

# Detailed description of the Project Achievements

## A. Main scientific findings in detail

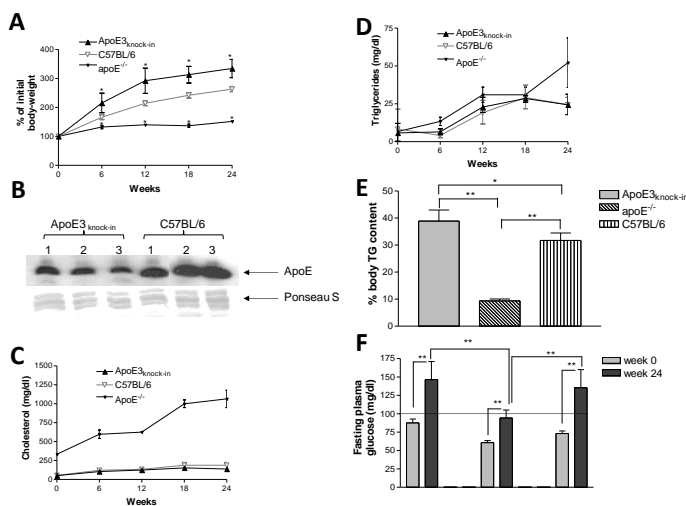
### A1.1. Role of the chylomicron metabolic pathway in diet-induced obesity and glucose metabolism (FEBS J. 275(19):4796-809).

**ApoE promotes diet-induced weight-gain in mice while apoE-deficiency prevents it.** To test the effects of apoE on weight gain in mice, groups of 4-6 week-old female apoE3<sub>knock-in</sub>, apoE<sup>-/-</sup>, and wild-type (WT) C57BL/6 mice were placed on western-type or normal chow diet for a total period of 24 weeks. Mice in each group were weighed immediately before the initiation of the experiment (week 0) and every six weeks thereafter up to week 24, using a Mettler® precision microscale.

It became apparent that as early as 6 weeks on high fat diet, apoE3-expressing mice gained weight and were significantly heavier than the WT C57BL/6 mice on the same diet (Fig. 1A). The weight of the apoE3<sub>knock-in</sub> mice was 31.37±3.98 grams (115±34% higher as compared to their initial weight of 17.03±0.94 grams, p<0.05) (Fig. 1A). During the same period, C57BL/6 mice on high-fat diet had an average body weight of 26.08±1.89 grams (66±8% higher as compared to their initial weight of 16.26±0.61 grams, p<0.05) (Fig. 1A). There were no statistical differences between the weights of the apoE3<sub>knock-in</sub> and C57BL/6 control groups fed chow diet for 6 weeks (data not shown).

At week 24 on high fat diet, apoE3-expressing mice showed a dramatic increase in body weight, with an average value of 50.23±2.22 grams (235±32% higher as compared to their starting weight at week 0, p<0.05) (Fig. 1A). The body weight of the C57BL/6 mice was 43.10±0.94 grams (164±9% higher as compared to their starting weight at week 0, p<0.05) (Fig. 1A). The control apoE3<sub>knock-in</sub> and C57BL/6 mouse groups on chow diet showed a much smaller increase in body weight, ranging between 22 and 24 grams (29±6% increase as compared to their starting weight at week 0, p<0.05) (data not shown). In contrast to the apoE3<sub>knock-in</sub> and the C57BL/6 mice, apoE<sup>-/-</sup> mice that were fed western-type diet showed only a modest increase in body-weight during the course of the experiment (Fig. 1A). At week 6 of the experiment, the apoE<sup>-/-</sup> mouse group had an average body-weight of 20.36±1.37 grams (32±6% increase, as compared to their starting weight of 16.26±0.61 at week 0, p<0.05). At week 24, their body weight was 24.58±1.07

grams (41±4% increase, as compared to their starting weight at week 0, p<0.05) (Fig. 1A). Similar increases in body-weight were observed in the control apoE<sup>-/-</sup> mice fed chow diet (data not shown).



**Fig. 1.** Percent of initial body-weight (*panel A*), plasma apoE levels (*panel B*), plasma cholesterol levels (*panel C*) and plasma triglyceride levels (*panel D*), of C57BL/6, apoE3<sub>knock-in</sub> and apoE-deficient mice fed western-type diet for a period of 24 weeks. (*Panel E*) Percent body fat content of apoE3<sub>knock-in</sub>, apoE<sup>-/-</sup>, and C57BL/6 at week 24. (*Panel F*) Fasting plasma glucose levels of apoE3<sub>knock-in</sub>, apoE<sup>-/-</sup>, and C57BL/6 mice on week 0, and 24. Each point on the graphs represents the mean value of the group and error bars indicate the standard error of the mean. The statistical significance of the observed differences among groups at each time point

is as indicated (\*corresponds to p<0.05 and \*\* corresponds to p<0.005). In *panel B*, plasma apoE levels in apoE3<sub>knock-in</sub> and apoE<sup>-/-</sup> mice were determined by western blot analysis using an antibody that recognizes equally the mouse and human apoE (Santa-cruz biotech, cat. # sc-31821). Ponceau S staining of the nitrocellulose membrane was used to confirm equal loading and efficient transfer of proteins on the membrane.

To compare the steady-state plasma apoE levels between apoE3<sub>knock-in</sub> and C57BL/6 mice, at week 0 of the experiment plasma samples were isolated from three mice from each group and 5 µl of plasma were analyzed by western blotting using a polyclonal antibody that recognizes both the human and mouse apoE (Santa-cruz biotech, Santa Cruz, CA., USA, cat. # sc-31821). This analysis showed that C57BL/6 mice have approximately 4-fold higher steady-state plasma apoE levels than apoE3<sub>knock-in</sub> mice, suggesting that the increased sensitivity of apoE3<sub>knock-in</sub> mice to obesity is not due to higher plasma apoE levels in these mice compared to C57BL/6 mice (Fig. 1B).

To determine if body-weight differences among groups fed western-type diet could be explained by differences in their average daily food consumption, at week 12 of the experiment we determined the average daily food consumption for each mouse group. It was found that apoE3<sub>knock-in</sub> mice consumed 3.04±0.13 grams/mouse/day, C57BL/6 mice consumed 3.31±0.15 grams/mouse/day, apoE<sup>-/-</sup> mice consumed 3.19±0.17 grams/mouse/day, and there was no statistical difference among groups (p>0.05).

**Plasma lipid levels of the mice fed western-type diet for 24 weeks.** During the 24-week period of feeding mice with western-type diet, fasting plasma samples were isolated every 6 weeks and cholesterol, triglyceride and free-fatty acid levels were measured as described in Experimental Procedures. As shown in Fig. 1C, at week 24 both apoE3<sub>knock in</sub> and C57BL/6 mice on high fat diet had slightly elevated fasting cholesterol levels (138±10 mg/dl and 188±14 mg/dl respectively) as compared to their starting cholesterol levels at week 0 (50±3 mg/dl and 54±6 mg/dl respectively), while their plasma triglyceride levels remained normal (24±7 mg/dl and 24±5 mg/dl respectively) (Fig. 1D). FPLC analysis of plasma from these mice showed that the small increases in the cholesterol levels of these mice at week 24 were due to a minor accumulation of chylomicron and VLDL remnants (not shown). In contrast, apoE<sup>-/-</sup> mice showed a dramatic increase in their plasma cholesterol levels during the course of the experiment (Fig. 1C). At week 24 of the experiment plasma cholesterol levels of the apoE<sup>-/-</sup> mice were 1064±198 mg/dl (Fig. 1C) while their plasma triglyceride levels remained normal (52±28 mg/dl at week 24) (Fig. 1D). FPLC analysis showed that the hypercholesterolemia of these mice was due to increased accumulation of triglyceride-containing cholesterol-rich chylomicron remnants (not shown). No significant difference in the plasma free fatty-acid levels among groups was observed during the course of the experiment. At week 24 plasma free fatty-acid levels of the apoE3<sub>knock-in</sub>, C57BL/6 and apoE<sup>-/-</sup> were 0.89±0.08 mmole Equiv, 0.81±0.05 mmole Equiv, and 0.99±0.08 mmole Equiv, respectively.

**Body composition analysis of the mice fed western-type diet for 24 weeks.** At the end of the 24-week period on western-type diet, at least six mice from each group (ApoE3<sub>knock-in</sub>, apoE<sup>-/-</sup>, and C57BL/6 mice) were sacrificed. As shown in Fig. 1E, body composition analysis established that apoE3<sub>knock-in</sub> mice had a total body lipid content of 39±4% . The WT C57BL/6 mice had a significantly lower total body lipid content of 32±3% , (p<0.05). Thus, the increased body-weight of the apoE3<sub>knock-in</sub> and C57BL/6 mice reflects excess accumulation of adipose tissue in these mice. In contrast, apoE<sup>-/-</sup> mice remained lean during the course of the experiment with a total body fat content of 11±1% (Fig. 1E, p<0.005). The complete body composition analysis of the mice fed western-type diet for 24 weeks is summarized in Table 1.

**TABLE 1.** Body-composition of apoE3<sub>knock-in</sub>, C57BL/6 and apoE<sup>-/-</sup> mice fed western-type diet for 24 weeks.

Mouse Strain	Wet body-weight	Dry body-weight	LBM	Body-fat	Water
apoE3 <sub>knock-in</sub>	50.2±2.2	33.2±2.0	28.9±3.3	21.3±1.5	17.0±3.9
C57BL/6	43.1±0.9	34.8±1.7	28.1±1.9	15.0±1.6	8.3±2.6
ApoE <sup>-/-</sup>	24.6±1.1	9.2±0.7	22.0±0.8	2.5±0.3	14.4±0.4

Values are in grams expressed as mean ± standard error of the mean.

**Diet-induced obesity in apoE3<sub>knock-in</sub> and C57BL/6 mice is associated with elevated plasma glucose, insulin and leptin levels.** Epidemiological and animal studies have established that central obesity is associated with glucose intolerance and insulin resistance (1) . In addition, obesity is accompanied by increased leptin levels (2) , a hormone that reduces appetite and may function as the link between obesity and hypertension in individuals with the metabolic syndrome (3, 4) .

To determine if the obesity observed in apoE3<sub>knock-in</sub> and C57BL/6 mice is associated with hyperglycemia, at weeks 0 and 24 of the experiment mice were fasted for 16 hours and then plasma glucose levels were measured. Immediately prior to switching mice to western-type diet (week 0), the fasting plasma glucose levels of the apoE3<sub>knock-in</sub>, apoE<sup>-/-</sup>, and C57BL/6, were 84.2±3.7 mg/dl, 60.7±2.8 mg/dl, and 75.2±3.5 mg/dl respectively (Fig. 1F, p<0.05). After 24 weeks on western-type diet, apoE3<sub>knock-in</sub> and the control C57BL/6 mice developed hyperglycemia with fasting glucose levels of 146.5±9.1 mg/dl (p<0.05) and 135.4±7.9 mg/dl (p<0.05) respectively (Fig. 1F). In contrast, apoE<sup>-/-</sup> mice which remained lean during the course of the experiment had only slightly elevated fasting glucose levels that were within physiological levels (94.3±3.22 mg/dl, p<0.005). To determine plasma insulin and leptin levels in these mice, serum samples were isolated at weeks 0 and 24 of the experiment and analyzed for insulin and leptin. At week 0, all three mouse groups had similar plasma insulin levels (0.17±0.05 ng/ml for apoE3<sub>knock-in</sub> mice,

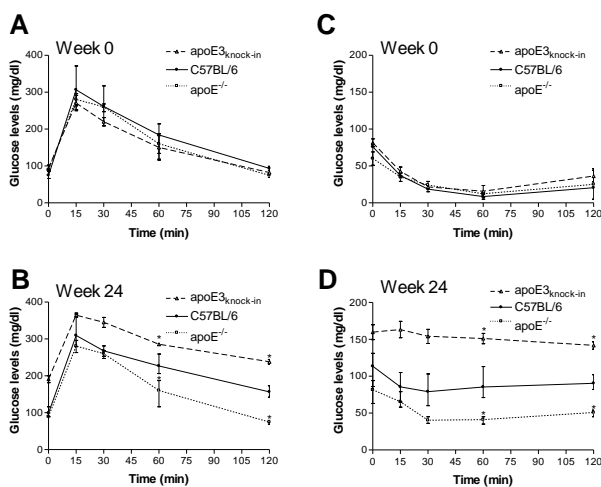
0.12±0.05 ng/ml for apoE<sup>-/-</sup> mice, and 0.16±0.01 ng/ml for C57BL/6 mice). At week 24, apoE3<sup>knock-in</sup> and C57BL/6 mice had elevated plasma insulin levels with concentrations in the range of 4.73±1.03 ng/ml (p<0.05) and 1.28±0.32 ng/ml (p<0.05), respectively. In contrast, apoE<sup>-/-</sup> mice fed western-type diet for 24 weeks had insulin levels of 0.23±0.07 ng/ml that were similar to the levels of apoE<sup>-/-</sup> mice (0.317±0.17 ng/ml) on chow-diet for the same period of time.

Analysis of plasma leptin levels showed that at week 0, mice had similar leptin levels of 5.90±0.40 ng/ml for apoE3<sup>knock-in</sup> mice, 4.51±0.32 ng/ml for apoE<sup>-/-</sup> mice, and 9.2±0.30 ng/ml for C57BL/6 mice. At week 24, the plasma leptin levels of the apoE<sup>-/-</sup> mice were reduced to 2.1±0.4 ng/ml. In contrast in apoE3<sup>knock-in</sup> mice fed western-type diet for 24 weeks leptin levels increased dramatically to 41.14±1.20 ng/ml (p<0.005). A similar but lower increase was also observed in the plasma leptin levels of C57BL/6 mice fed western-type diet for 24 weeks with a concentration of 34.70±1.50 ng/ml (p<0.005).

**Diet-induced obesity in apoE3<sup>knock-in</sup> and C57BL/6 mice is associated with reduced glucose tolerance and insulin sensitivity.** To determine the role of apoE in the development of obesity-associated insulin resistance and glucose intolerance, we performed the standard glucose tolerance (GTT) and insulin

sensitivity (IST) tests. GTT established that at week 0 all three mouse groups (apoE3<sup>knock-in</sup>, apoE<sup>-/-</sup>, and C57BL/6 mice) had similar normal responses to intraperitoneal administration of glucose (Fig. 2A).

**Fig. 2.** Glucose tolerance curves (Panels A, B) and insulin sensitivity curves (Panels C, D) of apoE3<sup>knock-in</sup>, C57BL/6, and apoE<sup>-/-</sup> mice at weeks 0 and 24. Values indicate the average plasma glucose levels expressed as mean ± standard error of the mean. The statistical significance of the observed differences among groups at each time point is as indicated (\*corresponds to p<0.05).



C57BL/6 mice (Fig. 2A, B, p<0.05). Remarkably however, apoE<sup>-/-</sup> mice fed western-type diet for 24 weeks (which are resistant to diet-induced obesity) clear glucose from the circulation more efficiently in comparison to the two other groups, and there was no significant difference in their response to intraperitoneal glucose load between weeks 0 and 24 on western-type diet (compare Fig. 2A to 2B, p>0.05).

In a similar fashion, when an IST was performed at week 0 all three mouse groups exhibited a similar response to intraperitoneal administration of insulin (Fig. 2C). However, at week 24 apoE3<sup>knock-in</sup> mice fed with western-type diet for 24 weeks displayed the poorest response to insulin administration as compared to C57BL/6 and apoE<sup>-/-</sup> mice (Fig. 2D, p<0.05). C57BL/6 mice also exhibited reduced insulin sensitivity at week 24 that was less severe than in apoE3<sup>knock-in</sup> mice (Fig. 2D, p<0.05). In contrast, apoE<sup>-/-</sup> mice fed western-type diet for 24 weeks exhibited the highest sensitivity to insulin of all three mouse groups (p<0.05). In addition, there was no significant difference in their insulin sensitivity curves between weeks 0 and 24 of the experiment (compare Fig. 2C to 2D, p>0.05).

**LDLr<sup>-/-</sup> mice are more sensitive to diet-induced obesity and hyperglycemia than apoE-deficient mice but less sensitive than C57BL/6 mice.** The LDLr is the major receptor involved in the clearance of apoE-containing lipoproteins in the circulation (5). Therefore, one mechanistic explanation for the role of apoE

in the development of obesity would be that LDLr-receptor mediated uptake of apoE-containing chylomicron remnants promotes the direct deposition of dietary fat to the adipose tissue. In such case, deficiency in LDLr would prevent obesity and hyperglycemia. To address this possibility, groups of 8-10 female LDLr<sup>-/-</sup> or C57BL/6 or apoE<sup>-/-</sup> mice were placed on western-type diet for a period of 15 weeks and their body-weight and composition, and plasma cholesterol, triglyceride, and glucose levels were determined during the course of the experiment. At week 5 of the experiment the average weight of the LDLr<sup>-/-</sup> mice was 22.28±1.32 grams, (29.7±4.1% higher as compared to their initial weight of 17.13±0.65 grams, p<0.05) (Fig. 3A). This increase was comparable to the 27.4±3.8% increase observed in the apoE<sup>-/-</sup> mice (from 16.83±0.24 grams to 21.43±0.56 grams, p<0.05) but lower than the 55.4±3.8% increase in the C57BL/6 mice (from 16.26±0.28 grams to 25.25±0.50 grams, p<0.005) (Fig. 3A). At week 15 of the

experiment however, LDLr<sup>-/-</sup> mice showed an 84.5±8.7% increase in body weight (with average final weight of 31.63±2.10 grams, p<0.05) (Fig. 3A). This increase was higher than the 51.4± 4.5% increase (p<0.05) observed in the weight of the apoE<sup>-/-</sup> mice (with average final weight of 25.46±0.55 grams). However, it is still significantly lower than the 119.8±7.6% increase observed in the weight of C57BL/6 mice (with average final weight of 37.28±0.72 grams) fed western type diet for the same period of time (Fig. 3A, p<0.05)

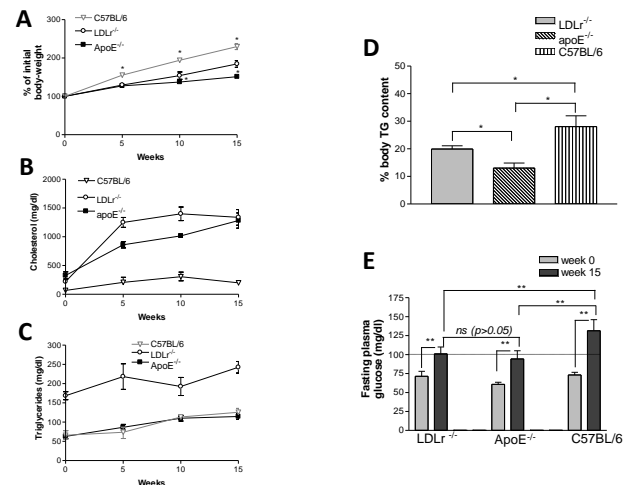
Body composition analysis at the end of the experiment revealed that at week 15, LDLr<sup>-/-</sup> mice had a body fat content of 19.9±1.2% that was much higher than the body fat content of the apoE<sup>-/-</sup> mice (13±1.9%, p<0.05) but still lower than the body fat content of the C57BL/6 mice (28.06±3.92%, p<0.05) fed western-type diet for the same period of time (Fig. 3D). The complete body composition analysis of the mice fed western-type diet for 15 weeks is summarized in Table 2.

