# Effects of heterozygous lipoprotein lipase deficiency on diet-induced atherosclerosis in mice

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**Abstract Heterozygous lipoprotein lipase deficiency (LPL+/−) is common and has been implicated in premature atherosclerosis in epidemiologic studies. However, in vitro data suggest that LPL deficiency in the vascular wall may be antiatherogenic. To address the role of LPL in atherosclerosis, LPL+/− mice in the C57BL/6J background were fed an atherogenic diet for 8 months. LPL+/− mice were more dyslipidemic than +/+ animals due to increased concentrations of non-HDL lipoproteins. There was no difference in aortic origin atherosclerosis between LPL+/− (n = 56) and +/+ (n = 55) mice. LPL+/− mice in the low density lipoprotein receptor knockout (LDLR−/−) background were fed the same atherogenic diet for 3 months. LPL+/−LDLR−/− mice were more dyslipidemic than LPL+/+LDLR−/− animals. There was no difference in atherosclerosis assayed for the entire aorta and no difference in aortic sterol content between LPL+/−LDLR−/− (n = 28) and LPL+/+LDLR−/− (n = 15) mice. LPL protein was detected in murine lesions in a consistent layered pattern. More luminal, lipid-laden macrophages generally did not stain for LPL, but deeper, lipid-poor macrophages as well as necrotic core regions contained immunoreactive LPL. LPL protein was more abundant in lesions from LPL+/+ LDLR−/− than LPL+/−LDLR−/− mice. After eating an atherogenic diet, LPL+/− as compared to LPL+/+ mice have more dyslipidemia, but no more atherosclerosis, and less LPL protein in atherosclerotic lesions.▮ These data suggest that lipoprotein lipase deficiency in the vascular wall could prevent the retention of atherogenic lipoproteins.**—Semenkovich, C. F., T. Coleman, and A. Daugherty. **Effects of heterozygous lipoprotein lipase deficiency on diet-induced atherosclerosis in mice.** *J. Lipid Res.* **1998.** 39: 1141–1151.

**Supplementary key words** triglyceride • LDL receptor knockout mice • macrophage • vascular wall • cholesterol

Whether hypertriglyceridemia, either directly or indirectly, promotes atherosclerosis is unresolved. A major enzyme in triglyceride metabolism is lipoprotein lipase (LPL). Understanding its role in atherosclerosis is clinically relevant because genetic defects of this enzyme are surprisingly common. More than 40 coding region mutations and at least three promoter mutations that decrease LPL enzyme activity (1) have been described. Homozy-

gous LPL deficiency (LPL−/−), characterized by extreme hypertriglyceridemia and recurrent pancreatitis, is uncommon. But heterozygous LPL deficiency (LPL+/−) is estimated to be present in 3–7% of the general population (2). The frequency of a single mutation known to impair LPL activity, Asn291Ser, is 1.88 to 3.37% in unselected, healthy control subjects of different ethnic backgrounds (3). Five percent of humans with known atherosclerosis and low HDL cholesterol carry the same mutation (4). Given the high frequency of the LPL+/− state, understanding how LPL affects atherosclerosis is important.

LPL is expressed at the capillary endothelium, mostly in adipose tissue and muscle (5), where its activity affects lipid levels. It is also expressed in the vascular wall. LPL at these two sites may have opposing effects on atherosclerosis.

Some evidence in humans suggests that LPL is antiatherogenic. LPL+/− humans have atherogenic lipoproteins, especially in the postprandial state (6). In 948 Danish people with angiographically proven disease, the Asn291Ser mutation was associated with ischemic heart disease in females (7). Rodent studies also suggest that LPL activity is antiatherogenic. NO-1886 is a compound that increases LPL activity. Its administration to rats decreases diet-induced atherosclerosis (8). When mice transgenic for LPL are crossed with LDLR−/− mice, diet-induced vascular lesions are decreased (9).

Another body of evidence suggests that LPL is proatherogenic. LPL is expressed in macrophages in human and rabbit atherosclerotic lesions (10, 11). LPL dimer mass is associated with calcific coronary artery disease in humans with homozygous familial hypercholesterolemia (12). LPL promotes the retention of LDL particles by sub-

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endothelial matrix through an interaction with apolipo-protein (apo)B (13, 14). Inbred mice with the highest levels of macrophage LPL expression have the most diet-induced atherosclerosis (15). The LPL dimer reduces macrophage secretion of apoE (16), suggesting that the presence of LPL in the vascular wall could antagonize the antiatherogenic effects of apoE. LPL stimulates the uptake of triglyceride-rich lipoproteins by macrophages (17). Large LPL+/− kindreds have been described with impressive dyslipidemia but no premature atherosclerosis (18, 19), suggesting that certain mutations may promote dyslipidemia (through decreased enzyme activity at the endothelium) but attenuate the retention of lipoproteins in the vessel wall (through decreased macrophage LPL mass).

LPL knockout mice provide an animal model for studying the role of this enzyme in atherosclerosis (20). The LPL−/− state is lethal in the early postnatal period, making it impossible to study the effects of LPL absence on diet-induced atherosclerosis in mice. LPL+/− mice are viable, have decreased LPL enzyme activity and protein mass in all tissues, and manifest fasting hypertriglyceridemia exacerbated by the postprandial state (21, 22), features consistent with the LPL+/− genotype in humans. Because the human LPL+/− state is extremely common and the exact role of LPL in atherosclerosis unclear, this study specifically addresses the question: Does heterozygous LPL deficiency promote diet-induced atherosclerosis?

## METHODS

### Animals

LPL+/− mice were generated by disrupting the carboxyl-terminus of the LPL protein using homologous recombination as previously described (20). These mice have lower LPL enzyme activity and LPL protein mass in all tissues as compared to wildtype mice. Animals (originally C57BL/6J-129Sv hybrids) were continually mated with inbred C57BL/6J mice. N4 and N5 generation descendants from these crosses into the C57BL/6J background were used for experiments comparing LPL+/− versus +/+ genotypes.

LDL receptor knockout mice (LDLR−/−) were purchased from the Jackson Laboratory (Bar Harbor, ME) and crossed with N5 LPL+/− (LDLR+/+) mice. The progeny of this cross, all LDLR+/−, were genotyped at the LPL locus. LPL+/−LDLR+/− were crossed with each other and the progeny were genotyped at both the LPL and LDLR loci. As expected, 25% of these progeny were LDLR−/−. As the LDLR and LPL genes are located on different chromosomes, LDLR−/− animals from this cross should have the following LPL genotypes: 25% LPL+/+, 50% LPL+/−, 25% LPL−/−. The LPL−/− genotype is lethal, so of the viable animals from this cross, the ratio of LPL+/−LDLR−/− to LPL+/+LDLR−/− mice should be 2:1. The observed ratio was 3:1 (95 LPL+/−LDLR−/− vs. 31 LPL+/+LDLR−/−), suggesting that LPL+/− animals may have a modest survival advantage compared to their LPL+/+ littermates. Previous data also suggest that LPL overproduction decreases mouse fetal viability (23).

Genotyping was performed by polymerase chain reaction (PCR). For identification of LPL heterozygotes, the following primers were used in a reaction containing 2 mm MgCl$_2$ at an annealing temperature of 55°C: LPL1-5′ TTT ACA CGG AGG TGG ACA TCG GA, LPL2-5′ TCG CCT TCT ATC GCC TTC TTG AC.

LDLR genotyping was performed by multiplex PCR exactly as described by Gaw, Mancini, and Ishibashi (24) using four separate primers (two LDLR gene specific and two neo gene specific) in the same reaction.

Mice were maintained on a chow diet (50/50 mixture of PicoLab rodent chow 20 and mouse chow 20 with a total fat content of 6.75%) until the age of 8 weeks. At that time, they were either continued on the chow diet or started on an atherogenic diet that contained 1.25% cholesterol, 0.5% cholate, 7.5% milk fat, 1.625% dextrin, 1.625% sucrose, 2.5% dextrose, 7.5% casein, 1.25% cellulose, in addition to minerals and vitamins (Harlan Teklad, TD 95046).

