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Human apoB contributes to increased serum total apo(a) level in *LPA* transgenic mice

Päivi A Teivainen*[1,2], Knut A Eliassen[3], Edward M Rubin[4], Srdjan Djurovic[1,2] and Kåre Berg[1,2]

Address: [1]Institute of Medical Genetics, University of Oslo, Oslo, Norway, [2]Department of Medical Genetics, Ullevål University Hospital, Oslo, Norway, [3]Department of Basic Sciences and Aquatic Medicine, The Norwegian School of Veterinary Science, Oslo, Norway and [4]Genome Sciences Department, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

Email: Päivi A Teivainen* - p.a.teivainen@ioks.uio.no; Knut A Eliassen - knut.eliassen@veths.no; Edward M Rubin - emrubin@lbl.gov; Srdjan Djurovic - srdjan.djurovic@ioks.uio.no; Kåre Berg - k.berg@genova.no

* Corresponding author

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Abstract

Background: The Lp(a) lipoprotein (Lp(a)) consists of the polymorphic glycoprotein apolipoprotein(a) (apo(a)), which is attached by a disulfide bond to apolipoprotein B (apoB). Apo(a), which has high homology with plasminogen, is present only in primates and hedgehogs. However, transgenic mice and rabbits with high serum apo(a) levels exist. Liver is the main site for apo(a) synthesis, but the site of removal is uncertain. To examine differences between transgenic mice expressing the *LPA* gene and mice capable of forming Lp(a) particles, *LPA*-YAC transgenic mice and *hAPOB* transgenic mice were crossed and their offspring examined.

Results: Comparison of *LPA*-YAC with *LPA*-YAC/*hAPOB* transgenic mice showed that *LPA*-YAC/*hAPOB* transgenic mice have higher serum total apo(a) and total cholesterol level than mice lacking the *hAPOB* gene. However, hepatic apo(a) mRNA level was higher in *LPA*-YAC transgenic mice than in *LPA*-YAC/*hAPOB* transgenic mice. Feeding of a high-cholesterol/high-fat diet to male *LPA*-YAC transgenic mice with or without the *hAPOB* gene resulted in reduced serum total apo(a) and hepatic apo(a) mRNA level.

Conclusion: In conclusion, the higher serum total apo(a) level in *LPA*-YAC/*hAPOB* transgenic mice than in *LPA*-YAC transgenic mice is not caused by increased apo(a) synthesis. Lower hepatic apo(a) mRNA level in *LPA*-YAC/*hAPOB* than in *LPA*-YAC transgenic mice may suggest that the increase in total apo(a) level is a result of apo(a) accumulation in serum. Furthermore, observed higher serum total cholesterol level in *LPA*-YAC/*hAPOB* transgenic mice than either in wild type or *LPA*-YAC transgenic mice may further suggest that human *APOB* transgenicity is a factor that contributes to increased serum total apo(a) and cholesterol levels. Our results on reduced serum total apo(a) and hepatic apo(a) mRNA levels in HCHF fed male *LPA*-YAC transgenic mice confirm earlier findings in females, and show that there are no sex difference in mechanisms for lowering apo(a) level in response to HCHF feeding.

Background

Association between Lp(a) lipoprotein (Lp(a) [1], and coronary heart disease was first demonstrated in 1974 [2]. Afterwards, numerous studies have confirmed that high plasma level of Lp(a) is an independent risk factor for atherosclerotic and thrombotic disease [3]. The intact Lp(a) molecule consists of the highly polymorphic glycoprotein apolipoprotein(a) (apo(a)), which is attached through a disulfide bond to apolipoprotein B-100 (apoB-100) of low density lipoprotein (LDL).

Gene encoding for apo(a) (*LPA*), which expression is mainly confined to the liver, is tightly linked to plasminogen (*PLG*) gene on chromosome 6 and is homologous to *PLG* [4,5]. This sequence similarity indicates that the human *LPA* gene evolved from a duplicated *PLG* gene during primate evolution [6,7]. Interestingly, in addition to humans, Old World monkeys, great apes and hedgehogs are the only species, which express *LPA* naturally [8-10].

Varying numbers of KIV structures account for the size variation of apo(a) whose size is inversely related to plasma Lp(a) level [11]. Furthermore, recent studies in transgenic animal models have shown that 50% of human Lp(a) is catabolized by the liver [12,13], but the mechanism for uptake is unclear. Study of WHHL transgenic rabbits expressing human apo(a) showed that defect LDL receptor (LDLR) lead to marked accumulation of plasma Lp(a) [14]. However, despite structural similarity of Lp(a) to LDL, the LDLR does not seem to be involved significantly in Lp(a) removal in humans [15-17].

Studies in female mice transgenic for *LPA*-yeast artificial chromosome (*LPA*-YAC), which contains a 40 kilobase (kb) *LPA-PLG* intergenic region [18], have shown that feeding of a high-fat diet reduces serum apo(a) and hepatic apo(a) mRNA level [19,20]. In contrast, serum apo(a) levels in mice transgenic for human apo(a) cDNA are stable despite high-fat feeding [21], which suggests that the effect of fat-rich diet on apo(a) levels in *LPA*-YAC transgenic mice is most likely exerted through regulatory elements located within the *LPA-PLG* intergenic region.

