An ELISA for apolipoprotein M reveals a strong correlation to total cholesterol in human plasma

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Abstract **Apolipoprotein M (apoM) is a 188 amino acid, 25 kDa protein belonging to the lipocalin protein superfamily. Although predominantly associated with high density lipoprotein, apoM is found in all major lipoprotein classes. To facilitate clinical studies of apoM, we have developed a sandwich ELISA for the measurement of apoM in human plasma. This method has been used to investigate normal apoM variation and to establish reference values for healthy individuals through the measurement of 598 samples from the Nordic Reference Interval Project Bio-bank and Database (NOBIDA) biobank. For women 18–49 years old, the reference interval for apoM was 0.58–1.18 μmol/l, whereas for women 50+ years and for men, the reference range was 0.61–1.30 μmol/l. Correlation studies of apoM with 26 common clinical chemical analytes from the NOBIDA database revealed a marked positive correlation with plasma total cholesterol ($r = 0.52$) and LDL and HDL cholesterol ($r = 0.43$ and 0.36, respectively). There was no statistically significant correlation with HDL/total cholesterol ratio or body mass index. In conclusion, a sandwich ELISA for the measurement of apoM in human plasma shows that apoM concentration is strongly correlated to total cholesterol in healthy individuals.**—Axler, O., J. Ahnström, and B. Dahlbäck. **An ELISA for apolipoprotein M reveals a strong correlation to total cholesterol in human plasma.** ***J. Lipid Res.*** **2007.** 48: **1772–1780.**

Supplementary key words high density lipoprotein • low density lipoprotein • reference interval • reference range • biobank • Nordic Reference Interval Project Bio-bank and Database • Nordic Reference Interval Project • lipocalin • enzyme-linked immunosorbent assay

In blood, cholesterol and triglycerides are transported in the form of lipoproteins. Lipoproteins have a hydrophobic core of triglyceride and esterified cholesterol surrounded by cholesterol, phospholipid, and apolipoproteins (1, 2). The apolipoproteins provide structural stability and functional specificity to the lipoprotein particle (3). They bind to specific cellular receptors, regulate lipolytic enzymes, and take part in the process of interparticle exchange and transfer. By tradition, lipoproteins are classified according to density as high, low, intermediate, and very low density lipoproteins and chylomicrons.

Apolipoprotein M (apoM) was first described in 1999 (4). The protein is composed of 188 amino acids, with an apparent size of 25 kDa. It belongs to the lipocalin protein superfamily (5, 6). The signal peptide remains uncleaved in the circulating protein, a feature that to our knowledge only has been described for two other plasma proteins, paraoxonase 1 and haptoglobin-related protein, both of which are lipoprotein-associated (7, 8). Although apoM was originally found in chylomicrons and is found in all major lipoprotein classes, the majority of apoM is found in HDL in normal human and mouse plasma (4).

The biological role of the protein is not fully understood. An important role for apoM was relatively recently described in preβ HDL formation, as small inhibitory RNA silencing of apoM expression resulted in the accumulation of cholesterol in large HDL particles, whereas the conversion of HDL to preβ HDL was impaired (9). In the same study, overexpression of apoM in LDL receptor knockout mice protected against atherosclerosis when the mice were challenged with a cholesterol-enriched diet (9).

ApoM-containing particles from human plasma were recently isolated and described (10). ApoM was associated with plasma lipoproteins that are heterogeneous in size and charge as well as in protein and lipid composition. Compared with apoM-free HDL, human apoM-containing HDL particles had an enhanced ability to reduce LDL oxidation and promote cholesterol efflux. The actual biological implications of these findings are uncertain, however, as apoM-containing HDL represents only a small fraction of total HDL in plasma (10).

Quantification of apoM has hitherto been done by semiquantitative Western blot or dot blot-based methods in mouse and human plasma. Significantly reduced plasma apoM levels have been described in mice lacking apoA-I (11), which had a mean plasma level of 33% of normal concentration. Reduced plasma levels of apoM have also been described in leptin- and leptin receptor-

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deficient mice (12). Most dramatically, mice homozygous for mutations in the transcription factor hepatic nuclear factor (HNF)-1α had undetectable levels of apoM (13), showing that HNF-1α is required for apoM expression in vivo. Mutations in HNF-1α, in heterozygous form, are responsible for the development of maturity-onset diabetes of the young (MODY) type 3 in humans, and reduced plasma apoM levels were found in MODY 3 patients (13).

To confirm these findings and expand apoM research in the direction of clinical studies, a specific, quantitative method for the measurement of apoM in humans is desirable. Here, we present a sandwich ELISA method for the quantification of apoM in human plasma. We also present reference values for healthy individuals, based on measurements from 598 samples from the Nordic Reference Interval Project Bio-bank and Database (NOBIDA) biobank, together with results from studies of correlation between plasma apoM concentration and 26 common clinical chemical analytes.