**TABLE 2.** Body-composition of LDLr<sup>-/-</sup>, apoE<sup>-/-</sup>, and C57BL/6 mice fed western-type diet for 15 weeks.

Mouse Strain	Wet body-weight	Dry body-weight	LBM	Body-fat	Water
LDLr <sup>-/-</sup>	31.6±2.1	14.5±2.3	25.7±2.5	5.3±0.7	16.4±1.0
ApoE <sup>-/-</sup>	25.5±0.6	15.3±1.5	19.9±1.4	3.4±0.6	8.0±2.3
C57BL/6	37.3±0.7	23.9±1.2	26.9±1.8	10.4±1.3	13.4±1.5

Values are in grams expressed as mean ± standard error of the mean

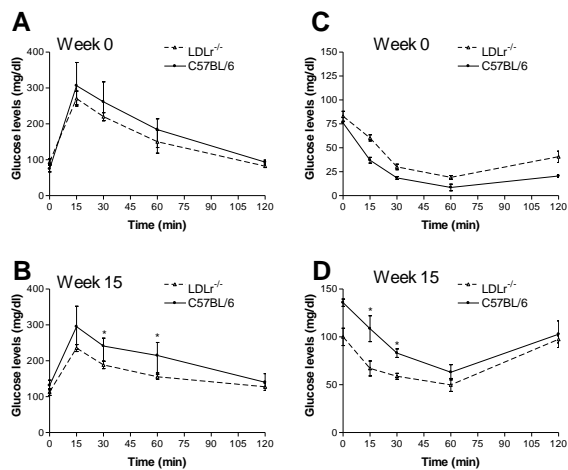
**Fig. 3.** Percent of initial body-weight (*panel A*), plasma cholesterol levels (*panel B*) and plasma triglyceride levels (*panel C*), of C57BL/6, LDLr-deficient (LDLr<sup>-/-</sup>), and apoE-deficient (apoE<sup>-/-</sup>) mice fed western-type diet for a period of 15 weeks. (*Panel D*) Percent body fat content of apoE<sub>3</sub><sup>knock-in</sup>, apoE<sup>-/-</sup>, and C57BL/6 at week 15. (*Panel E*) Fasting plasma glucose levels of apoE<sub>3</sub><sup>knock-in</sup>, apoE<sup>-/-</sup>, and C57BL/6 mice at weeks 0, and 15. Each point on the graphs represents the mean value of the group and error bars indicate the standard error of the mean. The statistical significance of the observed differences among groups at each time-point is indicated (\* corresponds to p<0.05 and \*\* corresponds to p<0.005).



Plasma lipid and glucose analysis showed that during the 15-week period, LDLr<sup>-/-</sup> mice developed severe hypercholesterolemia (1338±135 mg/dl) that was accompanied by a moderate hypertriglyceridemia (242.6±14.9 mg/dl) (Fig. 3B, C). Plasma glucose levels were increased moderately (from 71.3±6.7 mg/dl to 101±4.9 mg/dl, p<0.05) but were still lower than the levels of C57BL/6 mice (131±5.3 mg/dl) at week 15 of the experiment (Fig. 3E, p<0.005).

GTT and IST revealed that the LDLr<sup>-/-</sup> mice fed western-type diet for 15 weeks had similar tolerance to glucose and sensitivity to insulin, as compared to their starting state (week 0) (Fig. 4, p>0.05). In addition, there was no significant difference in the response to intraperitoneal administration of glucose or insulin between LDLr<sup>-/-</sup> and C57BL/6 mice fed western-type diet for 15 weeks (Fig. 4). Interestingly, in our studies feeding western-type diet to C57BL/6 for 15 weeks did not result in insulin resistance or glucose intolerance (Fig. 4B and 4D). Taken together, these data indicate that female LDLr<sup>-/-</sup> mice fed western-type diet for 15 weeks appear to be more sensitive than female apoE<sup>-/-</sup> mice but still more resistant than female C57BL/6 mice in the development of diet induced obesity and its related disorders.

**ApoE promotes diet-induced accumulation of excess triglycerides in the liver while apoE- or LDLr-deficiency does not.** If apoE and the LDLr are involved in the direct delivery of dietary lipids to tissues, one would expect that feeding apoE<sub>3</sub><sup>knock-in</sup> and C57BL/6 mice western-type diet would result in excess accumulation of triglycerides in the liver, while apoE<sup>-/-</sup> and LDLr<sup>-/-</sup> mice would be resistant to excess hepatic triglyceride accumulation. To test this hypothesis, we isolated liver samples from mice fed western-type diet for 15 weeks and determined their triglyceride content (Fig. 5A).



**Fig. 4.** Glucose tolerance curves (*Panels A, B*) and insulin sensitivity curves (*panel C, D*) of  $LDLr^{-/-}$  and C57BL/6 mice at weeks 0 and 15. Values indicate the average plasma glucose levels expressed as mean  $\pm$  standard error of the mean. The statistical significance of the observed differences among groups at each time point is as indicated (\*corresponds to  $p < 0.05$ ).

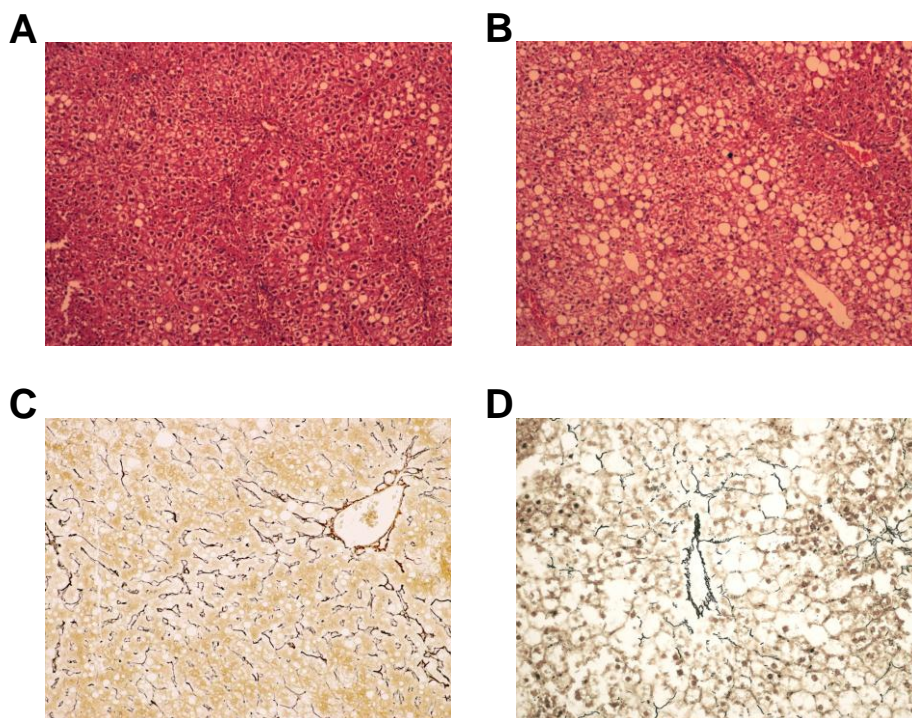
Liver samples from  $apoE^{-/-}$  and  $LDLr^{-/-}$  mice fed western-type diet for 15 weeks had a similar triglyceride content of  $60.67 \pm 4.12$  mg/gram and  $58.40 \pm 5.11$  mg/gram of hepatic tissue, respectively (Fig. 5A,  $p > 0.05$ ). In contrast,  $apoE3_{\text{knock-in}}$  and C57BL/6 mice had a much higher hepatic triglyceride content ( $215.00 \pm 33.56$  mg/gram and  $213.72 \pm 11.89$  mg/gram respectively,  $p < 0.05$ ) confirming that human apoE3, murine apoE, and the LDLr contribute to the

accumulation of excess lipids to the liver, in response to western-type diet (Fig. 5A).

### A1.2 Effects of apoE and the LDLr deficiency on the deposition of dietary lipids to adipose tissue and the development of nonalcoholic fatty liver disease (FEBS J. 278(17):3119-29)

**ApoE deficient mice are less sensitive to hepatic triglyceride accumulation compared to control C57BL/6 mice.** To test the effects of apoE on hepatic triglyceride accumulation, groups of 10-12 week-old male apoE-deficient ( $apoE^{-/-}$ ) and wild-type (WT) C57BL/6 mice were placed on western-type for a total period of 24 weeks. As shown in Fig. 5A, H&E staining of liver sections revealed that deficiency in apoE did not result in any significant distortion of liver microscopic morphology as well as accumulation of triglycerides to the livers of  $apoE^{-/-}$  mice fed western type diet for 24 weeks. In contrast, control C57BL/6 mice fed western-type diet for the same period exhibited remarkable steatosis, characterized by excessive accumulation of lipids within the liver cells (Fig. 5B). The observed steatosis was diffuse and of the macrovesicular type. Statistical analysis following histomorphometric evaluation of the H&E sections uncovered that the number of lipid droplets within the liver hepatocytes was significantly elevated in the C57BL/6 mice as compared to the  $apoE^{-/-}$  mice ( $p = 0.0001$ ). In agreement with these data, staining of hepatic sections with reticulin showed that in C57BL/6 mice fed western-type diet for 24 weeks, NAFLD was much more progressed and

resulted in a significant disruption in the normal architecture of the liver extracellular reticulin fibrils (Fig. 5D), as compared to  $apoE^{-/-}$  mice (Fig. 5C), which displayed a normal hepatic histology.



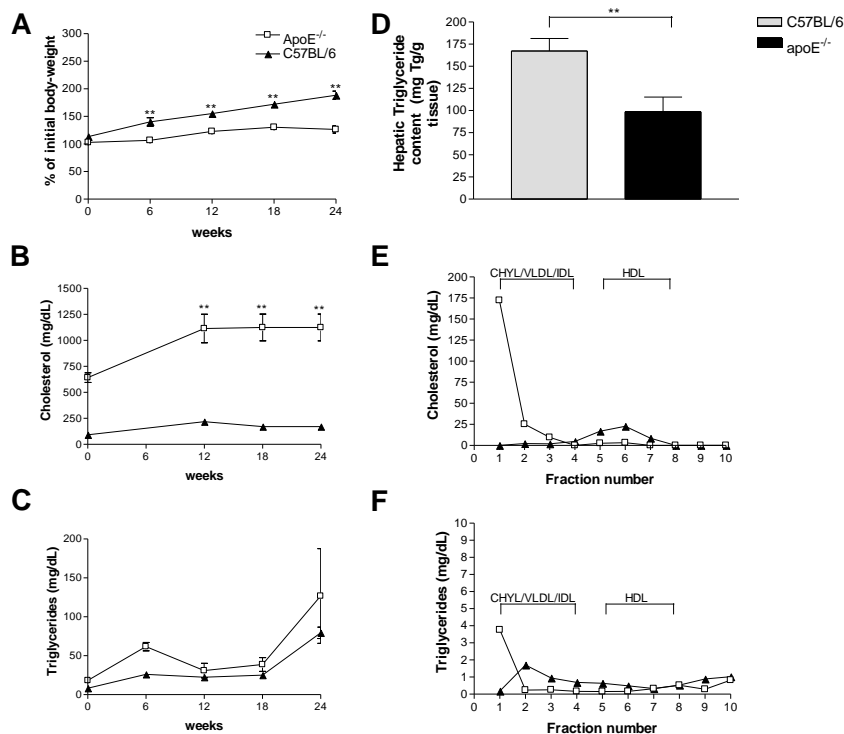
**Fig.5.** Histological analyses of liver sections from  $apoE^{-/-}$  and C57BL/6 mice. (A, B) Representative pictures of eosin and hematoxylin (H&E) stained hepatic sections from  $apoE^{-/-}$  (A) and C57BL/6 (B) mice at week 24 on western-type diet. (C, D) Representative pictures of reticulin stained hepatic sections of  $apoE^{-/-}$  (C) and C57BL/6 (D) mice fed western-type diet for 24 weeks. All pictures were taken at 20X original magnification.

No significant differences in the size and shape of visceral adipocytes were detected between the two mouse groups (data not shown). To further confirm that deficiency in apoE prevented the accumulation of hepatic triglycerides to the livers of mice fed western-type diet for 24 weeks, liver samples were isolated from apoE<sup>-/-</sup> and C57BL/6 mice and their triglyceride content was determined biochemically, as described in Materials and Methods. This analysis showed that apoE<sup>-/-</sup> mice fed western-type diet for 24 weeks had a triglyceride content of 98.6±16.7 mg per gram of hepatic tissue while C57BL/6 mice had a much higher hepatic triglyceride content (155.7±10 mg per gram of hepatic tissue, p<0.005) further confirming that apoE possesses a central role in the deposition of dietary triglycerides to the liver of mice and the development of diet-induced NAFLD.

### Body-weight measurements and body-composition analysis of mice fed western-type diet for 24 weeks.

As expected from previously published results, apoE<sup>-/-</sup> mice less sensitive to the development of diet-induced obesity compared to the C57BL/6 mice (6, 7). Specifically, during the course of the experiment apoE<sup>-/-</sup> mice showed only a modest increase in body-weight (Fig. 5A). At week 6 of the experiment, the apoE<sup>-/-</sup> mouse group had an average body-weight of 26.7±0.6 grams (5.52±1.45% increase as compared to their starting weight of 25.7±0.2 grams at week 0, p<0.05). At week 12, their average body-weight was 30.7±1.1 grams while at week 24, it further increased slightly to 31.6±1.7 grams (19.7±7.3% increase, as compared to their starting weight at week 0, p<0.05) (Fig. 5A). In contrast, the C57BL/6 mice showed a significant increase in their body weight during the course of the experiment. At week 6, the C57BL/6 mice had an average body-weight of 31.8±1.7 grams (23.5±3.9% increase, as compared to their starting weight of 25.8±1 at week 0, p<0.05), at week 12 their body-weight was already 35.3±0.6 grams, while at week 24 it increased further to 42.8±1.7 grams (66.7±5.6% increase, as compared to their starting weight at week 0, p<0.05) (Fig. 2A). In agreement with our findings described above, the increased body-weight of the

C57BL/6 mice corresponds to increased body fat mass.



**Fig. 6.** Biochemical parameters of apoE<sup>-/-</sup> and C57BL/6 mice fed western-type diet for a period of 24 weeks. (A) Changes in average body weight. (B, C) Changes in average plasma cholesterol and plasma triglycerides, respectively. (D) Average hepatic triglyceride content of mice fed western-type diet for 24 weeks (\*\* corresponds to p<0.005). (E, F) Cholesterol and triglyceride content respectively, of the different density fractions following separation of plasma lipoproteins by density gradient ultracentrifugation (UCF). Fraction 1 corresponds to the top fraction (containing chylomicrons and VLDL).

### Plasma lipid levels and average daily food consumption of mice fed western-type diet for

**24 weeks.** To determine how plasma lipid levels may reflect differences in hepatic triglyceride accumulation in apoE<sup>-/-</sup> and C57BL/6 mice, fasting plasma samples were isolated every 6 weeks and cholesterol, triglyceride and free-fatty acid levels were measured as described in Materials and Methods. As shown in Fig. 6B, apoE<sup>-/-</sup> mice showed a dramatic increase in their plasma cholesterol levels during the course of the experiment. At week 24 of the experiment plasma cholesterol levels of the apoE<sup>-/-</sup> mice were

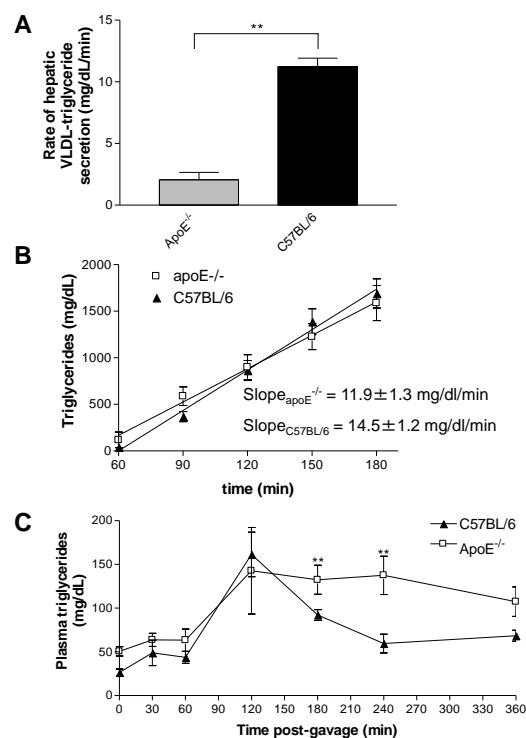
1475±48 mg/dl (Fig. 6B) while their plasma triglyceride levels increased but remained within physiological range (126.7±60.9 mg/dl at week 24 versus 18.3±1.9 mg/dl at week 0) (Fig. 6C). Ultracentrifugation analysis of plasma samples showed that the hypercholesterolemia of these mice was due to increased accumulation of triglyceride-containing cholesterol-rich chylomicron remnants (Fig. 6E, F). However, C57BL/6 mice on high fat diet for 24 weeks had slightly elevated fasting cholesterol levels (224.6±21 mg/dl) as compared to their starting cholesterol levels at week 0 (91.9±10 mg/dl) (Fig. 6B), while their plasma triglyceride levels remained normal (79.4±7.4 mg/dl at week 24 vs. 58.2±1.1 mg/dl at week 0) (Fig. 6C). Ultracentrifugation analysis of plasma samples showed that the cholesterol of these mice was mainly distributed in the HDL density fractions (Fig. 6E, F).

