*Clinical Chemistry* 51:2
351–359 (2005)



# The Novel Apolipoprotein A5 Is Present in Human Serum, Is Associated with VLDL, HDL, and Chylomicrons, and Circulates at Very Low Concentrations Compared with Other Apolipoproteins

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***Background:*** **The recently discovered apolipoprotein A5 (ApoA5) is fast gaining attention as a key regulator of serum triglyceride concentrations. An ApoA5 mouse knock-out model produced an approximately fourfold increase in serum triglycerides, whereas a knock-in model with human ApoA5 produced 50–70% lower concentrations of mouse serum triglycerides. In addition, peroxisome proliferator-activated receptor-$\alpha$ agonists, which are used clinically to lower serum triglyceride concentrations, cause increased ApoA5 mRNA expression. Despite these compelling molecular biology data, relatively little is known about ApoA5 protein in human serum.**

***Methods:*** **To better understand circulating concentrations and lipoprotein particle distribution of ApoA5, we expressed the recombinant human ApoA5 protein and raised antibodies against both the $NH_2$ and COOH termini.**

***Results:*** **Using the above reagents, we demonstrate for the first time that ApoA5 is present in human serum, although at much lower concentrations than other apolipoproteins such as ApoA1. Using a dual-antibody sandwich ELISA that we developed, we observed ApoA5 concentrations in human serum ranging from 24 to 406 $\mu$g/L compared with ~1 g/L for ApoA1. We also examined the lipoprotein particle distribution of ApoA5 and found that ApoA5 was detectable in VLDL, HDL, and chylomicrons, but not LDL.**

***Conclusions:*** **These data demonstrate for the first time that ApoA5 is a secreted protein present in human serum and is associated with specific lipoprotein particles. In addition, our data indicate that the circulating concentration of human ApoA5 is very low compared with other apolipoproteins.**



The recently discovered apolipoprotein A5 (ApoA5)[3] is rapidly being recognized as a key regulator of serum triglyceride concentrations [for a complete review, see Refs. *(1, 2)*]. The gene for this novel apolipoprotein was originally identified by experiments looking for new open reading frames in the ApoA1-ApoC3-ApoA4 gene cluster, which is located on human chromosome 11q23 *(3, 4)*. What emerged from this search was a new gene that appeared to code for an apolipoprotein with greatest homology to ApoA4; the new protein was named ApoA5 *(3, 4)*.

When the corresponding human gene for ApoA5 was expressed in transgenic mice, triglyceride concentrations decreased by 50–70%, and when the mouse ApoA5 gene itself was knocked out, triglyceride concentrations in-

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Received July 27, 2004; accepted October 15, 2004.
Previously published online at DOI: 10.1373/clinchem.2004.040824

[3] Nonstandard abbreviations: Apo, apolipoprotein; PPAR-$\alpha$, peroxisome proliferator-activated receptor-$\alpha$; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LC/MS/MS, liquid chromatography–tandem mass spectrometry; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; and TBST, Tris-buffered saline-Tween.

creased approximately fourfold *(4, 5)*. These data suggested that ApoA5 expression may be highly and inversely correlated with triglyceride concentrations. In addition, in humans, identified polymorphisms in the ApoA5 gene have been shown to correlate with increases in circulating triglyceride concentrations *(6–21)*. It was also recently shown that ApoA5 mRNA expression is regulated by peroxisome proliferator-activated receptor-$\alpha$ (PPAR-$\alpha$) agonists, suggesting that ApoA5 may be the long-sought mechanism by which this class of compounds decreases serum triglycerides *(22, 23)*.

Despite these compelling data, very little is known about ApoA5 expression at the protein level in human serum. To determine whether ApoA5 is present in human serum, we expressed the recombinant human protein and raised antibodies against its $NH_2$ and COOH termini for use as reagents for immunoprecipitations, Western blotting, and the development of a dual-antibody sandwich ELISA. Using these techniques, we demonstrate here for the first time that ApoA5 is present in human serum, estimate its circulating concentration, and describe its lipoprotein particle distribution pattern.

## Materials and Methods

### SERUM SAMPLES AND LIPOPROTEIN FRACTIONS

Human serum samples were obtained from Bioreclamation, Inc. Highly purified lipoprotein fractions were obtained from Athens Research and Technology. Lipoproteins were isolated stepwise by sequential flotation ultracentrifugation. Plasma density was adjusted by adding KBr, and centrifugation was performed with a fixed-angle rotor (Type 50.2 Ti) in a Beckman L8-M ultracentrifuge. The isolated lipoprotein fractions were dialyzed exhaustively in 150 mmol/L NaCl–0.1 g/L EDTA, pH 7.4, to remove KBr and then sterile-filtered (0.2 $\mu$m Whatman 25 mm GD/X disposable filter). A second step was required for the preparation of HDL. After ultracentrifugation and dialysis, HDL was chromatographed on a Superdex 200 column (HiLoad 26/60; previously Amersham Biosciences, now GE Health Care) to remove albumin and other contaminants. Lipoprotein classes were verified, and freedom from contamination by other lipoprotein classes was determined by agarose electrophoresis and staining with Fat Red 7B (Titan Gel Lipoprotein Kit; Helena Laboratories). With this method, VLDL was collected at a density of 0.95–1.006 kg/L and LDL at a density of 1.02–1.063 kg/L.

