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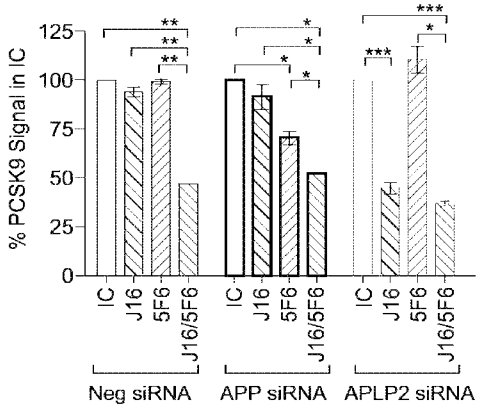
(54) Title: METHODS FOR REDUCING LDL-CHOLESTEROL

FIG. 2D



(57) Abstract: The present invention relates to methods for reducing LDL-cholesterol levels in blood comprising the administration of a combination of at least two different anti- PCSK9 antagonist antibodies or an anti-PCSK9 antagonist antibody (e.g., bispecific or bifunctional antibody) directed to different epitopes of PCSK9 protein. The subject single or combination treatment can be used in the prevention and/or treatment of cholesterol and lipoprotein metabolism disorders, including hypercholesterolemia, dyslipidemia, hyperlipidemia, atherosclerosis, acute coronary syndrome and, more generally, cardiovascular disease (CVD).

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METHODS FOR REDUCING LDL-CHOLESTEROL

Cross-Reference To Related Applications

This application claims the benefit of U.S. Provisional Application No. 62/034,028 filed August 6, 2014, and U.S Provisional Application No. 62/192,771 filed July 15, 2015, all of which are hereby incorporated by reference in their entireties.

Field

The present invention relates to methods for the treatment of reducing LDL-cholesterol levels in blood comprising the administration of a combination of at least two different anti-PCSK9 antagonist antibodies directed to different epitopes of the PCSK9 protein. Also provided are methods for the treatment of reducing LDL-cholesterol levels in blood comprising the administration of an anti-PCSK9 antagonist comprising at least two different epitopes of the PCSK9 protein. The subject combination treatment can be used in the prevention and/or treatment of cholesterol and lipoprotein metabolism disorders, including hypercholesterolemia, dyslipidemia, hyperlipidemia, atherosclerosis, acute coronary syndrome and, more generally, cardiovascular disease (CVD).

Background

High serum LDL-cholesterol (LDL-C) levels correlate strongly with hypercholesterolemia and coronary artery disease (CAD). Thus, multitudes of CAD prevention therapeutics focus on lowering LDL-C levels. One such approach aims to increase expression of the LDL receptor (LDLR), a protein that clears LDL-C from the blood. LDL binds LDLR on the cell surface, and following internalization, LDLR undergoes a pH dependent conformational change upon entering endosomes. This allows LDLR to release the bound LDL particle, which is then delivered to lysosomes, while LDLR itself is recycled back to the cell surface to repeat the process (Jeon et al., Annu Rev. Biochem. 74:535-565 (2005)).

PCSK9 is a soluble, secreted protein that regulates LDLR protein levels by binding LDLR on the plasma membrane and redirecting it towards lysosomes (Nassoury et al., Traffic, 8:718-732 (2007); Kwon et al., Pro. Natl. Acad. Sci. U.S.A.

105:1820-1825 (2008); Legace et al., J. Clin. Invest. 116:2995-3005 (2006); and McNutt et al., J. Biol. chem. 284:10561-10570 (2007)). In addition to LDLR, PCSK9 mediates lysosomal degradation of a number of receptors, including VLDLR, ApoER2, and BACE1 (Jonas et al., EMBO Rep, 9:916-922 (2008); Shan et al., Biochem. Biophys. Res. Commun, 9:916-922 (2008); Poirier et al., J. Biol. Chem., 283:2363-2372 (2008); and Canuel et al., PLoS One, 8:e64145). PCSK9 likely utilizes its C-terminal Cis-His Rich Domain (CHRD) to mediate post-endocytic lysosomal delivery of its targets (Nassoury et al., (2007); Luna Saavedra et al., J. Biol. Chem. (2012); Ni et al., J. Biol. Chem., 285:12882-12891 (2010); Surdo et al., EMBO Rep. (2011); and Holla et al., J. Lipid Res. 52: 1787-1794 (2011)). Importantly, the CHRD interacts in a pH dependent manner with APLP2, a member of the amyloid precursor protein (APP) family. This interaction allows PCSK9 to bridge LDLR to APLP2 (Amyloid Precursor-Like Protein 2), which in turn transports the entire complex to lysosomes (DeVay et al., J. Biol. Chem., 288:10805-10818 (2013)).

Human genetics studies demonstrate that people who harbor loss of function PCSK9 mutations have low LDL-C levels and decreased risk of CAD (Cohen et al., Nat. Genet. 37:161-165 (2005); Cohen et al., N. Engl. J. Med. 354:1264-1272 (2006)), while gain of function PCSK9 carriers show the opposite effects (Abifadel et al., Nat. Genet. 34:154-156 (2003); Maxwell et al., Curr. Opin. Lipidol. 15:167-1172 (2005)). PCSK9 has therefore become a promising target for treating hypercholesterolemia. Indeed, PCSK9 can be effectively attenuated using monoclonal antibody therapeutics that inhibit its interactions with LDLR, and endocytosed PCSK9 is effectively delivered to lysosomes when bound to either LDLR or a PCSK9 antagonist antibody (see, e.g., US Pat. No. 8,080,243, Chaparro-Riggers et al., J. Biol. Chem. (2012), and Liang et al., J. Pharmacol. Exp. Ther. (2012)).

Prior to the present invention, it still remained unclear with respect to 1) the indirect role of LDLR in regulating PCSK9 endocytosis in the absence of direct interaction of LDLR and PCSK9, 2) the endocytic epitopes on the PCSK9 utilized by a variety of internalization mechanisms, and 3) whether targeting these epitopes would provide an effective treatment for reducing hypercholesterolemia and the associated incidence of CVD.

Summary

The present invention relates to methods for reducing LDL-cholesterol levels in blood comprising the administration of a combination of at least two different anti-PCSK9 antagonist antibodies directed to different epitopes of PCSK9 protein. Further, the present invention also relates to methods for reducing LDL-cholesterol levels in blood comprising the administration of an anti-PCSK9 antagonist antibody (e.g., a bispecific or bifunctional antibody) directed to at least two different epitopes of PCSK9 protein. The inventors have discovered that two distinct epitopes on human PCSK9 protein are utilized by two independent PCSK9 internalization mechanisms and that administration of two distinct anti-PCSK9 antagonist antibodies or one anti-PCSK9 antibody (e.g., bispecific or bifunctional antibody) directed to these different epitopes significantly blocked PCSK9 internalization. More specifically, the two distinct epitopes include 1) an epitope interacting with the EGF-like domain of the LDLR (e.g., SEQ ID NO: 17 or amino acid residues 314-353 of SEQ ID NO: 18) and having the function of blocking LDLR binding to the human PCSK9 protein; and 2) the other epitope recognizing the C-terminal Cys-His Rich Domain (CHRD) of the human PCSK9 protein (e.g., APLP2 binding epitope) and having the function of blocking the binding of PCSK9 to APLP2. In the case of anti-PCSK9 antagonist antibody combination treatment method, the combination of the two specific anti-PCSK9 antagonist antibodies with two independent PCSK9 internalization mechanisms can extend the half-life of either anti-PCSK9 antagonist antibodies and thereby prolong the effect of LDL-cholesterol reduction.

In one aspect, this invention provides a method of reducing a level of LDL-cholesterol in blood of a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a first and second antagonist antibodies or antigen binding fragment thereof specific to human proprotein convertase subtilisin kexin type 9 (PCSK9) amino acid sequence of SEQ ID NO: 1, wherein the first anti-PCSK9 antibody blocks LDLR binding to the human PCSK9, and wherein the second anti-PCSK9 antibody binds to C-terminal Cis-His Rich domain (CHRD) of the human PCSK9.

In some embodiments, the first antibody is alirocumab (PRALUENT™), evolocumab (REPATHA™), REGN728, LGT209, RG7652, LY3015014, J16, L1L3, 31H4, 11F1, 12H11, 8A3, 8A1, 3C4, 300N, or 1D05. In some embodiments, the first anti-PCSK9 antibody is a full antagonist of the PCSK9-mediated effect LDL receptor

(LDLR) levels as measured in vitro using an LDLR down-regulation assay in Huh7 cells. In some embodiments, the first anti-PCSK9 antibody comprises a heavy chain variable region (VH) comprising complementarity determining region one (CDR1), CDR2, and CDR3 of the amino acid sequence shown in SEQ ID NO: 2; and a light chain variable region (VL) comprising CDR1, CDR2, and CDR3 of the amino acid sequence shown in SEQ ID NO: 3. In some embodiments, the first anti-PCSK9 antibody comprises a VH CDR1 having the amino acid sequence shown in SEQ ID NO: 4, 5, or 6, a VH CDR2 having the amino acid sequence shown in SEQ ID NO:7 or 8, a VH CDR3 having the amino acid sequence shown in SEQ ID NO: 9, a VL CDR1 having the amino acid sequence shown in SEQ ID NO:10, a VL CDR2 having the amino acid sequence shown in SEQ ID NO:11, and a VL CDR3 having the amino acid sequence shown in SEQ ID NO: 12. In some embodiments, the first anti-PCSK9 antibody comprises a light chain having SEQ ID NO: 13 and a heavy chain having SEQ ID NO: 14, with or without the C-terminal lysine of SEQ ID NO: 14.

In some embodiments, the second anti-PCSK9 antibody (e.g., 5F6 or 11B6) blocks binding of PCSK9 to amyloid precursor-like protein 2 (APLP2).

In some embodiments, both the first and the second anti-PCSK9 antibodies are administered intravenously or subcutaneously. In some embodiments, the first and second anti-PCSK9 antibodies are administered sequentially or simultaneously. In some embodiments, the first and the second anti-PCSK9 antibodies are both administered at least every four weeks or every 2 weeks to the subject. In some embodiments, the half-life of the first anti-PCSK9 antibody is extended by the administration of the second-anti-PCSK9 antibody

In some embodiments, the method described herein comprises administering about 10 mg to about 2000 mg of the first and the second anti-PCSK9 antibody to the subject.

In some embodiments, a statin can be administered prior to the initial dose of the first and second anti-PCSK antibodies. In some embodiments, a daily dose of a statin is administered. In other embodiments, stable doses of the statin have been administered for at least about two, three, four, five, or six weeks prior to the initial dose of both the first and second anti-PCSK9 antibodies. Examples of a statin include atorvastatin, cerivastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin,

rosuvastatin, simvastatin, or any pharmaceutically acceptable salts, or stereoisomers thereof.

In another aspect, the invention also provides a method of reducing LDL-cholesterol levels in blood comprising the administration of a therapeutically effective amount of a bispecific antibody directed to two different epitopes of the PCSK9 protein, wherein the first arm of the antibody blocks the LDLR binding to the PCSK9 protein (e.g., SEQ ID NO: 1), and the second arm of the antibody binds to CHRD of the PCSK9 protein (e.g., SEQ ID NO: 1).

In some embodiments, provided is a bispecific antibody directed to two different epitopes of the PCSK9 protein, wherein the first arm of the antibody blocks the LDLR binding to the PCSK9 protein (e.g., SEQ ID NO: 1), and the second arm of the antibody binds to CHRD of the PCSK9 protein (e.g., SEQ ID NO: 1).

In another aspect, the invention also provides a method of reducing a level of LDL-cholesterol levels in blood of a subject need thereof, comprising administering to the subject a therapeutically effective amount of an antibody specific to a PCSK9 protein, wherein the antibody blocks the LDLR binding to the PCSK9 amino acid sequence of SEQ ID NO: 1 and binds to C-Terminal Cys-His Rich Domain (CHRD) of the PCSK9 amino acid sequence of SEQ ID NO: 1.

In some embodiments, provided is an isolated antibody, or an antigen binding fragment thereof, which specifically binds to PCSK9 and comprises: a) a heavy chain variable region (VH) comprising complementarity determining region one (CDR1), CDR2, and CDR3 of the amino acid sequence shown in SEQ ID NO: 21 or 34; and/or a light chain variable region (VL) comprising CDR1, CDR2, and CDR3 of the amino acid sequence shown in SEQ ID NO: 22 or 35. In some embodiments, the antibody or antigen binding fragment comprises: a) VH CDR1 having the amino acid sequence shown in SEQ ID NO: 23, 24, or 25, a VH CDR2 having the amino acid sequence shown in SEQ ID NO:26 or 27, and a VH CDR3 having the amino acid sequence shown in SEQ ID NO: 28; and/or a VL CDR1 having the amino acid sequence shown in SEQ ID NO:29, a VL CDR2 having the amino acid sequence shown in SEQ ID NO:30, and a VL CDR3 having the amino acid sequence shown in SEQ ID NO: 31; or b) a VH CDR1 having the amino acid sequence shown in SEQ ID NO: 36, 24, or 37, a VH CDR2 having the amino acid sequence shown in SEQ ID NO:38 or 39, and a VH CDR3 having the amino acid sequence shown in SEQ ID NO: 40; and/or a VL CDR1 having

the amino acid sequence shown in SEQ ID NO:41, a VL CDR2 having the amino acid sequence shown in SEQ ID NO:42, and a VL CDR3 having the amino acid sequence shown in SEQ ID NO: 43.

The methods described herein can be used for treating a subject suffering from dyslipidemia, hyperlipidemia, hypercholesterolemia, atherosclerosis, cardiovascular disease, and/or coronary heart disease, or prophylactically treating a subject having a high risk of developing any of the diseases listed above.

In some embodiments, provided is a use of an anti-PCSK9 antibody (e.g., bispecific or bifunctional antibody) or a combination of anti-PCSK9 antibodies in a method of the invention, as set forth in any one of the preceding embodiments.

In some embodiments, provided is a use of an anti-PCSK9 antibody (e.g., bispecific or bifunctional antibody) or a combination of anti-PCSK9 antibodies in a method of manufacture of a medicament for use in a method as set forth in any one of the preceding embodiments.

In some embodiments, provided is an anti-PCSK9 antagonist antibody (e.g., bispecific or bifunctional antibody) or a combination of anti-PCSK9 antibodies for use as set forth in any one of the preceding embodiments.

In another aspect, this invention provides a kit or an article of manufacture, comprising a container, a composition within the container comprising an anti-PCSK9 antagonist antibody (e.g., bispecific or bifunctional antibody) or a combination of anti-PCSK9 antagonist antibodies, and a package insert containing instructions to administer a therapeutically effective amount of the PCSK9 antagonist antibod(ies) for reducing a level of LDL-cholesterol.

Brief Description of the Figures/Drawings

Figure 1 depicts PCSK9 internalization with antibodies 5F6, J16, or combination of both. Antibody 5F6 is directed to the CHRD of the PCSK9 protein, and antibody J16 interacts with the EGF-like domain of the LDLR. Figures 1A and 1B show PCSK9-488 internalization in the presence of mouse isotype control (mIC) and human isotype control (hIC) antibodies (top row), mIC and J16 (middle top row), hIC and 5F6 (middle bottom row), or 5F6 and J16 (bottom row) in (A) HepG2 cells or (B) primary mouse hepatocytes. (Figure 1C) PCSK9-488 internalization alone (top), in the presence of J16 Fab (middle top), 5F6 Fab (middle bottom), or 5F6 Fab and J16 Fab (bottom). Scale bars, 10μM. Figures 1D-1F depict siRNA knockdown and effect of 12E3 on

PLP2/PCSK9 interactions. (Figure 1D) Representative western blots showing TFNR, APLP2, and APP levels in negative control and siRNA treated cell lysates, as indicated. LDLR western shown for APP siRNA. (Figure 1E) ELISA showing binding of 1μg/ml biotinylated PCSK9 (Bio-PCSK9) to APLP2 ECD coated plates (coated at 5μg/ml), with increasing concentrations of the anti-APLP2 antibody 12E3. Shown as average of triplicate samples with SD. (Figure 1F) ELISA of 1μg/ml bio-PCSK9 to LDLR ECD coated plates (coated at 5μg/ml), with increasing RD-LDLR, as indicated. Shown as average of triplicate samples with SD. J16 effect shown as dotted line.

Figure 2 depicts which of APP or APLP2 affects PCSK9 internalization. Figures 2A, 2B, and 2C): PCSK9-488 internalization in the presence of mIC and hIC antibodies (top row), mIC and J16 (middle top row), hIC and 5F6 (middle bottom row), or 5F6 and J16 (bottom row) in (A) Neg, (B) APLP2, or (C) APP siRNA treated HepG2 cells. Scale bars, 10μM. (D) Quantification of (Figures 2A, 2B, and 2C) shown as average fluorescent signal of PCSK9-488 per cell normalized to IC with SEM of 3 independent experiments. (Figure 2E) PCSK9-488 internalization in the presence of 12E3 and hIC (top row), mIC and J16 (middle row) or 12E3 and J16 (bottom row). Scale bars, 10μM. (Figure 2F) Quantification of (Figure 2E) shown as average percent signal of PCSK9-488 per cell of PCSK9-488/J16 (first two bars from the left) or average percent signal of J16 per cell of PCSK9-488/J16 (last two bars from the left) with SEM from 3 independent experiments. (Figure 2G) PCSK9-488 internalization in the presence of RD-LDLR (top row), hIC and 5F6 (middle row) or RD-LDLR and 5F6 (bottom row). Scale bars, 10μM. (Figure 2H) Quantification of (Figure 2G) shown as average percent signal of PCSK9-488 per cell of PCSK9-488/5F6 or average percent signal of 5F6 per cell of PCSK9-488/5F6 with SEM from 3 independent experiments.

Figure 3 depicts coimmunoprecipitation of APLP2 with PCSK9 at neutral pH. (Figures 3A and 3B) Western blot showing APLP2, PCSK9, or Transferrin receptor (TFNR) levels in input fraction (I), IC or J16 immunoprecipitated samples (IP Ab.) in the absence or presence of 5F6 Fab or 12E3 Fab, as indicated. (Figure 3B) Quantification of (Figure 3A); shown as average APLP2 normalized to PCSK9 of 3 independent experiments with SEM. (Figures 3C and 3D) J16 coIPs of PCSK9 from Neg or LDLR siRNA treated HepG2 cells with IC control, as indicated. (D) Quantification of (C);

shown as average APLP2 normalized to PCSK9 from 3 independent experiments with SEM. (Figures 3E, 3F, and 3G) Western blot of LDLR, ApoER2, or TFNR in siRNA treated cells following treatment with PCSK9 at 0, 20, 50, or 100 μg/ml. (Figure 3F) LDLR levels from (Figure 3E) quantified as percent LDLR degradation of untreated cells and normalized to Neg siRNA samples. Shown as average with SEM from 4 independent experiments. (Figure 3G) Same as Figure 3F, but measuring ApoER2 levels. Figures 3H-3K show characterization of LDLR/APLP2 associations: (Figure 3H) Western blot showing APLP2, PCSK9, or TFNR levels in input fraction (I) or J16, IC, or LDLR IPs following Accutase treatment or direct lysis, as indicated. (Figure 3I) ELISA showing APLP2-ECD at varying concentrations binding to LDLR-ECD coated plates. Shown as average of triplicate samples with SD. (Figure 3J) ELISA of APLP2-ECD binding to LDLR-ECD coated plates, with increasing concentrations of PCSK9. Shown as average of triplicate samples with SD. (Figure 3K) Western blots of APLP2, LDLR, or TFNR in coIPs. I=Input, N=negative control antibody. IP Ab. represents the antibody used for immunoprecipitation. 5F6 or J16 Fab were added as indicated.

Figure 4 depicts ApoB/LDL effects on PCSK9 internalization and function. (Figures 4A and 4B) PCSK9-488 internalization in the presence of J16 (top row), LDL and J16 (middle row), or LDL+5F6+J16 (bottom row) in APLP2 siRNA treated cells. Dotted line indicates background signal, as measured by IC alone. Scale bars, 10μM. (Figure 4B) Quantification of (Figure 4A) shown as average+SEM of J16 fluorescent signal per cell in APLP2 siRNA treated cells. Dotted line indicates average IC background levels. (Figure 4C) Western blot showing APLP2, LDLR, ApoB, Transferrin receptor (TFNR) levels in input fraction (I), IC or J16 immunoprecipitated samples (IP Ab.) under pH 7.4 or pH 6.0 conditions with increasing concentrations of ApoB, as indicated. (Figure 4D) Western blot showing recombinant ApoB, LDLR-ECD, or PCSK9 in anti-LDLR immunoprecipitated samples at pH 6.0, with or without 5F6 Fab, as indicated. Figures 4E-4G show APLP2 internalization in LDLR knockdown cells and PCSK9 mediated J16 internalization in mouse liver. (Figure 4E) APLP2 internalization in negative control (left), LDLR (middle), or APLP2 (right) siRNA treated DAPI stained HepG2 cells. Scale Bars, 10μM. (Figure 4F) Quantification of (Figure 4E), calculated average fluorescence intensity, normalized against negative control cells. Shown as Average with SEM from 3 independent experiments. (Figure 4G) Internalization of J16, IC, or J16/PCSK9 in mouse liver. Human antibodies, DAPI; scale bars 10μM.

Figure 5 depicts PCSK9-488 internalization and LDLR levels *in vivo*. PCSK9-488 internalization in mouse liver in the presence of mIC and hIC (top row), mIC and J16 (middle top row), hIC and 5F6 (middle bottom row), or 5F6 and J16 (bottom row). LDLR and DAPI staining shown, as indicated. Scale bars, 10μM.

Figure 6 depicts one-shot kinetic analysis of hPCSK9 binding to immobilized mAb34 and mAb44 using a ProteOn SPR biosensor. Each overlay plot shows an example of the measured data (noisy lines) and the global fit (smooth lines) to a simple Langmuir model. The reported rate and affinity constants are from two independent experiments, of which one is shown here.

Figure 7 depicts Biacore blocking analysis against hLDLR and J16 showing the results from a COINJECT strategy (A and B) and a PREMIX strategy (C and D). In the COINJECT strategy, mAbs were screened for binding to hPCSK9 that was first captured via (A) immobilized hLDLR or (B) immobilized J16. In the PREMIX strategy, mAbs were first premixed with hPCSK9 (or buffer) and these complexes were injected over (C) immobilized hLDLR or (D) immobilized J16. The control mAb analyte confirms that a sandwiching signal (indicating a non-blocker) was easily detectable on these surfaces.

Figure 8 depicts Biacore blocking analysis against hAPLP2 and control mAb36 showing the results from a COINJECT strategy (A and B) and a PREMIX strategy (C and D). In the COINJECT strategy, mAbs were tested for binding to hPCSK9 that was first captured via (A) immobilized hAPLP2 or (B) immobilized control mAb36. In the PREMIX strategy, mAbs were first premixed with hPCSK9 (or buffer) and these complexes were injected over (C) immobilized hAPLP2 or (D) immobilized control mAb36. The control mAb36 provided a positive sandwiching signal for the mAb analytes, thereby confirming that they were active.

Figure 9 depicts Biacore blocking analysis against immobilized hLDLR, hAPLP2 and J16 compared in the same experiment using a COINJECT strategy (top panel) and a

PREMIX strategy (bottom panel). The running buffer was 25mM bis/tris pH 6.0, 150 mM NaCl, 1 mM $CaCl_2$, and 0.05% Tween-20.

Detailed Description

The present invention relates to methods for the treatment of reducing LDL-cholesterol levels in blood comprising the administration of a combination of at least two different anti-PCSK9 antagonist antibodies directed to different epitopes of PCSK9 protein. The inventors have discovered that two distinct epitopes on human PCSK9 protein are utilized by two independent PCSK9 internalization mechanisms and that administration of two distinct anti-PCSK9 antagonist antibodies directed to these different epitopes significantly blocked PCSK9 internalization. More specifically, the two distinct epitopes include 1) an epitope interacting with the EGF-like domain of the LDLR (e.g., SEQ ID NO: 17 or amino acid residues 314-353 of SEQ ID NO: 18) and having the function of blocking LDLR binding to the human PCSK9 protein; and 2) the other epitope recognizing the C-terminal Cys-His Rich Domain (CHRD) of the human PCSK9 protein (e.g., APLP2 binding epitope) and having the function of blocking the binding of PCSK9 to APLP2. The combination of these two specific anti-PCSK9 antagonist antibodies with two independent PCSK9 internalization mechanisms can extend the half-life of either anti-PCSK9 antagonist antibodies and thereby prolong the effect of LDL-cholesterol reduction. The combination methods described herein can be used in the prevention and/or treatment of cholesterol and lipoprotein metabolism disorders, such as hypercholesterolemia (e.g., heterozygous familial hypercholesterolemia (HetFH) or homozygous familial hypercholesterolemia (HoFH)), dyslipidemia (e.g., mixed dyslipidemia), hyperlipidemia (e.g., heterozygous or homozygous familial and non-familial), atherosclerosis, acute coronary syndrome and, more generally, and cardiovascular disease (CVD).

In one variation, the invention also relates to methods for the treatment of reducing LDL-cholesterol levels in blood comprising the administration of a therapeutically effective amount of a bispecific antibody directed to two different epitopes of the PCSK9 protein.

In another variation, the invention also relates to methods for reducing LDL-cholesterol levels in blood comprising the administration of an anti-PCSK9 antagonist

antibody (e.g., a bifunctional antibody) directed to at least two different epitopes of PCSK9 protein.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J.E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R.I. Freshney, ed., 1987); Introduction to Cell and Tissue Culture (J.P. Mather and P.E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-1998) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Handbook of Experimental Immunology (D.M. Weir and C.C. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller and M.P. Calos, eds., 1987); Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Current Protocols in Immunology (J.E. Coligan et al., eds., 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C.A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: a practical approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal antibodies: a practical approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); Using antibodies: a laboratory manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J.D. Capra, eds., Harwood Academic Publishers, 1995).

Definitions

An “antibody” is an immunoglobulin molecule capable of specific binding to a target, such as a carbohydrate, polynucleotide, lipid, polypeptide, etc., through at least one antigen recognition site, located in the variable region of the immunoglobulin molecule. As used herein, the term “antibody” encompasses not only intact polyclonal or monoclonal antibodies, but also any antigen binding fragment (i.e., "antigen-binding

portion") or single chain thereof, fusion proteins comprising an antibody, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site including, for example without limitation, scFv, single domain antibodies (*e.g.*, shark and camelid antibodies), maxibodies, minibodies, intrabodies, diabodies, triabodies, tetrabodies, v-NAR bis-scFv (see, e.g., Hollinger and Hudson, 2005, Nature Biotechnology 23(9): 1126-1136), and bispecific antibody. An antibody includes an antibody of any class, such as IgG, IgA, or IgM (or sub-class thereof), and the antibody need not be of any particular class. Depending on the antibody amino acid sequence of the constant region of its heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy-chain constant regions that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antigen binding portion" or “antigen binding fragment” of an antibody, as used herein, refers to one or more fragments of an intact antibody that retain the ability to specifically bind to a given antigen (e.g., PCSK9). Antigen binding functions of an antibody can be performed by fragments of an intact antibody. Examples of binding fragments encompassed within the term "antigen binding portion" of an antibody include Fab; Fab’; $F(ab')_2$; an Fd fragment consisting of the VH and CH1 domains; an Fv fragment consisting of the VL and VH domains of a single arm of an antibody; a single domain antibody (dAb) fragment (Ward et al., 1989, Nature 341:544-546), and an isolated complementarity determining region (CDR).

The term "monoclonal antibody" (Mab) refers to an antibody that is derived from a single copy or clone, including e.g., any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced. Preferably, a monoclonal antibody of the invention exists in a homogeneous or substantially homogeneous population.

"Humanized" antibody refers to forms of non-human (*e.g.* murine) antibodies that are chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) that contain minimal sequence derived from non-human immunoglobulin. Preferably, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a

complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity.

As used herein, "human antibody" means an antibody having an amino acid sequence corresponding to that of an antibody that can be produced by a human and/or which has been made using any of the techniques for making human antibodies known to those skilled in the art or disclosed herein. This definition of a human antibody includes antibodies comprising at least one human heavy chain polypeptide or at least one human light chain polypeptide. One such example is an antibody comprising murine light chain and human heavy chain polypeptides. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan et al., 1996, Nature Biotechnology, 14:309-314; Sheets et al., 1998, Proc. Natl. Acad. Sci. (USA) 95:6157-6162; Hoogenboom and Winter, 1991, J. Mol. Biol., 227:381; Marks et al., 1991, J. Mol. Biol., 222:581). Human antibodies can also be made by immunization of animals into which human immunoglobulin loci have been transgenically introduced in place of the endogenous loci, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. This approach is described in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016. Alternatively, the human antibody may be prepared by immortalizing human B lymphocytes that produce an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or may have been immunized in vitro). See, e.g., Cole et al. Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77, 1985; Boerner et al., 1991, J. Immunol., 147 (1):86-95; and U.S. Patent No. 5,750,373.