All mouse protocols were approved by the Washington University Animal Studies Committee.

### Lipids and lipoproteins

Mice were fasted for 4 h, then blood was collected from the retro-orbital venous plexus with a capillary tube under metofane anesthesia. On the same day of collection, the concentration of total cholesterol and triglycerides in serum was determined enzymatically using reagents in kit form purchased from Sigma (St. Louis). HDL cholesterol was determined after precipitation of apoB-containing lipoproteins with polyethylene glycol. For determination of lipoproteins, serum from 6–10 animals was pooled and 1.0 ml was injected into the sample loop of an FPLC containing a single Superose column. Fractions (0.5 ml) were collected the same day of sample collection, stored at 4°C, and assayed for cholesterol and triglyceride content the following day.

### LPL enzyme activity

Mice were fasted for 4 h, killed, then hearts were homogenized in assay buffer as previously described (20). Enzyme activity was assayed as the salt-inhibitable ability of duplicate samples to hydrolyze a radiolabeled triolein emulsion as described previously (25).

### Atherosclerosis quantitation

For studies of the LPL+/− state in the C57BL/6J background, the degree of lipid staining of cross-sections of the aortic origin was quantitated by a modification of the technique of Paigen et al. (26). Mice were exsanguinated under metofane anesthesia, and the heart and aortic arch were removed en bloc. After perfusion with cold phosphate buffered saline, they were placed in a cryostat mold and frozen immediately in tissue freeze medium (TBS, Durham, NC). Hearts were cut using a Jung 1800 cryostat. For each sample, 64 sections were made beginning just caudal to the aortic sinus and extending into the proximal aorta at 10 μm intervals. Slides were fixed with 60% isopropanol, stained with Oil Red O, and counterstained with hematoxylin. For each heart, lesions in eight sections at 80 μm intervals were quantitated by image analysis using Image-Pro Plus 2.0 software. Color thresholding was used to identify regions of Oil Red O staining. Before each microscopy session, the image processing program was calibrated using a hemocytometer grid. Data are reported as mean lesion area (in μm$^2$) per section.

For studies in the LDLR−/− background, the extent of lesion involvement of the entire aorta was assayed by the technique of Tangirala, Rubin, and Palinski (27) as modified by Daugherty et al. (28). After exsanguination, the left ventricle was cannulated and perfused with phosphate buffered saline, then fixed by perfusion with 4% paraformaldehyde. The aorta was incised longitudinally and dissected free from the adventitia; a second longitudinal incision was made at the arch to allow the intimal surface to be pinned flat. The heart was separated at the aortic sinus and frozen in tissue freeze media as described above. Pinned aortas were photographed with a digital camera, and the pictures were subjected to image analysis. Each individual image was edited by

two observers to ensure that artefacts were not scored as plaques. Determinations of the two observers routinely differed by less than 5%. Lesions are reported as percent intimal involvement for the arch (extending from the origin to a point 4 mm distal to the left subclavian artery), thoracic aorta (ending at the final intercostal artery), and the abdominal aorta (ending at the ileal artery bifurcation). For mice in the LDLR−/− background, atherosclerosis was not rigorously quantitated in cross sections of the aortic origin. However, sections through the aortic valve were made for a subset (10 mice) of these animals, and there was no trend suggesting that lesion extent in this part of the aorta was affected by the LPL genotype differently than lesion extent for the entire intimal surface (data not shown).

Cholesterol content of the aorta was determined by gas chromatography. For each sample, 5α cholestane (dissolved in methylene chloride) was added as an internal standard, then lipids were extracted with chloroform–methanol 2:1. Lipids were dried under nitrogen gas, resuspended in methylene chloride, and injected into a gas chromatograph for determination of free cholesterol. For total cholesterol, the same process was repeated after saponification of samples with tetramethylammonium hydroxide. Cholesteryl ester was calculated as the difference between total cholesterol and free cholesterol. Results are reported as sterol content normalized to intimal surface area ($\mu$g sterol per mm$^2$).

### Immunocytochemistry

Three primary antibodies were used: MOMA-2, a rat antibody raised against mouse macrophages purchased from Serotec/Harlan (Indianapolis); a rabbit anti-mouse macrophage antibody purchased from Accurate (San Diego); and a rabbit anti-human LPL antibody. The latter has been described previously (25) and recognizes mouse LPL on Western blots (not shown). Immunostaining was done using frozen sections and Vectastain kits (Vector Labs, Burlingame, CA) as described by the manufacturer. Sections were air dried and fixed with acetone. Endogenous peroxidase was quenched with $H_2O_2$ in methanol. Blocking was performed using nonimmune serum. Both primary and secondary antibodies were diluted 1:1000 in reagent buffer. Each experiment included a slide incubated with nonimmune serum instead of the primary antibody. Peroxidase was visualized with the chromogen 3-amino-9-ethyl carbazole and sections were counterstained with hematoxylin.

## RESULTS

To study the effects of the LPL+/− state in the C57BL/6J background, mice were fed an atherogenic diet for 8 months in four separate experiments over 2 years. Serum lipid results at the time of killing are shown in **Table 1**. For the first two feeding experiments, lipids were measured 1 month after initiating the diet, then at 2-month intervals. At each of these time points, lipids were identical to those measured at the time of killing (not shown).

As shown in Table 1, fasting triglycerides were 42% higher in LPL+/− compared to +/+ females ($P <$ 0.0001) and 55% higher in LPL+/− compared to +/+ males ($P <$ 0.0001). Fasting cholesterol was 15% higher ($P =$ 0.0330) in LPL+/− versus +/+ females. There was no genotype effect on cholesterol levels in males. In females, the increase in total cholesterol was due to non-HDL cholesterol as HDL-cholesterol tended to be lower in female LPL+/− mice (77 mg/dl for female +/− vs. 84.1 for female +/+). The first two feeding experiments included control animals of both sexes fed a chow diet. At the time of killing, fasting cholesterol levels (mg/dl) in these animals were: LPL+/− 47 ± 6, LPL+/+ 52 ± 5. Triglycerides (mg/dl) in the same animals were: LPL+/− 95 ± 8, LPL+/+ 59 ± 2 ($P =$ 0.0018). Thus the atherogenic diet elevated cholesterol about 4-fold in all animals, had more of an effect on females with the LPL+/− genotype, and decreased triglyceride levels in all animals while maintaining the relative effect of the LPL genotype on triglycerides.

An FPLC analysis of lipoproteins from female mice on the atherogenic diet is shown in **Fig. 1**. LPL+/− animals had higher concentrations of very low density lipoprotein (VLDL) triglycerides (top panel) and VLDL cholesterol (bottom panel). There was a consistently higher shoulder to the right of the VLDL peak in cholesterol profiles from LPL+/− animals representing intermediate density/low density lipoprotein (IDL/LDL) particles.