Surprisingly, apoE<sup>-/-</sup> mice which do not develop NAFLD had a higher plasma concentration of free fatty acids (FFA) compared to C57BL/6 mice. Steady-state free fatty-acid levels of the apoE<sup>-/-</sup> mice were 7.6±1.2 mmole Equiv, while C57BL/6 mice had a much lower steady-state plasma FFA concentration of 1.4±0.1 mmole Equiv (p=0.0001).

To determine if differences in hepatic triglyceride accumulation could be explained by differences in average daily food consumption between the two mouse groups, at weeks 12 and 24 of the experiment we determined the average daily food consumption for each mouse group. It was found that apoE<sup>-/-</sup> mice consumed 3.3±0.2 and 3.5±0.6 grams/mouse/day on week 12 and 24 respectively (p>0.05). Similarly, C57BL/6 mice consumed 3.8±0.2 and 3.4±0.2 grams/mouse/day at week 12 and 24 respectively (p>0.05), and there was no statistical difference between groups (p>0.05). Thus, in the present study (n=5) we were not able to determine a statistically significant difference in the average daily food consumption between the two mouse strains at week 12 of the experiment (3.3±0.2 grams/mouse/day versus 3.8±0.2 grams/mouse/day for the apoE<sup>-/-</sup> and C57BL/6 mice respectively, (p=0.0833).

### Rate of hepatic triglyceride secretion and intestinal triglyceride absorption in apoE<sup>-/-</sup> and C57BL/6 mice.

One mechanism that could affect hepatic triglyceride content is the secretion of hepatic triglycerides in the circulation. To determine the contribution of VLDL-triglyceride secretion in the apoE-mediated hepatic lipid accumulation we compared the rate of hepatic VLDL triglyceride secretion between apoE<sup>-/-</sup> and C57BL/6 mice. In accordance with previous studies (8–10), we found that the rate of hepatic triglyceride secretion was significantly decreased in apoE<sup>-/-</sup> compared to C57BL/6 mice. Specifically, secretion rates were 2.1±0.4 mg/dl/min (min=1.7, max=3.5, SEM=0.4, n=5) for apoE<sup>-/-</sup> mice versus 11.2±0.9 mg/dl/min (min=9.8, max=13.7, SEM=0.9, n=5) for C57BL/6 mice, p=0.0001 (Fig. 7A). Thus, based on these results it appears that hepatic triglyceride secretion cannot account for the differences in hepatic triglyceride deposition seen between apoE<sup>-/-</sup> and C57BL/6 mice.



**Fig.7.** Analysis of kinetic parameters associated with hepatic triglyceride content. (A) Rate of hepatic VLDL triglyceride secretion. (B) Rate of total triglyceride supply in plasma. (C) Kinetics of post-prandial triglyceride clearance, in apoE<sup>-/-</sup> (open squares) and C57BL/6 (closed triangles) mice.

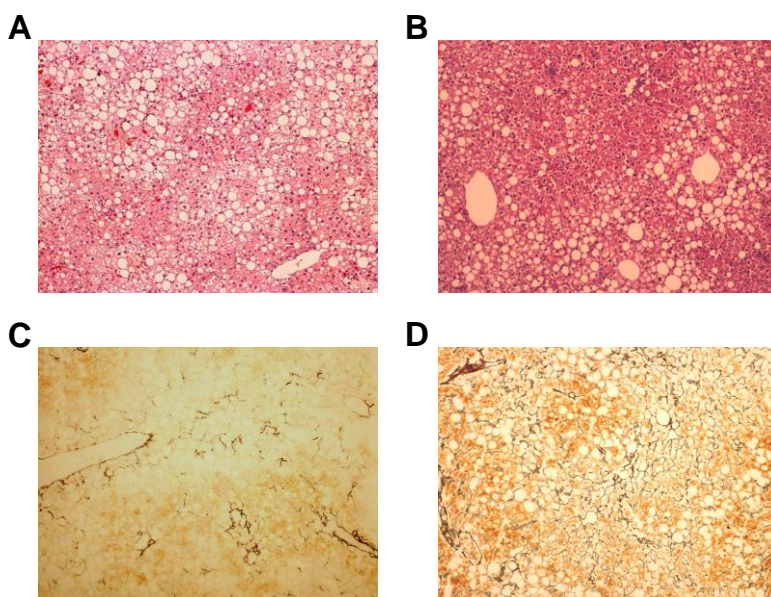
One additional mechanism that could explain the increased sensitivity of apoE<sup>-/-</sup> mice to diet-induced NAFLD could be increased intestinal secretion of triglyceride-rich lipoproteins to the plasma of these mice.

To determine the rate of intestinal triglyceride secretion, next we determined the total rate (intestinal and hepatic) of plasma triglyceride input in apoE<sup>-/-</sup> and C57BL/6 mice fed western-type diet, following an oral fat load. Groups of 5 apoE<sup>-/-</sup> and C57BL/6 mice each were fasted for 16 hours, and then administered an oral fat load of 300 μl of olive oil, as described in Materials and Methods. One hour post-gavage, mice were injected with Triton WR1339 and then plasma triglyceride levels were determined as a function of time. As shown in Fig. 7B, apoE<sup>-/-</sup> mice showed a lower rate of total triglyceride input compared to C57BL/6. Specifically, the rates were 11.9±1.3 mg/dl/min for apoE<sup>-/-</sup>

and  $14.5 \pm 1.2$  mg/dl/min for C57BL/6 mice, ( $n=5$ ,  $p=0.023$ ). Then, by subtracting the rate of hepatic triglyceride secretion (determined above) from the total rate of plasma triglyceride supply, the rate of intestinal triglyceride secretion was determined as  $9.8 \pm 1.3$  mg/dl/min for the apoE<sup>-/-</sup> mice and  $2.0 \pm 0.7$  mg/dl/min for the C57BL/6 mice ( $n=5$ ,  $p=0.023$ ). The data suggest that differences in intestinal triglyceride absorption or hepatic triglyceride secretion cannot account for the observed histological differences between apoE<sup>-/-</sup> and C57BL/6 mice.

**Kinetics of post-prandial triglyceride clearance in apoE<sup>-/-</sup> and C57BL/6 mice.** Another potential mechanism that could explain the reduced sensitivity of apoE<sup>-/-</sup> mice to diet-induced NAFLD could be reduced clearance of plasma triglycerides in these mice. Thus, in the next set of experiments we sought to determine the kinetics of post-prandial triglyceride clearance. As shown in Fig. 7C following gavage administration of olive oil both mouse groups reached a maximum plasma concentration of  $142.7 \pm 29.6$  mg/dl and  $161.4 \pm 21.5$  mg/dl respectively at 120 minutes post-gavage ( $n=5$ ,  $p=0.2195$ ) (Fig. 7C). However, there was a significant difference in post-prandial triglyceride clearance in apoE<sup>-/-</sup> mice compared to C57BL/6 mice. In particular, in C57BL/6 mice the rapid increase in their plasma triglyceride levels at 120 min after olive oil administration was followed by an immediate and steep decline. At 240 min post-gavage plasma triglycerides of C57BL/6 mice reached baseline levels ( $59.5 \pm 10.7$  mg/dl, min=20.7, max=80.8, SEM=10.7). However, in apoE<sup>-/-</sup> mice a similar increase in plasma triglyceride levels at the 2 hour time-point, persisted over the period of the next four hours (360 min) suggesting that in the absence of apoE, post-prandial triglycerides are cleared from the circulation at a significantly slower rate. At 240 min post-gavage plasma triglycerides of apoE<sup>-/-</sup> mice were still significantly elevated ( $137.5 \pm 21.9$  mg/dl, min=106.5, max=184.0, SEM=21.9).

**LDLr<sup>-/-</sup> mice fed western-type diet for 24 weeks developed significant accumulation of hepatic triglycerides and NAFLD.** To address the potential role of LDLr in the apoE-mediated deposition of dietary triglycerides to the liver, LDLr-deficient mice were fed western-type diet for 24 weeks and liver specimens were isolated and analyzed for their triglyceride content by biochemical and histological analyses. In agreement with our studies above, LDLr<sup>-/-</sup> mice were more susceptible to diet-induced obesity than apoE<sup>-/-</sup> mice, but more resistant than C57BL/6 mice (6). Surprisingly however, we found that hepatic specimens from LDLr<sup>-/-</sup> mice had higher triglyceride content than control C57BL/6 mice ( $233.0 \pm 19$  vs.  $155.7 \pm 10$  mg per gram of hepatic tissue, respectively). Our biochemical results were in agreement with data from our histological analyses showing that LDLr<sup>-/-</sup> mice developed NAFLD that was even more progressed than control C57BL/6 mice. Liver steatosis was diffuse and both the micro- and macro-vesicular type was observed (Fig. 8A, B). A few lymphocytes were detected within the liver parenchyma. Reticulin stain revealed that liver architecture was disturbed, mainly because of the extensive steatosis (Fig. 8C, D).



**Fig.8.** Histological analyses of liver sections from LDLr<sup>-/-</sup> and C57BL/6 mice. (A, B) Representative pictures of eosin and hematoxylin (H&E) stained hepatic sections from LDLr<sup>-/-</sup> (A) and C57BL/6 (B) mice at week 24 on western-type diet. (C, D) Representative pictures of reticulin stained hepatic sections of LDLr<sup>-/-</sup> (C) and C57BL/6 (D) mice fed western-type diet for 24 weeks. All pictures were taken at 20X original magnification.

### **A1.3. Discussion of the data on the role of the chylomicron metabolic pathway in diet-induced obesity and glucose metabolism**

The prevalence of obesity and its related pathologies is a major cause of death with rates increasing at an alarming pace [1]. By the beginning of the millennium, overweight adults accounted for over 15% of the world's population (BMI>30, World Health Organization) [2] with these numbers increasing to 50% within the US and Europe [3]. Obesity develops as a result of the disruption of the homeostasis between food intake and energy expenditure, and therefore factors affecting these processes are the focus of extensive research for the development of effective anti-obesity drugs, with only limited success thus far [4]. Aging, hormonal imbalance, and genetic predisposition may also contribute to obesity [5-9;9-16]. However, very few cases of human obesity are reported to be caused by genetic factors [17] leaving western-type diet and sedentary lifestyle, physical inactivity and imbalance in caloric load as the most common contributors to the development of central obesity and the metabolic syndrome [2;18].

The risk of developing all other components of the metabolic syndrome increases with obesity, supporting the hypothesis that obesity is the central feature of the syndrome [19]. It is well-established that abdominal obesity may result in insulin resistance and hyperinsulinemia [19;20]. Epidemiological and population studies have established a direct correlation between obesity and the development of cardiovascular disease [21;22]. Despite the pivotal role of obesity and dyslipidemia in the development of the metabolic syndrome and heart disease, the functional interactions between adipose tissue and the lipid and lipoprotein transport system have not yet been investigated thoroughly.

Apolipoprotein E (apoE) is a 34.2kDa glycoprotein synthesized by the liver and other peripheral tissues. In humans there are three major natural isoforms, apoE2, apoE3, and apoE4 [23] with apoE3 being the most common of the three isoforms [23-29]. ApoE is a major protein component of chylomicron remnants and VLDL [23]. The importance of this protein in the maintenance of plasma lipid homeostasis and atheroprotection was first established with the generation of the apoE-deficient mouse [30;31], which develops hypercholesterolemia and spontaneous atherosclerosis [30;31]. Lipid-bound apoE is the natural ligand of the low density lipoprotein receptor (LDLr) [32-34], a cell surface receptor that is responsible for the catabolism of atherogenic lipoproteins [32;35-37].

In this set of our studies we investigated the role of apoE in the development of obesity, glucose intolerance and nonalcoholic fatty liver disease (NAFLD) in mice. Since western-type diet and sedentary lifestyle that result in excess body fat, physical inactivity and imbalance in caloric load, are the most common contributors to these conditions we focused our studies on diet-induced obesity, glucose intolerance and NAFLD. We report that deficiency in apoE has a protective effect against diet-induced obesity, glucose intolerance and NAFLD, which correlates mainly with reduced clearance of post-prandial triglycerides from the circulation.

We found that apoE3knock-in mice are more sensitive to diet-induced obesity and related metabolic dysfunctions than WT C57BL/6 mice, while apoE<sup>-/-</sup> mice are resistant to the development of these conditions. Furthermore, deficiency in the LDLr results in reduced sensitivity towards obesity in response to western-type diet raising the possibility that the effects of apoE may be mediated, at least in part, via its interactions with the LDLr.

Interestingly, there were no significant differences in plasma free fatty-acid levels among mouse groups (apoE3knock-in vs. C57BL/6 vs. LDLr<sup>-/-</sup> vs. apoE<sup>-/-</sup>), although previous studies suggested that increased plasma levels of free fatty acids are closely associated with obesity-induced insulin resistance [42;43]. Moreover, daily food consumption of the apoE3knock-in, C57BL/6, and apoE<sup>-/-</sup> mice was similar among groups, suggesting that different responses to western type diet cannot be attributed to differences in appetite.

One possible explanation for the increased sensitivity of the apoE3knock-in mice towards diet-induced obesity would be that higher plasma apoE levels in these mice compared to C57BL/6 mice, are responsible for the enhanced deposition of dietary lipids to adipose tissue. To address this possibility we compared the apoE levels in plasma samples isolated from apoE3knock-in and C57BL/6 mice at week 0 of the experiment, using western blotting. This analysis showed that steady-state plasma apoE levels in the apoE3knock-in mice used in our study are approximately four times lower than those in WT C57BL/6 mice. Thus, the increased sensitivity of apoE3knock-in mice towards diet-induced obesity is merely the result of elevated plasma apoE levels in these mice and the difference in the ability of the human apoE3 and the

murine apoE to promote obesity in response to high-fat diet may be due to intrinsic differences between these two peptides.

In a previous work, Sullivan and coworkers [44] observed significant differences in the composition of the lipoprotein particles that are formed in the plasma of the apoE3knock-in mice, and their wild-type littermates. Based on these findings, the differences in obesity between apoE3knock-in and C57BL/6 mice that we report here cannot be attributed to differences in apoE expression in adipose tissue or total plasma apoE levels between the two groups. The data presented in Fig. 6B, raise the possibility that chylomicron and VLDL remnants containing the human apoE3 isoform are taken up more avidly by adipose tissue than the lipoproteins containing mouse apoE.

Human apoE has three natural isoforms in humans: apoE2, apoE3 and apoE4. In vitro receptor binding studies established that lipid bound apoE3 and apoE4 have a similar affinity for the LDLr while lipid bound apoE2 has a much lower affinity [45;46]. In this study, we focused on apoE3 mainly because it is the most common apoE genetic polymorphism in humans [24-29]. If the effects of apoE3 on obesity are mediated by its lipid lowering potential via the LDLr, then we expect that both apoE3 and apoE4 will predispose to a similar extent to diet-induced obesity and insulin resistance in mice, while apoE2 may have a much lower potential to promote these conditions. Further studies are needed in order to address this point, and other mechanisms of apoE-promoted diet-induced obesity should not be excluded.

It is quite interesting that in all our experiments, plasma cholesterol levels correlate inversely with body-weight gain and body-fat accumulation (Fig. 1 and 4). In the apoE<sup>-/-</sup> mice, failure to clear chylomicron remnants due to deficiency in apoE resulted in a steady increase in plasma cholesterol levels and rendered these mice resistant to diet-induced obesity. In contrast, in the apoE3knock-in mice, the efficient catabolism of chylomicron remnants resulted in only slightly elevated plasma cholesterol levels, but promoted obesity, insulin resistance and glucose intolerance. Similar to the apoE3knock-in mice, C57BL/6 mice which express the mouse apoE, developed only a mild hypercholesterolemia but became obese and insulin resistant following western-type diet for 24 weeks.

Plasma triglyceride levels of the LDLr<sup>-/-</sup> mice were moderately elevated at week 0, and remained elevated during the 15 weeks on high-fat diet, while plasma triglyceride levels of the other animal groups remained normal for the duration of the experiment. This is not surprising because in the absence of the LDLr, reduced clearance of apoE-containing lipoproteins from the circulation results in elevated steady-state plasma apoE levels. Since apoE is a known inhibitor of LpL [47], and plasma triglyceride levels correlate with plasma apoE levels [48], accumulation of apoE in the blood of LDLr<sup>-/-</sup> mice results in reduced LpL-mediated lipolysis of plasma triglycerides and hypertriglyceridemia.

In our studies, LDLr<sup>-/-</sup> mice became more obese than the apoE<sup>-/-</sup> mice but less obese than C57BL/6 mice, raising the possibility that in addition to the LDLr, other apoE-recognizing receptors may also promote the deposition of postprandial lipids to adipose tissue, thus contributing to diet-induced obesity and related metabolic dysfunctions. Indeed, a recent study showed that adipose-tissue specific deletion of the LDLr-related protein (LRP) makes mice less sensitive to obesity [49]. In the case of the LDLr<sup>-/-</sup> mice, LRP and possibly other apoE-recognizing "scavenger" receptors may promote to some extent delivery of apoE-containing chylomicron remnants to adipose tissue. However, in the case of the apoE<sup>-/-</sup> mice which lack the endogenous apoE all these apoE-mediated receptor processes are blocked, and apoE<sup>-/-</sup> mice are more resistant to body fat gaining compared to the LDLr<sup>-/-</sup> mice. Even though the relative expression of LDLr in adipose tissue is much lower compared to liver, our data support a functional role for this receptor in the apoE-mediated mechanism of diet-induced obesity.