A "bispecific," "dual-specific" or "bifunctional" antibody is a hybrid antibody having two different antigen binding sites. The two antigen binding sites of a bispecific antibody bind to two different epitopes, which may reside on the same or different protein targets. In the present invention, for example, the bispecific antibody as described herein comprises an antigen binding site that interacts with the EGF-like domain of the LDLR (e.g., SEQ ID NO: 17 or amino acid residues 314-353 of SEQ ID NO: 18) in one arm and a second antigen binding site that recognizes the CHRD of the human PCSK9 protein in another arm.

A “variable region” of an antibody refers to the variable region of the antibody light chain or the variable region of the antibody heavy chain, either alone or in combination. As known in the art, the variable regions of the heavy and light chain each consist of four framework regions (FRs) connected by three complementarity determining regions (CDRs) also known as hypervariable regions, contribute to the formation of the antigen binding site of antibodies. If variants of a subject variable region are desired, particularly with substitution in amino acid residues outside of a CDR region (i.e., in the framework region), appropriate amino acid substitution, preferably, conservative amino acid substitution, can be identified by comparing the subject variable region to the variable regions of other antibodies which contain CDR1 and CDR2 sequences in the same canonincal class as the subject variable region (Chothia and Lesk, J Mol Biol 196(4): 901-917, 1987). When choosing FR to flank subject CDRs, e.g., when humanizing or optimizing an antibody, FRs from antibodies which contain CDR1 and CDR2 sequences in the same canonical class are preferred.

A “CDR” of a variable domain are amino acid residues within the variable region that are identified in accordance with the definitions of the Kabat, Chothia, the accumulation of both Kabat and Chothia, AbM, contact, and/or conformational definitions or any method of CDR determination well known in the art. Antibody CDRs may be identified as the hypervariable regions originally defined by Kabat et al. See, e.g., Kabat et al., 1992, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, NIH, Washington D.C. The positions of the CDRs may also be identified as the structural loop structures originally described by Chothia and others. See, e.g., Chothia et al., 1989, Nature 342:877-883. Other approaches to CDR identification include the “AbM definition,” which is a compromise between Kabat and Chothia and is derived using Oxford Molecular's AbM antibody modeling software (now ACCELRYS®), or the “contact definition” of CDRs based on observed antigen contacts, set forth in MacCallum et al., 1996, J. Mol. Biol., 262:732-745. In another approach, referred to herein as the “conformational definition” of CDRs, the positions of the CDRs may be identified as the residues that make enthalpic contributions to antigen binding. See, e.g., Makabe et al., 2008, Journal of Biological Chemistry, 283:1156-1166. Still other CDR boundary definitions may not strictly follow one of the above approaches, but will nonetheless overlap with at least a portion of the Kabat CDRs, although they may be shortened or lengthened in light of prediction or experimental findings that

particular residues or groups of residues or even entire CDRs do not significantly impact antigen binding. As used herein, a CDR may refer to CDRs defined by any approach known in the art, including combinations of approaches. The methods used herein may utilize CDRs defined according to any of these approaches. For any given embodiment containing more than one CDR, the CDRs may be defined in accordance with any of Kabat, Chothia, extended, AbM, contact, and/or conformational definitions.

As known in the art a "constant region" of an antibody refers to the constant region of the antibody light chain or the constant region of the antibody heavy chain, either alone or in combination.

As used herein, the term "PCSK9" refers to any form of PCSK9 and variants thereof that retain at least part of the activity of PCSK9. Unless indicated differently, such as by specific reference to human PCSK9, PCSK9 includes all mammalian species of native sequence PCSK9, e.g., human, canine, feline, equine, and bovine. One exemplary human PCSK9 is found as Uniprot Accession Number Q8NBP7 (SEQ ID NO: 1).

As used herein, an "anti-PCSK9 antagonist antibody" or "PCSK9 antagonist antibody" refers to an anti-PCSK9 antibody that is able to inhibit PCSK9 biological activity and/or downstream pathway(s) mediated by PCSK9 signaling, including PCSK9-mediated down-regulation of the LDLR, and PCSK9-mediated decrease in LDL blood clearance. A PCSK9 antagonist antibody encompasses antibodies that block, antagonize, suppress or reduce (to any degree including significantly) PCSK9 biological activity, including downstream pathways mediated by PCSK9 signaling, such as LDLR interaction, elicitation of a cellular response to PCSK9, and/or interaction with APLP2. For purpose of the present invention, it will be explicitly understood that the term "PCSK9 antagonist antibody" encompasses all the previously identified terms, titles, and functional states and characteristics whereby the PCSK9 itself, a PCSK9 biological activity (including but not limited to its ability to mediate any aspect of interaction with the LDLR, down regulation of LDLR, and decreased blood LDL clearance), or the consequences of the biological activity, are substantially nullified, decreased, or neutralized in any meaningful degree. In some embodiments, a PCSK9 antagonist antibody binds PCSK9 and prevents interaction with the LDLR. Examples of PCSK9 antagonist antibodies are provided in, e.g., U.S. Patent Application Publication No. 20100068199 and Devay et al., J. Biol. Chem. 288: 10805-10818 (2013), which are

herein incorporated by reference in its entirety. In some embodiments, a PCSK9 antagonist antibody binds to PCSK9 and prevents interaction with APLP2, APP, and ApoB/LDL.

As used herein a “full antagonist” is an antagonist which, at an effective concentration, essentially completely blocks a measurable effect of PCSK9. By a partial antagonist is meant an antagonist that is capable of partially blocking a measurable effect, but that, even at a highest concentration is not a full antagonist. By essentially completely is meant at least about 80%, preferably, at least about 90%, more preferably, at least about 95%, and most preferably, at least about 98% or 99% of the measurable effect is blocked. The relevant “measurable effects” are described herein and include down regulation of LDLR by a PCSK9 antagonist as assayed in Huh7 cells in vitro, in vivo decrease in blood (or plasma) levels of total cholesterol, and in vivo decrease in LDL levels in blood (or plasma).

The term "epitope" refers to that portion of a molecule capable of being recognized by and bound by an antibody at one or more of the antibody's antigen-binding regions. Epitopes often consist of a surface grouping of molecules such as amino acids or sugar side chains and have specific three-dimensional structural characteristics as well as specific charge characteristics. In some embodiments, the epitope can be a protein epitope. Protein epitopes can be linear or conformational. In a linear epitope, all of the points of interaction between the protein and the interacting molecule (such as an antibody) occur linearly along the primary amino acid sequence of the protein. A "nonlinear epitope" or "conformational epitope" comprises noncontiguous polypeptides (or amino acids) within the antigenic protein to which an antibody specific to the epitope binds. The term "antigenic epitope" as used herein, is defined as a portion of an antigen to which an antibody can specifically bind as determined by any method well known in the art, for example, by conventional immunoassays. Once a desired epitope on an antigen is determined, it is possible to generate antibodies to that epitope, e.g., using the techniques described in the present specification. Alternatively, during the discovery process, the generation and characterization of antibodies may elucidate information about desirable epitopes. From this information, it is then possible to competitively screen antibodies for binding to the same epitope. An approach to achieve this is to conduct competition and cross-competition studies to find antibodies

that compete or cross-compete with one another for binding to PCSK9, e.g., the antibodies compete for binding to the antigen.

As used herein, the term "Cys-His Rich Domain (CHRD)" of the PCSK9 refers to a C-terminal region on the PCSK9 protein comprising three repeat modules M1 (amino acids 453-531), M2 (amino acids 530-605), and M3 (amino acids 604-692). The CHRD of PCSK9 is not required for PCSK9/LDLR binding, but is required for APLP2 (Amyloid Precursor protein-Like Protein-2) binding and postendocytic PCSK9 lysosomal trafficking.

As used herein, the term "clinically meaningful" means at least a 15% reduction in blood LDL-cholesterol levels in humans or at least a 15% reduction in total blood cholesterol in mice. It is clear that measurements in plasma or serum can serve as surrogates for measurement of levels in blood.

The terms "polypeptide", "oligopeptide", "peptide" and "protein" are used interchangeably herein to refer to chains of amino acids of any length, preferably, relatively short (e.g., 10-100 amino acids). The chain may be linear or branched, it may comprise modified amino acids, and/or may be interrupted by non-amino acids. The terms also encompass an amino acid chain that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. It is understood that the polypeptides can occur as single chains or associated chains.

As known in the art, "polynucleotide," or "nucleic acid," as used interchangeably herein, refer to chains of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a chain by DNA or RNA polymerase. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the chain. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps", substitution of

one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, alpha- or beta-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), (O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

An antibody that "preferentially binds" or "specifically binds" (used interchangeably herein) to an epitope is a term well understood in the art, and methods to determine such specific or preferential binding are also well known in the art. A molecule is said to exhibit "specific binding" or "preferential binding" if it reacts or associates more frequently, more rapidly, with greater duration and/or with greater

affinity with a particular cell or substance than it does with alternative cells or substances. An antibody “specifically binds” or “preferentially binds” to a target if it binds with greater affinity, avidity, more readily, and/or with greater duration than it binds to other substances. For example, an antibody that specifically or preferentially binds to a PCSK9 epitope is an antibody that binds this epitope with greater affinity, avidity, more readily, and/or with greater duration than it binds to other PCSK9 epitopes or non-PCSK9 epitopes. It is also understood by reading this definition that, for example, an antibody (or moiety or epitope) that specifically or preferentially binds to a first target may or may not specifically or preferentially bind to a second target. As such, “specific binding” or “preferential binding” does not necessarily require (although it can include) exclusive binding. Generally, but not necessarily, reference to binding means preferential binding.

As used herein, “substantially pure” refers to material which is at least 50% pure (i.e., free from contaminants), more preferably, at least 90% pure, more preferably, at least 95% pure, yet more preferably, at least 98% pure, and most preferably, at least 99% pure.

A “host cell” includes an individual cell or cell culture that can be or has been a recipient for vector(s) for incorporation of polynucleotide inserts. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in genomic DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. A host cell includes cells transfected in vivo with a polynucleotide(s) of this invention.

As known in the art, the term "Fc region" is used to define a C-terminal region of an immunoglobulin heavy chain. The "Fc region" may be a native sequence Fc region or a variant Fc region. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The numbering of the residues in the Fc region is that of the EU index as in Kabat. Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md., 1991. The Fc region of an immunoglobulin generally comprises two constant domains, CH2 and CH3.

As used in the art, "Fc receptor" and “FcR” describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR.

Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. FcRs are reviewed in Ravetch and Kinet, 1991, Ann. Rev. Immunol., 9:457-92; Capel et al., 1994, Immunomethods, 4:25-34; and de Haas et al., 1995, J. Lab. Clin. Med., 126:330-41. "FcR" also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., 1976 J. Immunol., 117:587; and Kim et al., 1994, J. Immunol., 24:249).

The term "compete", as used herein with regard to an antibody, means that a first antibody, or an antigen-binding portion thereof, binds to an epitope in a manner sufficiently similar to the binding of a second antibody, or an antigen-binding portion thereof, such that the result of binding of the first antibody with its cognate epitope is detectably decreased in the presence of the second antibody compared to the binding of the first antibody in the absence of the second antibody. The alternative, where the binding of the second antibody to its epitope is also detectably decreased in the presence of the first antibody, can, but need not be the case. That is, a first antibody can inhibit the binding of a second antibody to its epitope without that second antibody inhibiting the binding of the first antibody to its respective epitope. However, where each antibody detectably inhibits the binding of the other antibody with its cognate epitope or ligand, whether to the same, greater, or lesser extent, the antibodies are said to "cross-compete" with each other for binding of their respective epitope(s). Both competing and cross-competing antibodies are encompassed by the present invention. Regardless of the mechanism by which such competition or cross-competition occurs (e.g., steric hindrance, conformational change, or binding to a common epitope, or portion thereof), the skilled artisan would appreciate, based upon the teachings provided herein, that such competing and/or cross-competing antibodies are encompassed and can be useful for the methods disclosed herein.

By an antibody with an epitope that "overlaps" with another (second) epitope or with a surface on PCSK9 that interacts with the EGF-like domain of the LDLR is meant the sharing of space in terms of the PCSK9 residues that are interacted with. To calculate the percent of overlap, for example, the percent overlap of the claimed

antibody's PCSK9 epitope with the surface of PCSK9 which interacts with the EGF-like domain of the LDLR, the surface area of PCSK9 buried when in complex with the LDLR is calculated on a per-residue basis. The buried area is also calculated for these residues in the PCSK9:antibody complex. To prevent more than 100% possible overlap, surface area for residues that have higher buried surface area in the PCSK9:antibody complex than in LDLR:PCSK9 complex is set to values from the LDLR:PCSK9 complex (100%). Percent surface overlap is calculated by summing over all of the LDLR:PCSK9 interacting residues and is weighted by the interaction area.

A "functional Fc region" possesses at least one effector function of a native sequence Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity; phagocytosis; down-regulation of cell surface receptors (e.g., B cell receptor), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g., an antibody variable domain) and can be assessed using various assays known in the art for evaluating such antibody effector functions.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification, yet retains at least one effector function of the native sequence Fc region. Preferably, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, e.g., from about one to about ten amino acid substitutions, and preferably, from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will preferably possess at least about 80% sequence identity with a native sequence Fc region and/or with an Fc region of a parent polypeptide, and most preferably, at least about 90% sequence identity therewith, more preferably, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% sequence identity therewith.

As used herein, the terms "atorvastatin", "cerivastatin", "fluvastatin", "lovastatin", "mevastatin", "pitavastatin", "pravastatin", "rosuvastatin" and "simvastatin" include atorvastatin, cerivastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin, respectively, and any pharmaceutically acceptable salts, or

stereoisomers, thereof. As used herein, the term "pharmaceutically acceptable salt" includes salts that are physiologically tolerated by a patient. Such salts are typically prepared from inorganic acids or bases and/or organic acids or bases. Examples of these acids and bases are well known to those of ordinary skill in the art.

As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, one or more of the following: enhancement of LDL clearance and reducing incidence or amelioration of aberrant cholesterol and/or lipoprotein levels resulting from metabolic and/or eating disorders, or including hypercholesterolemia (e.g., HetFH or HoFH), dyslipidemia (e.g., mixed dyslipidemia), hyperlipidemia (e.g., heterozygous or homozygous familial and non-familial), atherosclerosis, acute coronary syndrome (ACS), and, more generally, cardiovascular disease (CVD).

"Reducing incidence" means any of reducing severity (which can include reducing need for and/or amount of (e.g., exposure to) other drugs and/or therapies generally used for this condition. As is understood by those skilled in the art, individuals may vary in terms of their response to treatment, and, as such, for example, a "method of reducing incidence" reflects administering the combination of anti-PCSK9 antagonist antibody as described herein based on a reasonable expectation that such administration may likely cause such a reduction in incidence in that particular individual.

"Ameliorating" means a lessening or improvement of one or more symptoms as compared to not administering a PCSK9 antagonist antibody. "Ameliorating" also includes shortening or reduction in duration of a symptom.

As used herein, an "effective dosage", "effective amount", or "therapeutically effective amount" of drug, compound, or pharmaceutical composition is an amount sufficient to effect any one or more beneficial or desired results. For prophylactic use, beneficial or desired results include eliminating or reducing the risk, lessening the severity, or delaying the outset of the disease, including biochemical, histological and/or behavioral symptoms of the disease, its complications and intermediate pathological phenotypes presenting during development of the disease. For therapeutic use, beneficial or desired results include clinical results such as reducing hypercholesterolemia or one or more symptoms of dyslipidemia, hyperlipidemia,

atherosclerosis, cardiovascular disease, or coronary heart disease, decreasing the dose of other medications required to treat the disease, enhancing the effect of another medication, and/or delaying the progression of the disease of patients. An effective dosage can be administered in one or more administrations. For purposes of this invention, an effective dosage of drug, compound, or pharmaceutical composition is an amount sufficient to accomplish prophylactic or therapeutic treatment either directly or indirectly. As is understood in the clinical context, an effective dosage of a drug, compound, or pharmaceutical composition may or may not be achieved in conjunction with another drug, compound, or pharmaceutical composition. Thus, an “effective dosage” may be considered in the context of administering one or more therapeutic agents, and a single agent may be considered to be given in an effective amount if, in conjunction with one or more other agents, a desirable result may be or is achieved.

An “individual” or a "subject" is a mammal, more preferably, a human. Mammals also include, but are not limited to, farm animals, sport animals, pets, primates, horses, dogs, cats, mice and rats.

As used herein, “a subject having a high risk of developing” a disease (e.g., dyslipidemia, hyperlipidemia, hypercholesterolemia, atherosclerosis, cardiovascular disease, and/or coronary heart disease) refers to those who have pre-existing conditions that make them more likely than the others to develop a condition associated with high levels of LDL-cholesterol. These pre-existing conditions include, but are not limited to, type I or type II diabetes, renal failure, HIV (human immunodeficiency virus), and metabolic syndrome, as well as transplant patients receiving cyclosporine or tacrolimus.

As used herein, "vector" means a construct, which is capable of delivering, and, preferably, expressing, one or more gene(s) or sequence(s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells.

As used herein, "expression control sequence" means a nucleic acid sequence that directs transcription of a nucleic acid. An expression control sequence can be a promoter, such as a constitutive or an inducible promoter, or an enhancer. The

expression control sequence is operably linked to the nucleic acid sequence to be transcribed.

As used herein, "pharmaceutically acceptable carrier" or "pharmaceutical acceptable excipient" includes any material which, when combined with an active ingredient, allows the ingredient to retain biological activity and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Preferred diluents for aerosol or parenteral administration are phosphate buffered saline (PBS) or normal (0.9%) saline. Compositions comprising such carriers are formulated by well known conventional methods (see, for example, Remington's Pharmaceutical Sciences, 18th edition, A. Gennaro, ed., Mack Publishing Co., Easton, PA, 1990; and Remington, The Science and Practice of Pharmacy, 20th Ed., Mack Publishing, 2000).

The term "k_{on}", as used herein, refers to the rate constant for association of an antibody to an antigen. Specifically, the rate constants (k_{on} and k_{off}) and equilibrium dissociation constants are measured using Fab antibody fragments (i.e., univalent) and PCSK9.

The term "k_{off} ", as used herein, refers to the rate constant for dissociation of an antibody from the antibody/antigen complex.

The term "K_D", as used herein, refers to the equilibrium dissociation constant of an antibody-antigen interaction.

Reference to “about” a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter *per se*. For example, description referring to “about X” includes description of “X.” Numeric ranges are inclusive of the numbers defining the range.

It is understood that wherever embodiments are described herein with the language “comprising,” otherwise analogous embodiments described in terms of “consisting of” and/or “consisting essentially of” are also provided.

Where aspects or embodiments of the invention are described in terms of a Markush group or other grouping of alternatives, the present invention encompasses not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group, but also the main group absent one or

more of the group members. The present invention also envisages the explicit exclusion of one or more of any of the group members in the claimed invention.

Unless otherwise defined, 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. Exemplary methods and materials are described herein, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention. All publications and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. Although a number of documents are cited herein, this citation does not constitute an admission that any of these documents forms part of the common general knowledge in the art. Throughout this specification and claims, the word "comprise," or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The materials, methods, and examples are illustrative only and not intended to be limiting.

Antibodies of the invention can be produced using techniques well known in the art, *e.g.*, recombinant technologies, phage display technologies, synthetic technologies or combinations of such technologies or other technologies readily known in the art (see, for example, Jayasena, S.D., Clin. Chem., 45: 1628-50, 1999 and Fellouse, F.A., et al, J. Mol. Biol., 373(4):924-40, 2007).

Published information related to anti-PCSK9 antibodies includes the following publications: PCT/IB2009/053990, published March 18, 2010 as WO 2010/029513, U.S. Patent Application No. 12/558312, published December 20, 2011 as US 8,080,243, DeVay et al., J. Biol. Chem. 288:10805-10818 (2013), each of which is herein incorporated by reference in its entirety.

Exemplary methods and materials are described herein, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention. The materials, methods, and examples are illustrative only and not intended to be limiting.

Methods for preventing or treating disorders associated with high LDL-cholesterol

In one aspect, the invention provides a method of reducing a level of LDL-cholesterol in blood of a subject in need thereof comprising administering to the subject a therapeutically effective amount of a first and second antagonist antibodies or antigen binding fragments thereof specific to PCSK9, wherein the first anti-PCSK9 antagonist antibody blocks LDLR binding to the PCSK9 of SEQ ID NO: 1, and wherein the second anti-PCSK9 antagonist antibody binds to C-Terminal Cys-His Rich Domain (CHRD) of the PCSK9 of SEQ ID NO: 1.

In some embodiments, provided is a first and second antagonist antibody or antigen binding fragments thereof specific to PCSK9 for use in reducing a level of LDL-cholesterol in blood of a subject in need thereof, wherein the first anti-PCSK9 antagonist antibody blocks LDLR binding to the PCSK9 of SEQ ID NO: 1, and wherein the second anti-PCSK9 antagonist antibody binds to C-Terminal Cys-His Rich Domain (CHRD) of the PCSK9 of SEQ ID NO: 1.

In some embodiments, provided is a use of a first and second antagonist antibody or antigen binding fragments thereof specific to PCSK9 in the manufacture of a medicament for reducing a level of LDL-cholesterol in blood of a subject, wherein the first anti-PCSK9 antagonist antibody blocks LDLR binding to the PCSK9 of SEQ ID NO: 1, and wherein the second anti-PCSK9 antagonist antibody binds to C-Terminal Cys-His Rich Domain (CHRD) of the PCSK9 of SEQ ID NO: 1.

In some embodiments, the first anti-PCSK9 antagonist antibody interacts with the EGF-like domain of the LDLR (e.g., SEQ ID NO: 17 or amino acid residues 314-353 of SEQ ID NO: 18). In some embodiments, the first anti-PCSK9 antibody is alirocumab (PRALUENT™); evolocumab (REPATHA™); REGN728; LGT209; RG7652; LY3015014; J16, L1L3 (see, e.g., US8,080,243); 31H4, 11F1, 12H11, 8A3, 8A1, or 3C4 (see, e.g., US8,030,457); 300N (see, e.g., US8,062,640); or 1D05 (see, e.g., US8,188,234). In some embodiments, the first anti-PCSK9 antibody is bococizumab, evolocumab (REPATHA™), or alirocumab (PRALUENT™). In some embodiments, the antibody recognizes 1) an epitope on human PCSK9 comprising amino acid residues 153-155, 194, 195, 197, 237-239, 367, 369, 374-379 and/or 381 of the PCSK9 amino acid sequence of SEQ ID NO: 1; or 2) an epitope on human PCSK9 comprising amino acid residues 153, 154 194, 238, 369, 374, 377, and/or 379 of the PCSK9 amino acid sequence of SEQ ID NO: 1.

In some embodiments, the method or use comprises administering an initial dose of about 0.025 mg/kg to about 20 mg/kg of the first anti-PCSK9 antagonist antibody that blocks LDLR binding to PCSK9 amino acid sequence of SEQ ID NO: 1 and an initial dose of about 0.025 mg/kg to about 20 mg/kg of the second anti-PCSK9 antagonist antibody that binds to the CHRD of the PCSK9 amino acid sequence of SEQ ID NO: 1. For example, the initial dose for both anti-PCSK9 antagonist antibodies is about any of 0.025 mg/kg, 0.25 mg/kg, 0.5 mg/kg, 0.75 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 3.5 mg/kg, 4 mg/kg, 4.5 mg/kg, 5mg/kg, 5.5 mg/kg, 6 mg/kg, 6.5 mg/kg, 7 mg/kg, 7.5 mg/kg, 8 mg/kg, 8.5 mg/kg, 9 mg/kg, 9.5 mg/kg, 10 mg/kg, 11 mg/kg, 12 mg/kg, 13 mg/kg, 14 mg/kg, 15 mg/kg, 16 mg/kg, 17 mg/kg, 18 mg/kg, 19 mg/kg, or 20 mg/kg. In some embodiments, the maintenance dose is administered at least any of weekly, every other week, every three weeks, every four weeks, every five weeks, every six weeks, every seven weeks, every eight weeks, every nine weeks, every ten weeks, every eleven weeks, or every twelve weeks. In some embodiments, the initial dose and the first subsequent and additional subsequent doses are separated in time from each other by at least about two weeks or about four weeks.

In some embodiments, the method or use comprises administering a fixed dose of about 0.25 mg to about 2000 mg of the first anti-PCSK9 antagonist antibody that blocks LDLR binding to the PCSK9 amino acid sequence of SEQ ID NO: 1 and a fixed dose of about 0.25 mg to about 2000 mg of the second anti-PCSK9 antagonist antibody that binds to the CHRD of the PCSK9 amino acid sequence of SEQ ID NO: 1. In some embodiments, the first anti-PCSK9 antagonist antibody is alirocumab (PRALUENT™), evolocumab (REPATHA™), or bococizumab. For example, the fixed dose for both anti-PCSK9 antagonist antibodies is about any of 0.25 mg, 0.25 mg, 0.5 mg, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 11 mg, 12 mg, 13 mg, 14 mg, 15 mg, 16 mg, 17 mg, 18 mg, 19 mg, 20 mg, 21 mg, 22 mg, 23 mg, 24 mg, 25 mg, 26 mg, 27 mg, 28 mg, 29 mg, 30 mg, 31 mg, 32 mg, 33 mg, 34 mg, 35 mg, 36 mg, 37 mg, 38 mg, 39 mg, 40 mg, 41 mg, 42 mg, 43 mg, 44 mg, 45 mg, 46 mg, 47 mg, 48 mg, 49 mg, 50 mg, 51 mg, 52 mg, 53 mg, 54 mg, 55 mg, 56 mg, 57 mg, 58 mg, 59 mg, 60 mg, 61 mg, 62 mg, 63 mg, 64 mg, 65 mg, 66 mg, 67 mg, 68 mg, 69 mg, 70 mg, 71 mg, 72 mg, 73 mg, 74 mg, 75 mg, 76 mg, 77 mg, 78 mg, 79 mg, 80 mg, 81 mg, 82 mg, 83 mg, 84 mg, 85 mg, 86 mg, 87 mg, 88 mg, 89 mg, 90 mg, 91 mg, 92 mg, 93 mg, 94 mg, 95 mg, 96

mg, 99 mg, 98 mg, 99 mg, 100 mg, 101 mg, 102 mg, 103 mg, 104 mg, 105 mg, 106 mg, 107 mg, 108 mg, 109 mg, 110 mg, 111 mg, 112 mg, 113 mg, 114 mg, 115 mg, 116 mg, 117 mg, 118 mg, 119 mg, 120 mg, 121 mg, 122 mg, 123 mg, 124 mg, 125 mg, 126 mg, 127 mg, 128 mg, 129 mg, 130 mg, 131 mg, 132 mg, 133 mg, 134 mg, 135 mg, 136 mg, 137 mg, 138 mg, 139 mg, 140 mg, 141 mg, 142 mg, 143 mg, 144 mg, 145 mg, 146 mg, 147 mg, 148 mg, 149 mg, 150 mg, 151 mg, 152 mg, 153 mg, 154 mg, 155 mg, 156 mg, 157 mg, 158 mg, 159 mg, 160 mg, 161 mg, 162 mg, 163 mg, 164 mg, 165 mg, 166 mg, 167 mg, 168 mg, 169 mg, 170 mg, 171 mg, 172 mg, 173 mg, 174 mg, 175 mg, 176 mg, 177 mg, 178 mg, 179 mg, 180 mg, 181 mg, 182 mg, 183 mg, 184 mg, 185 mg, 186 mg, 187 mg, 188 mg, 189 mg, 190 mg, 191 mg 192 mg, 193 mg, 194 mg, 195 mg, 196 mg, 199 mg, 198 mg, 199 mg, 200 mg, 250 mg, 300 mg, 350 mg, 400 mg, 450 mg, 500 mg, 600 mg, 700 mg, 800 mg, 900 mg, 1000 mg, 1100 mg, 1200 mg, 1300 mg, 1400 mg, 1500 mg, 1600 mg, 1700 mg, 1800 mg, 1900 mg, or 2000 mg. In some embodiments, both the first and the second anti-PCSK9 antagonist antibodies are administered weekly, every other week, every three weeks, every four weeks, every five weeks, every six weeks, every seven weeks, every eight weeks, every nine weeks, every ten weeks, every eleven weeks, or every twelve weeks. In some embodiments, both the first and the second anti-PCSK9 antagonist antibodies are administered every two weeks or every four weeks.

In some embodiments, the first and the second anti-PCSK9 antagonist antibodies are administered sequentially or simultaneously. For example, the first anti-PCSK9 antagonist antibody can be administered about any of 10 seconds, 30 seconds, 1 minute, 5 minutes, 10 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days earlier than the second anti-PCSK9 antagonist antibody.

In some embodiments, the dosing regimen described herein results in extending the half-life of the first anti-PCKS9 antagonist antibody and/or the second anti-PCSK9 antagonist antibody by at least about any of 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, or 30 days.