Recent studies on PPARα suggest that PPARα is of major importance for regulation of serum levels of apoB and production of apoB-containing lipoproteins [22,23], but the possible role of PPARα in determining Lp(a) serum level is unclear. The DNase I hypersensitive site (DH) II enhancer part of the *LPA-PLG* intergenic region contains the gene for peroxisome proliferators-activated receptor (PPAR) α binding site [24,25]. DH II increases the activity of the *LPA* promoter by over 7-fold in reporter-gene assays in HepG2 cells *in vitro* [26]. This together with recent co-transfection assays, which showed that PPARα increased the effect of the DHII enhancer on *LPA* transcriptional activity may indicate that PPARα plays a role also in determining plasma Lp(a) level [26].

In order to examine differences between transgenic mice only expressing the *LPA* gene and transgenic mice capable of forming Lp(a) particles, *LPA*-YAC transgenic and mice transgenic for the human *APOB (hAPOB)* gene were crossed and their offspring examined. The study revealed that serum total apo(a) levels in *LPA*-YAC trangenic mice also expressing the *hAPOB* gene were higher than in *LPA*-YAC transgenic animals lacking *hAPOB*. However, hepatic apo(a) mRNA level in *LPA*-YAC transgenic mice was higher than in *LPA*-YAC/*hAPOB* transgenic mice. Furthermore, we showed that feeding of a high-cholesterol/high-fat (HCHF) diet to male *LPA*-YAC transgenic mice, which on a standard mouse diet exhibit much lower serum total apo(a) level than female mice, reduced serum total apo(a) and hepatic apo(a) mRNA level in a similar manner as previously shown in female mice [19,20]. The lack of significant variation in hepatic PPARα mRNA levels between wild type (WT) and *LPA*-YAC transgenic mice or between mice fed either a semi-synthetic mouse diet (AIN-76) or the HCHF diet, may indicate that neither *LPA* transgenicity nor high-fat feeding have a significant impact on expression of PPARα.

Results

Serum apo(a) level

On a regular RMI(E)SQC mouse diet serum total apo(a) level was significantly higher in *LPA*-YAC/*hAPOB* transgenic mice than in *LPA*-YAC transgenic mice ($P < 0.05$, figure 1) and serum total apo(a) levels were higher in female transgenic mice than in respective male transgenic mice ($P < 0.005$, figure 1).

Male *LPA*-YAC transgenic mice (n= 4) had a mean serum total apo(a) level of 7.6 ± 2.5 mg/dL at the start and of 6.4 ± 3.9 mg/dL at the end of feeding the AIN-76 diet for 7 weeks. Corresponding levels in *LPA*-YAC/*hAPOB* transgenic mice (n= 6) were 9.2 ± 1.0 mg/dL and 9.5 ± 2.2 mg/dL, respectively. Serum total apo(a) level in male *LPA*-YAC/*hAPOB* transgenic mice was significantly reduced ($P < 0.001$, figure 2) after two weeks of HCHF feeding and the level remained low during 7 weeks of feeding. Feeding of the HCHF diet to male *LPA*-YAC transgenic mice resulted in reduced mean serum total apo(a) level from 2.9 ± 1.3 mg/dL to below background level, but because of the small number the difference did not reach statistical significance ($P= 0.08$).

Serum total cholesterol and triglycerides in male mice

LPA-YAC transgenic mice had similar serum total cholesterol (TC) and serum triglycerides (TG) levels as WT mice, independently of the diets (table 1). *LPA*-YAC/*hAPOB* transgenic mice fed the AIN-76 diet had higher serum TG

