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(54) Title: ULTRA-SMALL APOB-CONTAINING PARTICLES AND METHODS OF USE THEREOF

(57) Abstract: The present disclosure provides an isolated particle comprising very high density, ultra small, lipid depleted apo B containing particles, and may also contain cytokeratin 8. The isolated particle is useful in diagnostic assays, which are also provided.

ULTRA-SMALL APOB-CONTAINING PARTICLES AND METHODS OF USE THEREOF

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 61/497,847, filed June 16, 2011, which application is incorporated herein by reference in its entirety.

BACKGROUND

[0002] Lipoproteins function to transport lipids around the body. Lipids are generally hydrophobic, while the extracellular environment is generally aqueous. Apolipoproteins bind to lipids, such as cholesterol and triglycerides, and facilitate their transport through the aqueous environment. Apolipoprotein B (apoB) represents most of the protein content in low density lipoprotein (LDL), and is also present in intermediate-density lipoproteins (IDL) and very low density lipoproteins (VLDL). Apolipoprotein AI (apoAI) is the principal protein in high density lipoprotein (HDL) and represents about 70%.

[0003] The total cholesterol/HDL cholesterol ratio and the LDL/HDL cholesterol ratio are two indicators of vascular disease risk, including cardiovascular disease (CVD) risk. For example, an increase in the total cholesterol concentration, and specifically LDL cholesterol, is an atherogenic lipid marker. Reduced HDL cholesterol concentration is associated with various risk factors, including components of the metabolic syndrome.

Literature

[0004] Millán et al. (2009) *Vascular Health and Risk Management* 5:757; Superko and Gadesam (2008) *Curr. Atheroscler. Rep.* 10:377; USPN 7,781,219; U.S. Patent Publication No. 20100183607; U.S. Patent Publication No. 20100179066; U.S. Patent Publication No. 20090155915; WO 2010/115200; WO 2010/115094.

SUMMARY

[0005] The present disclosure describes a very high density, ultra small lipid depleted apolipoprotein B containing particle that may be indicative of increased cardiovascular disease risk. These particles may contain other proteins, such as cytokeratins. The isolated particles are useful in diagnostic assays, which are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] Figure 1 depicts the ion mobility (IM) analysis of particles isolated from plasma from a representative individual using a specific anti-apoB antibody conjugated to magnetic beads.

[0007] Figure 2 depicts ion mobility analysis of the d<1.21 g/ml ultracentrifugal fraction of plasma (blue) and the d>1.21 g/ml ultracentrifugal fraction of plasma (black) from a representative individual.

[0008] Figure 3 depicts ion mobility analysis of the apoB-containing lipoproteins isolated from the ultracentrifugal fractions described in Figure 2 using a specific anti-apoB antibody conjugated to magnetic beads.

[0009] Figure 4 displays a 2-14% gradient polyacrylamide gel, showing the elution window with pre-stained lipoprotein standards.

[0010] Figure 5 depicts ion mobility analysis of particles isolated by electrophoresis in fraction 1 from the elution window shown in Figure 4.

[0011] Figure 6 depicts ion mobility analysis of particles isolated by electrophoresis in fraction 2 from the elution window shown in Figure 4.

[0012] Figure 7 ion mobility analysis of particles isolated by electrophoresis in fraction 3 from the elution window shown in Figure 4.

[0013] Figure 8 depicts the results of 2-14% gradient gel electrophoresis (GGE), followed by lipid staining with Sudan Black, of fractions 2 and 3 described in Figures 6 and 7. Lanes 1,2, 8, 17 & 18, lipoprotein standards; lanes 3-5, fraction 2; lanes 6 & 7, fraction 3; lanes 9-12, empty; lane 13, high molecular weight protein standards; lanes 14-16, empty.

[0014] Figure 9 depicts the densitometric scan of lane 3 in Figure 8.

[0015] Figure 10 depicts the densitometric scan of lane 4 in Figure 8.

[0016] Figure 11 depicts a 2-14% GGE stained with Coomassie blue of fractions 1, 2 and 3 from the elution window shown in Figure 4. Lanes 1,2, 9, 17 & 18, lipoprotein standards; lanes 4-6, fraction 2, 3 and 4; lanes 3, 7, 8, 15 and 16, empty; lane 10, bovine serum albumin; lane 13, LDL control; lanes 11, 12 and 14, ultra small apo B containing particles.

[0017] Figure 12 depicts a sodium dodecyl sulfate (SDS) polyacrylamide of fraction 1 from the elution window shown in Figure 4. Lane 1, molecular weight standard, lane 2, apo B-100 control, lane 3 empty; lane 4 and 5, ultra small apo B containing particles.

[0018] Figure 13 depicts apoB-100 immunoblotting of fraction 1 samples derived from two individuals (lanes 2 and 3),

[0019] Figure 14 depicts apoB-100 immunoblotting of fraction 1 samples derived from a third individual.

[0020] Figure 15 depicts cytokeratin 8 (CK8) immunoblotting of fractions 1-5 (F1 to F5) isolated from a single individual using the elution window described in Figure 4. First left lane, CK standard; lanes F1 to F5, fractions 1 to 5.

[0021] Figure 16 depicts cytokeratin 8 immunoblotting of fraction 1 isolated from 5 individuals as described in Figure 4.

[0022] Figures 17A-D depicts an amino acid sequence of apoB-100.

[0023] Figure 18 depicts an amino acid sequence of cytokeratin 8.

DEFINITIONS

[0024] As used herein, the terms "determining," "measuring," and "assessing," and "assaying" are used interchangeably and include both quantitative and qualitative determinations.

[0025] A "biological sample" encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as polynucleotides. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

[0026] As used herein the term "isolated" is meant to describe a compound of interest that is in an environment different from that in which the compound naturally occurs. "Isolated" is meant to include compounds that are within samples that are substantially enriched for the compound of interest and/or in which the compound of interest is partially or substantially purified.

[0027] As used herein, the term "substantially pure" refers to a compound that is removed from its natural environment and is at least 80% free, at least 85% free, at least 90%, at least 95%, at least 98%, or at least 99%, free from other components with which it is naturally associated.

[0028] "Predisposition" as used herein is substantially synonymous with risk, inclination, tendency, predilection, or susceptibility.

[0029] The terms "individual," "subject," "host," and "patient," used interchangeably herein, refer to a mammal, including, but not limited to, murines (rats, mice), non-human primates, humans, canines, felines, ungulates (e.g., equines, bovines, ovines, porcines, caprines), etc. In some cases, the term refers to a human.

[0030] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to

be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0031] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0032] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0033] It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an apoB particle" includes a plurality of such particles and reference to "the diagnostic assay" includes reference to one or more diagnostic assays and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

[0034] It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments pertaining to the invention are specifically embraced by the present invention and are disclosed herein just as if each and every combination was individually and explicitly disclosed, to the extent that such

combinations embrace subject matter that are, for example, compounds that are stable compounds (i.e., compounds that can be made, isolated, characterized, and tested for biological activity). In addition, all sub-combinations of the various embodiments and elements thereof (e.g., elements of the chemical groups listed in the embodiments describing such variables) are also specifically embraced by the present invention and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

[0035] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DETAILED DESCRIPTION

[0036] The present disclosure describes a species of very high density, ultra small, lipid-depleted apolipoprotein B containing particles. These particles may contain other proteins including cytokeratins. The isolated particles are useful in diagnostic assays, which are also provided.

APOLIPOPROTEIN PARTICLE

[0037] The present disclosure provides an isolated particle comprising: a) apolipoprotein B (apoB); and b) a cytokeratin-8 polypeptide having a molecular weight of about 52 kDa. A subject isolated particle is referred to herein as a "very high density, ultra small, de-lipidated apolipoprotein B containing particle". A subject "very high density, ultra small, de-lipidated apolipoprotein B containing particle" is characterized by having an average particle diameter in the range of from about 7.1 nm to about 22 nm; having a density greater than 1.21 g/mL; and having undetectable content of cholesterol and triglycerides by sensitive assays.

[0038] A subject "very high density, ultra small, de-lipidated apolipoprotein B containing particle" can have an average particle diameter in the range of from approximately 7.1 nm to 22 nm, e.g., from about 7.1 nm to about 15 nm, from about 15 nm to about 18 nm, or from about 18 nm to about 22 nm. Thus, e.g., at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99%, of the particles in a population of "very high density, ultra small, de-lipidated apolipoprotein B containing

particles" has an average particle diameter in a range of from about 7.1 nm to about 15 nm, from about 15 nm to about 18 nm, or from about 18 nm to about 22 nm.

[0039] A subject "very high density, ultra small, de-lipidated apolipoprotein B containing particle" can have a size in the range of from about 71 Å to about 220 Å, e.g., from about 71 Å to about 160 Å, from about 160 Å to about 175 Å, from about 175 Å to about 200 Å, from about 200 Å to about 210 Å, from about 210 Å to about 220 Å.

[0040] A subject "very high density, ultra small, de-lipidated apolipoprotein B containing particle" generally has a density greater than 1.21 g/mL, e.g., a subject "very high density, ultra small, de-lipidated apolipoprotein B containing particle" generally has a density of from about 1.21 g/mL to about 1.3 g/mL, or from about 1.3 g/mL to about 1.35 g/mL. Thus, e.g., at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99%, of the particles in a population of "very high density, ultra small de-lipidated apolipoprotein B containing particles" has a density of from about 1.21 g/mL to about 1.3 g/mL, or from about 1.3 g/mL to about 1.35 g/mL.

[0041] An isolated "very high density, ultra small, de-lipidated apolipoprotein B containing particle" of the present disclosure has substantially no lipid. For example, a subject "very high density, ultra small, de-lipidated apolipoprotein B containing particle" has substantially no cholesterol, e.g., a subject "very high density, ultra small, de-lipidated apolipoprotein B containing particle" will have less than about 10%, less than about 5%, less than about 3%, less than about 2%, less than about 1%, less than about 0.5%, less than about 0.2%, less than about 0.1%, or less than about 0.01%, by weight, cholesterol. In some cases, a subject "very high density, ultra small, de-lipidated apolipoprotein B containing particle" has no detectable cholesterol.

[0042] As another example, a subject "very high density, ultra small, de-lipidated apolipoprotein B containing particle" has substantially no triglycerides, e.g., a subject "very high density, ultra small, de-lipidated apolipoprotein B containing particle" will have less than about 10%, less than about 5%, less than about 3%, less than about 2%, less than about 1%, less than about 0.5%, less than about 0.2%, less than about 0.1%, or less than about 0.01%, by weight, triglycerides. In some cases, a subject "very high density, ultra small, de-lipidated apolipoprotein B containing particle" has no detectable triglycerides.

Apolipoprotein-B 100

[0043] Amino acid sequences of apoB-100 polypeptides are known in the art. For example, the following GenBank accession numbers provide amino acid sequences of apoB-100 polypeptides; 1) GenBank Accession No. NP_000375.2 (*Homo sapiens* apoB-100); 2)

GenBank Accession No. XP_515323.2 (*Pan troglodytes* apoB-100); 3) GenBank Accession No. XP_001097500.1 (*Macaca mulatta* apoB-100); 4) GenBank Accession No. XP_001501729.1 (*Equus caballus* apoB-100); 5) GenBank Accession No. NP_033823.2 (*Mus musculus* apoB-100); and 6) GenBank Accession No. NP_062160.2 (*Rattus norvegicus* apoB-100).

[0044] In some embodiments, an apoB-100 polypeptide that is included in a subject "very high density, ultra small de-lipidated apolipoprotein B containing particle" comprises an amino acid sequence having at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99%, or 100%, amino acid sequence identity with amino acids 28-4563 of the amino acid sequence set forth in Figures 17A-D and SEQ ID NO:1.

Cytokeratin 8

[0045] Amino acid sequences of cytokeratin 8 polypeptides are known in the art. For example, the following GenBank accession numbers provide amino acid sequences of cytokeratin-8 polypeptides: 1) GenBank Accession No. AAA35763 (*Homo sapiens* cytokeratin 8); 2) GenBank Accession No. AAA19668.1 (*Rattus norvegicus* cytokeratin 8); 3) GenBank Accession No. AAI06155.1 (*Mus musculus* cytokeratin 8); 4) GenBank Accession No. AAI54778.1 (*Danio rerio* cytokeratin 8); and 5) GenBank Accession No. XP_002742819 (*Callithrix jacchus* cytokeratin 8).

[0046] In some embodiments, a cytokeratin-8 polypeptide that is included in a subject "very high density, ultra small, lipid depleted apolipoprotein B containing particle" comprises an amino acid sequence having at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99%, or 100%, amino acid sequence identity with the amino acid sequence set forth in Figure 18 and SEQ ID NO:2.

METHODS OF ISOLATING A VERY HIGH DENSITY, ULTRA SMALL, LIPID DEPLETED APOLIPOPROTEIN B CONTAINING PARTICLE

[0047] The present disclosure provides a number of methods to isolate the ultra-small apo B containing particles which may contain cytokeratin 8. An immunoaffinity method can be used. For example anti-apoB antibody that is immobilized (e.g., on a column, a magnetic bead, and the like) can be contacted with a sample (e.g., plasma, such as human plasma) containing the particle, where the particle binds to the immobilizied anti-apoB antibody, forming an immobilized anti-apoB-particle complex. The particle in the immobilized complex can be eluted.

[0048] For example, Apo B antibody (antibody specific for apoB) was conjugated to the Dynabeads® M-280 Tosylactivated using manufacturer recommended procedure with

slight modification such as replacing the bovine serum albumin (BSA) in buffer D with non-fat dry milk, replacing BSA in buffer E with Tween 20 and antibody conjugation temperature from 37°C for 12-18 hours to 22-25°C (room temperature) for 24 hours. Serum sample was diluted 1:200 in buffer D and incubated with apo B-specific monoclonal antibody conjugated to magnetic Dynabeads at 25°C with continuous rocking/mixing for 30 minutes. At the end of the incubation period, the magnetic field was applied to the tubes, then supernatant was removed followed by 3 wash with phosphate buffer saline (PBS). Glycine buffer (pH 2.8) was used to elute/release the apo B particles from their respected antibody. Then the pH was immediately adjusted to around 7.5 with 2-2.5 µL of the 2.5mmol NaOH. The eluted particles were dialyzed over night against 25mmol ammonium acetate before analysis with ion mobility (IM), see **Figure 1**. Furthermore, the eluted particles were assayed using an enzyme-linked immunosorbent assay (ELISA) to estimate the recovery of this method.