In one variation, the invention also provides a method for reducing LDL-cholesterol levels in blood of a subject in need thereof comprising the administration of a therapeutically effective amount of a bispecific antibody directed to two different epitopes of the PCSK9 protein, wherein the first arm of the antibody blocks the LDLR binding to the PCSK9 protein (e.g., SEQ ID NO: 1), and the second arm of the antibody binds to CHRD of the PCSK9 protein (e.g., SEQ ID NO: 1). In some embodiments, the first arm of the bispecific antibody interacts with the EGF-like domain of the LDLR (e.g., SEQ ID NO: 17 or amino acid residues 314-353 of SEQ ID NO: 18), or recognizes 1) an epitope on human PCSK9 comprising amino acid residues 153-155, 194, 195, 197, 237-239, 367, 369, 374-379 and/or 381 of the PCSK9 amino acid sequence of SEQ ID NO: 1; or 2) an epitope on human PCSK9 comprising amino acid residues 153, 154 194, 238, 369, 374, 377, and/or 379 of the PCSK9 amino acid sequence of SEQ ID NO: 1.

In some embodiments, provided is a bispecific antagonist antibody or antigen binding fragments thereof specific to PCSK9 for use in reducing a level of LDL-cholesterol in blood of a subject in need thereof, wherein the first arm of the antibody blocks LDLR binding to the PCSK9 of SEQ ID NO: 1, and wherein the second arm of the antibody binds to C-Terminal Cys-His Rich Domain (CHRD) of the PCSK9 of SEQ ID NO: 1.

In some embodiments, provided is a use of a bispecific antibody or antigen binding fragments thereof specific to PCSK9 in the manufacture of a medicament for reducing a level of LDL-cholesterol in blood of a subject, wherein the first arm of the antibody blocks LDLR binding to the PCSK9 of SEQ ID NO: 1, and wherein the second arm of the antibody binds to C-Terminal Cys-His Rich Domain (CHRD) of the PCSK9 of SEQ ID NO: 1.

In another variation, the invention further provides a method for reducing LDL-cholesterol levels in blood of a subject in need thereof comprising the administration of a therapeutically effective amount of an antibody (e.g., bifunctional antibody) specific to a PCSK9 protein, wherein the antibody blocks the LDLR binding to the PCSK9 protein (e.g., SEQ ID NO: 1), and binds to CHRD of the PCSK9 protein (e.g., SEQ ID NO: 1). In some embodiments, the antibody has the dual function of a) interacting with the EGF-like domain of the LDLR (e.g., SEQ ID NO: 17 or amino acid residues 314-353 of SEQ ID NO: 18), or recognizing 1) an epitope on human PCSK9 comprising amino acid

residues 153-155, 194, 195, 197, 237-239, 367, 369, 374-379 and/or 381 of the PCSK9 amino acid sequence of SEQ ID NO: 1; or 2) an epitope on human PCSK9 comprising amino acid residues 153, 154 194, 238, 369, 374, 377, and/or 379 of the PCSK9 amino acid sequence of SEQ ID NO: 1; and b) blocking binding of PCSK9 to APLP2 protein. In some embodiments, the bifunctional PCSK9 antibody is a full antagonist of the PCSK9-mediated effect on LDLR levels as measured in in vivo or in vitro methods (e.g., LDLR down-regulation assay in Huh7 cells).

In some embodiments, provided is an antagonist antibody (e.g., bifunctional antibody) or antigen binding fragments thereof specific to PCSK9 for use in reducing a level of LDL-cholesterol in blood of a subject in need thereof, wherein the antibody blocks the LDLR binding to the PCSK9 protein (e.g., SEQ ID NO: 1), and binds to CHRD of the PCSK9 protein (e.g., SEQ ID NO: 1).

In some embodiments, provided is a use of an antagonist antibody (e.g., bifunctional antibody) or antigen binding fragments thereof specific to PCSK9 in the manufacture of a medicament for reducing a level of LDL-cholesterol in blood of a subject, wherein the antibody blocks the LDLR binding to the PCSK9 protein (e.g., SEQ ID NO: 1), and binds to CHRD of the PCSK9 protein (e.g., SEQ ID NO: 1).

In some embodiments, the method or use comprises administering an initial dose of about 0.025 mg/kg to about 20 mg/kg of the anti-PCSK9 bispecific or bifunctional antibody as described herein. For example, the initial dose for the anti-PCSK9 bispecific or bifunctional antibody is about any of 0.025 mg/kg, 0.25 mg/kg, 0.5 mg/kg, 0.75 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 3.5 mg/kg, 4 mg/kg, 4.5 mg/kg, 5mg/kg, 5.5 mg/kg, 6 mg/kg, 6.5 mg/kg, 7 mg/kg, 7.5 mg/kg, 8 mg/kg, 8.5 mg/kg, 9 mg/kg, 9.5 mg/kg, 10 mg/kg, 11 mg/kg, 12 mg/kg, 13 mg/kg, 14 mg/kg, 15 mg/kg, 16 mg/kg, 17 mg/kg, 18 mg/kg, 19 mg/kg, or 20 mg/kg. In some embodiments, the maintenance dose is administered at least any of weekly, every other week, every three weeks, every four weeks, every five weeks, every six weeks, every seven weeks, every eight weeks, every nine weeks, every ten weeks, every eleven weeks, or every twelve weeks. In some embodiments, the initial dose and the first subsequent and additional subsequent doses are separated in time from each other by at least about two weeks or about four weeks.

In some embodiments, the method or use comprises administering a fixed dose of about 0.25 mg to about 2000 mg of the anti-PCSK9 bispecific or bifunctional antibody

as described herein. For example, the fixed dose for the anti-PCSK9 bispecific or bifunctional antibody is about any of 0.25 mg, 0.25 mg, 0.5 mg, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 11 mg, 12 mg, 13 mg, 14 mg, 15 mg, 16 mg, 17 mg, 18 mg, 19 mg, 20 mg, 21 mg, 22 mg, 23 mg, 24 mg, 25 mg, 26 mg, 27 mg, 28 mg, 29 mg, 30 mg, 31 mg, 32 mg, 33 mg, 34 mg, 35 mg, 36 mg, 37 mg, 38 mg, 39 mg, 40 mg, 41 mg, 42 mg, 43 mg, 44 mg, 45 mg, 46 mg, 47 mg, 48 mg, 49 mg, 50 mg, 51 mg, 52 mg, 53 mg, 54 mg, 55 mg, 56 mg, 57 mg, 58 mg, 59 mg, 60 mg, 61 mg, 62 mg, 63 mg, 64 mg, 65 mg, 66 mg, 67 mg, 68 mg, 69 mg, 70 mg, 71 mg, 72 mg, 73 mg, 74 mg, 75 mg, 76 mg, 77 mg, 78 mg, 79 mg, 80 mg, 81 mg, 82 mg, 83 mg, 84 mg, 85 mg, 86 mg, 87 mg, 88 mg, 89 mg, 90 mg, 91 mg, 92 mg, 93 mg, 94 mg, 95 mg, 96 mg, 99 mg, 98 mg, 99 mg, 100 mg, 101 mg, 102 mg, 103 mg, 104 mg, 105 mg, 106 mg, 107 mg, 108 mg, 109 mg, 110 mg, 111 mg, 112 mg, 113 mg, 114 mg, 115 mg, 116 mg, 117 mg, 118 mg, 119 mg, 120 mg, 121 mg, 122 mg, 123 mg, 124 mg, 125 mg, 126 mg, 127 mg, 128 mg, 129 mg, 130 mg, 131 mg, 132 mg, 133 mg, 134 mg, 135 mg, 136 mg, 137 mg, 138 mg, 139 mg, 140 mg, 141 mg, 142 mg, 143 mg, 144 mg, 145 mg, 146 mg, 147 mg, 148 mg, 149 mg, 150 mg, 151 mg, 152 mg, 153 mg, 154 mg, 155 mg, 156 mg, 157 mg, 158 mg, 159 mg, 160 mg, 161 mg, 162 mg, 163 mg, 164 mg, 165 mg, 166 mg, 167 mg, 168 mg, 169 mg, 170 mg, 171 mg, 172 mg, 173 mg, 174 mg, 175 mg, 176 mg, 177 mg, 178 mg, 179 mg, 180 mg, 181 mg, 182 mg, 183 mg, 184 mg, 185 mg, 186 mg, 187 mg, 188 mg, 189 mg, 190 mg, 191 mg 192 mg, 193 mg, 194 mg, 195 mg, 196 mg, 199 mg, 198 mg, 199 mg, 200 mg, 250 mg, 300 mg, 350 mg, 400 mg, 450 mg, 500 mg, 600 mg, 700 mg, 800 mg, 900 mg, 1000 mg, 1100 mg, 1200 mg, 1300 mg, 1400 mg, 1500 mg, 1600 mg, 1700 mg, 1800 mg, 1900 mg, or 2000 mg. In some embodiments, the anti-PCSK9 bispecific or bifunctional antibody as described herein is administered weekly, every other week, every three weeks, every four weeks, every five weeks, every six weeks, every seven weeks, every eight weeks, every nine weeks, every ten weeks, every eleven weeks, or every twelve weeks. In some embodiments, the anti-PCSK9 bispecific or bifunctional antibody is administered every two weeks or every four weeks.

PCSK9 antagonist antibodies, including the bispecific or bifunctional antibody, can further be administered according to one or more dosing regimens disclosed herein to an individual on stable doses of a statin. The stable doses can be, for example without limitation, a daily dose or an every-other-day dose of a statin. A variety of

statins known to those of skill in the art, and include, for example without limitation, atorvastatin, simvastatin, lovastatin, pravastatin, rosuvastatin, fluvastatin, cerivastatin, mevastatin, pitavastatin, and statin combination therapies. Non-limiting examples of statin combination therapies include atorvastatin plus amlodipine (CADUET™), simvastatin plus ezetimibe (VYTORIN™), lovastatin plus niacin (ADVICOR™), and simvastatin plus niacin (SIMCOR™).

In some embodiments, an individual has been on stable doses of a statin for at least one, two, three, four, five or six weeks prior to administration of an initial dose of the combination of two different PCSK9 antagonist antibodies or the anti-PCSK9 bispecific or bifunctional antibody as described herein. Preferably, the individual on stable doses of a statin has a fasting LDL-C greater than or equal to about 70 mg/dL prior to administration of an initial dose of the PCSK9 antagonist antibody or antibodies. In some embodiments, the individual on stable doses of a statin has a fasting LDL-C greater than or equal to about 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or 200 mg/dL prior to administration of an initial dose of the PCSK9 antagonist antibody or antibodies.

In some embodiments, an individual had been on stable doses of a statin (e.g., 1 day, 14 days, 1 month, 2 months, 3 months, 1 year, 2 years ago, etc.) prior to administration of an initial dose of the combination of two different PCSK9 antagonist antibodies or the anti-PCSK9 bispecific or bifunctional antibody as described herein, and initiate the statin doses with the anti-PCSK9 antagonist antibody dosing regimen at the same time.

For the purpose of the present invention, a typical statin dose might range from about 1 mg to about 80 mg, depending on the factors mentioned above. For example, a statin dose of about any of 0.3 mg, 0.5 mg, 1 mg, 2.5 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 11 mg, 12 mg, 13 mg, 14 mg, 15 mg, 16 mg, 17 mg, 18 mg, 19 mg, 20 mg, 21 mg, 22 mg, 23 mg, 24 mg, 25 mg, 26 mg, 27 mg, 28 mg, 29 mg, 30 mg, 30 mg, 31 mg, 32 mg, 33 mg, 34 mg, 35 mg, about 36 mg, about 37 mg, about 38 mg, 39 mg, 40 mg, 41 mg, 42 mg, 43 mg, 44 mg, 45 mg, 46 mg, 47 mg, 48 mg, 49 mg, 50 mg, 51 mg, 52 mg, 53 mg, 54 mg, 55 mg, about 56 mg, about 57 mg, about 58 mg, 59 mg, 60 mg, 61 mg, 62 mg, 63 mg, 64 mg, 65 mg, 66 mg, 67 mg, 68 mg, 69 mg, 70 mg, 71 mg, 72 mg, 73 mg, 74 mg, 75 mg, 76 mg, 77 mg, 78 mg, 79 mg, or 80 mg may be used.

In preferred embodiments, a dose of 40 mg or 80 mg atorvastatin is used. In other embodiments, a dose of 20 mg or 40 mg rosuvastatin is used. In other embodiments, a dose of 40 mg or 80 mg simvastatin is used.

The method described herein can be used for treating a subject (e.g., a patient) suffering conditions associated with high levels of LDL-cholesterol, including, but not limited to, from dyslipidemia, hyperlipidemia (e.g., heterozygous or homozygous familial and non-familial), hypercholesterolemia (e.g., HetFH or HoFH), atherosclerosis, cardiovascular disease, and/or coronary heart disease, and/or prophylactically treating a subject having a high risk of developing one or more conditions associated with high levels of LDL-cholesterol.

Advantageously, administration of two different anti-PCSK9 antagonist antibodies or the anti-PCSK9 bispecific or bifunctional antibody directed to two distinct epitopes on the PCSK9 protein results in lower blood LDL-cholesterol. Preferably, blood LDL-cholesterol is at least about 10% or 15% lower than before administration. More preferably, blood LDL-cholesterol is at least about 20% lower than before administration of the antibody. Yet more preferably, blood LDL-cholesterol is at least 30% lower than before administration of the antibody. Advantageously, blood LDL-cholesterol is at least 40% lower than before administration of the antibody. More advantageously, blood LDL-cholesterol is at least 50% lower than before administration of the antibody. Very preferably, blood LDL-cholesterol is at least 60% lower than before administration of the antibody. Most preferably, blood LDL-cholesterol is at least 70% lower than before administration of the antibody.

The anti-PCSK9 antagonist antibodies or the anti-PCSK9 bispecific or bifunctional antibody described herein can be administered to a subject via any suitable route. It should be apparent to a person skilled in the art that the examples described herein are not intended to be limiting but to be illustrative of the techniques available. Accordingly, in some embodiments, the anti-PCSK9 antagonist antibodies (e.g., including the bispecific antibody and the bifunctional antibody) are administered to an individual in accord with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by subcutaneous, intramuscular, intraperitoneal, intracerebrospinal, transdermal, intra-articular, sublingually, intrasynovial, via insufflation, intrathecal, oral, inhalation or topical routes. Administration can be systemic, e.g., intravenous administration, or localized.

Commercially available nebulizers for liquid formulations, including jet nebulizers and ultrasonic nebulizers are useful for administration. Liquid formulations can be directly nebulized and lyophilized powder can be nebulized after reconstitution. Alternatively, the anti-PCSK9 antagonist antibody can be aerosolized using a fluorocarbon formulation and a metered dose inhaler, or inhaled as a lyophilized and milled powder.

In some embodiments, in the case of combination treatment method, the first anti-PCSK9 antagonist antibody is administered via a different route than the second anti-PCSK9 antagonist antibody. For example, the first anti-PCSK9 antagonist antibody is administered subcutaneously, whereas the second anti-PCSK9 antagonist antibody is administered intravenously.

In some embodiments, the first and/or second anti-PCSK9 antagonist antibodies, or the anti-PCSK9 bispecific or bifunctional antibody, are administered via site-specific or targeted local delivery techniques. Examples of site-specific or targeted local delivery techniques include various implantable depot sources of the anti-PCSK9 antagonist antibodies or local delivery catheters, such as infusion catheters, indwelling catheters, or needle catheters, synthetic grafts, adventitial wraps, shunts and stents or other implantable devices, site specific carriers, direct injection, or direct application. See, e.g., PCT Publ. No. WO 00/53211 and U.S. Patent No. 5,981,568.

With respect to all methods described herein, reference to any anti-PCSK9 antagonist antibody also includes compositions comprising 1) both the first anti-PCSK9 antagonist antibody or the second anti-PCSK9 antagonist antibody; or 2) the bispecific or bifunctional anti-PCSK9 antagonist antibody and, additionally, one or more additional agents. These compositions may further comprise suitable excipients, such as pharmaceutically acceptable excipients including buffers, which are well known in the art. The present invention can be used alone or in combination with other conventional methods of treatment.

Various formulations of an anti-PCSK9 antagonist antibody may be used for combination administration. In some embodiments, the anti-PCSK9 antagonist antibody can be administered neat. In some embodiments, the anti-PCSK9 antagonist antibody can also be administered via inhalation. In some embodiments, the anti-PCSK9 antagonist antibody and a pharmaceutically acceptable excipient may be in various formulations. Pharmaceutically acceptable excipients are known in the art, and are relatively inert substances that facilitate administration of a pharmacologically

effective substance. For example, an excipient can give form or consistency, or act as a diluent. Suitable excipients include but are not limited to stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, buffers, and skin penetration enhancers. Excipients as well as formulations for parenteral and nonparenteral drug delivery are set forth in Remington, The Science and Practice of Pharmacy, 21st Ed., Mack Publishing (2005).

These agents can be combined with pharmaceutically acceptable vehicles such as saline, Ringer's solution, dextrose solution, and the like. The particular dosage regimen, i.e., dose, timing and repetition, will depend on the particular individual and that individual's medical history.

Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and may comprise buffers such as phosphate, citrate, and other organic acids; salts such as sodium chloride; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens, such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

Liposomes containing the anti-PCSK9 antagonist antibody are prepared by methods known in the art, such as described in Epstein, et al., 1985, Proc. Natl. Acad. Sci. USA 82:3688; Hwang, et al., 1980, Proc. Natl Acad. Sci. USA 77:4030; and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556. Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-

PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington, The Science and Practice of Pharmacy, 21st Ed., Mack Publishing (2005).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or 'poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and 7 ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT ™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), sucrose acetate isobutyrate, and poly-D-(-)-3-hydroxybutyric acid.

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by, for example, filtration through sterile filtration membranes. Therapeutic anti-PCSK9 antagonist antibodies are generally placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Suitable emulsions may be prepared using commercially available fat emulsions, such as INTRALIPID™, LIPOSYN™, INFONUTROL™, LIPOFUNDIN™ and LIPIPHYSAN™. The active ingredient may be either dissolved in a pre-mixed emulsion composition or alternatively it may be dissolved in an oil (e.g., soybean oil, safflower oil, cottonseed oil, sesame oil, corn oil or almond oil) and an emulsion formed upon mixing with a phospholipid (e.g., egg phospholipids, soybean phospholipids or soybean lecithin) and water. It will be appreciated that other ingredients may be added, for example glycerol or glucose, to adjust the tonicity of the emulsion. Suitable emulsions will typically contain up to 20% oil, for example, between 5 and 20%. The fat emulsion

can comprise fat droplets between 0.1 and 1.0 μm, particularly 0.1 and 0.5 μm, and have a pH in the range of 5.5 to 8.0.

The emulsion compositions can be those prepared by mixing the multiple anti-PCSK9 antagonist antibodies with INTRALIPID™. or the components thereof (soybean oil, egg phospholipids, glycerol and water).

Compositions for inhalation or insufflation include solutions and suspensions in pharmaceutically acceptable, aqueous or organic solvents, or mixtures thereof, and powders. The liquid or solid compositions may contain suitable pharmaceutically acceptable excipients as set out above. In some embodiments, the compositions are administered by the oral or nasal respiratory route for local or systemic effect. Compositions in preferably sterile pharmaceutically acceptable solvents may be nebulised by use of gases. Nebulised solutions may be breathed directly from the nebulising device or the nebulising device may be attached to a face mask, tent or intermittent positive pressure breathing machine. Solution, suspension or powder compositions may be administered, preferably orally or nasally, from devices which deliver the formulation in an appropriate manner.

PCSK9 Antagonist Antibodies

A description follows as to an exemplary technique for the production of the antibodies used in accordance with the present invention. The PCSK9 antigen to be used for production of antibodies may be, e.g. full-length human PCSK9, full length mouse PCSK9, and various peptides fragments of PCSK9. Other forms of PCSK9 useful for generating antibodies will be apparent to those skilled in the art.

As will be appreciated, antibodies for use in the present invention may be derived from hybridomas but can also be expressed in cell lines other than hybridomas. Sequences encoding the cDNAs or genomic clones for the particular antibodies can be used for transformation of suitable mammalian or nonmammalian host cells. Mammalian cell lines available as hosts for expression are well known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to Chinese hamster ovary (CHO) cells, NSO, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), and human hepatocellular carcinoma cells (e.g., Hep G6). Non-mammalian cells can also be employed, including bacterial, yeast, insect, and plant cells. Site directed

mutagenesis of the antibody CH6 domain to eliminate glycosylation may be preferred in order to prevent changes in either the immunogenicity, pharmacokinetic, and/or effector functions resulting from non-human glycosylation. The glutamine synthase system of expression is discussed in whole or part in connection with European Patents 616 846, 656 055, and 363 997 and European Patent Application 89303964.4. Further, a dihydrofolate reductase (DHFR) expression system, including those known in the art, can be used to produce the antibody.

In some embodiments, the invention is practiced using an anti-PCSK9 antagonist antibody that interacts with the EGF-like domain of the LDLR. In some embodiments, the anti-PCSK9 antibody is alirocumab (PRALUENT™); evolocumab (REPATHA™); REGN728; LGT209; RG7652; LY3015014; J16, L1L3 (see, e.g., US8,080,243); 31H4, 11F1, 12H11, 8A1, 8A3, or 3C4 (see, e.g., US8,030,457); 300N (see, e.g., US8,062,640); or 1D05 (see, e.g., US8,188,234). In some embodiments, the anti-PCSK9 antibody is bococizumab, evolocumab, or alirocumab. In some embodiments, the antibody recognizes an epitope on human PCSK9 comprising amino acid residues 153-155, 194, 195, 197, 237-239, 367, 369, 374-379 and/or 381 of the PCSK9 amino acid sequence of SEQ ID NO: 1. In other embodiments, the antibody recognizes an epitope on human PCSK9 comprising amino acid residues 153-155, 194, 238, 369, 374, 377, and/or 379 of the PCSK9 amino acid sequence of SEQ ID NO: 1.

In some embodiments, the invention is practiced using an antibody comprising three CDRS from a heavy chain variable region having the amino acid sequence shown in SEQ ID NO: 2 and three CDRS from a light chain variable region having the amino acid sequence shown in SEQ ID NO: 3.

In some embodiments, the invention is practiced using an antibody that specifically binds PCSK9 comprising a VH complementary determining region one (CDR1) having the amino acid sequence shown in SEQ ID NO: 4 (SYYMH), SEQ ID NO: 5 (GYTFTSY), or SEQ ID NO: 6 (GYTFTSYYMH); a VH CDR2 having the amino acid sequence shown in SEQ ID NO: 7 (EISPFGGRTNYNEKFKS) or SEQ ID NO: 8 (SPFGGR), and/or VH CDR3 having the amino acid sequence shown in SEQ ID NO: 9 (ERPLYASDL), or a variant thereof having one or more conservative amino acid substitutions in said sequences of CDR1, CDR2, and/or CDR3, wherein the variant retains essentially the same binding specificity as the CDR defined by said sequences.

Preferably, the variant comprises up to about ten amino acid substitutions and, more preferably, up to about four amino acid substitutions.

In some embodiments, the invention is practiced using an antibody comprising a VL CDR1 having the amino acid sequence shown in SEQ ID NO: 10 (RASQGISSALA), a CDR2 having the amino acid sequence shown in SEQ ID NO: 11 (SASYRYT), and/or CDR3 having the amino acid sequence shown in SEQ ID NO: 12 (QQRYSLWRT), or a variant thereof having one or more conservative amino acid substitutions in said sequences of CDR1, CDR2, and/or CDR3, wherein the variant retains essentially the same binding specificity as the CDR1 defined by said sequences. Preferably, the variant comprises up to about ten amino acid substitutions and, more preferably, up to about four amino acid substitutions.

In some embodiments, the invention is practiced using an antibody having a heavy chain sequence comprising or consisting of SEQ ID NO: 14, with or without the C-terminal lysine of SEQ ID NO: 14, and a light chain sequence comprising or consisting of SEQ ID NO: 13.

In some embodiments, the invention is practiced using an antibody having a heavy chain variable region comprising or consisting of the amino acid sequence shown in SEQ ID NO: 11 and a light chain variable region comprising or consisting of the amino acid sequence shown in SEQ ID NO: 12.

In some embodiments, the invention is practiced using an antibody that recognizes a first epitope of PCSK9 that is the same as or overlaps with a second epitope that is recognized by a monoclonal antibody selected from the group consisting of 5A10, which is produced by a hybridoma cell line deposited with the American Type Culture Collection and assigned accession number PTA-8986; 4A5, which is produced by a hybridoma cell line deposited with the American Type Culture Collection and assigned accession number PTA-8985; 6F6, which is produced by a hybridoma cell line deposited with the American Type Culture Collection and assigned accession number PTA-8984, and 7D4, which is produced by a hybridoma cell line deposited with the American Type Culture Collection and assigned accession number PTA-8983. In preferred embodiments, the invention is practiced using the PCSK9 antagonist antibody L1L3 (see, PCT/IB2009/053990, published March 18, 2010 as WO 2010/029513, and U.S. Patent Application No. 12/558312, published March 18, 2010 as US 2010/0068199).

In some embodiments, the invention is practiced using the anti-PCSK9 antagonist antibody that binds to the C-terminal CHRD of the human PCSK9 (e.g., SEQ ID NO:1). The antibodies directed to the C-terminal CHRD of the PCSK9 protein (e.g., SEQ ID NO: 1) have the properties of blocking the binding of PCSK9 to APLP2 by at least about any of 50-60%, 60-70%, 70-80%, 80-85%, 85-90%, 90%-95%, 95-97%, 97-98%, 98-99%, or more (as measured in an *in vitro* competitive binding assay such as ELISA or immunoprecipitation, see, e.g., DeVay et al. (*supra*. 2013)). Examples of this type of anti-PCSK9 antagonist antibody include, but are not limited to, 5F6 or 11B6. Accordingly, in some embodiments, the invention is practiced using an antibody comprising three CDRS from a heavy chain variable region having the amino acid sequence shown in SEQ ID NO: 15 or 19 and three CDRS from a light chain variable region having the amino acid sequence shown in SEQ ID NO: 16 or 20.

In some embodiments, the invention is practiced using the bifunctional anti-PCSK9 antagonist antibody that has any one of partial light chain sequence as listed in Table 1 and/or any one of partial heavy chain sequence as listed in Table 1.

Table 1

mAb	Light Chain	Heavy Chain
34	EIVLTQSPGTLSLSPGERATLSC**R** **ASQSVSSNYLA**WYQQKPGQAP RLLIY**GASRRAS**GIPDRFSGSGS GTDFTLTISRLEPEDFAVYYC**QQ** **FSTSPS**FGQGTKVEIK (SEQ ID NO: 22)	QVQLVQSGAEVKKPGSSVKVSCK AS**GGTFSSY**AINWVRQAPGQGLE WMGG**IIPMFGT**ANYAQKFQGRVTI TADESTSTAYMELSSLRSEDTAVY YCAR**DSVDDDWSLDWHYGMDH** WGQGTLVTVSS (SEQ ID NO: 21)
44	EIVLTQSPGTLSLSPGERATLSC**R** **ASQSVSSNSLA**WYQQKPGQAP RLLIY**GASTRAT**GIPDRFSGSGS GTDFTLTISRLEPEDFAVYYC**QQ** **YGSSPYT**FGQGTKVEIK (SEQ ID NO: 35)	QVQLVQSGAEVKKPGSSVKVSCK AS**GGTFSSY**AISWVRQAPGQGLE WMGR**IIPFFGI**ANYAQKFQGRVNIT ADKSTSTAYMELSSLRSEDTAVYY CAR**DYGIYGSTDPMAL**WGQGTLV TVSS (SEQ ID NO: 34)

In Table 1, the underlined sequences are CDR sequences according to Kabat and in bold according to Chothia.

In some embodiments, the invention also provides CDR portions of the bifunctional antibodies to PCSK9 (including Chothia, Kabat CDRs, and CDR contact regions). Determination of CDR regions is well within the skill of the art. It is understood that in some embodiments, CDRs can be a combination of the Kabat and Chothia CDR (also termed "combined CRs" or "extended CDRs"). In some embodiments, the CDRs are the Kabat CDRs. In other embodiments, the CDRs are the Chothia CDRs. In other words, in embodiments with more than one CDR, the CDRs may be any of Kabat, Chothia, combination CDRs, or combinations thereof. Table 2 provides examples of CDR sequences provided herein.