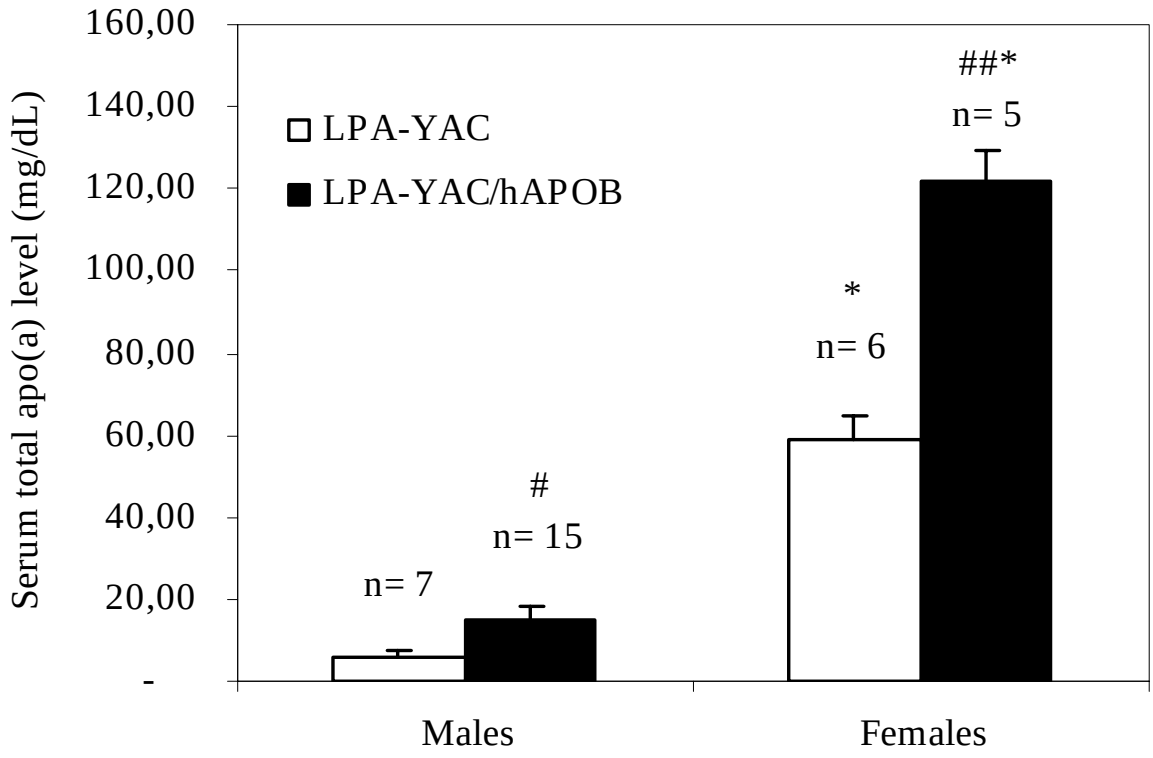

Figure 1
Serum total apo(a) level in male and female *LPA*-YAC and *LPA*-YAC/*hAPOB* transgenic mice on a regular RMI(E)SQC mouse diet. Values shown are mean ± SEM (mg/dL). Statistically significant difference between *LPA*-YAC and *LPA*-YAC/*hAPOB* transgenic mice is indicated by # ($P < 0.05$) or by ## ($P < 0.01$). Statistically significant difference between respective male and female mice is indicated by * ($P < 0.005$).

level than either WT or *LPA*-YAC transgenic mice ($P < 0.05$). The HCHF fed *LPA*-YAC/*hAPOB* transgenic mice had significantly higher serum TC level than the AIN-76 fed mice ($P < 0.005$). In comparison, feeding of the HCHF diet to WT or *LPA*-YAC transgenic mice had no significant effect on serum TC levels compared to the AIN-76 fed mice. Serum TG levels were lower in the HCHF fed *LPA*-YAC and *LPA*-YAC/*hAPOB* transgenic mice compared to the AIN-76 fed transgenic mice ($P < 0.05$ and $P < 0.005$ respectively, for the two categories of mice). The comparable difference on serum TG level between the AIN-76 and the HCHF fed WT did not reach statistical significance.

Apo(a), PPARα and LDLR mRNA levels in male mice

Apo(a) mRNA was mainly detected in liver of *LPA*-YAC transgenic mice and to a very low degree in kidneys in 3 out of 4 mice (table 2). Apo(a) mRNA was not detected in spleen, heart, brain and intestine.

On the AIN-76 diet hepatic apo(a) mRNA levels were lower in *LPA*-YAC/*hAPOB* than in *LPA*-YAC transgenic mice ($P< 0.05$, figure 3). Furthermore, feeding of the HCHF diet to transgenic mice reduced hepatic apo(a) mRNA level significantly ($P<0.05$) in both strains.

Hepatic PPARα mRNA levels did not vary significantly between WT and transgenic mice, or between the AIN-76

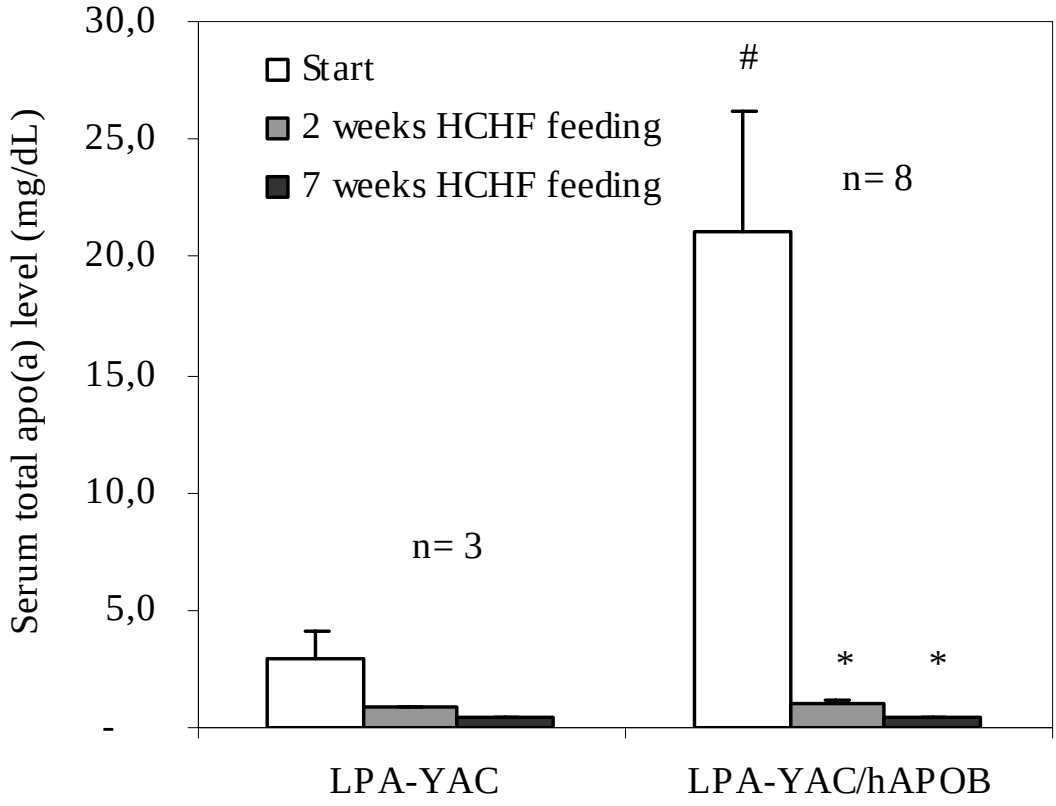

Figure 2
Effect of 7 weeks HCHF feeding on serum total apo(a) level in male *LPA*-YAC and *LPA*-YAC/*hAPOB* transgenic mice. Mice were fed the HCHF diet for 7 weeks. Mice were bled at the start, after 2 weeks of feeding and when the animals were sacrificed. Values shown are mean ± SEM (mg/dL) of serum total apo(a) level. According to manufacturer, values <1.2 mg/dL reflect background noise. The * indicates statistically significant difference ($P < 0.001$) in comparison to the start level. Statistically significant difference ($P < 0.05$) between *LPA*-YAC and *LPA*-YAC/*hAPOB* transgenic mice is indicated by #.

fed and the HCHF fed mice of the same category (figure 4).

