**Acid Sphingomyelinase Promotes Lipoprotein Retention within Early Atheromata and Accelerates Lesion Progression**

Cecilia M. Devlin, Andrew R. Leventhal, George Kuriakose, Edward H. Schuchman, Kevin Jon Williams, Ira Tabas

From the Departments of Medicine (C.M.D., A.R.L., G.K., I.T.), Pathology & Cell Biology (I.T.), and Physiology & Cellular Biophysics (I.T.), Columbia University, New York, NY 10032; the Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, New York, NY 10029 (E.H.S.); and the Division of Endocrinology, Diabetes, and Metabolic Diseases, Department of Medicine, Jefferson Medical College of Thomas Jefferson University, Philadelphia, PA 19107 (K.J.W.)

**Online publication information**

**Supplementary Materials and Methods**

**Acid SMase Activity Assay**

Concanavalin A-elicited macrophages were assayed for acid SMase activity using [choline-methyl-$^{14}$C] sphingomyelin as substrate as previously described.[1]

**Plasma Lipoprotein Analysis and Aortic Root Atherosclerosis Assay**

Mice were fasted for 16 h prior to euthanasia, at which point they were anesthetized with isoflurane and exsanguinated through cardiac puncture. Total plasma cholesterol concentrations were determined using enzymatic kits from Wako Chemicals GmbH (Neuss, Germany). Plasma high-density lipoprotein (HDL) cholesterol concentration was determined after dextran sulfate-$Mg^{2+}$ precipitation of apolipoprotein B-containing lipoproteins. Plasma lipoproteins were also analyzed using a fast protein liquid chromatography (FPLC) system equipped with two Superose 6 columns connected in series (Amersham Pharmacia). The cholesterol content of each fraction was measured by enzymatic assay. For lesion analysis, the heart and aorta were removed, perfused with PBS, embedded in OCT compound (Sakura Finetek, Torrance, CA), snap-frozen in an ethanol-dry ice bath, and stored at -70$^{o}$C. Ten-micron sections were prepared at -20$^{o}$C using a Microm microtome cryostat HM505E (Walldorf, Germany). Starting from the aortic

valve leaflets, every eighth section was used for analysis for a total distance of ~400 µm. To evaluate lesion area, the sections were stained with Oil Red O for neutral lipid and with Harris hematoxylin for nuclei. Stained lesions were viewed with a Nikon Labophot-2 microscope equipped with a Sony CCD-Iris/RGB color video camera. Lesion area was quantified using a computerized imaging system and IMAGE-PRO PLUS 4.5.1.29 software. The mean Oil Red O-positive area per section from six sections was determined in a blinded fashion for individual animals.

**Fluorescent Labeling of Lipoproteins**

Lipoproteins of *d*<1.063 were isolated by preparative ultracentrifugation of plasma from either chow-fed *Apoe*-/- mice or Western diet-fed *Ldlr*-/- mice. The lipoproteins were labeled with Alexa Fluor 568 (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Labeled lipoproteins were separated from unincorporated dye by gel filtration chromatography using a Bio Gel P-30 column and then dialysis at 4°C against 150 mM NaCl, 0.3 mM EDTA, pH 7.4. For an additional experiment, *d*<1.063 lipoproteins from *Apoe*-/- mice were labeled with 1,1´-dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) by the method of Pitas *et al.*[2] Labeled lipoproteins were stored at 4°C under argon gas and used within 2 weeks of preparation.

**Co-localization of retained lipoproteins with macrophages and perlecan**

Frozen sections (10-µm thick) of the aortic root were fixed for 10 min in 4% paraformaldehyde in PBS and then blocked for 1 h in 10% normal goat serum in PBS at room temperature. Sections were incubated for 1 h in 1.2 µg rat anti-mouse Mac-3 antibody/ml (BD Biosciences, San Jose, CA) or rat anti-mouse perlecan antibody (Thermo Fisher Scientific Inc., Fremont, CA) in blocking solution at room temperature. After the sections were washed repeatedly in PBS, they were incubated for 1 h at room temperature with 4 µg Alexa Fluor 488-labeled goat anti-rat IgG per ml blocking solution. The sections were then washed with PBS and mounted with the anti-fading reagent, ProLong Gold. Images were obtained with a Zeiss LSM-510 Meta scanning confocal microscope using a 40x objective. Retained lipoproteins were visualized by 543-nm excitation and a BP560-615 emission filter. The presence of macrophages was detected by 488-nm excitation and a BP505-550 emission filter.