In a previous study by Schreyer et. al., [50], it was suggested that LDLr<sup>-/-</sup> mice fed a diabetogenic diet for 16 weeks were more susceptible to diet-induced obesity and hyperglycemia than C57BL/6 mice, while apoE<sup>-/-</sup> mice appeared to be as susceptible to the development of these conditions as the C57BL/6 mice. Furthermore, in that study LDLr<sup>-/-</sup> mice developed severe hypertriglyceridemia during the course of the experiment. The diabetogenic diet used in that study contained a very high fat content of 35.5% (derived mainly from lard) (Bioserve, Frenchtown, NJ, cat#F1850). In all our experiments mice were fed the standard western-type diet containing 21.1% fat (Harlan Teklad, cat# TD88137). It is possible that the high fat content of the diabetogenic diet predisposed apoE<sup>-/-</sup>, LDLr<sup>-/-</sup> and C57BL6 mice to the development of diet-induced obesity, and resulted in saturation of the metabolic pathways that control body-fat deposition and plasma lipid and glucose homeostasis. Under such conditions, apoE or LDLr-deficiency may not be sufficient to prevent obesity, since other mechanisms contributing to obesity may override the protective

effect of apoE or LDLr deletion that we observed in our experiments. Our data on LDLr<sup>-/-</sup> mice are in agreement with the data of MacDonald et al., [51] showing that female LDLr<sup>-/-</sup> mice do not become excessively obese, and do not develop hyperglycemia and glucose intolerance in response to western-type diet.

In our experiments we studied the role of apoE in the development of obesity in response to dietary consumption of fat, one of the major causes of human obesity [2;18]. Our findings are supported by previous observations by Gao et al., [52] and Chiba et al., [53] showing that deficiency in apoE renders genetically predisposed obese mice less sensitive to spontaneous development of obesity. Furthermore, in vitro studies suggested that apoE promotes triglyceride uptake and deposition in in vitro differentiated adipocytes and in freshly isolated adipose-tissue explants [54] while VLDL induces adipocyte differentiation in an apoE-dependent manner [53]. In the present study, we report for the first time that human apoE3 increases susceptibility towards diet induced obesity compared to mouse apoE. The functional role of apoE-containing chylomicron and VLDL remnants in the development of diet-induced obesity is further supported by the observation that apoE<sup>-/-</sup> mice remain lean when fed western-type diet for 15 or 24 weeks.

One of the hallmarks of obesity-associated insulin resistance is the increase in the circulating levels of insulin [55]. Such change is also evident in our studies, further supporting the hypothesis that the beneficial effects of apoE deletion on weight loss extend to increased insulin sensitivity. Furthermore, our data demonstrate a significant increase in the plasma leptin levels in the apoE3 knock-in and to a lesser extent in C57BL/6 mice, an observation that is also consistent with their increased adiposity [56].

Histological evaluation following H&E staining of liver sections from the control mice, revealed increased levels of steatosis as demonstrated by the existence of a large number of lipid droplets within the vast majority of the examined hepatocytes. Steatosis was diffuse and of the macrovesicular type, in which a large fat vacuole within the hepatocyte pushed the nucleus towards the edge of the cell. In contrast however, H&E stained liver sections from apoE<sup>-/-</sup> mice, had normal microscopic appearance, the liver architecture was normal, and there was no evidence of lipid accumulation within hepatocytes. Our histological findings were in harmony with the results obtained by the reticulin stain showing that in the livers of apoE-deficient mice the reticulin network was not distorted, in contrast to livers of C57BL/6 mice that were heavily loaded with fat. Reticulin stain is a classical histopathological marker for identification of hepatic architecture and of structural damage within the liver parenchyma. Therefore, the presence of more reticulin in Fig. 5C indicates that in ApoE<sup>-/-</sup> mice the reticulin network is better preserved, further confirming that the structural damage in the liver of these animals is minimal following feeding with high fat diet. In contrast, the destruction of the reticulin network (visualized as reduced reticulin stain) in the livers of the C57BL/6 mice (Fig. 5D) corresponds to an extensive destruction in hepatic architecture primarily due to lipid accumulation within the hepatocytes and the development of NAFLD in these mice.

In order to identify the molecular basis for this phenomenon we determined a number of parameters which could affect the delivery and deposition of intestinal dietary triglycerides to the liver of the experimental mice. In general, hepatic triglyceride content is a function of three parameters: a) dietary triglyceride deposition to the liver, b) endogenous triglyceride synthesis and turnover, and c) hepatic VLDL-triglyceride secretion to the circulation. Endogenous triglyceride clearance and turnover cannot account for the observed differences between apoE<sup>-/-</sup> and C57BL/6 mice since it is well established that intracellular triglyceride turnover and synthesis, as well as the activities of diacylglycerol acyltransferase (DGAT) and microsomal triglyceride transfer protein (MTP) are comparable between ApoE<sup>-/-</sup> and wild type C57BL/6 mice [22]. Similarly, differences in the rate of hepatic VLDL-triglyceride secretion between apoE<sup>-/-</sup> and C57BL/6 mice could not explain the observed resistance of apoE<sup>-/-</sup> mice towards diet-induced NAFLD. Consistent with previous data [19-21;21;23], we found that apoE<sup>-/-</sup> mice display approximately 5 times slower hepatic VLDL-triglyceride secretion compared to control C57BL/6 mice ( $2.1 \pm 0.4$  mg/dl/min for apoE<sup>-/-</sup> mice versus  $11.2 \pm 0.9$  mg/dl/min for C57BL/6 mice). Thus, we were led to hypothesize that the resistance of apoE<sup>-/-</sup> mice towards diet-induced NAFLD must be due to either decreased rate of intestinal absorption of dietary lipids or reduced hepatic deposition of plasma triglycerides. Kinetic analysis showed that apoE<sup>-/-</sup> mice exhibit reduced rates of intestinal absorption of dietary triglycerides compared to C57BL/6 mice ( $2.0 \pm 0.7$  mg/dl/min in C57BL/6 mice vs.  $9.8 \pm 1.3$  mg/dl/min in apoE<sup>-/-</sup> mice,  $p < 0.05$ ). However, apoE<sup>-/-</sup> mice displayed a significantly slower clearance of post-prandial triglycerides from the circulation consistent with a slower rate of dietary lipid deposition to the liver and other peripheral tissues.

Previously, it was suggested that 3-4 month-old apoE<sup>-/-</sup> mice on chow diet had slightly higher hepatic triglyceride content compared to control mice [22]. Our results show that the slightly higher baseline hepatic triglyceride content of apoE<sup>-/-</sup> mice fed chow diet does not predispose these mice to increased sensitivity towards NAFLD. In contrast, we find that apoE-deficiency renders these mice less sensitive towards hepatic triglyceride accumulation following feeding with a high-fat diet. A more recent study suggested that hypercholesterolemia sensitizes apoE<sup>-/-</sup> mice to carbon tetrachloride mediated liver injury [24]. Our data show that the hypercholesterolemia of apoE<sup>-/-</sup> mice is not causal for diet-induced NAFLD in these mice. Rather, our results establish that deficiency in apoE has a rather protective effect against hepatic triglyceride accumulation despite the apparent increase in plasma cholesterol levels of the apoE<sup>-/-</sup> mice. It is interesting that in our experiments plasma cholesterol levels are inversely related to hepatic accumulation of dietary triglycerides in mice. Although in our study apoE-deficient mice appear less sensitive to hepatic lipid deposition compared to control apoE-expressing C57BL/6 mice, previous work by Ma et al., [25] showed that artificially-induced low grade inflammatory stress triggered by subcutaneous injection of 10% casein increases the sensitivity of these mice towards NAFLD development. In the future it would be interesting to compare how casein-induced inflammation affects the sensitivities of apoE<sup>-/-</sup> and C57BL/6 mice to develop NAFLD.

Despite the enhanced intestinal absorption and the reduced deposition of post-prandial triglycerides to the liver and other peripheral tissues, steady-state plasma triglyceride levels of apoE<sup>-/-</sup> mice fed western-type diet remained within normal levels (<150 mg/dl), though they were elevated compared to C57BL/6 mice for the duration of the experiment. It is well-established that apoE is a potent inhibitor of plasma LpL [26-28], and that lipolysis-mediated release of FFAs is more efficient in apoE<sup>-/-</sup> mice than in apoE-expressing C57BL/6 mice [27]. In agreement with these studies, apoE<sup>-/-</sup> mice had elevated plasma FFA levels compared to C57BL/6 mice (apoE<sup>-/-</sup> mice had a steady-state FFA levels of 7.6±1.2 mmole Equiv, while C57BL/6 mice had a much lower steady-state plasma FFA concentration of 1.4±0.1 mmole Equiv, p<0.005). Despite this apparent increase in LpL-mediated FFA production and in steady-state plasma FFA levels, our apoE<sup>-/-</sup> mice are resistant to diet-induced NAFLD and obesity. Thus, our data do not support the notion that elevated plasma FFAs are pivotal for the accumulation of triglycerides in the liver of experimental mice [29;30] and that enhanced plasma LpL activity promotes deposition of plasma triglycerides to peripheral tissues, including hepatic and adipose tissues [31]. In our experiments it is apoE and not plasma FFAs that plays a central role in the deposition of post-prandial triglycerides to the liver, a process that over long periods of time may lead to NAFLD.

In vitro and in vivo studies have shown that lipoprotein-bound apoE is the natural ligand for the LDL-receptor [26;32], which is the main receptor involved in the clearance of apoE-containing lipoproteins in vivo [33]. Our data indicate that the apoE-mediated mechanism of hepatic triglyceride accumulation in mice is independent of the LDLr since LDLr<sup>-/-</sup> mice fed western-type diet for 24 weeks developed significant NAFLD that was more severe than in C57BL/6 mice. One possibility is that the effects of apoE on hepatic lipid accumulation are mediated by LRP-1 or CD36, or potentially other apoE-receptors. However, other alternative mechanisms should also be investigated. A recent epidemiological study showed that the ε2 allele may be protective against NAFLD in humans, while another epidemiological study supported a correlation of the ε4 allele with increased pathogenesis of fatty-liver disease [34]. Since the human apoE2 isoform of apoE is far less efficient in removing triglyceride-rich lipoproteins from the circulation than apoE3 and apoE4 [28], it is possible that the ability of apoE to promote the deposition of hepatic triglycerides to the liver is associated with its lipoprotein clearing function.

Our data extend our current knowledge on NAFLD development. Though additional experiments will be needed in order to determine if receptors mediate the effects of apoE, our data clearly support a new function of apoE as a key peripheral contributor to hepatic lipid deposition and the development of diet-induced NAFLD in mice.

## **A2. Role of the HDL metabolic pathway in diet-induced obesity and related metabolic disorders**

### **A2.1. Overall strategy**

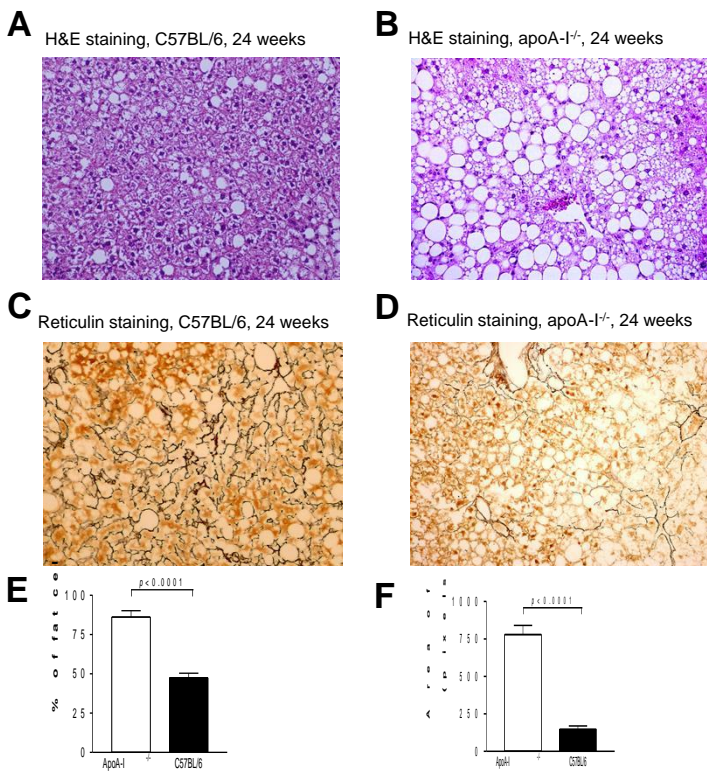
During the biogenesis of HDL, lipid free or minimally lipidated apoA-I interacts functionally with the lipid transporter ABCA1 to form immature discoidal HDL which are then converted into mature spherical particles by the action of lecithin:cholesterol acyl transferase (LCAT). Here we investigated the mechanistic relationship between low and dysfunctional HDL and diet-induced NAFLD development using mouse models. We employed male apoA-I-deficient (apoA-I<sup>-/-</sup>) mice that lack classical apoA-I containing HDL and male deficient (LCAT<sup>-/-</sup>) mice that have immature discoidal HDL. Mice were fed the standard western-type diet for 24 weeks and then histological and biochemical analyses were performed. ApoA-I<sup>-/-</sup> mice showed increased diet-induced hepatic triglyceride deposition and disturbed hepatic histology while they exhibited reduced glucose tolerance and insulin sensitivity. Quantification of FASN-1, DGAT-1, and PPAR $\gamma$  mRNA expression suggested that the increased hepatic triglyceride content of the apoA-I<sup>-/-</sup> mice was not due to de novo synthesis of triglycerides. Similarly, metabolic profiling did not reveal differences in the energy expenditure between the two mouse groups. However, apoA-I<sup>-/-</sup> mice exhibited enhanced intestinal absorption of dietary triglycerides, accelerated clearance of postprandial triglycerides, and a reduced rate of hepatic very low density lipoprotein triglyceride secretion. In agreement with these findings, adenovirus-mediated gene transfer of apoA-IMilano in apoA-I<sup>-/-</sup> mice fed western-type diet for 12 weeks resulted in a significant reduction in hepatic triglyceride content and an improvement of hepatic histology and architecture. Similar to apoA-I<sup>-/-</sup> mice, LCAT<sup>-/-</sup> mice were characterized by increased diet-induced hepatic triglyceride deposition and impaired hepatic histology and architecture. Adenovirus-mediated gene transfer of LCAT in LCAT<sup>-/-</sup> mice that were fed western-type diet for 12 weeks resulted in a significant reduction in hepatic triglyceride content and a great improvement of hepatic histology and architecture. Taken together, our data establish that the HDL metabolic pathway is a central contributor to the deposition of dietary triglycerides to the liver and the development of NAFLD. Our data further support that the coexistence of reduced HDL levels and NAFLD in an individual with metabolic syndrome may not be a mere coincidence, rather it underlays a strong causative relationship between these two conditions.

### **A2.2. Apolipoprotein A-I modulates Processes Associated with Diet-Induced Obesity, Glucose Intolerance and Nonalcoholic Fatty Liver Disease in Mice (Mol Med. 2012 Sep 7;18(9):901-12).**

#### **ApoA-I/- deficiency enhances hepatic triglyceride accumulation and the development of NAFLD in mice.**

To test the effects of apoA-I on hepatic triglyceride accumulation, groups of 10-12 week-old male apoA-I-deficient (apoA-I<sup>-/-</sup>) and wild-type (WT) C57BL/6 mice were placed on western-type for 24 weeks. Hematoxylin and eosin (H&E) staining of liver sections from wild-type (WT) C57BL/6 and apoA-I<sup>-/-</sup> mice at the beginning of the experiment (week 0) did not reveal any significant accumulation of hepatic lipids in the form of lipid droplets within the hepatocytes of these mice (data not shown). However, at week 24, both C57BL/6 and apoA-I<sup>-/-</sup> mice showed accumulation of hepatic lipids (Figure 10A, B). Statistical analysis following histomorphometric evaluation of the H&E sections revealed that the number of lipid droplets within hepatocytes was significantly elevated in the apoA-I<sup>-/-</sup> mice than in the C57BL/6 mice ( $p=0.0001$ ) (Figure 10A, B, E, F). The observed steatosis was diffuse and of the mixed (macro- and micro-vesicular) type. In agreement with these data, staining of hepatic sections with reticulin showed that in apoA-I<sup>-/-</sup> mice fed western-type diet for 24 weeks, NAFLD was much more progressed and resulted in a significant disruption of the normal architecture of the liver extracellular reticulin fibrils (Figure 10D), as compared to C57BL/6 mice (Figure 10C).

Biochemical measurement of total hepatic cholesterol (free + esterified) and triglyceride content showed that apoA-I<sup>-/-</sup> mice fed western-type diet for 24 weeks had a total hepatic cholesterol content of  $4.0\pm 3.9$  mg per gram of liver while C57BL/6 mice had  $10.5\pm 0.9$  mg per gram of liver (Figure 11A) ( $p<0.05$ ). In contrast, apoA-I<sup>-/-</sup> mice had a triglyceride content of  $295.5\pm 15.8$  mg per gram of hepatic tissue while C57BL/6 mice had a significantly lower hepatic triglyceride content ( $155.7\pm 10$  mg per gram of hepatic tissue,  $p<0.005$ ) (Figure 11A).



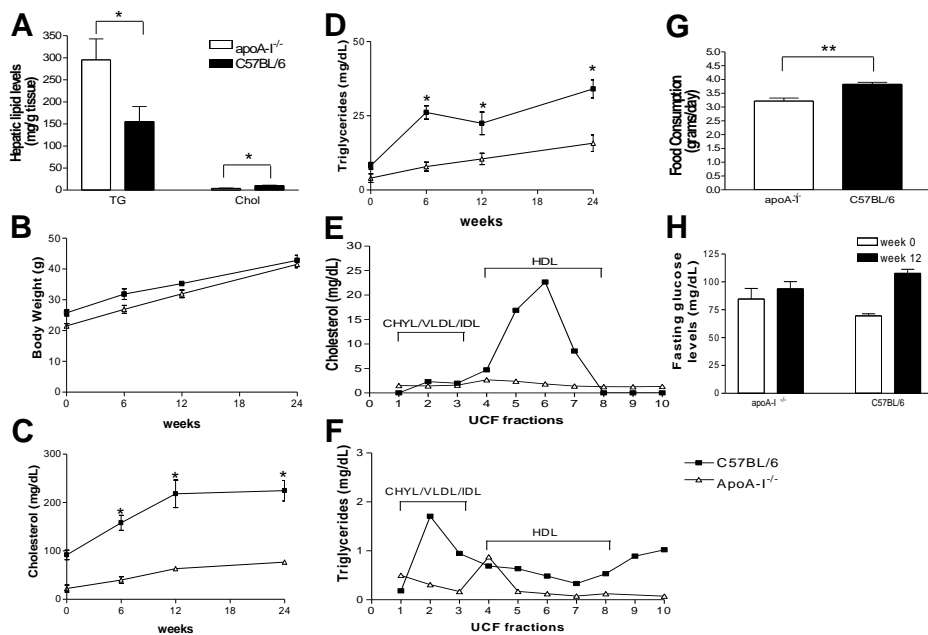
**Fig. 10.** Histological analyses of liver sections from apoA-I<sup>-/-</sup> and C57BL/6 mice. Panels A and B are representative pictures of hematoxylin and eosin stained hepatic sections from C57BL/6 (A) and apoA-I<sup>-/-</sup> mice (B) at week 24 on western-type diet. Panels C, D show representative pictures of reticulin stained hepatic sections from C57BL/6 (C) and apoA-I<sup>-/-</sup> mice (D) at week 24 of the experiment. All pictures were taken under original magnification 20X. Panels (E) and (F) show the percentage (E) and the average surface area (F) of lipid loaded cells in the livers of apoA-I<sup>-/-</sup> and C57BL/6 mice fed western-type diet for 24 weeks.