[0049] The density gradient is one of the methods used. The density of the plasma was adjusted to 1.21 g/mL (by adding 1.91 gram NaBr to 6.503 mL plasma) and to 1.25 g/L (by adding 2.294 gram NaBr to 6.4 mL plasma). After completely dissolving the NaBr into the plasma, 6 mL was taken and added to the ultracentrifuge tubes, and then 6 µL 10mmol trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was added to each tube to prevent oxidation of lipoproteins. Ultracentrifugation was carried out at 40,000 rpm (115,046 g force) and 15 °C for 24 hours. At the end of the ultracentrifugation, the tubes were carefully removed and the top 1.5 mL was harvested from the all tubes. The top and bottom fractions were dialyzed against 25mmol ammonium acetate for approximately 24 hours at 4°C. Following the dialysis of the plasma fractions, Ion Mobility was used to measure particles size (**Figure 2**). Also the top and bottom density fractions were subject to above described immunoassay isolation procedure to isolate the very high density ultra-small dense apo B containing particles (**see Figure 3**). In addition, the concentration of apo B was measured by ELISA assay using monoclonal specific antibody (see, e.g., **Table 1**, below, for apo B ultracentrifugation recovery by ELISA).

[0050] The present disclosure also provides a gradient gel electrophoresis method of isolating a very high density, ultra small apolipoprotein B containing particle described above. A subject method generally involves applying a sample (e.g., a plasma sample) comprising the very high density, ultra small apolipoprotein B containing particles as described above, on a 2-14% non-denaturing gradient polyacrylamide gel; separating the components of the

sample on the gel by applying increasing voltage to the gel; and collecting fractions that advance beyond an LDL-IV standard band in the gel.

[0051] Pre-stained lipoprotein standards can be run alongside the sample, to provide an indication as to where to elute the sample. Suitable standards include: Lp(a); Large LDL; LDL-III; and LDL-IV. "Lp(a)" refers to biological particles consisting of LDL covalently attached to the protein lipoprotein A.

[0052] For example, a plasma (e.g., human plasma) sample is applied to a slot in a 2-14% non-denaturing gradient polyacrylamide gel; and pre-stained standard lipoproteins (e.g., Lp(a); Large LDL; LDL-III; and LDL-IV) are applied to sample application slots on either side of the plasma application slot. The gel can be run at 125 V 12-18 hours, i.e., 125 volts can be applied to the gel for 12-18 hours. Alternatively, the following voltage gradient can be applied: 20V for 15 minutes, 40V for 15 minutes, 60V for 15 minutes, 80V for 15 minutes, and 125V for 15 minutes, for a total of 1 hour 15 minutes; and the voltage gradient re-applied continuously over the course of 12 hours. After application of the voltage for 12-18 hours, a portion of the gel that is in a position beyond (toward the cathode side of the gel) the position of the LDL-IV band is excised, creating a trough (**Figure 4**); the trough is filled with buffer; and voltage (250V) is applied to the gel for approximately 30-60 minutes to allow components of the sample to enter the buffer-filled trough, forming an elution sample. The elution sample is then analyzed for the presence of the very high density, ultra small apolipoprotein B containing particles. Any convenient method, e.g., ion mobility analysis, can be used to test the "very high density, ultra small lipid depleted apolipoprotein B containing particle" present in the elution sample. Ion mobility analysis is described in, e.g., U.S. Patent Publication No. 2010/0213061. The presence in the particle of apoB-100 and cytokeratin 8 can be detected using antibodies specific for these components.

DETECTION METHODS

[0053] The present disclosure provides various detection methods involving detection of a very high density, ultra small, lipid depleted apolipoprotein B containing particles. The present disclosure provides methods for detecting a very high density, ultra small, lipid depleted apolipoprotein B containing particle, as described herein, in a biological sample obtained from an individual. The methods generally involve contacting the biological sample with an antibody specific for apoB100 and/or an antibody specific for cytokeratin-8; and detecting binding of the antibody to molecules in the sample. A subject isolated very high density, ultra small, lipid depleted apolipoprotein B containing particle can be used as a positive control in a subject detection method.

[0054] The presence in the biological sample of an amount of the very high density, ultra small, lipid depleted apolipoprotein B containing particle and/or cytokeratin 8 that is higher than a normal control amount can indicate that the individual from whom the biological sample was obtained has, or is at higher risk than the general population of developing, a disorder such as cardiovascular disease, atherosclerosis, myocardial infarction, or atherosclerotic plaque rupture, or is at risk of atherosclerotic plaque rupture. In some cases, the level of the very high density, ultra small, lipid depleted apolipoprotein B containing particle and/or cytokeratin 8 can provide an indication of the individual's prognosis following placement in the individual of a stent. In some cases, the level of the very high density, ultra small, lipid depleted apolipoprotein B containing particle and/or cytokeratin 8 can provide an indication of the individual's prognosis following coronary artery bypass graft surgery (CABG).

[0055] The present disclosure provides diagnostic assays for determining whether an individual has cardiovascular disease (CVD); assays for assessing an individual's response to therapy for a CVD or other drug treatment; and prognostic assays for determining the risk that an individual will develop CVD. A subject isolated very high density, ultra small, lipid depleted apolipoprotein B containing particle can be used as a positive control in a subject detection method. A subject isolated very high density, ultra small, lipid depleted apolipoprotein B containing particle can be used to generate a standard curve, for use in a subject detection method, e.g., where the detection is qualitative.

Detecting an ultra-small apo B containing particles

[0056] A very high density, ultra small, lipid depleted apolipoprotein B containing particle as described above can be detected in a biological sample (e.g., blood, or a blood fraction such as serum or plasma). A very high density, ultra small, lipid depleted apolipoprotein B containing particle as described above, can be detected using, e.g., antibody specific for apoB and antibody specific for cytokeratin 8. An antibody specific for a component (e.g., an antibody specific for apoB-100; an antibody specific for cytokeratin 8) can comprise a detectable label. Suitable detectable labels include any composition detectable by ion Mobility, spectroscopic, photochemical, biochemical, immunochemical, electrical, optical, or chemical means. Suitable detectable labels include, but are not limited to, magnetic beads (e.g. Dynabeads™), fluorescent dyes (e.g., fluorescein isothiocyanate, texas red, rhodamine, a green fluorescent protein, a red fluorescent protein, a yellow fluorescent protein, and the like), a radiolabel (e.g., ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), an enzyme (e.g., horse radish peroxidase, alkaline phosphatase, luciferase, and others commonly used in an

enzyme-linked immunosorbent assay (ELISA)), and colorimetric labels such as colloidal gold or colored glass or plastic (e.g. polystyrene, polypropylene, latex, etc.) beads.

[0057] An antibody specific for a component (e.g., an antibody specific for apoB-100; an antibody specific for cytokeratin 8) can be immobilized on a on a solid support. Suitable supports are well known in the art and comprise, inter alia, polystyrene beads, latex beads, magnetic beads, colloid metal particles, glass and/or silicon chips and surfaces, nitrocellulose strips, nylon membranes, sheets, duracytes, wells of reaction trays (e.g., multi-well plates), plastic tubes, etc.

[0058] In some cases, the average particle diameter size and the mass of the particle are determined following detection of the particle. The average particle diameter and mass of the particle can be determined using ion mobility analysis. See, e.g., U.S. Patent Publication No. 2010/0213061. Non-denaturing polyacrylamide gradient gel electrophoresis (see, U.S. Patent Publication No. 5,925,229). Agarose gel electrophoresis. Nuclear Magnetic Resonance (NMR), see U.S. Patent No. 20110004453. Density gradient ultracentrifugation. Electron microscope. Any method cable to count and measuring particles size not listed here.

[0059] The substantial lack of triglycerides and cholesterol can be determined using standard assays for these compounds. For example, methods involving use of enzymatic hydrolysis of triglycerides to glycerol and free fatty acids, followed by either colorimetric or fluorometric measurement of the glycerol released, can be used. For standard assays for triglycerides see, e.g., Bucolo and David (1973) *Clin. Chem.* 19:476; Fossati and Prencipe (1982) *Clin. Chem.* 28:2077; McGowan et al. (1983) *Clin. Chem.* 29:538; and Mendez et al. (1986) *Anal. Biochem.* 156:386. Various colorimetric and fluorometric assays for cholesterol are known in the art; and any such assay method can be used to determine substantial lack of cholesterol in an apoB/apoA-I particle as described herein. See, e.g., Kishi et al. (2002) *Clin. Chem.* 48:737, for an example of an assay for cholesterol.

[0060] The detection can be quantitative or qualitative. In some embodiments, e.g., where quantitative detection is desired, a standard curve using known amounts (e.g., 1 ng, 10 ng, 50 ng, 100 ng, 1 μg, 10 g, 50 μg, 100 μg, etc.) of a subject very high density ultra-small apo B containing particles is used.

Assessing risk of CVD

[0061] The present disclosure method for assessing risk of CVD in an individual, the method comprising: detecting (e.g., measuring, determining, or assessing) a level of a very high density, ultra small, lipid-depleted apo B containing particle as described above in a

biological sample from the individual; and assessing the risk based on the detected level of the particle. A level of the very high density, ultra small, lipid depleted apo B containing particle that is higher than a normal control level indicates that the individual has an increased risk of CVD. For example, a level of the "very high density, ultra small, lipid depleted apo B containing particle" that is at least 15% higher, at least 25% higher, at least 50% higher, at least 75% higher, at least 2-fold higher, at least 5-fold higher, or greater than 5-fold higher, than a normal control level indicates that the individual has an increased risk of CVD. CVD includes atherosclerosis, coronary artery disease (which may result in myocardial infarction), angina, stroke, hypertension, and heart failure. In some instances, the individual (e.g., a human) exhibits at least one clinical symptom or sign of cardiovascular disease.

[0062] A subject method of assessing risk of CVD can involve use of a subject kit (as described below), where the kit can include a positive control (e.g., a purified very high density ultra-small apo B containing particle, as described above) and/or components for generating a standard curve (e.g., a subject isolated very high density ultra-small apo B containing particle in defined amounts, e.g., 1 ng, 10 ng, 50 ng, 100 ng, 1 μg, 10 μg, 50 μg, 100 μg, etc.). For example, the level of the very high density, ultra small, lipid depleted apo B containing particle can be determined by comparison to a standard curve generated using a subject isolated very high density ultra-small apo B containing particle in defined amounts.

[0063] Based on a subject detection method, a certain therapeutic regimen may be recommended by a physician or other qualified medical personnel. For example, where the outcome of subject detection method indicates that the individual has an increased risk, compared to a healthy individual who has no signs of CVD, of developing CVD, a recommendation as to pharmaceutical intervention, diet alteration, exercise regimen, and the like, may be made.

[0064] In some cases, a subject method of assessing risk of CVD can further include communicating to the individual from whom the biological sample was obtained (in which biological sample the level of the very high density ultra-small apo B containing particle was detected) the results of the assessment and/or suggested treatment regimens. Thus, in some embodiments, a subject method comprises detecting a level of a very high density, ultra small, lipid depleted apo B containing particle as described above in a biological sample from the individual; assessing the risk that the individual has or will develop CVD based on the detected level; and communicating a recommended treatment regimen to the individual. The recommended treatment regimen can be based on a therapy decision tree that sets forth various treatment options, depending on the results of the subject method,

and optionally other patient information (e.g., results of other tests, such as other tests for CVD; patient medical history; any prior or ongoing treatment the patient is undergoing; etc.).

[0065] In some embodiments, a subject method of assessing risk of CVD can further include treating the individual for CVD. For example, an individual determined to be a higher risk of CVD than the general population can be treated with a blood pressure-lowering drug (e.g., a diuretic; a beta blocker), an anti-coagulant drug, or a cholesterol-lowering drug. For example, an individual determined to be a higher risk of CVD than the general population can be treated with: 1) a diuretic, e.g., a thiazide diuretic; 2) a beta blocker (e.g., Sectral (acebutolol); Zebeta (bisoprolol); Bevibloc (esmolol); Inderal (propranolol); Tenormin (atenolol); Normodyne (labetalol); Coreg (carvedilol); Lopressor (metoprolol)); 3) an anti-coagulant such as Coumadin (warfarin), Heparin, Lovenox, or Fragmin; or 4) a cholesterol-lowering drug such as a HMG-CoA reductase inhibitor (a statin) (e.g., atorvastatin (Lipitor), fluvastatin (Lescol), lovastatin (Mevacor), pitavastatin (Pitava), pravastatin (Pravachol), rosuvastatin (Crestor), simvastatin (Zocor)), or a fibrate (e.g., gemfibrozil (Lopid), fenofibrate (Tricor), or fenofibric acid (Trilipix).

[0066] A subject method of assessing risk of CVD can further include generating a report that provides an indication of the risk that the individual will develop CVD. A "report," as described herein, is an electronic or tangible document that includes report elements that provide information of interest relating to a likelihood assessment and its results. A subject report includes at least a likelihood assessment, e.g., an indication as to the risk that an individual will develop CVD. A subject report can be completely or partially electronically generated, e.g., presented on an electronic display (e.g., computer monitor). A report can further include one or more of: 1) information regarding the testing facility; 2) service provider information; 3) patient data; 4) sample data; 5) an interpretive report, which can include various information including: a) indication; b) test data, where test data can include the level of very high density, ultra small, lipid depleted apo B containing particles and a normal control level of very high density, ultra small, lipid depleted apo B containing particles and 6) other features.

