(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 March 2003 (06.03.2003)

PCT

(10) International Publication Number
WO 03/019142 A2

(51) International Patent Classification[7]: G01N

(21) International Application Number: PCT/US02/26913

(22) International Filing Date: 22 August 2002 (22.08.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/314,329 23 August 2001 (23.08.2001) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/314,329 (CIP)
Filed on 23 August 2001 (23.08.2001)

(71) Applicants *(for all designated States except US)*: **TRUSTEES OF TUFTS COLLEGE** [US/US]; 136 Harrison Avenue, Boston, MA 02111 (US). **UNIVERSITEIT MAASTRICHT** [NL/NL]; Universiteitssingel 50, NL-6229 ER Maastricht (NL).

(72) Inventors; and

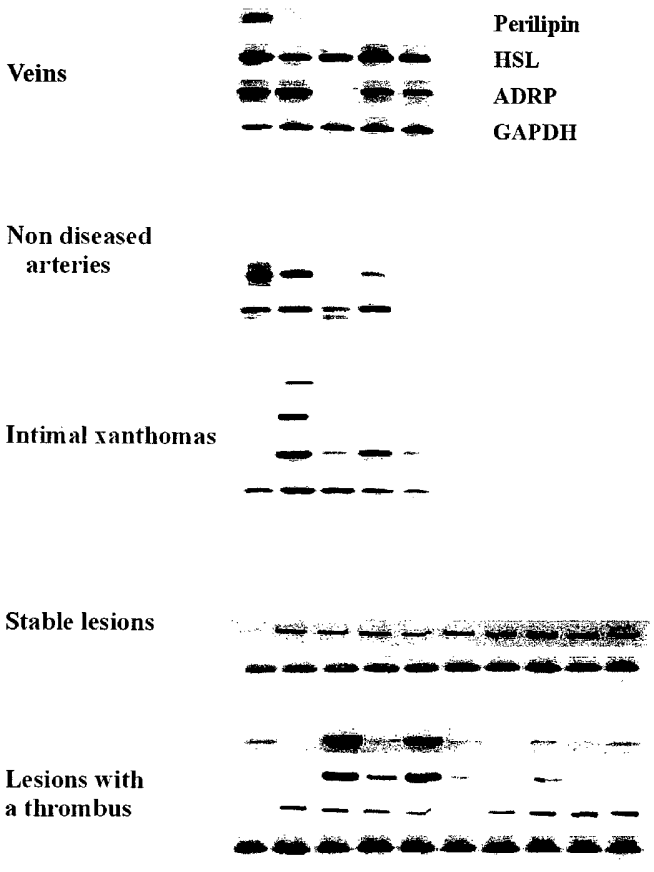
(75) Inventors/Applicants *(for US only)*: **GREENBERG, Andrew, S.** [US/US]; 110 Rachel Rd, Newton, MA 02459 (US). **DAEMEN, Mat, J., A., P.** [NL/NL]; Boviersdaal 3, NL-6228 GP Maastricht (NL).

(74) Agent: **GUTERMAN, Sonia, K.**; Mintz, Levin, Cohn, Ferris, Glovsky and Popeo, P.C., One Financial Center, Boston, MA 02111 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,

[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS FOR DETECTION AND REGULATION OF GENES ASSOCIATED WITH RUPTURED ATHEROSCLEROTIC PLAQUE



(57) Abstract: Compositions and methods for detecting tissue specificity for expression of perilipin, Hormone Sensitive Lipase (HSL) and Adipocyte Differentiation Related Protein (ADRP) mRNA and polypeptide are provided, and show that perilipin mRNA is located in plaques containing a thrombus, and perilipin polypeptide is found in foam cells of intimal xanthomas, in the shoulder area of stable lesions, and near the site of cap rupture in lesions containing a thrombus. HSL expression increases during plaque progression, and co-localizes to the same sites as perilipin. ADRP is expressed in all stages of human atherosclerosis, and is most important during the initial phases of lipid uptake. Perilipin expression increases in more lipid-laden macrophage derived foam cells, and replaces ADRP on the surface of the lipid droplet, thereby regulating accessibility to the lipolytic enzyme HSL. Perilipin and HSL are markers for progression of atherosclerosis associated with ruptured plaque.