Table 2

Heavy Chain			
mAb	**CDRH1**	**CDRH2**	**CDRH3**
34	SYAIN (SEQ ID NO: 23) (Kabat); GGTFSSY (SEQ ID NO: 24) (Chothia); GGTFSSYAIN (SEQ ID NO: 25) (extended)	GIIPMFGTANYAQKFQG (SEQ ID NO: 26) (Kabat) IPMFGT (SEQ ID NO: 27) (Chothia)	DSVDDDWSLDW HYGMDH (SEQ ID NO: 28)
44	SYAIS (SEQ ID NO: 36) (Kabat); GGTFSSY (SEQ ID NO: 24) (Chothia); GGTFSSYAIS (SEQ ID NO: 37) (extended)	RIIPFFGIANYAQKFQG (SEQ ID NO: 38) (Kabat) IPFFGI (SEQ ID NO: 39) (Chothia)	DYGIYGSTDPMA L (SEQ ID NO: 40)

Light Chain			
mAb	**CDRL1**	**CDRL2**	**CDRL3**
34	RASQSVSSNSLA (SEQ ID NO: 29)	GASRRAS (SEQ ID NO: 30)	QQFSTSPS (SEQ ID NO: 31)
44	RASQSVSSNYLA (SEQ ID NO: 41)	GASTRAT (SEQ ID NO: 42)	QQYGSSPYT (SEQ ID NO: 43)

Accordingly, in some embodiments, the bifunctional anti-PCSK9 antibody as described herein comprises: a) a heavy chain variable region (VH) comprising complementarity determining region one (CDR1), CDR2, and CDR3 of the amino acid sequence shown in SEQ ID NO: 21 or 34; and/or a light chain variable region (VL) comprising CDR1, CDR2, and CDR3 of the amino acid sequence shown in SEQ ID NO: 22 or 35. In some embodiments, the antibody or antigen binding fragment comprises: a) VH CDR1 having the amino acid sequence shown in SEQ ID NO: 23, 24, or 25**,** a VH CDR2 having the amino acid sequence shown in SEQ ID NO:26 or 27**,** and a VH CDR3 having the amino acid sequence shown in SEQ ID NO: 28**;** and/or a VL CDR1 having the amino acid sequence shown in SEQ ID NO:29**,** a VL CDR2 having the amino acid sequence shown in SEQ ID NO:30, and a VL CDR3 having the amino acid sequence shown in SEQ ID NO: 31; or b) a VH CDR1 having the amino acid sequence shown in SEQ ID NO: 36, 24, or 37**,** a VH CDR2 having the amino acid sequence shown in SEQ ID NO:38 or 39**,** and a VH CDR3 having the amino acid sequence shown in SEQ ID NO: 40; and/or a VL CDR1 having the amino acid sequence shown in SEQ ID NO:41**,** a VL CDR2 having the amino acid sequence shown in SEQ ID NO:42, and a VL CDR3 having the amino acid sequence shown in SEQ ID NO: 43.

In some embodiments, the bifunctional anti-PCSK9 antibody as described herein comprises polynucleotides encoding antibody mAb34 and mAb44, and vectors and host cells comprising the polynucleotide. In some embodiments, the antibody comprises a nucleic acid comprising: a) the nucleic acid sequence of SEQ ID NO: 32, and/or the nucleic acid sequence of SEQ ID NO: 33; or b) the nucleic acid sequence of SEQ ID NO: 44;and/or

the nucleic acid sequence of SEQ ID NO: 45.

In some embodiments, the bifunctional anti-PCSK9 antibody as described herein competes with the bifunctional anti-PCSK9 antibody mAb34 and/or mAb44 as described herein.

In some embodiments, a variant of the anti-PCSK9 antagonist antibody as described herein comprises up to about twenty amino acid substitutions and more preferably, up to about eight amino acid substitutions. Preferably, the antibody further comprises an immunologically inert constant region, and/or the antibody has an isotype that is selected from the group consisting of IgG_2, IgG_4, $IgG_{2\Delta a}$, $IgG_{4\Delta b}$, $IgG_{4\Delta c}$, IgG_4 S228P, $IgG_{4\Delta b}$ S228P and $IgG_{4\Delta c}$ S228P. In another preferred embodiment, the constant region is aglycosylated Fc.

The antibodies useful in the present invention can encompass monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, Fab', F(ab')2, Fv, Fc, etc.), chimeric antibodies, bispecific antibodies, heteroconjugate antibodies, single chain (ScFv), mutants thereof, fusion proteins comprising an antibody portion (e.g., a domain antibody), human antibodies, humanized antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity, including glycosylation variants of antibodies, amino acid sequence variants of antibodies, and covalently modified antibodies. The antibodies may be murine, rat, human, or any other origin (including chimeric or humanized antibodies).

In some embodiments, the PCSK9 antagonist antibody is a monoclonal antibody. The PCSK9 antagonist antibody can also be humanized. In other embodiments, the antibody is human.

The anti-PCSK9 bispecific antibodies as described herein can be prepared using methods known in the art (see, e.g., Suresh et al., Methods in Enzymology 121:210, 1986). Traditionally, the recombinant production of bispecific antibodies was based on the coexpression of two immunoglobulin heavy chain-light chain pairs, with the two heavy chains having different specificities (Millstein and Cuello, Nature 305, 537-539, 1983).

According to one approach to making bispecific antibodies, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant region sequences. The fusion preferably is with an immunoglobulin heavy chain constant region, comprising at least part of the hinge, CH2

and CH3 regions. It is preferred to have the first heavy chain constant region (CH1), containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are cotransfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In one approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. This asymmetric structure, with an immunoglobulin light chain in only one half of the bispecific molecule, facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations. This approach is described in PCT Publication No. WO 94/04690.

In another approach, the bispecific antibodies are composed of amino acid modification in the first hinge region in one arm, and the substituted/replaced amino acid in the first hinge region has an opposite charge to the corresponding amino acid in the second hinge region in another arm. This approach is described in International Patent Application No. PCT/US2011/036419 (WO2011/143545).

In another approach, the bispecific antibodies can be generated using a glutamine-containing peptide tag engineered to the antibody directed to an epitope (e.g., Trop-2) in one arm and another peptide tag (e.g., a Lys-containing peptide tag or a reactive endogenous Lys) engineered to a second antibody directed to a second epitope in another arm in the presence of transglutaminase. This approach is described in International Patent Application No. PCT/IB2011/054899 (WO2012/059882).

In some embodiments, the antibody comprises a modified constant region, such as a constant region that is immunologically inert, that is, having a reduced potential for provoking an immune response. In some embodiments, the constant region is modified

as described in Eur. J. Immunol., 1999, 29:2613-2624; PCT Publ. No. WO99/58572; and/or UK Patent Application No. 9809951.8. The Fc can be human IgG_2 or human IgG_4. The Fc can be human IgG_2 containing the mutation A330P331 to S330S331 ($IgG_{2\Delta a}$), in which the amino acid residues are numbered with reference to the wild type IgG2 sequence. Eur. J. Immunol., 1999, 29:2613-2624. In some embodiments, the antibody comprises a constant region of IgG_4 comprising the following mutations (Armour et al., 2003, Molecular Immunology 40 585-593): E233F234L235 to P233V234A235 ($IgG_{4\Delta c}$), in which the numbering is with reference to wild type IgG4. In yet another embodiment, the Fc is human IgG_4 E233F234L235 to P233V234A235 with deletion G236 ($IgG_{4\Delta b}$). In another embodiment the Fc is any human IgG_4 Fc (IgG_4, $IgG_{4\Delta b}$ or $IgG_{4\Delta c}$) containing hinge stabilizing mutation S228 to P228 (Aalberse et al., 2002, Immunology 105, 9-19). In another embodiment, the Fc can be aglycosylated Fc.

In some embodiments, the constant region is aglycosylated by mutating the oligosaccharide attachment residue (such as Asn297) and/or flanking residues that are part of the glycosylation recognition sequence in the constant region. In some embodiments, the constant region is aglycosylated for N-linked glycosylation enzymatically. The constant region may be aglycosylated for N-linked glycosylation enzymatically or by expression in a glycosylation deficient host cell.

The anti-PCSK9 antagonist antibodies as described herein can also be used in conjunction with other PCSK9 antagonists or PCSK9 receptor antagonists. For example, one or more of the following PCSK9 antagonists may be used: an antisense molecule directed to a PCSK9 (including an anti-sense molecule directed to a nucleic acid encoding PCSK9), a PCSK9 inhibitory compound, and a PCSK9 structural analog. A PCSK9 antagonist antibody can also be used in conjunction with other agents that serve to enhance and/or complement the effectiveness of the agents.

Kits

The invention also provides kits or an article of manufacture comprising a combination of at least two anti-PCSK9 antagonist antibodies, or an anti-PCSK9 bispecific or bifunctional antibody, and instructions for use. Accordingly, in some embodiments, provided is a kit or an article of manufacture, comprising a container, a composition within the container comprising an anti-PCSK9 antagonist antibody (e.g., bispecific or bifunctional antibody) or a combination of anti-PCSK9 antagonist

antibodies, and a package insert containing instructions to administer a therapeutically effective amount of the anti-PCSK9 antagonist antibodies for reducing a level of LDL-cholesterol in blood of a subject in need thereof.

Kits of the invention include one or more containers comprising at least two different anti-PCSK9 antagonist antibodies, or an anti-PCSK9 bispecific or bifunctional antibody, described herein and instructions for use in accordance with the methods of the invention described herein. Generally, these instructions comprise a description of administration of the anti-PCSK0 antagonist antibodies for the above described therapeutic treatments. In some embodiments, kits are provided for producing a single-dose administration unit. In certain embodiments, the kit can contain both a first container having a dried protein and a second container having an aqueous formulation. In certain embodiments, kits containing single and multi-chambered pre-filled syringes (e.g., liquid syringes and lyosyringes) are included.

The instructions relating to the use of a combination of anti-PCSK9 antagonist antibodies, or an anti-PCSK9 bispecific antibody, generally include information as to dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (e.g., multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (e.g., a paper sheet included in the kit), but machine-readable instructions (e.g., instructions carried on a magnetic or optical storage disk) are also acceptable.

The kits of this invention are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging (e.g., sealed Mylar or plastic bags), and the like. Also contemplated are packages for use in combination with a specific device, such as an inhaler, nasal administration device (e.g., an atomizer) or an infusion device such as a minipump. A kit may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The container may also have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is a combination of at least two anti-PCSK9 antagonist antibodies, or an anti-PCSK9 bispecific antibody. The container may further comprise a second pharmaceutically active agent.

Kits may optionally provide additional components such as buffers and interpretive information. Normally, the kit comprises a container and a label or package insert(s) on or associated with the container.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

Examples

Example 1: APLP2 and LDLR mediate PCSK9 internalization *in vitro*

This example illustrates PCSK9 endocytosis can be mediated by either LDLR or APLP2.

To interrogate the roles of LDLR and APP family members in PCSK9 endocytosis, monoclonal antibody J16 that specifically inhibits PCSK9 binding to LDLR, and 5F6, a monoclonal antibody that specifically blocks PCSK9 binding to APLP2 and its closely related family member APP, were used in the experiments. More specifically, recombinant human PCSK9 conjugated with Alexa Fluor 488 (PCSK9-488) was premixed with the following combinations of antibodies: (1) mouse isotype control antibody (mIC) + human isotype control antibody (hIC), (2) J16 + mIC, (3) hIC + 5F6, or (4) J16 + 5F6. All antibodies were used at a molar excess to saturate PCSK9-488, and the complexes were monitored for internalization in HepG2 cells or mouse primary hepatocytes.

Methods

Protein Purification

PCSK9, isotype control antibodies (mIC and hIC), and J16 were purified as described in Liang et al. (J. Pharmacol. Exp. Ther., 340(2):228-36 (2012)). 5F6 and APLP2-ECD were purified as described in DeVay et al. (J. Biol. Chem., 288(15):10805-18 (2013)). 12E3 was identified in an anti-APLP2-ECD antibody screen from hybridoma cultures according to methods described by Liang et al. (*supra*. (2012)) and purified using monoclonal antibody select protein A beads (GE Healthcare, Pittsburgh, PA). LDLR-ECD-6xHis was cloned into pAPLP2ECD (see, e.g., DeVay et al. (*supra*. 2013))

using the Nhe1/Age1 sites, and purified using standard techniques. PCSK9 was labeled with Alexa Fluor 488, according to manufacturer's instructions (Life Technologies, Carlsbad, CA) with an average of 2 dye molecules per PCSK9 molecule.

Cell Culture and siRNA knockdown

HepG2 cells were cultured in DMEM supplemented with 10%FBS, L-glutamine, and pen-strep. For siRNA knockdown, HepG2 cells were transfected using RNAiMax lipofectamine reagent and Ambion silencer select siRNA oligos (Life Technologies, Carlsbad, CA), according to manufacturer's reverse transcription protocol.

Internalization assays and immunofluorescence

Media was exchanged for DMEM with 10% LPDS at least 16 hours prior to assays. 7.5 μg/ml PCSK9-488 was premixed with 25 μg/ml of antibodies or Fab fragments and added to HepG2 cells or primary mouse hepatocytes plated on uncoated or collagen coated glass coverslips, respectively. Fab fragments were produced by digesting IgG molecules with Pierce mouse IgG1 Fab Digestion Kit (Thermo-Fisher Scientific, Rockford, IL). LDL (Life Technologies, Carlsbad, CA) was added at 2.5 μg/ml in indicated assays. After 3 hour incubation at 37 degrees, cells were fixed with 4% formaldehyde, permeabilized, and blocked. 2μg/ml goat anti-mouse IgG1 647 secondary antibody, goat anti-Human 546, donkey anti-mouse 647, or donkey anti-goat 546 secondary antibodies (Life Technologies, Carlsbad, CA) were used to detect internalized antibodies. siRNA internalization assays were performed identically 72 hours following transfection.

For APLP2 internalization, HepG2 cells were incubated with anti-APLP2 monoclonal antibody (R&D systems, Minneapolis, MN) for 30 minutes at 4 degrees. Cells were washed twice with cold media, shifted to warm media, and incubated at 37 degrees for 15 minutes. Cells were then fixed, permeabilized, blocked, and stained for internalized mouse antibody (goat anti-mouse IgG2b 488; Life Technologies) before being subjected to confocal microscopy.

Confocal Microscopy

Microscopy images from z stacks with 0.5 μm increments were collected using a 63x, 1.4NA objective lens at room temperature on a Leica SP3 laser scanning confocal microscope (Leica, Buffalo Grove, IL). All images shown are projections of optical sections. Data analysis was performed using Leica LAS AF software. Internalization was quantified as intensity of fluorescence signal per cell, from at least 45 cells for each of 3 experiments. Average intensity was normalized against negative control or IC, as indicated in figure legends.

CoImmunoprecipitation:

CoIPs were performed similarly as described in DeVay et al. (*supra*. 2013). Briefly, cells were removed from plates directly using coIP buffer (20mM Hepes, 7.4 or 6.0, 20mM CaCl2, 150mM NaCl, 0.1% Triton x-100) or by Accutase treatment followed by resuspension in coIP buffer. Cells were passed through a 27g needle twice and pelleted for 15 minutes at 16,100xg. Lysates were precleared with mAb select protein A beads (GE Healthcare), and then incubated with 10μg/ml anti-LDLR, anti-APLP2, J16, or IC antibodies and mAb select beads overnight. 10μg/ml of 12E3 or 5F6 Fab fragments were added to IPs where indicated. Recombinant ApoB (Sigma) was added at indicated concentrations to lysates prior to preclearing step. Beads were washed 3x with coIP buffer and complexes were eluted with sample buffer before running on a 4-12% Bis-Tris gel and transferred to nitrocellulose membranes for western blot analysis. Recombinant coIPs were performed using pH 6.0 coIP buffer with 5μg/ml ApoB, 5μg/ml LDLR-ECD, and 5μg/ml PCSK9. After 1 hour incubation at room temperature, Protein A beads saturated with anti-LDLR were added for 1 hour before wash and elution steps.

PCSK9 sensitivity assay

72 hours after siRNA transfection, 0, 20, 50, or 100μg/ml PCSK9 was added to HepG2 cells that had been incubated overnight in 10%LPDS media. After 4 hours, lysates were harvested and run onto a 4-12% Bis-Tris gel, and transferred to nitrocellulose membranes for western blot analysis.

Western blot analysis

Nitrocellulose membranes were blocked with Odyssey blocking buffer (Licor Biotechnologies), incubated with primary antibodies (LDLR (goat anti-Human; R&D

Systems), TFNR (mouse anti-Human; Life Technologies), PCSK9 (sheep anti-Human; R&D Systems), APLP2 (goat anti-Human; R&D Systems), APP (goat anti-mouse; Life Technologies), ApoER2 (Abcam)) for at least 1 hour, washed, and incubated with secondary antibodies (donkey anti-mouse 680, donkey anti-goat 800, goat anti-rabbit 680, goat anti-mouse 800 (Licor), or donkey anti-sheep 680 (Life Technologies)) before imaging on Licor Odyssey imaging system. Integrated intensity signals were measured using Odyssey software and normalized against loading controls (as indicated in figure legends).

ELISA

ELISA of PCSK9 binding to APLP2-ECD or LDLR-ECD was performed as described in DeVay et al. (*supra*. 2013), but using 12E3, J16, or RD-LDLR (R&D Systems) at concentrations indicated in the figures. LDLR/APLP2 ELISA was performed by coating MaxiSorp™ plates (Thermo-Fisher) with 5 μg/ml LDLR, blocking, and binding APLP2-ECD at the indicated concentrations. PCSK9 was added at indicated concentrations with 5 μg/ml APLP2-ECD. APLP2-ECD was detected using mouse anti-human APLP2 and goat anti-Mouse HRP antibody (R&D Systems), followed by TMB (Thermo-Fisher) according to manufacturer's instructions.

Statistical Analyses

Paired, two-tailed Student's t-test of at least 3 experiments was performed to assess statistical significance in all experiments.

Results

Consistent with previous report that PCSK9 can be internalized in the absence of direct interactions with LDLR, APP, or APLP2 (see DeVay et al., *supra* (2013)), PCSK9-488 complexed with J16 or 5F6 alone was endocytosed in both HepG2 cells and primary mouse hepatocytes as efficiently as PCSK9-488 combined with IC antibodies (Figures 1A and 1B). However, PCSK9-488 bound by both 5F6 and J16 was significantly blocked for internalization and barely visible above background (Figures 1A and B). Similarly, the combination of 5F6 and J16 Fab fragments inhibited PCSK9 endocytosis in HepG2 cells, while either Fab alone did not have an appreciable effect

(Figure 1C). This rules out the possibility that simultaneous binding of two full IgG molecules sterically hindered PCSK9 internalization.

The PCSK9 internalization in HepG2 cells, whose expression of APLP2 or APP was lowered by 89% or 87% respectively by siRNA, was then tested (Figure 1E). As expected, loss of either APP or APLP2 did not alter PCSK9 endocytosis relative to Negative control (Neg) cells, and PCSK9-488 internalization was significantly blocked when complexed with both J16 and 5F6 under all conditions (Figures 2A, 2B, 2C, and 2D). Interestingly, J16 significantly inhibited PCSK9-488 internalization in *APLP2*, but not *APP* or Neg, siRNA treated cells (Figures 2A, 2B, 2C, and 2D). Furthermore, PCSK9-488/J16 complex internalization in HepG2 cells was inhibited in the presence of 12E3 (Figures 2E and 2F), an anti-APLP2 antibody that blocks PCSK9 binding (Supp. Fig. 1B; measured at pH 6.0). These results suggest that APLP2, and not APP, mediates PCSK9/J16 complex internalization.

During these studies, it was also observed that PCSK9-488/5F6 complex endocytosis was attenuated in *APP* knockdown cells (29.35±3.29% inhibition; Figures 2C and 2D). This finding may be attributed to the transcriptional suppression and corresponding diminished LDLR protein levels in *APP* knockdown cells (Figure 1E). Accordingly, without being bound by theory, it is hypothesized that in the absence of APLP2 binding, PCSK9 relies on LDLR binding for endocytosis, and that as a result, PCSK9/5F6 complex internalization correlates strongly with LDLR levels.

To determine whether LDLR binding is indeed required for APLP2 independent endocytosis, an LDLR antibody and RD-LDLR which inhibits LDLR interactions with PCSK9 were utilized (Figure 1F). Importantly, RD-LDLR blocked internalization of PCSK9-488/5F6 complexes, but not PCSK9-488 alone (Figures 2G and H). Together, these data indicate that LDLR and APLP2 are each sufficient to mediate PCSK9 endocytosis, and that PCSK9 internalization can be blocked by inhibiting both of these interactions.

Example 2: PCSK9 Interacts Specifically with APLP2 at Neutral pH

This example illustrates PCSK9/APLP2 interaction at neutral pH.

It was previously reported that PCSK9/APLP2 interactions by co-immunoprecipitation (coIP) was not detected at neutral pH, a finding that is inconsistent with APLP2 playing a direct role in PCSK9 internalization (DeVay et al., *supra* (2013)).

Importantly, cell lysates for that study were prepared by first using Accutase to remove adherent HepG2 cells so the same cell population could be divided into varying pH buffers. Here, a weak APLP2 signal was detected in PCSK9/J16 coIPs from HepG2 cells directly lysed with IP buffer, but not in cells that were first treated with Accutase (Figure 3H).

To confirm the specificity of this interaction, PCSK9/J16 coIPs were performed in the presence of 5F6 Fab or 12E3 Fab, which reduced the intensity of the APLP2 band by 45.24±3.45% and 52.11±6.51%, respectively (Figures 3A and 3B). However, a direct interaction between PCSK9 and APLP2 using recombinant proteins at neutral pH were not detected, possibly due to lack of other co-factors.

Example 3: LDLR Facilitates PCSK9 Interactions with APLP2 at Neutral pH

This example illustrates the regulatory role of LDLR in APLP2-mediated PCSK9 internalization.

During the experiments, an interaction between LDLR and APLP2 at neutral pH that is Accutase sensitive was observed (Figure 3H). This interaction is direct, since recombinant APLP2-ECD bound LDLR-ECD by ELISA at pH7.4. Furthermore, the presence of PCSK9 is not required for LDLR to bind APLP2 by ELISA, (Figures 3I and 3J), and the presence of 5F6 Fab and/or J16 Fab does not affect the coIPed complex in HepG2 cell lysates at pH 7.4 (Figure 3K). Thus, contrary to pH 6.0, PCSK9 does not facilitate APLP2/LDLR interactions at neutral pH. Notably, these interactions may be weak and transient since there was no detectable LDLR in J16/PCSK9 coIPs at neutral pH (Figure 3C, Neg siRNA).

Given these data and the previous findings, it was hypothesized that in the absence of a direct interaction, LDLR facilitates APLP2-mediated PCSK9 internalization. To address the mechanism by which this occurs, APLP2 internalization and PCSK9 interactions in *LDLR* siRNA treated cells was observed. APLP2 was internalized efficiently in *LDLR* knockdown cells (Figures 4E and 4F), but the APLP2/PCSK9 interaction in these cells was significantly reduced (Figures 3C and 3D). Indeed, APLP2 levels normalized to the amount of immunoprecipitated PCSK9 were 41.26±5.38% lower in coIPs from *LDLR* knockdown lysates relative to negative control lysates (Figures 3C and 3D). It is possible that LDLR induces a conformational change in APLP2 that allows PCSK9 binding, or alternatively that LDLR delivers a third, as of

yet unknown binding partner to mediate this interaction. Importantly, these data shed light on the required regulatory role of LDLR in APLP2-mediated PCSK9 internalization, and that these two proteins work together to regulate PCSK9.

To test whether LDLR and/or APLP2 are generally required for PCSK9 function, we examined the ability of PCSK9 to degrade ApoER2, another known target of PCSK9, in *LDLR* or *APLP2* knockdown cells. As previously reported (see, e.g. DeVay et al., *supra* (2013)), at saturating concentrations of PCSK9, LDLR degradation is significantly attenuated in *APLP2* knockdown cells (Figures 3E and 3F). Importantly, PCSK9-mediated ApoER2 degradation was inhibited in both *LDLR* and *APLP2* knockdown cells over a series of PCSK9 concentrations (Figures 3E and 3G). These results suggest that under the conditions tested, LDLR and APLP2 are each required for efficient PCSK9-mediated degradation of ApoER2.

Example 4: ApoB/LDL Can Indirectly Facilitate PCSK9 Internalization

This example illustrates the role of ApoB and LDL in facilitating PCSK9 internalization.

Thus far, studies have been limited to cell cultures lacking lipoproteins. Intriguingly, recent studies have shown that 25-40% of plasma PCSK9 is bound to LDL, and that LDL plays a role in PCSK9 clearance (see, e.g., Fan et al., Biochemistry, 47:1631-1639 (2008); Tavori et al., Circulation, 127:2403-2413 (2013); Kosenko et al., J. Biol. Chem., 288:8279-8288 (2013)). One report showed that physiological concentrations of LDL (greater than 100μg/ml) inhibit PCSK9 internalization and subsequent LDLR degradation. Notably, the authors of this study also found that PCSK9 does not affect LDL/LDLR interactions (Kosenko et al., *supra* (2013)), suggesting that LDL could bridge PCSK9 to LDLR. The effect of LDL on PCSK9 trafficking was therefore tested. Addition of 2.5 μg/ml LDL allowed PCSK9/J16 complex internalization in *APLP2* knockdown cells, indicating that LDL can facilitate PCSK9 internalization in the absence of both LDLR and APLP2 direct interactions (Figures 4A and 4B).

Interestingly, 5F6 inhibited LDL-mediated PCSK9 internalization, which indicates the APLP2 binding epitope on PCSK9 is also important for interacting with LDL (Figures 4A and 4B). In further support of this, recombinant ApoB added to HepG2 cell lysates was coIPed with PCSK9/J16 and competed with APLP2 in a dose-dependent manner

(Figure 4C). The coIPed ApoB was complexed with LDLR, confirming that ApoB interacts with PCSK9 and LDLR via unique epitopes (Figure 4C).

ApoB similarly competed with APLP2 for PCSK9 binding at pH 6.0, while its interaction with LDLR was expectedly lost (Figure 4C). It was therefore hypothesized that PCSK9 could bridge LDLR to ApoB/LDL at endosomal pH. Consistent with this idea, PCSK9 enhanced ApoB interactions with LDLR-ECD by coIP in a recombinant system at pH 6.0. Moreover, this interaction was reversible with 5F6 Fab, indicating that at endosomal pH, ApoB interacts directly with the APLP2 binding epitope on PCSK9 (Figure 4D). Since LDLR is transported to lysosomes when it cannot release LDL (Strom et al., Biochem. Biophys. Res. Commun., 408:642-646 (2011)), PCSK9-mediated LDL reassociation with LDLR could provide an APLP2 independent means by which PCSK9 degrades LDLR.

Example 5: PCSK9 internalization *in vivo*

This example illustrates multiple PCSK9 internalization mechanisms through APLP2 or LDLR

To understand whether the findings described herein are physiologically relevant, *Pcsk9* $^{-/-}$ mice were injected with PCSK9-488 pre-mixed with hIC+mIC, J16+mIC, hIC+5F6 or J16+5F6 to monitor trafficking and internalization of PCSK9/antibody complexes *in vivo*.

Methods

Mouse Studies

About 6-10 week old PCSK9$^{-/-}$ or wild type *C57BL/6* mice were licensed and purchased from Jackson Laboratories. Mice were bred at the facility or acclimated for at least 10 days prior to experiments. Animals were housed under ambient conditions with access to water and standard chow. Primary hepatocytes were isolated from male *C57Bl6* mice. After mice were anesthetized, livers were perfused through the hepatic portal vein with Hepatocyte Perfusion Buffer (Life Technologies) until blood cleared through the inferior vena cava. Livers were then perfused with 25mL of collagenase solution (1mg/mL collagenase; Sigma) in Hepatocyte Wash Buffer (Life Technologies). Livers were extracted and filtered through a 70µm filter. Cells were washed twice and resuspended in 20% Histodenz (Sigma) to collect hepatocytes. After centrifugation,

hepatocytes were resuspended in Hepatocyte Wash Buffer with 5%FBS and 1%P/S. ~3x10^5 cells were seeded onto collagen-coated coverslips BD Bioscience) in 6-well plates and left for 1-2 hours at 37°C in 5% CO_2. Media was replaced with SFM (Life Technologies) with 1% P/S, 1% L-glutamine. Cells were used within 24 hours.

For *in vivo* internalization, IC, J16, or 5F6 (15mg/kg) alone or premixed with PCSK9 (3mg/kg) were injected by tail vein into age and gender matched Pcsk9 $^{-/-}$ or wild type mice. After 1 hour, mice were anesthetized and livers were perfused through the hepatic portal vein with PBS until the blood cleared, harvested, and fixed in 4%PFA. After incubating in 40% sucrose overnight, livers were embedded in OCT and flash frozen. 12μm sections were prepared by cryostat sectioning (CM1850, Leica).

Immunofluorescence of liver sections

Liver sections were incubated in blocking buffer (0.3% TX-100, 300mM Glycine, 2mg/ml BSA, 10% goat or donkey serum in PBS-T) for at least 1 hour, followed by incubation overnight at 4 degrees with 3μg/ml anti-Alexa-488 antibody (Life Technologies) or LDLR antibody (R&D Systems) diluted in incubation buffer (0.3% Triton x-100, 2% BSA, 1% serum, PBS-T). All secondary antibodies were from Life Technologies and diluted 1:1000. Sections were mounted using Prolong Gold with DAPI (Life Technologies) and imaged by confocal microscopy.

Results

PCSK9-488 was readily taken into hepatocytes when coinjected with ICs, 5F6, or J16 alone. However, PCSK9-488 was not internalized and remained in the sinusoids when both epitopes that are required for endocytosis were blocked by J16 and 5F6 (Figure 5). Similarly, J16 complexed with PCSK9 injected in wild type *C57BL/6* mice was internalized in hepatocytes 1 hr after injection, while no visible internalized human antibody was observed in J16 alone, hIC alone, or hIC complexed with PCSK9 (Figure 4G).