[0067] Thus, in some embodiments, the methods of the present disclosure further include generating a report that includes information regarding the patient's likely clinical outcome, e.g. risk of CVD. For example, the methods disclosed herein can further include a step of generating or outputting a report providing the results of a subject risk assessment, which report can be provided in the form of an electronic medium (e.g., an electronic

display on a computer monitor), or in the form of a tangible medium (e.g., a report printed on paper or other tangible medium).

Assessing efficacy of treatment

[0068] The present disclosure provides a method of assessing the efficacy of a treatment for a cardiovascular disease in an individual. In some cases, the method comprises: a) analyzing the level of a very high density, ultra small, lipid depleted apo B containing particles (as described above) in a biological sample obtained from the individual following the treatment; and b) comparing the post-treatment level to a pre-treatment level. A post-treatment level that is lower than the pre-treatment level indicates that the treatment was efficacious.

[0069] In some cases, a method of assessing efficacy of therapy involves analyzing the level of very high density, ultra small, lipid depleted apo B containing particles (as described above) in a biological sample obtained from an individual at a first time point during treatment for a CVD; analyzing the level of very high density, ultra small, lipid depleted apo B containing particles (as described above) in a biological sample obtained from an individual at a second time point (where the second time point is later than the first time point) during treatment for a CVD; and comparing the level from the first and second time points. A level at the second time point that is lower than the level at the first time point indicates that the treatment was efficacious. The second time point can be from one day to one week, from one week to one month, from one month to three months, from three months to six months, or more than six months, later than the first time point.

Determining risk of mortality

[0070] The present disclosure provides a method of determining the risk of mortality due to a CVD in an individual. The method comprises detecting a level of very high density, ultra small, lipid depleted apo B containing particles (as described above) in a biological sample from the individual. A level of the particle that is higher than a normal control level indicates that the individual has an increased risk of mortality due to a CVD.

KITS

[0071] The present disclosure provides a kit (e.g., a test kit) for use in carrying out a subject detection method. A subject kit includes an antibody specific for apoB-100 and an antibody specific for cytokeratin 8. The antibodies can be in separate containers. The antibodies can be immobilized on a solid support. The antibodies can be detectably labeled.

[0072] The antibodies can be immobilized on a solid support. Suitable supports are well known in the art and comprise, inter alia, commercially available column materials, polystyrene

beads, latex beads, magnetic beads, colloid metal particles, glass and/or silicon chips and surfaces, nitrocellulose strips, nylon membranes, sheets, duracytes, wells of reaction trays (e.g., multi-well plates), plastic tubes, etc. A solid support can comprise any of a variety of substances, including, e.g., glass, polystyrene, polyvinyl chloride, polypropylene, polyethylene, polycarbonate, dextran, nylon, amylose, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. Suitable methods for immobilizing a subject antibody onto a solid support are well known and include, but are not limited to ionic, hydrophobic, covalent interactions and the like. Solid supports can be soluble or insoluble, e.g., in aqueous solution. In some embodiments, a suitable solid support is generally insoluble in an aqueous solution.

[0073] In some embodiments, a subject kit includes an antibody specific for apoB-100 and an antibody specific for cytokeratin 8, where each antibody is immobilized on a solid support, such as a test strip.

[0074] An antibody included in a subject kit will in some embodiments comprise a detectable label. Suitable detectable labels include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Suitable include, but are not limited to, magnetic beads (e.g. Dynabeads™), fluorescent dyes (e.g., fluorescein isothiocyanate, texas red, rhodamine, a green fluorescent protein, a red fluorescent protein, a yellow fluorescent protein, and the like), radiolabels (e.g., ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase, luciferase, and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (e.g. polystyrene, polypropylene, latex, etc.) beads.

[0075] A subject kit can further include reagents for detecting triglycerides; reagents for detecting cholesterol; etc. Other optional components of the kit include: a buffer; a protease inhibitor; a detectable label; etc. The various components of the kit may be present in separate containers or certain compatible components may be pre-combined into a single container, as desired.

[0076] In addition to above-mentioned components, a subject kit can include positive controls (e.g., a purified very high density ultra-small apo B containing particle); and/or components for generating a standard curve (e.g., a subject isolated very high density ultra-small apo B containing particles in defined amounts, e.g., 1 ng, 10 ng, 50 ng, 100 ng, 1 μg, 10 μg, 50 μg, 100 μg, etc.).

[0077] In addition to above-mentioned components, a subject kit can include instructions for using the components of the kit to practice a subject method. The instructions for practicing a subject method are generally recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or subpackaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. compact disc-read only memory (CD-ROM), digital versatile disk (DVD), diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

EXAMPLES

[0078] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); and the like.

Example 1: Isolation and characterization of very high density, ultra small, lipid depleted apo B containing particles.

Materials and Methods

Materials

[0079] Monoclonal apo B antibody M-035 (cat# H45640M, concentration 4.61mg/mL, lot# 7821) and M-036 (Cat# H45161M, concentration 2.1mg/mL, lot# 4A03107) from Meridian Life Science, Saco, ME 04072, USA. Dynabeads M-280 Tosylactivated magnetic beads cat# 142-04 from Invitrogen, Carlsbad, CA 92008, USA.

[0080] Polyacrylamide gradient gels (2-14%), with 18 sample loading lanes, were produced locally at the Children's Hospital Oakland Research Institute (CHORI), Oakland, CA 94609, USA. Electrophoresis chamber with power supply and cooling device was from Pharmacia Company, Stockholm, Sweden. Concentrated electrophoresis buffer was made in-house according to a standard operating procedure; 217.93 grams (gm) trizma base (Tris-hydroxymethyl aminomethane), 98.88 gm boric acid, 20.20 gm di-sodium EDTA in 4000 mL double deionized water. The working solution was made by diluting the concentrated buffer 1 in 5 (600 ml concentrated buffer + 2400 double deionized water).

[0081] Sudan black stain was from Beckman-Coulter, Fullerton, CA, USA. Lipoprotein particle size calibrator or quality control (LPCAL: AE/AG) was made in-house, and provided the following range of particle sizes: 315, 275.8, 248.7, and 225.2Å. Sample application comb was from Pharmacia, Stockholm, Sweden. Centricon-10 (cat# 4206) 2ml-concentrator was from Amicon, Billerica, MA, USA.

[0082] Other reagents and product were obtained from the following sources: 1) Coomassie blue (cat# 6104-58-1) (Sigma, St. Louis, MO, USA); 2) Airborne ion mobility analyzer (TSI incorporation, Minnesota, USA); 3) Cholesterol reagent (cat# E33940), (Polestar Laboratory, Escondido, CA, USA); 4) Free Glycerol Reagent A (Sigma,cat. No. F6428, 40 ml); 5) Triglyceride Reagent B (Sigma, cat. No. T2449, 10 ml); and 6) Bradford protein assay (Bio-Rad, cat. No. #500-0202, Hercules, CA 94547).

[0083] A monoclonal specific antibody against apoB-100, (catalogue No. K90086P), and horseradish peroxidase (HRP) conjugated secondary (anti-IgG) antibody, were obtained from Biodesign International, a division of Meridian Life Science Inc, Saco, ME 04072, USA.

[0084] Additional reagents and products were obtained from the following sources: 1) Super-signal developing reagent kit, (catalogue No. 34096), from Pierce, Rockford, IL 61105, USA; 2) X-ray film, (catalogue No. EK8FL), Belgium; 3) X-ray film developing

equipment from Kodak, Rochester, New York; 4) Bovine albumin (> 99% purity), (catalogue No. A-6003) from Sigma, St. Louis, MO, USA; 5) Glycine, (catalogue No. G7126) from Sigma; 6) Methanol, (catalogue No. M1775) from Sigma-Aldrich, St Louis, MO, USA; 7) Trizma-base (Hydroxymethyl aminomethane), (catalogue No. T1503) from Sigma-Aldrich; 8) sodium dodecyl sulfate (SDS) gel 3-8% and 4-20% (catalogue No. EC60385) from Invitrogen, Carlsbad, CA 92008, USA; 9) SDS reducing agent (catalogue No. NP0009) from Invitrogen; 10) SDS tracking dye (catalogue No. NP0007) from Invitrogen; 11) SDS running buffer, (catalogue No. NP0001) from Invitrogen; 12) Antioxidant, (catalogue No. NP0005) from Invitrogen; 13) Nitrocellulose transfer membrane, (catalogue No. 13849) from Bio-Rad, Hercules, CA, 94547, USA; 14) Tween-20 detergent, (catalogue No. P1379) from Sigma-Aldrich; 15) Phosphate buffered saline (PBS) made according to the laboratory standard operating protocol, CHORI, Oakland, CA, USA; 17) Pre-stained proteins molecular weight standard (10-250 KDA), (catalogue No. 161-0375) from Bio-Rad, Hercules, CA, USA and 27-180 KDA from Sigma, catalogue # MW-SDS-Blue; 18) Unstained proteins molecular weight (40-500 KDA) standard, (catalogue No. LC5688) from Invitrogen; 19) ApoB-100 and apoB-48 standard prepared in-house; 20) Brilliant Blue G (Coomassie blue) stain, (catalogue No. B0770) from Sigma; 21) SDS gels de-staining solution made according to the laboratory standard operating protocol; 22) Polyclonal specific antibody against cytokeratin 8, (catalogue No. RB-9095-PO) from Labvission, Fremont, California, USA; and 23) Cytokeratin 8 positive control, (catalogue No. RB-9095-PCL) from Labvission, Fremont, California, USA.

Methods

Separation of very high density, ultra small, lipid depleted apo B containing particles by immunoaffinity

[0085] Apo B was conjugated to the Dynabeads® M-280 Tosylactivated using manufacturer recommended procedure with slight modification such as replacing the bovine serum albumin (BSA) in buffer D with non-fat dry milk powder, replacing BSA in buffer E with Tween 20 and antibody conjugation temperature from 37°C for 12-18 hours to 22-25°C for 24 hours. Samples were diluted 1:200 in buffer D and incubated with beads conjugated with apo B-specific antibody at 25°C with continuous rocking for 30 minutes. At the end of the incubation period, a magnetic field was applied to the tubes, then the supernatant was removed, followed by 3 washes with phosphate buffer saline (PBS). Glycine buffer (pH 2.8) was used to elute the apo B particles. Then the pH was immediately adjusted to around

7.0 with 2 µL of the 2.5mmol NaOH. The eluted particles were dialyzed over night against 25mmol ammonium acetate before analysis by ion mobility.

Isolation of very high density, ultra small, lipid depleted apoB containing particles by ultracentrifugation

[0086] Plasma density adjustment: The density of the plasma was adjusted to 1.21 g/mL (by adding 1.91 gram NaBr to 6.503 mL plasma) and to 1.25 g/L (by adding 2.294 gram NaBr to 6.4 mL plasma). After completely dissolving NaBr into the plasma, 6 mL was taken and added to the ultracentrifuge tubes, then 6 µL 10mmol trolox was added to each tube to prevent lipoproteins oxidation. Mock solution density adjustment: The density of the mock solution was adjusted to 1.21 g/mL (by adding 1.91 gram NaBr to 6.503 mL plasma) and to 1.25 g/L (by adding 2.294 gram NaBr to 6.4 mL plasma). After complete dissolution of NaBr into the mock solution, 6 mL was taken and added to the ultracentrifuge tubes, then 6 µL 10mmol trolox was added to each tubs for reason of treating these tubes equally to the plasma tubes. These tubes served as balance in the ultracentrifuge and also for density verification for the plasma tubes. Ultracentrifugation was carried out at 40,000 rpm (average 115,046 g force), at 15 °C for 24 hours. At the end of the ultracentrifugation, the tubes were carefully removed and the top 1.5 mL was harvested from the all tubes (plasma and mock solution). Densities of the top and bottom fractions of the mock solution tubes were measured by the densitometer. The top and bottom fractions were dialyzed against 25mmol ammonium acetate for approximately 24 hours at 4°C. Following the dialysis of the plasma fractions, ion mobility was used to measure particle size. In addition, the concentration of apoB was measured by ELISA assay using monoclonal specific antibody.

Isolation of very high density, ultra small, lipid-depleted apo B containing particles by gradient gel electrophoresis (GGE)

[0087] A pre-staining lipoprotein standard with known peak particles size (Å) was prepared as follows prior to carrying out the procedure: Sudan black 'Lipostain' (Beckman Coulter) was added to the lipoprotein standard to make a 4% (v/v) solution (4µl Lipostain + 96µl lipoprotein standard). This was then incubated overnight (~12h) and was used within one week.

[0088] A pre-staining lipoprotein standard with known peak particles size (Å) was prepared as follows prior to carrying out the procedure: Sudan black 'Lipostain' (Beckman Coulter) was added to the lipoprotein standard to make a 4% (v/v) solution (4µl Lipostain + 96µl lipoprotein standard). This was then incubated overnight (~12h) and was used within one week.

[0089] The electrophoresis buffer was cooled to a temperature between 8 -16 °C. The gel (2-14%) was pre-electrophoresed at 125 volts for at least 30 minutes to remove any particulates and to condition the gel with electrophoresis buffer. A pre-stained lipoprotein standard with known particle size was applied (10 µL) to lanes 1, 2, 17 and 18. Plasma samples (10 µL) were applied to lanes 3 through 16. Electrophoresis was carried for 15 minute intervals at 20, 40, 60, and 80V, and at 125V overnight (12 hours).

[0090] A scalpel or a razor blade was used to remove the top section of the gel (~1 - 3mm) to remove any proteins or albumin that might have been trapped with the VLDL fraction in this part of the gel. The gel was then re-loaded using fresh electrophoresis buffer and electrophoresis was carried out at 250V for an additional 2 hours.

[0091] At the end of the 2 hours, a scalpel or razor blade and a ruler was used to excise the gel and create a window below the LDL-IV band (225 Å) with an approximate width of 0.5cm. A length of about 0.5 cm was left on the side of the window to hold the upper and lower parts of the gel together (see **Figure 4**). The window was filled with 1X Trizma Borate EDTA (TBE) buffer and was covered with a dialysis membrane to prevent buffer leakage.