### RECOMBINANT ApoA5 CLONING AND EXPRESSION

Human ApoA5 (Clone LI1138_D12; NM_052968) was purchased form OriGene Technologies, Inc. Plasmid DNA was propagated in *Escherichia coli* XL-1 Blue (Stratagene) and used as a template for mutagenic PCR to create the bacterial expression plasmid pWA25. The complete coding sequence of human ApoA5 was amplified by use of the forward primer 5′-GTAATGGCAAGCCATATGGCTGCCGTG-3′ and the reverse primer 5′-GGCAGGTGGATCCTCAGGGGTCCCC-3′ with the following temperature profile: one 5-min cycle at 94 °C followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. The ~1 kb PCR product was subcloned into pCRScript (Stratagene), then into pET16b (Novagen), by use of *Bam*HI and *Nde*I.

The new construct contained the entire ApoA5 coding region, with a $His_{10}$ epitope tag inserted upstream. We next transformed *E. coli* BL21 (DE3) pLysS (Novagen) with pWA25 and propagated an isolated colony overnight at 37 °C with aeration in Terrific Broth (per liter, 12 g of Bacto tryptone, 24 g of Bacto yeast extract, 4 mL of glycerol, 0.17 mol of $KH_2PO_4$, and 0.72 mol $K_2HPO_4$) supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. We diluted 1 mL of the overnight culture 1:250 in fresh Terrific Broth supplemented with 100 mg/L ampicillin and incubated the sample at 37 °C with shaking at 200 rpm. When the $A_{600}$ reached 0.400, we added isopropyl-$\beta$-D-thiogalactopyranoside to a final concentration of 1.0 mmol/L. After 3 h, cells were harvested by centrifugation, and the pellet was stored at −80 °C until needed.

### PURIFICATION OF ApoA5

We suspended a 5-g cell pellet in 12 mL of B-PER (Bacterial Protein Extraction Reagent; Pierce Chemical Co.) supplemented with 10 mg/L lysozyme and 10 mg/L DNase. The cell suspension was sonicated for 2 min with interspersed cooling cycles and centrifuged at 10 000*g* for 30 min at 4 °C to pellet unbroken cells and debris. The supernatant was mixed with an equal volume of 2× binding buffer (80 mmol/L phosphate, 1.0 mol/L NaCl, 12 mol/L urea, pH 7.40) and passed through a 0.45 $\mu$m filter. The lysate was added to a 5.0-mL $Ni^{+2}$ column and eluted with a linear gradient of elution buffer (40 mmol/L phosphate, 0.5 mol/L NaCl, 6 mol/L urea, and 0.5 mol/L imidazole) that increased from 0% to 100% over 60 min. Fractions were analyzed for ApoA5 protein by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and combined for dialysis overnight against distilled water at 4 °C. The resulting precipitate was dissolved in 0.1 mol/L HCl and, after quantification, was stored at −80 °C until needed.

### LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY ANALYSIS OF PURIFIED ApoA5 AND LIPOPROTEIN FRACTIONS

After one-dimensional electrophoresis (as described below), the gel was stained with colloidal Coomassie Blue (Novex), and the putative ApoA5 band was excised. In-gel digestion and liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis were performed as described previously *(24)* to confirm the identity of the recombinant protein. In addition, highly purified lipoprotein fractions were also analyzed by LC/MS/MS to determine whether any ApoA5 peptides could

be detected in the individual fractions. Data from the mass spectrometer were processed for signal quality, and protein identification was performed with Sequest software (Thermofinigan).

### preparation of ApoA5 peptides and conjugation to carrier proteins

Peptides corresponding to the N-terminal hydrophilic region and the C-terminal hydrophilic region of the human ApoA5 amino acid sequence were synthesized (Anaspec, Inc.). Terminal cysteine residues were added at the time of synthesis to allow directional conjugation to carrier proteins and chromatography beads. Chicken egg albumin (Sigma), keyhole limpet hemocyanin (Sigma), and chicken globulin (Sigma) were activated for conjugation with a 10-fold molar excess of Sulpho-SMCC (Pierce). Free SMCC was removed from Carrier-SMCC conjugates by chromatography through Sephadex G-50 equilibrated in phosphate-buffered saline (PBS; 138 mmol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L $KH_2PO_4$, 8.0 mmol/L $Na_2HPO_4$). The first peak was collected, and the protein concentration was determined by measuring absorbance at 280 nm. Peptides were conjugated to activated carriers with a 10-fold molar excess of peptide by incubation at room temperature for 2 h.

### production and affinity purification of anti-ApoA5 antisera

Four rabbits per antigen were immunized every 21 days with 100 μg of peptide carrier conjugates in Freund's adjuvant. Animals were bled 10 days after booster injections beginning after the third injection. All polyclonal antisera were tested by Western blotting against recombinant ApoA5 protein and found to be approximately equally reactive. As a result, polyclonal anti-C-terminal ApoA5 antisera were pooled, and polyclonal anti-N-terminal ApoA5 antisera were pooled. Afterward, anti-C- and anti-N-terminal ApoA5 polyclonal antibodies were affinity-purified separately against their respective immunizing peptides. For affinity column preparation, 5 mg of each peptide was conjugated to 5 mL of hydrated activated thiol Sepharose 4B beads (Amersham) in a total volume of 10 mL of 0.1 mol/L Tris-HCl–0.2 mol/L NaCl, pH 7.50. The conjugation reaction mixture was incubated overnight at 4 °C. The column was then washed thoroughly before being loaded with serum. Approximately 10 mL of rabbit sera was mixed with an equal volume of PBS, incubated with the affinity beads overnight at 4 °C, washed extensively, and eluted with low-pH buffer (60% 0.6 mol/L acetic acid, 40% 1× PBS). The eluate was neutralized with 2 mol/L Tris base and dialyzed four times with 1 L of PBS. The purified antibody was then concentrated with an Amicon stir cell concentrator (YM30 membrane), and the yield was determined by absorbance at 280 nm. After affinity purification of anti-C- and anti-N-terminal ApoA5 antibodies, a portion of each was labeled with horseradish peroxidase (HRP) with use of Pierce reagents.