Feeding the atherogenic diet for 8 months resulted in early atherosclerotic lesions. Panel A of **Fig. 2** shows lipid staining at the base of the aorta in a female LPL+/− animal on the atherogenic diet. The aortas from 8 chow-fed mice (2 LPL+/− females, 2 LPL+/+ females, 2 LPL+/− males, and 2 LPL+/+ males) were also assayed for atherosclerosis. As expected, no lesions were detected (Fig. 2, panel B). Thus, LPL+/− animals do not develop atherosclerosis after 8 months on a chow diet. Lipid-staining lesions from atherogenic-fed LPL+/− females contained

TABLE 1. Lipid concentrations of LPL +/+ and +/− mice on an atherogenic diet

| | Female +/+ | Female +/− | Male +/+ | Male +/− |
|---|---|---|---|---|
| | *mg/dl ± SEM* | | | |
| Total cholesterol | 183 ± 8.5[a] <br> n = 27 | 211 ± 9.6[a] <br> n = 25 | 190 ± 8.0 <br> n = 27 | 198 ± 8.4 <br> n = 25 |
| Total triglycerides | 22.3 ± 0.9[b] <br> n = 27 | 31.6 ± 1.8[b] <br> n = 25 | 40.6 ± 2.4[c] <br> n = 35 | 62.8 ± 5.2[c] <br> n = 26 |
| HDL cholesterol | 84.1 ± 5.1 <br> n = 22 | 77 ± 5.2 <br> n = 17 | 97.1 ± 5.2 <br> n = 31 | 97.3 ± 4.8 <br> n = 25 |

Lipids and HDL cholesterol were determined for each of the four groups in the fasting state after 32 weeks on an atherogenic diet. Significant differences between animals of the same sex but different genotype are indicated by the following symbols:
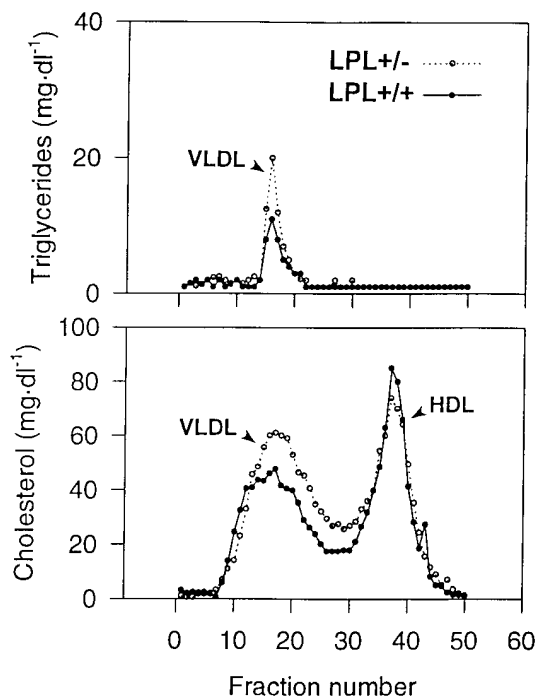
[a] $P =$ 0.0330.
[b] $P <$ 0.0001.
[c] $P <$ 0.0001.

**Fig. 1.** FPLC analysis of lipoproteins in female LPL+/− and LPL+/+ mice. Mice were fed an atherogenic diet for 8 months. Before killing, animals were fasted for 4 h. Serum from 6–10 animals per genotype was pooled and 1.0 ml was injected into the sample loop of an FPLC containing a single Superose column. Fractions of 0.5 ml each were assayed for triglyceride (top panel) and cholesterol content (bottom panel). The same results were seen in four independent experiments over 2 years. LPL+/− mice (open circles) had higher concentrations of VLDL triglyceride and cholesterol as well as a higher shoulder to the right of the VLDL cholesterol peak representing IDL/LDL. Profiles of control chow-fed animals showed VLDL triglyceride peaks but essentially no VLDL/IDL/LDL cholesterol (not shown).

macrophages (Fig. 2, panel C), confirming that these animals develop early atherosclerosis.

The aortas of 56 LPL+/− mice (31 females and 25 males) and 55 LPL+/+ mice (29 females and 26 males) were assayed for atherosclerosis (**Fig. 3**). Lesions were more extensive in females than males: female +/− median 15,630 μm² versus male +/− median 915 μm² ($P < 0.001$ by Dunn's multiple comparisons test); female +/+ median 16,691 μm² versus male +/+ median 969 μm² ($P < 0.001$ by Dunn's multiple comparisons test). However, there was no effect of LPL genotype on the extent of atherosclerosis in the C57BL/6J background.

The LPL+/− mutation was also studied in the LDLR−/− background. To verify that the LPL+/− state in the LDLR−/− background is associated with impaired LPL enzyme activity, chow-fed mice were killed after a 4-h fast and LPL enzyme activity was quantitated in heart (the murine tissue with the highest LPL enzyme activity, reference 20). LPL enzyme activity (nmol FFA/mg/min) was 12.98 ± 0.59 (mean ± sem) for LPL+/+LDLR−/− mice and 7.78 ± 1.48 for LPL+/−LDLR−/− mice (n = 4 for each group, $P = 0.0173$ by two-tailed $t$ test).

Consistent with the finding that LPL activity was lower

in LPL+/−LDLR−/− mice, triglycerides were 34 to 40% higher in LPL+/−LDLR−/− versus LPL+/+LDLR−/− mice on a chow diet (**Table 2**). After 3 months on an atherogenic diet, the LPL mutation was associated with proportionally more dyslipidemia in females than males. Triglycerides were 147% higher in female LPL+/−LDLR−/− vs. LPL+/+LDLR−/− mice and 55% higher in male LPL+/−LDLR−/− vs. LPL+/+LDLR−/− mice. Total cholesterol was 14% higher in LPL+/−LDLR−/− compared to LPL+/+LDLR−/− females, nearly identical to the 15% cholesterol elevation seen in LPL+/− females in the C57BL/6J background (see Table 1). As for the C57BL/6J background, there was no effect of LPL genotype on cholesterol levels in LDLR−/− males.

An FPLC analysis of lipoproteins in female LPL+/−LDLR−/− and LPL+/+LDLR−/− animals on the atherogenic diet is shown in **Fig. 4**. Female LPL+/−LDLR−/− animals had higher levels of VLDL triglycerides (top panel) and VLDL cholesterol (bottom panel) compared to LPL+/+LDLR−/− mice. HDL cholesterol was undetectable by FPLC techniques. It was also undetectable after precipitation of apoB-containing lipoproteins (not shown). Others have shown that the same diet fed to LDLR−/− mice decreases HDL cholesterol to very low but still detectable levels (29), a difference perhaps explained by assay sensitivity.

Aortas from these animals were mounted en face and lesion extent was quantitated as described in Methods. Atherosclerosis extent expressed as percent involvement of aortic surface area is shown in **Fig. 5**, panel A. The most extensive involvement was detected in the arch. There was no effect of sex on lesion extent. Despite having higher concentrations of both triglycerides and cholesterol, female LPL+/− mice had no more lesions at any site than female LPL+/+ mice. As lesion extent was greatest in the arch, these samples were assayed for cholesteryl ester content (panel B). There was no LPL genotype effect on cholesteryl ester content. Male +/− compared to male +/+ mice had a higher lesion area that was not statistically significant (panel A). However, cholesteryl ester content for the two genotypes in males was identical (panel B). These data suggest that although there was a trend for more surface area involvement in male +/− mice, the lesions in male +/+ mice probably extended further toward the lumen (the dimension not measured when en face aortic images are captured using a digital camera).

LDLR−/− mice develop extensive lesions at the aortic origin on an atherogenic diet which are correlated with the degree of lesion involvement of the entire aorta (27). Antibodies directed against LPL and mouse macrophages were used in immunocytochemistry studies of aortic origin lesions in ten LPL+/−LDLR−/− and LPL+/+LDLR−/− mice.

