Supplement Material

Expanded Materials and Methods

Isolation and stimulation of mouse peritoneal macrophages

Macrophages were collected from the peritoneum of the *apo E-/-x gclm-/-, apo E-/-/Gclc-Tg,* and *apo E-/-* mice four days after i.p. injection of 2 ml of 4% thioglycollate. Peritoneal exudate cells were harvested from the peritoneal cavity four times with 5 ml each of RPMI-1640-5% FBS. The cells were washed twice with 30 ml of ice-cold PBS after centrifugation at 250 x g at 4°C. Total cell numbers were determined with a hemocytometer after staining with Turk solution (Wako Pure Chemical Industries). Cells (3.2×10^6 cells/cm^2) were cultured in RPMI 1640 medium supplemented with 10% FBS (RPMI 1640–10% FBS) at 37°C in 5% CO2. After incubation for 2 h, the medium was changed and cultured in RPMI 1640–10% FBS for an additional 20–22 h.

Measurement of cellular and tissue GSH content

The total glutathione content (GSH+GSSG) in mouse peritoneal macrophages, liver, spleen and lung from 3 mice/group was determined in a 96-well fluorescent microtiter plate assay[1,2]. Briefly, the cells or tissue aliquots were homogenized in TES/SB buffer (20 mM Tris, 1 mM EDTA, 250 mM sucrose, 20 mM sodium borate, 2 mM L-serine, PH 7.4) and an equal volume of 10% of sulfosalicylic acid was added to the homogenates, and precipitated protein was removed by centrifuging at 4 °C at 12,000 rpm for 5 min. 25 μl of the resulting

supernatant was added to 100 μl of 0.2 M NEM (n-ethylmorpholine) in 0.02 M KOH and 10 μl of tris carboxyethylphosphine (TCEP) were pipetted to 96-well flat-bottom fluorescent microtiter plate and incubated for 15 min at room temperature. To bring the solution to a pH of 12.5, 50 μl of NaOH were added, followed by 10 μl of 10 mM naphthaline dicarboxyaldheyde. After 30 min incubation in the dark at room temperature, the fluorescence was detected using 472 nm excitation and 528 nm emission (Molecular Devices, Spectra Max M2). All assays were performed in triplicate. Standard curves were run simultaneously with 0–25 nmol of GSSG per well and the GSH levels were calculated as nmole per microgram of soluble protein (nmole/mg) in the original cell extract.

1.Ou YC, White CC, Krejsa CM, Ponce RA, Kavanagh TJ, Faustman EM. The roles of intracellular glutathione in methylmercury-induced toxicity in embryonic neuronal cells. *Neurotoxicology*. 1999;20:793-804.

2. White CC, Viernes H, Krejsa CM, Botta D, Kavanagh TJ. Fluorescence-based microtiter plate assay for glutamate-cysteine ligase activity. *Anal Biochem*. 2003;318:175-80.

A

	Male							
	20 weeks of age		30 weeks of age		40 weeks of age		50 weeks of age	
	ApoE-/-/,Gclm-/- (n=5)	***ApoE-/- (n=14)***	***ApoE-/-/Gclm-/- (n=8)***	***ApoE-/- (n=14)***	***ApoE-/-/Gclm-/- (n=7)***	***ApoE-/- (n=10)***	***ApoE-/-/Gclm-/- (n=9)***	***ApoE-/- (n=3)***
Foam cells	57.5% ± 19.2%	62.5% ± 11.2%	92.5% ± 7.5% *	65% ± 11.5%	81.2% ± 11.6%	68.3% ± 12.7%	43% ± 15.9%	81.9% ± 7.2%
Thin fibrous cap	3.3% ± 3.3%	0%	33.1% ± 9.9%*	0%	7.1% ± 7.1%	10% ± 10%	29.7% ± 13.1%	42.9% ± 0%
Large necrotic core	0%	0%	14.7% ± 10.4%*	0%	50.4% ± 13.7%	18.3% ± 10.7%	52.4% ± 13.3%	21.5% ± 21.5%
Cholesterol clefts	0%	0%	27.0% ± 11.4% *	1% ± 1%	67.7% ± 13.8%	32.7% ± 11.7%	82.6% ±11.3%	35.7% ± 35.7%
Lateral xanthomas	3.3% ± 3.3%	0%	46.9% ± 16%*	1% ± 1%	47.7% ± 11.3%	23% ± 12.3%	47.6% ± 12.1%	14.3% ± 14.3%
Hemorrhage	0%	0%	30.5% ± 13.8%*	0%	14.3% ± 12.1%	14% ± 10.3%	5.6% ± 3.9%	0,0%
Medial thickening	50.8% ± 20.9%	80% ± 10.3%	100% *	76.8% ± 10.6%	100%	100%	100%	100,0%
Chondrocytes	0%	0%	19.5% ± 8.1%*	0%	67.9% ± 12.1%*	13.5% ± 7.4%	57.8% ± 16.8%	35.7% ± 35.7%
Calcification	0%	0%	0%	0%	4.1% ± 4.1%	0%	0%	7.2% ± 7.2%