### immunoprecipitation of ApoA5

For each immunoprecipitation, 100 μL of serum or lipoprotein fraction was added to 900 μL of lysis buffer (50 mmol/L HEPES, pH 7.40; 150 mmol/L NaCl; 10 mL/L Triton X-100; 5 mmol/L EDTA; 5 mmol/L EGTA; 20 mmol/L NaF, 20 mmol/L $Na_4P_2O_7$) in 1.5-mL Eppendorf tubes. ApoA5 was immunoprecipitated overnight with 1 μg of anti-ApoA5 (C- and N-terminal) antibodies covalently coupled to protein A beads. Afterward, beads were washed twice with lysis buffer, and 40 μL of 2× sample buffer (100 mmol/L Tris, pH 6.80; 40 g/L SDS; 20 mL/L glycerol; 20 μg/L bromphenol blue; 15 g/L dithiothreitol) was added to each tube. Samples were vortex-mixed, boiled for 5 min, and stored at −20 °C before subsequent analysis.

### western blotting

Samples were loaded on SDS-polyacrylamide gels. Colored molecular weight markers (Amersham) were run on each gel. Proteins were separated for 1 h at 175 V at room temperature and transferred to ECL nitrocellulose paper (Amersham) for 1 h (100 V at 4 °C). Nitrocellulose blots were blocked for 1 h at room temperature in Tris-buffered saline-casein blocking buffer (Pierce) containing Tween 20 (TBST; 10 mmol/L Tris, pH 7.40; 150 mmol/L NaCl; 1 mL/L Tween 20). After blocking, blots were probed with HRP-labeled anti-ApoA5 antibodies (1 mg/L C- and N-terminal) in blocking buffer for 1 h at room temperature. Blots were washed three times (10 min each) with TBST. After washing, blots were developed with ECL reagent. After air-drying, blots were exposed to Bio-Max x-ray film (Kodak).

### elisa design

Two different ELISAs were constructed with the reagents described above. In the first version, wells were coated overnight (Pierce carbonate-bicarbonate coating buffer, pH 9.40) with anti-C-terminal ApoA5 antibody at a concentration of 4 mg/L. The following day, wells were aspirated, washed three times with TBST, and blocked for 1 h with TBS-casein blocking buffer (Pierce). Human serum or recombinant ApoA5 calibrator (100 μL, containing various concentrations of recombinant protein without bound lipid) were diluted 1:2 in assay buffer (50 mmol/L HEPES, pH 7.40; 150 mmol/L NaCl; 10 mL/L Triton X-100; 5 mmol/L EDTA; 5 mmol/L EGTA; 20 mmol/L NaF; 20 mmol/L $Na_4P_2O_7$). After the plate was washed three times with TBST, 200 μL of each diluted sample was added to individual wells (final serum dilution, 1:2) and allowed to incubate for 2 h at room temperature. After aspiration, wells were washed six times with TBST, and 100 μL of a 1:1000 dilution of conjugate antibody (HRP-labeled anti-N-terminal ApoA5 antibody, 1 mg/L) was added to the wells for a 1-h