In every animal examined, lesions contained LPL-immunoreactive material in a consistent layered pattern. Luminal lipid-laden foam cells generally did not stain for LPL but they were recognized by an anti-macrophage antibody (see asterisk in **Fig. 6**, panel A and compare this re-
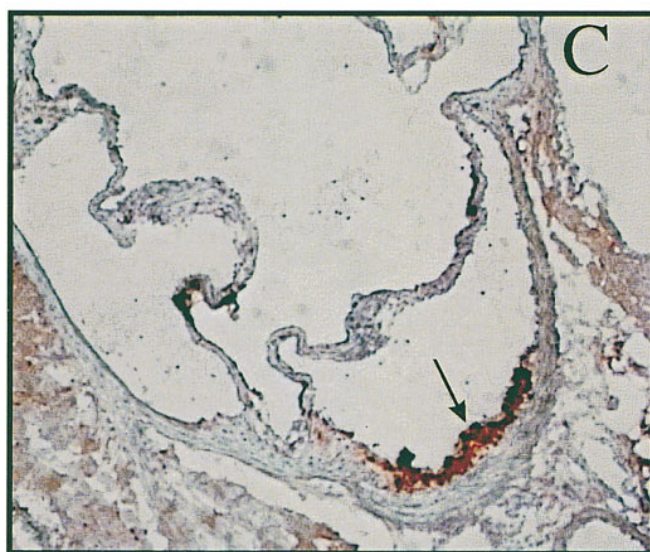
## Atherogenic Diet

## Chow Diet
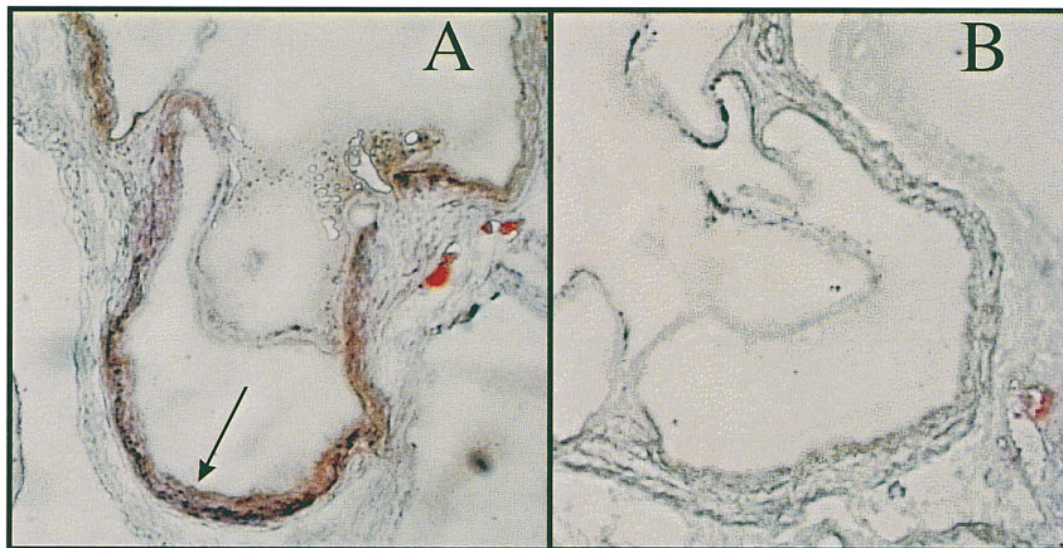


## Oil Red O and Hematoxylin

## Atherogenic Diet



## Anti-Macrophage Ab

**Fig. 2.** C57BL/6J aortic origin cross sections. At the time of killing, hearts were processed as described in Methods. Frozen sections of the aortic origin were stained with Oil Red O and hematoxylin. Shown are representative photomicrographs of tissue from a female LPL+/− mouse on an atherogenic diet for 8 months (panel A) and tissue from a female LPL+/− mouse on a chow diet for 8 months (panel B). The arrow in panel A denotes a region of intimal lipid staining. To confirm that this staining represents early atherosclerosis, sections from atherogenic diet-fed female LPL+/− mice were also stained with MOMA-2, an anti-macrophage antibody (panel C). The arrow in panel C indicates a region of antibody staining indicating the presence of macrophages. Staining was negative using nonimmune serum as the primary antibody (not shown).

gion with the same area for panel B). Lipid-poor macrophages just beneath the layer of luminal foam cells did stain for LPL (see panel A). Proceeding toward the adventitia, the next layer did not stain for either LPL or macrophages (see arrow in panel B) and presumably represents smooth muscle cells. The deepest layer of the neointima contained necrotic debris and cholesterol crystals. This region stained intensely for LPL.

There was more LPL immunoreactive material in the aortic origin lesions from LPL+/+ than LPL+/− mice. In eight of nine comparisons, a blinded observer correctly identified the LPL genotype based on the abundance of LPL protein. **Figure 7** shows representative comparisons of the LPL content of lesions from LPL+/+ (panels A, C, E) and LPL +/− (panels B, D, and F) mice. When lesions of similar size and location were compared from animals of the same genotype, there was little variation in LPL staining.
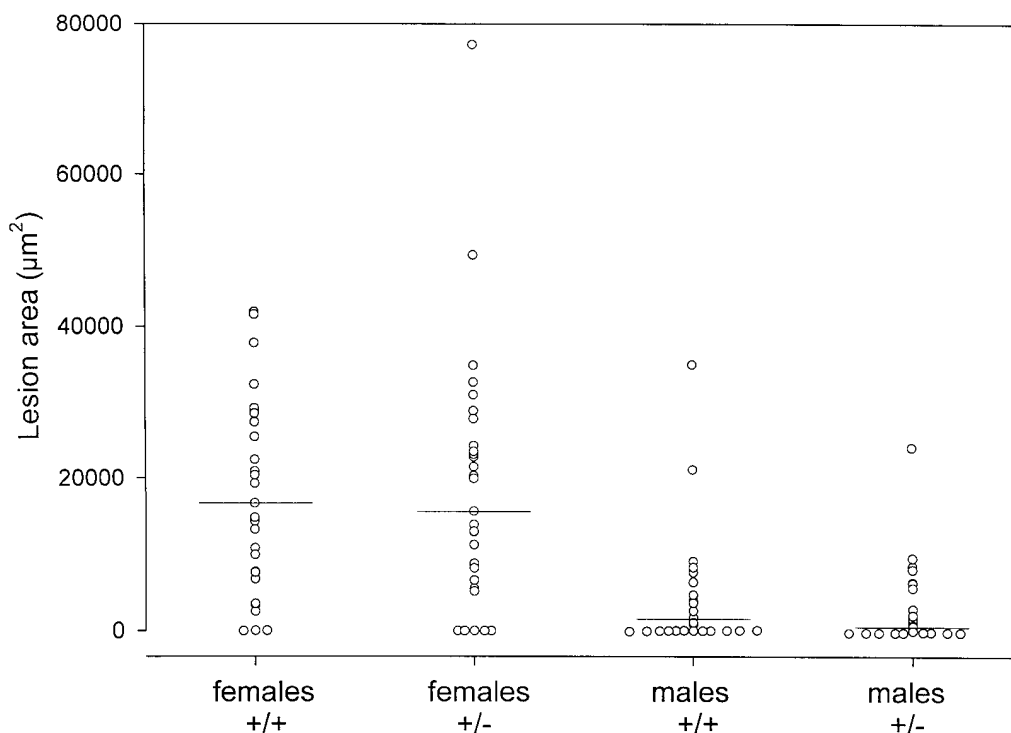
**Fig. 3.** Quantitation of atherosclerosis at the aortic origin in LPL+/+ and +/− mice. After 8 months of treatment with an atherogenic diet, the extent of atherosclerosis at the aortic origin (see Figure 2, Panel A) was quantitated. A total of 56 LPL+/− (31 females and 25 males) and 55 LPL+/+ (29 females and 26 males) mice were studied. Each data point represents the mean lesion area measured for 8 sections at 80 μm intervals for one mouse. For each sex/genotype group, no lesions were detected in several animals and these data are shown at the 0 point of the vertical axis. The horizontal lines within each data set represent the median for that set.

## DISCUSSION

LPL deficiency is extremely common but the role of LPL in atherogenesis is unknown. Given the sheer frequency of the LPL+/− state, characterizing the effects of this mutation on diet-induced dyslipidemia and atherosclerosis in an animal model has value.

