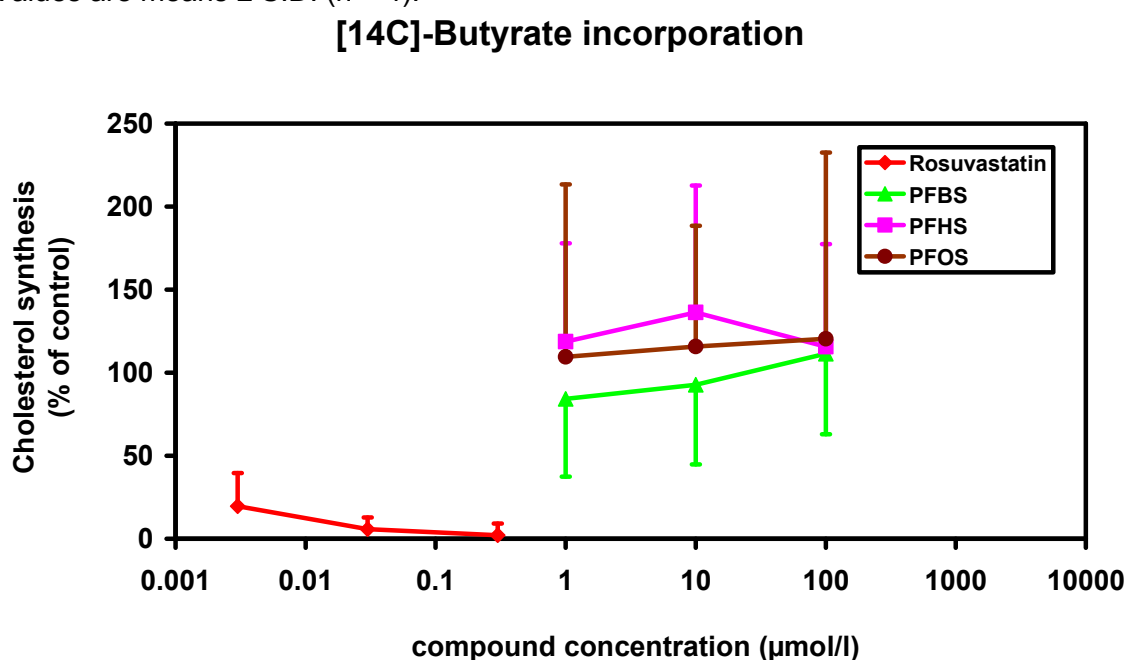


Figure 4.2 Incorporation of [14 C]-butyrate into cholesterol in rat hepatocytes *in vitro*
Values are means $\pm$ S.D. (n = 4).



Rosuvastatin strongly inhibited the incorporation of the short chain fatty acids acetate and butyrate into cholesterol (Figs. 4.1 and 4.2). Although there is a large variation in the data obtained for the test compounds in the individual experiments, on average there seems to be hardly an effect of these compounds on cholesterol synthesis from acetate and butyrate.

The incorporation of the longer fatty acid [^{14}C]-laurate into cholesterol is also comparable to control values in the presence of the PFAS's (Fig. 4.3); however, there might be a slight concentration dependent decreasing effect of PFHS and PFOS. Rosuvastatin is again a much stronger inhibitor if the cholesterol synthesis from [^{14}C]-laurate and also from [^{14}C]-palmitate (Fig. 4.4).

Figure 4.3 Incorporation of [^{14}C]-laurate into cholesterol in rat hepatocytes *in vitro*
Values are means $\pm$ S.D. (n = 3).