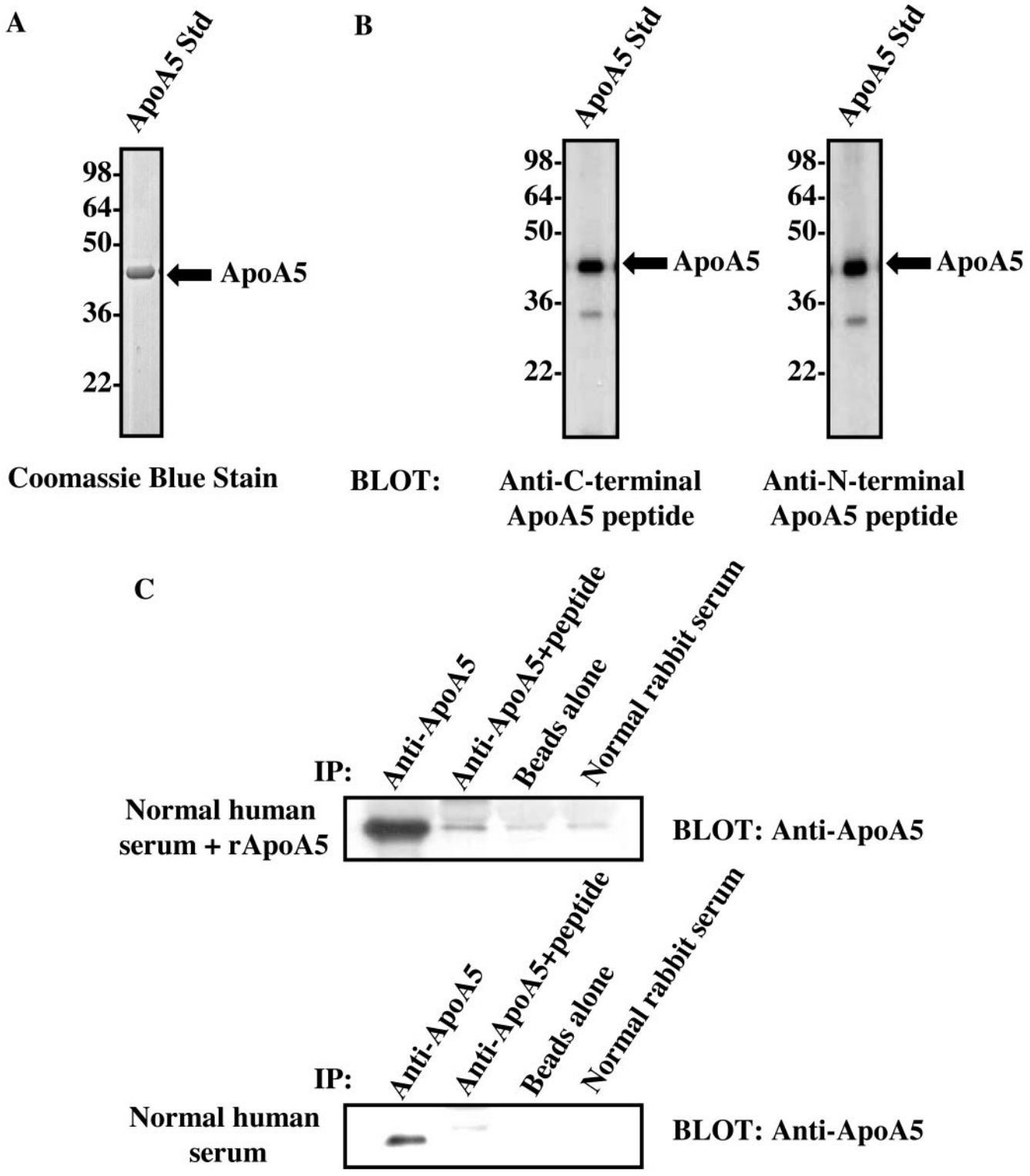


Fig. 1. Expression and purification of recombinant human ApoA5 and generation and characterization of anti-ApoA5 antibodies

(*A*), recombinant ApoA5 was expressed, purified, and separated electrophoretically. The gel was subsequently stained with colloidal Coomassie Blue. (*B*), recombinant ApoA5 protein was separated electrophoretically and subsequently transferred to nitrocellulose for Western blotting with anti-ApoA5 antisera raised against N- and C-terminal peptides. Typical results for N- and C-terminal antisera are shown. Results are representative of two independent experiments. In *A* and *B*, molecular weights (× 1000) are indicated on the *y axis*. (*C*), *top*, 1 μg of recombinant ApoA5 (*rApoA5*) was added to 100 μL of human serum and immunoprecipitated (*IP*) with affinity-purified anti-ApoA5 antibodies in the absence and presence of 10 mg/L immunizing ApoA5 peptide (*lanes 1* and *2*). Immunoprecipitation was also carried out in the absence of any anti-ApoA5 antibody (*lane 3*) and with 5 μL of normal rabbit serum as control (*lane 4*). Afterward, immunoprecipitates were separated electrophoretically and transferred to nitrocellulose for subsequent Western blotting with anti-ApoA5 antibodies. Results are representative of two independent experiments. *Bottom*, endogenous ApoA5 was immunoprecipitated from 100 μL of normal human serum in the absence and presence of 10 mg/L immunizing ApoA5 peptide (*lanes 1* and *2*). Immunoprecipitation was also carried out in the absence of any anti-ApoA5 antibody (*lane 3*) and with 5 μL of normal rabbit serum as control (*lane 4*). Afterward, immunoprecipitates were separated electrophoretically and transferred to nitrocellulose for subsequent Western blotting with anti-ApoA5 antibodies. Results are representative of two independent experiments.

incubation at room temperature. After aspiration, wells were washed six times with TBST. After the last aspiration of TBST, 100 μL of tetramethylbenzidine development substrate (Pierce) was added to each well and allowed to incubate for 30 min at room temperature. The reaction was stopped with an equal volume of 0.67 mol/L phosphoric acid, and plates were read at 450 nm. The second version of the ELISA was identical to the first except that the orientation of the anti-C-terminal ApoA5 antibodies and anti-N-terminal ApoA5 antibodies was reversed. In all ELISA experiments, SigmaPlot (Ver. 8.0) was used for fitting of the calibration curves. ApoA5 ELISA dilution curves for the recombinant calibrator and human serum samples were determined to be parallel.

## Results

The overall strategy for raising anti-ApoA5 antibodies is shown in Fig. 1. Recombinant ApoA5 protein was analyzed by SDS-PAGE followed by Coomassie Blue staining. Expression and subsequent purification of recombinant ApoA5 yielded a single band with an approximate $M_r$ of 40 000, consistent with the predicted $M_r$ of ApoA5 (Fig. 1A). The identity of the recombinant ApoA5 protein was confirmed by excision of a Coomassie-stained gel slice for LC/MS/MS analysis.