WO 03/019142 A2

SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

COMPOSITIONS AND METHODS FOR DETECTION AND REGULATION OF GENES ASSOCIATED WITH RUPTURED ATHEROSCLEROTIC PLAQUE

Technical field

The present invention relates to expression of genes associated with ruptured and stable atherosclerotic plaque, and compositions and methods for detecting and distinguishing stable and ruptured plaque.

Background

Rupture of atherosclerotic plaques is the predominant underlying process in the pathogenesis of acute coronary syndromes and peripheral vascular disease (Libby P. 2000 *J. Intern. Med.* Vol. 247 pp.349-358; Zaman A. et al. 2000 *Atherosclerosis* Vol. 149 pp.251-266). There is a lack of plaque type markers which are specific for each of stable plaque and ruptured plaque. Further, the exact molecular mechanisms underlying the process of plaque rupture are still largely unknown.

The ability to diagnose the presence of ruptured plaque and to pinpoint its location is medically important with respect to a recommendation for surgery, such as bypass surgery. Ruptured plaque has been associated with sequelae such as blood clots leading to heart attack, stroke, arrhythmia, and claudication, i.e., loss of circulation to a limb and possible loss of limb. The potential type of damage depends on the location, for example, ruptured plaque in a carotid artery might result in stroke, and in a coronary artery, might result in a heart attack.

In an attempt to shed more light on the possible molecular mechanisms involved in the onset and progression of atherosclerosis, studies have compared gene expression of activated human umbilical vein endothelial cells, vascular smooth muscle cells and cholesterol loaded macrophages to non-activated cells (Lu K. et al. 1998 *Biochem. Biophys. Res. Commun.* Vol. 253 pp. 828-833; de Vries C. et al. 2000 *J Biol. Chem.* Vol. 275 pp. 23939-23947; Shiffman D. et al. 2000 *J. Biol. Chem.* Vol. 275 pp. 37324-37332). Regulation of expression of these genes *in vivo*, however, remains to be determined.

Summary

A feature of the present invention is a method of identifying an unstable atherosclerotic plaque, the method comprising introducing into the vascular lumen an agent wherein the agent binds to a polypeptide which is upregulated in an atherosclerotic

plaque, and determining the presence of the unstable plaque by imaging a location of the agent bound to the plaque, wherein binding of the agent to the plaque identifies the unstable plaque having the upregulated polypeptide. The term "vascular lumen" as used herein means the endothelial portion of the cirulatory system and its contents, blood cells and surrounding fluid with dissolved components.

In general according to this method, the agent is an antibody. The polypeptide is all or a portion of perilipin or hormone sensitive lipase (HSL), and the antibody further comprises a covalently linked adduct. The adduct is an imaging agent, and the imaging agent can be an adduct that is detectible by virtue of being magnetic, chemiluminescent, bioluminescent, radioactive, or fluorogenic. The magnetic molecule is in one embodiment one or more paramagnetic iron atoms. Further, the radioactive molecule can be, without limitation, ^{18}fluorine, ^{76}bromine, ^{77}bromine, ^{124}iodine, ^{125}iodine, ^{94}technetium, or ^{99}technetium.

For the purpose of covalent bonding to an antibody, the adduct can be an imaging agent such as a chelator molecule which is complexed with ^{99}technetium. A bioluminescent molecule can be for example a green fluorescent protein (GFP) or enhanced GFP (EFGP), and a subunit of the antibody or a single chain antibody can be expressed as a fusion to the GFP or EGFP. Further, the determining step can include use of a device for imaging the vascular lumen, the device being positive emission tomography (PET), magnetic resonance imaging (MRI), computerized axial tomography (CAT), and micro-photography.

Also featured among the embodiments of the invention herein is a method for identifying a perilipin binding composition. The method includes providing a sample containing a potential binding composition; adding an antibody specific for all or a portion of perilipin; and analyzing components of a resulting complex, thereby identifying the perilipin binding composition. This method can similarly be used to identify an HSL binding composition, or an adipocyte differentiation related protein (ADRP) binding composition.

According to this method, providing a sample can be providing a biological fluid. The biological fluid is a cell lysate, a spent culture medium, or a bodily fluid. A typical bodily fluid is blood, serum, plasma, urine, saliva, cerebrospinal fluid, pleural fluid, perspiration, lachrymal fluid, lymph, bile, or gastric fluid. Alternatively, providing a sample is providing a chemical composition, for example, the chemical composition is a component of a library of chemical compounds.