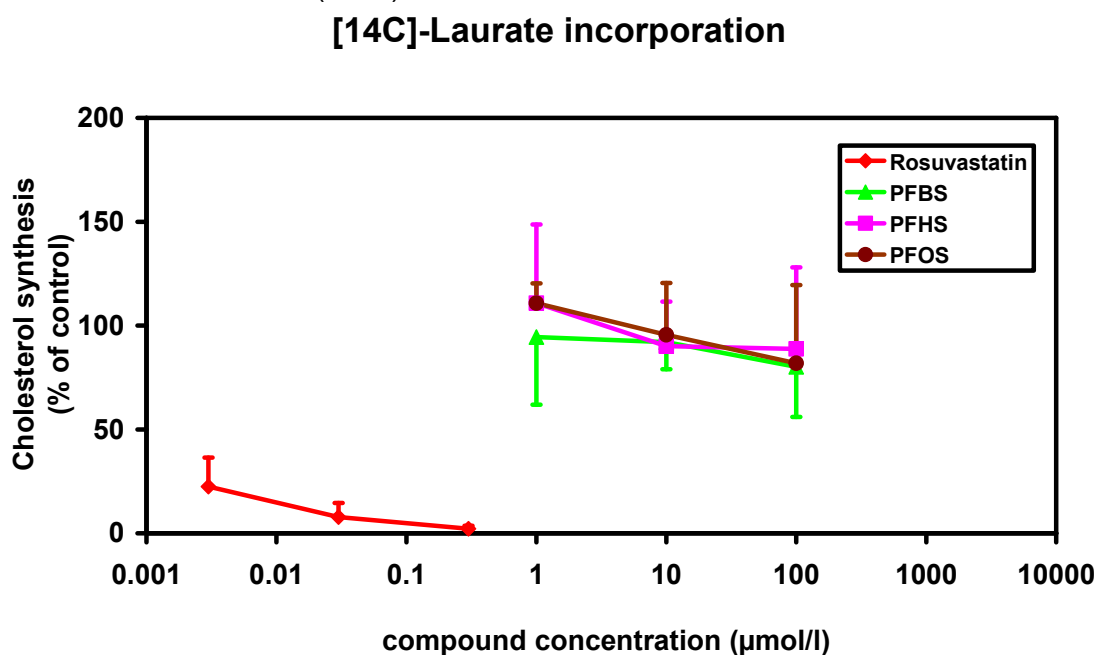
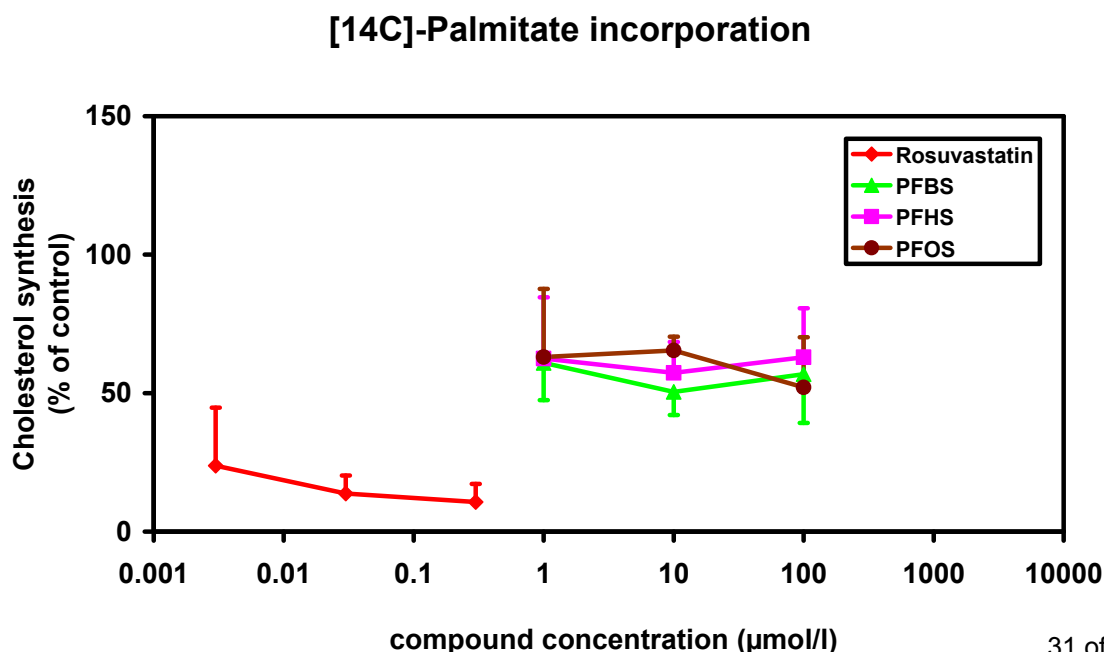


Figure 4.4 Incorporation of [^{14}C]-palmitate into cholesterol in rat hepatocytes *in vitro*
Values are means $\pm$ S.D. (n = 3).