The recombinant ApoA5 protein from Fig. 1A was next used as a calibrator to assess anti-N- and C-terminal anti-ApoA5 polyclonal antisera raised against the respective ApoA5 peptides. Antisera raised against both the C- and N-terminal ApoA5 peptides were able to recognize the recombinant ApoA5 protein in Western blotting. The results obtained with two representative antisera (one anti-C-terminal and one anti-N-terminal) are shown in Fig. 1B. As a result, anti-C-terminal ApoA5 antisera were pooled, and anti-N-terminal ApoA5 antisera were pooled. After pooling, anti-N- and C-terminal apoA5 antibodies were affinity-purified and labeled with HRP.

As shown in the top panel of Fig. 1C, the anti-ApoA5 antibodies were able to immunoprecipitate recombinant ApoA5 added to human serum. Importantly, immunoprecipitation of the recombinant ApoA5 was blocked by the addition of excess immunizing peptides. We next performed similar experiments with normal human serum. As shown in the lower panel of Fig. 1C, endogenous ApoA5 could also be successfully immunoprecipitated from 100 μL of normal human serum. The immunoprecipitation was again blocked by the addition of excess immunizing peptides, confirming the specificity of the reactivity.

We then used the affinity-purified antibodies described above to design an ELISA capable of quantifying ApoA5 in human serum. During development of the assay, we found that the preferred orientation of the antibodies was anti-N-terminal ApoA5 as the capture antibody and anti-C-terminal ApoA5 as the conjugate antibody (Fig. 2). On the basis of ELISAs performed with recombinant ApoA5 calibrator with this orientation of antibodies, we originally estimated that the detection limit of the assay was ~10 μg/L. Subsequently, we determined that the limit of detection of the assay was ~17 μg/L based on +3 SD for a zero calibrator (assay buffer only).

To correlate human serum ApoA5 concentrations measured with the ELISA with those obtained by immunoprecipitation and Western blotting, we expanded the original calibration curve obtained with recombinant ApoA5 by first diluting the protein to a concentration of 50 000 μg/L and making serial 1:5 dilutions. We then assayed 10 human serum samples with this final ELISA format (Fig. 3A) and also assayed them by immunoprecipitation with anti-ApoA5 antibodies followed by West-

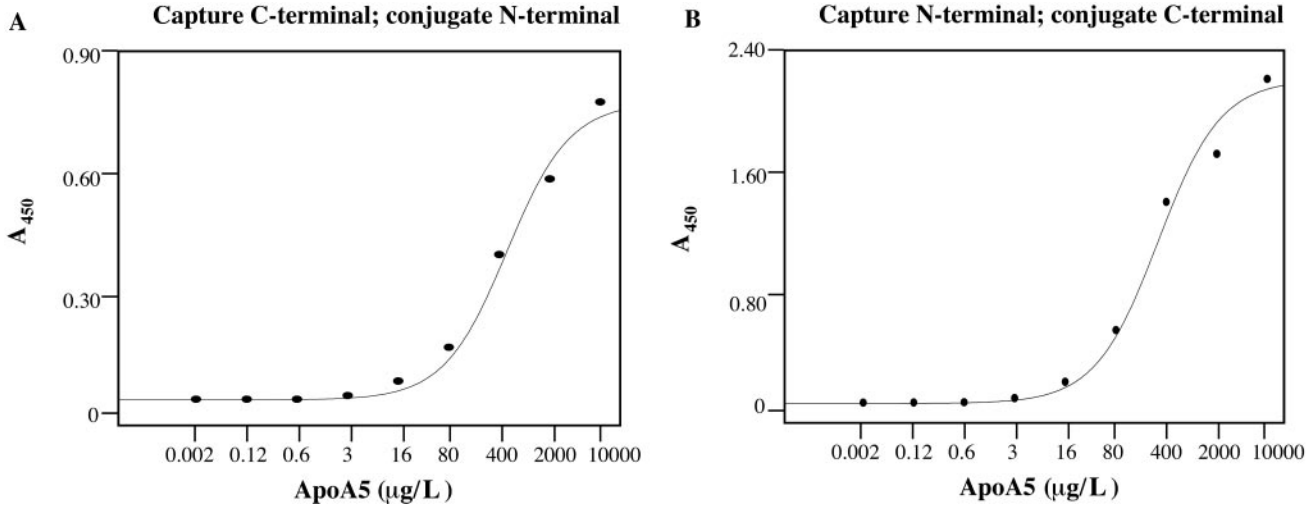


Fig. 2. Anti-ApoA5 antibody orientation is important in ELISA design.

(*A*), a dual-antibody sandwich ELISA was developed to measure ApoA5. Recombinant ApoA5 was used to construct a calibration curve starting at a concentration of 10 000 μg/L with serial 1:5 dilutions. Anti-ApoA5 antibodies were oriented as anti-C-terminal for capture and anti-N-terminal as conjugate. Results are representative of two independent experiments. (*B*), an alternative dual-antibody sandwich ELISA for ApoA5 was developed as above except that the antibody orientation was anti-N-terminal for capture and anti-C-terminal as conjugate. Results are representative of two independent experiments.