**Body-weight, body-fat content and plasma lipid levels of mice fed western-type diet for 24 weeks.** To test the effects of apoA-I deficiency on body weight gain, the weights of apoA-I<sup>-/-</sup> and C57BL/6 mice fed western-type diet were monitored every six weeks for a total period of 24 weeks.

As expected, C57BL/6 mice showed an increase in their body-weight during the course of the experiment (26). At week 6, C57BL/6 mice had an average body-weight of

31.8±1.7 grams (23.5±3.9% increase, as compared to their starting weight of 25.8±1 at week 0,  $p < 0.05$ ), at week 12 their body-weight was already 35.3±0.6 grams, while at week 24 it increased further to 42.8±1.7 grams (66.7±5.6% increase, as compared to their starting weight at week 0,  $p < 0.05$ ) (Figure 11B).

ApoA-I<sup>-/-</sup> mice showed a similar increase in their body weights during the course of the experiment (Figure 11B). Specifically, at week 6 of the experiment, the apoA-I<sup>-/-</sup> mouse group had an average body-weight of 26.9±1.3 grams (24.8±2.6% increase as compared to their starting weight of 21,53±0,741grams at week 0,  $p < 0.05$ ). At week 12, their average body-weight was 31.9±1.3 grams while at week 24 their body-weight further increased to 41.5±2.2 grams (98.1±3.2% increase, as compared to their starting weight at week 0,  $p < 0.05$ ) (Figure 11B).



**Fig. 11.** Biochemical parameters of apoA-I<sup>-/-</sup> and C57BL/6 mice fed western-type diet for a period of 24 weeks. (Panel A) Changes in hepatic triglycerides (TG) and cholesterol (Chol), (Panel B) body weight, (Panel C) plasma cholesterol, and (Panel D) plasma triglycerides of mice fed western-type diet for 24 weeks. Panels E and F show the cholesterol and triglyceride content respectively, of the different density lipoprotein fractions following separation of plasma lipoproteins by density gradient ultracentrifugation. The different lipoprotein fractions are indicated. Panel G shows the average

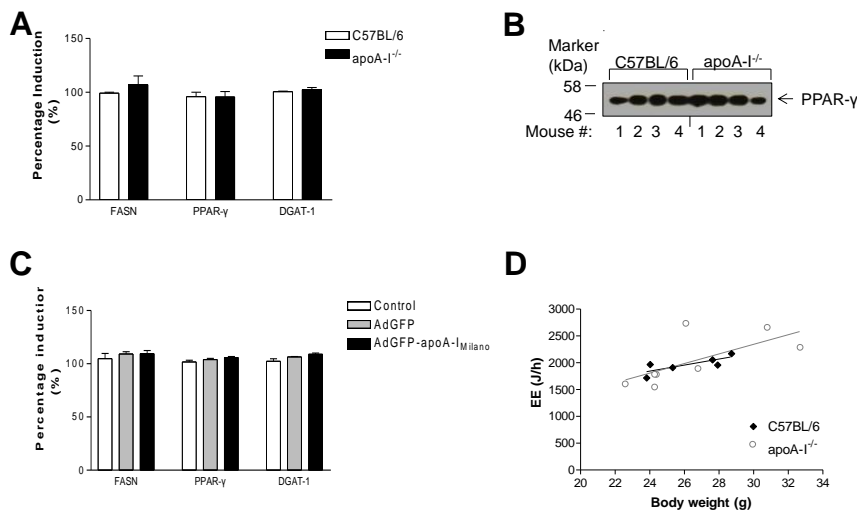
food consumption of apoA-I<sup>-/-</sup> and C57BL/6 mice at week 0 of the experiment. Panel H shows the fasting plasma glucose levels of the mice at weeks 0 and 24 of the experiment.

To our surprise, apoA-I-deficient mice were found to consume significantly less food than the control C57BL/6 mice. Average daily food consumption, measured daily during a 7 day period, showed that apoA-I<sup>-/-</sup> mice consumed 3.2±0.1 grams/day/mouse while C57BL/6 mice consumed 3.8±0.1 grams/day/mouse (p<0.001). (Figure 11G). At week 24 apoA-I<sup>-/-</sup> mice a mean body weight than was similar to that of C57BL/6 mice (p>0.05) (Figure 11B), though they had a higher body fat content than the control mice (26±2 grams of fat for ApoA-I<sup>-/-</sup> mice and 22±2 grams of fat for C57BL/6 mice, p<0.05).

C57BL/6 mice on high fat diet for 24 weeks had significantly elevated fasting cholesterol levels (224.6±21 mg/dl) as compared to their starting cholesterol levels at week 0 (91.9±10 mg/dl) (Figure 11C), while their plasma triglyceride levels remained normal (34.1±3.0 mg/dl at week 24 vs. 8.2±1.1 mg/dl at week 0) (Figure 11D). ApoA-I<sup>-/-</sup> mice also showed a modest increase in their plasma cholesterol levels during the course of the experiment. At week 24 of the experiment plasma cholesterol levels of the apoA-I<sup>-/-</sup> mice were elevated (76.9±1.4 mg/dl at week 24 versus 22.4±7.3 mg/dl at week 0) (Figure 11C), while their plasma triglyceride levels remained within normal range (15.8±2.8 mg/dl at week 24 versus 4.0±1.4 mg/dl at week 0, p<0.001) (Figure 11D).

Fractionation of plasma lipoproteins by density gradient ultracentrifugation followed by determination of total cholesterol and triglycerides in the different density fractions revealed that deficiency in apoA-I had a profound effect on the plasma lipoproteins of mice fed western-type diet for 24 weeks. Specifically, in apoA-I deficient mice HDL cholesterol was significantly reduced (Figure 11E). In contrast, in C57BL/6 mice the vast majority of cholesterol was distributed in the HDL density fractions (Figure 11E). Both apoA-I<sup>-/-</sup> and C57BL/6 mice had also low levels of triglyceride rich chylomicrons and VLDL (Figure 11F).

**Expression of lipogenic genes and indirect calorimetry analysis.** To determine if the increased hepatic triglyceride levels of the apoA-I<sup>-/-</sup> mice is due to increased de novo fatty acid and triglyceride synthesis in the liver of these mice, we measured the mRNA expression of key lipogenic enzymes such as FASN-1, DGAT-1 and PPAR $\gamma$  by real-time PCR. As shown in Figure 12A, we did not observe any significant differences in the expression of these lipogenic markers between apoA-I<sup>-/-</sup> and C57BL/6 mice. In agreement with these results, western blot analysis for PPAR- $\gamma$  (Figure 12B) followed by scanning densitometry did not reveal any significant differences in the average PPAR $\gamma$  protein abundance between C57BL/6 and apoA-I<sup>-/-</sup> mice fed western-type diet for 24 weeks.



**Fig. 12.** Expression levels of lipogenic genes and indirect calorimetry analysis. Panels A shows the relative mRNA expression of FASN-1, PPAR- $\gamma$  and DGAT-1 genes. Panel B indicates the protein levels of hepatic PPAR- $\gamma$  in the livers of C57BL/6 and apoA-I<sup>-/-</sup> mice fed western-type diet for 24 weeks. Panel C shows the relative mRNA expression of FASN-1, PPAR- $\gamma$  and DGAT-1 in untreated apoA-I<sup>-/-</sup> mice or apoA-I<sup>-/-</sup> mice treated with the AdGFP-apoA-I-Milano expressing or a control AdGFP adenovirus. Error bars indicate the standard error of the mean. Panel D is a linear regression plot of energy expenditure (J/h) versus mouse body weight (g) for the C57BL/6 and apoA-I<sup>-/-</sup> mouse groups.

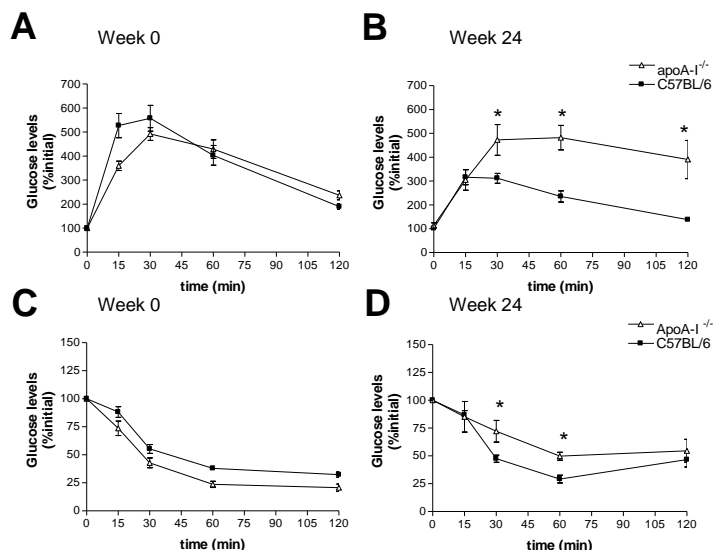
In an effort to determine whether differences in the energy expenditure (EE) may be a causal factor for the differences in adipose and liver lipid levels observed between the two mouse groups, indirect calorimetry analysis was performed. We chose to characterize our mice at the beginning of the experiment (week 0), based on the recommendations of Tschop and co-workers (11) that such metabolic phenotyping should be ideally performed early in an experiment when body weight and body fat content are identical between mouse groups. Average EE was 2030±164 J/h for apoA-I<sup>-/-</sup> mice and 1930±65 J/h for C57BL/6 mice

( $p > 0.05$ ). ANCOVA analysis indicated that when controlled for body weight, apoA-I<sup>-/-</sup> and C57BL/6 mouse groups exhibit similar EE ( $F = 0.368$ ,  $p = 0.556$ ,  $n = 8$ ) (Figure 12D).

**Increased hepatic deposition of triglycerides in the livers of apoA-I<sup>-/-</sup> mice correlates with reduced glucose tolerance and insulin sensitivity.**

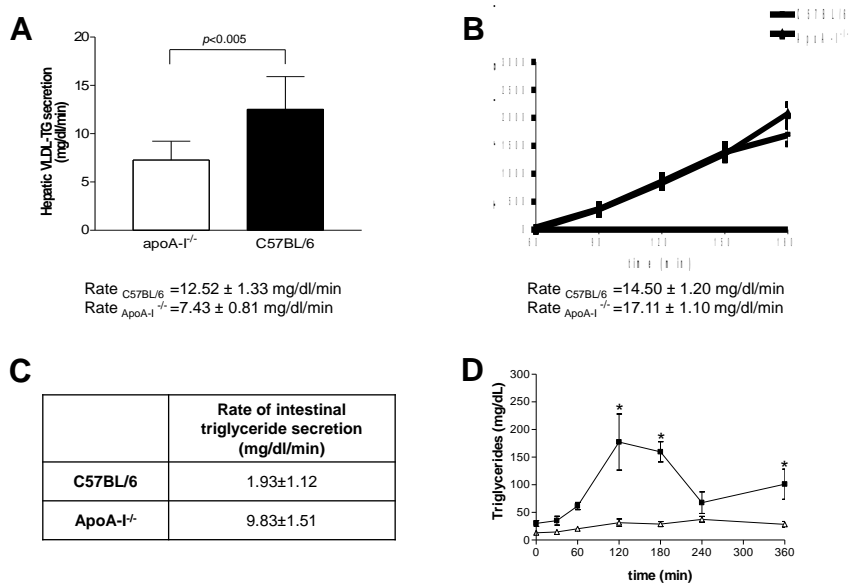
To determine if the increased hepatic triglyceride content of the apoA-I<sup>-/-</sup> mice correlates with disturbances in plasma glucose homeostasis we performed the standard glucose tolerance (GTT) and insulin sensitivity (IST) tests. At week 0 both mouse groups had similar fasting plasma glucose levels ( $84.6 \pm 9.5$  mg/dl for apoA-I<sup>-/-</sup> mice and  $69.5 \pm 1.9$  mg/dl for C57BL/6 mice,  $p > 0.05$ )

(Figure 11H) and normal responses to intraperitoneal administration of glucose and insulin (Figure 13A, C). Similarly, at week 24 of the experiment, the apoA-I<sup>-/-</sup> mice had fasting glucose levels of  $93.8 \pm 6.5$  mg/dl while the C57BL/6 mice had fasting plasma glucose of  $107.6 \pm 3.7$  mg/dl ( $p = 0.054$ ) (Figure 11H). However, apoA-I<sup>-/-</sup> mice showed a significant deterioration in their ability to clear plasma glucose in a GTT (Figure 13B) and to respond to intraperitoneal administration of insulin in an IST, compared to C57BL/6 (Figure 13D).



**Fig. 13.** Glucose tolerance test (A, B) and insulin sensitivity test (C, D) of apoA-I<sup>-/-</sup> and C57BL/6 mice at weeks 0 (A, C) and 24 (B, D) of the experiment.

**Rate of hepatic triglyceride secretion in apoA-I<sup>-/-</sup> and C57BL/6 mice.** One mechanism that could affect hepatic triglyceride content is the secretion of VLDL triglycerides in the circulation. Thus, to determine the effects of apoA-I deficiency on the secretion of hepatic triglycerides, we next performed a classical VLDL triglyceride secretion assay in apoA-I<sup>-/-</sup> and C57BL/6 mice. We found that apoA-I<sup>-/-</sup> mice had a reduced rate of hepatic VLDL-triglyceride secretion when compared to C57BL/6 mice. Specifically, secretion rates were  $7.43 \pm 0.81$  mg/dl/min versus  $12.52 \pm 1.33$  mg/dl/min for apoA-I<sup>-/-</sup> and C57BL/6 mice respectively ( $p < 0.005$ ). (Figure 14A).



**Fig. 14.** Kinetic parameters of apoA-I<sup>-/-</sup> and C57BL/6 mice at the initiation of the experiment (week 0). Panel A shows the rate of hepatic VLDL triglyceride secretion in apoA-I<sup>-/-</sup> and C57BL/6 mice at week 0 of the experiment. The bar-graph represents the mean  $\pm$  standard deviation, of the individual rates of VLDL-triglyceride secretion per virus group. Panel B shows the total rate of triglyceride input in the plasma of apoA-I<sup>-/-</sup> and C57BL/6 mice. Panel C indicates the rate of intestinal triglyceride secretion of apoA-I<sup>-/-</sup> and C57BL/6 mice that results by subtracting the rate of hepatic triglyceride secretion (determined in panel A) from the total rate of plasma triglyceride supply (determined in panel B). Panel D shows the kinetics of post-prandial triglyceride clearance in apoA-I<sup>-/-</sup> and C57BL/6 mice. Values were expressed in mg/dl  $\pm$  standard error of the

mean.

**Rate of intestinal triglyceride secretion in apoA-I<sup>-/-</sup> and C57BL/6 mice.** One additional mechanism that could explain the increased sensitivity of apoA-I<sup>-/-</sup> mice to diet-induced NAFLD, could be increased intestinal secretion of triglyceride-rich lipoproteins to the plasma of these mice. To determine the effects of apoA-I deficiency on the rate of intestinal triglyceride secretion we first determined the total rate (intestinal and hepatic) of plasma triglyceride supply in apoA-I<sup>-/-</sup> and C57BL/6 mice fed western-type diet, following an oral fat load. Groups of 5 apoA-I<sup>-/-</sup> and C57BL/6 mice each were fasted for 16 hours, and then administered an oral fat load of 300  $\mu$ l of olive oil, as described in Materials and Methods. One hour post-gavage, mice were injected with triton WR1339, then plasma triglyceride levels were measured as a function of time and a linear graph of plasma triglycerides versus time was created. As shown in Figure 14B, both mouse strains exhibited comparable rates of plasma triglyceride increase ( $14.5 \pm 1.2$  mg/dl/min for C57BL/6 vs.  $17.11 \pm 1.1$  mg/dl/min for apoA-I<sup>-/-</sup> mice,  $p > 0.05$ ) as determined from the slopes of the graphs.

Then, by subtracting the rate of hepatic triglyceride secretion (determined above) from the total rate of plasma triglyceride supply, the rate of intestinal triglyceride secretion was determined as  $9.83 \pm 1.51$  mg/dl/min for the apoA-I<sup>-/-</sup> mice and  $1.93 \pm 1.12$  mg/dl/min for the C57BL/6 mice ( $p < 0.05$ ) (Figure 14C).