Another aspect of the invention is an antibody composition having affinity for an epitope of a polypeptide in a human ruptured atherosclerotic plaque. For example, the antibody binds to an epitope from perilipin or hormone sensitive lipase. In one embodiment, the polypeptide is perilipin, for example, human perilipin in a ruptured plaque in vivo in a subject, or in an ex vivo plaque from a subject.

The antibody that binds to human perilipin in a ruptured plaque is obtained by immunizing an animal with a peptide selected from all or a portion of amino acid sequences consisting of: MSMNKGPTLLDGDLPEQENVC (SEQ ID NO: 13); CLLGDLPEQENVL (SEQ ID NO: 14); PRELPARRVSDSFFRPSV (SEQ ID NO: 15); PREKPARRVSDSFFPSV (SEQ ID NO: 16); and RLAQPRRSLRSAQSPGC (SEQ ID NO: 17); and MSMNKGPTLLDGDLPEQENVC (SEQ ID NO: 22). A preferred aspect of the invention is a perilipin fragment comprising the amino acid sequence MSMNKGPTLLDGDLPEQENVC (SEQ ID NO: 13).

Yet another aspect of the invention is antibody that binds to human ADRP (also known as adipophilin) in a plaque. The antibody is obtained by immunizing an animal with a peptide which is all or a portion of the ADRP amino acid sequence fragment comprising the amino acid sequence CAQDQGAEMDKSSQETQR (SEQ ID NO: 23). The antibody obtained by immunizing an animal with the ADRP fragment can be used to bind to a ruptured atherosclerotic plaque or to a stable plaque.

Further, the antibody is polyclonal, and can fall into any of the immunoglobin classes of IgA, IgG, or IgM. Further, the antibody can be fragment such as a Fab, an Fab', an Fv, and a single-chain antibody, produced chemically or by recombinant technology. The antibody can be engineered to be bi-specific or trispecific.

In certain embodiments, binding of the antibody to ruptured plaques is at least two-fold greater than to stable plaques, for example, the binding to ruptured plaques is at least five-fold or even at least ten-fold greater than to stable plaques.

In certain embodiments, the antibody is monoclonal. Further, the antibody is substantially purified. Further, embodiments of the invention herein comprise a cell producing a monoclonal antibody made by immunizing an animal with any of the above peptide fragments of perilipin or ADRP, for example, the cell is a hybridoma. A nucleotide sequence encoding any of the antibodies herein, and a trangenic animal carrying any of the encoding sequences, are further embodiments of the invention.

Brief Description of the Drawings

Figure 1 is a flow chart of the various screening steps showing reduction of the number of candidate clones at each step.

Figure 2 is an autoradiogram of a macro array analysis of cDNA clones generated by suppressive subtractive by bridization (SSH). Two identical macro arrays were made by transfer of PCR products to nylon membranes. The Dot Blots were hybridized to: panel A) a ^{32}P-labeled cDNA pool of three different stable plaques, and panel B) a ^{32}P-labeled cDNA pool of three different ruptured plaques as described in Materials and Methods. Position A1 represents a clone upregulated in ruptured plaques and position B1 represents a clone downregulated in ruptured plaques. Position B12 contains RNA polymerase 2, while positions D6 and H4 contain respectively genomic DNA and empty vector.

Figure 3 is an RT-PCR analysis of expression of three genes that are differentially expressed in ruptured human atherosclerotic plaques compared to stable human atherosclerotic plaques. The perilipin expression patterns of (SSH1/11), SSH6 and SSH42 in 10 different stable plaques (left panel) and 10 different ruptured plaques (right panel) are shown. Expression of the housekeeping gene GAPDH is shown in the lower panel.

Figure 4 is a set of photomicrographs of stained tissue showing localization of perilipin mRNA expression within the different plaque types by RNA *in situ* hybridization. Panel A: Representative ruptured plaque hybridized with an anti-sense digoxygenin (DIG)-labeled perilipin RNA probe. Panel B: Section of the same ruptured plaque at a higher magnification. Dark purple staining indicates expression of perilipin mRNA. Panel C: Serial section of the ruptured plaque hybridized with the sense RNA probe. Panel D: Hematoxylin and eosin staining of a serial section. Panel E: Representative non diseased artery. Panel F: Representative stable plaque hybridized with an anti-sense DIG-labeled RNA probe. The scale bar represents 100μm.