METHODS

Expression and purification of recombinant apoM

Human truncated apoM (amino acids 22–188; i.e., without the signal peptide), originally amplified from a human liver 5′-stretch plus cDNA library (Clontech), was cloned into pET-30Xa/LIC vector (Novagen) containing N-terminal His and S tags. *Escherichia coli* strain BL21/DE3 (Stratagene) bearing the plasmid was cultured at 37°C in Luria-Bertani medium containing 100 μg/ml ampicillin until absorbance measured at 600 nm was 0.8. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. The culture was centrifuged at 2,700 *g* for 20 min at 4°C, and the supernatant was discarded. The pellet was resuspended twice in 200 ml of ice-cold 20 mM Tris-HCl, pH 8.0, and 0.15 M NaCl and recentrifuged at 2,700 *g*. The pellet was then resuspended in 40 ml of 20 mM Tris, pH 8.0, and 0.15 M NaCl. Lysozyme and benzamidine were added to final concentrations of 100 μg/ml and 500 μM, respectively, and samples were incubated at 30°C for 15 min. The solution was then sonicated in intermittent 5 s bursts for 30 min until it was clear and nonviscous. The solution was centrifuged at 2,700 *g* for 15 min at 4°C, and the supernatant was discarded. The pellet was washed twice in 200 ml of ice-cold 20 mM Tris-HCl, pH 8.0, and 0.15 M NaCl with centrifugation at 2,700 *g*. The supernatant was discarded, and the pellet was dissolved in 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 6 M guanidine-HCl, and 10 mM reduced glutathione and subjected to gentle shaking at room temperature overnight.

The solution was centrifuged at 15,500 *g* for 90 min at 20°C, and the supernatant was loaded onto a 40 ml nickel-nitrilotriacetic acid agarose superflow column (Qiagen) equilibrated in the same buffer. The column was washed until absorbance, measured at 280 nm, returned to baseline. Bound protein was eluted by a 0–500 mM linear gradient of imidazole in the same buffer. The main protein-containing fractions were pooled and refolded through sequential dialysis. The initial refolding buffer contained 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 4 mM reduced glutathione, 0.4 mM oxidized glutathione, 10% glycerol, and 6 M guanidine-HCl. The second and third refolding buffers had identical composition apart from containing 3 M and 0 M guanidine-HCl, respectively. Iodacetamide was then added to a final concentration of 100 mM, and the protein was dialyzed extensively against 20 mM Tris-HCl, pH 8.0. To remove polymeric protein, refolded protein was purified by ion-exchange chromatography. The protein was loaded onto a 10 ml Q-Sepharose Fast Flow column (Pharmacia) equilibrated with 20 mM Tris-HCl, pH 8.0, and the protein was eluted by a 0–500 mM linear gradient of NaCl and analyzed by 12% SDS-PAGE followed by silver staining. Monomeric apoM eluted in early fractions, which were pooled and dialyzed against 20 mM Tris-HCl, pH 8.0, and 0.15 M NaCl and stored at −20°C.

Recombinant apoM used in the Biacore analysis (see below) lacked His and S tags and was produced as described (6).

Monoclonal antibodies

BALB/c mice were injected intracutaneously with 10 μg of purified recombinant apoM (see above) together with complete Freund's adjuvant and 0.15 M NaCl. This was followed by booster injections with incomplete Freund's adjuvant after 1 and 3 weeks. The mice were bled and serum was tested for immunoreactivity against recombinant apoM by ELISA. The mouse with the strongest anti-apoM response was injected intraperitoneally for 4 consecutive days with 200 μg of purified recombinant apoM. On the 5th day, the mice were euthanized, and splenocytes were extracted and fused with SP2/2AG14 myeloma cells using standard techniques (14). The fused cells were seeded with feeder cells onto 96-well microtiter plates containing Dulbecco's modified Eagle's medium supplemented with hypoxanthine, aminopterin, and thymidine. The hybridoma supernatants were screened by ELISA, and cells producing antibodies reacting with recombinant apoM were subcloned twice by the limiting dilution method on 96-well plates and subsequently cultured in a Tecnomouse (Integra Biosciences) hollow fiber chamber. Conditioned media were collected and stored at −20°C until used.

Purification and biotinylation of antibodies

A Protein G-coupled 5 ml HiTrap column (Amersham) was equilibrated in 20 mM Na-phosphate buffer, pH 7.0, and antibody-containing medium was applied at a flow rate of 1 ml/min. The column was washed with 20 mM Na-phosphate buffer, pH 7.0, until baseline returned to zero. Antibodies were eluted with 0.1 M glycine-HCl, pH 2.7, and collected in 1 ml fractions in tubes containing 50 μl of Tris-HCl, pH 9.0. Protein-containing fractions were pooled, and the preparation was dialyzed extensively against 0.1 M borate buffer, pH 8.0. *N*-(+)-Biotinyl-6-aminocaproic acid *N*-succinimidyl ester (Fluka) was dissolved in 100 μl of dimethyl sulfoxide (Duchefa) and added in a 100:1 molar ratio to protein. After incubation overnight at 4°C, glycine was added to a final concentration of 50 mM. The preparation was dialyzed overnight against TBS, pH 7.5, and stored at −20°C until used.

SDS-PAGE and Western blot

SDS-PAGE and Western blot analysis were performed according to a procedure described previously (11). In the experiments using monoclonal antibodies, a slightly modified protocol was used. Biotinylated antibody (see above) was added at a concentration of 0.1 μg/ml. After wash, the membranes were incubated with alkaline phosphate-conjugated StreptABComplex (Dako) prepared according to the manufacturer's instructions. After another round of washes, the membranes were developed with 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine salt and *p*-nitro-blue tetrazolium (Sigma-Aldrich).