**Statistical Analysis**

Data are reported as means ± SEM.  Numbers of samples are listed in the figure legends. Differences between groups were analyzed for statistical significance by the Mann-Whitney non-parametric test, which is valid for both normally and non-normally distributed data.

## References


1. Schissel SL, Schuchman EH, Williams KJ, Tabas I. $Zn^{2+}$-stimulated sphingomyelinase is secreted by many cell types and is a product of the acid sphingomyelinase gene. *Journal of Biological Chemistry*. 1996;271:18431-18436.

2. Pitas RE, Innerarity TL, Weinstein JN, Mahley RW. Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages *in vitro* by fluorescence microscopy. *Arteriosclerosis*. 1981;1:177-185.

## Supplementary Figure Legends

**Figure I. Acid SMase activity in macrophages from wild-type and Asm-/- mice.** Peritoneal macrophages from *Asm*+/+ wild-type mice and from *Asm*-/- mice were harvested and assayed for acid SMase activity as described in Materials and Methods.

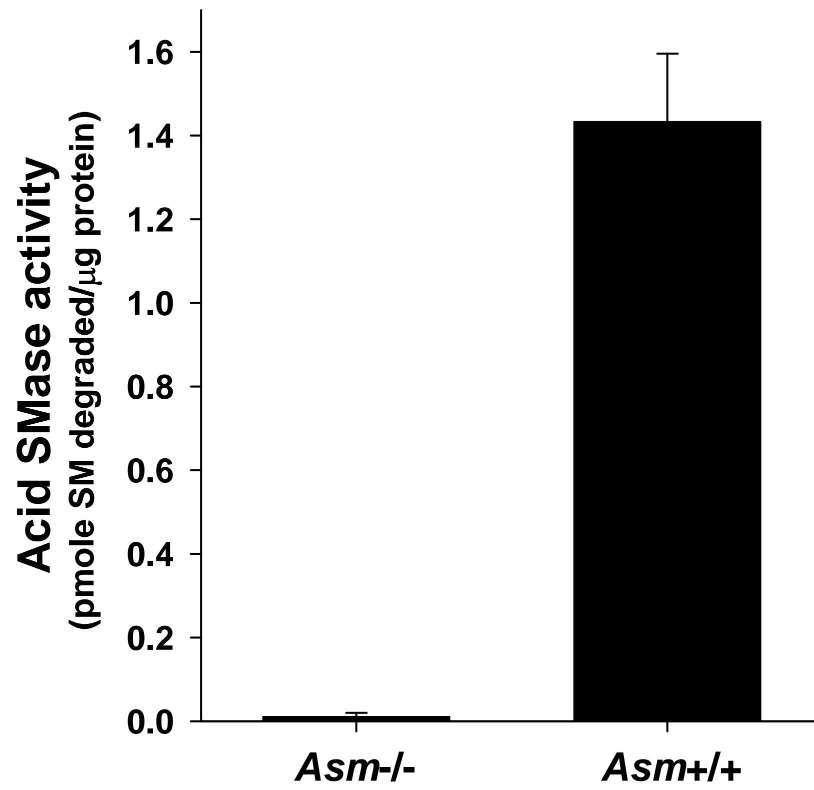
**Figure II. Acid SMase deficiency does not affect plasma cholesterol or lipoprotein profile in chow-fed *Apoe*-/- mice.** ***A***, Total plasma cholesterol values and FPLC lipoprotein profiles for littermate female *Asm*+/+;*Apoe*-/- (n = 10) and *Asm*-/-;*Apoe*-/- (n = 15) mice. ***B***, As in *A*, for male mice, where n = 9 for *Asm*+/+;*Apoe*-/- mice and n = 14 for *Asm*-/-;*Apoe*-/- mice. None of the differences between groups were statistically significant. ***C***, Body weights of the mice described in *A* and *B*. The differences between the two genotypes within each sex group were not statistically significant.