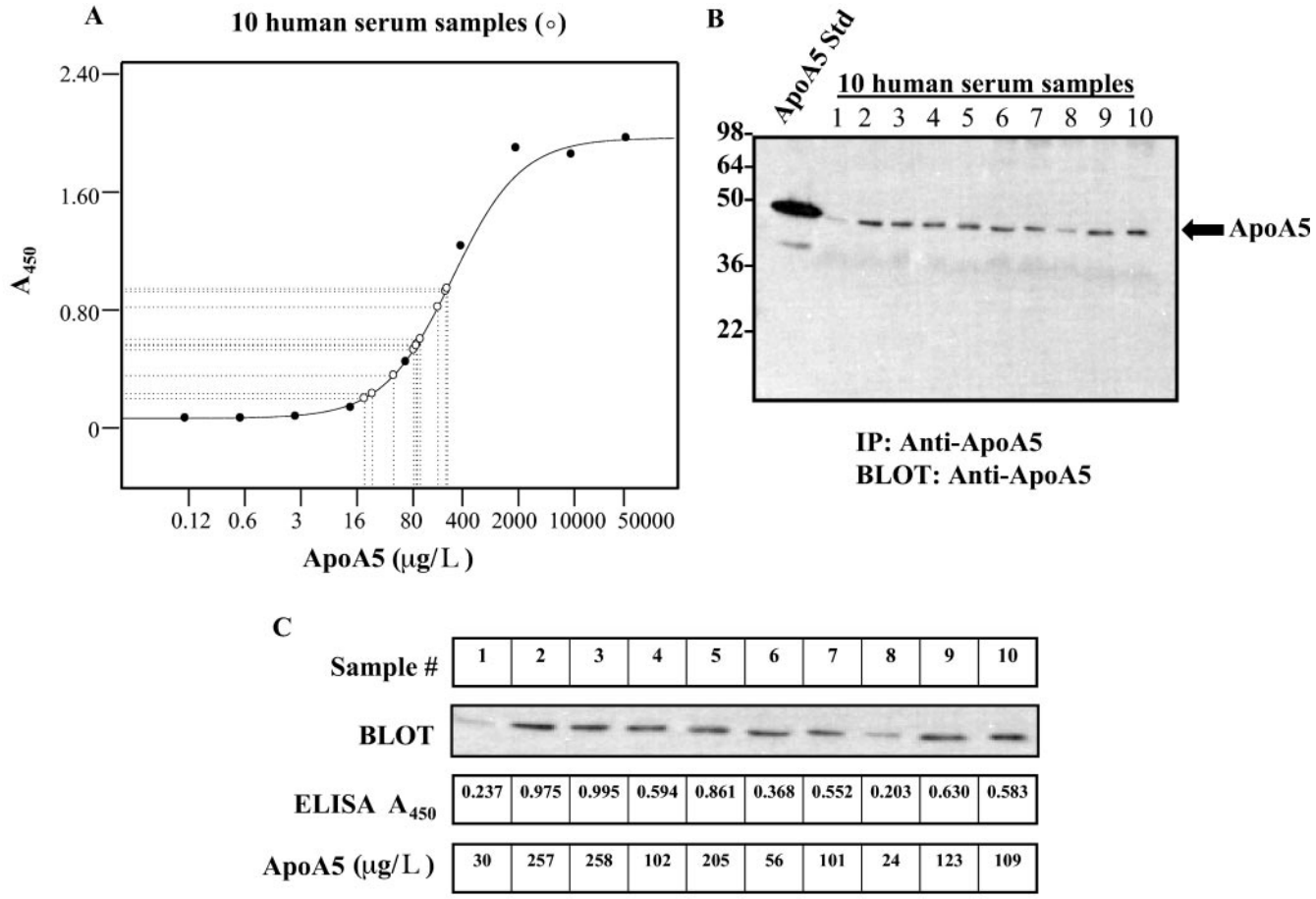


Fig. 3. Correlation of ApoA5 ELISA with immunoprecipitation and Western blotting.

(*A*), based on the results shown in Fig. 2, the antibody orientation of anti-N-terminal for capture and anti-C-terminal as conjugate was chosen, and an additional 50 000 μg/L point was added to the ELISA calibration curve. Ten serum samples were run on the ELISA with absorbances plotted on the curve. Results are representative of two independent experiments. (*B*), ApoA5 was immunoprecipitated (*IP*) from 100 μL of each of the 10 serum samples from *panel A*. Afterward, immunoprecipitates were separated electrophoretically and transferred to nitrocellulose for subsequent Western blotting with anti-ApoA5 antibodies. Recombinant ApoA5 was run as a positive control. Results are representative of two independent experiments. Molecular weights (× 1000) are indicated on the *y axis*. (*C*), direct comparison of the Western blotting results shown in *panel B* and the ELISA results in *panel A*. Results are representative of two independent experiments.

ern blotting with anti-ApoA5 antibodies (Fig. 3B). The concentrations of all samples were on the calibration curve of the ELISA, with individual absorbances plotted in Fig. 3A. The immunoprecipitation and Western blotting results for the same 10 human serum samples are shown in Fig. 3B. These data confirmed a variation in ApoA5 concentrations from person to person and provided a basis for comparison with the ELISA results. Shown in Fig. 3C are direct comparisons for the 10 serum samples of the immunoprecipitation/Western blotting results, the raw absorbance data from the ELISA, and the calculated serum concentration from the ELISA.