Figure 5 is a set of photomicrographs of stained tissue showing localization of perilipin protein expression in different human atherosclerotic plaque types. Perilipin immunoreactivity (red stain) in ruptured plaques was observed in panel A showing cells surrounding some cholesterol clefts (arrow) and foam cells (arrowhead), and in panel B showing endothelial cells in newly formed vessels. Combined immunohistochemical staining is shown in ruptured plaques for perilipin (brown stain); and blue stain shows CD68 (macrophage specific; panel C), and Factor VIII (endothelium specific; panel D), respectively. Perilipin immunoreactivity was also tested in non-diseased arteries (panel

E) and in stable plaques (panel F). The scale bar represents 25μm in each of panels A, B, C and D or 100μm in panels E and F.

Figure 6 is a photograph of RT-PCR analysis of perilipin, HSL, and ADRP during human atherogenesis. The figure shows perilipin, HSL and ADRP mRNA expression in veins, non-diseased arteries, intimal xanthomas, stable plaques and lesions with a thrombus. Expression of GAPDH as a control is shown in the bottom panel of each set.

Figure 7 is a photograph of immunohistochemical staining of perilipin (left panels, indicated by an arrow) HSL (middle panels, indicated by an arrowhead) and ADRP (right panels, indicated by an asterix) during human atherogenesis. Immunoreactivity is depicted in red. The figure shows perilipin, HSL and ADRP protein expression in an intimal xanthoma (panels A, B and C), a fibrous lesion containing an area of calcification (panels D,E and F), an inflamed stable lesion (panels G,H and I), a lesion with a thrombus (panels J,K and L) and a lateral xanthoma (panels M, N and O). Bar represents 100μm in panels A,B,C,M,N and O and 200μm in panels D-L.

Figure 8 is a photograph of immunohistochemical staining of perilipin (left panels), HSL (middle panels) and ADRP (right panels) during human atherogenesis. Immunoreactivity is depicted in red. The upper panel shows co-localization of perilipin (panel A, indicated with an arrow) and HSL (panel B, indicated with an arrowhead) in foam cells of a fibrous cap atheroma showing only faint ADRP expression (panel C, indicated with an asterix). The lower panel shows faint perilipin (panel D, indicated with an arrow) and HSL (panel E, indicated with an arrowhead) staining in a fibrous cap atheroma that shows extensive ADRP (panel F, indicated with an asterix) protein expression in foam cells. Bar represents 100μm.

Figure 9 is a photograph of a western blot analysis with antibodies directed towards perilipin, HSL and ADRP. Lanes 1-4 contain protein lysate of four stable plaques. Lanes 5-8 represent protein lysate of four plaques containing a thrombus. The top panel represents perilipin polypeptide expression (65kDa), the middle panel represents HSL polypeptide expression (84kDa) and the bottom panel represents ADRP polypeptide expression (53kDa).

Description of Specific Embodiments

Research has in the past focused on differences in gene expression between fatty streaks and advanced lesions (Adams L. et al. 2000 *Circ. Res.* Vol. 87 pp. 623-631) and intima and media of human atherosclerotic plaques (McCaffrey T. et al. 2000 *J. Clin.*

Invest. Vol. 105 pp. 653-662). Adams et al. revealed different gene expression patterns between veins and arteries in macaques.

The morphology of ruptured plaques is known (Stary H. et al.1995 *Arterioscler. Thromb. Vasc. Biol.* Vol. 15 pp.1512-1531; Virmani R. et al. 2000 *Arterioscler. Thromb. Vasc. Biol.* Vol. 20 pp.1262-1275). However, there remains a presently unmet medical need for markers and methods of use of such markers, which can be used to identify specifically those plaques which are ruptured or prone to rupture *in vivo*.

The amino acid and the nucleotide sequences of the perilipin protein and its encoding gene are described in U.S. patent numbers 5,585,462, issued Dec. 17, 1996, and 6,074,842, issued June 13, 2000.

The gene encoding human perilipin was cloned (Nishiu J, et al., 1998 *Genomics.*, Vol. 48, pp. 254-257), however, studies of its function have been limited heretofore to rat adipocytes in culture. Perilipin is a phosphoprotein present on the surface layer of intracellular lipid droplets in adipocytes and sterogenic cells (Greenberg A. et al. 1991 *J. Biol. Chem.* Vol. 266 pp. 11341-11346; Blanchette-Mackie E. et al., 1995 *J. Lipid Res.* Vol. 36 pp. 1211-1226), and is phosphorylated upon lipolytic stimulation (Greenberg A. et al. 1991 *J. Biol. Chem.* Vol. 266 pp. 11341-11346).