**Figure III. SDS-PAGE and agarose gels of *d*<1.063 unlabeled and labeled lipoproteins from *Apoe*-/- mice.** Lipoproteins (*d*<1.063) from the plasma of *Apoe*-/- mice were labeled as described in Materials and Methods and analyzed by SDS-PAGE (***A***) and agarose gel electrophoresis (***B***). The SDS-PAGE gel was stained with Coomassie Brilliant Blue G-250. The agarose gel electrophoresis was run in sodium barbital buffer and stained with Sudan red 7B.

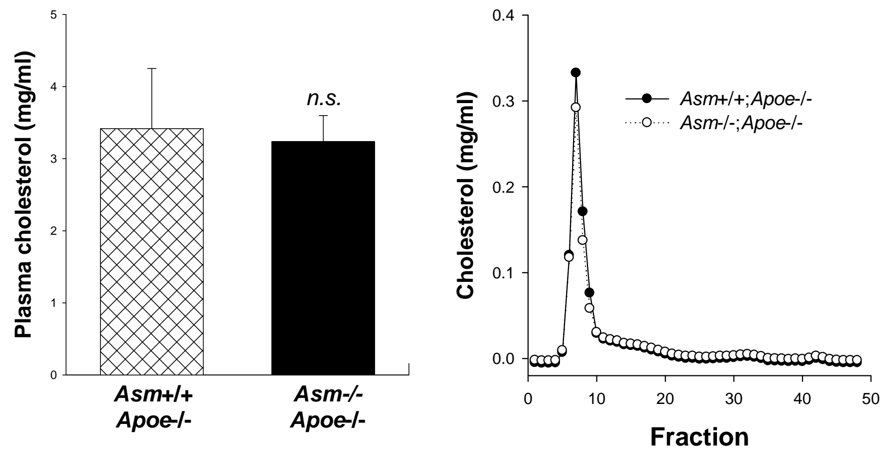
**Figure IV. Body weights of *Asm*+/+;*Ldlr*-/- and *Asm*-/-;*Ldlr*-/- mice.** The differences between the two genotypes within each sex group were not statistically significant.

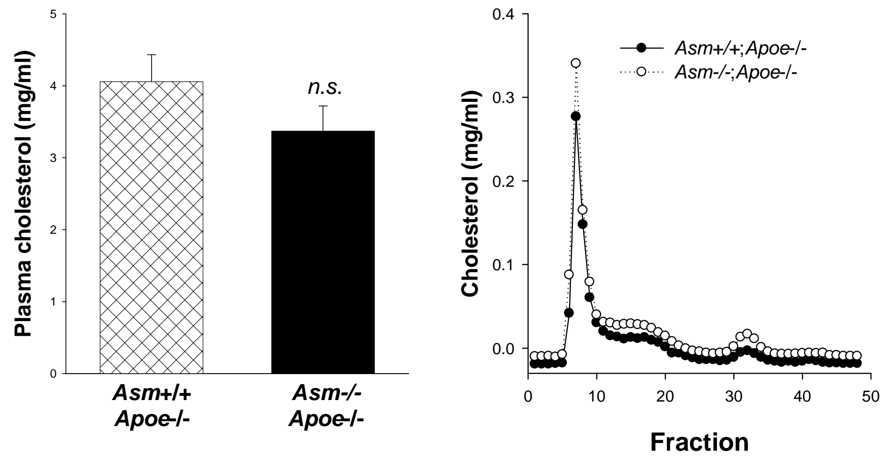
**Figure V. SDS-PAGE and agarose gels of *d*<1.063 unlabeled lipoproteins from *Asm*+/+;*Ldlr*-/- and *Asm*-/-;*Ldlr*-/- mice.** Lipoproteins (*d*<1.063) from the plasma of *Asm*+/+;*Ldlr*-/- and *Asm*-/-;*Ldlr*-/- mice were analyzed by SDS-PAGE (*A*) and agarose gel electrophoresis (*B*). In *A*, the samples delineated by the brackets were run in duplicate. The SDS-PAGE gel was stained with Coomassie Brilliant Blue G-250. The agarose gel electrophoresis was run in sodium barbital buffer and stained with Sudan red 7B.