Feeding an atherogenic diet produced more dyslipi-demia in LPL+/− mice in both the C57BL/6J and LDLR−/− backgrounds. Despite having higher concentrations of fasting lipids, LPL+/− mice had no more atherosclerosis at the aortic origin in C57BL/6J animals and throughout the aorta in LDLR−/− animals. One potential explanation for this observation is that detrimental effects of circulating dyslipidemia caused by LPL deficiency are offset by beneficial effects of de-

TABLE 2. Lipid concentrations of LPL +/+LDLR−/− and LPL+/−LDLR−/− mice on both chow and atherogenic diets

|  | Female +/+ (n = 9) | Female +/− (n = 16) | Male +/+ (n = 6) | Male +/− (n = 12) |
|---|---|---|---|---|
|  | *mg/dl ± SEM* | | | |
| Chow diet |  |  |  |  |
| Total cholesterol | 171 ± 11 | 169 ± 6.2 | 161 ± 6.5 | 170 ± 6.2 |
| Total triglycerides | 98 ± 12[a] | 131 ± 9.2[a] | 117 ± 14[b] | 164 ± 11[b] |
| Atherogenic Diet |  |  |  |  |
| Total cholesterol | 1,171 ± 53[c] | 1,339 ± 28[c] | 1,273 ± 27 | 1,323 ± 33 |
| Total triglycerides | 81 ± 19[d] | 200 ± 20[d] | 252 ± 42[e] | 392 ± 37[e] |

Fasting lipids were determined for LPL +/+ and +/− mice in the LDLR −/− background receiving a chow diet and after 12 weeks on an atherogenic diet. Significant differences between animals of the same sex but different genotype are indicated by the following symbols:
[a]$P = 0.0407$.
[b]$P = 0.0217$.
[c]$P = 0.0049$.
[d]$P = 0.0007$.
[e]$P = 0.0327$.

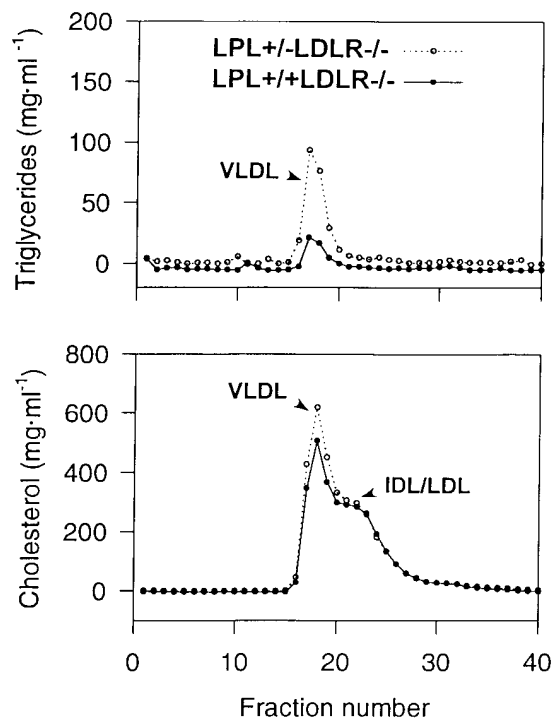**Fig. 4.** FPLC analysis of lipoproteins in female LPL+/−LDLR−/− and LPL+/+LDLR−/− mice. Mice were fed an atherogenic diet for 3 months. Before killing, animals were fasted for 4 h. Serum from several animals per genotype was pooled and separated by FPLC as described in the legend to Fig. 1. Female LPL +/− LDLR−/− mice (open circles) had higher concentrations of VLDL triglycerides (top panel) and VLDL cholesterol (bottom panel).



**Fig. 5.** Quantitation of atherosclerosis (panel A) and determination of aortic arch sterol content (panel B) in LPL+/+ and +/− mice in the LDLR−/− background. Mice were fed an atherogenic diet for 3 months, then aortas were excised and pinned en face. Percent involvement of each section of the aorta was determined by image analysis and data are shown in panel A as mean ± SEM. As most lesions were in the arch, cholesteryl ester content (normalized to intimal surface area) for the arch in each animal was determined by gas chromatography; data are shown in panel B as mean ± SEM.

creased LPL protein mass in the vascular wall caused by LPL deficiency.

It is never easy to prove a negative finding, in this case, the absence of an effect on atherosclerosis. In an ideal experiment, genetic LPL−/− mice would have been compared to LPL+/+ mice after atherogenic feeding to determine whether extreme dyslipidemia might be neutralized by the complete absence of vascular wall LPL protein. However, LPL−/− mice, unlike humans with complete LPL deficiency, are not viable (20, 21). Although LPL+/− mice have less dyslipidemia and only a partial deficiency of vascular wall LPL protein, several lines of evidence suggest that our results with these mice are important.

The dyslipidemia associated with the LPL+/− genotype was interesting. Although the mechanism is unknown, feeding an atherogenic diet to mice decreases serum triglycerides. There is clear evidence that increasing dietary fat content alone without adding cholesterol or cholate also decreases triglycerides in mice (30). In the current study, high fat feeding to C57BL/6J mice also decreased triglycerides without affecting the relative hypertriglyceridemia associated with the LPL+/− state. However, high fat feeding to LDLR−/− mice did not decrease triglycerides (Table 2); in LDLR−/− males, triglycerides clearly increased with atherogenic feeding. The simplest explanation for this observation is that in mice, the LDL
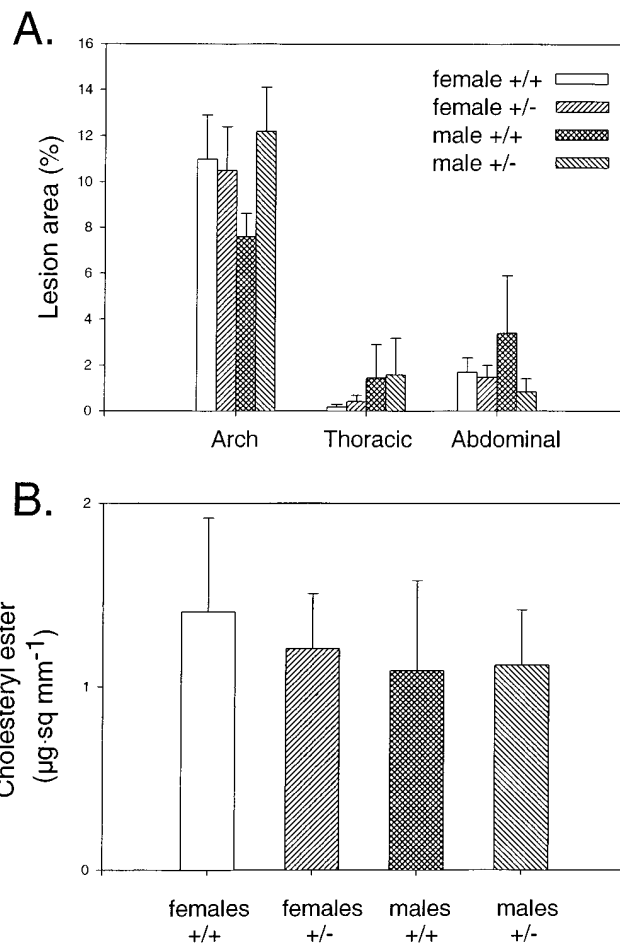
receptor is involved in the decrease of triglyceride-rich lipoproteins induced by high fat feeding.

In the setting of an atherogenic diet, the LPL+/− state had a greater relative effect on lipids in females than males. Fasting cholesterol was elevated to the same degree (about 15%) in female LPL+/− versus +/+ mice in both C57BL/6J and LDLR−/− models. There was no genotype effect on cholesterol levels in males.