[0092] The gel cassette was then re-assembled and re-inserted into the upper electrophoresis chamber, and electrophoresis was continued at 250 volts for 45 minutes to collect fraction 1. Then the gel cassette was opened to collect the first fraction by aspiration using plastic transfer pipette. This aspiration was repeated a few times to ensure that all particles belonging to that fraction were collected.

[0093] The cassette was re-assembled and re-loaded again into the GGE chamber and electrophoresis was continued at 250 volts for intervals of 1 hour until all of the LDL fractions of interest had been separated and collected. The volume of each fraction was approximately 500µl from each gel and the total volume from 4 gels was 2mL.

[0094] The collected fractions were then concentrated by centrifugation at 7,000 rpm for 60 minutes at a temperature of 4°C using the 2ml concentrator Centricon-10 to reduce the volume to approximately 500µl (4X concentration). To verify the uniformity of the final collected fractions, they were separated a second time by electrophoresis on a new 2-14% gradient gel, and by an ion mobility analyzer, for the measurements of their particles size diameter.

Ion Mobility (IM)

[0095] An ion mobility analyzer was used as an additional tool to test the eluted lipoprotein particles as described by Caulfield *et al* 2008.

Molecular weight determination of proteins by SDS-PAGE

[0096] SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify proteins associated with the LDL-IV fraction according to their molecular weight. First the protein concentration was measured with Bradford protein assay (0.158 μg/mL; assay sensitivity 0.125–2.0 mg/ml) then the proteins of interest were separated and stained on SDS-PAGE as described by the kit manufacturer (Invitrogen, CA, USA).

Protein transfer procedure

[0097] Following the separation of proteins by SDS-PAGE as described above, the proteins were transferred to a nitrocellulose membrane for immunoblotting as described by the kit manufacturer (Invitrogen, CA, USA.

Immunoblotting

[0098] The manufacturer procedure was modified/optimized and carried out as follows.

[0099] Bovine Serum Albumin (BSA) (3%) was made with PBS buffer, then 25 μl of Tween-20 was added to 50 ml of 3% bovine albumin (0.05%) and the membrane was incubated with gentle shaking at room temperature or overnight at 4°C.

[00100] The membrane was incubated, with shaking, with an apoB-specific primary antibody diluted 1: 10,000 with 3% BSA containing Tween-20 at a concentration of 0.05% at 300 revolutions for 2 hours at RT or overnight in the cold room. The 0.3% BSA containing 0.05% Tween-20 was used as a washing buffer to wash the membrane on three occasions, at 10 minutes for each wash, to remove all non-specific binding of antibodies. The membrane was incubated with secondary apoB HRP conjugated antibody (diluted 1:5000) for 1 hour at room temperature (RT). The membrane was washed 5 times, with shaking, using the washing buffer described above, at 300 revolutions for 10 minutes for each wash to remove any non-specific binding.

[00101] The membrane was developed using the super signal reagent, prepared by adding 1ml Reagent One to 1ml Reagent Two in 8ml distilled water. The membrane was then soaked in the super signal reagent for 2-5 minutes, after which, the developed membrane was placed between two layers of clear thin plastic. The membrane was exposed to x-ray film for 10, 20, 30, 60, and 90 seconds. The exposed films were then developed and examined.

Determination of molecular mass of the 52kDa band by mass spectrometry (MS)

[00102] Mass spectrometry was performed at Stanford University, Palo Alto, California, USA, according to their laboratory standard operating procedure (Shevchenko et al, 1996, 2007). The very high density, ultra small, lipid depleted apo B containing particles were

separated and stained after SDS-PAGE as described above and in-gel trypsin digestion and protein analysis of the ≈52 kDa band by MS was performed.

RESULTS

[00103] The immunoaffinity procedure was employed using monoclonal apo B antibody to isolate very high density, ultra small, lipid depleted apo B containing particles directly from plasma. The isolated particles were analyzed by ion Mobility as shown in **Figure 1**.

[00104] Following the ultracentrifugal separation, dialysis and appropriate dilution of the top and bottom fractions of the very high density solution containing the sample of interest, the following results were obtained by ion Mobility as shown in **Figure 2**. Also the immunoaffinity procedure was used to isolated apo B containing particles from the top 1.5mL of the 1.25g/mL density (black line) and from the bottom 4.5mL of the 1.25g/mL density (blue line) ultracentrifuged sample and then the eluted particles were analyzed by ion Mobility as shown in **Figure 3**. The ELISA results for apoB in the top and bottom density fractions are shown in **Table 1**.

Table 1

Density 1.21g/mL	Dilution factor	Concentration factor	apo B concentration	final apo B result
Top fraction	1.08	4	219.6	59.3
Bottom fraction	1.08	1.3	7.2	6.0
Top + Bottom				65.3
Original Plasma	N/A	N/A	80	80
Recovery				82%

[00105] **Table 2:** apo B recovery calculation in comparison to the original plasma concentration;some losses may be attributed to adhesion to the ultracentrifuge tube.

[00106] **Figure 1**. IM profile showing the total apo B containing particles following immunoaffinity isolation (directly from the plasma) using monoclonal apo B antibody.

[00107] **Figure 2.** IM profile showing LDL and HDL (blue line) isolated from the 1.5 mL top fraction of 1.21g/mL and the very high density ultra small particles (black line) isolated from the 4.5 mL bottom 1.21g/mL.

[00108] **Figure 3**. IM profile showing immunoaffinity isolated apo B containing particles from the top 1.5mL of the 1.25g/mL density (black line) and from the bottom 4.5mL of the 1.25g/mL density (blue line) ultracentrifuged sample.

[00109] Since the gel elution method released the smaller and denser particles first, the fraction numbers were inversely related to LDL fractions as defined by increasing density in the ultracentrifugation i.e. I to IV. The gel elution fractions start at 1, 2, 3, 4, and 5, moving

from smaller and denser particles to the larger and more buoyant particles, in which fraction 1 corresponds to the very high density, ultra small, lipid depleted apo B containing particles) (F1), fraction 2 to LDL-IV (F2), fraction 3 to LDL-III (F3), while fractions 4 and 5 correspond to the larger LDL's (F4 and F5).

Ion Mobility analysis

[00110] The characteristics of the particles in the separated fractions, namely very high density, ultra small, lipid depleted apo B containing particles), LDL-IV and LDL-III, were confirmed by ion mobility analysis as shown in **Figure 5, Figure 6, and Figure 7**.

[00111] Figure 5 depicts ion mobility analysis of particles isolated by electrophoresis in fraction 1 from the elution window shown in Figure 4.

[00112] Figure 6 depicts ion mobility analysis of particles isolated by electrophoresis in fraction 2 from the elution window shown in Figure 4.

[00113] Figure 7 ion mobility analysis of particles isolated by electrophoresis in fraction 3 from the elution window shown in Figure 4.

Fractions on 2-14% gradient gels

[00114] Figure 8 depicts the results of 2-14% gradient gel electrophoresis (GGE), followed by lipid staining with Sudan Black, of fractions 2 and 3 described in Figures 6 and 7. Lanes 1,2, 8, 17 & 18, lipoprotein standards; lanes 3-5, fraction 2; lanes 6& 7, fraction 3; lanes 9-12, empty; lane 13, high molecular weight protein standards; lanes 14-16, empty.

[00115] Figure 9 depicts the densitometric scan of lane 3 in Figure 8.

[00116] Figure 10 depicts the densitometric scan of lane 4 in Figure 8.

[00117] Figure 11 depicts a 2-14% GGE stained with Coomassie blue of fractions 1, 2 and 3 from the elution window shown in Figure 4. Lanes 1,2, 9, 17 & 18, lipoprotein standards; lanes 4-6, fraction 2, 3 and 4; lanes 3, 7, 8, 15 and 16, empty; lane 10, bovine serum albumin; lane 13, LDL control; lanes 11, 12 and 14, ultra small apo B containing particles.

[00118] Figure 12 depicts a sodium dodecyl sulfate (SDS) polyacrylamide of fraction 1 from the elution window shown in Figure 4. Lane 1, molecular weight standard, lane 2, apo B-100 control, lane 3 empty; lane 4 and 5, ultra small apo B containing particles.

Western immunoblotting for apoB-100 identification

[00119] Figure 13 depicts apoB-100 immunoblotting of fraction 1 samples derived from two individuals (lanes 2 and 3),

[00120] Figure 14 depicts apoB-100 immunoblotting of fraction 1 samples derived from a third individual.

Cytokeratin 8

[00121] The specific antibody against cytokeratin "8" reacted, to a variable extent, with ≈ 53 KDA protein band blotted from the very high density, ultra small, lipid depleted apo B containing particles (F1), LDL-IV (F2), LDL-III (F3) and Lp(a), but not with LDL-II (F4) and LDL-I (F5) fractions eluted with GGE method and separated on SDS-PAGE (**Figure 15**). Cytokeratin "8" was blotted from the 53 kDa bands of the very high density, ultra small, lipid depleted apo B containing particles eluted from 5 different plasma samples, and against the whole plasma. The first two subjects on the left side in **Figure 16** were recognized as LDL subclass pattern 'B' with predominantly small dense LDL, while the other three subjects were pattern 'A'. In the whole plasma sample, the cytokeratin "8" antibody reacted with the proteins corresponding to cytokeratin "8" in terms of its molecular weight and the molecular weight standard (STD), in addition to the presence of some other bands on the same, whole plasma lane. The appearance of the other additional bands may have been due to non-specific binding or to the presence of other cytokeratins with a homologous peptide sequence to cytokeratin "8".

[00122] **Figure 15** depicts cytokeratin 8 (CK8) immunoblotting of fractions 1-5 (F1 to F5) isolated from a single individual using the elution window described in Figure 4. First left lane, CK standard; lanes F1 to F5, fractions 1 to 5.

[00123] **Figure 16** depicts cytokeratin 8 immunoblotting of fraction 1 isolated from 5 individuals as described in Figure 4. Additionally, the CK "8" antibody was tested against whole plasma. The first two subjects on the left side are recognized as having LDL subclass pattern B, with respect to the distribution of their LDL particles.

[00124] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

What is claimed is:

1. An isolated, very high density, ultra small, lipid-depleted particle, the particle comprising:

a) apolipoprotein B (apoB);

b) a cytokeratin 8 polypeptide,

wherein the isolated particle has an average particle diameter in a range of from about 7.1 nm to about 22 nm,

wherein the isolated particle has a density >1.21 g/mL, and

wherein the isolated particle has substantially no cholesterol and substantially no triglyceride.

2. The isolated particle of claim 1, wherein the apoB comprises an amino acid sequence having at least about 95% amino acid sequence identity with the amino acid sequence set forth in SEQ ID NO:1.

3. The isolated particle of claim 1, wherein the cytokeratin 8 comprises an amino acid sequence having at least about 95% amino acid sequence identity with the amino acid sequence set forth in SEQ ID NO:2.

4. The isolated particle of claim 1, wherein the particle is at least 85% pure.

5. The isolated particle of claim 1, wherein the particle is at least 95% pure.

6. A method for assessing risk of cardiovascular disease (CVD) in an individual, the method comprising:

detecting a level of the particle of claim 1 in a biological sample from the individual; and

assessing the risk of CVD based on the results of said detecting, wherein a level of the particle that is higher than a normal control level indicates that the individual has an increased risk of CVD.

7. The method of claim 6, wherein the biological sample is blood or a blood fraction.

8. The method of claim 6, further comprising generating a report that provides an indication of the risk that the individual will develop CVD.

9. The method of claim 6, wherein the individual is a human.

10. The method of claim 6, wherein the individual exhibits at least one clinical symptom or sign of cardiovascular disease.

11. The method of claim 6, further comprising communicating to the individual various treatment options based on the results of the detecting step.

12. The method of claim 6, further comprising treating the individual for CVD.

13. A method of assessing the efficacy of a treatment for a cardiovascular disease in an individual, the method comprising:

a) analyzing the level of the particle of claim 1 in a biological sample obtained from the subject following the treatment; and

b) comparing the post-treatment level to a pre-treatment level,

wherein a post-treatment level that is lower than the pre-treatment level indicates that the treatment was efficacious.

14. The method of claim 13, wherein the individual is a human.

15. A method of determining the risk of mortality due to a cardiovascular disease (CVD) in an individual, the method comprising detecting a level of the particle of claim 1 in a biological sample from the individual, wherein a level of the particle that is higher than a normal control level indicates that the individual has an increased risk of mortality due to a CVD.

16. A kit for assessing risk of cardiovascular disease, the kit comprising:

a) a reagent that specifically binds apolipoprotein B-100; and

b) a reagent that specifically binds cytokeratin 8.

17. The kit of claim 16, further comprising an isolated particle of claim 1.

18. The kit of claim 16, further comprising instructions for use.

19. The kit of claim 16, wherein each of (a) and (b) is in a separate container.

20. The kit of claim 16, wherein each of (a) and (b) is an antibody.

21. The kit of claim 20, wherein each of the antibodies is immobilized on an insoluble support or is detectably labeled.

22. A method of detecting the particle of claim 1 in a biological sample, the method comprising:

a) contacting the biological sample with an antibody that binds apolipoprotein B-100 and/or an antibody that binds cytokeratin 8; and

b) detecting binding of the antibody to molecules in the biological sample.

23. The method of claim 22, wherein the biological sample is obtained from an individual who is being evaluated for possible cardiovascular disease (CVD) or CVD risk.

24. A method of isolating the particle of claim 1, wherein the method comprises subjecting a sample comprising the particle to an immunoaffinity method, a density gradient method, or a gradient gel electrophoresis method.

25. The method of claim 24, wherein the method comprises contacting a sample comprising the particle with an immobilized antibody specific for apoB; and eluting particles bound to the apoB.