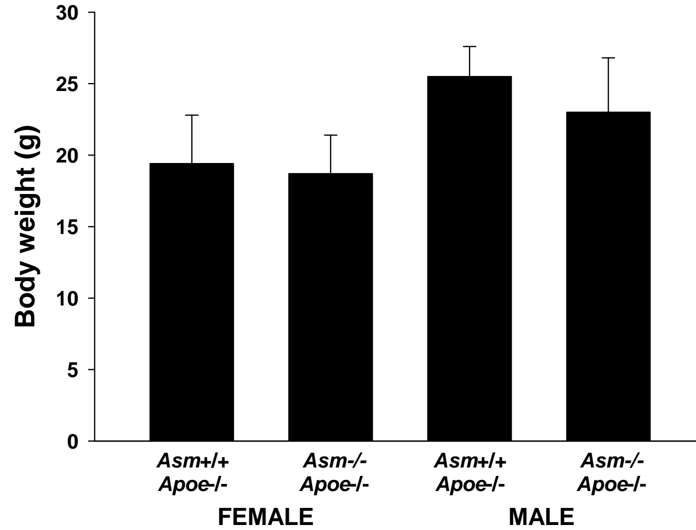
**Figure VI. Partial co-location of lesional LDL with perlecan in *Ldlr*-/- lesions.** A lesional section from an Alexa Fluor 568-labeled LDL-perfused *Ldlr*-/- mouse from the same experiment shown in Fig. 5 was stained with anti-perlecan antibody and Alexa Fluor 488-labeled (green) secondary antibody. The section was viewed for Alexa 488 (perlecan), Alexa 568 (LDL), and phase. Another section stained with non-immune rat IgG2a antibody (control for the anti-perlecan antibody) showed no green signal.