Surface plasmon resonance analysis

Binding of recombinant apoM (lacking His and S tags) to immobilized monoclonal antibodies M23, M42, and M58 was

studied with surface plasmon resonance analysis in a Biacore 2000 instrument (Biacore). Immobilization of the antibodies to a sensor chip through amine coupling was performed with 10 mM HEPES, 0.15 M NaCl, and 0.005% Tween 20, pH 7.4, as flow buffer. Immobilization levels of 1,500 and 3,000 resonance units were tested and gave identical results. The sensor chip CM5 and amine coupling kit containing *N*-hydroxysuccinimide, *N*-ethyl-*N*′-(3-[diethylamino]propyl)carboxydiimide and ethanolamine hydrochloride were purchased from Pharmacia Biosensor AB.

Binding of apoM to monoclonal antibodies M23, M42, and M58 was performed using 10 mM HEPES, 0.15 M NaCl, and 0.005% Tween 20, pH 7.4, as flow buffer. A total of 250 μl of apoM was injected at concentrations of 3.2–25.8 nM, with a flow rate of 30 μl/min. The dissociation into pure buffer was followed for 800 s. The remaining bound apoM was removed by washing two times with 10 μl of 0.1 M HCl and 2 M NaCl. The binding kinetics were analyzed using BIAevaluation software, version 4.1 (Biacore). The association and dissociation rate constants were fitted separately using global settings for parameters.

Purification of plasma apoM

Purification of apoM-containing particles from human plasma was performed as described previously (10). Particles were then reloaded onto a 5 ml HiTrap N-hydroxysuccinimide-activated column (Amersham Biosciences AB) to which monoclonal antibodies against apoM (M23) were coupled at 10 mg/ml according to the manufacturer's instructions. The column was washed with 25 ml of TBS (50 mM Tris-HCl and 150 mM NaCl, pH 7.4) followed by 25 ml of 50 mM Tris-HCl, 0.5 M NaCl, pH 7.4, and 10 ml of TBS. The particles were then dissolved on-column by washing with 5 ml of TBS containing 100 mM octyl glucoside (OG; *N*-octyl-β-D-glucopyranoside) (Calbiochem, EMD Biosciences) followed by 5 ml of TBS containing 50 mM OG and finally 5 ml of TBS containing 10 mM OG. ApoM was eluted with 0.1 M glycine, pH 2.2, containing 10 mM OG, and 1 ml fractions were collected in tubes containing 50 μl of 1 M Tris (pH 9.0). Purity was assessed by SDS-PAGE followed by silver staining, which showed no band other than those expected for apoM. The amino acid composition was determined and the molar concentration of apoM calculated after acid hydrolysis (6 M HCl, 24 h, 110°C in vacuum) using an amino acid analyzer (Beckman 6300).

ELISA procedure

Costar 3590 96-well plates (Corning) were coated with 100 μl of monoclonal M58 at a concentration of 10 μg/ml in carbonate buffer, pH 9.6. The plates were quenched with 200 μl of 2% BSA (Sigma-Aldrich) in TBS overnight at 4°C. Samples, together with a plasma standard curve, were diluted in TBS with 1% Triton X-100 (Sigma-Aldrich) and 1% BSA, and 100 μl was added to each well. After incubation overnight at room temperature, plates were washed in washing buffer (TBS with 0.1% Triton X-100), after which 100 μl of biotinylated M42, diluted to a final concentration of 0.5 μg/ml in TBS with 0.1% Triton X-100 and 1% BSA, was added and plates were incubated for 2 h at room temperature followed by wash. Streptavidin-avidin complex with horseradish peroxidase (Dako) was prepared according to the manufacturer's instructions, diluted in TBS with 0.1% Triton X-100 and 1% BSA, and 100 μl was added. After wash, peroxide and o-phenylenediamine dihydrochloride (Dako) was prepared, and 100 μl was added according to the manufacturer's instructions. After 9 min, the reaction was terminated and absorbance at 490 nm was read by plate reader (EL808; BioTek Instruments) with Deltasoft 3 software.

The final standard dilution of the plasma samples in the assay was 1:3,249. A standard curve was prepared from a Li-heparin plasma pool stored at −80°C from 16 healthy individuals. The range was from 1:9,177 (35% of standard concentration) to 1:1,197 (271%). Because of the sigmoidal shape of the standard plasma curve, this was fitted by applying least-squares principles to a five-parameter sigmoidal model (equation 1) in which x represents apoM concentration and y represents measured absorbance at 490 nm:

$$x = \frac{a-d}{1 + \left(\frac{y}{c}\right)^b} + d + e \qquad \textit{(Eq. 1)}$$

Calculated apoM concentration was expressed as μmol/l based on the concentration of the plasma pool (0.94 μmol/l).

Recovery

ApoM purified from human plasma (see above) in TBS with 10 mM OG was added to plasma samples diluted 1:10 in TBS from six individuals, and apoM was quantified by ELISA. Recovery was calculated as the difference between spiked and unspiked plasma samples, expressed as a percentage of the measured level of purified apoM.