B

	Female							
	20 weeks of age		30 weeks of age		40 weeks of age		50 weeks of age	
	ApoE-/-/Gclm-/- (n=5)	***ApoE-/- (n=21)***	***ApoE-/-/Gclm-/- (n=8)***	***ApoE-/- (n=14)***	***ApoE-/-/Gclm-/- (n=6)***	***ApoE-/- (n=11)***	***ApoE-/-/Gclm-/- (n=5)***	***ApoE-/- (n=14)***
Foam cells	97.5% ± 10.9% *	48.9% ± 7.3%	76% ± 12.5%	85.4% ± 6.1%	48% ± 19.6%	53% ± 12.3%	13% ± 8.6%*	47.2% ± 8.9%
Thin fibrous cap	7.5% ± 0.6% *	1.7% ± 1.2%	28.3% ± 9.8%	29.2% ± 9.7%	4.8% ± 4.8%	18.2% ± 10.9%	46.1% ± 18.2%	33.2% ± 9.6%
Large necrotic core	7.5% ± 1.2% *	0%	36.7% ± 12.3% *	3.8% ± 2.6%	62.7% ± 14.2% *	27.3% ± 7.3%	73.4% ± 16.2%	44.7% ± 9.2%
Cholesterol clefts	10% ± 1.6% *	0%	57.3% ± 14.8% *	14.8% ± 5.3%	100% *	47% ± 11.2%	100%*	73.8% ± 7.7%
Lateral xanthoma	32.5% ± 3.3% *	0%	58.4% ±15.2% *	13.2% ± 6.2%	61.1% ± 15.9%	55.2% ± 12.1%	44.8% ± 13.5%	41.5% ± 9.4%
Hemorrhage	5% ± 0.8% *	0%	30.7% ± 13.2%*	7.5% ±4.6%	26.2% ± 17.5%	15.6% ± 9.5%	0%	0%
Medial thickening	87.5% ± 7.1%	68.7% ± 8.7%	94.3% ± 4.2%	95% ± 3.7%	100%	96.1% ± 3.9%	100%	100%
Chondrocytes	12.5% ± 2% *	0%	31.8% ± 15.2%	23.7% ± 9%	93.5% ± 4.4% *	57% ± 12.9%	100% *	78.7% ± 8.8%
Calcification	0%	0%	7.5% ± 7.5%	0%	37.3% ± 17.7%	20.3% ± 11.2%	38.4% ± 17.2%	44.9% ± 10.5%

C

	Male							
	20 weeks of age		30 weeks of age		40 weeks of age		50 weeks of age	
	ApoE-/-/Gclc-Tg (n=9)	*ApoE-/- (n=10)*	*ApoE -/-/Gclc-Tg (n=8)*	*ApoE-/- (n=6)*	*ApoE-/-/Gclc-Tg (n=6)*	*ApoE-/- (n=10)*	*ApoE-/-/Gclc-Tg (n=10)*	*ApoE-/- (n=11)*
Foam cells	88% ± 5.2%	86.1% ± 7.5%	81.8% ± 8.1%*	52.2% ± 19.3%	66.7% ± 12.9%	63.8% ± 12.3%	46.1% ± 11.8%*	35.1% ± 10.3%
Thin fibrous cap	1.6% ± 1.6%	0%	0%*	23.9% ± 12.8%	0%*	15.4% ± 9.1%	9.5% ± 7.7%	8.6% ± 5.2%
Large necrotic core	2.2% ± 2.2%	0%	22.7% ± 10.9%	31.7% ± 15.2%	34.4% ± 19.5%	31% ± 13.1%	31.5% ± 12.1%*	48.8% ± 9.8%
Cholesterol clefts	2.2% ± 2.2%	0%	23.8% ± 7%	33.5% ± 16.7%	67.8% ± 18.9%	76.8% ± 10%	84.7% ± 6.1%*	96.4% ± 2.7%
Lateral xanthomas	3.2% ± 3.2%	0%	32.1% ± 10.9%	42.9% ± 16.4%	67.8% ± 18.9%	54.9% ± 13.1%	62% ± 10.5%	54.5% ± 9.8%
Hemorrhage	5.6% ± 5.6%	0%	24.6% ± 9.2%	27.8% ± 18.1%	6.7% ± 5.2%	4% ± 4%	6.1% ± 4.1%	10.6% ± 9%
Medial thickening	97.2% ± 2.8%	100%	96.4% ± 3.3%	100%	100%	100%	100%	100%
Chondrocytes	3.2% ± 3.2%	0%	31.33% ± 12.8%	41.1% ± 16.6%	61.1% ± 24%	51.2% ± 13.8%	89.2% ± 4%*	98.2% ± 1.8%
Calcification	0%	0%	0%	11.1% ± 11.1%	27.8% ± 15.5%	28.2% ± 12.2%	51.9% ± 11.9%*	71% ± 8.9%