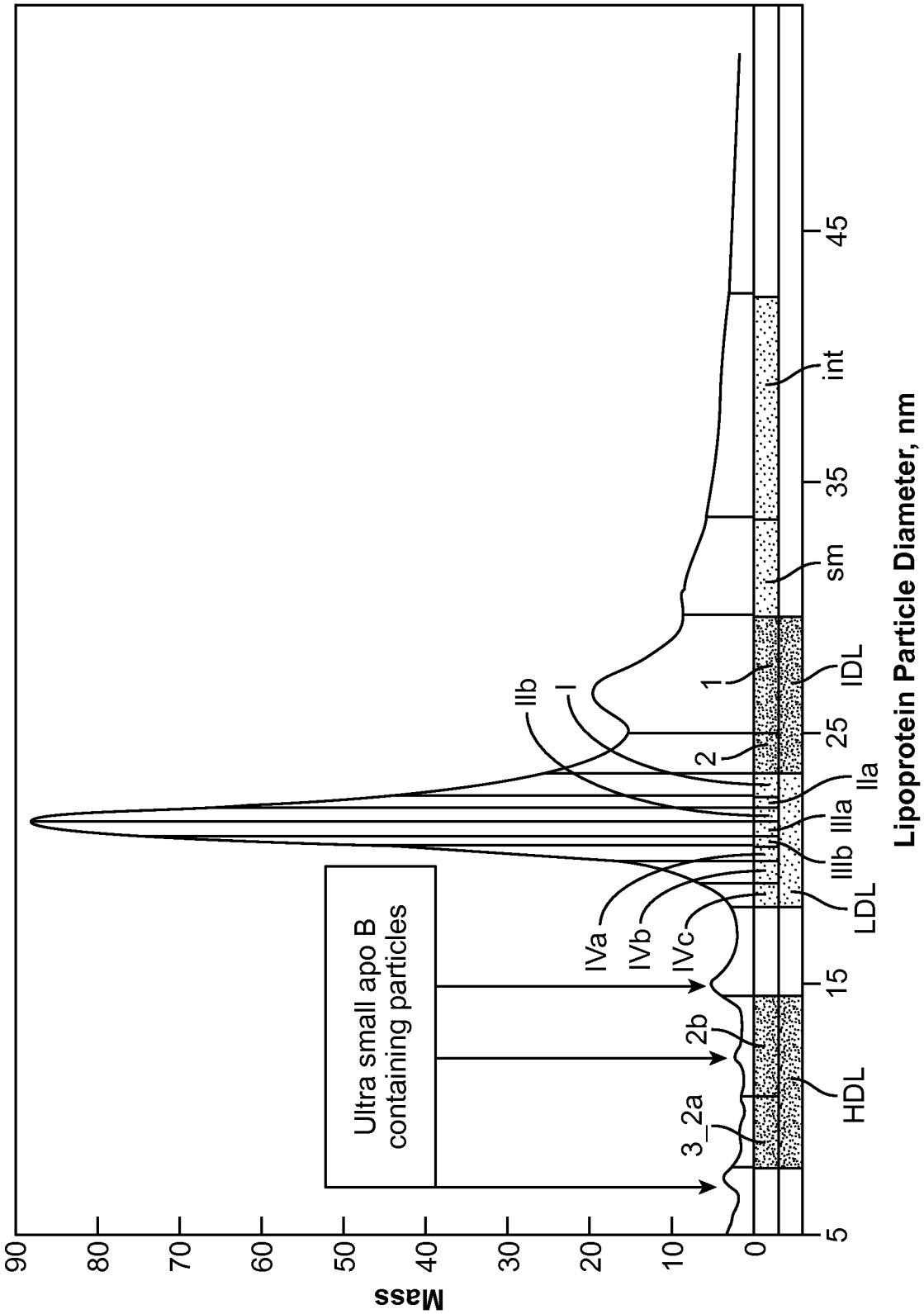

Mass
90
80
70
60
50
40
30
20
10
0
5
15
25
35
45
Lipoprotein Particle Diameter, nm
Ultra small apo B containing particles
3_2a
2b
IVa
IVb
IVc
HDL
LDL
IIIb
IIIa
IIa
2
1
I
IIb
sm
int
IDL

FIG. 1

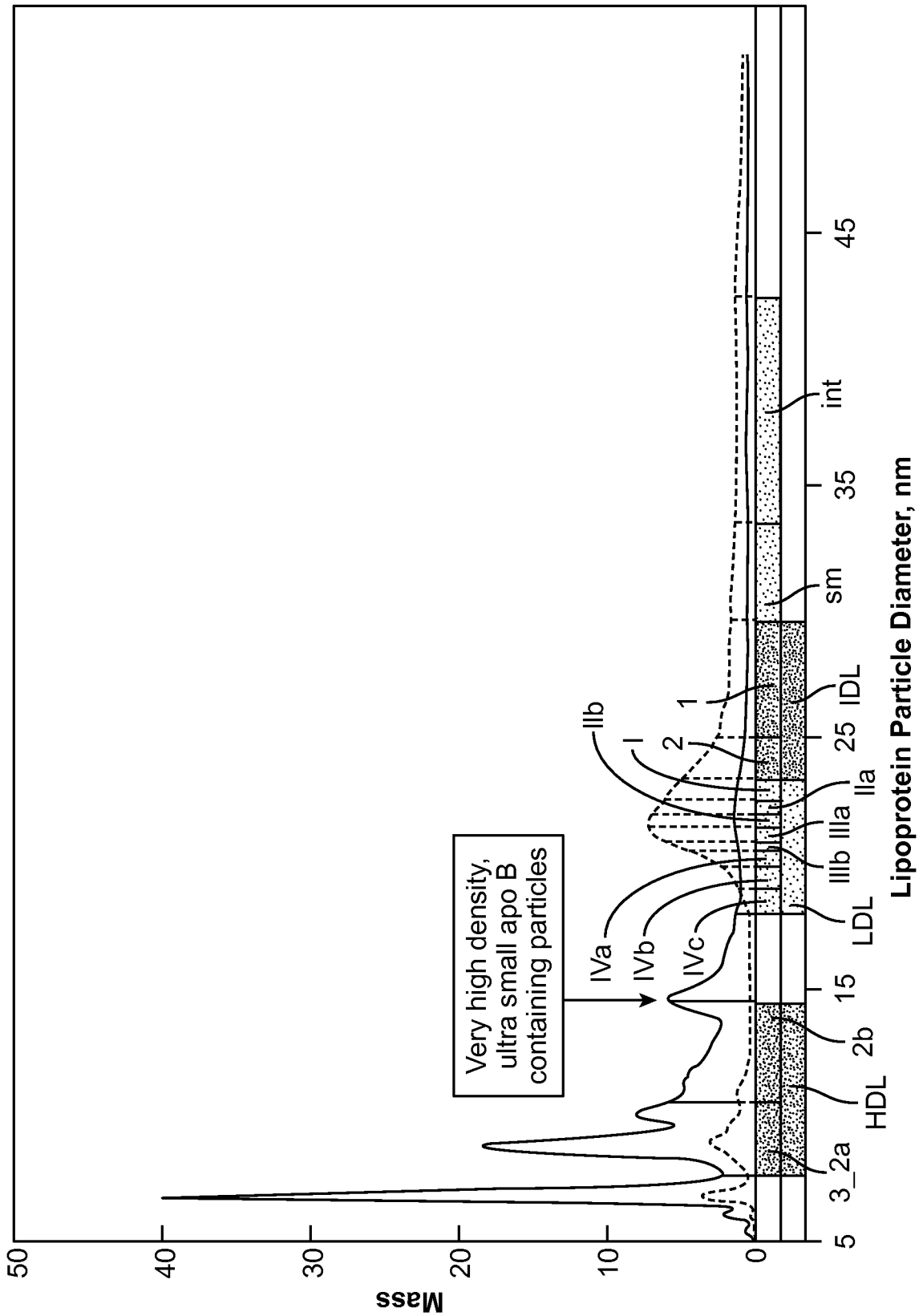

Mass
50
40
30
20
10
0
5
3_2a
HDL
2b
15
LDL
IVc
IVb
IVa
Very high density, ultra small apo B containing particles
IIIb
IIIa
IIa
25
IDL
1
2
I
IIb
sm
35
int
45
Lipoprotein Particle Diameter, nm

FIG. 2

FIG. 3

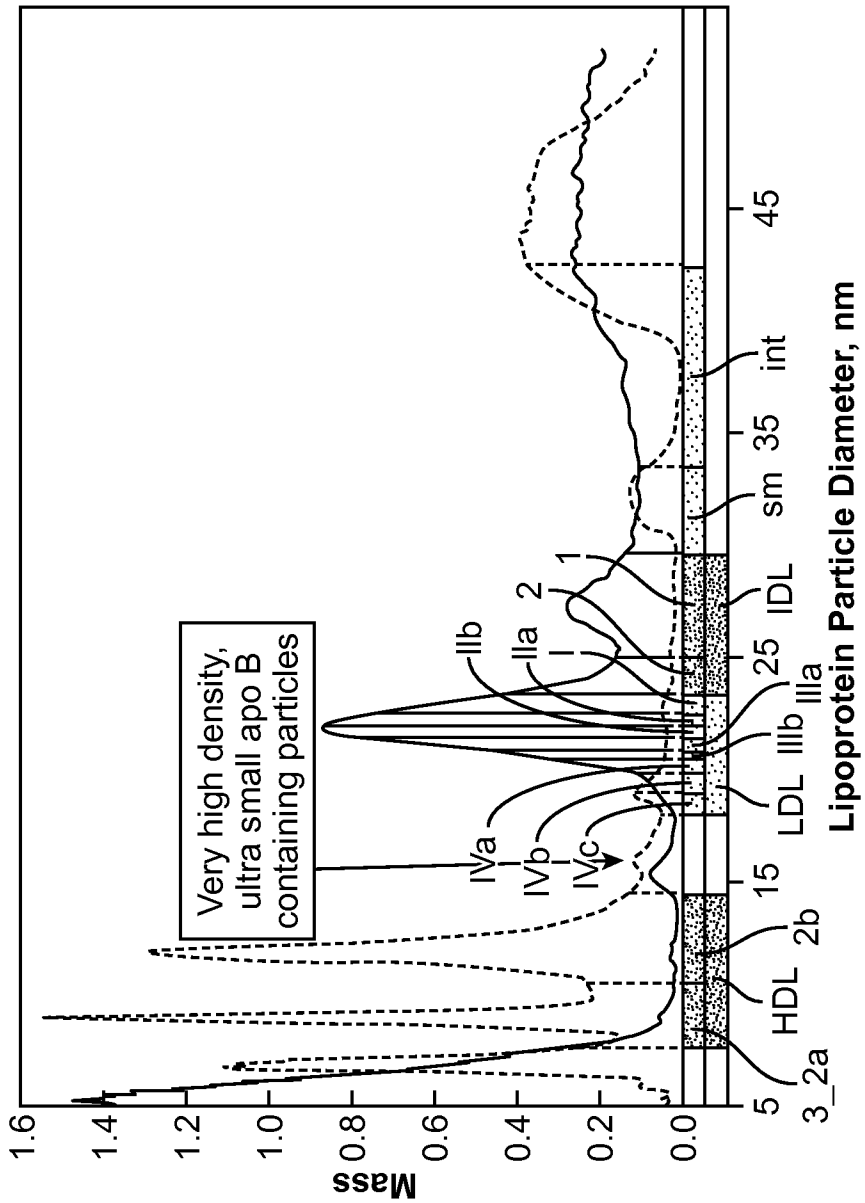
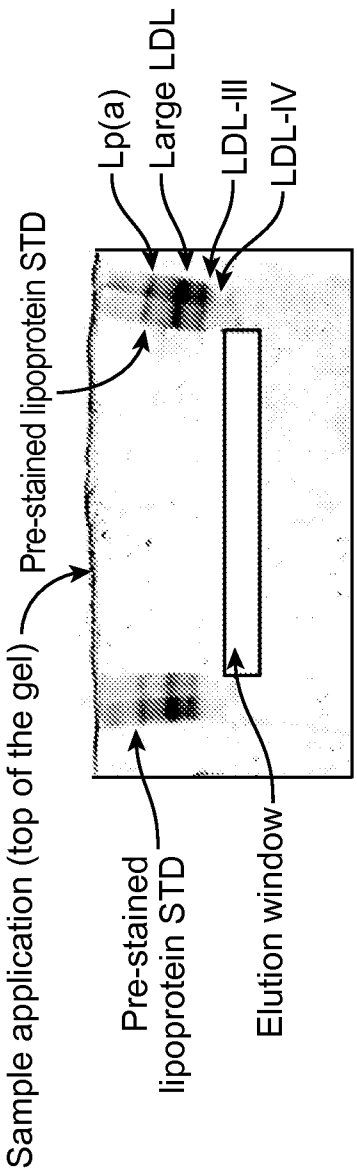

Mass
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Very high density, ultra small apo B containing particles
IVa
IVb
IVc
IIb
IIa
I
1
2
IDL
sm
35
int
45
25
IIIa
IIIb
LDL
15
2b
HDL
5
3_2a
Lipoprotein Particle Diameter, nm

FIG. 4


Lp(a)
Large LDL
LDL-III
LDL-IV
Pre-stained lipoprotein STD
Sample application (top of the gel)
Pre-stained lipoprotein STD
Elution window