Hepatic LDLR mRNA level did not vary significantly between the AIN-76 fed WT, *LPA*-YAC and *LPA*-YAC/*hAPOB* transgenic mice, or between the HCHF fed and the AIN-76 fed *LPA*-YAC transgenic mice (figure 5). HCHF feeding reduced LDLR mRNA levels in WT and *LPA*-YAC/*hAPOB* transgenic mice, compared to the levels in the AIN-76 fed mice ($P < 0.05$).

Discussion

Observation of higher serum levels of total apo(a) (figure 1 & 2) in *LPA*-YAC/*hAPOB* transgenic mice than in *LPA*-YAC transgenic mice are in agreement with the results of Callow *et al* [27]. Our finding of lower hepatic apo(a) mRNA levels (figure 3) in *LPA*-YAC/*hAPOB* transgenic mice than in mice having the *LPA* gene only, suggest that the elevated serum Lp(a) levels in *LPA*-YAC/*hAPOB* transgenic mice are not due to increased hepatic apo(a) mRNA transcription rate. Furthermore, analysis of *LPA* expression in different tissues (table 2) indicates that no other organs than the liver, can produce significant amounts of apo(a).

LDL can be removed from the circulation by the LDL receptor (LDLR), which binds to the apoB-100 part of LDL [28]. The efficient assembly of Lp(a) in mice transgenic for

Table 1: Serum triglycerides (TG) and serum total cholesterol (TC) levels in male WT, *LPA*-YAC transgenic and *LPA*-YAC/*hAPOB* transgenic mice

	AIN-76 diet		HCHF diet	
Mice	n	TG (mmol/L)	n	TG (mmol/L)
WT	5	1.8 ± 0.3	5	1.2 ± 0.3
LPA-YAC	4	1.9 ± 0.2	3	[2)]1.1 ± 0.2
LPA-YAC/*hAPOB*	6	[1)]3.1 ± 0.2	8	[2)]1.4 ± 0.1
Mice	n	TC (mmol/L)	n	TC (mmol/L)
WT	5	5.2 ± 0.4	5	6.3 ± 0.2
LPA-YAC	4	5.3 ± 0.2	3	5.7 ± 0.2
LPA-YAC/*hAPOB*	6	6.0 ± 0.3	8	[3)]15.0 ± 0.9

Concentrations are presented as mean ± SEM (mmol/L). 1): Statistically significant difference ($P < 0.05$) in serum TG levels as compared to the AIN-76 fed WT or *LPA*-YAC transgenic mice. 2): Statistically significant difference in serum TG levels as compared to the respective AIN-76 fed mice ($P < 0.05$ for *LPA*-YAC, $P < 0.005$ for *LPA*-YAC/*hAPOB*). 3): Statistically significant difference ($P < 0.005$) in serum TC levels as compared to the AIN-76 fed *LPA*-YAC/*hAPOB* transgenic mice.

Table 2: *LPA* expression level in different tissues of 1-year old male *LPA*-YAC transgenic mice on a regular RMI(E)SQC mouse diet

		LPA expression					
Mice	n	Liver	Spleen	Heart	Brain	Intestine	Kidney
LPA-YAC	4	High	-	-	-	-	Low
WT	2	-	-	-	-	-	-

both *LPA* and *hAPOB* and higher efficiency of Lp(a) assembly with the human apoB-100 than with mouse apoB-100 [29,30] may suggest that measured total apo(a) level in *LPA*-YAC/*hAPOB* transgenic mice consist mainly of human Lp(a) particles.

Because mouse LDLR has low capacity to bind human apoB-100 containing particles [31], elevated serum total apo(a) level may theoretically be secondary effect of reduced clearance rate of human apoB containing particles via the LDLR dependent pathway in *LPA*-YAC/*hAPOB* transgenic mice. Support for this comes from study, which showed that overexpression of LDLR in mice leads to accelerated metabolism of injected human Lp(a) particles [32]. Furthermore, it is notable that serum Lp(a) levels in LDLR$^{-/-}$/*LPA* transgenic mice are similar whether or not the human *APOB* gene is also present [33]. Since triglyceride-rich apoB particles have increased affinity to apo(a) in man [34,35], it seems likely that the human apoB part of the Lp(a) molecule in mouse serum may associate with TG containing particles, which may explain why *hAPOB* transgenicity also contributes to increased serum TG levels.

Suggestion for a role of LDLR in Lp(a) clearance comes also from studies in rabbits, where apoB, in contrast to mice, associates with human apo(a) forming Lp(a) particles [36]. A defect in LDLR in *LPA* transgenic WHHL rabbits is accompanied by accumulation of Lp(a) in plasma [14]. Since mice or rabbits do not normally have apo(a), the mechanism of Lp(a) removal in these animals may well differ from that in man, where the LDLR does not seem to affect Lp(a) metabolism [15-17].