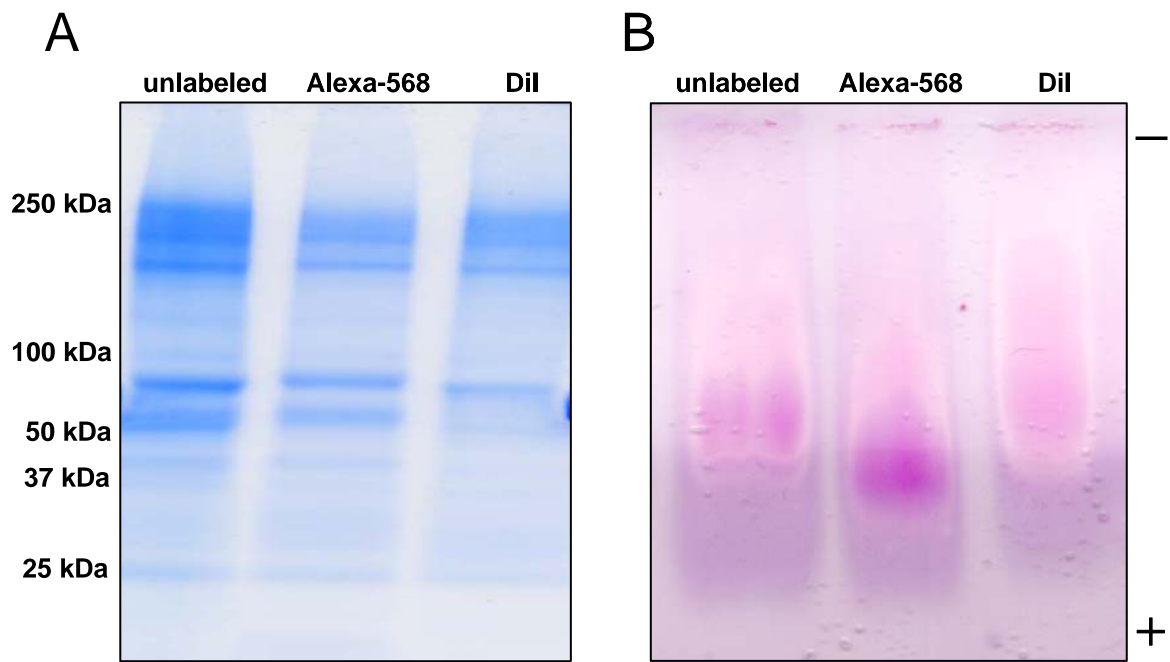

**Devlin *et al.* Figure I**




**Devlin *et al.* Figure II**

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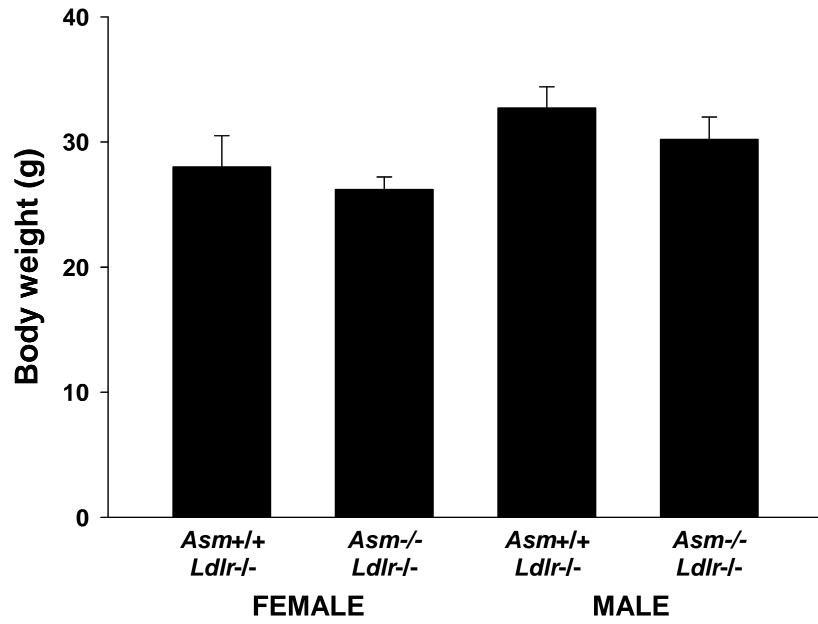
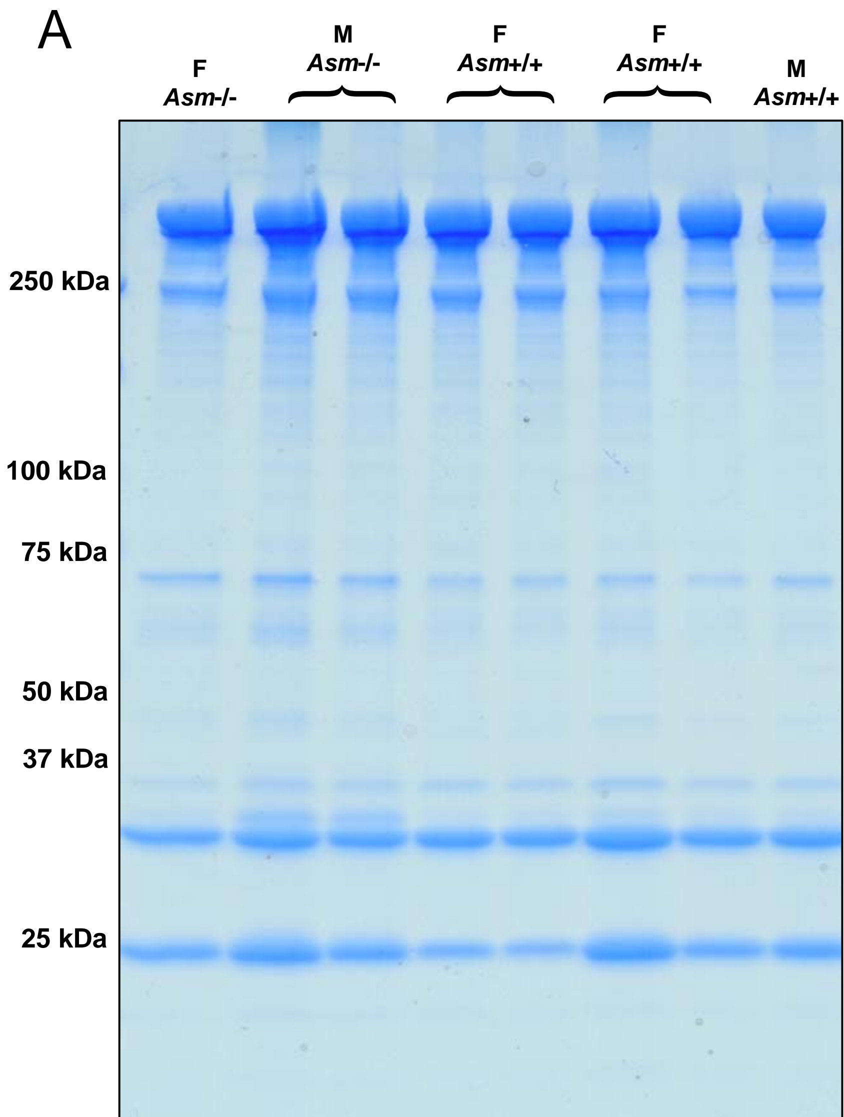
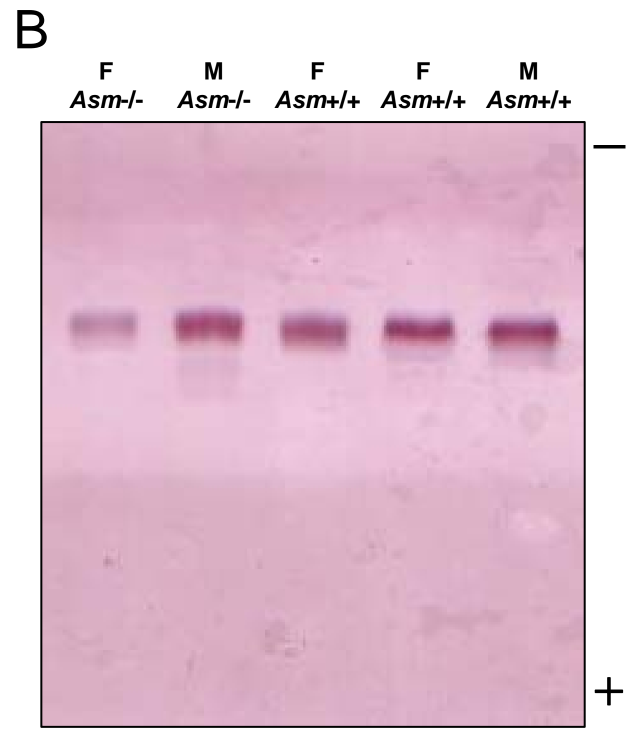