Preparation of apoM-depleted human plasma

Human pooled Li-heparin plasma from 16 healthy individuals was diluted 1:10 in TBS with or without 1% Triton X-100 to a total volume of 1 or 1.5 ml and applied to a 1 ml HiTrap *N*-hydroxysuccinimide-activated column (Amersham Biosciences) to which monoclonal antibodies against apoM (M23, M42, or M58) had been coupled at 10 mg/ml according to the manufacturer's instructions. Flow-through was collected in 300 μl fractions, and absorbance at 280 nm was measured. The top fractions were analyzed for apoM with ELISA and Western blot using polyclonal antiserum. Additional dilution of the plasma during the depletion, according to the absorbance at 280 nm, was corrected for.

Determination of molar apoM concentration

Two fractions of purified plasma apoM for which the molar concentration of apoM had been calculated were used to determine the molar concentration of the standard plasma pool. The fractions were quantified by ELISA against the standard plasma pool using the standard procedure. Each fraction was analyzed at least twice in independent runs, and the molar apoM concentration of the plasma pool was calculated.

NOBIDA plasma samples

Plasma samples were from the NOBIDA biobank, a resource established as part of the Nordic Reference Interval Project (NORIP) administered by Dansk Institut for Ekstern Kvalitetsikring for laboratorier i Sundhetssektoren, Herlev Hospital, in Denmark (15, 16). In this project, blood samples from 3,035 individuals were collected at 103 clinical laboratories in the five Nordic countries. To participate in the study, subjects had to be subjectively healthy and older than 18 years of age. The exclusion criteria were as follows: hospitalization in the month before sampling, having had more than two glasses of wine or two bottles of strong beer within the last 24 h, having smoked less than 1 h before sampling, having donated blood in the last 5 months, being pregnant or breast-feeding a child, and having taken prescription medication in the last 2 weeks (contraceptives and estrogens excluded). In conjunction with blood sampling, subjects also gave information on age, sex, smoking habits, medication, physical activity, etc. In the NORIP project, a number of clinical chemical analytes had been measured by the participating laboratories, with results recalculated after harmonization with reference samples (17).

The samples for this study were chosen on the basis of randomization within country, within age group (18–29, 30–39, 40–49, 50–59, 60–69, and 70+ years), and within sex to achieve an even distribution for these three parameters. Icelandic samples were few (n = 39), and all were included in the study. For several individuals, analytes had been measured for serum as well as plasma. As the majority of individuals (91%) had been analyzed using thawed serum, this was preferred when there were several entries in the database; thereafter, fresh serum and thawed plasma were used in that order of priority.

Mathematical analysis

All mathematical analyses were performed in Microsoft Excel for Mac, version 10.0.0, except for Spearman's rank-based correlation and multiple regression analyses, which were performed using Wessa Statistics Software, version 1.1.20 http://www.wessa.net), and GraphPad Instat 3.

Ethics approval

This study, including the manufacture of monoclonal antibodies and the biobank analysis, was approved by the local ethics committee. Informed consent was received from all participants in the study. The consent form is included in Ref. 15.

RESULTS

Monoclonal antibodies

Three monoclonal antibodies (M23, M42, and M58) reacting against human native apoM were obtained. Binding of purified recombinant apoM to immobilized antibody was analyzed with surface plasmon resonance using Biacore 2000. The mean dissociation constants for the binding of apoM to M42 and M58 were 4.8 ± 0.61 and 0.89 ± 0.46 nM, respectively. The dissociation constant for monoclonal M23 was of an order of magnitude larger than those for M42 and M58, and it was not used in the ELISA. On Western blot analysis of plasma, M42 and M58 showed reaction against a protein with a size corresponding to the molecular mass of apoM (**Fig. 1**). An assay using M58 as immobilized antibody and M42 as the labeled antibody showed a dose-dependent response to apoM with good sensitivity.

Three detergents were evaluated: Tween 20, Triton X-100, and CHAPS. Of these, Triton X-100 gave the best signal-to-noise ratio and was chosen for the assay. As apoM retains its hydrophobic signal peptide, a relatively high detergent concentration of 1% Triton X-100 was used for plasma dilution in the assay.

Because of the sigmoidal shape of the dose-response curve, this was fitted by applying least-squares principles to a sigmoidal model. ApoM concentration was expressed as μmol/l based on the concentration of apoM in the standard plasma pool (see below). A typical standard curve for the measurements is shown in **Fig. 2**.

Method evaluation

Determination of molar apoM concentration. ApoM concentration in the plasma pool was determined by measurement against apoM purified from human plasma. The concentration of apoM in the plasma pool was 0.94 μmol/l.