An important question is whether the observed increases in serum lipids due to the LPL+/− state are sufficient to cause an increase in the magnitude of atherosclerosis. A difference in total cholesterol of 15% is modest, but other investigators have detected significant increases in aortic atherosclerosis with similar increases in total cholesterol. For example, total cholesterol in apoB[48/48] apoE−/− mice was ~16% higher than in apo^{+/+} apoE−/− mice after about 6 months ($P = 0.035$); the animals with higher serum cholesterol had significantly more aortic atherosclerosis (31). Total cholesterol in our
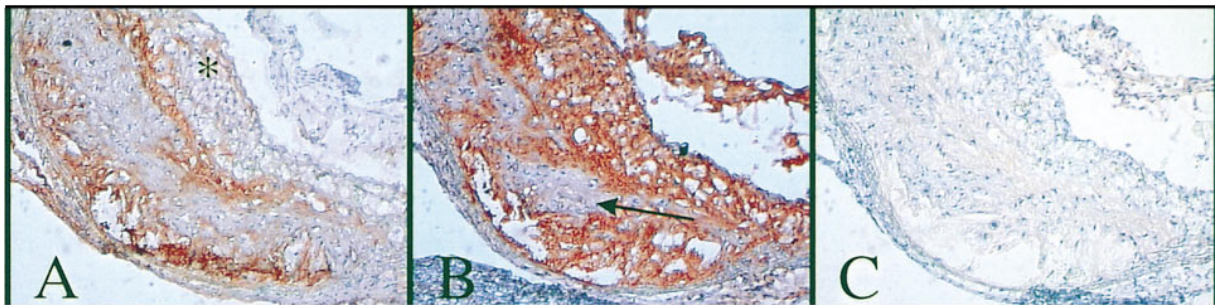
Anti-LPL        Anti-Macrophage        Control

**Fig. 6.** Detection of LPL in murine atherosclerotic lesions. Serial frozen sections of lesions at the aortic origin from LDLR−/− mice fed an atherogenic diet for 3 months were subjected to immunostaining using an anti-LPL antibody (panel A), an anti-macrophage antibody (panel B), and nonimmune serum (Panel C). This lesion was from a female LPL+/−LDLR−/− mouse. The asterisk in panel A denotes foam cells that generally do not react with the LPL antibody but react strongly with the macrophage antibody (see corresponding region of panel B). The arrow in panel B denotes a region that does not react with either the LPL or the macrophage antibody.

female LPL+/−LDLR−/− mice was ~14% higher than in LPL+/+LDLR−/− mice after 3 months ($P = 0.0049$); the animals with higher serum cholesterol had fewer aortic arch lesions and lower aortic cholesteryl ester content although these differences were not significant (Fig. 5).

Fasting triglycerides were similarly elevated in LPL+/− vs. +/+ mice of both sexes in the C57BL/6J model (Table 1). The LPL+/− mutation had a proportionately greater triglyceride effect on females in the LDLR−/− background (Table 2). Female LPL+/− mice in the LDLR−/− background had triglycerides that were ~250% of their LPL+/+ littermates in addition to their significantly
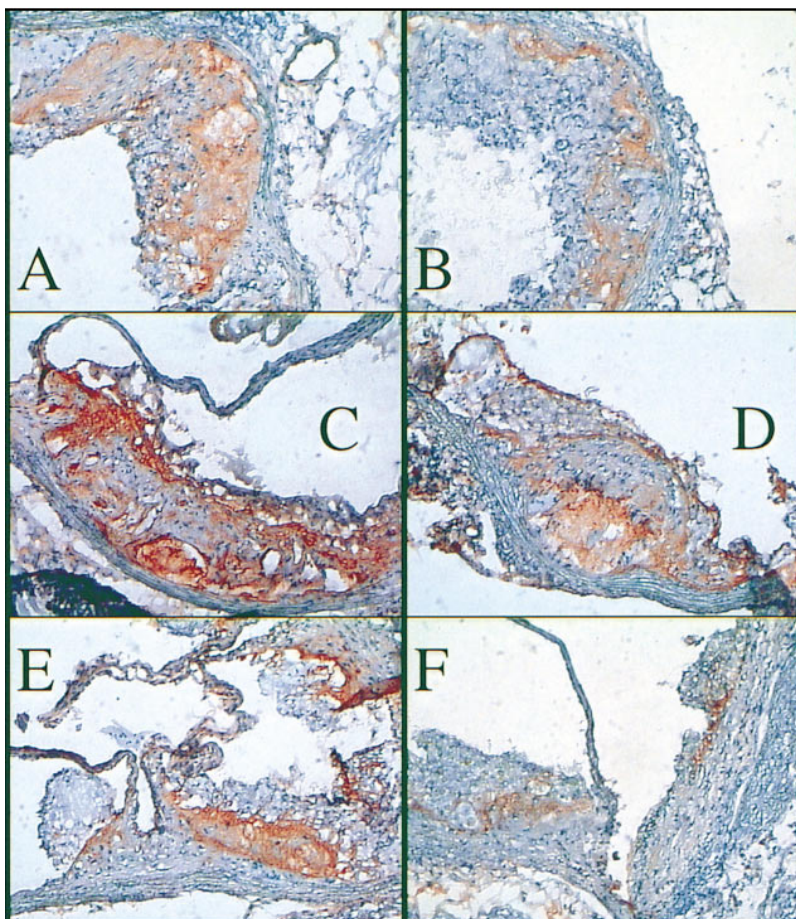


**Fig. 7.** Comparison of LPL immunostaining in atherosclerotic lesions from LPL+/+LDLR−/− (left panels) and LPL+/− LDLR−/− (right panels) mice. Mice were fed an atherogenic diet for 3 months. Frozen sections of lesions at the aortic origin were subjected to LPL immunostaining. Shown are comparisons of lesions at similar sites and of similar sizes to show that lesions from LPL+/+ mice contained more immunoreactive LPL (compare panels A and B, C and D, E and F). For each panel, the letter (e.g. "A") is located in the lumen of the vessel. Panels C, D, and E show the same layered pattern of LPL staining seen in Fig. 6.

LPL +/+        LPL +/-

higher cholesterol levels but had no more atherosclerosis. The relative hypertriglyceridemia measured in the fasting state in LPL+/− mice almost certainly underestimates the degree of hypertriglyceridemia to which the vascular wall is exposed during most of the lives of these mice. LPL+/− mice have striking degrees of hypertriglyceridemia relative to LPL+/+ mice during both oral and intravenous fat tolerance tests (21, 22). Because mice, like humans, spend most of their lives in the postprandial state, LPL+/− mice in the current study were more hypertriglyceridemic than suggested by fasting triglyceride data.

The finding that female LPL+/− mice are more dyslipidemic in the setting of an atherogenic diet has parallels with human data. In a large epidemiologic study of a population known to eat an atherogenic diet, females, but not males, with heterozygous LPL deficiency had elevated triglycerides compared to those without this mutation (7). Female LPL+/− mice did not develop more atherosclerosis; human LPL+/− females were reported to have an increased risk for ischemic heart disease (7). However, if this mutation actually promotes atherosclerosis, one would expect carriers to die prematurely and not be included in older age groups. There was no difference in allele frequencies up to the age of 80 (7).

We detected less immunoreactive LPL in the lesions of LPL+/−LDLR−/− as compared to LPL+/+LDLR−/− mice. This finding, in concert with observations that these mice had more dyslipidemia but no more atherosclerosis, raises the possibility that LPL deficiency in the vascular wall may be beneficial. There is evidence supporting this hypothesis.

LPL activity is increased in atherosclerotic regions of rabbit aorta (32) where it may locally generate atherogenic remnants. Balloon injury rapidly increases LPL activity in rabbit aorta (33). LPL expression has been detected in both human and rabbit atherosclerotic lesions (10, 11). In reductionist systems, LPL appears to form a bridge between apoB-containing lipoproteins and matrix proteoglycans, indicating that the LPL protein may retain atherogenic lipoproteins in the vessel wall (34). Perfusion studies using hamster aorta suggest that LPL promotes LDL retention in intact vessels, an effect blocked by anti-apoB antibodies (35).