D

	Female							
	20 weeks of age		30 weeks of age		40 weeks of age		50 weeks of age	
	ApoE-/-/Gclc-Tg (n=13)	*ApoE-/- (n=11)*	*ApoE-/-/Gclc-Tg (n=13)*	*ApoE-/- (n=10)*	*ApoE-/-/Gclc-Tg (n=7)*	*ApoE-/- (n=7)*	*ApoE -/-/Gclc-Tg (n=7)*	*ApoE-/- (n=18)*
Foam cells	82.5% ± 6%	77.6% ± 7.7%	73.9% ± 10.7%	77.2% ± 9.7%	40% ± 24.5%	58% ± 17.5%	32.7% ± 16.3%	33.7% ± 9.5%
Thin Fibrous cap	0%	5.6% ± 3%	4.5% ± 3.1%*	34.1% ± 11.3%	0%*	14.3% ± 14.3%	6.5% ± 3.9%*	11.9% ± 5.2%
Large Necrotic core	7.9% ± 6.1%	0%	24.8% ± 6.3%	21.3% ± 9.5%	39.7% ± 16.5%	28.6% ± 8.6%	48.9% ± 17.9%*	60.9% ± 10.1%
Cholesterol clefts	6.7% ± 6.4%	0%	35.6% ± 11.2%	30.3% ± 10.6%	88% ± 12%*	63.8% ± 10.9%	97.2% ± 2.6%*	89.9% ± 6%
Lateral xanthoma	6.2% ± 6%	1.8% ± 1.8%	29.5% ± 12.3%	32.8% ± 9.4%	20% ± 20%*	75.2% ± 14.2%	52.1% ± 19.9%	48% ± 10.8%
Hemorrhage	5% ± 3.5%	0%	14.9% ± 8.1%	9.6% ± 4.5%	16% ± 16%	24.5% ± 14.2%	22.2% ± 13.6%	0%
Medial thickening	96.7% ± 3.2%	72% ± 9.6%	100%	100%	100%	100%	100%	100%
Chondrocyte	3.2% ± 3.2%	0%	31.8% ± 12.5%*	44.3% ± 11.7%	66% ± 18.9%	72.4% ± 13.5%	79% ± 8.9%*	88.1% ± 7.6%
Calcification	0%	0%	6.5% ± 3.6%	8.6% ± 8.6%	23.7% ± 11.3%	27.5% ± 14.7%	43.7% ± 12.5%*	60.6% ± 8.1%

Supplemental Table I. Composition of the Lesions in the Innominate Arteries.

The frequency of features of plaque composition were measured in male (A,C) and female (B,D) mice at 20, 30, 40, and 50 weeks of age. Data are presented as the mean percentage of the total Movat's stained sections per lesion per mouse exhibiting the listed marker ± SE, $p<0.05$ vs littermate controls.