Various etiological factors in development of atherosclerosis, the structure of plaque, and a model presenting this condition as an inflammmatory disease, are reviewed by Ross, R. 1999 *New Engl. J. Med.* Vol. 340 pp. 115-126. Stable plaques are lesions known to have fibrous caps. Plaque rupture and subsequent thrombosis may be responsible for as many as 50% of acute coronary syndromes and myocardial infarction. In most patients, infarction occurs as a result of erosion or thinning and rupture of the cap. Little is known of gene expression in plaques *in vivo*.

An important characteristic of atherosclerosis is the presence of lipids and cholesterol in the vessel wall (Libby, P., 2000, *J Intern Med.* 247:349-358; Lusis, A. J., 2000, *Nature* 407:233-241). Excessive accumulation of lipids and cholesterol is considered to be a risk factor for plaque rupture (Davies, M. J. et al., 1993, *Br Heart J.* 69:377-381).

Examples herein and shown in Faber et al., 2001 Circ Res. 89:547-554, the entire contents of which are hereby incorporated by reference herein, show that the gene encoding perilipin, a protein involved in lipid metabolism, was found to be expressed primarily in ruptured plaques. Perilipin protein expression was observed in foam cells and in microvascular endothelial cells of lesions containing a thrombus, while only few

foam cells in the shoulder region of stable plaques expressed perilipin.

Perilipin is a phosphoprotein present on the surface layer of intracellular lipid droplets in adipocytes and sterogenic cells (Greenberg, A. S., et al., 1991, *J Biol. Chem.* 266:11341-11346; Servetnick, D. A., et al., 1995, *J Biol. Chem.* 270:16970-16973). Four isoforms of perilipin arise by alternative splicing from a single copy gene (Lu, X., et al., 2001, *Mamm Genome.* 12:741-749). Perilipin A is the predominant form in adipocytes and sterogenic cells. Perilipin B is found primarily in adipocytes, while isoforms C and D are restricted to sterogenic cells (Servetnick, D. A., et al., 1995, *J Biol. Chem.* 270:16970-16973; Greenberg, A. S., et al., 1993, *Proc Natl Acad Sci U S A.* 90:12035-12039).

In adipocytes, perilipin is hyperphosphorylated by cAMP dependent protein kinase A (PKA) upon lipolytic stimulation (Greenberg, A. S., et al., 1991, *J Biol. Chem.* 266:11341-11346; Souza, S. C., et al., 2002, *J Biol. Chem.* 277:8267-8272). As a consequence, the surface of the lipid droplet is altered, resulting in an increased accessibility for the lipolytic enzyme hormone sensitive lipase (HSL), which, if phosphorylated by PKA, is capable of hydrolyzing triacylglycerol and cholesteryl esters (Clifford, G. M., et al., 2000, *J Biol. Chem.* 275:5011-5015; Belkner, J., et al., 2000, *Biochem J.* 352 Pt 1:125-133). In mice, absence of perilipin leads to elevated lipolysis which results in smaller white adipocytes (Martinez-Botas, J., et al., 2000, *Nat Genet.* 26:474-479; Tansey, J. T., et al., 2001, *Proc Natl Acad Sci U S A.* 98:6494-6499). In adipocytes, overexpression of perilipin decreases triacylglycerol hydrolysis (Souza, S. C., et al., 1998, *J Biol. Chem.* 273:24665-24669; Brasaemle, D. L., et al., 2000, *J Biol. Chem.* 275:38486-38493). Thus perilipin, when not hyperphosphorylated, provides a barrier for enzymes involved in lipolysis.

In human atherosclerotic plaques, macrophage derived foam cells express a low level of HSL mRNA (approximately two percent of the level found in human adipocytes) (Harte, R. A., et al., 2000, *Atherosclerosis.* 149:343-350; Reue, K., et al., 1997, *Arterioscler Thromb Vasc Biol.* 17:3428-3432). In a transgenic mouse model, macrophages specifically overexpress HSL. Surprisingly, these mice developed more advanced lesions, probably due to an increase in free cholesterol within the lesion resulting in a pro-atherogenic inflammatory response (Escary, J. L., et al., 1999, *J Lipid Res.* 40:397-404).