As shown in Figure 5, liver LDLR was almost completely degraded in PCSK9-488+IC treated mice while J16 or J16 combined with 5F6 inhibited PCSK9-mediated LDLR degradation. Surprisingly, 5F6 alone only partially attenuated LDLR degradation. Incomplete protection may have been due either to insufficient affinity of 5F6 for PCSK9

or PCSK9 merging with the intracellular route and mediating degradation of LDLR independently of the endocytic pathways. Alternatively, since 5F6 bound PCSK9 binds LDLR, the complex could shift LDLR towards lysosomes by sterically inhibiting it from entering the sorting endosome.

These findings show that PCSK9 can be internalized in hepatic cells directly by APLP2 or LDLR, or indirectly through interactions with ApoB/LDL. Interestingly, both of these routes utilize either the LDLR or APLP2 (ApoB) binding epitopes on PCSK9. APLP2 and LDLR are each sufficient, while at least one of these partners is necessary, for mediating PCSK9 endocytosis in the absence of lipoproteins. Moreover, LDLR is required for APLP2-mediated PCSK9 internalization, likely by modulation of PCSK9/APLP2 interactions.

In the presence of LDL, ApoB can simultaneously bind LDLR and PCSK9, and thereby indirectly facilitate PCSK9 internalization by bridging it LDLR. Intriguingly, LDL may also dictate an alternate lysosomal trafficking route for LDLR degradation that bypasses APLP2. PCSK9/LDL complex uptake *in vivo* is highly dependent on the relative molar ratio of PCSK9 to LDL. Under high, physiological LDL:PCSK9 ratios, PCSK9 bound LDL particles would be in competition with free LDL particles, which in turn would limit the internalization efficiency of PCSK9. Thus, it was hypothesized that PCSK9 normally enters cells through LDLR and/or APLP2 binding. Altogether, this study defines distinct, conserved routes for internalization of PCSK9, and indicates that PCSK9 may carry out its functions through a multitude of mechanisms.

Example 6: Combination Treatment with anti-PCSK9 Antagonist Antibody J16 and a Second anti-PCSK9 Antagonist 5F6 is Effective for Reducing Serum Cholesterol and LDL Cholesterol Levels

This example illustrates efficacy of the combination treatment of two anti-PCSK9 antagonist antibodies in reducing serum cholesterol and LDL cholesterol levels in animal models.

In cynomolgus monkeys on a normal diet, J16 and 5F6 are co-administered as a single IV dose at 0.1, 1, 3, 5, and 10 mg/kg (n=4/group). Administration of these doses causes a reduction in LDL-C ranging from at least about 50% to about 90% between day 2 and day 30. Both the magnitude and duration of the LDL-C lowering effect of J16

and 5F6 correlate with drug exposure. HDL-C levels are not affected by this combination treatment in all dose groups.

PK studies are also conducted by a single bolus i.v. injection of 0.1, 1.0, 3.0, 10.0 and 100.0 mg/kg of 1) both J16 and 5F6 and 2) J16 alone in cynomolgus monkeys, and the total antibody concentration is measured. The estimated β-phase half-life for J16 increases by at least 1 fold in the presence of 5F6 in comparison to a single bolus i.v. injection of J16 without 5F6 of equivalent dosage. The estimated β-phase half-life for J16 is also the same in comparison to a single bolus i.v. injection of J16 having the combined dosage of J16 and 5F6. Thus, in cynomolgus monkeys, co-administration of both J16 and 5F6 demonstrate an increase in half-life of J16.

Example 7: Biosensor Characterization of Anti-PCSK9 Bifunctional Antibodies mA34 and mAb44

This example illustrates that bifunctional anti-PCSK9 antibodies mAb34 and mAb44 block both hPCSK9 binding to human LDLR and human APLP2.

Methods

Binding Affinity for hPCSK9

The binding affinities of hPCSK9 towards mAb34 and mAb44 were determined at 25°C and in a running buffer of PBS pH 7.4 + 0.01% Tween 20 using a ProteOn equipped with neutravidin (NLC) chip (BioRad, Hercules, CA). A capture-based one-shot kinetic analysis was employed (see Nahshol et al, Anal. Biochem. 383(1):52-60 (2008)). To prepare the capture surface, an anti-hFc IgG (Cappel) was biotinylated using a 10:1 molar ratio of linker (EZ-Link™ Sulfo-NHS-LC-LC-Biotin, Thermo Scientific, catalog number 21338) and the purified product was captured to a level of approximately 2000 RU along the Analyte channels. Anti-PCSK9 mAbs 34 and 44 were captured at 5 µg/ml along separate Ligand channels for two or three min to levels of approximately 300 RU. hPCSK9 was injected at 100, 20, 4, 0.8, 0.16, and 0 nM in a one-shot kinetic manner along the Analyte channels for three min, allowing a 15 min dissociation phase. Capture surfaces were regenerated with 75 mM phosphoric acid. The data were processed and analyzed in the ProteOn Manager software as follows; the binding data were double-referenced (see Myszka, J. Mol. Recognit. 12(5): 279-84 (1999)) by subtracting the responses from the interspots (representing the naked

capture surface) and subtracting the responses from the in-line buffer injection. The double-referenced data were fit globally to a simple Langmuir model and the affinity (or K_D value) determined from the quotient of the kinetic rate constants, where $K_D = k_d/k_a$.

Blockade of hLDLR

The ability of anti-PCSK9 mAbs (34 and 44) to block hPCSK9 binding to hLDLR, hAPLP2, and J16 was tested using a Biacore 2000 equipped with streptavidin (SA) chips. Interaction analysis was performed at 25°C in a running buffer of 25 mM tris/bis pH 6.0, 150 mM NaCl, 1 mM CaCl2, 0.05% Tween20, 1 g/l BSA; PCSK9's interaction with hLDLR was also studied in a running buffer of HBS pH 7.4, 2 mM CaCl2, 0.05% Tween20. The reaction surfaces for these experiments were prepared by capturing on separate flow cells biotinylated receptors, hLDLR-ECD-His and hAPLP2-ECD-His, where ECD-His refers to their expression as monomeric extracellular domain (ECD) with terminal His-tag. Flow cell 1 of each chip was left unmodified to provide a reference surface. Blocking experiments were performed in two different assay formats; coinject or premix. In the coinject strategy, a one-min injection of 100 nM (or in some experiments, 300 nM) hPCSK9 was followed immediately by a one-min injection of mAb at approximately 20 µg/ml, varying the identity of the mAb per cycle (e.g., buffer, mAb34, mAb44, J16, or a control mAb that was chosen because it exhibited a sandwiching signal with both hLDLR and J16). In the premix strategy, 100 nM (or in some experiments, 300 nM) hPCSK9 was premixed with a large molar excess of each mAb (typically 1 µM binding sites) and these hPCSK9/mAb mixtures were injected over the surfaces. Their binding responses were compared to those of hPCSK9 alone, which was injected in triplicate binding cycles to confirm that the assay was reproducible. A positive control (mAb36) that did NOT block hPCSK9 binding to mAb34, mAb44, or J16 was also used as both an analyte and surface in the premix assay format to confirm that the mAb analytes were active. Regardless of the assay format used, the surfaces were regenerated with a 2:1 v/v blend of Pierce IgG elution buffer (pH 2.8)/4 M NaCl.

Results

The kinetic analysis of hPCSK9 binding to immobilized mAb34 and mAb44 reveals that the two mAbs have similar K_D values of about 2 nM at 25°C, with mAb34 showing a slightly slower dissociation rate constant than mAb44. See Figure 6.

Further, the data from a Biacore experiment in which mAb34, mAb44, J16, and a control mAb (chosen because it sandwiches with both hLDLR and J16) were tested for their ability to block hPCSK9 binding to immobilized hLDLR and immobilized J16. The results show that mAbs 34, 44, and J16 blocked hPCSK9 binding to immobilized hLDLR. MAb34 did not block binding to immobilized J16, whereas mAb44 partially blocked binding to immobilized J16. These results were consistent across two different assay formats, namely coinject and premix, where the order of addition was reversed. In the coinject assay format, hLDLR-captured hPCSK9 was tested for binding to a mAb analyte, whereas in the premix assay format, a preformed solution phase hPCSK9/mAb complex was tested for binding to immobilized hLDLR. See Figure 7.

Further, the Biacore results from a similar blocking experiment performed on immobilized hAPLP2 and a control mAb36, chosen because it does not block mAb34, mAb44, and J16 and thereore provided a positive control to confirm that all mAb analytes were active. The results show that mAb34 and mAb44 both block PCSK9 binding to hAPLP2, whereas J16 does not block PCSK9 binding to hAPLP2. See Figure 8. Within the same experiment, the ability of mAb34, mAb44, and J16 to block PCSK9 binding to immobilized hLDLR, hAPLP2, and J16 was also compared. Taken together, the results show that mAb34 and mAb44 both block hPCSK9 binding to hLDLR and hAPLP2. MAb34 does not block hPCSK9 binding to J16, whereas mAb44 partially blocks hPCSK9 binding to J16. J16 blocks hPCSK9 binding to hLDLR but does not block hPCSK9 binding to hAPLP2. See Figure 9.

Although the disclosed teachings have been described with reference to various applications, methods, kits, and compositions, it will be appreciated that various changes and modifications can be made without departing from the teachings herein and the claimed invention below. The foregoing examples are provided to better illustrate the disclosed teachings and are not intended to limit the scope of the teachings presented herein. While the present teachings have been described in terms of these exemplary embodiments, the skilled artisan will readily understand that numerous variations and modifications of these exemplary embodiments are possible without undue experimentation. All such variations and modifications are within the scope of the current teachings.

All references cited herein, including patents, patent applications, papers, text books, and the like, and the references cited therein, to the extent that they are not

already, are hereby incorporated by reference in their entirety. In the event that one or more of the incorporated literature and similar materials differs from or contradicts this application, including but not limited to defined terms, term usage, described techniques, or the like, this application controls.

The foregoing description and Examples detail certain specific embodiments of the invention and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the invention may be practiced in many ways and the invention should be construed in accordance with the appended claims and any equivalents thereof.

Claims

It is claimed:

1. A method of reducing a level of LDL-cholesterol in blood of a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a first and second antagonist antibodies or antigen binding fragments thereof specific to proprotein convertase subtilisin kexin type 9 (PCSK9), wherein the first anti-PCSK9 antagonist antibody blocks the LDLR binding to the PCSK9 amino acid sequence of SEQ ID NO: 1, and wherein the second anti-PCSK9 antagonist antibody binds to C-Terminal Cys-His Rich Domain (CHRD) of the PCSK9 amino acid sequence of SEQ ID NO: 1.

2. The method of claim 1, wherein the first anti-PCSK9 antibody is alirocumab (PRALUENT™), evolocumab (REPATHA™), REGN728, LGT209, RG7652, LY3015014, J16, L1L3, 31H4, 11F1, 12H11, 8A1, 8A3, 3C4, 300N, or 1D05.

3. The method of claim 1 or 2, wherein the first anti-PCSK9 antibody comprises a heavy chain variable region (VH) comprising complementarity determining region one (CDR1), CDR2, and CDR3 of the amino acid sequence shown in SEQ ID NO: 2; and a light chain variable region (VL) comprising CDR1, CDR2, and CDR3 of the amino acid sequence shown in SEQ ID NO: 3.

4. The method of any one of claims 1-3, wherein the first anti-PCSK9 antibody comprises a VH CDR1 having the amino acid sequence shown in SEQ ID NO: 4, 5, or 6, a VH CDR2 having the amino acid sequence shown in SEQ ID NO:7 or 8, a VH CDR3 having the amino acid sequence shown in SEQ ID NO: 9, a VL CDR1 having the amino acid sequence shown in SEQ ID NO:10, a VL CDR2 having the amino acid sequence shown in SEQ ID NO:11, and a VL CDR3 having the amino acid sequence shown in SEQ ID NO: 12.

5. The method of any one of claims 1-4, wherein the first anti-PCSK9 antibody comprises a light chain having SEQ ID NO: 13 and a heavy chain having SEQ ID NO: 14, with or without the C-terminal lysine of SEQ ID NO: 14.

6. The method of any one of claims 1-5, wherein the first anti-PCSK9 antibody is a full antagonist of the PCSK9-mediated effect on LDL receptor (LDLR) levels as measured in vitro using an LDLR down-regulation assay in Huh7 cells.

7. The method of any one of claims 1-6, wherein the second anti-PCSK9 antibody blocks binding of PCSK9 to amyloid precursor-like protein 2 (APLP2).

8. The method of claim 7, wherein the second anti-PCSK9 antibody is 5F6 or 11B6.

9. The method of claim 7 or 8, wherein the second anti-PCSK9 antibody comprises a light chain having SEQ ID NO: 20 or 16 and a heavy chain having SEQ ID NO: 19 or 15.

10. The method of any one of claims 1-9, wherein the subject suffers from dyslipidemia, hyperlipidemia, hypercholesterolemia, atherosclerosis, cardiovascular disease, and/or coronary heart disease, and/or are at high risk of developing any of the diseases thereof.

11. The method of any one of claims 1-10, wherein both the first and the second anti-PCSK9 antibodies are administered intravenously or subcutaneously.

12. The method of any one of claims 1-11, wherein the first and the second anti-PCSK9 antibodies are administered sequentially or simultaneously.

13. The method of any one of claims 1-12, wherein the half-life of the first anti-PCSK9 antibody is extended by at least about 1 day by the administration of the second-anti-PCSK9 antibody.

14. The method of any one of claims 1-13, wherein the first and the second anti-PCSK9 antibodies are both administered at least every four weeks or every 2 weeks to the subject.

15. The method of any one of claims 1-14, wherein the method comprises administering about 10 mg to about 2000 mg of the first anti-PCSK9 antibody to the subject.

16. The method of any one of claims 1-15, wherein the method comprises administering about 10 mg to about 2000 mg of the second anti-PCSK9 antibody to the subject.

17. The method of any one of claims 1-16, wherein a statin has been administered prior to the initial dose of the first and second anti-PCSK9 antibodies.

18. A method of reducing a level of LDL-cholesterol levels in blood of a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a bispecific antibody directed to two different epitopes of a PCSK9 protein, wherein the first arm of the antibody blocks the LDLR binding to the PCSK9 amino acid sequence of SEQ ID NO: 1, and the second arm of the antibody binds to C-terminal Cys-His Rich Domain (CHRD) of SEQ ID NO: 1.

19. The method of claim 17 or 18, wherein a daily dose of a statin is administered.

20. The method of claim 19, wherein stable doses of the statin have been administered for at least about two, three, four, five, or six weeks prior to the initial dose of both the first and second anti-PCSK9 antibodies or the anti-PCSK9 bispecific antibody.

21. The method of claim 20, wherein the statin is atorvastatin, cerivastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin, or any pharmaceutically acceptable salts, or stereoisomers thereof.

22. An isolated antibody, or an antigen binding fragment thereof, which specifically binds to PCSK9 and comprises:

a heavy chain variable region (VH) comprising complementarity determining region one (CDR1), CDR2, and CDR3 of the amino acid sequence shown in SEQ ID NO: 21 or 34; and/or
a light chain variable region (VL) comprising CDR1, CDR2, and CDR3 of the amino acid sequence shown in SEQ ID NO: 3 or 35.

23. The antibody or the antigen binding fragment of claim 22, comprises:

a) a VH CDR1 having the amino acid sequence shown in SEQ ID NO: 23, 24, or 25, a VH CDR2 having the amino acid sequence shown in SEQ ID NO:26 or 27, a VH CDR3 having the amino acid sequence shown in SEQ ID NO: 28; and/or a VL CDR1 having the amino acid sequence shown in SEQ ID NO:29, a VL CDR2 having the amino acid sequence shown in SEQ ID NO:30, and a VL CDR3 having the amino acid sequence shown in SEQ ID NO: 31; or

b) a VH CDR1 having the amino acid sequence shown in SEQ ID NO: 36, 24, or 37, a VH CDR2 having the amino acid sequence shown in SEQ ID NO:38 or 39, a VH CDR3 having the amino acid sequence shown in SEQ ID NO: 40; and/or a VL CDR1 having the amino acid sequence shown in SEQ ID NO:41, a VL CDR2 having the amino acid sequence shown in SEQ ID NO:42, and a VL CDR3 having the amino acid sequence shown in SEQ ID NO: 43.

24. The method of claim 22 or 23, wherein the antibody comprises a nucleic acid comprising:

a) the nucleic acid sequence of SEQ ID NO: 32, and/or
the nucleic acid sequence of SEQ ID NO: 33; or

b) the nucleic acid sequence of SEQ ID NO: 45;and/or
the nucleic acid sequence of SEQ ID NO: 46.

25. A method of reducing a level of LDL-cholesterol levels in blood of a subject need thereof, comprising administering to the subject a therapeutically effective amount of an antibody specific to a PCSK9 protein, wherein the antibody blocks the LDLR binding to the PCSK9 amino acid sequence of SEQ ID NO: 1 and binds to C-Terminal Cys-His Rich Domain (CHRD) of the PCSK9 amino acid sequence of SEQ ID NO: 1.

26. The method of claim 25, wherein the antibody blocks binding of PCSK9 to amyloid precursor-like protein 2 (APLP2).

27. The method of claim 25 or 26, wherein the subject suffers from dyslipidemia, hyperlipidemia, hypercholesterolemia, atherosclerosis, cardiovascular disease, and/or coronary heart disease, and/or are at high risk of developing any of the diseases thereof.

28. The method of any one of claims 25-27, wherein the anti-PCSK9 antibody is administered intravenously or subcutaneously.

29. The method of any one of claims 25-28, wherein the anti-PCSK9 antibody is administered at least every four weeks or every 2 weeks to the subject.

30. The method of any one of claims 25-29, wherein the method comprises administering about 10 mg to about 2000 mg of the anti-PCSK9 antibody to the subject.

31. The method of any one of claims 25-30, wherein a statin has been administered prior to the initial dose of the anti-PCSK9 antibody.

32. The method of claim 31, wherein the statin is atorvastatin, cerivastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin, or any pharmaceutically acceptable salts, or stereoisomers thereof.

33. Use of a first and second antagonist antibody or antigen binding fragments thereof specific to PCSK9 in the manufacture of a medicament for reducing a level of LDL-cholesterol in blood of a subject, wherein the first anti-PCSK9 antagonist antibody blocks LDLR binding to the PCSK9 of SEQ ID NO: 1, and wherein the second anti-PCSK9 antagonist antibody binds to C-Terminal Cys-His Rich Domain (CHRD) of the PCSK9 of SEQ ID NO: 1.

34. Use of a bispecific antibody or antigen binding fragments thereof specific to PCSK9 in the manufacture of a medicament for reducing a level of LDL-cholesterol in blood of a subject, wherein the first arm of the antibody blocks LDLR binding to the PCSK9 of SEQ ID NO: 1, and wherein the second arm of the antibody binds to C-Terminal Cys-His Rich Domain (CHRD) of the PCSK9 of SEQ ID NO: 1.

35. Use of an antagonist antibody or antigen binding fragments thereof specific to PCSK9 in the manufacture of a medicament for reducing a level of LDL-cholesterol in blood of a subject, wherein the antibody blocks the LDLR binding to the PCSK9 protein (e.g., SEQ ID NO: 1), and binds to CHRD of the PCSK9 protein (e.g., SEQ ID NO: 1).

36. An article of manufacture, comprising a container, a composition within the container comprising an anti-PCSK9 antagonist antibody or a combination of anti-PCSK9 antagonist antibodies, and a package insert containing instructions to administer a therapeutically effective amount of the anti-PCSK9 antagonist antibody or antibodies for reducing a level of LDL-cholesterol in blood of a subject in need thereof.