	BONE MARROW TRANSPLANT			
	20 weeks post transplant		30 weeks post transplant	
	***Apo E-/-/Gclm-/-* donor (n=16)**	***ApoE-/-* donor (n=15)**	***Apo E-/-/Gclm-/-* donor (n=8)**	***ApoE-/-* donor (n=8)**
Foam cells	31.4% ± 8.9%	51.8% ± 11.6%	24.6% ± 9%	36.1% ± 14.5%
Thin fibrous cap	39.3% ± 9.3% *	11.4% ± 5.7%	23.7% ± 10%	14.3% ± 8.6%
Large necrotic core	40% ± 9.6%	26.8% ± 7.9%	54.4% ± 11.6%	56.4% ± 13.9%
Cholesterol clefts	63.6% ± 10.4%	43.4% ± 9%	93% ± 3.7% *	61.4% ± 10.6%
Lateral xanthoma	53.9% ± 12%	38.2% ± 10.7%	90.5% ± 5.4% *	59.4% ± 13.3%
Hemorrhage	4.4% ± 3.6%	2.7% ± 2.7%	20% ± 8.2%	10.1% ± 8.3%
Medial thickening	100%	100%	100%	100%
Chondrocytes	64.1% ± 10.6% *	32.7% ± 11.1%	68.5% ± 12.1%	68.3% ± 13.1%
Calcification	29.1% ± 10%	16.1% ± 8.2%	33.7% ± 12.5% *	16.7% ± 12.2%

	***ApoE-/-/Gclc--Tg* donor (n=16)**	***ApoE-/-* donor (n=15)**	***ApoE-/-/Gclc-Tg* donor (n=12)**	***ApoE-/-* donor (n=8)**
Foam cells	85% ± 7.2% *	51.8% ± 11.6%	56.7% ± 12.7%*	36.1% ± 14.5%
Thin fibrous cap	14.7% ± 5.3%	11.4% ± 5.7%	15.7% ± 9.2%	14.3% ± 8.6%
Large necrotic core	27.7% ± 7.5%	26.8% ± 7.9%	45.1% ± 10%*	56.4% ± 13.9%
Cholesterol clefts	39.2% ± 8.6%	43.4% ± 9%	59.4% ± 9.6%	61.4% ± 10.6%
Lateral xanthomas	15.3% ± 7.4%	38.2% ± 10.7%	75.1% ± 10.4%	59.4% ± 13.3%
Hemorrhage	0%	2.7% ± 2.7%	11.6% ± 5.7%	10.1% ± 8.3%
Medial thickening	100%	100%	100%	100%
Chondrocytes	19.3% ± 8.7% *	32.7% ± 11.1%	50.7% ± 12%*	68.3% ± 13.1%
Calcification	0% *	16.1% ± 8.2%	17.4% ± 6.6%	16.7% ± 12.2%

Supplemental Table II. Composition of the Lesions in the Innominate Arteries Following Bone Marrow Transplantation.

The frequency of features of plaque composition were measured in female *apoE-/- recipient mice* at 20 and 30 weeks post-transplant. Data are presented as the mean percentage of the total Movat's stained sections per lesion per mouse exhibiting the listed marker ± SE, $p<0.05$ vs littermate controls.

Supplemental Figure Legends

Supplemental Figure I. Western Blot of GCLC Protein in Peritoneal Macrophages from *apoE-/-/Gclc-Tg* and *apo E-/-* Littermate Control Mice.

Western Blot. Line 1: molecular weight markers. Lines 2 and 3: extracts of thioglycollate elicited peritoneal macrophages from 2 *apoE-/-/Gclc-Tg* mice. Line 4: extract of peritoneal macrophages from *apoE-/-* littermate control mouse.

Supplemental Figure II. Smooth Muscle Cell Content of the Lesions in the Innominate Arteries.

Area of smooth muscle actin staining as a percentage of the total area of the lesions in both male (A, B) and female (C, D) mice. Data are presented as means ± SE.

Supplemental Figure III. Macrophage and Smooth Muscle Imunostaining in the Innominate Arteries.

A*poE-/-/Gclm-/-* and *Apo E-/-* Littermate Controls (A-H). Representative examples of immunoperoxidase staining for smooth muscle actin and the macrophage-specific marker Mac2 in the innominate arteries of 40 week old mice.

Smooth muscle actin: a*poE-/-/Gclm-/-* male (A), a*poE-/-/Gclm-/-* female (B) a*poE-/-/Gclm*+/+ male (C), a*poE-/-/Gclm*+/+ female (D).

Mac2: a*poE-/-/Gclm-/-* male (E), a*poE-/-/Gclm-/-* female (F), a*poE-/-/Gclm*+/+ male (G), a*poE-/-/Gclm*+/+ female (H).