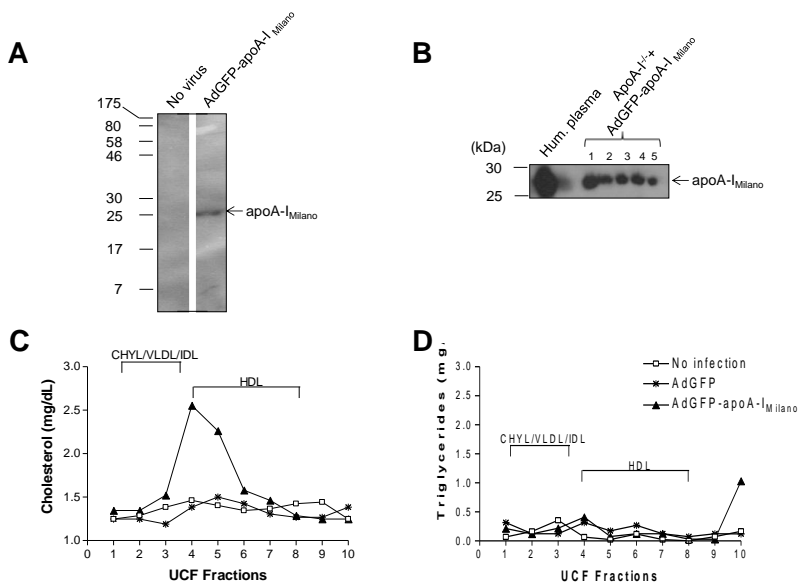
**Kinetics of post-prandial triglyceride clearance in apoA-I<sup>-/-</sup> and C57BL/6 mice.** Another potential mechanism that could explain the increased sensitivity of apoA-I<sup>-/-</sup> mice to diet-induced NAFLD, could be increased clearance of plasma triglycerides in these mice. To determine the effects of apoA-I deficiency on the kinetics of post-prandial triglyceride clearance, groups of five apoA-I<sup>-/-</sup> and five C57BL/6 mice were fasted for 16 hours, then administered an oral fat load of 300  $\mu$ l of olive oil and plasma samples were isolated at 30, 60, 120, 180, 240 and 360 min post gavage. As shown in Figure 14D, plasma triglyceride levels in apoA-I<sup>-/-</sup> mice started increasing at 120 min ( $31.4 \pm 6.819$  mg/dl), reached a modest peak at 240 min ( $37.4 \pm 5.409$  mg/dl) and rapidly declined back to baseline by 360 min ( $28.4 \pm 5.15$  mg/dl). In contrast, control C57BL/6 mice showed a much slower kinetics of catabolism of postprandial triglycerides. Specifically, plasma triglyceride levels in C57BL/6 started increasing as early as 60 min ( $61.6 \pm 6.6$  mg/dl) post-gavage. At 120 and 180 min, plasma triglyceride levels were  $177.4 \pm 50.8$  mg/dl and  $159.7 \pm 18.3$  mg/dl respectively, and then started declining slowly, reaching a concentration of  $67.7 \pm 19.5$  mg/dl at 180 min of the experiment (Figure 14D).

**Ectopic expression of apoA-I by a recombinant attenuated adenovirus significantly reduces hepatic triglyceride content and improves hepatic histology and architecture.** To evaluate the potential of ectopic apoA-I expression in reducing hepatic triglyceride load, as a proof of principle we used adenovirus-mediated gene transfer of apoA-IMilano in male apoA-I<sup>-/-</sup> mice fed western-type diet for a period of 12 weeks.

We generated a recombinant attenuated adenovirus expressing apoA-IMilano, designated as AdGFP-A-IMilano. The capacity of the adenovirus to infect cultured cells and produce apoA-IMilano was confirmed by western blot analysis of media from infected HTB-13 (human astrocytoma) cell cultures (Figure 15A). We next proceeded with the administration of adenoviruses to apoA-I<sup>-/-</sup> mice that were fed western-type diet for 12 weeks. Specifically, 5 mice were treated with the AdGFP-A-IMilano adenovirus, 5 mice were treated with the control AdGFP adenovirus, and 5 mice remained untreated. We chose to treat mice with a low dose of  $8 \times 10^8$  pfu of the adenoviruses in order to avoid infection-induced inflammation and liver damage that could affect the outcome of our experiment. Following infection, mice were switched to chow diet for 10 days, then sacrificed and plasma and livers were collected for further analyses.

The expression of apoA-IMilano in the plasma of the AdGFP-A-IMilano infected mice was confirmed by western blot analysis (Figure 15B). Furthermore, expression of apoA-IMilano resulted in a slight increase in the HDL cholesterol of these mice (Figure 15C) in agreement to previously published results (32), while no significant difference was observed in their triglyceride levels, compared to their AdGFP-infected and non-infected counterparts (Figure 15D).

As shown in Figure 7A, H&E staining of livers from uninfected apoA-I<sup>-/-</sup> mice fed western-type diet for 12 weeks revealed significant accumulation of lipids in the hepatocytes of these mice (not shown). Similarly apoA-I<sup>-/-</sup> mice infected with  $8 \times 10^8$  pfu of the control adenovirus AdGFP showed a similar accumulation of hepatic triglycerides and histological signs of inflammation (Figure 16A). In contrast, apoA-I<sup>-/-</sup> mice infected with  $8 \times 10^8$  pfu of the AdGFP-A-IMilano adenovirus had a profoundly reduced deposition of lipid droplets within the hepatocytes (Figure 16B), reflecting a significant reduction of the hepatic

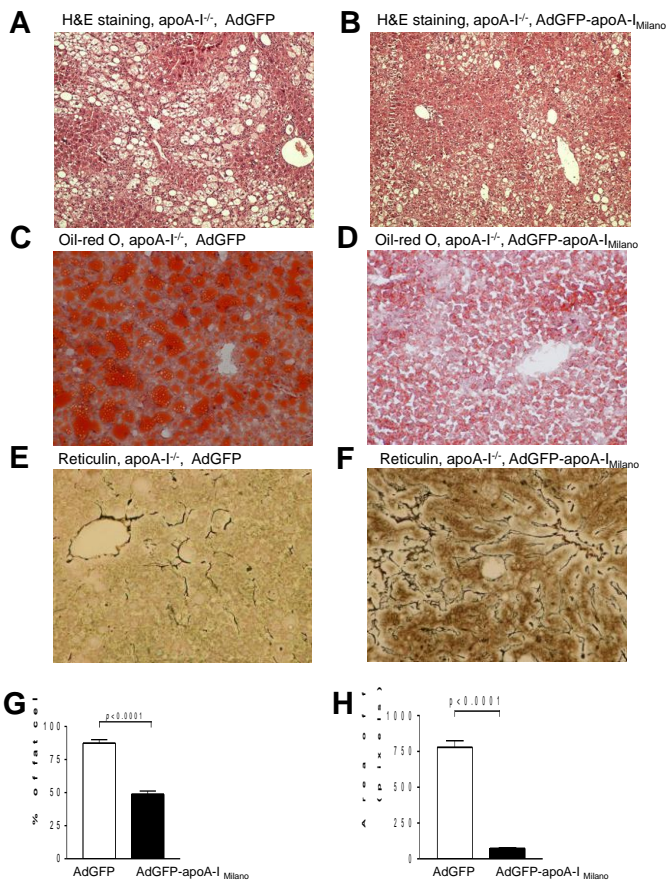


triglycerides and exhibited a significantly improved liver histology. Histomorphometry followed by statistical analysis showed that the number of lipid droplets and their average surface area were

**Fig. 15.** Effects of ectopic apoA-IMilano expression on hepatic lipid content of mice fed western-type diet for 24 weeks. (A) Western Blot analysis of media from infected HTB-13 (human astrocytoma) cells, confirming expression of apoA-IMilano. (B) Western Blot analysis of plasma of infected apoA-1<sup>-/-</sup> mice, confirming apoA-IMilano expression. UCF (C) cholesterol and (D) triglyceride profiles of uninfected, AdGFP-control infected and Ad-ApoA-IMilano-infected mice. The different lipoprotein fractions are indicated.

significantly reduced in the livers of apoA-1<sup>-/-</sup> mice infected with the apoA-IMilano-expressing adenovirus than the livers of uninfected or the control AdGFP adenovirus-infected apoA-1<sup>-/-</sup> mice ( $p=0.0001$ ) (Figure 16G, H). Oil-red O staining (Figure 16C, D) further confirmed that apoA-1<sup>-/-</sup> mice treated with the AdGFP-A-IMilano adenovirus (Figure 16D) had significantly reduced hepatic lipid content than the apoA-1<sup>-/-</sup> mice treated with the control adenovirus AdGFP (Figure. 7C). Staining with reticulin stain (Figure 16E, F) showed that treatment of apoA-1<sup>-/-</sup> mice with AdGFP-A-IMilano also resulted in a significant improvement in the hepatic architecture of these mice (Figure 16E, F). These differences could not be attributed to reduced de novo lipogenesis following infection with the apoA-IMilano-expressing adenovirus since no changes were observed in the hepatic mRNA expression of FASN-1, DGAT-1 and PPAR $\gamma$  between mouse groups (Figure 12C). Taken together, the

findings establish that ectopic expression of apoA-IMilano results in a significant reduction of hepatic triglyceride content and an improvement of hepatic histology and architecture in apoA-1<sup>-/-</sup> mice fed western-type diet for 12 weeks.

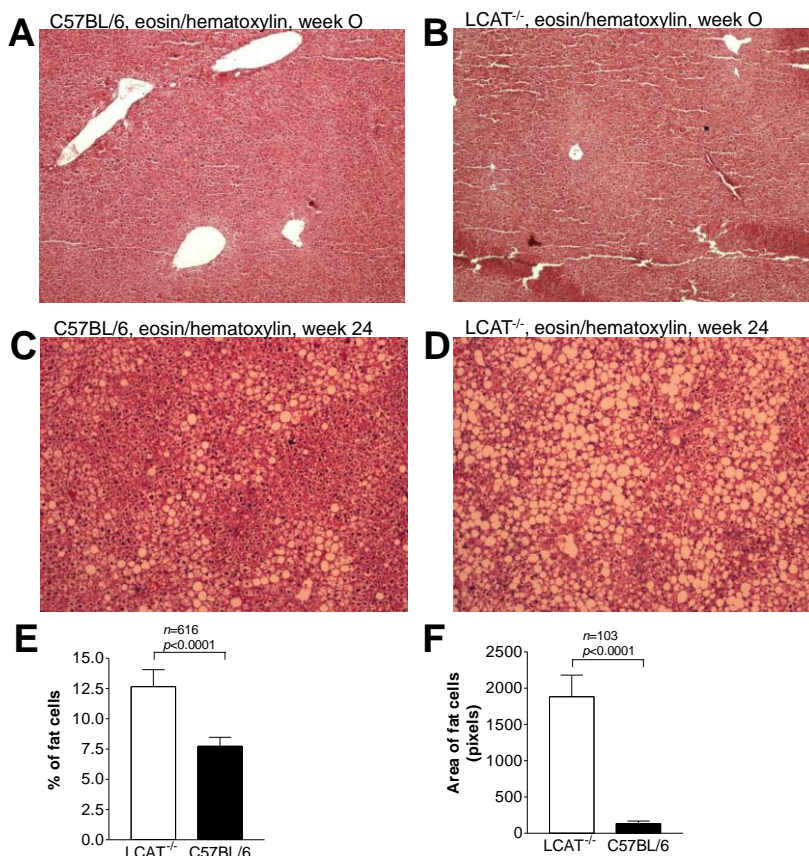


**Fig. 16.** Histological analysis of liver sections from mice infected with an AdGFP-apoA-IMilano expressing or a control adenovirus. Panels A, B are representative pictures of hematoxylin and eosin stained hepatic sections from apoA-1<sup>-/-</sup> mice, that were fed western-type diet for 12 weeks, and then infected with the control AdGFP adenovirus (panel A), or the AdGFP-apoA-IMilano adenovirus (panel B). Panels C, D are representative pictures of oil-red O stained hepatic sections from apoA-1<sup>-/-</sup> mice, that were fed western-type diet for 12 weeks, and then infected with the control AdGFP (panel C) or the AdGFP-apoA-IMilano (panel D) adenovirus. Panels E, F show representative pictures of reticulin stained hepatic sections from control AdGFP-infected (panel E) or the AdGFP-apoA-IMilano -infected (panel F) adenoviruses. All pictures were taken under original magnification 20X. Panels (G) and (H) indicate the % of fat cells and the average surface area of the lipid droplets in the livers of mice infected with AdGFP and AdGFP-AIMilano adenoviruses.

### A2.3. Lecithin:cholesterol acyltransferase modulates diet-induced obesity, glucose intolerance and hepatic deposition of triglycerides in mice (J Nutr Biochem. 2012 Jul 19).

#### LCAT deficiency enhances hepatic triglyceride accumulation in mice.

To test the effects of LCAT on hepatic triglyceride deposition, groups of 10-12 week-old male LCAT-deficient (LCAT<sup>-/-</sup>) and wild-type (WT) C57BL/6 mice were placed on western-type for 24 weeks. Hematoxylin and eosin (H&E) staining of liver sections from wild-type (WT) C57BL/6 and LCAT<sup>-/-</sup> mice at the beginning of the experiment (week 0) did not reveal any significant accumulation of hepatic triglycerides within the hepatocytes of these mice (Fig. 17A, B). However, at week 24 both C57BL/6 and LCAT<sup>-/-</sup> mice showed accumulation of hepatic triglycerides in the form of lipid droplets (Fig. 17C, D). Statistical analysis following histomorphometric evaluation of the H&E sections revealed that the number of lipid droplets within hepatocytes was significantly elevated in the LCAT<sup>-/-</sup> mice as compared to the C57BL/6 mice ( $p=0.0001$ ) (Fig. 17E, F). The observed lipid accumulation was diffuse and of the macrovesicular type. This finding was further confirmed by oil-red O staining of hepatic sections from C57BL/6 and LCAT<sup>-/-</sup> mice fed western-type diet for 24 weeks (Fig. 18C, D). As expected the average area, perimeter and maximum diameter of the lipid loaded cells were much greater (all  $p<0.0001$ ) in the LCAT<sup>-/-</sup> compared to the C57BL/6 mice (Fig. 18E, F, G respectively) In agreement with these data, staining of hepatic sections with reticulin showed that in LCAT<sup>-/-</sup> mice fed western-type diet for 24 weeks, hepatic lipid deposition was much more progressed and resulted in a significant disruption of the normal architecture of the liver extracellular reticulin fibrils (Fig. 18B), as compared to C57BL/6 mice (Fig. 18A). No significant differences in the size and shape of visceral adipocytes were detected between the two mouse groups (Fig. 19).

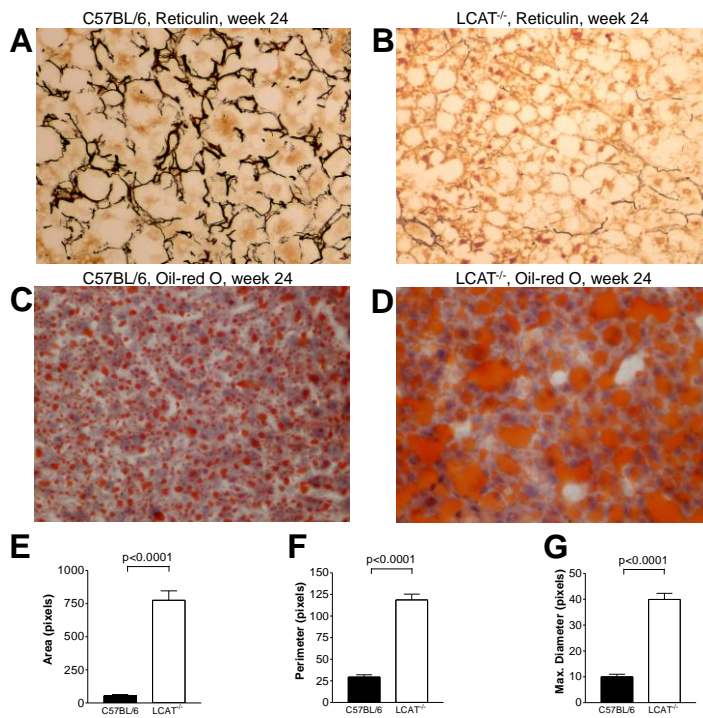


**Fig. 17.** H&E staining of liver sections from LCAT<sup>-/-</sup> and C57BL/6 mice. Representative pictures of H&E stained hepatic sections from C57BL/6 (A, C) and LCAT<sup>-/-</sup> mice (B, D) at week 0 (A, B) and 24 (C, D) on western-type diet. All pictures were taken under original magnification 40X. Panels (E) and (F) show the percentage (E) and the average area (F) of lipid loaded cells in the livers of LCAT and C57BL/6 mice fed western-type diet for 24 weeks.

Biochemical measurement of hepatic triglyceride content showed that LCAT<sup>-/-</sup> mice fed western-type diet for 24 weeks had a triglyceride content of  $197.3 \pm 10.6$  mg per gram of hepatic tissue while C57BL/6 mice had a significantly lower hepatic triglyceride content ( $155.7 \pm 10$  mg per gram of hepatic tissue,  $p<0.005$ ) further confirming that LCAT possesses a central role in the deposition of triglycerides to the liver of mice following feeding with western-type diet.

**Body-weight and plasma lipid levels of male mice fed western-type diet for 24 weeks.** Monitoring of animal body weight every six weeks during the course of the experiment showed that LCAT<sup>-/-</sup> mice became heavier than C57BL/6 mice. Specifically, at week 12 C57BL/6 mice had an average body-weight of  $35.3 \pm 0.6$  grams, while at week 24 it increased further to  $42.8 \pm 1.7$  grams ( $66.7 \pm 5.6\%$  increase, as compared to their starting weight at week 0,  $p<0.05$ ) (Fig. 20A). Interestingly, at week 12 of the experiment, the LCAT<sup>-/-</sup> mouse group had an average body-weight of  $42.2 \pm 1.1$  grams while at week 24, their body-weight further

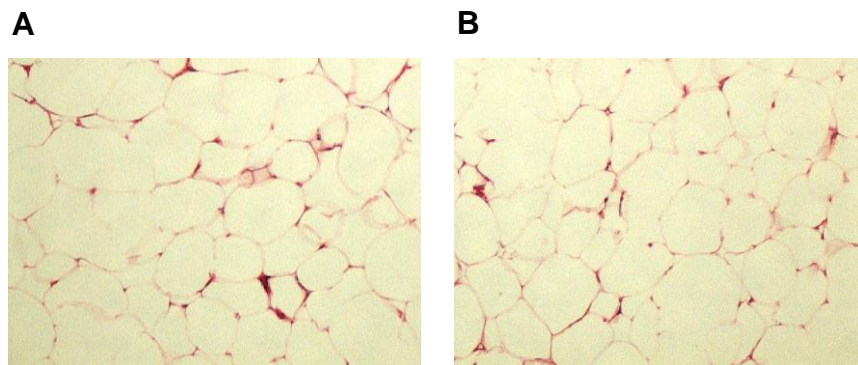
increased to  $50.5 \pm 1.9$  grams ( $116.5 \pm 8.4\%$  increase, as compared to their starting weight at week 0,  $p < 0.05$ ) (Fig. 20A). Body composition analysis showed that the increased body weight of the LCAT<sup>-/-</sup> mice was due to increased fat mass in these mice (Table I).



**Fig. 18.** Representative pictures of reticulin stained hepatic sections of C57BL/6 (A) and LCAT<sup>-/-</sup> mice (B) fed western-type diet for 24 weeks. Panels C and D are representative pictures of oil-red O stained hepatic sections from C57BL/6 (C) or LCAT<sup>-/-</sup> (D) mice that were fed western-type diet for 24 weeks. All pictures were taken under original magnification 40X. Panels (E), (F), and (G) indicate the average area, perimeter and maximum diameter of the lipid droplets in the livers of C57BL/6 and LCAT<sup>-/-</sup> mice.