The fall in serum apo(a) and hepatic apo(a) mRNA levels (figure 2 and 3) in male transgenic mice on the HCHF diet agrees with observations by Acquati *et al* and Huby *et al*, who showed that hepatic apo(a) mRNA and serum apo(a) levels fell in female *LPA* transgenic mice after two weeks fat feeding [19,20]. Man, who naturally express *LPA*, do not have significant variation in plasma Lp(a) levels despite of changes in diet [37]. This indicates that man possesses *LPA* control mechanisms that are different from

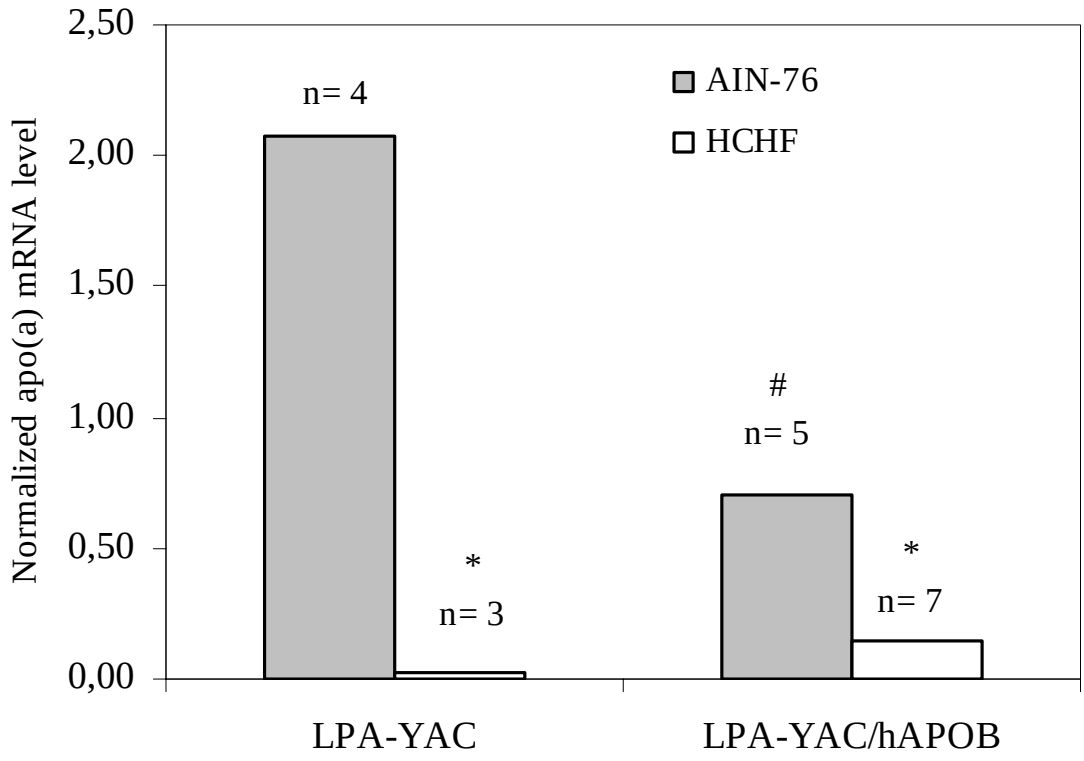

Figure 3
Median of normalized hepatic apo(a) mRNA level in male *LPA*-YAC and *LPA*-YAC/*hAPOB* transgenic mice fed the AIN-76 diet or the HCHF diet for 7 weeks. The * indicates statistically significant difference ($P < 0.05$) in comparison to the level in the AIN-76 fed mice. Statistically significant difference ($P < 0.05$) between *LPA*-YAC and *LPA*-YAC*hAPOB* transgenic mice is indicated by #.

those in mice. This agrees with the fact that CYP7A1, which regulates the initial step in the pathway of cholesterol metabolism and bile acid synthesis, is increased during cholesterol feeding in rats and mice, but not in humans [38]. Rodents' capacity to convert cholesterol into bile acids by liver X receptor-α mediated stimulation of CYP7A1 transcription has been suggested to account for some of the differences in response to fat intake between humans and rodents [39]. Furthermore, PPARα, which binds to the DHII enhancer part of *LPA-PLG* intergenic region [24], and is of importance for production of apoB and apoB-containing lipoproteins [22], was recently shown to be regulated by bile acids in humans but not in rodents [40]. Since cholesterol feeding promotes bile acid synthesis in rodents [41], our results showing similar hepatic PPARα mRNA levels between the AIN-76 and HCHF fed mice may give further support to the notion that bile acids do not increase PPARα mRNA levels in mice. Further studies will be needed to elucidate whether or not the species-specific differences in cholesterol and bile acid metabolism is a key to understanding the mechanism(s), which cause the reduction on apo(a) transcription in HCHF fed transgenic mice.