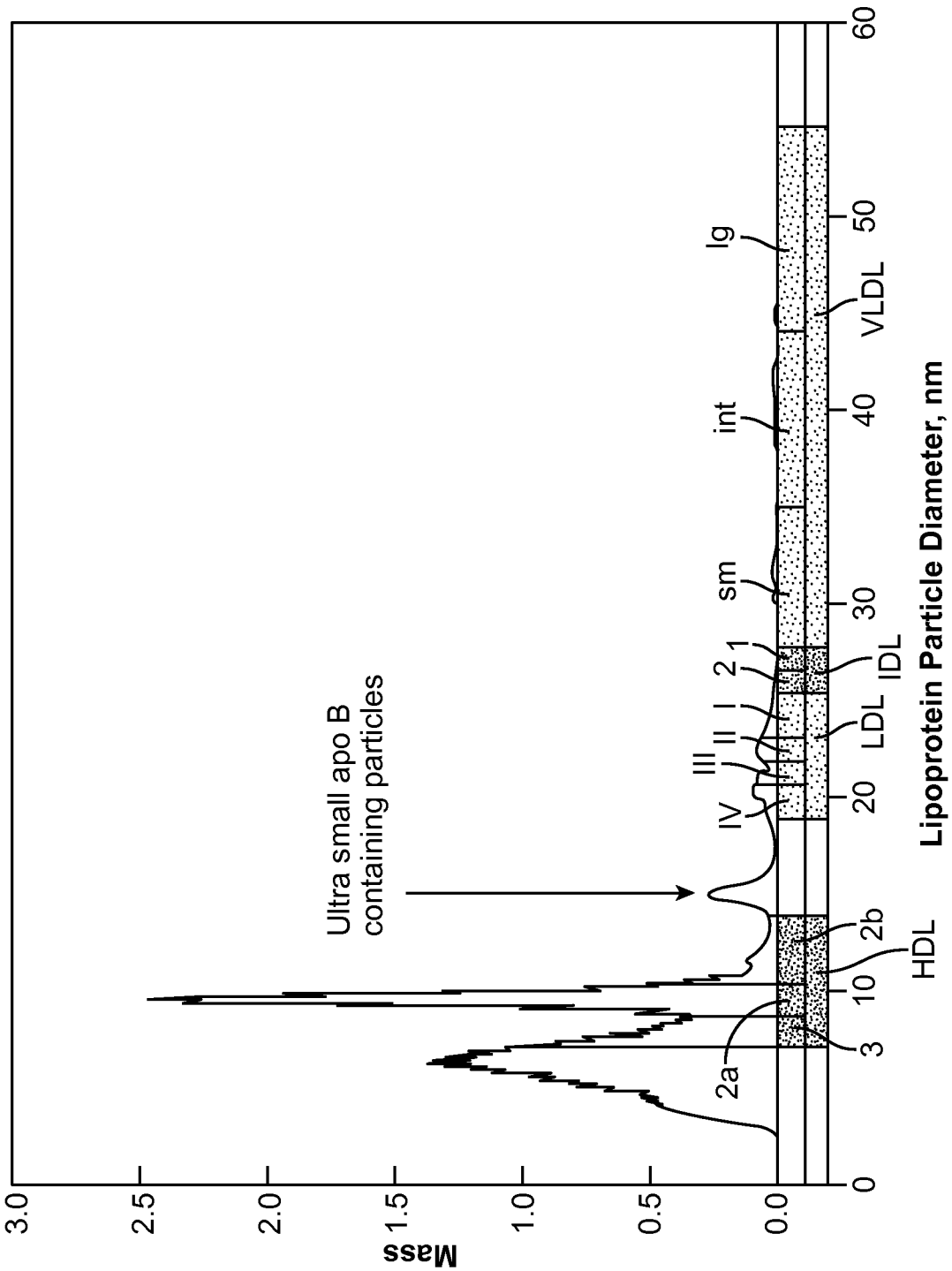
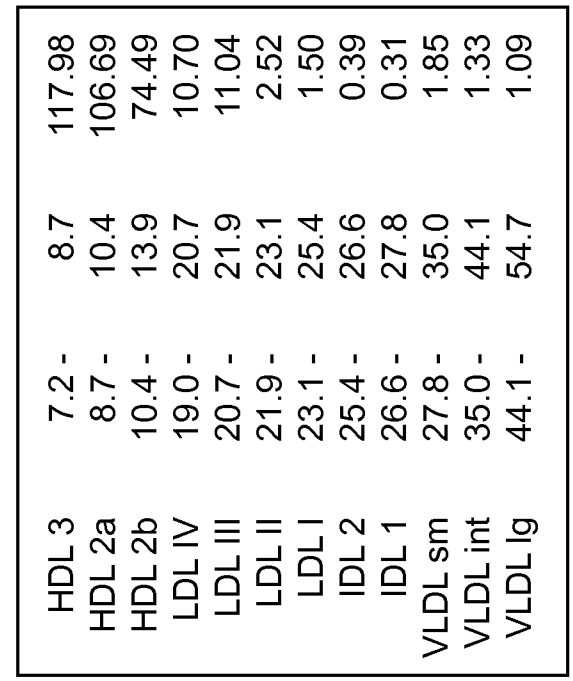

Mass
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ultra small apo B
containing particles
2a
3
2b
HDL
10
IV
III
II
I
2
1
IDL
LDL
20
sm
30
int
40
VLDL
lg
50
60
Lipoprotein Particle Diameter, nm
0

FIG. 5



HDL 3	7.2 -	8.7	117.98
HDL 2a	8.7 -	10.4	106.69
HDL 2b	10.4 -	13.9	74.49
LDL IV	19.0 -	20.7	10.70
LDL III	20.7 -	21.9	11.04
LDL II	21.9 -	23.1	2.52
LDL I	23.1 -	25.4	1.50
IDL 2	25.4 -	26.6	0.39
IDL 1	26.6 -	27.8	0.31
VLDL sm	27.8 -	35.0	1.85
VLDL int	35.0 -	44.1	1.33
VLDL lg	44.1 -	54.7	1.09

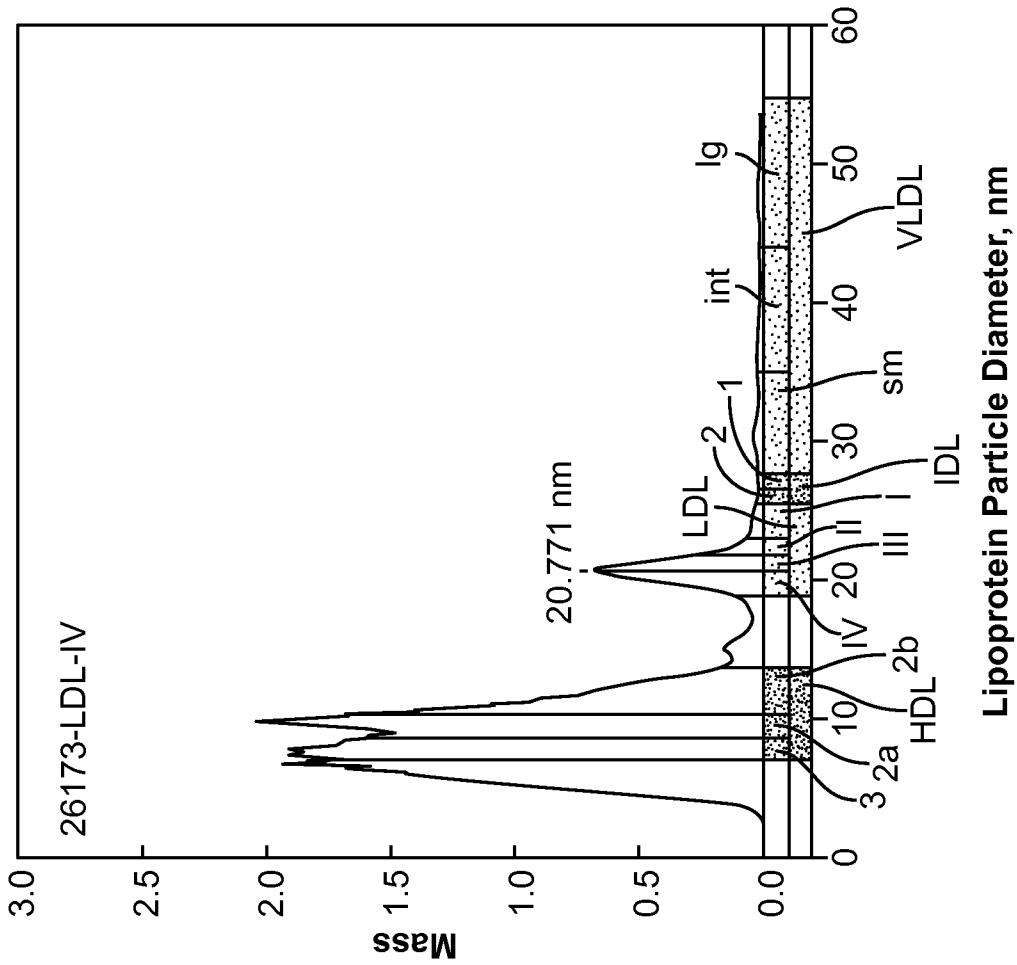


FIG. 6

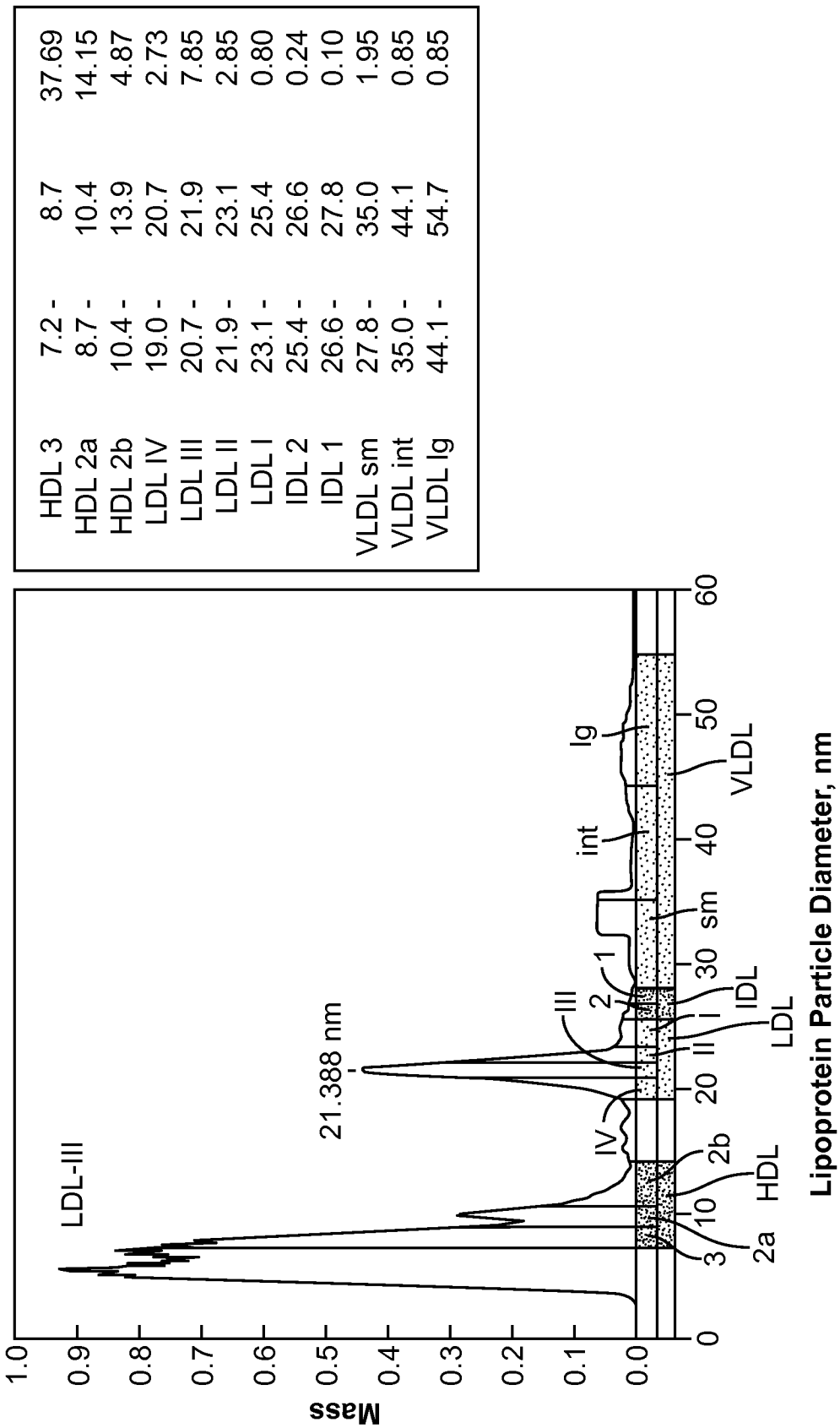

HDL 3 7.2 - 8.7 37.69
HDL 2a 8.7 - 10.4 14.15
HDL 2b 10.4 - 13.9 4.87
LDL IV 19.0 - 20.7 2.73
LDL III 20.7 - 21.9 7.85
LDL II 21.9 - 23.1 2.85
LDL I 23.1 - 25.4 0.80
IDL 2 25.4 - 26.6 0.24
IDL 1 26.6 - 27.8 0.10
VLDL sm 27.8 - 35.0 1.95
VLDL int 35.0 - 44.1 0.85
VLDL lg 44.1 - 54.7 0.85
LDL-III
21.388 nm
IV
III
2
1
int
lg
3
10
2b
2a
HDL
IDL
LDL
VLDL
sm
Mass
Lipoprotein Particle Diameter, nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
20
30
40
50
60