The incorporation of palmitate, the longest fatty acid used in this study, into cholesterol was influenced in a somewhat remarkable way. In the presence of the different concentrations of the test compounds on average all values are lower than control values. But there is no obvious concentration dependency in this decrease. So, it is difficult to understand that all three compounds are influencing palmitate uptake/transport/degradation in the same concentration independent way. Since the solubility of this longer fatty acid in the medium is depending on several factors and the radiolabelled compound was present in tracer amounts, it might be that the test compounds have influenced its solubility and hence its availability for uptake in the cells.

So, all together the test compounds have either no or only slight effects on the incorporation of short and longer radiolabelled fatty acids into cholesterol in rat hepatocytes under the conditions used.

5. SUMMARY AND CONCLUSIONS

Although the test compounds have similar chemical structures, they show differences in their biological behavior. For that reason they are discussed here separately.

5.1. Effects of PFBS in ApoE3Leiden mice

Both PFBS-treated groups 2 (0.01%) and 3 (0.03%) showed no or small differences with the control group 1. No differences were found in body weight, food intake, plasma ALAT and ketone bodies, metabolic parameters as measured in metabolic cages, fecal sterol and fatty acid excretion, intestinal cholesterol absorption, fat tissue weight and liver weight, lipid contents and enzyme activities.

Small differences have been seen in plasma lipid levels: a slight decrease in cholesterol and triglycerides, which was reflected in minor changes of the lipoprotein patterns; VLDL seemed to be increased a little, but IDL/LDL and HDL were somewhat lower than these fractions in the control patterns.

Interesting is the small but significant increase in fecal bile acid excretion in the group 2 mice. These mice showed also an increased hepatic cholesterol 7 α -hydroxylase activity which might support the preceding notion, but because of the large standard deviation in this value it was not statistically significant.

The suggested small increase in bile acid synthesis might be related to the slightly decreased plasma lipid levels.

5.2. Effects of PFHS in ApoE3Leiden mice

The group 4 animals have been treated with 0.006% of PFHS throughout the 10 weeks of the study period. The higher dosed group 5 (0.02% of PFHS) showed already from the start deviations in food intake, which led to a large loss of body weight in the 2nd week of treatment. Therefore, from week 2, these mice were put back on control diet and remained on that throughout the rest of the treatment period. This resulted in a recovery of these animals, which, after a shorter or longer period, showed similar values as measured in the control group; however, some differences remained.

The PFHS-treatment in group 4 had the following effects:

Although food intake did not change, the body weight steadily decreased in the last 3 weeks. This can be explained by the prominent loss in fat tissue (measured as perigonadal fat mass; it might be better to measure plasma leptin as a marker for whole body fat). The latter effect was probably been caused by increased fatty acid oxidation, what can be concluded from increased plasma ketone bodies and a preference for fat oxidation over carbohydrate oxidation, as measured in the metabolic cage analysis (tendency towards decreased RER; group size was rather small). Additionally, O₂-consumption and concomitant energy expenditure was enhanced in these animals. Another indication for increased oxidation is the increased quantity of mitochondria,

isolated from the significantly enlarged livers. For further support of increased fatty acid oxidation, hepatic activity and/or mRNA's of enzymes involved in fatty acid oxidation, e.g. Acyl-CoA oxidase, should be measured.

Plasma cholesterol and triglycerides were strongly decreased by PFHS throughout the treatment period, which was paralleled by a change in lipoprotein profile:

ApoB-containing fractions (VLDL, IDL and LDL) were decreased; whereas, a shift in HDL towards larger, less dense particles was observed. These phenomena can be explained by a decrease in hepatic VLDL production and decreased uptake/conversion of HDL by the liver.