The reduced hepatic LDLR mRNA level seen in HCHF fed WT mice and *LPA*-YAC/*hAPOB* transgenic mice (figure 5) is in agreement with previous results, which showed that hepatic LDLR level in mice is reduced in response to HCHF feeding [42]. This may suggest that the human apoB can interact sufficiently, although less than mouse

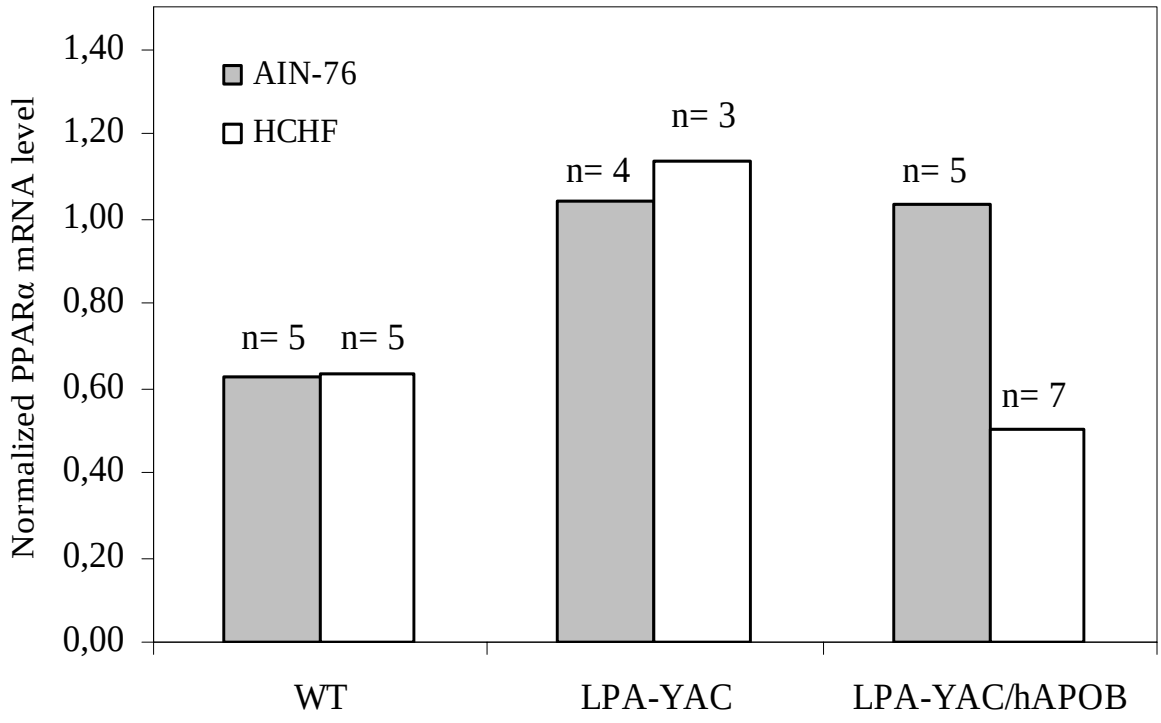

Figure 4
Median of normalized hepatic PPARα mRNA level in male WT, *LPA*-YAC and *LPA*-YAC/*hAPOB* transgenic mice fed the AIN-76 or the HCHF diet for 7 weeks.

apoB, with mouse LDLR to increase intracellular level of cholesterol and down regulate expression of the LDLR. Because of the small number of HCHF fed *LPA*-YAC transgenic mice in our study, the absence of an effect of feeding on LDLR mRNA level must be confirmed in further studies.

On the HCHF diet both WT mice and transgenic mice developed fatty livers (not shown). Since hepatic level either of housekeeping genes or PPARα mRNA was not reduced in response to the HCHF feeding, it seems clear that the decreased apo(a) mRNA level detected in the livers of HCHF fed transgenic mice was not due to a general decrease in liver function.

Conclusions

In conclusion, we have shown that serum total apo(a) level is higher in *LPA*-YAC/*hAPOB* transgenic mice than in mice that are transgenic for the *LPA* gene alone. The higher level is not caused by increased apo(a) synthesis. Lower hepatic apo(a) mRNA level in *LPA*-YAC/*hAPOB* than in *LPA*-YAC transgenic mice may suggest that the increase in total apo(a) level is a result of apo(a) accumulation in serum. Our results on reduced serum total apo(a) and hepatic apo(a) mRNA level in HCHF fed male *LPA*-YAC transgenic mice confirm earlier findings in females, and show that there are no sex difference in mechanisms for lowering apo(a) level in response to HCHF feeding.