A third protein involved in intracellular neutral lipid storage is adipocyte differentiation related protein (ADRP, also known as adipophilin). ADRP shares

sequence homology with the N-terminal region of perilipin (Londos, C., et al., 1999, *Semin Cell Dev Biol.* 10:51-58). ADRP mRNA was detected in foam cells of human atherosclerotic plaques, and expression is upregulated in oxLDL loaded macrophages (Wang, X., et al., 1999, *FEBS Lett.* 462:145-150; Shiffman, D., et al., 2000, *J Biol. Chem.* 275:37324-37332). In early stages of adipocyte differentiation, lipid droplets are coated with ADRP, which is thought to facilitate the uptake of fatty acids and/or stabilize the lipid droplet (Jiang, H. P., et al., 1992, *Proc Natl Acad Sci U S A.* 89:7856-7860; Brasaemle, D. L., et al., 1997, *J Lipid Res.* 38:2249-2263; Gao, J., et al., 1999, *J Biol. Chem.* 274:16825-16830). In mature adipocytes, the ADRP protein is replaced by perilipin, by a mechanism involving either competition or exclusion (Brasaemle, D. L., et al., 1997, *J Lipid Res.* 38:2249-2263).

Despite some homology between ADRP and perilipin, ADRP does not take over the 'barrier role' of perilipin, since basal lipolysis is enhanced in the perilipin null mice while ADRP protein is increased (Tansey, J. T., et al., 2001, *Proc Natl Acad Sci U S A.* 98:6494-6499). Without being limited by any particular mechanism, a role for ADRP may be to facilitate the uptake and storage of neutral lipids, and a role for perilipin may be to regulate HSL dependent lipolysis.

A role for neutral lipid metabolism in atherogenesis is also shown by targeted disruption of Acyl-CoA:Cholesterol Acyltransferase (ACAT), the enzyme involved in esterification of free cholesterol within foam cells. Disruption of ACAT resulted in increased atherosclerotic lesion progression in LDL deficient mice (Fazio, S., et al., 2001, *J Clin Invest.* 107:163-171). HSL has a role as a neutral cholesterol ester hydrolase in macrophages (Reue, K., et al., 1997, *Arterioscler Thromb Vasc Biol.* 17:3428-3432; Small, C. A., et al., 1989, *FEBS Lett.* 247:205-208).

The invention herein provides a method for identifying unstable atherosclerotic plaque in a subject, comprising: providing an agent that binds with a perilipin protein; administering the agent to the subject so as to access plaque; and determining a presence of unstable plaque by locating the agent in the subject. In a related embodiment, the invention provides a method for identifying unstable atherosclerotic plaque in a sample of tissue from a subject, comprising: providing a sample of tissue; adding an agent that binds a perilipin polypeptide; and determining the presence of unstable plaque by observing binding of the agent to the tissue. In general, the agent is an antibody obtained by immunization of an animal with an epitope from a perilipin, for example a human perilipin. Alternatively, the agent is a binding protein obtained by selecting a phage

library for a clone carrying a gene variant encoding a mutated polypeptide having affinity for a perilipin.

Further, the agent, for example, the antibody is labeled with an adduct selected from molecules that are colorimetric, epitopic, magnetic, radioactive, and fluorescent markers of the antibody. An embodiment of the magnetic molecule is paramagnetic iron. An embodiment of the radioactive molecule is ^{99}technetium. In accordance with a related embodiment, the adduct is a chelating agent covalently linked to the antibody, the chelating agent capable of complexation with ^{99}technetium.

The invention further provides a composition comprising all or a portion of an amino acid sequence MSMNKGPTLLDGDLPEQENVC (SEQ ID NO: 13). Moreover, the invention provides a composition comprising all or a portion of an amino acid sequence sequences selected from the group: CLLGDLPEQENVL (SEQ ID NO: 14); PRELPARRVSDSFFRPSV (SEQ ID NO: 15); PREKPARRVSDSFFPSV (SEQ ID NO: 16); and RLAQPRRSLRSAQSPGC (SEQ ID NO: 17).