A*poE-/-/Gclc-Tg* mice and *Apo E-/-* Littermate Controls (I-P). Representative examples of immunoperoxidase staining for smooth muscle actin and the macrophage-specific marker Mac2 in the innominate arteries of 30-week-old mice.

Smooth muscle actin; a*poE-/-/Gclc-Tg* male (I), a*poE-/-/Gclc-Tg* female (J), *apoE-/-/Gclc-WT* male (K), *apoE-/-/Gclc-WT* female (L).

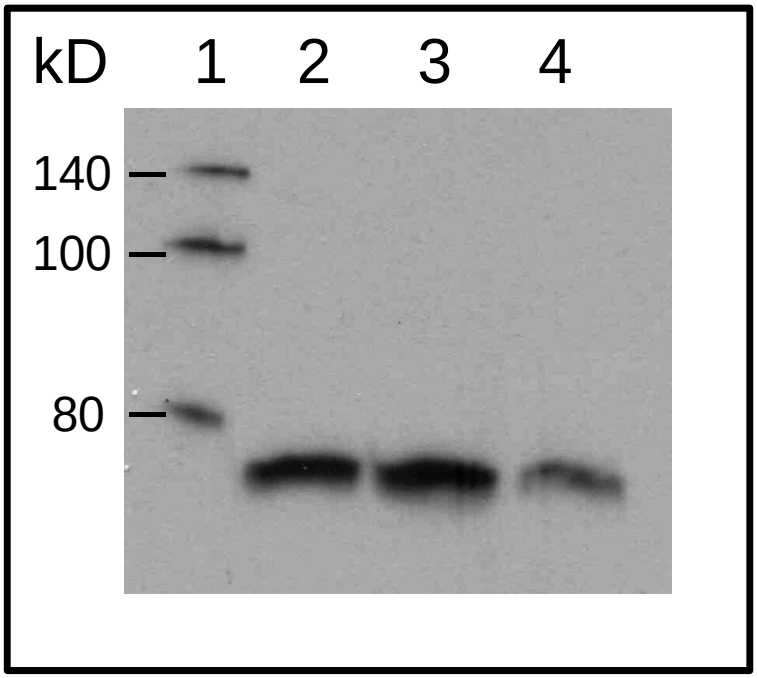
Mac2; a*poE-/-/Gclc-Tg* male (M), a*poE-/-/Gclc-Tg* female (N), *apoE-/-/Gclc-WT* male (O), *apoE-/-/Gclc-WT* female (P).

Supplemental Figure IV. Plasma Lipoprotein Cholesterol Profiles for A*poE-/-/Gclm-/-*, A*poE-/-/Gclc-Tg*, and Littermate Control *Apo E-/-* Mice.