In summary, liver functions have been changed, which is further supported by an increase in liver size/weight (liver lipid contents did not change), increased plasma ALAT values, increased HMG-CoA reductase activity and decreased cholesterol 7 α -hydroxylase, which are rate limiting enzymes in cholesterol and bile acid syntheses, respectively.

Mitochondrial sterol 27-hydroxylase activity was changed as well.

Changes in hepatic cholesterol/bile acid synthesis might have influenced the fecal lipid excretion and intestinal cholesterol absorption. PFHS increased fecal cholesterol and fatty acid excretion and decreased fecal bile acid excretion. On the other hand, changes in intestinal uptake/conversion and small changes in food intake cannot be ruled out in this respect. There is a trend observed towards increased intestinal cholesterol absorption by PFHS.

Several of the liver effects of PFHS are similar to those of a PPAR α agonist, e.g., like fibrates. For this reason it might be of use to further investigate the expression of other genes which are regulated by PPAR α .

As known, such compounds lower plasma lipids and decrease fat tissue as well, but their effect on intestinal functions is not known.

As mentioned above, in the group 5 animals with a 2 weeks-treatment at the highest dose of PFHS, followed by 8 weeks on control diet again, the effects by PFHS as seen in group 4 disappeared, and the parameters measured returned more or less rapidly to control values. That was not the case in all instances the case: The livers remained a little enlarged and plasma ALAT stayed somewhat higher than control. Ketone bodies remained significantly increased up to week 6, and fecal bile acid excretion was still reduced. So it might be that during this period PFHS was not yet totally eliminated from the mice. The values of plasma PFHS concentrations, which will be determined by 3M-company, will give inside in this matter.

5.3. Effects of PFOS in ApoE3Leiden mice

The effects of PFOS (group 6; 0.003%) on the parameters measured in ApoE3Leiden mice in this study were very similar to those of PFHS, however, the effects were in several instances more pronounced.

That means that group 6 mice showed:

Loss of (perigonadal) fat tissue, strongly increased plasma ketone bodies and a decreased RER, which showed a preference for fat oxidation over carbohydrate oxidation, and a concomitantly increased energy expenditure; all indications for an increased fatty acid oxidation. Additionally, increased quantities of mitochondria, have been isolated from the livers of these animals.

Plasma cholesterol and triglycerides were strongly decreased, with a similar change in lipoprotein profile as observed with PFHS. ApoB-containing fractions were decreased; whereas, a shift in HDL towards larger, less dense particles was observed. Interestingly, the "large"-HDL peak increased in time, suggesting that PFOS is accumulating during the treatment period in this group.

Furthermore, the liver weight increased almost 3 times with concomitantly increased plasma ALAT levels. Liver lipids, triglycerides and cholesteryl esters were significantly increased. Hepatic microsomal HMG-CoA reductase activity was increased and cholesterol 7 α -hydroxylase and mitochondrial sterol 27-hydroxylase activities were decreased.

PFOS did not change fecal cholesterol and fatty acid excretion, and decreased fecal bile acid excretion and composition. On the other hand, the ratio of different neutral sterols in the feces has been changed, and PFOS increased significantly intestinal cholesterol absorption. The latter might be a reflection of increased hepatic cholesterol synthesis, but other changes in intestinal cholesterol metabolism cannot be ruled out.

As with PFHS, the PFOS mechanism of action might be (partly) understood as working like a PPAR α agonist. This notion needs further investigation.

The observation in group 7 (2 weeks of a higher dose of PFOS followed by 10 weeks on control diet) are almost identical to those in group 6. And although cholesterol levels seem to recover after 4 weeks, the lipoprotein pattern continued to contain the same large "large"-HDL fraction. These all are indications that PFOS concentrations in the mice remained at a similar level throughout the whole period and that PFOS is not eliminated readily from the body. PFOS is known to have a long elimination half-life in rats, monkeys, and humans, and determination of the plasma PFOS-concentrations at the end of the treatment period, should confirm this presumption.