The above data suggested that human serum ApoA5 concentrations are <500 μg/L, indicating that ApoA5 circulates at much lower concentrations than other apolipoproteins, such as ApoA1. In light of these results, we conducted a series of independent LC/MS/MS experiments on purified lipoprotein fractions to confirm the low amounts of ApoA5. We hypothesized that if ApoA5 were truly present at such low concentrations compared with lipoproteins such as ApoA1, then ApoA5 peptides would be masked by peptides from other apolipoproteins present in the lipoprotein particles because of the finite dynamic range of the ion trap (~1000-fold or less). This is exactly what occurred: LC/MS/MS analysis of purified chylomicrons, VLDL, LDL, and HDL failed to detect the presence of any ApoA5 peptides. In contrast, peptides from the following apolipoproteins were identified in the lipoprotein particle fractions: ApoA1, ApoA2, ApoA4, ApoB, ApoC1, ApoC2, ApoC3, ApoC4, ApoD, ApoE, ApoF, ApoL, ApoM, and lipoprotein(a).

With these data consistent with the low serum concentrations of ApoA5, we next performed additional ELISA experiments to better quantify the range of ApoA5 in 40 normolipidemic individuals (total cholesterol <2400 mg/L and triglycerides <2000 mg/L). The results of this series of experiments (Fig. 4A) indicated that the mean serum concentration of ApoA5 was 157 μg/L. Only one

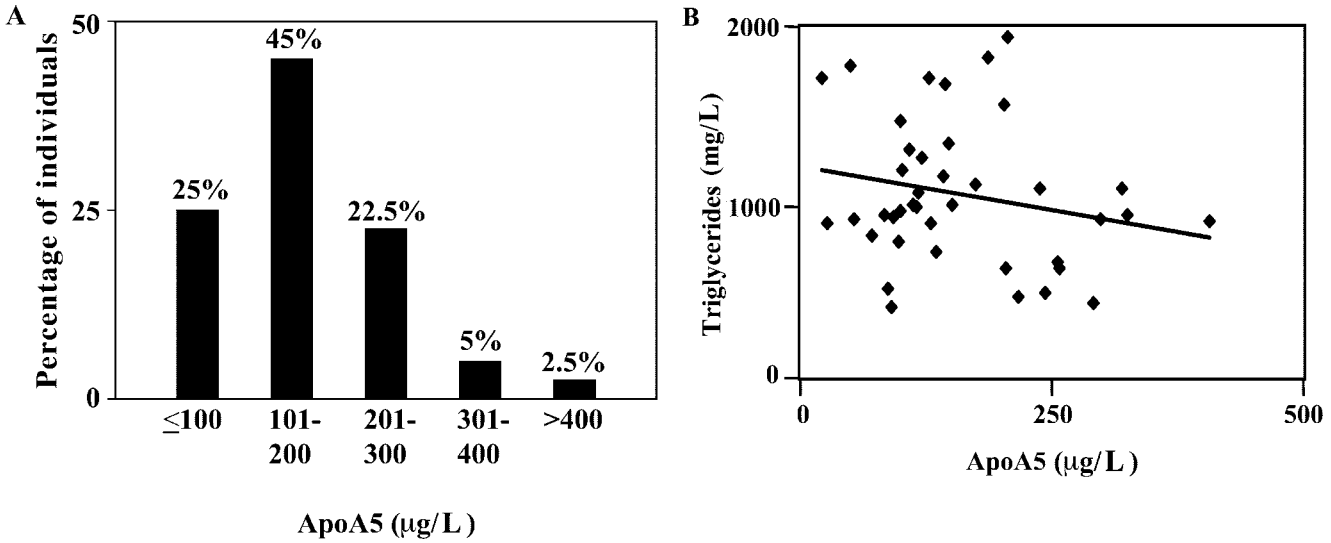


Fig. 4. Serum ApoA5 concentrations in normolipidemic individuals.

(*A*), ELISA experiments were performed to determine the range of ApoA5 in 40 normolipidemic individuals. The mean serum concentration of ApoA5 was 157 μg/L. Only one person had a serum ApoA5 concentration >400 μg/L (406 μg/L). (*B*), relationship between ApoA5 and triglyceride concentrations in the 40 individuals from *panel A*.

person had serum ApoA5 >400 μg/L (406 μg/L). Fig. 4B shows the relationship between ApoA5 and triglycerides concentrations in the 40 individuals from Fig. 4A.

Finally, we also determined the lipoprotein particle distribution pattern of ApoA5. To do this, we immunoprecipitated ApoA5 from highly purified fractions of chylomicrons, VLDL, LDL, and HDL with anti-ApoA5 antibodies. Afterward, we performed Western blotting with anti-ApoA5 antibodies. The results from these experiments are shown in Fig. 5. Interestingly, ApoA5 was present in VLDL, HDL, and chylomicrons, but was not observed in LDL, suggesting that if any ApoA5 is present in LDL, it is too little an amount to be easily detectable.

## Discussion

The above results demonstrate that ApoA5 is present in human serum and circulates at much lower concentrations than other apolipoproteins, such as ApoA1. Using the dual-antibody sandwich ApoA5 ELISA that we developed, we observed human serum ApoA5 concentrations ranging from 24 to 406 μg/L, with a mean value in normolipidemic persons of 157 μg/L. These are very low concentrations compared with ~1 g/L for ApoA1. To put this in perspective, the molar concentration of ApoA5 is ~4 nmol/L compared with ~40 μmol/L for ApoA1 and ~2 μmol/L for ApoB. Assuming that circulating ApoA5 is roughly equally split between VLDL and HDL (Fig. 5) in fasting serum (no chylomicrons), only ~1 in 1000 ApoB-containing particles would also contain a molecule of ApoA5. For ApoA1-containing particles, the difference is even more dramatic, with 20 000-fold less ApoA5 on a molar basis compared with ApoA1. This explains why ApoA5 was not discovered in serum, as was the case for related apolipoproteins coded for on the human chromosome 11q23 locus, but was instead identified at the molecular level in animal studies and via a search for open reading frames *(3, 4)*. Our observations are particu-