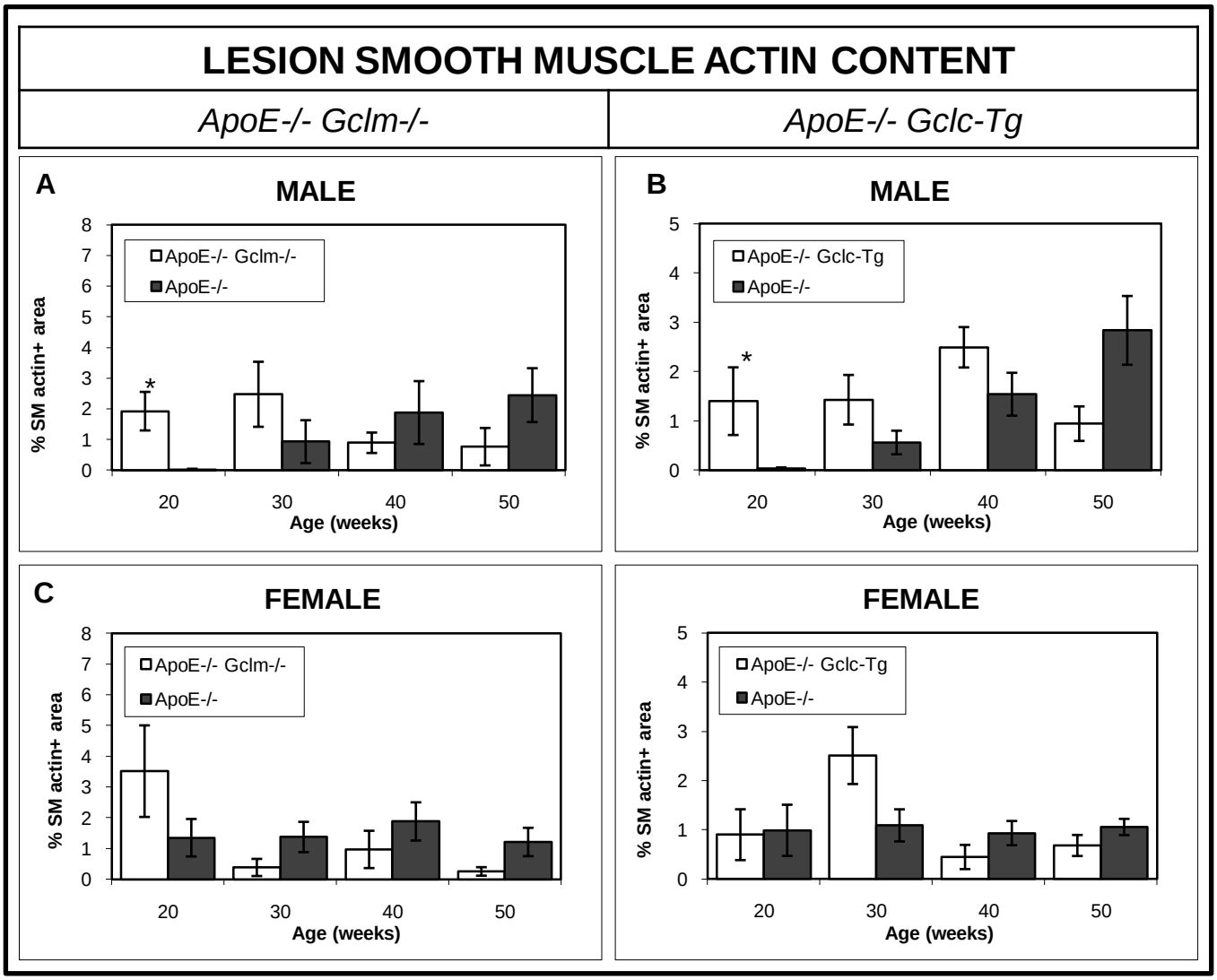
Lipoprotein cholesterol was measured in each fraction following FPLC separation of the plasma in 3 mice from each group at 20 and 50 weeks of age (A,B males; C,D females).

Supplemental Figure V. Plasma Lipoprotein Cholesterol Profiles for *Apo E-/-* Mice Transplanted with Bone Marrow from A*poE-/-/Gclm-/-* or *Apo E-/-* Littermate Control Mice.