FIG. 7

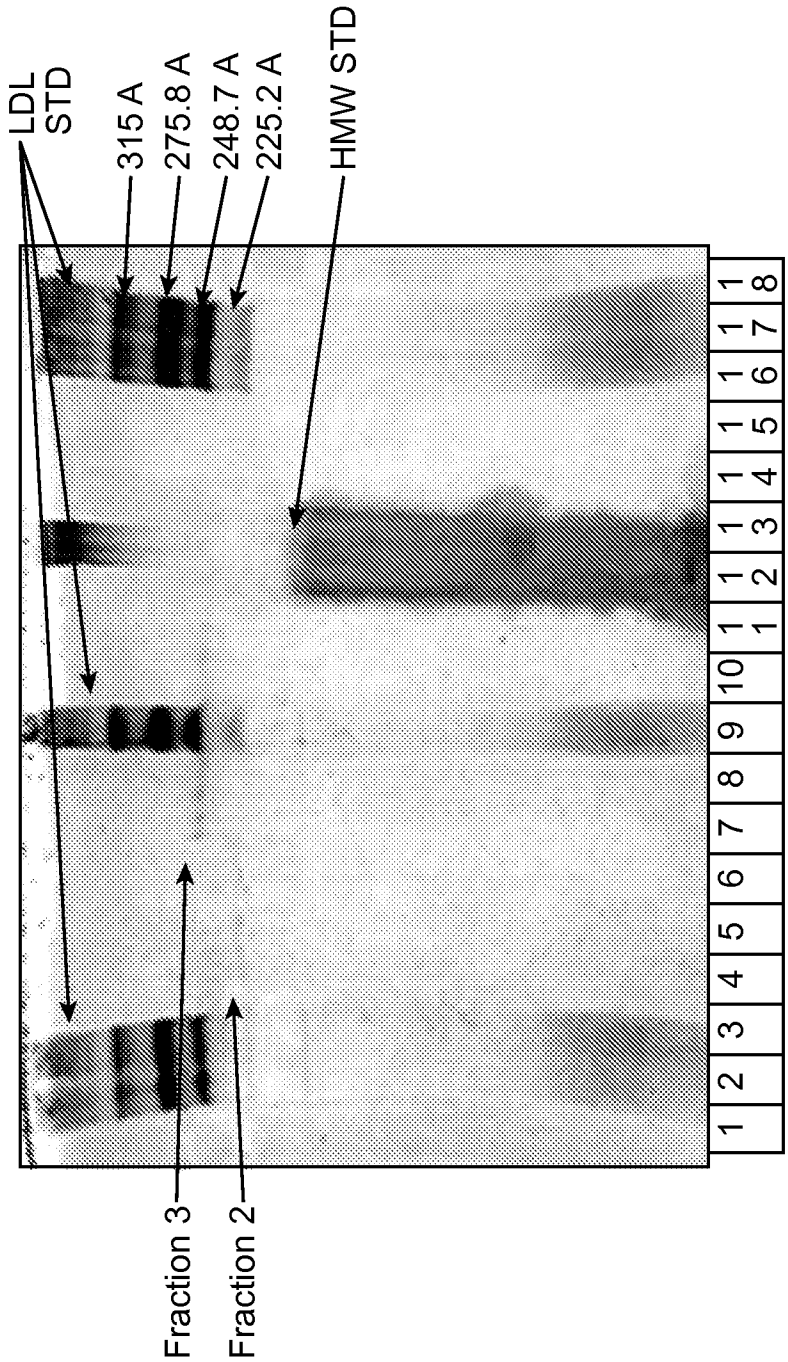

LDL STD
315 A
275.8 A
248.7 A
225.2 A
HMW STD
Fraction 3
Fraction 2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

FIG. 8

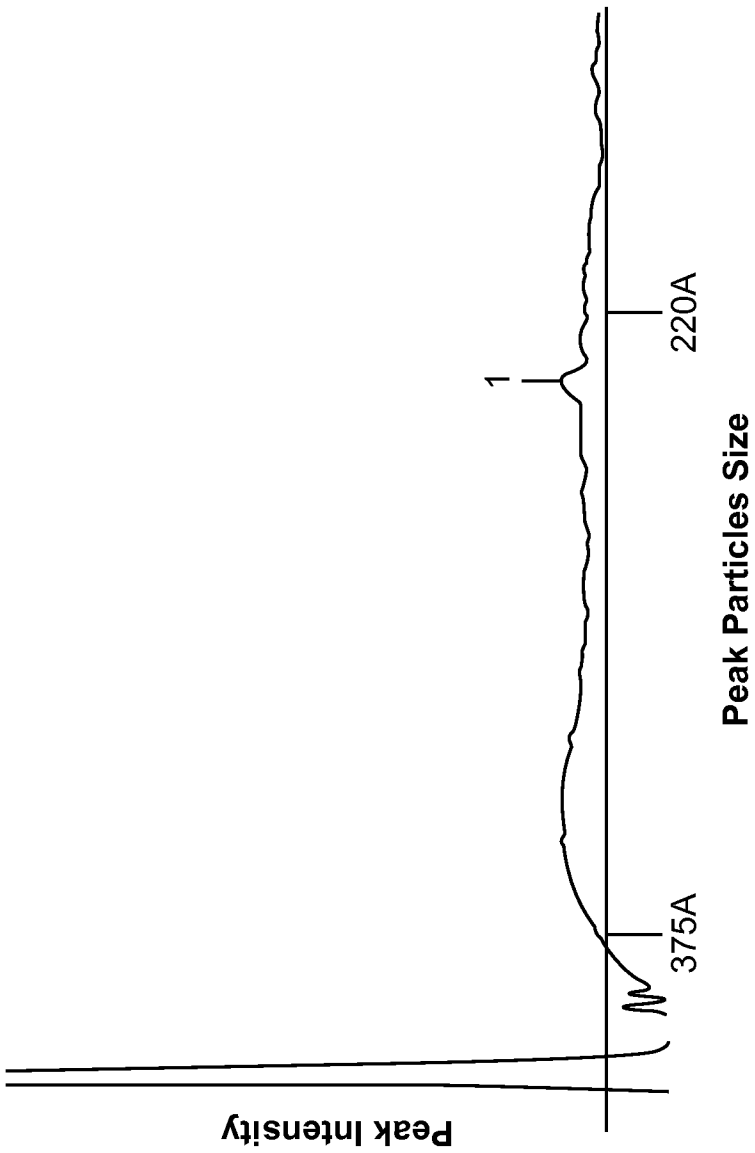


FIG. 9

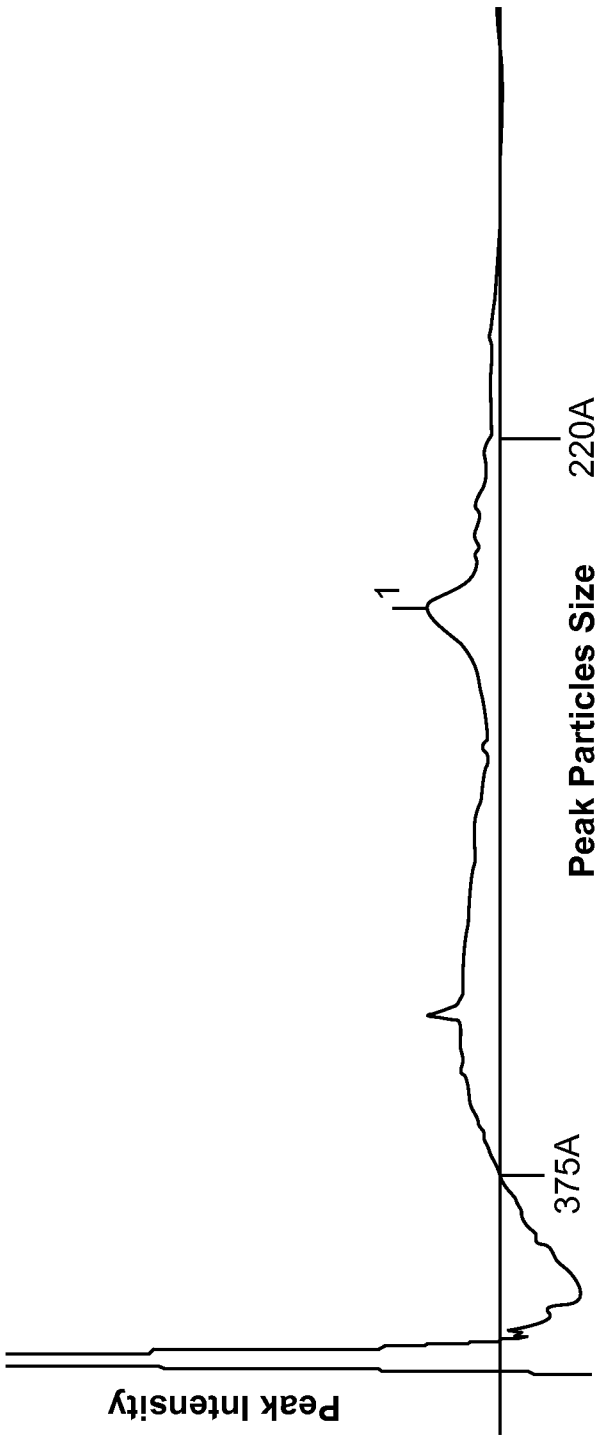


FIG. 10

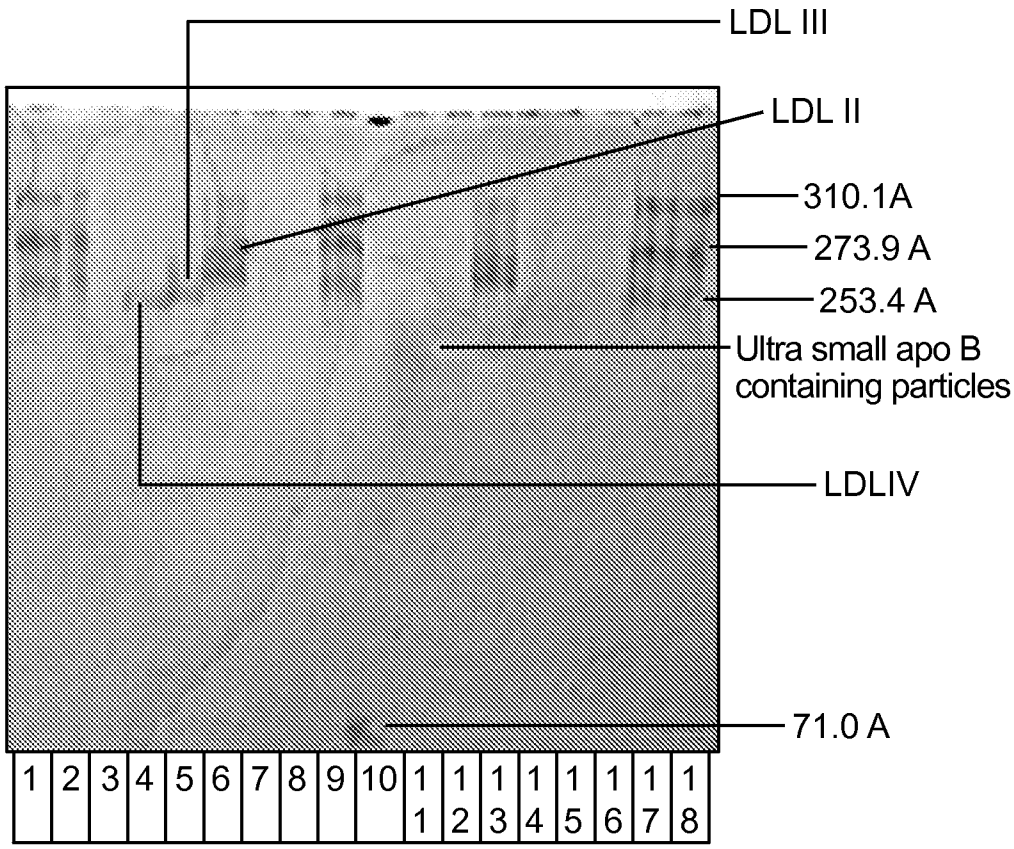

LDL III
LDL II
310.1A
273.9 A
253.4 A
Ultra small apo B containing particles
LDLIV
71.0 A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

FIG. 11

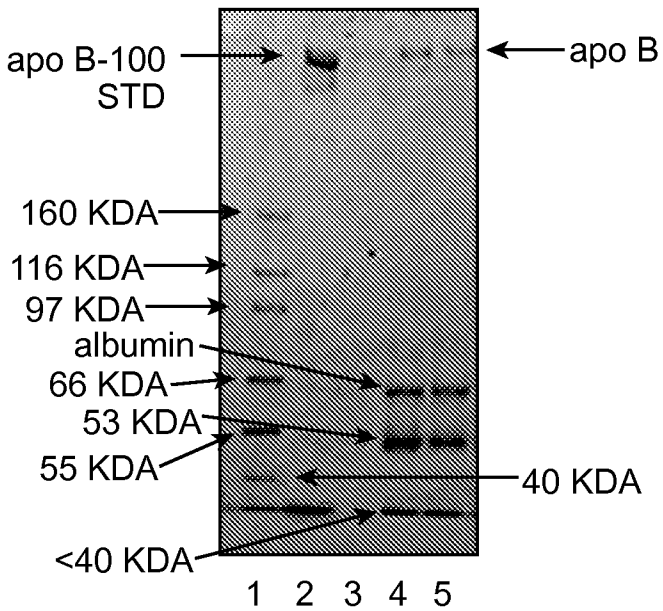

apo B-100 STD
apo B
160 KDA
116 KDA
97 KDA
albumin
66 KDA
53 KDA
55 KDA
40 KDA
<40 KDA
1 2 3 4 5