The only remarkable difference between group 6 and 7 is found in the fecal excretion data. Much less fatty acids have been found in the feces of group 7, which also possess a slightly different composition of fatty acids. So, intestinal fatty acid absorption (and/or conversion) seemed to be increased when PFOS is withdrawn from the food.

Table 5.1 gives an overview of the effects of PFBS, PFHS and PFOS treatment on the different measured parameters.

Table 5.1: Overview of effect of PFBS, PFHS and PFOS treatment on measured parameters

Measured parameter	PFBS 0.01%	PFBS 0.03%	PFHS 0.006%	PFHS 0.02% only 2 weeks on diet	PFOS 0.003%	PFOS 0.01% only 2 weeks on diet
Bodyweight	-	-	↓	↓↓	↓	↓↓
Food intake	-	-	-	↓↓	-	↓↓
Total Cholesterol	-	-	↓ (-36%)	↓ (-38%)*	↓ (-31%)	↓ (-46%)*
Total Triglycerides	-	-	↓ (-50%)	↓ (-54%)*	↓ (-22%)	↓ (-50%)*
Lipoprotein profile	slight decrease in LDL and HDL (n.s.)	slight decrease in LDL and HDL (n.s.)	decrease in HDL, shift to LDL, decrease in VLDL/IDL	decrease in HDL, shift to LDL, decrease in VLDL/IDL**	decrease in HDL, shift to LDL, decrease in VLDL/IDL	decrease in HDL, shift to LDL, decrease in VLDL/IDL**
ALAT	-	-	2-4.5 x ↑	1.5-2 x ↑	3-5 x ↑	3-5 x ↑
Ketone bodies	-	-	1.4 x ↑	1.5 x ↑	2.5 x ↑	2.7 x ↑
Fecal bile acids	1.3 x ↑	-	↓ (-25%)	↓ (-18%)	↓ (-48%)	↓ (-17%)
Fecal neutral sterols	-	-	1.5 x ↑	-	-	-
Fecal fatty acids	-	-	1.2 x ↑	-	-	↓ (-55%)
Fatty acid balance	-	-	-	-	-	1.2 x ↑
Metabolic cage analysis: O ₂ -consumption	-	-	↑	-	↑	-
CO ₂ -production	-	-	slight increase (n.s.)	-	slight increase (n.s.)	-
Respiratory exchange ratio	-	-	slight decrease (n.s.)	-	↓	-
Energy expenditure	-	-	↑	-	↑	-
Intestinal cholesterol absorption	-	-	-	-	1.2 x ↑	1.3 x ↑
Weight perigonadal fat	-	-	↓ (-53%)	-	↓ (-61%)	↓ (-54%)
Liver weight	-	-	2.1 x ↑	1.2 x ↑	2.4 x ↑	2.3 x ↑
Liver weight as % of bodyweight	-	1.2 x ↑	2.2 x ↑	1.2 x ↑	2.7 x ↑	2.5 x ↑
Liver lipids: Free cholesterol	-	-	-	-	-	-
Triglycerides	-	-	-	-	1.4 x ↑	1.5 x ↑
Cholesterol ester	-	-	-	-	1.8 x ↑	1.9 x ↑
Microsomes: amount	-	-	-	-	-	-
HMG CoA reductase per mg protein	-	-	2.7 x ↑	-	-	-
HMG CoA reductase per liver	-	-	5.9 x ↑	-	4.5 x ↑	3.8 x ↑
Cholesterol 7-α-hydroxylase per mg protein	-	-	↓ (-60%)	-	↓ (-77%)	↓ (-65%)
Cholesterol 7-α-hydroxylase per liver	-	-	↓ (-29%)	-	↓ (-46%)	-
Mitochondria: amount	-	-	1.7 x ↑	-	1.2 x ↑	1.2 x ↑
Cholesterol 27-hydroxylase per mg protein	-	-	↓ (-45%)	-	↓ (-41%)	↓ (-29%)
Cholesterol 27-hydroxylase per liver	-	-	2 x ↑	-	1.8 x ↑	2.1 x ↑

*: measured after 1 week of treatment

**: measured after 2 weeks after changing diet to control diet

n.s.: not significant