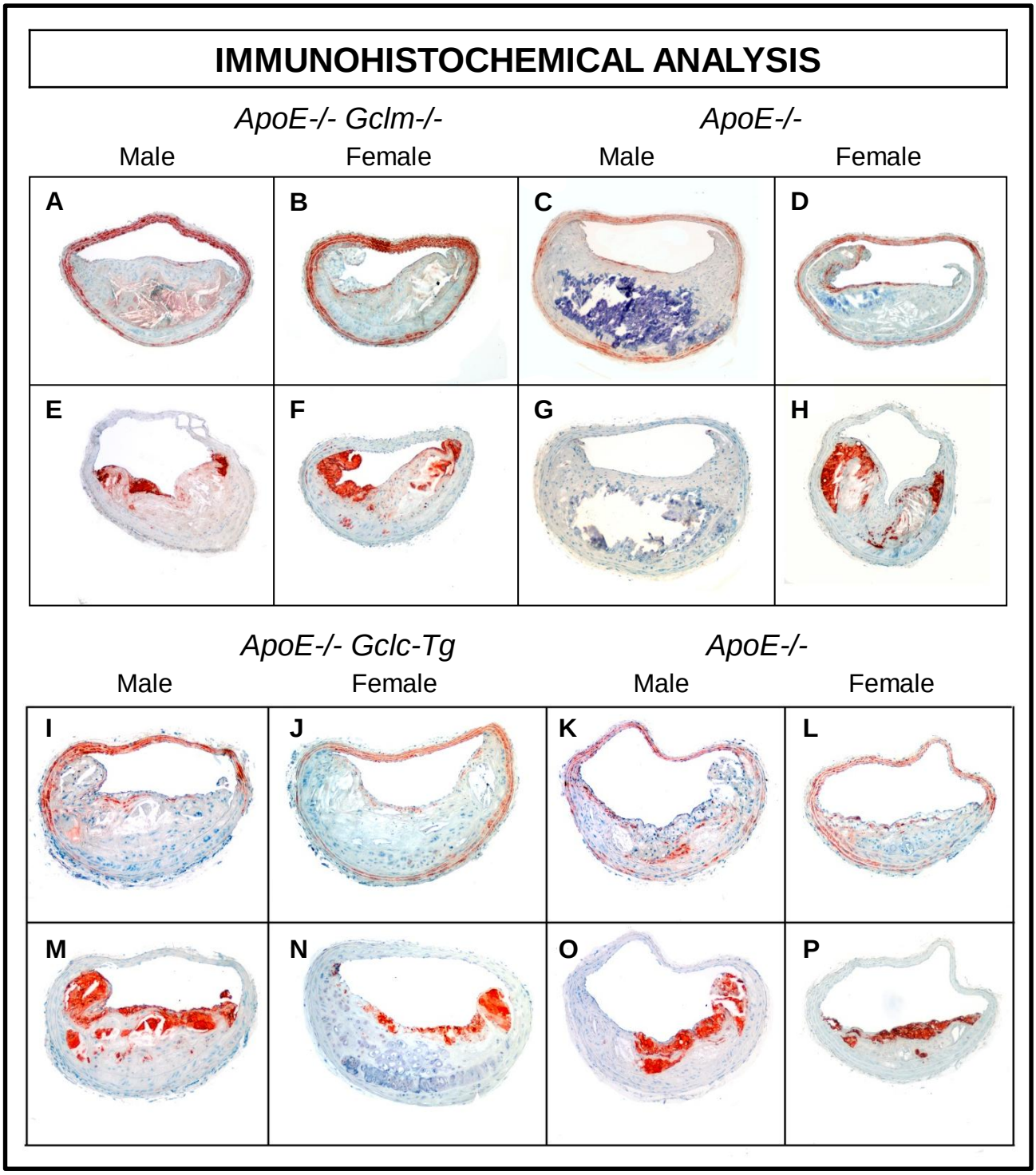
Lipoprotein cholesterol was measured in each fraction following FPLC separation of the plasma pooled from 7 apo E-/- mice transplanted with bone marrow from a*poE-/-/Gclm-/-* mice and 14 *apo E-/-* mice transplanted with bone marrow from littermate *apo E-/-* control mice. The plasma was obtained from mice sacrificed 20 weeks following bone marrow transplant.