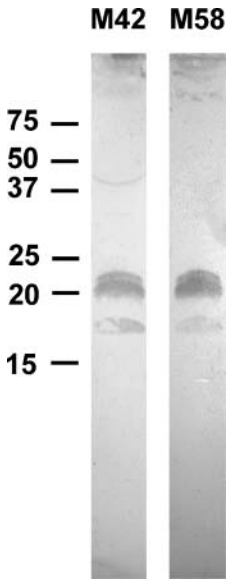

Fig. 1. Specific reaction of M42 and M58 to apolipoprotein M (apoM) in human plasma. Western blots are shown after separation on 15% SDS-PAGE under unreducing conditions. Pooled human plasma (0.5 μl) was loaded in each well, and apoM was probed with biotinylated monoclonal antibodies M42 and M58 at a concentration of 0.1 μg/ml. The markers represent apparent molecular mass in kilodaltons derived from a molecular mass standard run in an adjacent lane. In unreducing conditions, apoM migrates as a markedly lower molecular mass protein (~20 kDa) as a result of three internal disulfide bonds. The lower molecular mass band represents unglycosylated apoM.

Variability. Between-run variability (n = 11) was 4.9% at the 0.94 μmol/l level and 5.1% at the 0.47 μmol/l level. Within-run variability was 5.0% at the 0.94 μmol/l level (n = 14) and 4.5% at the 0.47 μmol/l level (n = 20).

Stability of samples. Plasma samples (n = 10) were stored at 4°C for 7 days without any significant change in measured apoM concentration.

Recovery of apoM. ApoM purified from human plasma was added at a concentration of 0.66 μmol/l to plasma

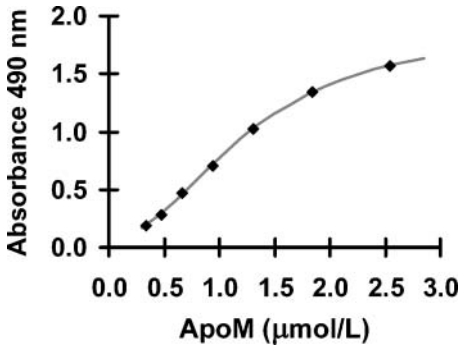

Fig. 2. A typical ELISA standard curve from the apoM quantification experiments in this study. Standard curve measurements are shown as black diamonds, and the sigmoidal curve fit is shown by the line.

TABLE 1. Recovery of apoM from plasma samples from six individuals

Sample No.	Unspiked	Spiked	Measured Difference	Expected Difference	Recovery
1	0.69	1.37	0.68	0.66	104%
2	0.86	1.51	0.65	0.66	99%
3	1.00	1.65	0.65	0.66	99%
4	0.71	1.34	0.63	0.66	97%
5	0.86	1.53	0.67	0.66	102%
6	0.97	1.58	0.61	0.66	93%

ApoM, apolipoprotein M. Plasma purified apoM was added to plasma samples from six individuals, increasing the concentration by 0.66 μmol/l. Their respective apoM concentrations before and after the addition of apoM are shown in the unspiked and spiked columns, respectively. Measured difference represents the difference in concentration between spiked and unspiked samples (i.e., the spike), and expected difference represents the concentration in the reference sample containing no plasma. Results are in μmol/l, except for recovery, which represents the measured spike expressed as a percentage of the expected value.

samples from six individuals. Recovery was calculated from the measured difference between spiked and unspiked samples. Mean recovery was 99.0% with a SD of 3.9%. The results of the individual measurements are presented in **Table 1**.

Specificity of the assay. To evaluate the specificity of the assay, the monoclonal antibodies M23, M42, and M58, the first of which was not used in the assay, were coupled to a HiTrap column, and pooled human plasma from 16 individuals was applied and collected. The resulting apoM-depleted plasma gave an apoM signal in the ELISA of 3% or less compared with the original sample for all three preparations. To ensure that no fraction of plasma apoM went undetected by the assay, Western blot using polyclonal rabbit anti-apoM was performed, showing no reaction against apoM in depleted plasma preparations (data not shown).

To ensure that individual plasma samples displayed the same dose-response relationship as the standard plasma pool, five individual samples (apoM concentration range 0.63–1.13 μmol/l) were analyzed at different dilutions. Two samples contained uncommonly high cholesterol concentrations (7.7 and 7.8 mmol/l), and two contained uncommonly low cholesterol concentrations (3.6 and 4.1 mmol/l). The individual and standard plasma log(dose)-response curves were found to be parallel (data not shown).

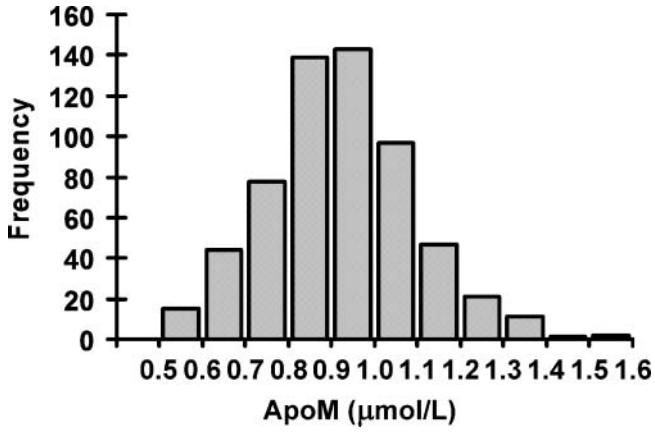

Fig. 3. Frequency distribution of apoM concentrations in samples from 598 individuals from the Nordic Reference Interval Project Bio-bank and Database (NOBIDA) biobank.