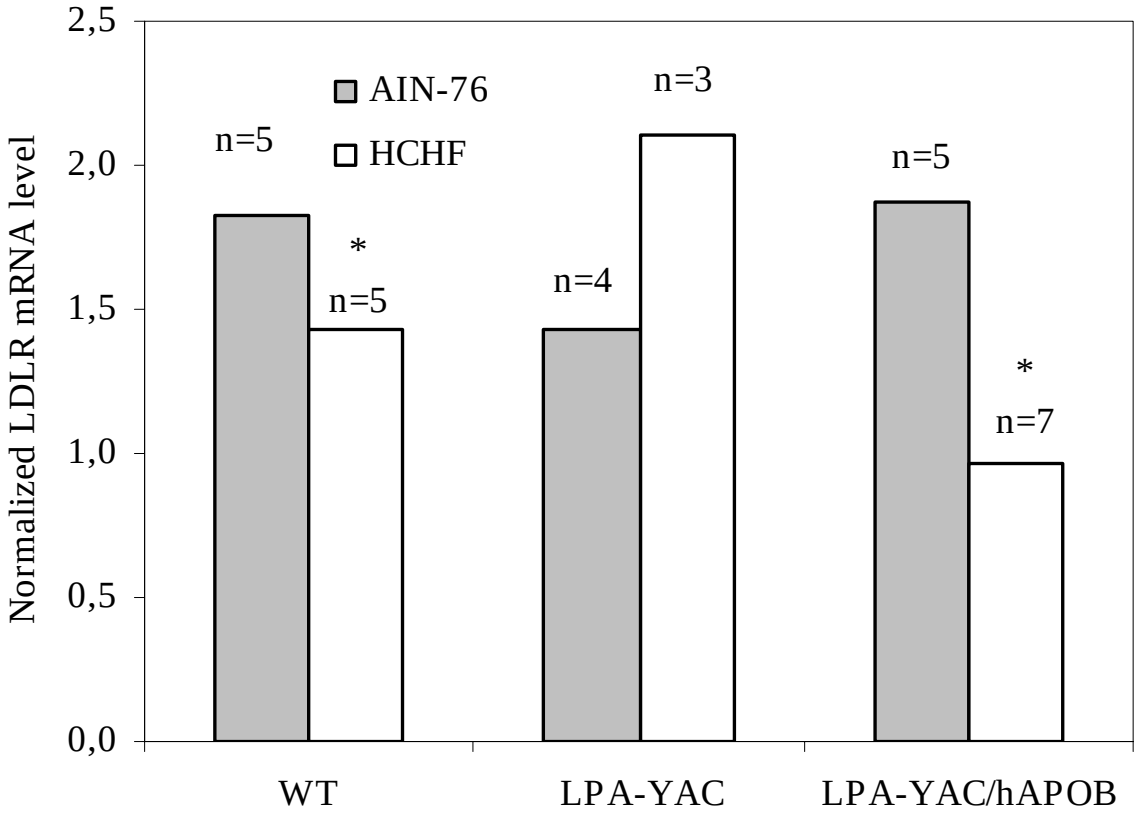

Figure 5
Median of normalized hepatic LDLR mRNA level in male WT, *LPA*-YAC or *LPA*-YAC/*hAPOB* transgenic mice fed the AIN-76 or the HCHF diet for 7 weeks. Statistically significant difference ($P < 0.05$) between the AIN-76 fed and the HCHF fed mice is indicated by *.

Materials and methods

Mice

Mice possessing a 270 kb YAC with a human genomic DNA clone containing the intact *LPA* gene and a 70 kb *LPA*-like gene with extensive 60 kb flanking regions on both sides and mice possessing the intact *hAPOB* gene in FVB genetic background have been described previously [18,27]. Non-transgenic FVB mice were purchased from Harland (Bicester, England). For experiments in Oslo, mice were bred at The Laboratory Animal Unit, which is fully accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care and Use), at The Norwegian School for Veterinary Science. The Norwegian Animal Research Authority approved the experiments and all animal experiments were performed in accordance with the Norwegian Gene Technology Act of 1994 and the European Community Directive of 24 November 1986.

To develop mice capable of forming Lp(a) particles, *LPA*-YAC transgenic mice and *hAPOB* transgenic mice were crossed. Sentinel animals were used to run a full felasa-style health-monitoring scheme and mice were found to be healthy.

The presence of the *LPA* gene and the *hAPOB* gene was demonstrated by PCR analysis [43]. For the analysis of *hAPOB* transgenicity sense primer TGGAAACGGA-GAAATTATGGA and antisense primer CACTTGGCAAAT-ACAATTCCTG were used.

Mice were housed in a room with 12 h light/dark cycle and 55% relative humidity at 21°C. Mice were kept on the regular RMI(E)SQC mouse diet (Special Diet Services, Witham, Essex, England) until the start of the feeding experiment.

Four 1-year old male *LPA*-YAC transgenic mice and two 1-year old male wild type mice were sacrificed for the study of *LPA* expression pattern. For the study of serum total apo(a) level prior to the feeding experiment, six female and seven male *LPA-YAC* transgenic as well as five female and fifteen male *LPA-YAC/hAPOB* transgenic mice were bled.

Feeding experiment

Ten male WT, seven *LPA*-YAC transgenic and fourteen *LPA*-YAC/*hAPOB* transgenic mice were divided into groups and fed with a semi-synthetic mouse diet (AIN-76, ICN, Asse-Relegem, Belgium) or a high-cholesterol/high-fat diet (Custom high-fat diet, ICN, Asse-Relegem, Belgium) for seven weeks (table 3). The HCHF diet contained 1.25% cholesterol, 18.4% regular butter, corresponding to approximately 9% saturated fat, and 1% corn oil, while the AIN-76 diet contained 5% corn oil. Corn oil contains approximately 1.7% saturated fat. Water and food were given ad libitum. In addition to the blood sampling before feeding, blood samples from the saphenous vein were drawn after two weeks feeding and when the animals were sacrificed after seven weeks feeding.

Table 3: Age at the start and number of mice fed different diets for 7 weeks

Mice	Age (weeks)	AIN-76 diet	HCHF diet
WT	44–47	5	5
LPA-YAC	49–52	4	3
LPA-YAC/*hAPOB*	49–52	6	8

Analysis of serum total apo(a), total cholesterol and triglycerides level

Serum total apo(a) level was measured using a radioimmuno assay (RIA) kit (Mercodia AB, Uppsala, Sweden). This method is based on direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants of apo(a). Results are given in units per liter (U/L). According to the manufacturer, results must be multiplied by 0.07 to obtain values in mg/dL, and levels below 1.2 mg/dL (17 U/L) are considered as background noise. For details of test's reliability to measure serum apo(a) level see Berg *et al* [43].

Serum TC and TG level was measured using enzymatic, colorimetric methods (Modular Analytics, Roche Diagnostics, F-Hoffmann-La Roche Ltd, Basel, Switzerland) at Fürst AS, Oslo, Norway.