Analysis of fasting plasma lipid levels showed that C57BL/6 mice on high fat diet for 24 weeks had significantly elevated fasting cholesterol levels ( $224.6 \pm 21$  mg/dl) as compared to their starting cholesterol levels at week 0 ( $91.9 \pm 10$  mg/dl) (Fig. 20B), while their plasma triglyceride levels remained normal ( $25.1 \pm 2.9$  mg/dl at week 24 vs.  $18.2 \pm 1.1$  mg/dl at week 0) (Fig. 20C). LCAT<sup>-/-</sup> mice also showed a modest increase in their plasma cholesterol levels during the course of

the experiment. At week 24 of the experiment plasma cholesterol levels of the LCAT<sup>-/-</sup> mice were elevated ( $271.6 \pm 16.8$  mg/dl at week 24 versus  $88.2 \pm 20.5$  mg/dl at week 0) (Fig. 20B), while their plasma triglyceride levels remained within normal range ( $53.2 \pm 10.3$  mg/dl at week 24 versus  $8.2 \pm 1.9$  mg/dl at week 0,  $p < 0.001$ ) (Fig. 20C).

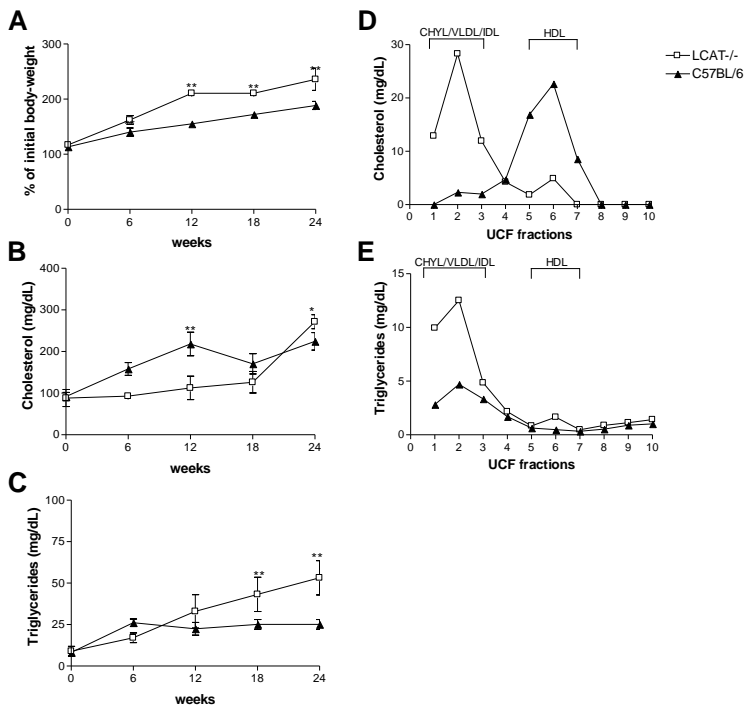


**Fig. 19.** H&E stained sections showing visceral fat from LCAT<sup>-/-</sup> (A) and C57BL/6 (B) mice fed western-type diet for twenty four weeks. All pictures were taken under original magnification 40X.

Interestingly, LCAT<sup>-/-</sup> mice had slightly lower steady-state plasma FFA levels compared to C57BL/6 mice. At week 24 of the experiment plasma free fatty-acid levels of the LCAT<sup>-/-</sup> mice were  $1.0 \pm 0.2$  mmole Equiv, while C57BL/6 mice had a concentration of  $1.4 \pm 0.7$  mmole Equiv ( $p < 0.005$ ).

Fractionation of plasma lipoproteins by density gradient ultracentrifugation followed by determination of total cholesterol, triglycerides, and phospholipids in the different density fractions revealed that deficiency in LCAT had a profound effect on the distribution and composition of plasma lipoproteins of mice fed western-type diet for 24 weeks. Specifically, in LCAT deficient mice most of the cholesterol was found in the chylomicron/VLDL and IDL density fractions while HDL cholesterol was significantly reduced (Fig. 20D). In contrast, in C57BL/6 mice the vast majority of cholesterol was distributed in the HDL density fractions while chylomicron/VLDL and IDL cholesterol levels were very low (Fig. 20D). The chylomicron/VLDL fraction of LCAT<sup>-/-</sup> mice had also a much higher concentration of triglycerides consistent with the higher steady-state triglyceride levels in the plasma of these mice (Fig. 20E). As expected, most of the cholesterol of LCAT deficient mice was unesterified.

**Rate of hepatic triglyceride secretion in male LCAT<sup>-/-</sup> and C57BL/6 mice.** One mechanism that could affect hepatic triglyceride content is the secretion of hepatic triglycerides in the circulation. Thus, we next



**Fig. 20.** Biochemical parameters of LCAT<sup>-/-</sup> and C57BL/6 mice fed western-type diet for a period of 24 weeks. Changes in (A) body weight, (B) plasma cholesterol, and (C) plasma triglycerides of mice fed western-type diet for 24 weeks. Panels (D) and (E) show the cholesterol and triglyceride content respectively, of the different density lipoprotein fractions following separation of plasma lipoproteins by density gradient ultracentrifugation (UCF). The different lipoprotein fractions are indicated.

Mouse Strain	Wet Body Weight	Dry Body Weight	Body Fat	Body Water
C57BL/6	35.7±1.5	25.5±1.6	21.8±2.3	10.2±0.2
LCAT <sup>-/-</sup>	41.9±1.7	32.0±1.8	30.6±5.8	9.9±0.5

**Table 1:** Body composition of C57BL/6 and LCAT<sup>-/-</sup> mice that were fed western-type diet for 24 weeks

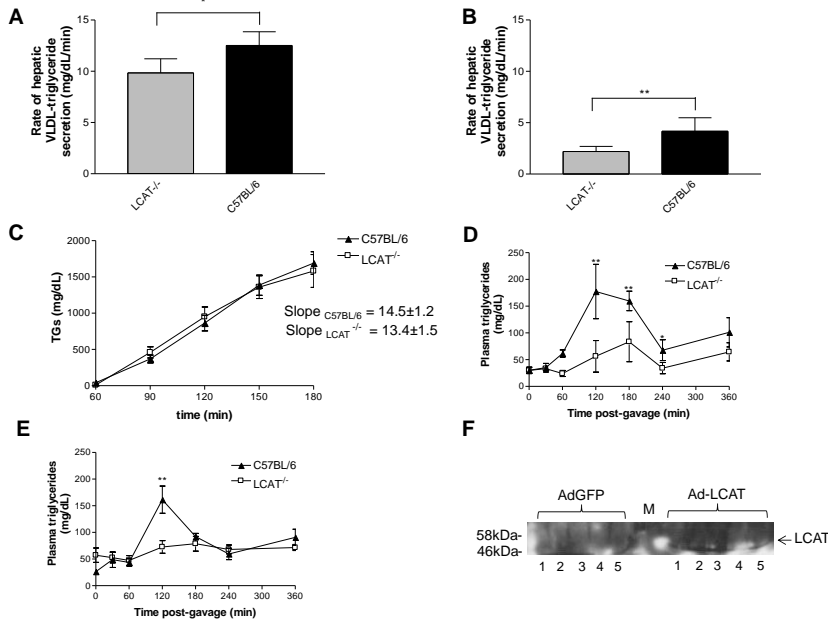
**Rate of intestinal triglyceride secretion in male LCAT<sup>-/-</sup> and C57BL/6 mice.** One additional mechanism that could affect hepatic triglyceride content is an increased intestinal secretion of triglyceride-rich lipoproteins to the plasma of these mice. To determine the rate of intestinal triglyceride secretion we first determined the total rate (intestinal and hepatic) of plasma triglyceride supply in LCAT<sup>-/-</sup> and C57BL/6 mice fed western-type diet, following an oral fat load. Groups of 5 LCAT<sup>-/-</sup> and C57BL/6 mice each were fasted for 16 hours, and then administered an oral fat load of 300  $\mu$ l of olive oil, as described in Materials and Methods. One hour post-gavage, mice were injected with triton WR1339, then plasma triglyceride levels were measured as a function of time and a linear graph of plasma triglycerides versus time was created. As shown in Fig. 21C, both mouse strains exhibited comparable rates of plasma triglyceride increase (14.5±1.2 mg/dl/min for C57BL/6 vs. 13.4±1.5 mg/dl/min for LCAT<sup>-/-</sup> mice,  $p>0.05$ ) as determined from the slopes of the graphs.

Then, by subtracting the rate of hepatic triglyceride secretion (determined above) from the total rate of plasma triglyceride supply, the rate of intestinal triglyceride secretion at week 0 of the experiment was determined as 3.6±0.5 mg/dl/min for the LCAT<sup>-/-</sup> mice and 2.0±0.7 mg/dl/min for the C57BL/6 mice ( $p<0.05$ ).

No significant differences in the average daily food consumption were found between the two mouse strains. Specifically, two independent measurements, one at week 12 and one at week 24, showed that LCAT<sup>-/-</sup> mice consumed 3.6±0.1 and 3.6±0.3 grams/mouse/day respectively ( $p>0.05$ ) while C57BL/6 mice consumed 3.8±0.2 and 3.4±0.2 grams/mouse/day ( $p>0.05$ ) respectively (measurements represent an average daily consumption during a 7 day period). No statistical difference was observed between the two mouse strains at each time-point ( $p>0.05$ ).

**Kinetics of post-prandial triglyceride clearance in male LCAT<sup>-/-</sup> and C57BL/6 mice.** Another mechanism that could affect hepatic triglyceride content is an increased clearance of postprandial plasma triglycerides

performed a classical VLDL triglyceride secretion assay in LCAT<sup>-/-</sup> and C57BL/6 mice at weeks 0 (immediately prior to diet initiation) and 12 following feeding with western-type diet. We found that LCAT<sup>-/-</sup> mice had a reduced rate of hepatic VLDL-triglyceride secretion at both time-points when compared to C57BL/6 mice. Interestingly, western-type diet for 12 weeks led to a reduction of hepatic VLDL-triglyceride secretion in both mouse strains. Specifically, at week 0, secretion rates were 9.8±1.1 mg/dl/min versus 12.5±1.3 mg/dl/min for LCAT<sup>-/-</sup> and C57BL/6 mice respectively ( $p<0.005$ ) (Fig. 21A). At week 12, secretion rates were reduced to 2.1±0.6 mg/dl/min versus 4.2±0.9 mg/dl/min for LCAT<sup>-/-</sup> and C57BL/6 mice respectively, ( $p<0.005$ ) (Fig. 21B).

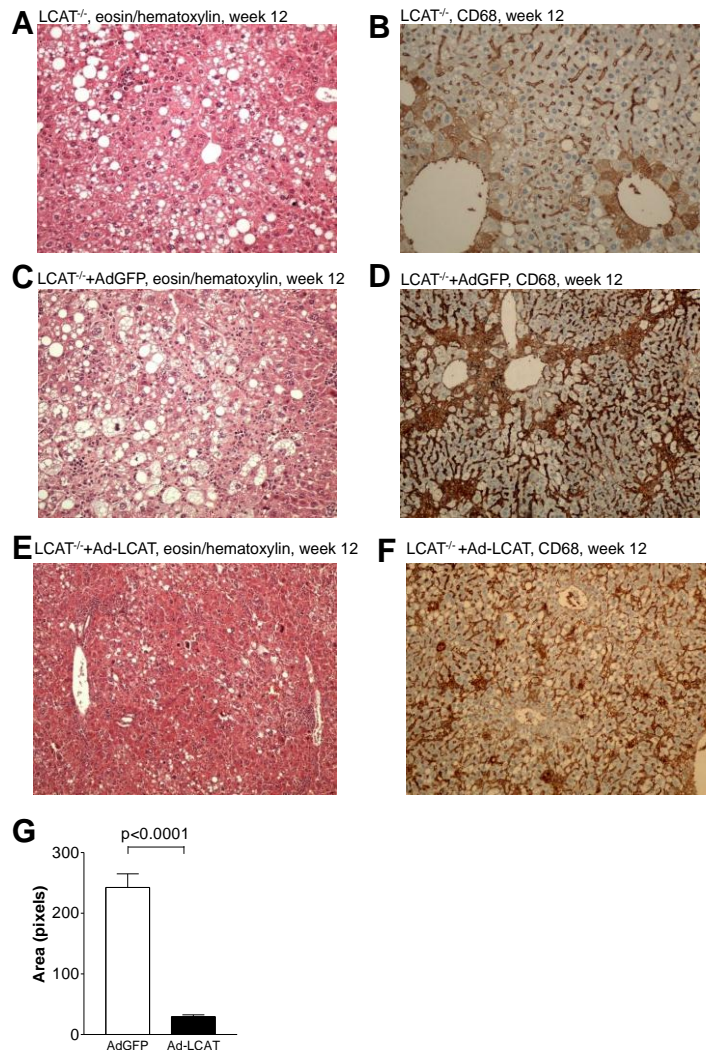


**Fig. 21.** Effects of LCAT deficiency on kinetic parameters of lipid metabolism. Panels A and B show the rate of hepatic VLDL triglyceride secretion in LCAT<sup>-/-</sup> and C57BL/6 mice at weeks 0 (A) and 12 (B) of the experiment. Panel C shows the rate of total triglyceride input in the plasma of LCAT<sup>-/-</sup> and C57BL/6 mice. Panels D, E represent the kinetics of post-prandial triglyceride clearance in LCAT<sup>-/-</sup> and C57BL/6 mice at week 0 (D) and 12 (E) of the experiment. Experiments were performed as described in Materials and Methods. Panel (F) is a western blot analysis of plasma samples from AdGFP and AdLCAT-infected mice isolated on day five post infection. Only the Ad-LCAT infected mice express LCAT.

and five C57BL/6 mice were fasted for 16 hours, then administered an oral fat load of 300  $\mu$ l of olive oil and plasma samples were isolated at 30, 60, 120, 180, 240 and 360 min post gavage. As shown in Fig. 21D, plasma triglyceride levels in LCAT<sup>-/-</sup> mice started increasing at 120 min (56.3 ± 29.5 mg/dl), reached a modest peak at 180 min (83.5 ± 37.4 mg/dl) and rapidly declined back to baseline by 240 min (34.2 ± 10.5 mg/dl).

in these mice. To determine the kinetics of post-prandial triglyceride clearance, groups of five LCAT<sup>-/-</sup>

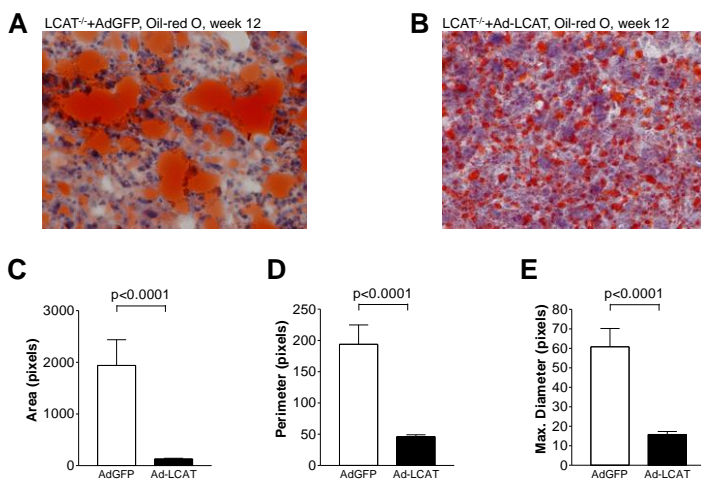
**Fig. 22.** Histological analysis of liver sections from mice infected with an LCAT-expressing or a control adenovirus. Panels (A), (C), and (E) are representative pictures of eosin/hematoxylin stained hepatic sections from uninfected LCAT<sup>-/-</sup> mice (A), LCAT<sup>-/-</sup> mice infected with the control AdGFP adenovirus (C), and LCAT<sup>-/-</sup> mice infected with the Ad-LCAT adenovirus (E), that were fed western-type diet for 12 weeks. Panels B, D, and F, are representative pictures of anti-CD68 antibody stained hepatic sections from uninfected LCAT<sup>-/-</sup> mice (B), LCAT<sup>-/-</sup> mice infected with the AdGFP adenovirus (D), and LCAT<sup>-/-</sup> mice infected with the Ad-LCAT adenovirus (F), that were fed western-type diet for 12 weeks. All pictures were taken under original magnification 40X. Panel (G) shows the average area of lipid loaded cells in the livers of mice treated with AdGFP and Ad-LCAT.



In contrast, control C57BL/6 mice showed a much slower kinetics of catabolism of postprandial triglycerides. Specifically, plasma triglyceride levels in C57BL/6 started increasing as early as 60 min (61.6 ± 6.6 mg/dl) post-gavage. At 120 and 180 min, plasma triglyceride levels were 177.4 ± 50.8 mg/dl and 159.7 ± 18.3 mg/dl respectively, and then started declining slowly, reaching a concentration of 67.7 ± 19.5 mg/dl at 180 min of the experiment (Fig. 21D). Similar post-prandial triglyceride kinetics was obtained for LCAT<sup>-/-</sup> and C57BL/6 mice fed western-type diet for 12 weeks (Fig. 21E). Given the

enhanced intestinal absorption of the LCAT<sup>-/-</sup> mice, the data support that these mice also display a very fast kinetics of post-prandial triglyceride clearance.

**Ectopic expression of LCAT by adenovirus-mediated gene transfer reduces hepatic triglyceride content in male LCAT<sup>-/-</sup> mice fed western-type diet for 12 weeks.** In the next set of experiments we investigated the effects of ectopic expression of LCAT on hepatic triglyceride deposition. The expression of LCAT was confirmed by western blot analysis of plasma samples isolated from the infected mice five days post-infection (Fig. 21F). As shown in Fig. 22A, H&E staining of livers from uninfected LCAT<sup>-/-</sup> mice fed western-type diet for 12 weeks reveal significant accumulation of triglycerides in the hepatocytes of these mice. Notably, the observed steatosis was of the microvesicular type. Similarly LCAT<sup>-/-</sup> mice infected with 8x10<sup>8</sup> pfu of the control adenovirus AdGFP showed a comparable accumulation of hepatic triglycerides and some signs of inflammation possibly due to the infection process (Fig. 22C). However, LCAT<sup>-/-</sup> mice infected with 8x10<sup>8</sup> pfu of the Ad-LCAT adenovirus had a profoundly reduced deposition of hepatic triglycerides and exhibited a significantly improved hepatic histology (Fig. 22E, G). Oil-red O staining (Fig. 23A, B) further confirmed that LCAT<sup>-/-</sup> mice treated with the Ad-LCAT adenovirus (Fig. 23B) had significantly reduced hepatic lipid content compared to the LCAT<sup>-/-</sup> mice treated with the control adenovirus AdGFP (Fig. 23A).