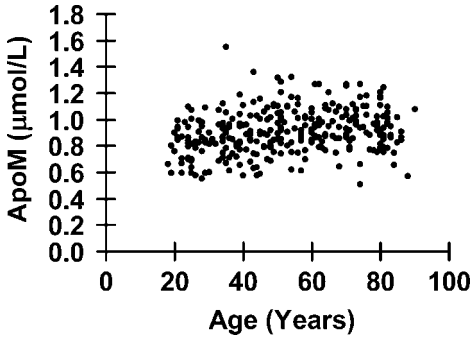

Fig. 4. Distribution of apoM concentrations for 303 women (age range, 18–90 years) from the NOBIDA biobank.

Analysis of NOBIDA samples

To establish reference values for healthy individuals, 598 plasma samples from the NOBIDA biobank, a resource established as a part of the NORIP, were analyzed for apoM by ELISA. The population consisted of 303 women and 295 men, and the age range was 18–91 years. The distribution of apoM concentrations in the population was essentially Gaussian (**Fig. 3**). The mean apoM concentration in the study population was 0.92 μmol/l, and the median value was 0.92 μmol/l.

Establishment of apoM reference intervals

We observed that for women, apoM concentration was positively correlated with age ($r = 0.24$, $P < 0.001$) (**Fig. 4**). Women aged 18–49 years had lower apoM plasma concentration (mean, 0.86 μmol/l; n = 136) than women aged 50 or older (mean, 0.95 μmol/l; n = 167, $P < 0.001$) or men (mean, 0.94 μmol/l). For men, there was no significant linear correlation with age ($r = 0.058$, $P > 0.05$) (**Fig. 5**). A common reference interval for the whole population was discarded, as an unacceptably large proportion

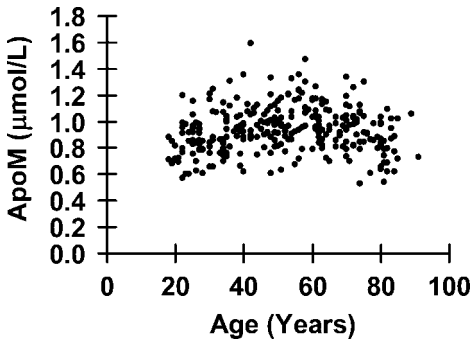

Fig. 5. Distribution of apoM concentrations for 295 men (age range, 18–91 years) from the NOBIDA biobank.

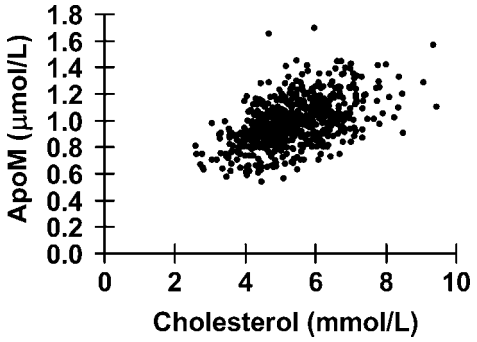

Fig. 6. Relationship between apoM concentration and total cholesterol ($r = 0.52$, n = 591). ApoM was measured by ELISA. Data for total cholesterol are from the NOBIDA database.

(>7%) of the younger group of women had an apoM concentration below the lower common reference limit. This is well outside the proportion limits 0.9–4.1% suggested by Lahti et al. (18) and used for partitioning decisions in the NORIP project. Calculations using the method of Harris and Boyd (19) using the original threshold z value of $3\sqrt{(n/120)}$ also supported partitioning into two subgroups. Reference intervals were calculated using the nonparametric method, which is recommended by the Clinical and Laboratory Standards Institute (20). For women 18–49 years, the reference interval was 0.58–1.18 μmol/l, and for women 50 years and older and men, the reference interval was 0.61–1.30 μmol/l.

Correlation analyses

In the NORIP project, several common clinical chemical analytes were measured for each sample by the participating laboratories. The results of these measurements were used for studies of covariation with apoM concentration. For seven of the analytes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, glucose, glutamyl transferase (GT), lactate dehydrogenase, and triglyceride, data distribution was significantly skewed, and the nonparametric Spearman rank-order correlation coefficient was calculated instead of the Pearson correlation coefficient used for the other analytes.

ApoM level correlated well with plasma total cholesterol ($r = 0.52$) (**Fig. 6**), LDL cholesterol ($r = 0.42$) (**Fig. 7**), and HDL cholesterol ($r = 0.36$) (**Fig. 8**). There was no statistically significant correlation between the calculated total cholesterol/HDL cholesterol ratio and apoM concentration ($P > 0.05$, n = 556). Less strong but still statistically significant ($P < 0.01$) correlations with apoM concentration were observed for apoM and albumin, ALT, AST, calcium, GT, total iron binding capacity (TIBC), and urate. Borderline significant correlations ($P = 0.01$–0.05) were observed for alkaline phosphatase (ALP) and sodium (**Table 2**).