FIG. 12

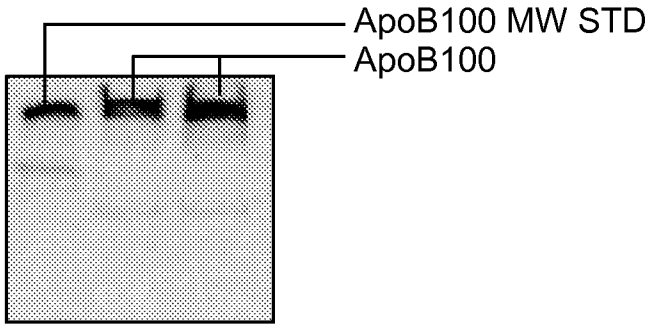


FIG. 13

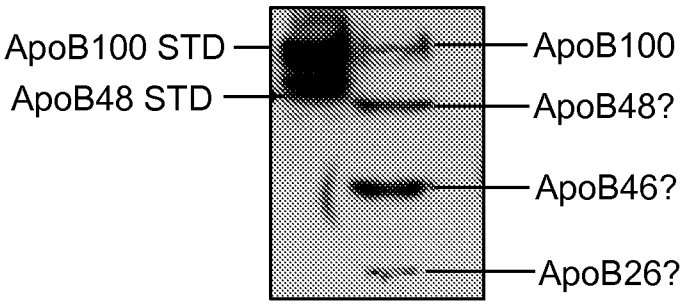


FIG. 14

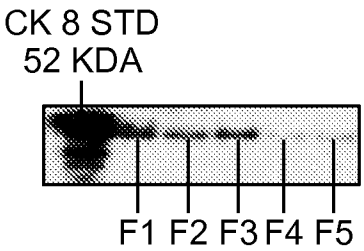


FIG. 15

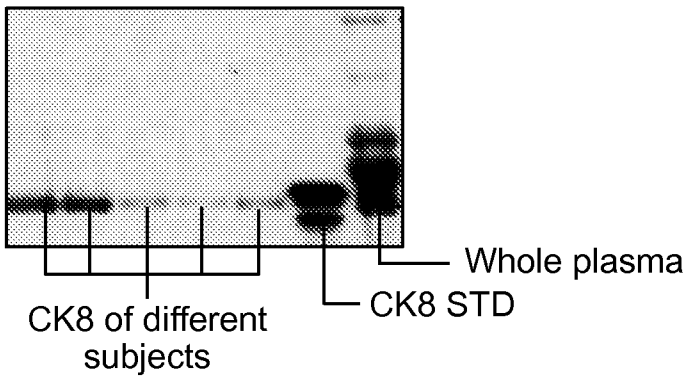


FIG. 16

apoB-100
GenBank NP_000375.2

```
   1 mdpprpalla llalpallll llagaraeee mlenvslvcp kdatrfkhlr kytynyeaes
  61 ssgvpgtads rsatrinckv elevpqlcsf ilktsqctlk evygfnpegk allkktknse
 121 efaaamsrye lklaipegkq vflypekdep tyilnikrgi isallvppet eeakqvlfld
 181 tvygncsthf tvktrkgnva teisterdlg qcdrfkpirt gisplalikg mtrplstlis
 241 ssqscqytld akrkhvaeai ckeqhlflpf syknkygmva qvtqtlkled tpkinsrffg
 301 egtkkmglaf estkstsppk qaeavlktlq elkkltiseq niqranlfnk lvtelrglsd
 361 eavtsllpql ievsspitlq alvqcgqpqc sthilqwlkr vhanpllidv vtylvalipe
 421 psaqqlreif nmardqrsra tlyalshavn nyhktnptgt qelldianyl meqiqddctg
 481 dedytylilr vignmgqtme qltpelkssi lkcvqstkps lmiqkaaiqa lrkmepkdkd
 541 qevllqtfld daspgdkrla aylmlmrsps qadinkivqi lpweqneqvk nfvashiani
 601 lnseeldiqd lkklvkealk esqlptvmdf rkfsrnyqly ksvslpsldp asakiegnli
 661 fdpnnylpke smlkttltaf gfasadliei glegkgfept lealfgkqgf fpdsvnkaly
 721 wvngqvpdgv skvlvdhfgy tkddkheqdm vngimlsvek likdlkskev pearaylril
 781 geelgfaslh dlqllgklll mgartlqgip qmigevirkg skndfflhyi fmenafelpt
 841 gaglqlqiss sgviapgaka gvklevanmq aelvakpsvs vefvtnmgii ipdfarsgvq
 901 mntnffhesg leahvalkag klkfiipspk rpvkllsggn tlhlvsttkt evipplienr
 961 qswsvckqvf pglnyctsga ysnasstdsa syypltgdtr lelelrptge ieqysvsaty
1021 elqredralv dtlkfvtqae gakqteatmt fkynrqsmtl ssevqipdfd vdlgtilrvn
1081 destegktsy rltldiqnkk itevalmghl scdtkeerki kgvisiprlq aearseilah
1141 wspaklllqm dssataygst vskrvawhyd eekiefewnt gtnvdtkkmt snfpvdlsdy
```

FIG. 17A

```
1201 pkslhmyanr lldhrvpqtd mtfrhvgskl ivamsswlqk asgslpytqt lqdhlnslke
1261 fnlqnmglpd fhipenlflk sdgrvkytln knslkieipl pfggkssrdl kmletvrtpa
1321 lhfksvgfhl psrefqvptf tipklyqlqv pllgvldlst nvysnlynws asysggntst
1381 dhfslraryh mkadsvvdll synvqgsget tydhkntftl scdgslrhkf ldsnikfshv
1441 eklgnnpvsk gllifdasss wgpqmsasvh ldskkkqhlf vkevkidgqf rvssfyakgt
1501 yglscqrdpn tgrlngesnl rfnssylqgt nqitgryedg tlsltstsdl qsgiikntas
1561 lkyenyeltl ksdtngkykn fatsnkmdmt fskqnallrs eyqadyeslr ffsllsgsln
1621 shglelnadi lgtdkinsga hkatlrigqd gistsattnl kcsllvlene lnaelglsga
1681 smklttngrf rehnakfsld gkaaltelsl gsayqamilg vdsknifnfk vsqeglklsn
1741 dmmgsyaemk fdhtnslnia glsldfsskl dniyssdkfy kqtvnlqlqp yslvttlnsd
1801 lkynaldltn ngklrleplk lhvagnlkga yqnneikhiy aissaalsas ykadtvakvq
1861 gvefshrlnt diaglasaid mstnynsdsl hfsnvfrsvm apftmtidah tngngklalw
1921 gehtgqlysk fllkaeplaf tfshdykgst shhlvsrksi saalehkvsa lltpaeqtgt
1981 wklktqfmnn eysqdldayn tkdkigvelt grtladltll dspikvplll sepiniidal
2041 emrdavekpq eftivafvky dknqdvhsin lpffetlqey fernrqtiiv vlenvqrnlk
2101 hinidqfvrk yraalgklpq qandylnsfn werqvshake kltaltkkyr itendiqial
2161 ddakinfnek lsqlqtymiq fdqyikdsyd lhdlkiaian iideiieklk sldehyhirv
2221 nlvktihdlh lfienidfnk sgsstaswiq nvdtkyqiri qiqeklqqlk rhiqnidiqh
2281 lagklkqhie aidvrvlldq lgttisferi ndvlehvkhf vinligdfev aekinafrak
2341 vhelieryev dqqiqvlmdk lvelahqykl ketiqklsnv lqqvkikdyf eklvgfidda
2401 vkklnelsfk tfiedvnkfl dmlikklksf dyhqfvdetn dkirevtqrl ngeiqalelp
2461 qkaealklfl eetkatvavy leslqdtkit liinwlqeal ssaslahmka kfretledtr
```

FIG. 17B

```
2521 drmyqmdiqq elqrylslvg qvystlvtyi sdwwtlaakn ltdfaeqysi qdwakrmkal
2581 veqgftvpei ktilgtmpaf evslqalqka tfqtpdfivp ltdlripsvq infkdlknik
2641 ipsrfstpef tilntfhips ftidfvemkv kiirtidqml nselqwpvpd iylrdlkved
2701 iplaritlpd frlpeiaipe fiiptlnlnd fqvpdlhipe fqlphishti evptfgklys
2761 ilkiqsplft ldanadigng ttsaneagia asitakgesk levlnfdfqa naqlsnpkin
2821 plalkesvkf sskylrtehg semlffgnai egksntvasl hteknt lels ngvivkinnq
2881 ltldsntkyf hklnipkldf ssqadlrnei ktllkaghia wtssgkgswk wacprfsdeg
2941 thesqisfti egpltsfgls nkinskhlrv nqnlvyesgs lnfskleiqs qvdsqhvghs
3001 vltakgmalf gegkaeftgr hdahlngkvi gtlknslffs aqpfeitast nnegnlkvrf
3061 plrltgkidf lnnyalflsp saqqaswqvs arfnqykynq nfsagnneni meahvginge
3121 anldflnipl tipemrlpyt iittpplkdf slwektglke flkttkqsfd lsvkaqykkn
3181 khrhsitnpl avlcefisqs iksfdrhfek nrnnaldfvt ksynetkikf dkykaekshd
3241 elprtfqipg ytvpvvnvev spftiemsaf gyvfpkavsm psfsilgsdv rvpsytlilp
3301 slelpvlhvp rnlklslpdf kelctishif ipamgnityd fsfkssvitl ntnaelfnqs
3361 divahllsss ssvidalqyk legttrltrk rglklatals lsnkfvegsh nstvslttkn
3421 mevsvatttk aqipilrmnf kqelngntks kptvsssmef kydfnssmly stakgavdhk
3481 lslesltsyf siesstkgdv kgsvlsreys gtiaseanty lnskstrssv klqgtskidd
3541 iwnlevkenf ageatlqriy slwehstknh lqleglfftn gehtskatle lspwqmsalv
3601 qvhasqpssf hdfpdlgqev alnantknqk irwknevrih sgsfqsqvel sndqekahld
3661 iagsleghlr flkniilpvy dkslwdflkl dvttsigrrq hlrvstafvy tknpngysfs
3721 ipvkvladkf iipglklndl nsvlvmptfh vpftdlqvps ckldfreiqi ykklrtssfa
3781 lnlptlpevk fpevdvltky sqpedslipf feitvpesql tvsqftlpks vsdgiaaldl
3841 navankiadf elptiivpeq tieipsikfs vpagivipsf qaltarfevd spvynatwsa
```

FIG. 17C

```
3901 slknkadyve tvldstcsst vqfleyelnv lgthkiedgt lasktkgtfa hrdfsaeyee
3961 dgkyeglqew egkahlniks paftdlhlry qkdkkgists aaspavgtvg mdmdedddfs
4021 kwnfyyspqs spdkkltifk telrvresde etqikvnwee eaasglltsl kdnvpkatgv
4081 lydyvnkyhw ehtgltlrev sskl rrnlqn naewvyqgai rqiddidvrf qkaasgttgt
4141 yqewkdkaqn lyqelltqeg qasfqglkdn vfdglvrvtq efhmkvkhli dslidflnfp
4201 rfqfpgkpgi ytreelctmf irevgtvlsq vyskvhngse ilfsyfqdlv itlpfelrkh
4261 klidvismyr ellkdlskea qevfkaiqsl kttevlrnlq dllqfifqli ednikqlkem
4321 kftylinyiq deintifsdy ipyvfkllke nlclnlhkfn efiqnelqea sqelqqihqy
4381 imalreeyfd psivgwtvky yeleekivsl iknllvalkd fhseyivsas nftsqlssqv
4441 eqflhrniqe ylsiltdpdg kgkekiaels ataqeiiksq aiatkkiisd yhqqfryklq
4501 dfsdqlsdyy ekfiaeskrl idlsiqnyht fliyitellk klqsttvmnp ymklapgelt
4561 iil (SEQ ID NO:1)
```

FIG. 17D

***Homo sapiens* cytokeratin 8**
GenBank AAA35763

```
   1 msirvtqksy kvstsgpraf ssrsytsgpg srissssfsr vgssnfrggl gggyggasgm
  61 ggitavtvnq sllsplvlev dpniqavrtq ekeqiktlnn kfasfidkvr fleqqnkmle
 121 tkwsllqqqk tarsnmdnmf esyinnlrrq letlgqeklk leaelgnmqg lvedfknkye
 181 deinkrteme nefvlikkdv deaymnkvel esrlegltde inflrqlyee eirelqsqis
 241 dtsvvlsmdn srsldmdsii aevkaqyedi anrsraeaes myqikyeelq slagkhgddl
 301 rrtkteisem nrnisrlqae ieglkgqras leaaiadaeq rgelaikdan aklseleaal
 361 qrakqdmarq lreyqelmnv klaldieiat yrkllegees rlesgmqnms ihtkttggya
 421 gglssayggs qaglsyslgs sfgsgagsss fsrtsssrav vvkkietrdg klvsesssdvl
 481 pk (SEQ ID NO:2)
```

FIG. 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/42513

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 14/775 (2012.01)
USPC - 530/359
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C07K 14/775 (2012.01)
USPC - 530/359

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
436/63, 424/9.1, 530/350

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST - DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=YES; OP=ADJ; Google Scholar
search terms: apob, apolipoprotein B, apo-b, apo B, FLDB, LDLCQ4, 100, dense, small, density, deplet$, delipi$, delipidated, lipoprotein, apolipoprotein, LDL, cytokeratin 8, cytokeratin-8, keratin 8, keratin-8, cyk8, k8, card2, ck8, k2c8, ck-8, hypercho$, arterioscl$, atheroscl

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010/0323376 A1 (CONTOIS J.) 23 December 2010 (23.12.2010) para [0009]; [0047]; [0063]; [0066]; [0067]; [0072]; [0121]; [0139]; [0140].	16, 18-21
Y	Katsuda et al., Human Atherosclerosis: Immunocytochemical Analysis of the Cell Composition of Lesions of Young Adults American Journal of Pathology, Vol. 140, No. 4, April 1992. p. 907, 2nd para; p. 907, last para - p. 908, 1st para; Table 1; Fig. 3.	16, 18-21
A	Koba et al., Significance of small dense low-density lipoproteins and other risk factors in patients with various types of coronary heart disease, Am. Heart. J. 144 (2002) 1026?1035. abstract; p. 207, 1st para; Fig. 1.	1-15, 22-25
A	US 2008/038829 A1 (KREMER et al.) 14 February 2008 (14.02.2008) para [0083]; Figs. 1 and 10.	1-15, 22-25
A	US 2009/0317819 A1 (TSIMIKAS et al.) 24 December 2009 (24.12.2009) para [0011]; [0044]; [0047]; [0049].	1-25
A	Al-Bahrani et al., A potential role of apolipoprotein B in the risk stratification of diabetic patients with dyslipidaemia Diabetes Research and Clinical Practice Volume 69, Issue 1 , Pages 44-51, July 2005. p. 45, 2nd para; p. 50, col. 2, 1st para.	1-25

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 August 2012 (26.08.2012)	21 SEP 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/42513

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 17
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.