Tissue preparation for mRNA analysis

For RNA preparations, tissue samples were washed in ice-cold saline, submerged in RNAlater™ buffer (Ambion Diagnostics, Cambridgeshire, England) and stored at -70°C until analysis.

Quantitative PCR

Level of mRNA was determined by quantitative PCR (qPCR), which was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers and probes (table 4) were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA).

Total RNA from 100–300 mg of tissues was isolated after homogenisation in 1 ml Trizol reagent according to the manufacturer's recommendations (Invitrogen Co, Carlsbad, CA, USA). RNA was treated with Dnase I (Ambion Ltd, Cambridgeshire, England) and 5 μg of total RNA was reverse-transcribed using $pd(N)_6$ random primers (First strand cDNA synthesis kit, Amersham Biosciences, Little Chalfont Buckinghamshire, England).

Table 4: Primer and probe sequences for cyclophilin, β-actin, *LPA*, PPARα and LDLR genes used in qPCR

Gene	Forward primer	Reverse primer	Probe
Cyclophilin	TGGAGAGCACCAAGACAGACA	TGCCGGAGTCGACAATGA	CGGGACAAGCCACTGAAGGATGT
β-actin	ACGAGGCCCAGAGCAAGAG	CGTCCCAGTTGGTAACAATGC	ATGTTCAATGGGGTACTTCAGGGTCAGGA
LPA	TTGTCTGCCATCCCCAGACTAC	AGGCCAGTC CCAAAGGTACCT	TGGTCACCGCCAGGACTGAATGTTACA
PPARα	TGAGATGGCAGGCCGTTAC	AAATCCCTGCTCTCCTGTATGG	ACTGTTCAGGGACCTCCGAGGCCT
LDLR	GCTCCATAGGCTATCTGCTCTTCA	CTGCGGTCCAGGGTCATC	CAACCGCCACGAGGTCCGGA

Table 5: Variation in A) β-actin and B) cyclophilin mRNA levels in five mouse livers submitted to qPCR. Calculations of variations were based on measured Ct value at the same threshold level.

A) β-actin	Within-run (SD)	Between-run (SD)	CV%	No. of observations
Mouse 1	0.02	0.17	0.78	9
Mouse 2	0.11	0.27	1.25	9
Mouse 3	0.09	0.36	1.63	9
Mouse 4	0.09	0.29	1.31	9
Mouse 5	0.23	0.27	1.24	9
All samples	0.22	0.36	1.66	45
B) Cyclophilin	Within-run (SD)	Between-run (SD)	CV%	No. of observations
Mouse 1	0.25	0.18	0.76	6
Mouse 2	0.02	0.06	1.24	6
Mouse 3	0.43	0.32	1.27	6
Mouse 4	0.06	0.26	1.06	6
Mouse 5	0.23	0.17	0.69	6
All samples	0.25	0.27	1.11	30

qPCR was performed using the TaqMan® Universal Master mix (Applied Biosystems, Foster City, CA, USA) containing 2 μl cDNA, 300 nM of each primer and probe in a 25 μl final volume. To evaluate the qPCR performances, cDNA samples, made as described above, from liver of five ten week's old WT male mice were analyzed for β-actin and cyclophilin mRNA levels. We run triplicates of 1:100 diluted cDNA samples for each mouse. Variance of Ct values at the same threshold for within-run and between-runs is presented in table 5. Analysis of genes of interest was carried out in a duplicate and relative mRNA level was determined by the Standard Curve Method (User Bulletin No. 2, Applied Biosystems). PCR cycle parameters were as follows: 1 cycle of 50°C for 2 min and 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. The level of mRNA was normalized to average of cyclophilin and β-actin RNA levels to compensate for variations in input RNA amounts and are presented as median level of normalized mRNA level. In a case of analysis of *LPA* expression pattern in different tissues, samples were screened qualitatively by qPCR and expression level is presented as high, low or not expressed (-).

LPA primers and probe (Eurogentech, Seraing, Belgium) were designed to bind specifically to the *KV* part of the *LPA* gene. Because of the high degree of sequence homology between the *LPA* and *PLG* genes, specificity of *LPA* primers and probes was tested by running PCR reactions with different concentrations of plasmid containing *PLG* gene or *LPA* plasmid construct without KV (a kind gift from Dr. S. Frank, University of Graz, Austria). There was no detectable PCR product when these plasmids were used as templates in the PCR reaction. cDNA made from RNA isolated from the liver of WT mouse was used as a negative control when analyzing the transgenes.

Because of poor RNA quality, two samples were excluded from analysis.

Statistical analyses

The non-parametric Mann-Whitney test was used to analyse differences between groups. All data from serum analysis are presented as mean ± SEM. In the case of mRNA data, results are presented as median value of normalized mRNA level.

List of abbreviations

Apo(a) = apolipoprotein(a)

HCHF = high-cholesterol/high-fat (diet)

LDL = low density lipoprotein

LDLR = LDL receptor

Lp(a) = lipoprotein(a)

LPA = gene encoding for apolipoprotein(a)

TG = triglycerides

TC = total cholesterol

WT = wild type (mouse)

Authors' contributions

EMR provided transgenic breeder animals. Breeding of transgenic mice in Norway was initiated and overseen by KB, KE and SD. PAT tested transgenicity of animals used in this study and carried out protein and expression studies, statistical analysis and drafted the manuscript. KB and KE conceived of the study, and participated in its design and coordination. All authors read and approved the final version.

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