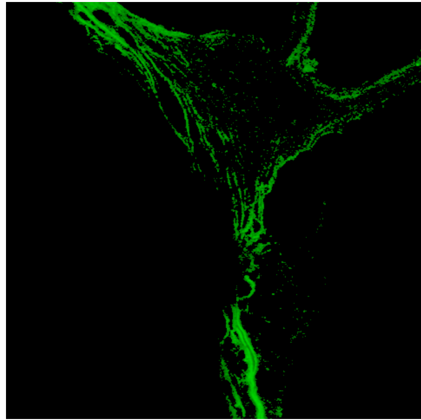

**Devlin *et al.* Figure IV**

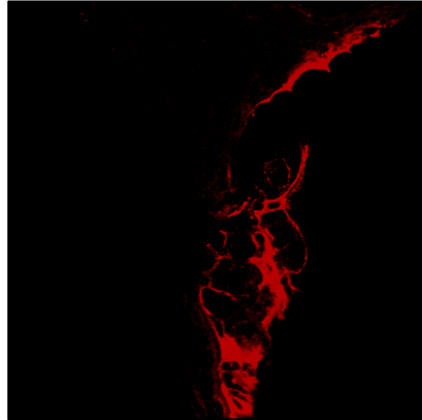


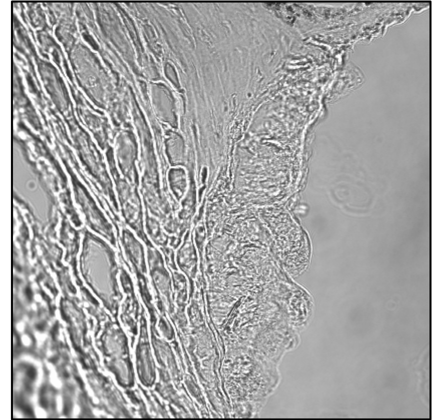




**Devlin *et al.* Figure V**

## *Ldlr-/-* Lesions







perlecan LDL phase

**Devlin *et al.* Figure VI**