FIG. 1A

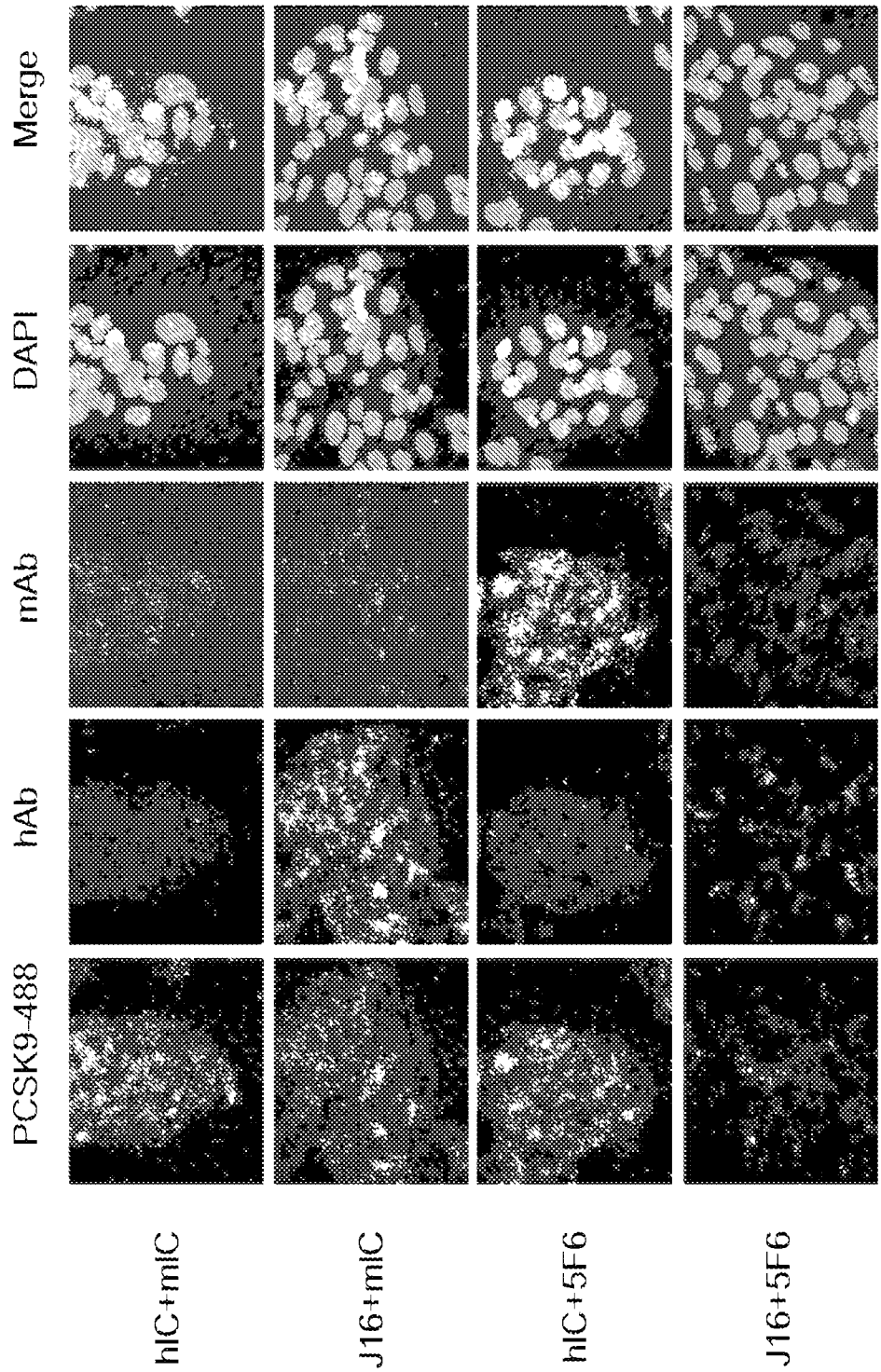


FIG. 1B

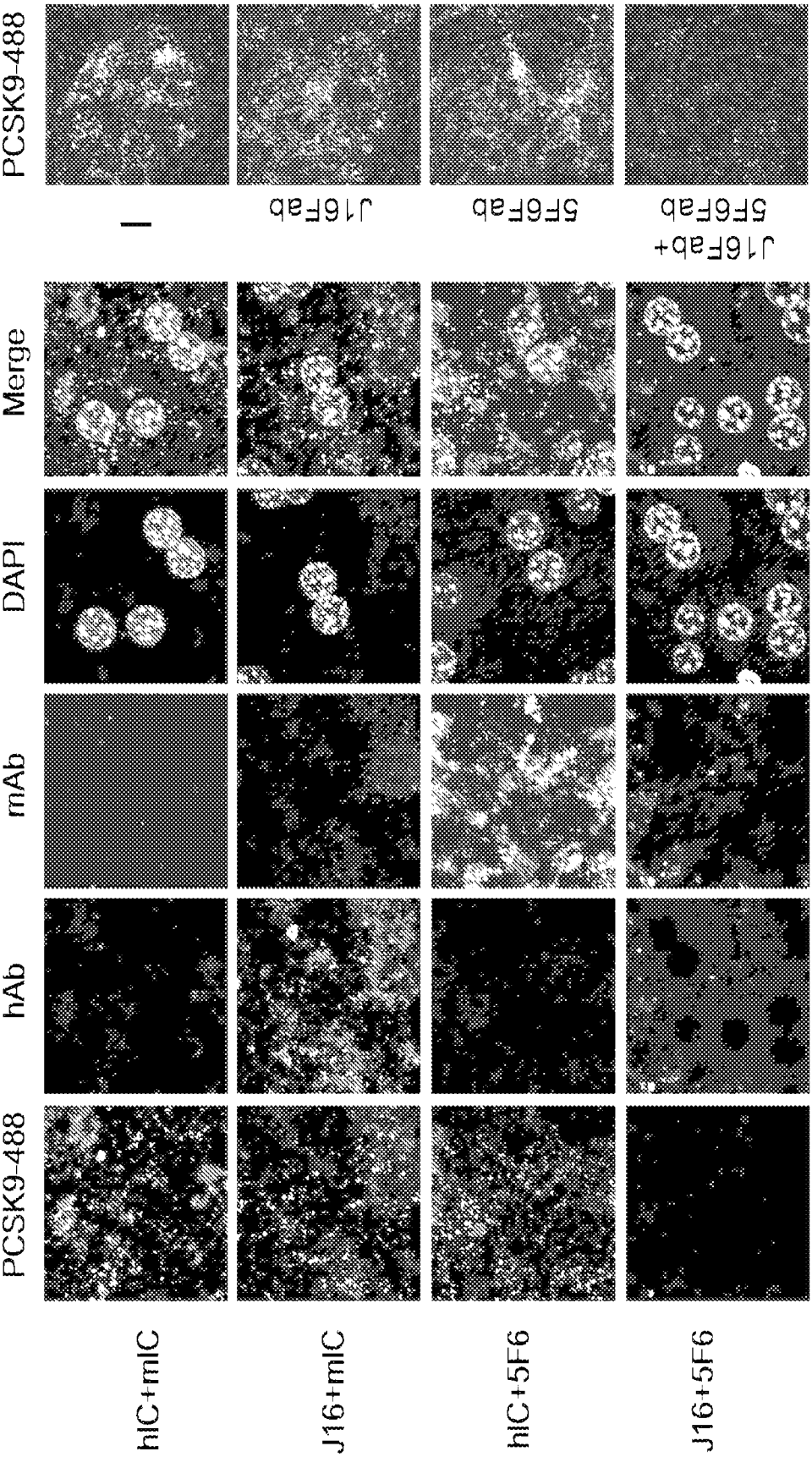

PCSK9-488
hAb
mAb
DAPI
Merge
hIC+mIC
J16+mIC
hIC+5F6
J16+5F6

FIG. 1C

PCSK9-488
-
J16Fab
5F6Fab
J16Fab+
5F6Fab

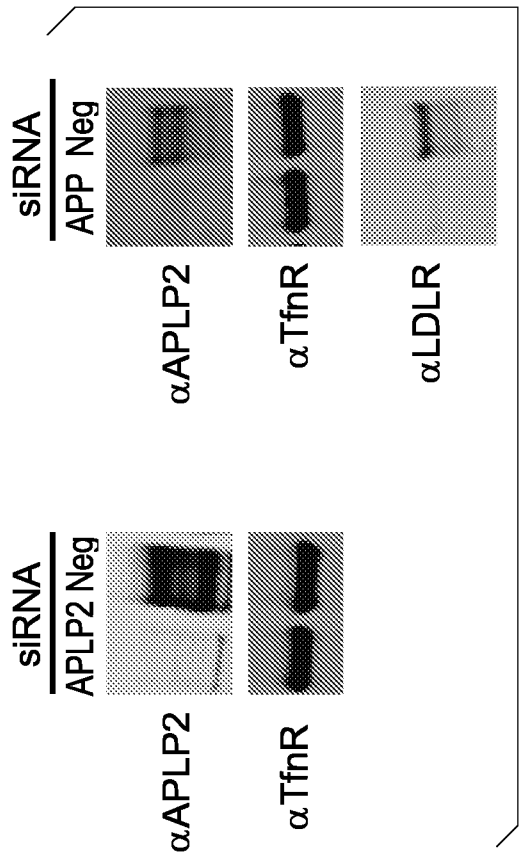


FIG. 1D

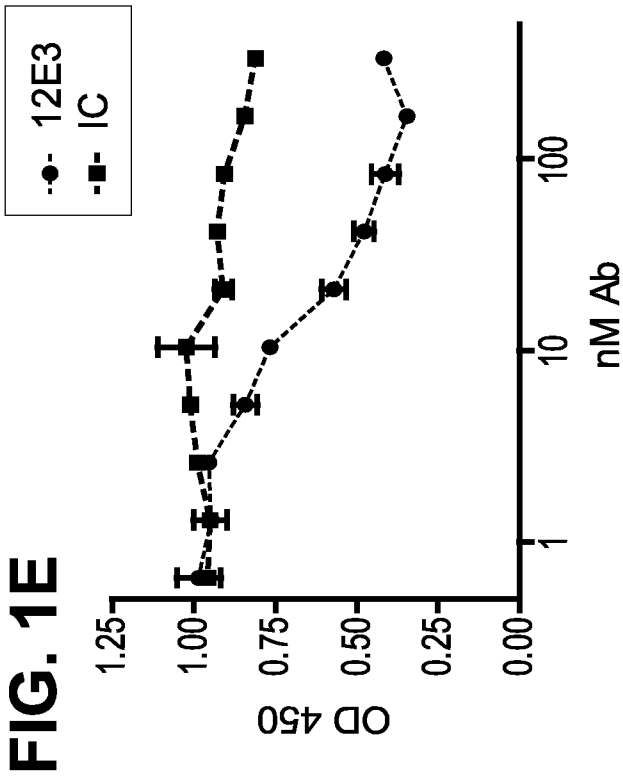


FIG. 1E

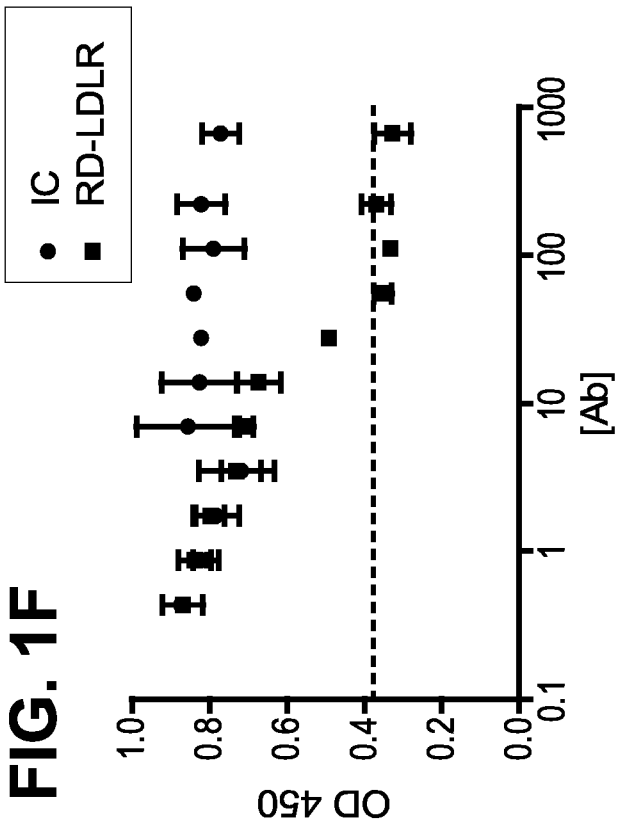


FIG. 1F

FIG. 2A

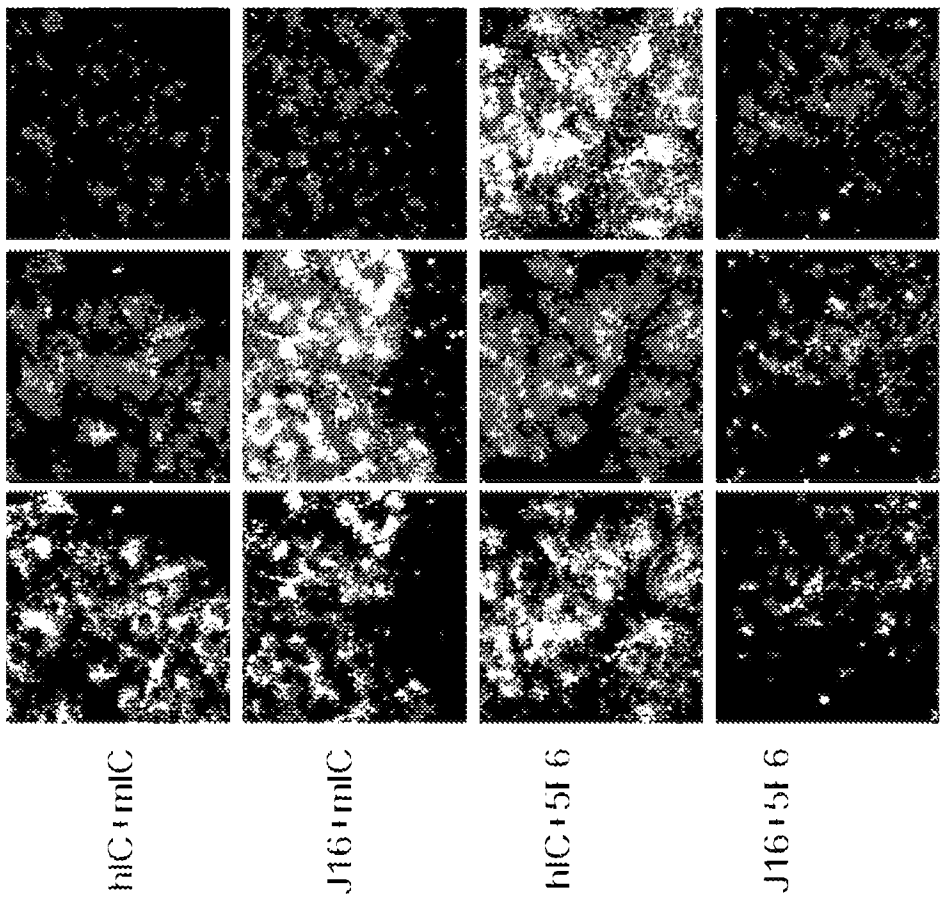

Neg siRNA
PCSK9 488
hAb
mAb
hIC+mIC
J16+mIC
hIC+5F6
J16+5F6

FIG. 2B

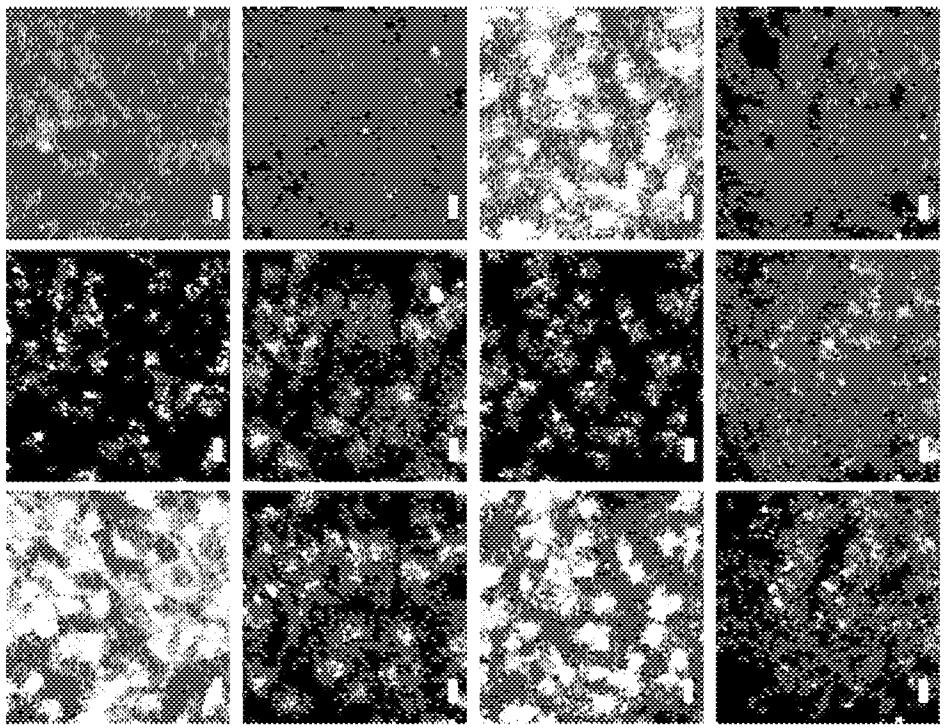

APLP2 siRNA
PCSK9 488
hAb
mAb

FIG. 2C

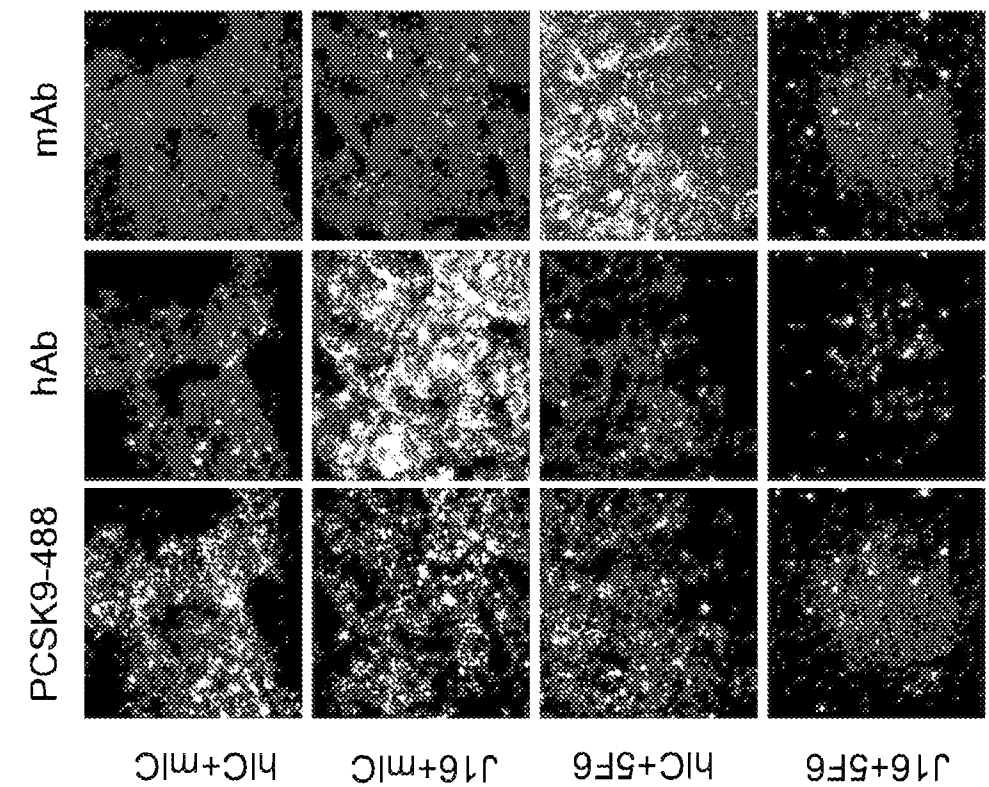

PCSK9-488
APP siRNA
hAb
mAb
hIC+mIC
J16+mIC
hIC+5F6
J16+5F6

FIG. 2D

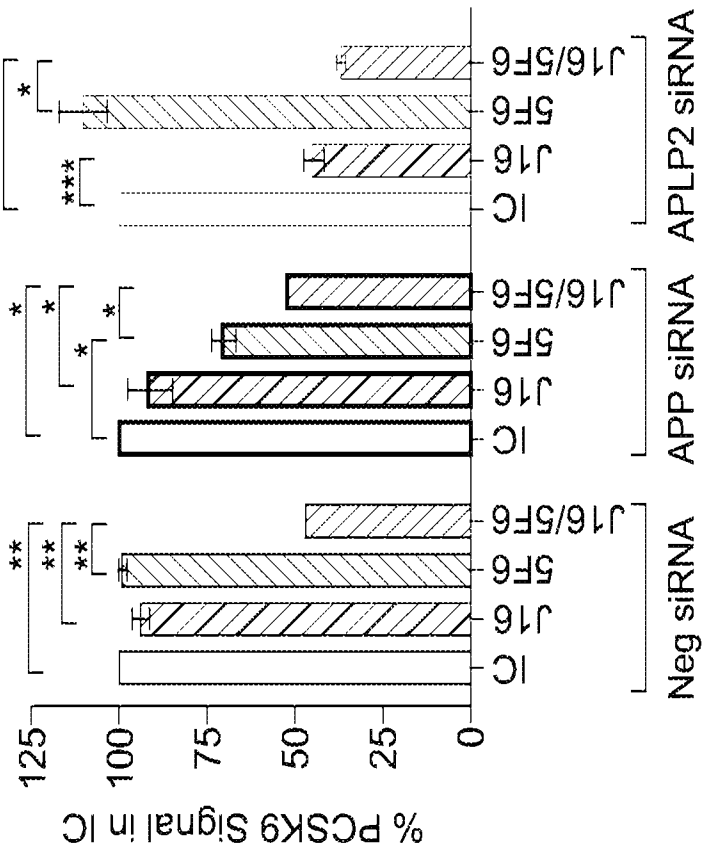

% PCSK9 Signal in IC
125
100
75
50
25
0
Neg siRNA
APP siRNA
APLP2 siRNA
IC
J16
5F6
J16/5F6
**
***
*

FIG. 2E

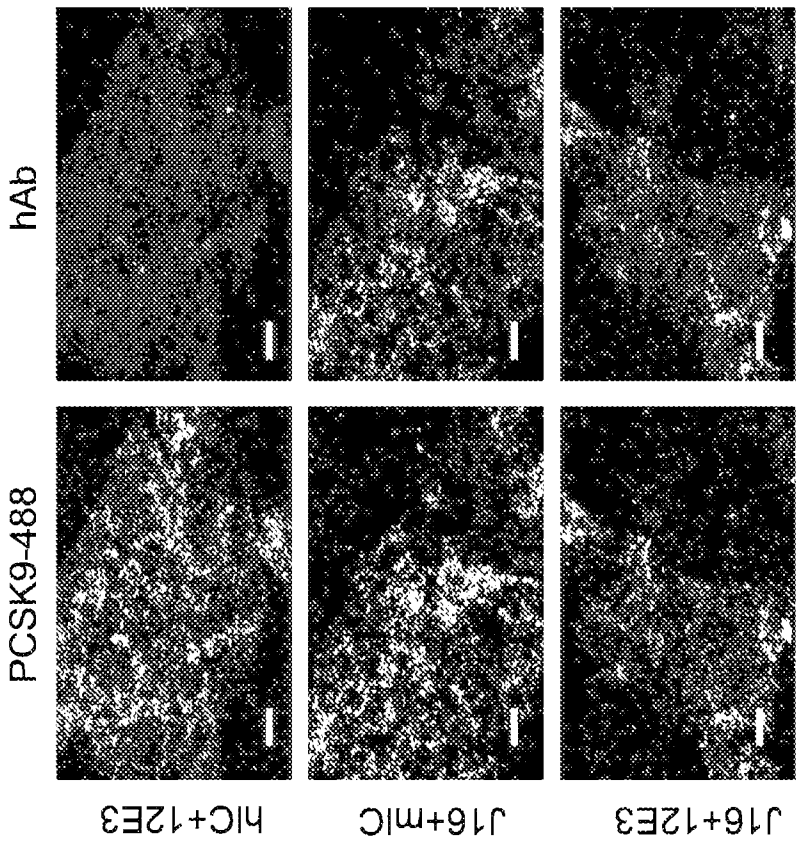

PCSK9-488
hAb
hIC+12E3
J16+mIC
J16+12E3

FIG. 2F

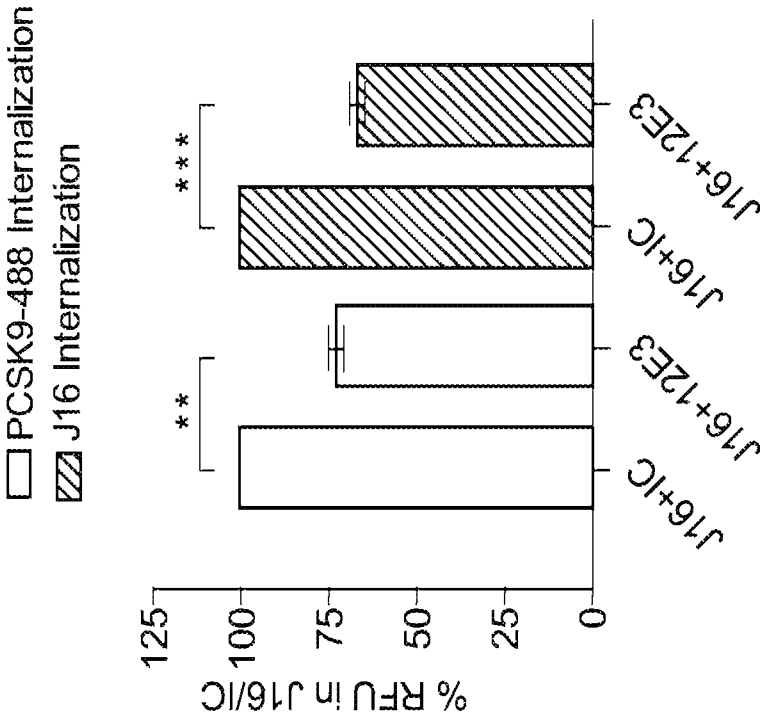

PCSK9-488 Internalization
J16 Internalization
% RFU in J16/IC
125
100
75
50
25
0
**
***
J16+IC
J16+12E3
J16+IC
J16+12E3

FIG. 2H

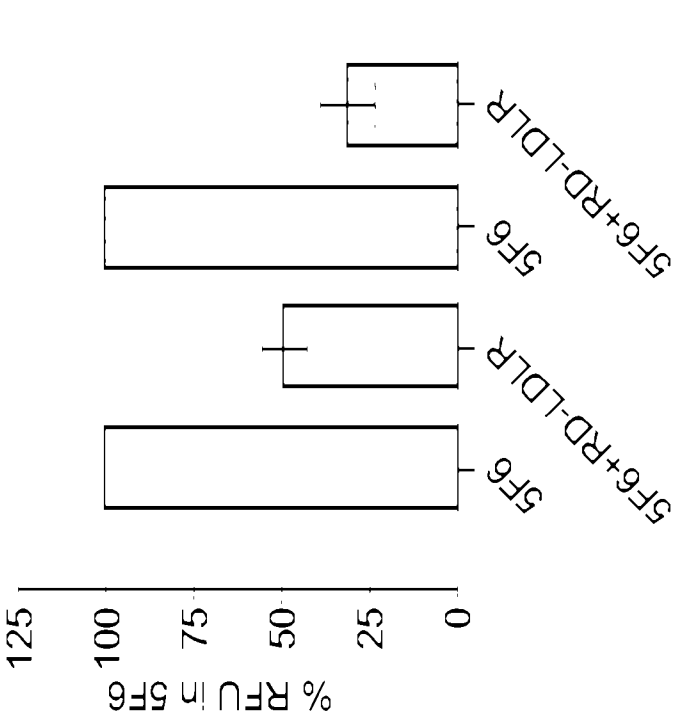

PCSK9-488 Internalization
5F6 Internalization
% RFU in 5F6
125
100
75
50
25
0
5F6
5F6+RD-LDLR
5F6
5F6+RD-LDLR

FIG. 2G

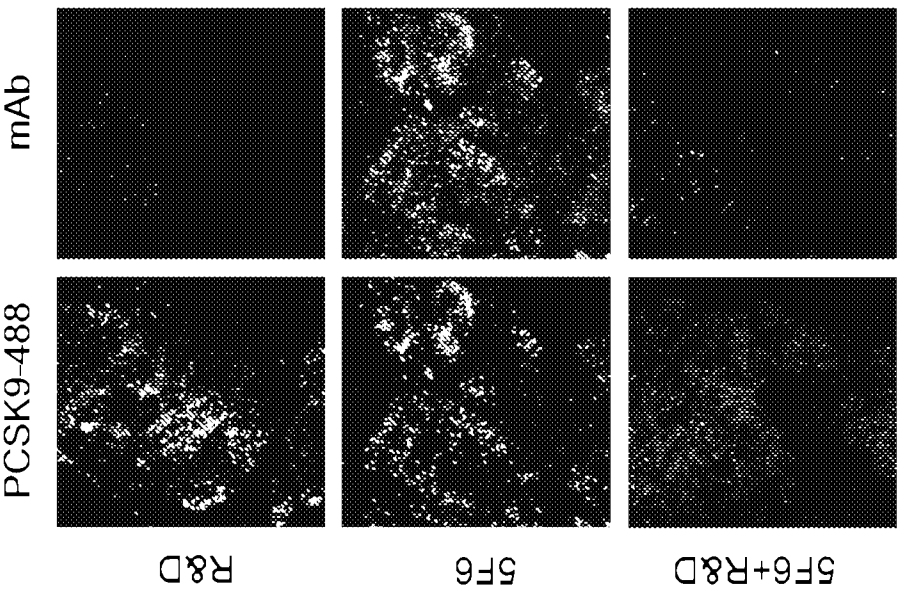

mAb
PCSK9-488
RαD
5F6
5F6+RαD

FIG. 3A

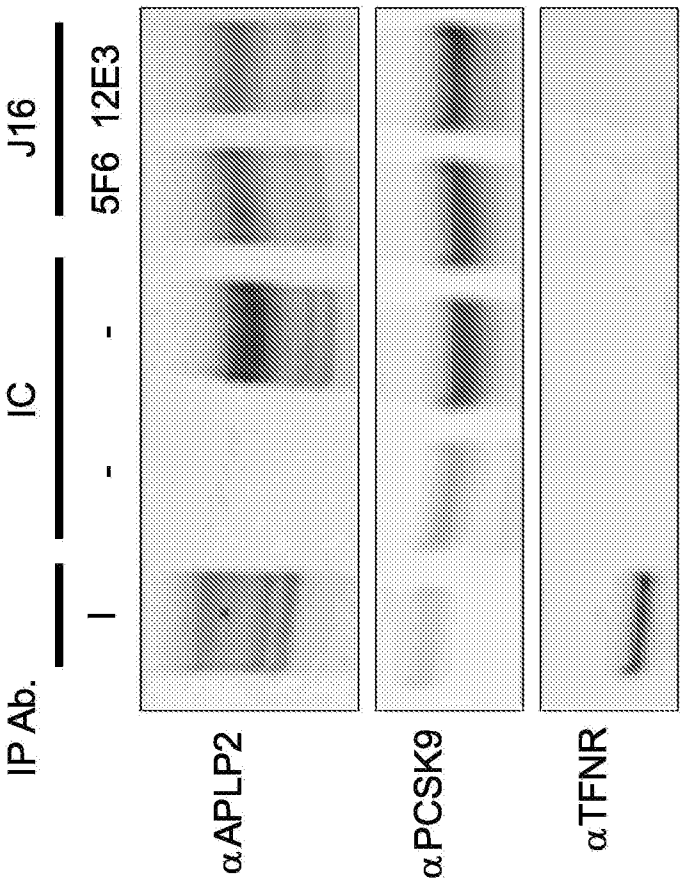

IP Ab.
I
IC
-
-
J16
5F6
12E3
αAPLP2
αPCSK9
αTFNR

FIG. 3B

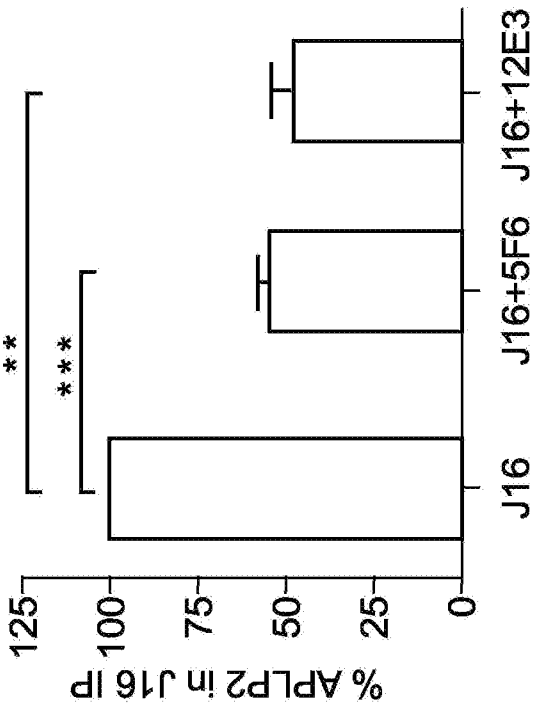

**
***
125
100
75
50
25
0
% APLP2 in J16 IP
J16
J16+5F6
J16+12E3

FIG. 3D

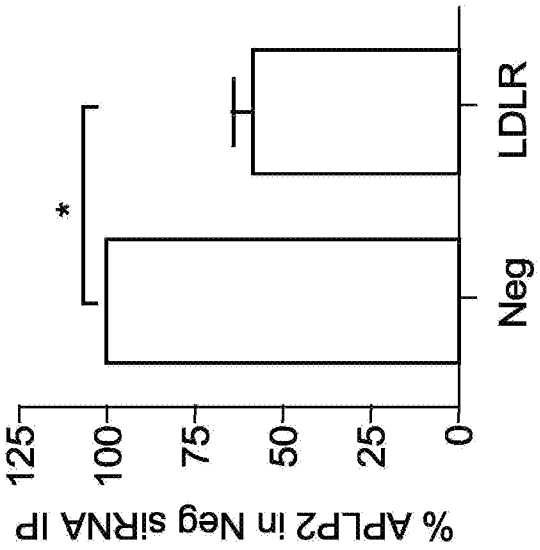
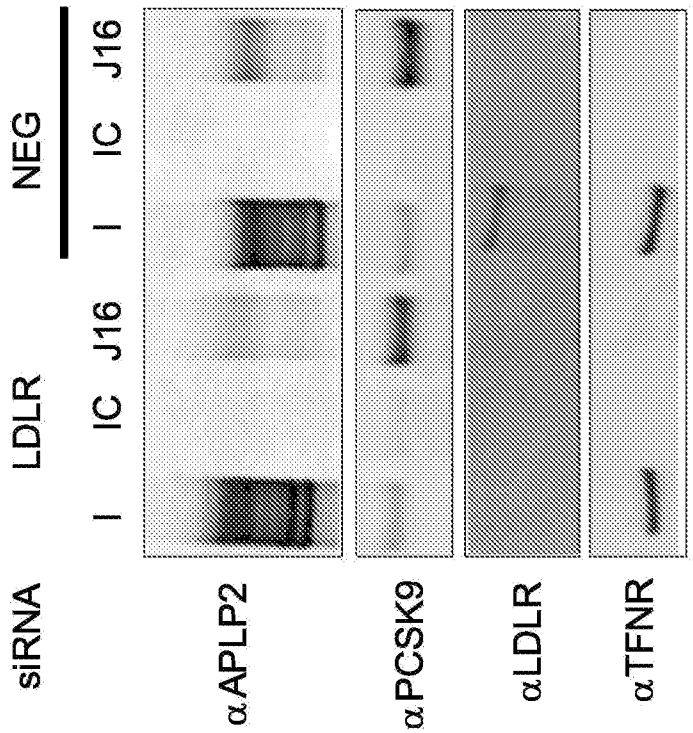