High levels of LPL in the vascular wall might also promote atherosclerosis by mechanisms independent of remnant generation and lipoprotein retention. The LPL dimer reduces secretion of macrophage apoE (16), which is probably antiatherogenic in the vessel wall. It can be associated with arterial calcification (12), a poorly understood phenomenon which is characteristic of atherosclerosis. It may promote arterial binding of monocytes, a critical component of lesions (36).

Our data also provide an initial description of the distribution of LPL in murine atherosclerotic lesions. There are several implications of our findings.

First, LPL was detected in a layered pattern (Fig. 6) reminiscent of the rings of a tree. The more luminal layer was found associated with lipid-poor macrophages while the deeper layer was associated with the lipid-rich core. Like tree rings, layers of LPL might correlate with lesion age.

Second, although immunostaining was present in consistent locations, these results do not prove that the LPL at these sites was produced by cells at these sites. LPL is a secreted protein which might diffuse within lesions. If this occurs, immunolocalization could reflect preferential association with specific matrix components rather than cell synthesis.

Third, if indeed LPL immunostaining identifies cells producing LPL, our data suggest an inverse relationship between macrophage lipid content and LPL production. More luminal macrophages in our studies had the morphology of lipid-rich foam cells but generally did not stain for LPL. Lipid-poor macrophages just beneath this foam cell layer were clearly immunoreactive for LPL. Our findings are consistent with previous studies in rabbits showing that only subsets of lesional macrophages stain for LPL (10), and with studies showing that human foam cells express low levels of LPL perhaps due to their oxysterol content (37, 38). The significance of different subsets of lesional macrophages and the trafficking of monocyte-macrophages within lesions are not well understood (39). If cells near the lumen are the newest inhabitants of lesions and cells near the media are the oldest, it is tempting to speculate that LPL expression in the mouse model might represent a marker for older macrophages that are mobilizing triglycerides (40) and cholesteryl esters (41) as a prelude to cell death.

Finally, we detected large differences in lesion extent between males and females in the C57BL/6J model confirming observations made by others (42). Recent data indicate that male C57BL/6J mice have higher numbers of aortic estrogen receptors and produce more endothelial-derived nitric oxide than females (43) which may help explain this sex difference in atherosclerotic susceptibility.

In summary, LPL+/− mice, especially females, become more dyslipidemic than LPL+/+ mice with atherogenic feeding but do not develop more atherosclerosis. An atherogenic diet lowers triglycerides in C57BL/6J but not in LDLR−/− mice. LPL protein is present in murine atherosclerotic lesions, associated with lipid-poor macrophages, and its level of expression is lower in LPL+/− mice. These results provide a basis for future studies directed at the role of vascular wall LPL protein in atherosclerosis progression. ▮▮

## REFERENCES

1. Yang, W.-S., D. N. Nevin, L. Iwasaki, R. Peng, R. G. Brown, J. D. Brunzell, and S. S. Deeb. 1996. Regulatory mutations in the human lipoprotein lipase gene in patients with familial combined hy-

perlipidemia and coronary artery disease. *J. Lipid Res.* 37: 2627–2637.

2. Benlian, P., J. L. De Gennes, L. Foubert, H. Zhang, S. E. Gagne, and M. R. Hayden. 1996. Premature atherosclerosis in patients with familial chylomicronemia caused by mutations in the lipoprotein lipase gene. *N. Engl. J. Med.* **335:** 848–854.

3. Fisher, R. M., F. Mailly, R. E. Peacock, A. Hamsten, M. Seed, J. S. Yudkin, U. Beisiegel, G. Feussner, G. Miller, S. E. Humphries, and P. J. Talmud. 1995. Interaction of the lipoprotein lipase asparagine 291-serine mutation with body mass index determines elevated plasma triacylglycerol concentrations: a study in hyperlipidemic subjects, myocardial infarction survivors, and healthy adults. *J. Lipid Res.* **36:** 2104–2112.

4. Reymer, P. W. A., E. Gagne, B. E. Groenmeyer, H. Zhang, I. Forsythe, H. Jansen, J. C. Seidel, D. Kromhout, K. E. Lie, J. J. Kastelein, and M. R. Hayden. 1995. A lipoprotein lipase mutation (Asn291Ser) is associated with reduced HDL cholesterol levels in premature atherosclerosis. *Nature Genet.* **10:** 28–34.

5. Semenkovich, C. F., S-H. Chen, M. Wims, C-C. Luo, W-H. Li, and L. Chan. 1989. Lipoprotein lipase and hepatic lipase mRNA tissue specific expression, developmental regulation and evolution. *J. Lipid Res.* **30:** 423–431.

6. Miesenbock, G., B. Holzl, B. Foger, E. Brandstatter, B. Paulweber, F. Sandhofer, and J. R. Patsch. 1993. Heterozygous lipoprotein lipase deficiency due to a missense mutation as the cause of impaired triglyceride tolerance with multiple lipoprotein abnormalities. *J. Clin. Invest.* **91:** 448–455.

7. Wittrup, H. H., A. Tybjaerg-Hansen, S. Abildgaard, R. Steffensen, P. Schnohr, and B. G. Nordestgaard. 1997. A common substitution (Asn291Ser) in lipoprotein lipase is associated with increased risk of ischemic heart disease. *J. Clin. Invest.* **99:** 1606–1613.

8. Tsutsumi, K., Y. Inoue, A. Shima, K. Iwasaki, M. Kawamura, and T. Murase. 1993. The novel compound NO-1886 increases lipoprotein lipase activity with resulting elevation of high density lipoprotein cholesterol, and long-term administration inhibits atherogenesis in the coronary arteries of rats with experimental atherosclerosis. *J. Clin. Invest.* **92:** 411–417.

9. Shimada, M., S. Ishibashi, T. Inaba, H. Yagyu, K. Harada, J. Osuga, K. Ohashi, Y. Yazaki, and N. Yamada. 1996. Suppression of diet-induced atherosclerosis in low density lipoprotein receptor knock-out mice overexpressing lipoprotein lipase. *Proc. Natl. Acad. Sci. USA.* **93:** 7242–7246.

10. Yla-Herttuala, S., B. A. Lipton, M. E. Rosenfeld, I. J. Goldberg, D. Steinberg, and J. L. Witztum. 1991. Macrophages and smooth muscle cells express lipoprotein lipase in human and rabbit atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA.* **88:** 10143–10147.

11. O'Brien, K. D., D. Gordon, S. Deeb, M. Ferguson, and A. Chait. 1992. Lipoprotein lipase is synthesized by macrophage-derived foam cells in human coronary atherosclerotic plaques. *J. Clin. Invest.* **89:** 1544–1550.

12. Dugi, K. A., I. M. Feuerstein, S. Hill, J. Shih, S. Santamarina-Fojo, H. B. Brewer, Jr., and J. M. Hoeg. 1997. Lipoprotein lipase correlates positively and hepatic lipase inversely with calcific atherosclerosis in homozygous familial hypercholesterolemia. *Arterioscl. Thromb. Vasc. Biol.* **17:** 354–364.

13. Saxena, U., M. G. Klein, T. M. Vanni, and I. J. Goldberg. 1992. Lipoprotein lipase increases low density lipoprotein retention by subendothelial matrix. *J. Clin. Invest.* **89:** 373–380.

14. Pang, L., P. Sivaram, and I. J. Goldberg. 1996. Cell-surface expression of an amino-terminal fragment of apolipoprotein B increases lipoprotein lipase binding to cells. *J. Biol. Chem.* **271:** 19518–19523.

15. Renier, G., E. Skamene, J. B. DeSanctis, and D. Radzioch. 1992. High macrophage lipoprotein lipase expression and secretion are associated in inbred murine strains with susceptibility to atherosclerosis. *Arterioscler. Thromb.* **13:** 190–196.