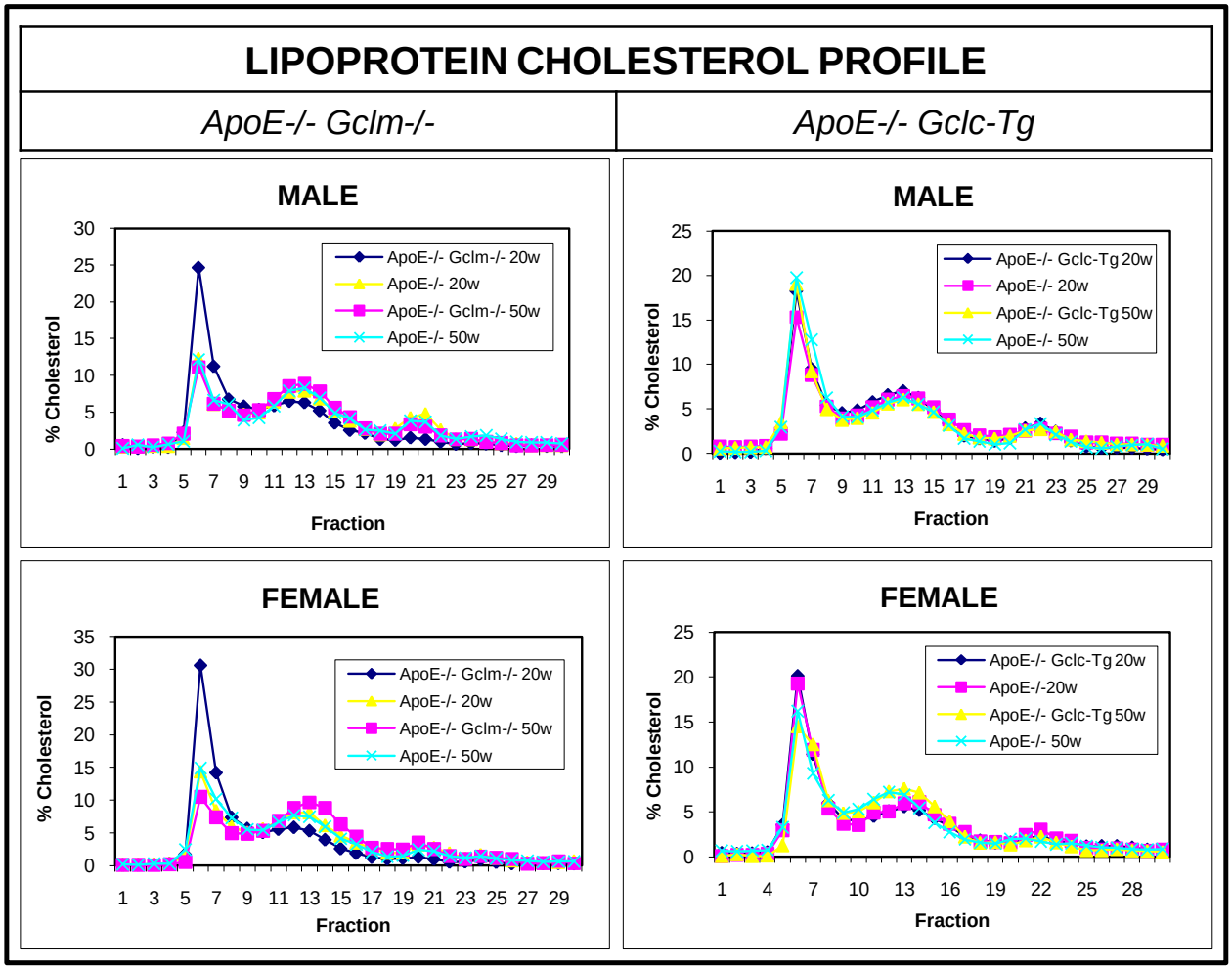
Supplemental Figure I



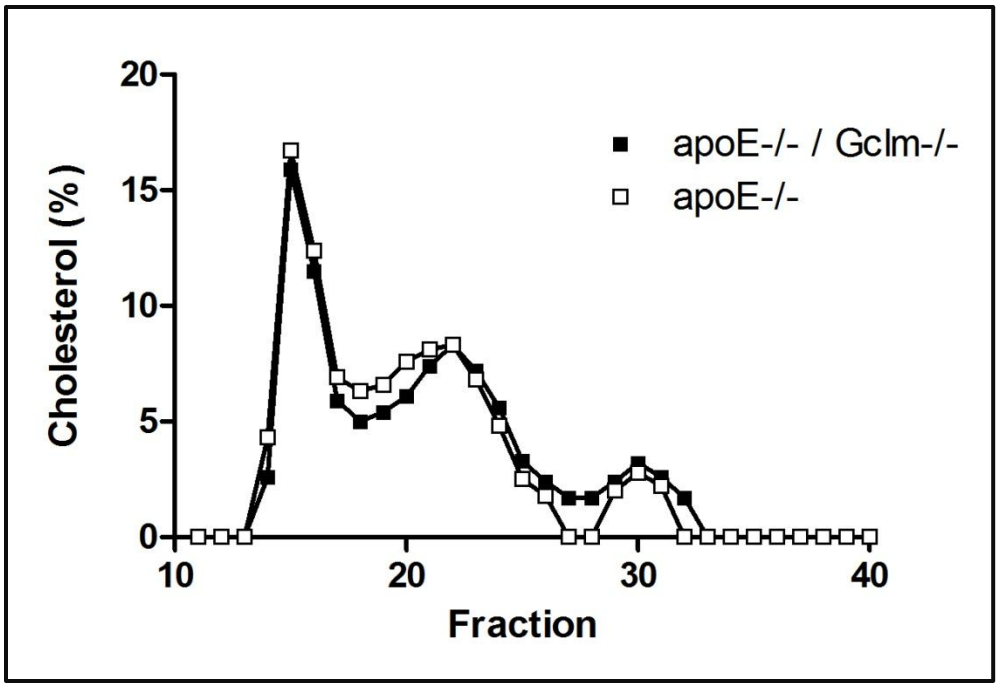
Supplemental Figure II.



Supplemental Figure III.



Supplemental Figure IV.



Supplemental Figure V.