Further, the invention in related embodiments provides a nucleotide sequence encoding any of the above amino acid sequences, wherein the nucleotide is selected from the group consisting of DNA, RNA, phosphorothioate-containing DNA, and peptide nucleic acid. The nucleotide sequence can be a part of a recombinant nucleic acid vector. The vector is in a recombinant cell. For example, the recombinant cell can be a microorganism, and the microorganism is selected from the group consisting of an *Escherichia coli,* a *Bacillus subtilis,* a *Saccharomyces cerevisiae,* and a *Pichia pastoris*, and other standard organisms used for optimal protein expression of recombinant genes. Alternatively, the recombinant cell is an animal cell, for example, the animal is an insect or a warm-blooded animal, for example, the cell is from a human.

In another embodiment, the invention provides a nucleotide sequence of a gene which is transcriptionally regulated in a ruptured atherosclerotic plaque compared to a stable atherosclerotic plaque. The gene is upregulated in ruptured plaque. Alternatively, the gene is downregulated in a ruptured plaque. For example, a difference in transcriptional level of the gene in the ruptured atherosclerotic plaque compared to the stable atherosclerotic plaque is at least about two fold, at least about four fold, or at least about six fold.

In another embodiment, the invention provides a method for obtaining a perilipin binding composition, the method comprising: providing a biological sample comprising a perilipin; adding an antibody specific for the perilipin; and identifying a complex

containing antibody and perilipin, wherein the perilipin binding protein is co-complexed with the antibody and the perilipin in the complex. The biological sample is selected from a cell lysate, a spent culture medium, and a bodily fluid, and the bodily fluid is selected from the group consisting of blood, urine, saliva, cerebrospinal fluid, pleural fluid, sweat, tears, lymph, bile, and gastric fluid.

The invention provides a method of obtaining a protein that associates with perilipin in an animal, the method comprising: providing a biological sample from the animal; reacting the sample with an antibody that binds with high affinity and specificity to perilipin under a condition that maintains the protein associated with perilipin; and collecting the complex of antibody bound to perilipin associated with the protein, to obtain the protein that associates with perilipin in the animal. The method further comprises a step of identifying the protein by subjecting the protein to amino acid analysis. Further, the antibody binds with high affinity and specificity to perilipin in a ruptured plaque. The biological material may be tissue from a biopsy, for example, may be cells from the tissue that are lysed. In a further embodiment, the invention provides a protein identified by these methods. Further, the protein may provide a target for identification of novel therapeutic agents for treatment of atherosclerotic plaque.

Antibodies and Antibody Therapeutics

Anti-PERILIPIN, HSL, AND ADRP Antibodies

Included in the invention are antibodies to perilipin, hormone sensitive lipase (HSL), and adipocyte differentiation related protein (ADRP), or fragments of these polypeptides. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin (Ig) molecules, *i.e.*, molecules that contain an antigen binding site that specifically binds (immunoreacts with) an antigen. Such antibodies include, but are not limited to, polyclonal, monoclonal, chimeric, bispecific, trispecific, single chain, F_{ab}, $F_{ab'}$ and $F_{(ab')2}$ fragments, and an F_{ab} expression library.

In general, antibody molecules obtained from humans relate to any of the classes IgG, IgM, IgA, IgE and IgD, which differ from one another by the nature of the heavy chain present in the molecule. Certain classes have subclasses as well, such as IgG_1, IgG_2, IgG3 and IgG4, and including allelic varients of the same. Furthermore, in humans, the light chain may be a kappa chain or a lambda chain. In other mammals, antibodies differ in isotypes, and one skilled in the art would recognize these and understand their application in view of the teachings provided herein. Additionally,

antibodies from non-human mammals can be engineered to remove immunoreactive polypeptide sequences and replace these with nonimmunoreactive polypeptide sequences, commonly referred to as "humanized" antibodies, described further below. Reference herein to antibodies includes a reference to all such classes, subclasses and types of mammalian antibody species, in particular mice, rat, rabbit, human or engineered humanized antibodies, whether produced by immunization of an animal or by recombinant means.

Isolated perilipin, HSL, and ADRP or a portion or fragment thereof intended to serve as an antigen (individually and collectively an "antigenic peptide"), can be used as an immunogen to generate antibodies that immunospecifically bind the antigen, using standard techniques for polyclonal and monoclonal antibody preparation. The full-length protein can be used or, alternatively, the invention provides antigenic peptide fragments of the antigen for use as immunogens. An antigenic peptide fragment comprises at least 6 amino acid residues of the amino acid sequence of the full length protein, and encompasses an epitope thereof such that an antibody raised against the peptide forms a specific immune complex with the full length protein or with any fragment that contains the epitope.