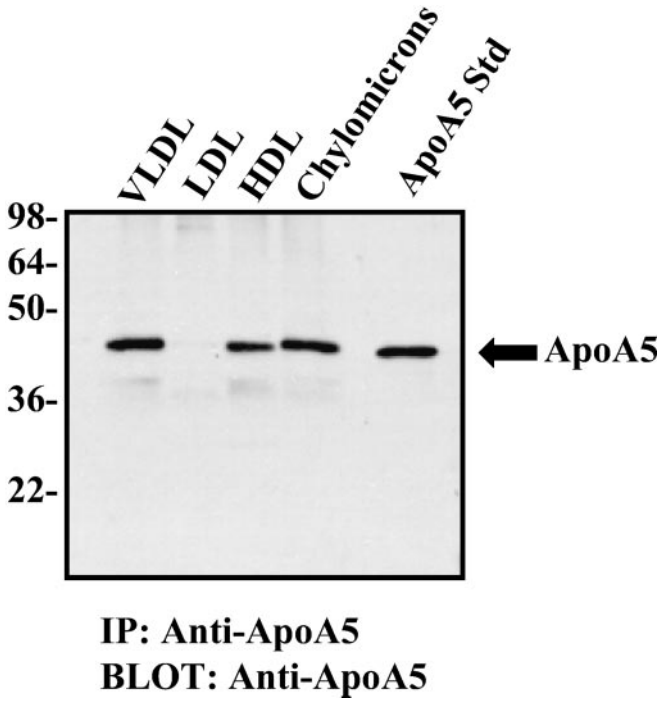


Fig. 5. ApoA5 is present in VLDL, HDL, and chylomicrons, but is not detectable in LDL.

To examine the lipoprotein particle distribution of ApoA5, 100 μL of highly purified VLDL, HDL, LDL, and chylomicron particle fractions were immunoprecipitated (*IP*) with anti-ApoA5 antibodies. Immunoprecipitates were analyzed by Western blotting with anti-ApoA5 antibodies, with recombinant ApoA5 run as a positive control. Results are representative of two independent experiments. Molecular weights (× 1000) are indicated on the *y axis*.

larly important in light of the paucity of any data in the literature for human serum ApoA5 concentrations.

Our data also indicate that the lipoprotein particle distribution of ApoA5 is remarkable in that ApoA5 is detectable in VLDL, HDL, and chylomicrons but not LDL. These data clearly confirm that, like other apolipoproteins, ApoA5 is indeed associated with lipoprotein particles, with the lipoprotein particle distribution of ApoA5 being most similar to that of ApoC3. The fact that the lipoprotein particle distribution of ApoA5 most closely resembles that of ApoC3 is interesting in light of recent data suggesting that ApoC3 and ApoA5 significantly affect triglyceride concentrations, albeit in opposite directions *(1, 25)*. Transgenic mice overexpressing human ApoC3 manifest significant hypertriglyceridemia, whereas those overexpressing ApoA5 manifest markedly decreased serum triglycerides *(1, 25)*. In the case of ApoC3, it has been thought for some time that the protein is an inhibitor of lipoprotein lipase. The mechanism by which ApoA5 lowers triglyceride concentrations is yet to be completely elucidated, although it has recently been demonstrated that it may act by inhibiting VLDL-triglyceride production and stimulating lipoprotein lipase-mediated VLDL-triglyceride hydrolysis *(26)*.

What is clear, however, is that only a small subset of VLDL lipoprotein particles contain any ApoA5. This idea is supported by structure-function studies *(27, 28)* and by comparison of the low concentrations of circulating human ApoA5 with human serum concentrations of ApoC3, which are ~100 mg/L. It has also been reported that transgenic mice that either had both genes knocked-out or overexpressed had essentially normal serum triglyceride concentrations, supporting the idea that ApoA5 may counteract the hypertriglyceridemic effects of ApoC3 *(25)*. These data may also indicate why the correlation between ApoA5 concentrations and serum triglycerides is not stronger and suggest why some normolipidemic individuals may have ApoA5 concentrations near the low end of the reference interval. It may be that the ratio of ApoA5 to ApoC3 correlates better with serum triglyceride concentrations than does an absolute measurement of ApoA5 alone.

This concept is particularly interesting in light of the recent observation that PPAR-$\alpha$ agonists (which decrease serum triglyceride concentrations) increase ApoA5 mRNA expression *(22, 23)*. In light of our data describing the circulating concentrations and lipoprotein particle distribution of human ApoA5 protein, it is likely that assays that measure human serum ApoA5 and that characterize lipoprotein particle distributions of ApoA5 will emerge as important efficacy biomarkers for PPAR-$\alpha$ agonists. We also anticipate that ApoA5 measurement in human serum will be increasingly used in the selection and development of PPAR-$\alpha$ agonists, as well as PPAR-$\alpha/\gamma$ coagonists, PPAR-$\alpha/\delta$ coagonists, PPAR-$\delta$ agonists, PPAR panagonists, and other types of molecules that are developed to treat dyslipidemia.

We thank Dr. Holger Schilske, Dr. David Robbins, and Nancy Hale for their support, Dr. John Hale and Jeff Fill for their assistance, and Dr. Mel Prince for advice and suggestions.

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