% APLP2 in Neg siRNA IP
125
100
75
50
25
0
*
Neg
LDLR

FIG. 3C


siRNA
LDLR
NEG
I
IC
J16
I
IC
J16
αAPLP2
αPCSK9
αLDLR
αTFNR

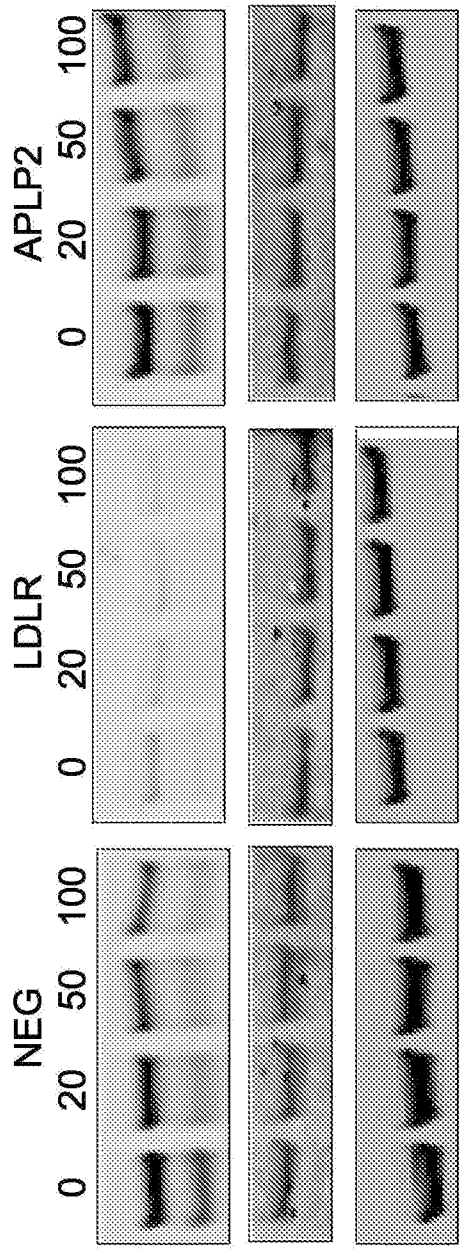


FIG. 3E

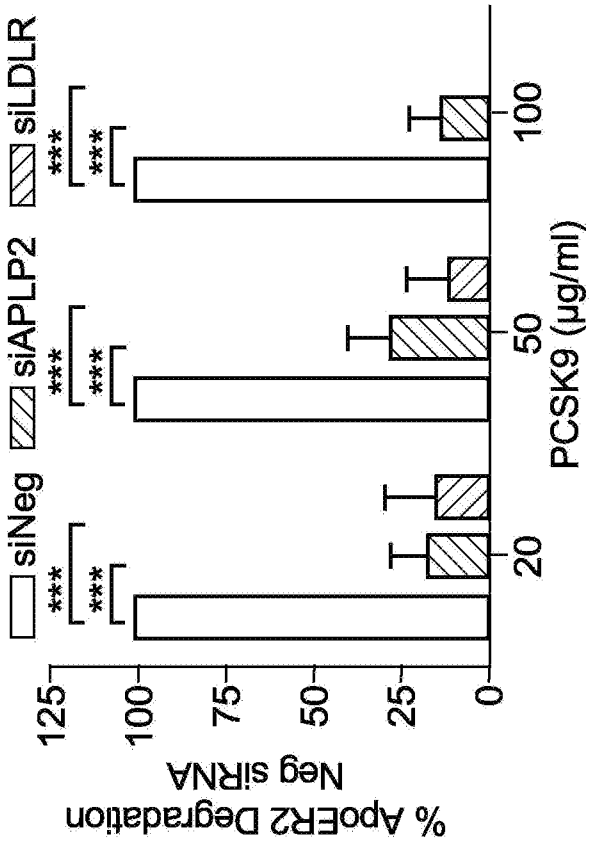


FIG. 3G

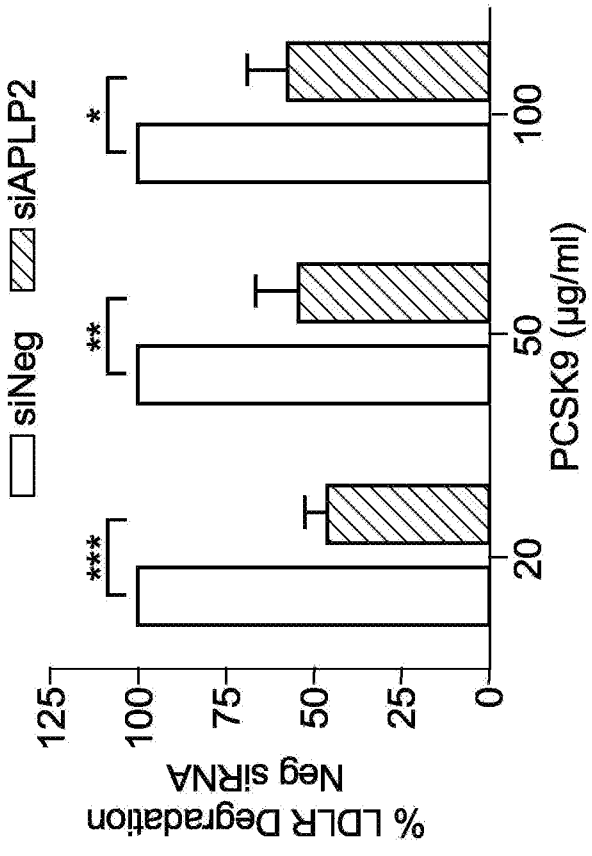


FIG. 3F

FIG. 3H

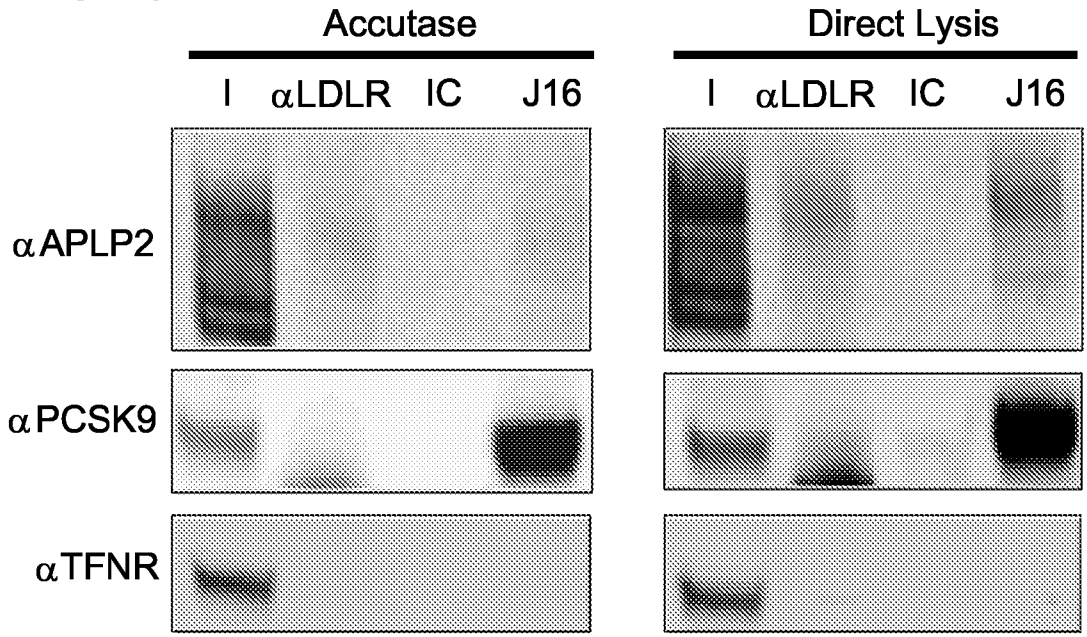

Accutase
Direct Lysis
I
αLDLR
IC
J16
I
αLDLR
IC
J16
αAPLP2
αPCSK9
αTFNR

FIG. 3I

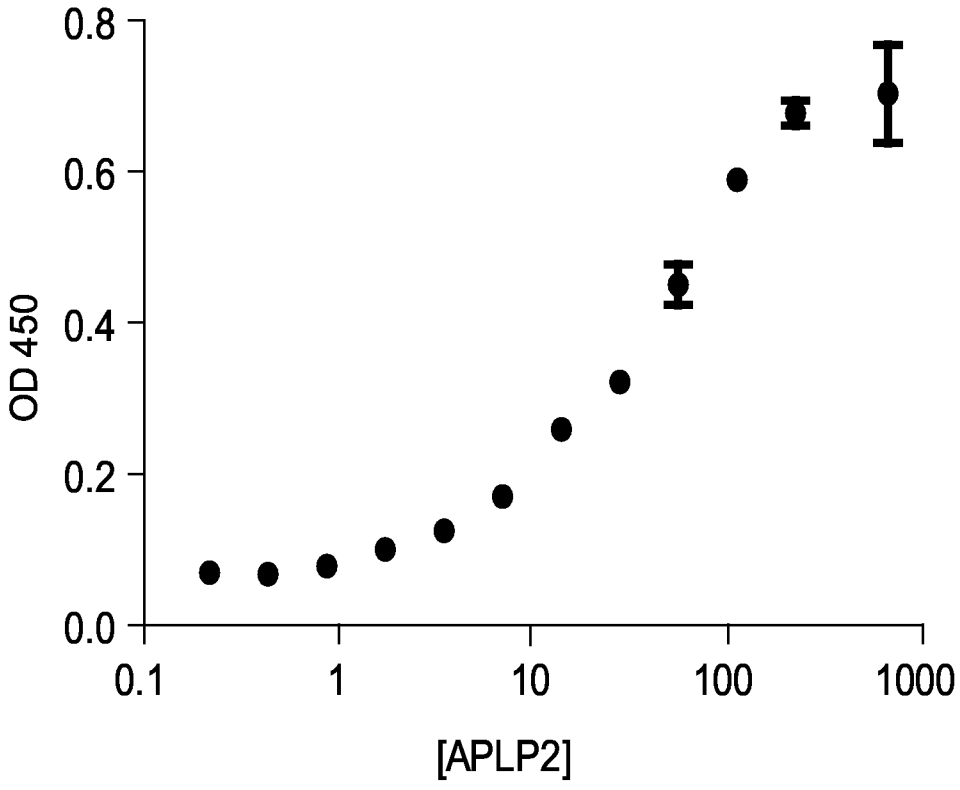

0.8
0.6
0.4
0.2
0.0
OD 450
0.1
1
10
100
1000
[APLP2]

FIG. 3J

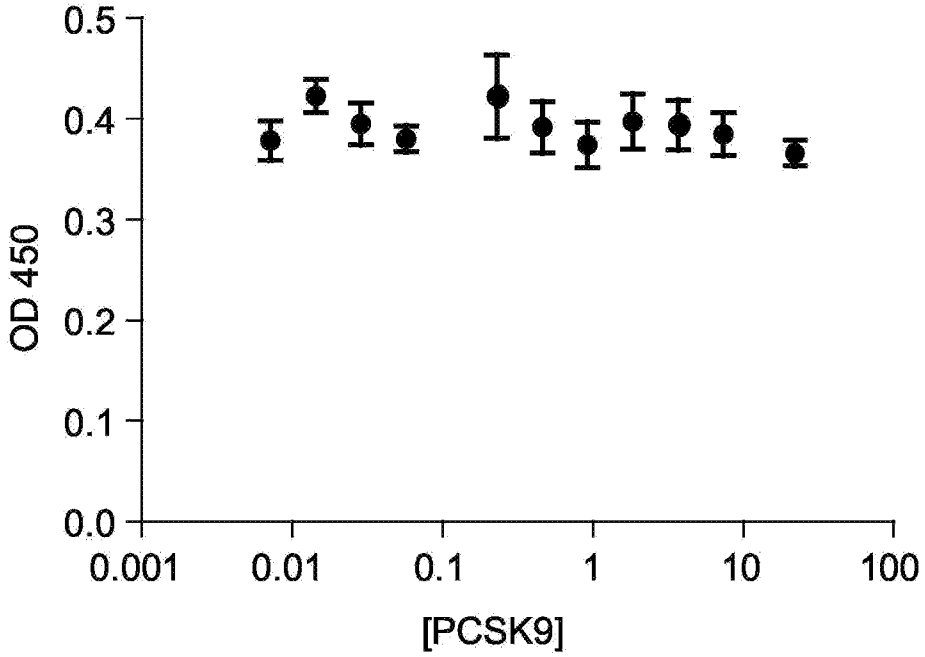

0.5
0.4
0.3
0.2
0.1
0.0
OD 450
0.001
0.01
0.1
1
10
100
[PCSK9]

FIG. 3K

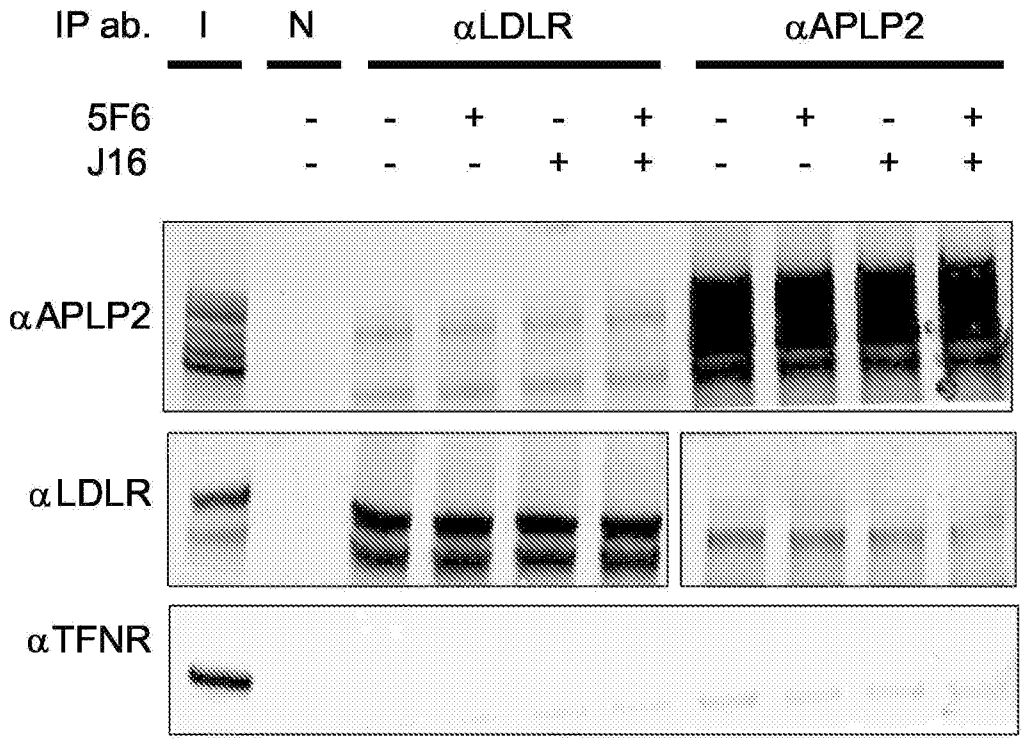

IP ab.
I
N
αLDLR
αAPLP2
5F6
J16
αAPLP2
αLDLR
αTFNR

FIG. 4A

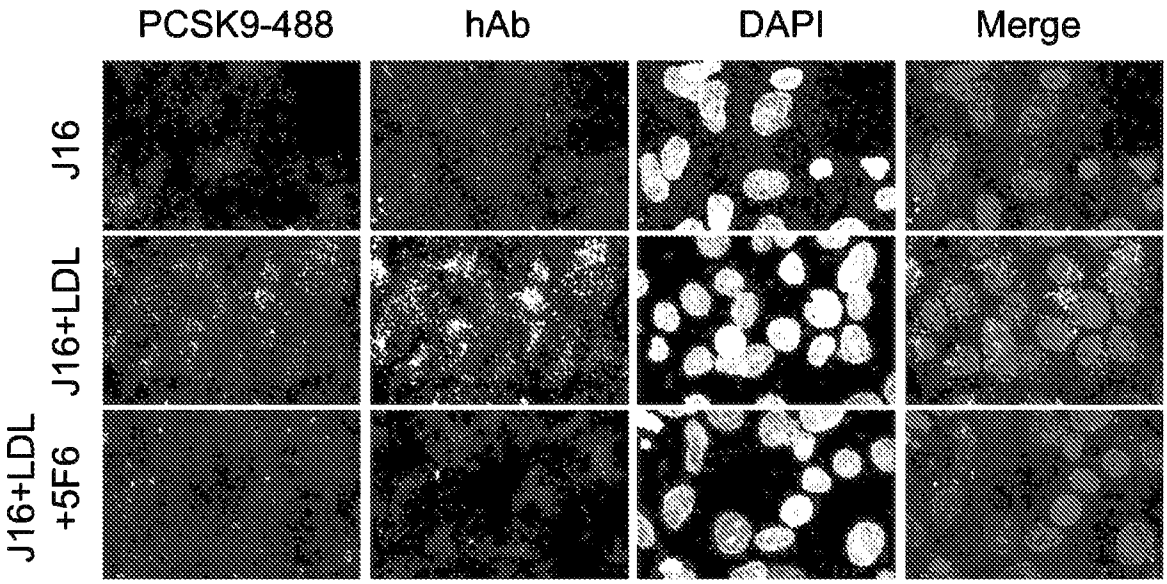

PCSK9-488
hAb
DAPI
Merge
J16
J16+LDL
J16+LDL +5F6

FIG. 4B

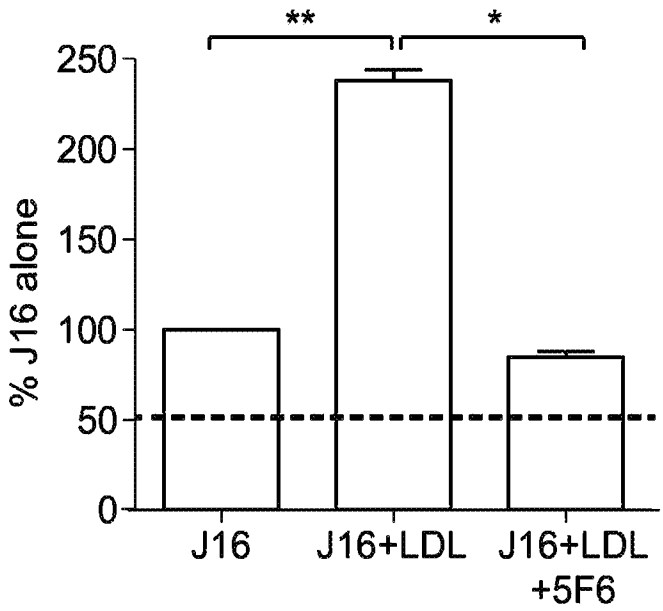

**
*
250
200
150
100
50
0
% J16 alone
J16
J16+LDL
J16+LDL +5F6

FIG. 4C

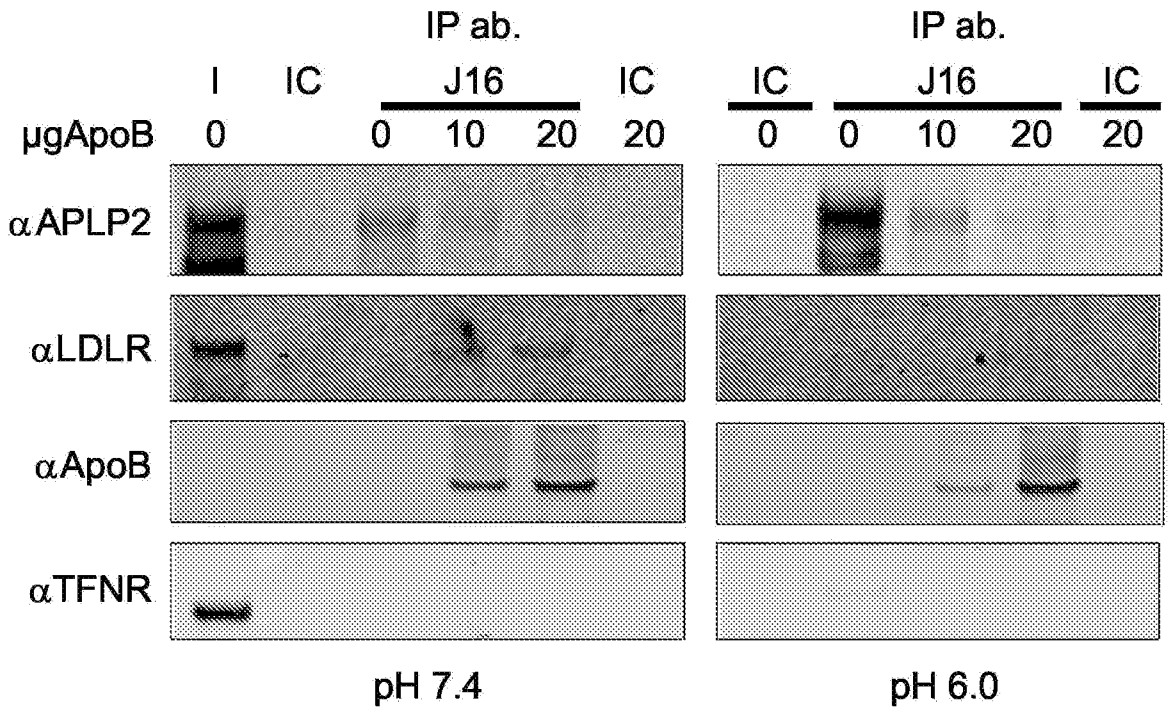

IP ab.
IP ab.
I
IC
J16
IC
IC
J16
IC
µgApoB
0
0
10
20
20
0
0
10
20
20
αAPLP2
αLDLR
αApoB
αTFNR
pH 7.4
pH 6.0