Apart from total, LDL, and HDL cholesterol, the analyte most significantly correlated with apoM concentration was GT (Spearman $r = 0.24$, $P < 0.001$). However, this analyte was significantly correlated to total and LDL cholesterol (Spearman $r = 0.31$ and 0.35 for total and LDL cholesterol, respectively). Multiple regression analysis of apoM, with GT/log GT and LDL cholesterol as independent variables, showed only small partial correlations of 0.08 and 0.07 for GT and its logarithm, respectively (n = 334, $P = 0.034$ and 0.041, respectively).

Additional data from the NOBIDA database were investigated for correlation with apoM. For smoking, the study participants had been separated according to daily consumption into zero (n = 519), one to five (n = 26), and more than five cigarettes a day (n = 53). Single-factor ANOVA demonstrated no significant difference between the three groups ($P = 0.29$).

In the original NORIP study, partitioning of alcohol consumption in the week before testing had been similarly done into zero, 1 to 21, and >21 units of alcohol (1 unit equals 10 ml or ~8 g of alcohol). However, as the highest consumption group only contained five individuals, apoM concentration was compared between zero-consumption individuals (n = 286) and others (n = 307). The alcohol consumption group showed a significantly higher mean apoM concentration (0.96 vs. 0.88 μmol/l; $P < 0.001$). There was no significant difference in total cholesterol concentration between the two groups (5.36 vs. 5.41 mmol/l; $P > 0.05$). Neither was the difference attributable to sex, as sex, when analyzed by multiple regression together with

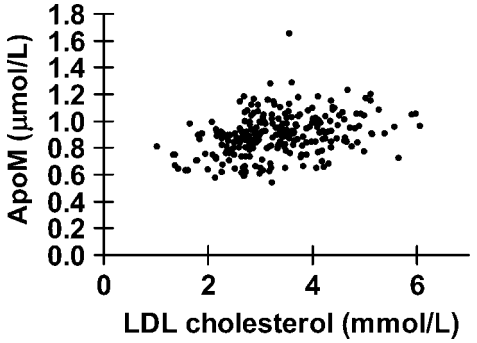

Fig. 7. Relationship between apoM concentration and LDL cholesterol ($r = 0.43$, n = 245). ApoM was measured by ELISA. Data for LDL cholesterol are from the NOBIDA database.

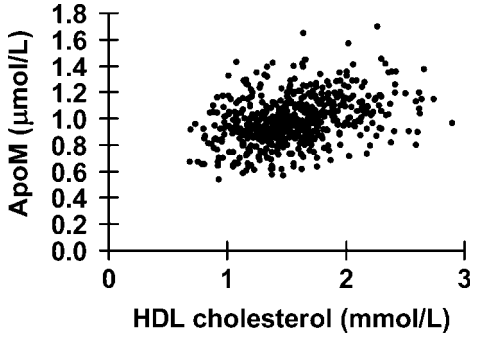

Fig. 8. Relationship between apoM concentration and HDL cholesterol ($r = 0.36$, n = 566). ApoM was measured by ELISA. Data for HDL cholesterol are from the NOBIDA database.

TABLE 2. Results from correlation studies of apoM plasma concentration as measured by ELISA and 26 analytes from the Nordic Reference Interval Project Bio-bank and Database

Analyte	n	*r*	*P*
Albumin	583	0.11	0.0091*
Alkaline phosphatase	227	0.14	0.033*
Alanine aminotransferase	516	0.15†	<0.001**
Amylase	149	0.069	>0.05
Pancreatic amylase	129	0.13	>0.05
Aspartate aminotransferase	478	0.13†	0.0058*
Bilirubin	582	0.064†	>0.05
Calcium	572	0.14	<0.001**
Carbamide	563	0.052	>0.05
Cholesterol	591	0.52	<0.001**
Creatine kinase	434	0.078	>0.05
Creatininium	556	0.076	>0.05
Glucose	191	0.10†	>0.05
Glutamyl transferase	336	0.24†	<0.001**
HDL cholesterol	566	0.36	<0.001**
Iron	514	0.024	>0.05
Lactate dehydrogenase	114	0.12†	>0.05
LDL cholesterol	245	0.43	<0.001**
Magnesium	472	−0.019	>0.05
Phosphate	565	0.029	>0.05
Potassium	587	0.068	>0.05
Protein	421	0.086	>0.05
Sodium	590	0.094	0.022*
Total iron binding capacity	164	0.21	0.0076*
Triglyceride	265	0.053†	>0.05
Urate	571	0.13	0.0018*

n denotes the number of individual measurements for the analyte in the database (maximum, 598). Analytes displaying a skewed distribution, for which Spearman rank-order correlation coefficient was calculated instead of the Pearson correlation coefficient, are marked with daggers. *P* values < 0.05 are marked with asterisks, and *P* values < 0.001 are marked with double asterisks.

age and apoM, did not make a significant contribution to the model. The subgroup of five individuals (all males) who had consumed >21 units had a substantially higher mean apoM concentration than the zero-consumption group, which was statistically significant (1.20 ± 0.23 μmol/l; $P = 0.037$, assuming unequal variances).

To investigate whether apoM levels are affected by fasting or time of day, the number of hours from the last meal (n = 598) and the hour of sampling (n = 595) were correlated with apoM. There was no statistically significant correlation of apoM with either parameter, nor was there any correlation with body mass index (n = 596).