16. Lucas, M., P-H. Iverius, D. K. Strickland, and T. Mazzone. 1997. Lipoprotein lipase reduces secretion of apolipoprotein E from macrophages. *J. Biol. Chem.* **272:** 13000–13005.

17. Hendricks, W. L., F. van der Sman-de Beer, B. J. M. van Vlijmen, L. C. van Vark, M. H. Hofker, and L. M. Havekes. 1997. Uptake by J774 macrophages of very-low-density lipoproteins isolated from apoE-deficient mice is mediated by a distinct receptor and stimulated by lipoprotein lipase. *Arterioscler. Thromb. Vasc. Biol.* **17:** 498–504.

18. Wilson, D. E., A. Hata, L. K. Kwong, A. Lingam, J. Shuhua, D. Ridinger, C. Yeager, K. C. Kaltenborn, P-H. Iverius, and J. M. Lalouel. 1993. Mutations in exon 3 of the lipoprotein lipase gene

segregating in a family with hypertriglyceridemia, pancreatitis, and noninsulin-dependent diabetes. *J. Clin. Invest.* **92:** 203–211.

19. Wilson, D. E., M. Emi, P-H. Iverius, A. Hata, L. L. Wu, E. Hillas, R. R. Williams, and J. M. Lalouel. 1990. Phenotypic expression of heterozygous lipoprotein lipase deficiency in the extended pedigree of a proband homozygous for a missense mutation. *J. Clin. Invest.* **86:** 735–750.

20. Coleman, T., R. L. Seip, J. M. Gimble, D. Lee, N. Maeda, and C. F. Semenkovich. 1995. COOH-terminal disruption of lipoprotein lipase in mice is lethal in homozygotes, but heterozygotes have elevated triglycerides and impaired enzyme activity. *J. Biol. Chem.* **270:** 12518–12525.

21. Weinstock, P. H., C. L. Bisgaier, K. Aalto-Setala, H. Radner, R. Ramakrishnan, S. Levak-Frank, A. D. Essenburg, R. Zechner, and J. L. Breslow. 1995. Severe hypertriglyceridemia, reduced high density lipoprotein, and neonatal death in lipoprotein lipase knockout mice. *J. Clin. Invest.* **96:** 2555–2568.

22. Ashbourne-Excoffon, K. J. D. A., G. Liu, L. Miao, J. E. Wilson, B. M. McManus, C. F. Semenkovich, T. Coleman, P. Benoit, N. Duverger, D. Branellec, P. Denefle, M. R. Hayden, and M. E. S. Lewis. 1997. Correction of hypertriglyceridemia and impaired fat tolerance in lipoprotein lipase-deficient mice by adenovirus-mediated expression of human lipoprotein lipase. *Arterioscler. Thromb. Vasc. Biol.* **17:** 2532–2539.

23. Zsigmond, E., E. Scheffler, T. M. Forte, R. Potenz, W. Wu, and L. Chan. 1994. Transgenic mice expressing human lipoprotein lipase driven by the mouse metallothionein promoter. a phenotype associated with increased perinatal mortality and reduced plasma very low density lipoprotein of normal size. *J. Biol. Chem.* **269:** 18757–18766.

24. Gaw, A., F. P. Mancini, and S. Ishibashi. 1995. Rapid genotyping of low density lipoprotein receptor knockout mice using a polymerase chain reaction technique. *Lab. Animals.* **29:** 447–449.

25. Seip, R. L., T. J. Angelopoulos, and C. F. Semenkovich. 1995. Exercise induces human lipoprotein lipase gene expression in skeletal muscle but not adipose tissue. *Am. J. Physiol.* **268:** E229–E236.

26. Paigen, B., A. Morrow, C. Brandon, D. Mitchell, and P. Holmes. 1985. Variation in susceptibility to atherosclerosis among inbred strains of mice. *Atherosclerosis.* **57:** 65–73.

27. Tangirala, R. K., E. M. Rubin, and W. Palinski. 1995. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J. Lipid Res.* **36:** 2320–2328.

28. Daugherty, A., E. Pure, D. Delfel-Butteiger, S. Chen, J. Leferovich, S. E. Roselaar, and D. J. Rader. 1997. The effects of total lymphocyte deficiency on the extent of atherosclerosis in apolipoprotein E−/− mice. *J. Clin. Invest.* **100:** 1575–1580.

29. Ishibashi, S., J. L. Goldstein, M. S. Brown, J. Herz, and D. K. Burns. 1994. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J. Clin. Invest.* **93:** 1885–1893.

30. LeBoeuf, R. C., M. Caldwell, and E. Kirk. 1994. Regulation by nutritional status of lipids and apolipoproteins A-I, A-II, and A-IV in inbred mice. *J. Lipid Res.* **35:** 121–133.

31. Veniant, M. M., V. Pierotti, D. Newland, C. M. Cham, D. A. Sanan, R. L. Walzem, and S. G. Young. 1997. Susceptibility to atherosclerosis in mice expressing exclusively apolipoprotein B-48 or apolipoprotein B-100. *J. Clin. Invest.* **100:** 180–188.

32. Zilversmit, D. B. 1979. Atherogenesis: a postprandial phenomenon. *Circulation.* **60:** 473–485.

33. Werba, J. P., L. A. Cuniberti, R. P. Laguens, L. D. Masnatta, R. H. Rey, R. Levy, and R. H. Pichel. 1996. Injury produces early rise in lipoprotein lipase activity in rabbit aorta. *Atherosclerosis.* **125:** 257–266.

34. Goldberg, I. J. 1996. Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. *J. Lipid Res.* **37:** 693–707.

35. Rutledge, J. C., M. M. Woo, A. A. Rezai, L. K. Curtiss, and I. J. Goldberg. 1997. Lipoprotein lipase increases lipoprotein binding to the artery wall and increases endothelial layer permeability by formation of lipolysis products. *Circ. Res.* **80:** 819–828.

36. Obunike, J. C., S. Paka, S. Pillarisetti, and I. J. Goldberg. 1997. Lipoprotein lipase can function as a monocyte adhesion protein. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1414–1420.

37. Mattson, L., H. Johansson, M. Ottosson, G. Bondjers, and O. Wiklund. 1993. Expression of lipoprotein lipase mRNA and secretion

in macrophages isolated from human atherosclerotic aorta. *J. Clin. Invest.* **92:** 1759–1765.

38. Hulten, L. M., H. Lindmark, U. Diczfalusy, I. Bjorkhem, M. Ottosson, Y. Liu, G. Bondjers, and O. Wiklund. 1996. Oxysterols present in atherosclerotic tissue decrease the expression of lipoprotein lipase messenger RNA in human monocyte-derived macrophages. *J. Clin. Invest.* **97:** 461–468.

39. Ross, R. 1995. Cell biology of atherosclerosis. *Annu. Rev. Physiol.* **57:** 791–804.

40. von Hodenberg, E., J. C. Khoo, D. Jensen, J. L. Witztum, and D. Steinberg. 1984. Mobilization of stored triglycerides from macrophages as free fatty acids. *Arteriosclerosis.* **4:** 630–635.

41. Brown, M. S., Y. K. Ho, and J. L. Goldstein. 1980. The cholesterol ester cycle in macrophage foam cells. *J. Biol. Chem.* **255:** 9344–9352.

42. Paigen, B., P. A. Holmes, D. Mitchell, and D. Albee. 1986. Comparison of atherosclerotic lesions and HDL-lipid levels in male, female, and testosterone-treated female mice from strains C57BL/6, BALB/c and C3H. *Atherosclerosis.* **64:** 215–221.

43. Rubanyi, G. M., A. D. Freay, K. Kauser, D. Sukovich, G. Burton, D. B. Lubahn, J. F. Couse, S. W. Curtis, and K. S. Korach. 1997. Vascular estrogen receptors and endothelium-derived nitric oxide production in the mouse aorta. *J. Clin. Invest.* **99:** 2429–2437.