FIG. 4D

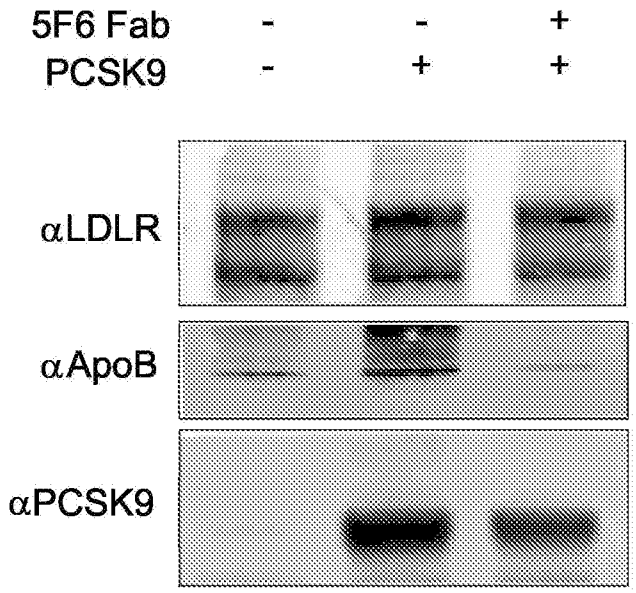

5F6 Fab
-
-
+
PCSK9
-
+
+
αLDLR
αApoB
αPCSK9

FIG. 4E

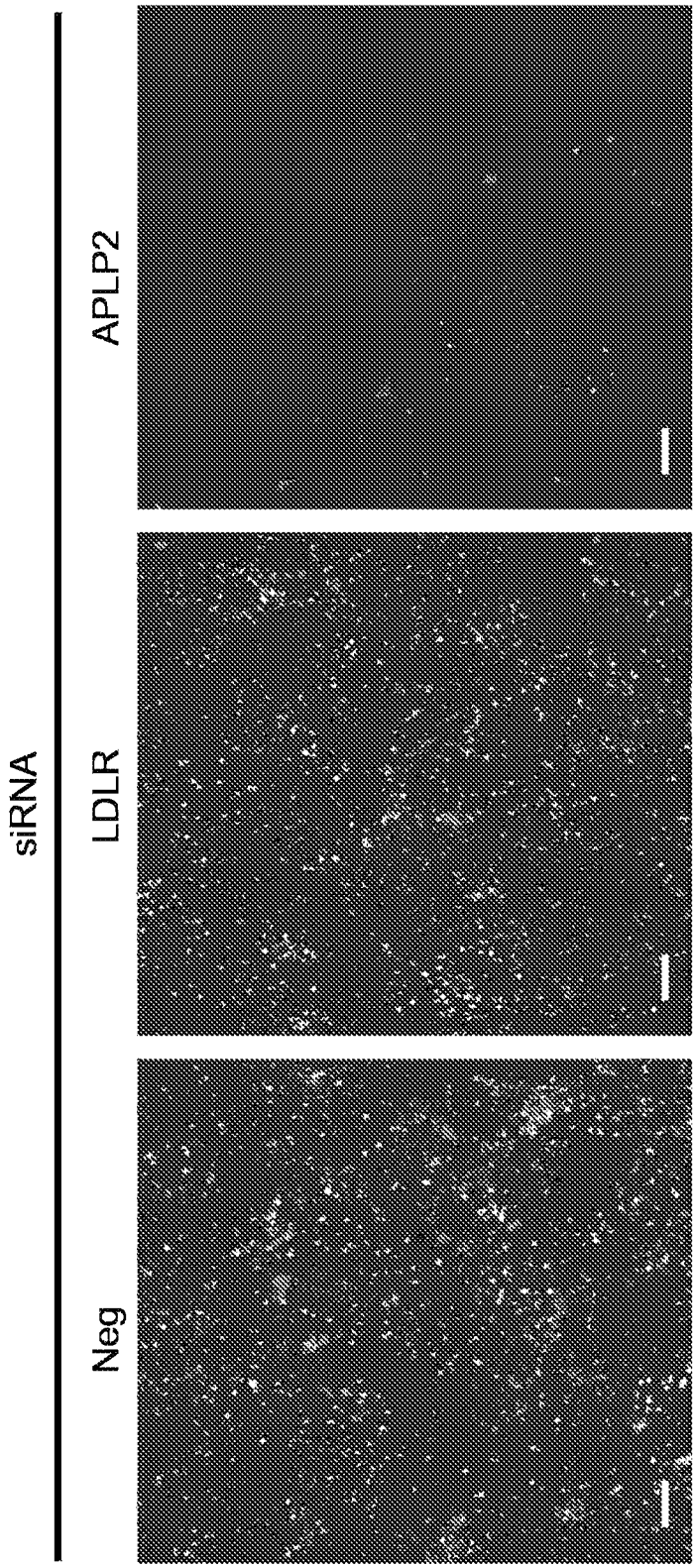


FIG. 4G

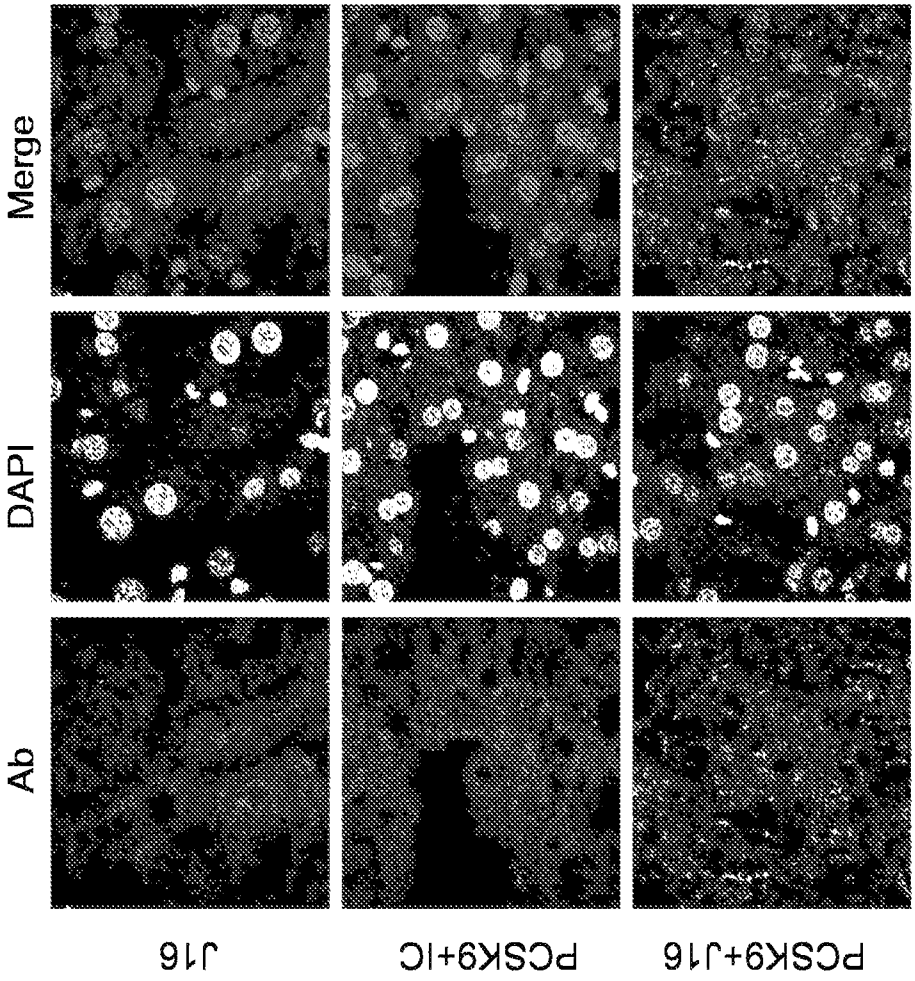

Ab
DAPI
Merge
J16
PCSK9+IC
PCSK9+J16

FIG. 4F

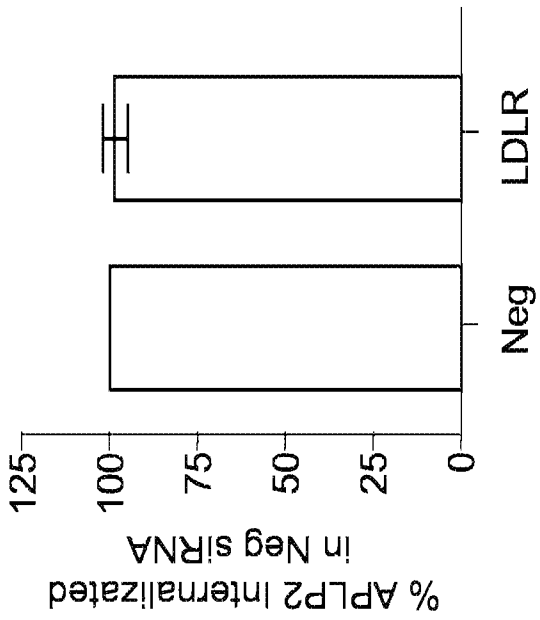

% APLP2 Internalizated
in Neg siRNA
125
100
75
50
25
0
Neg
LDLR

FIG. 5

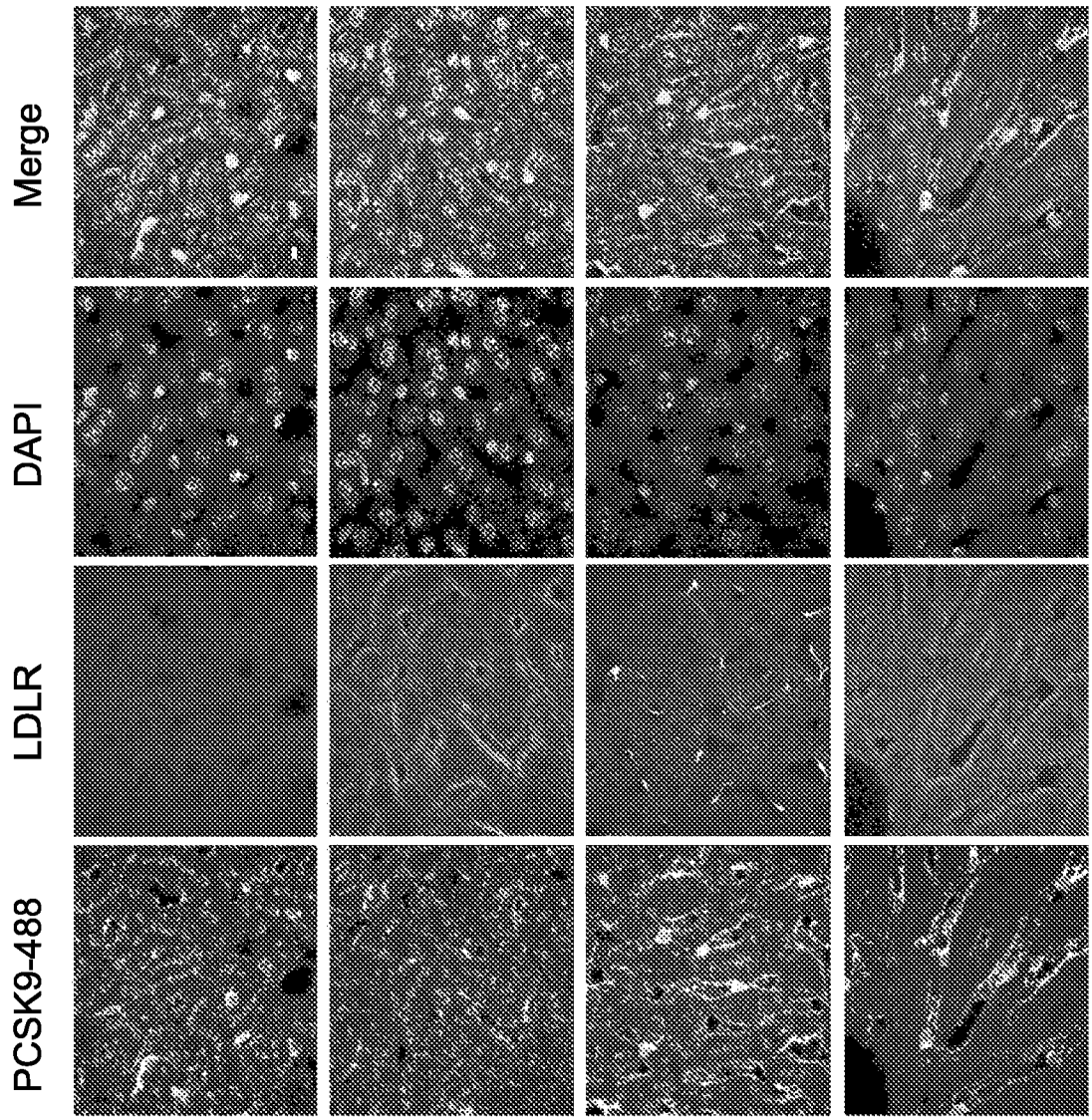


FIG. 6A

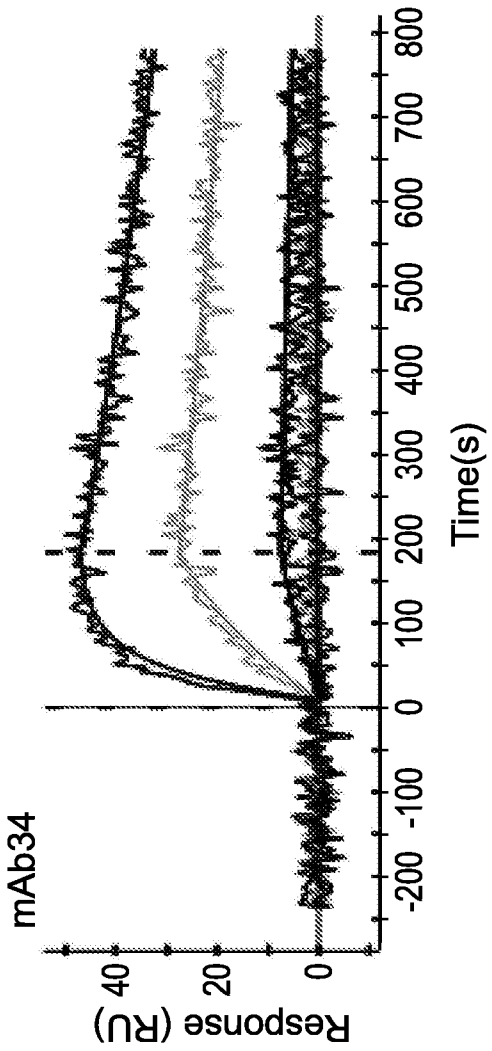

mAb34
k_a = 2.6 x 10^5, 3.3 x 10^5 ($M^{-1}s^{-1}$)
k_d = 6.2 x 10^{-4}, 5.0 x 10^{-4} (s^{-1})
k_D = 2.4, 1.5 nM (n=2)
Response (RU)
0
20
40
-200
-100
0
100
200
300
400
500
600
700
800
Time(s)

FIG. 6B

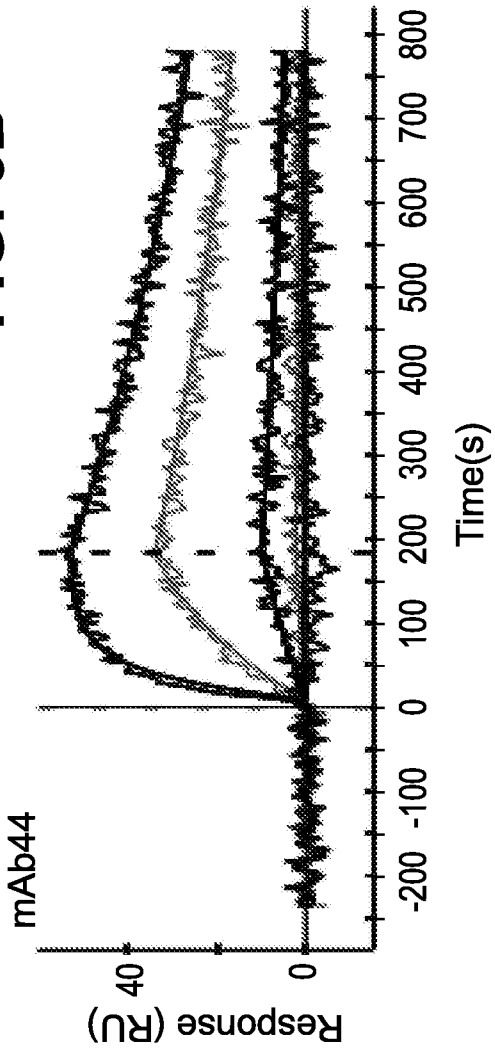

mAb44
k_a = 3.2 x 10^5, 4.4 x 10^5 ($M^{-1}s^{-1}$)
k_d = 1.2 x 10^{-3}, 1.1 x 10^{-3} (s^{-1})
k_D = 3.7, 2.6 nM (n=2)
Response (RU)
0
40
-200
-100
0
100
200
300
400
500
600
700
800
Time(s)

FIG. 7A Biotin-hLDLR on chip

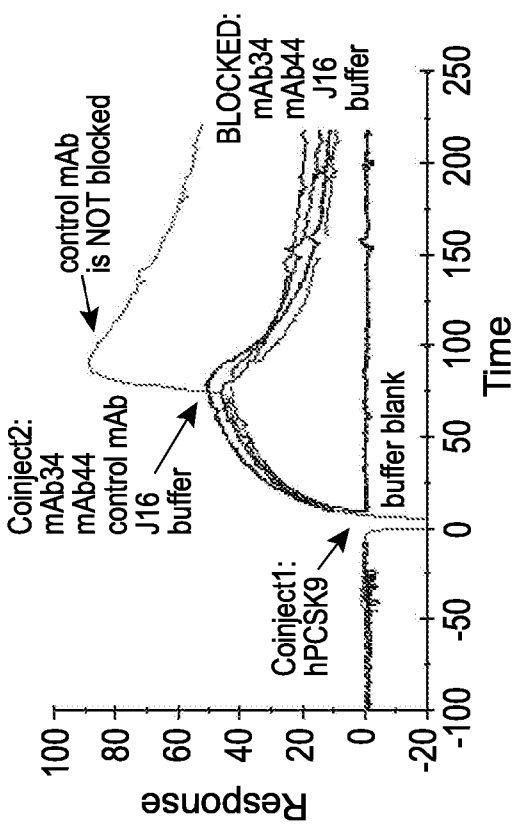


FIG. 7B Biotin-J16 on chip

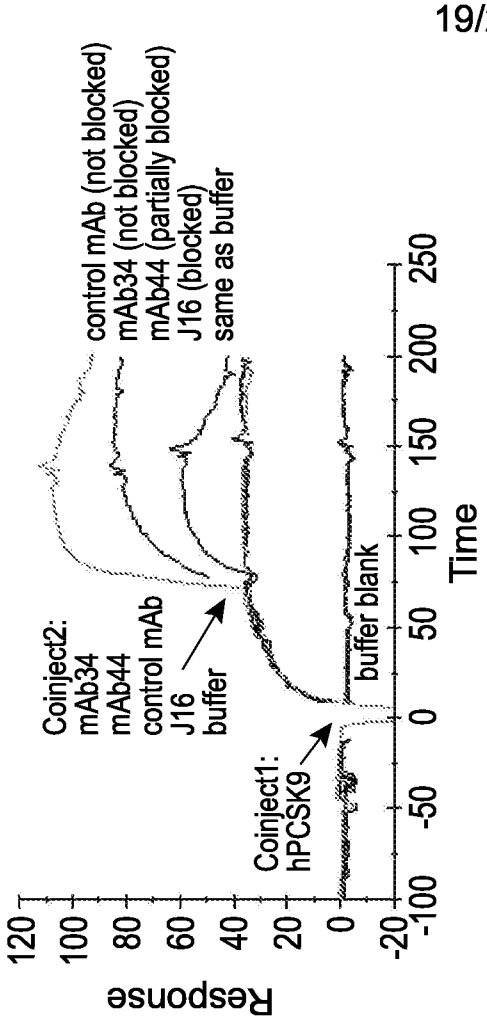


FIG. 7C

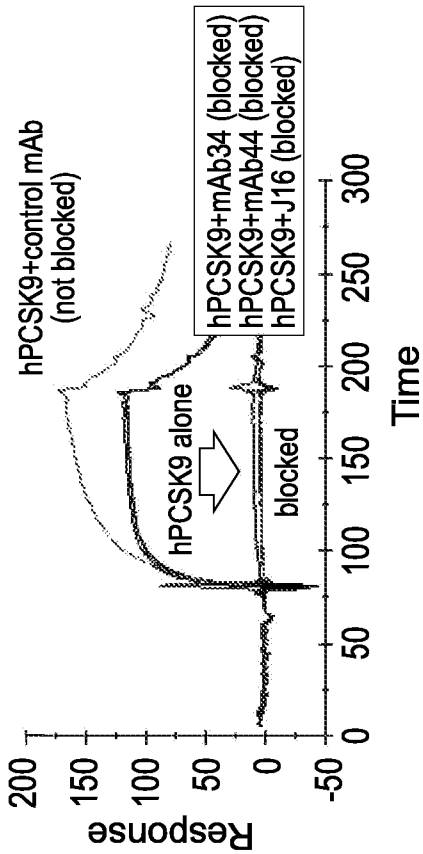


FIG. 7D

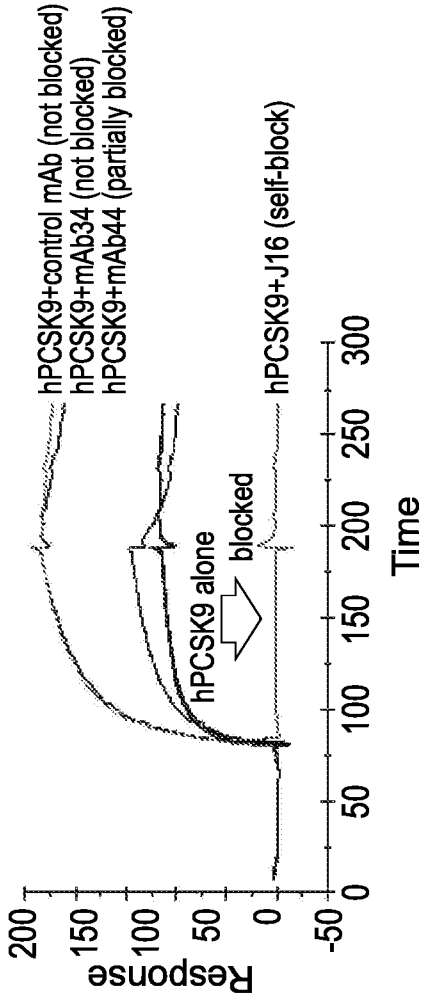


FIG. 8A Biotin-hAPLP2 on chip

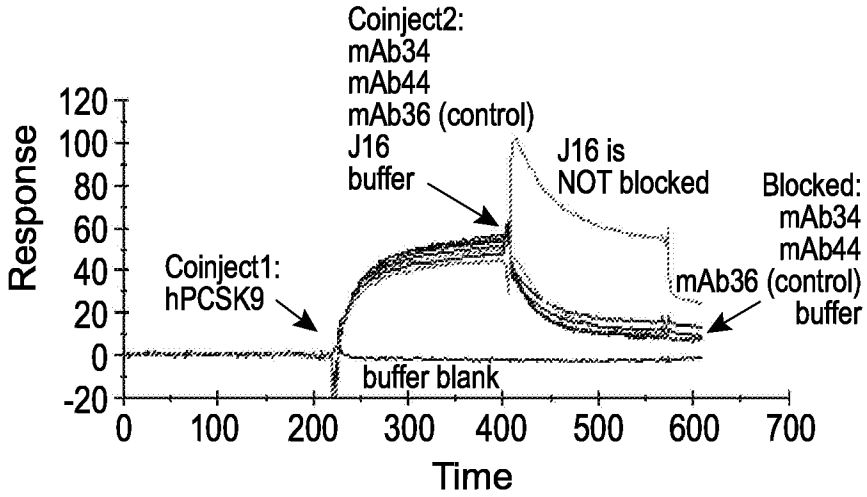


FIG. 8B Biotin-J16 on chip

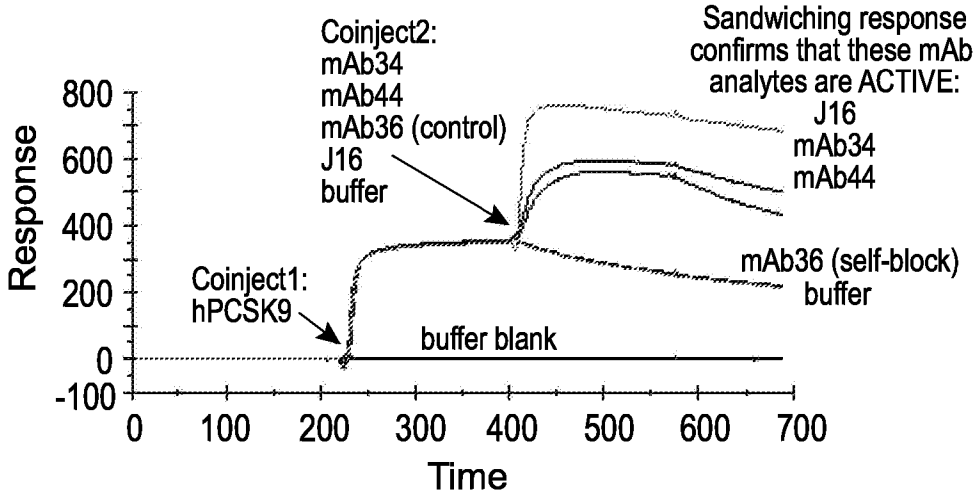


FIG. 8C

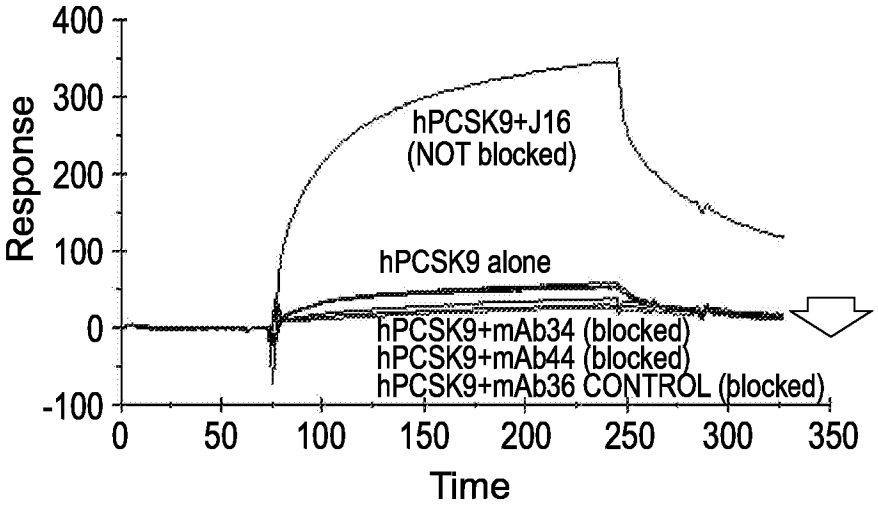
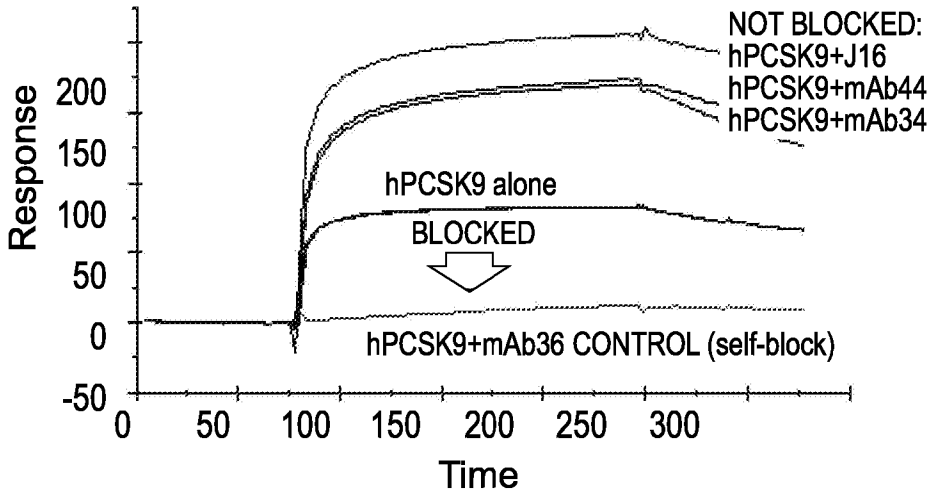


FIG. 8D



COINJECT

FIG. 9A

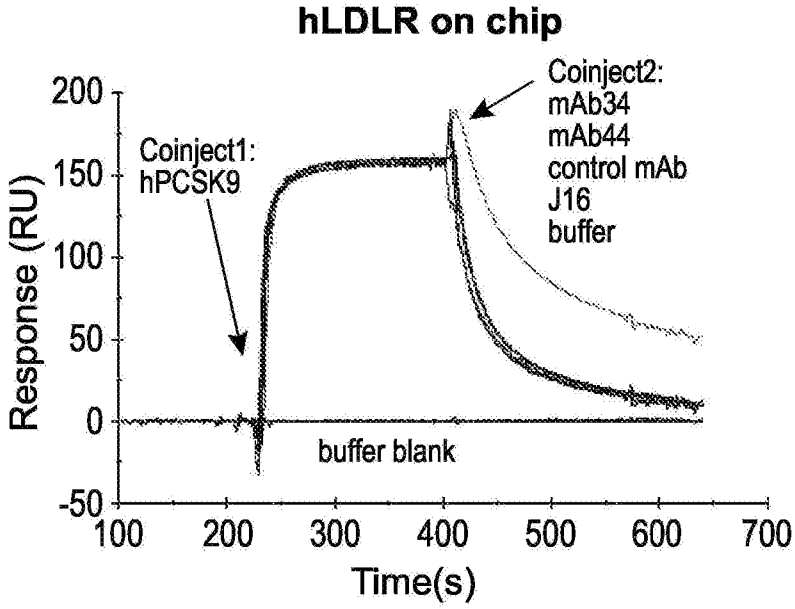


FIG. 9B

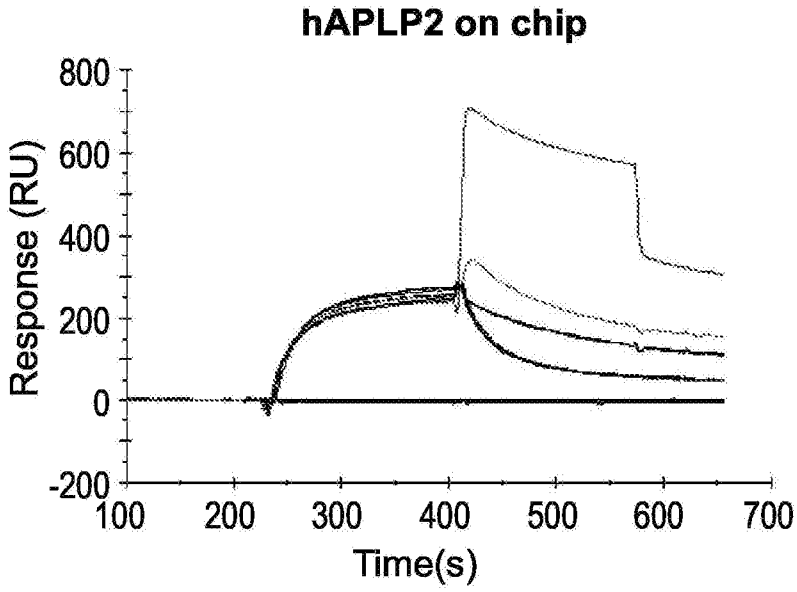
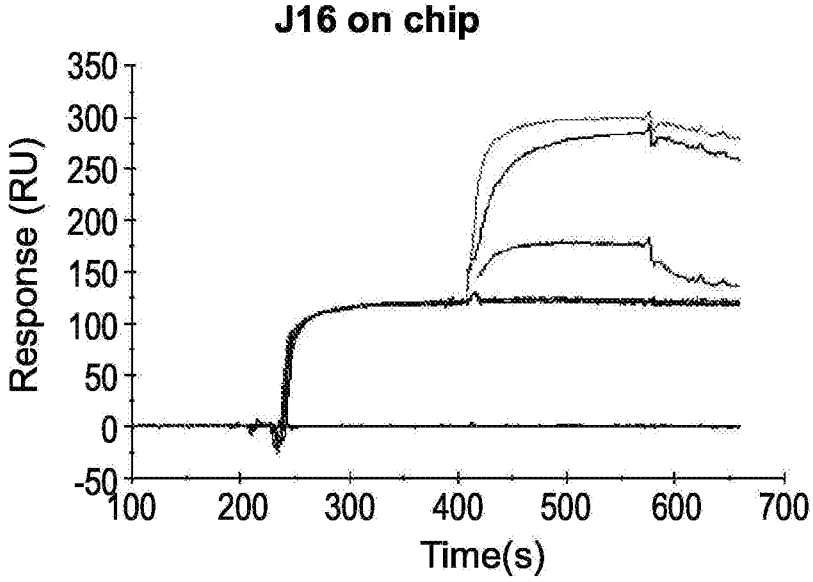


FIG. 9C



PREMIX

FIG. 9D

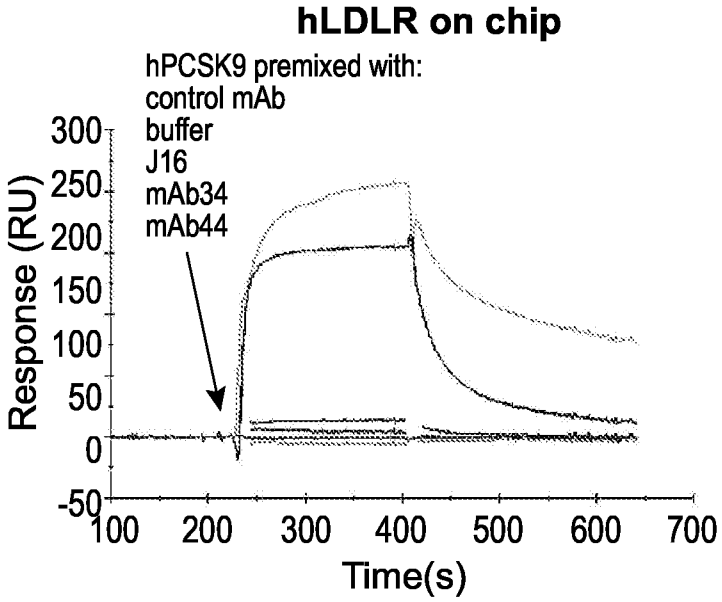


FIG. 9E

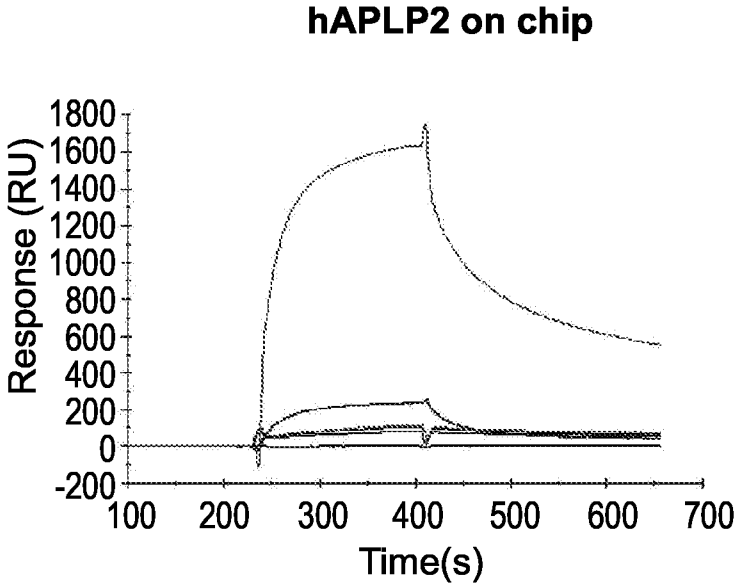
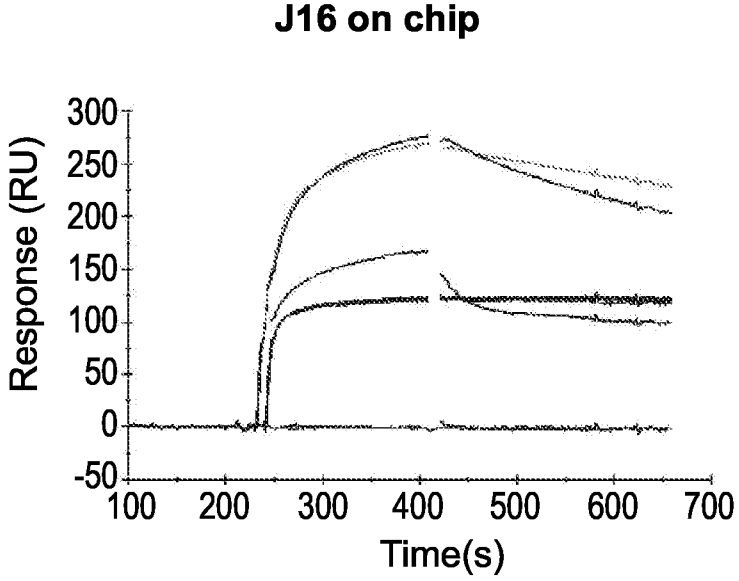


FIG. 9F



INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2015/055734

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/00 C07K16/40
ADD.

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)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/029513 A2 (RINAT NEUROSCIENCE CORP [US]; LIANG HONG [US]; ABDICHE YASMINA NOUBIA) 18 March 2010 (2010-03-18) cited in the application	22-24
Y	the whole document sequence 53 in particular, pages 3, 25 and 66	1-21, 25-35

X	WO 2013/008185 A1 (PFIZER [US]; UDATA CHANDRASEKHAR [US]) 17 January 2013 (2013-01-17)	22-24,36
Y	the whole document sequence 12	1-21, 25-35

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
26 October 2015	04/11/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pérez-Mato, Isabel

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2015/055734

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