**Fig. 6.** Panels A and B are representative pictures of oil-red O stained hepatic sections. LCAT<sup>-/-</sup> mice fed western-type diet for 12 weeks were infected with the control AdGFP (A) or the Ad-LCAT (B) adenovirus. Then, mice were switched to chow diet for ten days and hepatic sections were isolated and stained with Oil-red O. All pictures were taken under original magnification 40X. Panels (C), (D), and (E) indicate the average area, perimeter and maximum diameter of the lipid droplets in the livers of mice infected with AdGFP and Ad-LCAT adenoviruses.

As expected the average area, perimeter and maximum diameter of the lipid loaded cells were much greater (all  $p < 0.0001$ ) in

the AdGFP-infected compared to the Ad-LCAT-infected mice (Fig. 22C, D, E respectively). Immunohistochemical staining of hepatic sections with an anti-CD68 antibody revealed that both uninfected and AdGFP-infected LCAT<sup>-/-</sup> mice exhibited increased infiltration of macrophages, residing mainly around the hepatic venules and the lipid loaded hepatocytes (Fig. 22B, D). Control AdGFP-infected LCAT<sup>-/-</sup> mice showed the highest infiltration of macrophages, possibly due to an inflammatory response associated with the infection process. Interestingly, treatment of LCAT<sup>-/-</sup> mice with Ad-LCAT adenovirus significantly reduced the number of infiltrated macrophages accumulating in the liver of these mice (compare Fig. 22F to Fig. 22B, D). Histo-morphometry followed by statistical analysis showed that the number of lipid droplets was significantly reduced in the livers of LCAT<sup>-/-</sup> mice infected with the LCAT-expressing adenovirus as compared to the livers of uninfected or the control AdGFP adenovirus-infected LCAT<sup>-/-</sup> mice ( $p = 0.0001$ ) (Fig. 22G).

In agreement with these data, biochemical determination of hepatic triglyceride content showed that LCAT<sup>-/-</sup> mice treated with the control adenovirus had a triglyceride content of 121.2±5.9 mg of glycerol equivalents per gram of tissue, while LCAT<sup>-/-</sup> mice treated with Ad-LCAT had a significantly lower triglyceride content of 95.1±5.8 mg of glycerol equivalents per gram of tissue ( $p < 0.05$ ). Plasma cholesterol levels of LCAT<sup>-/-</sup> mice treated with AdGFP or Ad-LCAT were 185.5±21.3 mg/dl and 218.6±29.9 mg/dl respectively ( $p > 0.05$ ), while their plasma triglyceride levels were 42.9±3.1 mg/dl and 58.8±10 mg/dl respectively ( $p > 0.05$ ).

Taken together, the findings establish that ectopic expression of LCAT results in a significant reduction of hepatic triglyceride content and a great improvement of hepatic histology and architecture in LCAT<sup>-/-</sup> mice fed western-type diet for 12 weeks.

#### **A2.4 Discussion on the role of the HDL metabolic pathway in diet-induced obesity and related metabolic disorders**

To this date, the vast majority of the published studies have focused almost exclusively on the effects of apoA-I and LCAT expression on plasma lipid levels and atherosclerosis, while the involvement of apoA-I or LCAT in obesity, diabetes and hepatic triglyceride deposition and NAFLD has not been investigated thus far. To address this question, in the context of this FP7 research project we tested the sensitivity of apoA-I<sup>-/-</sup> and LCAT<sup>-/-</sup> mice towards diet-induced hepatic lipid deposition and NAFLD.

Histological evaluation of liver samples from C57BL/6 and apoA-I<sup>-/-</sup> mice fed western-type diet for 24 weeks revealed increased levels of steatosis in the apoA-I<sup>-/-</sup> mice. Biochemical analyses showed that apoA-I<sup>-/-</sup> mice had a much higher hepatic triglyceride content and significantly reduced hepatic cholesterol content than C57BL/6 mice, suggesting that the increased steatosis of the apoA-I<sup>-/-</sup> mice was due to hepatic accumulation of triglycerides. In an attempt to provide a mechanistic interpretation to our histological findings, we measured the mRNA expression levels of key lipogenic enzymes such as FASN-1, DGAT-1 and PPAR $\gamma$ . This analysis did not reveal significant differences between apoA-I<sup>-/-</sup> and C57BL/6 mice in the expression of these lipogenic markers. Since PPAR $\gamma$  is the master regulator of lipogenesis we also determined hepatic PPAR $\gamma$  protein expression levels. Again, no significant difference in PPAR $\gamma$  protein levels was obtained between the two mouse groups. The findings suggest that the increased deposition of triglycerides in the livers of apoA-I<sup>-/-</sup> mice is not due to increased *de novo* biogenesis of fatty acids and triglycerides in these mice. Similarly, indirect calorimetry analysis did not reveal any significant differences in the energy expenditure between the two mouse groups, suggesting that the observed phenotypic differences cannot be explained by differences in the EE of the mice. Taken together, these results suggest that the effects of apoA-I deficiency on hepatic triglyceride deposition may be mediated by its functions as modulator of lipoprotein metabolism in plasma.

Therefore we performed kinetic analyses which indicated that deficiency in apoA-I enhances intestinal absorption and secretion of dietary triglycerides in the plasma while it promotes a more efficient catabolism of post-prandial triglyceride-rich lipoproteins (TRLs) as determined by the rate of clearance of post-prandial triglycerides from the circulation (Figure 5A-D). It is possible that apoA-I, as a structural component of intestinally secreted chylomicrons, modulates their rate of assembly and subsequent secretion in the circulation. Likewise, since excess apoA-I is a known inhibitor of lipoprotein lipase (LpL) (12), it is possible that deficiency in apoA-I accelerates LpL mediated lipolysis of chylomicrons, thus promoting their catabolism. These possibilities warrant further investigation in future studies.

Our results indicate that mice deficient in apoA-I had significantly reduced hepatic secretion of triglyceride-rich VLDL than the control group. In line with our data, in a recent report Gambino and coworkers (13) identified a single nucleotide polymorphism in microsomal triglyceride transfer protein (MTTP) which is associated with increased NAFLD and reduced apoA-I and HDL cholesterol levels in carriers of this polymorphism. It would be very interesting to determine in the future if this polymorphism reduces MTTP activity.

Surprisingly, in our experimental setup apoA-I<sup>-/-</sup> mice had higher body fat content than control C57BL/6 mice, though the average body weights of the two groups were similar during the course of the experiment. Histological analysis of visceral adipocytes did not reveal any size difference between the two groups (not shown). Apparently, the slightly reduced food consumption of the apoA-I<sup>-/-</sup> mice is counteracted by an enhanced intestinal absorption and postprandial lipid deposition in these mice, eventually leading to higher hepatic lipid content and fat tissue hypertrophy.

As a proof of principle for the potential therapeutic role of apoA-I in NAFLD, ectopic expression of apoA-I<sub>Milano</sub> resulted in significantly reduced triglyceride content in the livers of these mice and in an improved hepatic histology than mice infected with the control AdGFP adenovirus or the non-infected mice. We selected to administer apoA-I<sub>Milano</sub> due its improved biological properties compared to wild-type apoA-I (14–16). The inclusion of the AdGFP-infected group was necessary in order to correct for any non-specific effects due to the infection process. Interestingly, only 10 days of apoA-I<sub>Milano</sub> expression were sufficient to improve the histopathological condition of the livers of these mice. We hypothesize that the natural ability of the liver to regenerate could be a factor in the improvement of hepatic histology following ectopic expression of apoA-I<sub>Milano</sub>.

Our data also show LCAT deficiency also promotes deposition of dietary triglycerides in the liver and results in significant histological and architectural alteration associated with NAFLD in response to feeding

western-type diet (17). Previous studies in mice (18) and in humans (19, 20) indicated that apoA-I is a strong activator of LCAT activity in plasma, while deficiency in apoA-I or mutations that affect its function result in a significant reduction in LCAT activity (21–25). In particular, it is well established in the literature that the apoA-I deficient mice that we used in our studies (26) have significantly reduced plasma LCAT activity (20-25% of their wild-type C57BL/6 counterparts) (25). Thus, it is possible that the enhanced deposition of hepatic triglycerides and the deteriorated hepatic pathology and architecture observed in the apoA-I deficient mice in response to high-fat diet could be due, at least in part, to the reduced LCAT activity of these mice.

In a recent report (27), it was found that in H-ras12V transgenic mice with steatosis, apolipoprotein A-I was elevated and accumulated around fatty vacuoles, leading these investigators to propose that apoA-I promotes steatosis. Our data allow for an alternative interpretation of those results, suggesting that the increased apoA-I presence around fat vacuoles may reflect rather a protective mechanism aiming at reducing the already elevated hepatic lipid content of H-ras12V transgenic mice.

Although obesity, and NAFLD and reduced HDL cholesterol levels coexist in individuals with metabolic syndrome (28–32), no clear mechanistic link between these two conditions has been established in the literature, thus far. Since expression of apoA-I is absolutely essential for the formation of HDL while LCAT is essential for the maturation of discoidal HDL into spherical, our data establish that the HDL metabolic pathway is a central contributor to the deposition of dietary triglycerides to the liver and to the development of NAFLD and other related metabolic dysfunctions associated with metabolic syndrome. Our data further support that the coexistence of reduced HDL levels and NAFLD in an individual with metabolic syndrome may not be a mere coincidence, rather it underlays a strong causative relationship between these two conditions. This observation extends the role of HDL beyond atherosclerosis, to the development of NAFLD, a pathological component found in patients with metabolic syndrome. Importantly, our findings raise the interesting possibility that expression of beneficial forms of apoA-I or LCAT by gene therapy approaches may find therapeutic applications for the treatment of NAFLD in the future.

**A3. Perturbations in the HDL metabolic pathway predispose to the development of osteoarthritis in mice following long-term exposure to Western-type diet (Osteoarthritis Cartilage. 2012 Nov 11. doi:pii: S1063-4584(12)01016-3. 10.1016/j.joca.2012.11.003).**

As a side project to the proposed work we came across the study of the role of HDL in the pathogenesis of osteoarthritis in mice. Since we had available material from the experimental mice used for the main studies, we proceeded to determine how deficiency in LCAT or apoA-I may be affecting the development of osteoarthritis in mice.

Recent data suggest that obesity and related metabolic aberrations are associated with osteoarthritis (OA) development, a phenomenon that is attributed at least in part to the consumption of lipid-rich diets. To date, the molecular mechanisms that govern the lipid-osteoarthritis connection remain largely unknown. Given the important role of high density lipoprotein (HDL) in plasma and tissue lipid metabolism, the main purpose of the present study was to investigate the role of HDL metabolism in the pathobiology of OA. We used apoA-I(-/-) mice that lack classical apoA-I containing HDL, LCAT(-/-) mice that have only immature HDL and relatively reduced HDL-cholesterol levels and control C57BL/6 mice. Mice were placed on chow or Western-Type (WTD) and monitored for 24 weeks. Knee joints were removed and articular cartilage was isolated for further analyses. The LCAT-/- mice were significantly more sensitive to the development of diet-induced obesity compared to the C57BL/6 and apoA-I-/- mice. Morphological, biochemical and molecular analyses revealed that the LCAT-/- obese mice developed OA, while the C57BL/6 mice that were fed WTD did not. Notably, apoA-I-/- mice that received WTD also developed OA although their body weight gain was similar to their wild-type counterparts. Interestingly, bone marrow from LCAT-/- and apoA-I-/- mice contained significantly increased number of adipocytes, compared to the other groups. Our findings suggested that perturbations in HDL metabolism predispose to OA following chronic insult with WTD and raise the challenging possibility that HDL has a causative relation to OA in patients with metabolic syndrome.

## Reference List

1. Gumbiner B et al. (1990) Effects of weight loss and reduced hyperglycemia on the kinetics of insulin secretion in obese non-insulin dependent diabetes mellitus. *J.Clin.Endocrinol.Metab* 70:1594-1602
2. Frederich RC et al. (1995) Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nat.Med.* 1:1311-1314
3. Hall JE, Hildebrandt DA, Kuo J (2001) Obesity hypertension: role of leptin and sympathetic nervous system. *Am.J.Hypertens.* 14:103S-115S
4. Bravo PE, Morse S, Borne DM, Aguilar EA, Reisin E (2006) Leptin and hypertension in obesity. *Vasc.Health Risk Manag.* 2:163-169
5. Kypreos KE, Zannis VI (2006) LDL receptor deficiency or apoE mutations prevent remnant clearance and induce hypertriglyceridemia in mice. *J.Lipid Res.* 47:521-529
6. Karagiannides I, Abdou R, Tzortzopoulou A, Voshol PJ, Kypreos KE (2008) Apolipoprotein E predisposes to obesity and related metabolic dysfunctions in mice. *FEBS J.* 275:4796-4809
7. Hofmann SM et al. (2008) Defective lipid delivery modulates glucose tolerance and metabolic response to diet in apolipoprotein E-deficient mice. *Diabetes* 57:5-12
8. Kuipers F et al. (1997) Impaired secretion of very low density lipoprotein-triglycerides by apolipoprotein E-deficient mouse hepatocytes. *J.Clin.Invest* 100:2915-2922
9. Huang Y et al. (1998) Overexpression and accumulation of apolipoprotein E as a cause of hypertriglyceridemia. *J.Biol.Chem.* 273:26388-26393
10. Tsukamoto K, Maugeais C, Glick JM, Rader DJ (2000) Markedly increased secretion of VLDL triglycerides induced by gene transfer of apolipoprotein E isoforms in apoE-deficient mice. *J.Lipid Res.* 41:253-259
11. Tschop MH et al. (2012) A guide to analysis of mouse energy metabolism. *Nat.Methods* 9:57-63
12. Yamamoto M et al. (2003) Effects of plasma apolipoproteins on lipoprotein lipase-mediated lipolysis of small and large lipid emulsions. *Biochim.Biophys.Acta* 1632:31-39
13. Gambino R, Cassader M, Pagano G, Durazzo M, Musso G (2007) Polymorphism in microsomal triglyceride transfer protein: a link

between liver disease and atherogenic postprandial lipid profile in NASH? *Hepatology* 45:1097-1107

14. Nissen SE et al. (2003) Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA* 290:2292-2300
15. Shah PK et al. (1998) Effects of recombinant apolipoprotein A-I (Milano) on aortic atherosclerosis in apolipoprotein E-deficient mice. *Circulation* 97:780-785
16. Ameli S et al. (1994) Recombinant apolipoprotein A-I Milano reduces intimal thickening after balloon injury in hypercholesterolemic rabbits. *Circulation* 90:1935-1941
17. Karavia EA, Papachristou DJ, Kotsikogianni I, Triantafyllidou I-E, Kypreos KE (2012) Lecithin:cholesterol acyltransferase modulates diet-induced hepatic deposition of triglycerides in mice. *J.Nutr.Biochem.* DOI: 10.1016/j.jnutbio.2012.02.007:
18. Plump AS et al. (1997) ApoA-I knockout mice: characterization of HDL metabolism in homozygotes and identification of a post-RNA mechanism of apoA-I up-regulation in heterozygotes  
1. *J.Lipid Res.* 38:1033-1047
19. Soutar AK et al. (1975) Effect of the human plasma apolipoproteins and phosphatidylcholine acyl donor on the activity of lecithin:cholesterol acyltransferase. *Biochemistry* 14:3057-3064
20. Santos RD et al. (2008) Characterization of high density lipoprotein particles in familial apolipoprotein A-I deficiency. *J Lipid Res.* 49:349-357
21. Utermann G et al. (1984) Apolipoprotein A-I Giessen (Pro143----Arg). A mutant that is defective in activating lecithin:cholesterol acyltransferase. *Eur.J.Biochem.* 144:325-331
22. Rall SC, Jr. et al. (1984) Abnormal lecithin:cholesterol acyltransferase activation by a human apolipoprotein A-I variant in which a single lysine residue is deleted. *J.Biol.Chem.* 259:10063-10070
23. Martin-Campos JM et al. (2002) ApoA-I (MALLORCA) impairs LCAT activation and induces dominant familial hypoalphalipoproteinemia  
1. *J.Lipid Res.* 43:115-123
24. Funke H et al. (1991) A frameshift mutation in the human apolipoprotein A-I gene causes high density lipoprotein deficiency, partial lecithin:cholesterol-acyltransferase deficiency, and corneal opacities. *J.Clin.Invest* 87:371-376
25. Parks JS, Li H, Gebre AK, Smith TL, Maeda N (1995) Effect of apolipoprotein A-I deficiency on lecithin:cholesterol acyltransferase activation in mouse plasma. *J.Lipid Res.* 36:349-355

26. Williamson R, Lee D, Hagaman J, Maeda N (1992) Marked reduction of high density lipoprotein cholesterol in mice genetically modified to lack apolipoprotein A-I. *Proc.Natl.Acad.Sci.U.S.A* 89:7134-7138
27. Wang AG et al. (2011) Steatosis induced by the accumulation of apolipoprotein A-I and elevated ROS levels in H-ras12V transgenic mice contributes to hepatic lesions. *Biochem.Biophys.Res.Commun.* 409:532-538
28. Preiss D, Sattar N (2008) Non-alcoholic fatty liver disease: an overview of prevalence, diagnosis, pathogenesis and treatment considerations. *Clin.Sci.(Lond)* 115:141-150
29. Angulo P (2002) Nonalcoholic fatty liver disease. *N.Engl.J.Med.* 346:1221-1231
30. Hamaguchi M et al. (2005) The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. *Ann.Intern.Med.* 143:722-728
31. Fan JG et al. (2005) Fatty liver and the metabolic syndrome among Shanghai adults. *J.Gastroenterol.Hepatol.* 20:1825-1832
32. Browning JD et al. (2004) Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 40:1387-1395