Whether strenuous sporting activity in the week before sampling affected apoM concentration was investigated with Student's *t*-test. The group that had performed strenuous exercise (mean concentration, 0.87 μmol/l; n = 71) showed a statistically significantly lower mean apoM concentration compared with the nonexercise group (mean concentration, 0.93 μmol/l; n = 524, $P = 0.019$). However, the exercise group also had a significantly lower mean plasma cholesterol level (4.8 vs. 5.5 mmol/l; $P < 0.001$), and in a multiple regression analysis of apoM concentration, with cholesterol as the other independent variable, exercise did not contribute significantly to the model.

There was no statistically significant difference in apoM level between subjects with and without first-degree relatives with diabetes.

DISCUSSION

The development of a simple, specific quantitative assay for the measurement of human apoM is important for the expansion of apoM research in the direction of clinical studies. Here, we describe a sandwich ELISA for the quantification of apoM in human plasma using two monoclonal antibodies. Between-assay and within-assay variability in the ELISA was ~5%, and mean recovery at the 0.66 μmol/l level was 99.0 ± 3.9%.

ApoM content in the healthy human plasma pool was 0.94 μM, or ~23 mg/l. This roughly corresponds to 1/50th of the mean molar concentration of apoA-I in plasma (21). As the majority of apoM is associated with HDL and two or three apoA-I molecules are associated with each HDL particle, this is in good agreement with earlier results from experiments based on apoM immunoaffinity chromatography of HDL prepared by ultracentrifugation, in which it was estimated that ~5% of HDL particles contain apoM (10).

To establish normal reference ranges, the assay has been used to quantify apoM in 598 plasma samples from healthy subjects. We observed significantly lower apoM levels in younger women compared with the other subjects (mean concentration, 0.86 vs. 0.94 μmol/l; $P < 0.001$). For this reason, a separate reference interval for women 18–49 years of age is necessary. The reason for the lower apoM levels observed in younger women is presumably the lower plasma cholesterol in this group. There is an increase in plasma cholesterol with age in women, occurring mainly during menopause (22–24), and in a multiple regression analysis of apoM concentration in all women, using cholesterol and age as independent variables, age did not contribute significantly to the model.

Indeed, the most striking finding from the correlation studies with other analytes is the clear correlation ($r = 0.52$) of apoM with plasma total cholesterol. The correlation of apoM with either LDL or HDL cholesterol was substantially weaker ($r = 0.43$ and 0.36, respectively). As the majority of plasma apoM-containing particles are of the HDL subclass, it is perhaps surprising to observe a weaker correlation of apoM with HDL cholesterol than total cholesterol. Mice that lack the LDL receptor or apoE have dramatically higher plasma cholesterol but do not exhibit significantly increased total apoM levels (25). This may suggest that high plasma cholesterol per se is insufficient to increase the level of apoM. Wolfrum, Poy, and Stoffel (9) measured increased plasma total cholesterol in mice in which apoM was overexpressed using an adenovirus vector and reduced cholesterol levels in mice in which apoM expression had been attenuated using small inhibitory RNA. However, the data indicate that this effect is mainly one of regulating HDL metabolism. In contrast to humans, rodent plasma cholesterol is present mainly in HDL (26). The results of the present study show that the correlation of apoM with HDL cholesterol is considerably weaker than the correlation with total cholesterol in healthy humans. Indeed, there was no statistically significant correlation between apoM and the HDL/total cholesterol ratio, further supporting the apparent

unimportance of which lipoprotein subclass is quantitatively dominant.

Of the other studied analytes, any correlation with apoM concentration observed was considerably weaker. The correlation coefficient range was ~0.1–0.2 for the nine other significantly correlated analytes, the highest being GT ($r = 0.24$). A majority of these analytes are connected to liver metabolism (albumin, ALP, ALT, AST, GT, and TIBC), which may imply a connection to liver apoM synthesis or metabolism, although these results should be interpreted with caution considering the low correlation coefficients.

There was a significantly higher (~9%) mean apoM level in the group of individuals who had consumed alcohol in the week before testing. The other two analytes for which a significant difference between groups was observed were GT and urate. Both analytes are known to increase with alcohol consumption (27, 28).

The absence of any correlation with time of day or number of hours since the last meal implies that apoM can be measured at any time of day and that a fasting sample is not needed. The intraindividual variability of apoM concentration is still unknown and will have to be studied separately.

In view of the report of a strong positive correlation of apoM and leptin in normal and obese subjects (29) and the well-established correlation between leptin and amount of body fat (30–32), the absence of any statistically significant correlation between apoM and body mass index in this study is somewhat surprising. Additionally, in the earlier study (27), a negative relationship with total cholesterol was found ($r = -0.41$), a result in stark contrast to the positive relationship found in the present study. The discrepancy may be attributable to differences in methodology or to the earlier report's inclusion of obese subjects in the study group.

There is a well-established relationship between plasma total cholesterol and the risk of development of myocardial infarction (33–35). The strong correlation with plasma cholesterol, together with the finding that overexpression of apoM in LDL receptor deficient mice can protect against atherosclerosis, make it interesting to investigate whether apoM is a predictor of cardiovascular disease.

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