Preferably, the antigenic peptide comprises at least 10 amino acid residues, or at least 15 amino acid residues, or at least 20 amino acid residues, or at least 30 amino acid residues. Preferred epitopes encompassed by the antigenic peptide are regions of the protein that are located on its surface; commonly these are hydrophilic regions. In a currently preferred embodiment, the invention provides an antibody obtained from an animal immunized with a peptide having at least 6 amino acid residues of a sequence of MSMNKGTPLLDGDLPEQENV (SEQ ID NO: 13), CLLGDLPEQENVL (SEQ ID NO: 14), PRELPARRVSDSFFRPSV (SEQ ID NO: 15), PREKPARRVSDSFFPSV (SEQ ID NO: 16), and/or RLAQPRRSLRSAQSPGC (SEQ ID NO: 17).

In certain embodiments of the invention, at least one epitope encompassed by the antigenic peptide is a region of the peptide that is located on the surface of the protein, *e.g.*, a hydrophilic region. A hydrophobicity analysis of the protein sequence will indicate which regions of the perilipin, HSL, and ADRP polypeptides are particularly hydrophilic and, therefore, are likely to encode surface residues useful for targeting antibody production. As a means for targeting antibody production, hydropathy plots showing regions of hydrophilicity and hydrophobicity may be generated by any method well known in the art, including, for example, the Kyte Doolittle or the Hopp Woods

methods, either with or without Fourier transformation. See, *e.g.*, Hopp and Woods, 1981, *Proc. Nat. Acad. Sci. USA* 78: 3824-3828; and Kyte and Doolittle 1982, *J. Mol. Biol.* 157: 105-142, each incorporated herein by reference in their entirety. Antibodies that are specific for one or more domains within an antigenic protein, or derivatives, fragments, analogs or homologs thereof, are also provided herein.

The term "epitope" includes any perilipin, HSL, and ADRP protein determinant capable of specific binding to an immunoglobulin or T-cell receptor. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. A perilipin, HSL, and ADRP polypeptide or a fragment thereof used for immunization comprises at least one antigenic epitope. An anti-perilipin, anti-HSL, and anti-ADRP antibody of the present invention is said to specifically bind to its antigen when the equilibrium binding constant (K_D) is ≤ 1 μM, preferably $\leq$ 100 nM, more preferably $\leq$ 10 nM, and most preferably $\leq$ 100 pM to about 1 pM, as measured by assays such as radioligand binding assays or similar assays known to those skilled in the art. The specificity of the antibody for ruptured atherosclerotic plaque is greater than for stable atherosclerotic plaque; for example, the relative affinity for ruptured compared to stable plaque is at least about two-fold, or at least about four-fold, or at least about six-fold greater.

Various procedures known within the art may be used for the production of polyclonal or monoclonal antibodies directed against perilipin, HSL, or ADRP, or against derivatives, fragments, analogs homologs or orthologs thereof (see, for example, Antibodies: A Laboratory Manual, Harlow E, and Lane D, 1988, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, incorporated herein by reference). Some of these antibodies are discussed below.

Polyclonal Antibodies

For the production of polyclonal antibodies, various suitable host animals (*e.g.*, rabbit, goat, mouse or other mammal) may be immunized by one or more injections with the native protein, the recombinant protein, a synthetic variant thereof, or a fragment derived from the foregoing perilipin, HSL, and ADRP. An appropriate immunogenic preparation can contain, for example, the naturally occurring immunogenic protein, a chemically synthesized polypeptide representing the immunogenic protein, or a recombinantly expressed immunogenic protein, or combinations of the above, or

including a suitable adjuvant. Furthermore, the protein may be conjugated to a second protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. The preparation can further include an adjuvant. Various adjuvants used to increase the immunological response include, but are not limited to, Freund's (complete and incomplete), mineral gels (*e.g.*, aluminum hydroxide), surface active substances (*e.g.*, lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, *etc.*), adjuvants usable in humans such as Bacille Calmette-Guerin and *Corynebacterium parvum*, or similar immunostimulatory agents. Additional examples of adjuvants which can be employed include MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate).

The resultant polyclonal antibody molecules directed against the immunogenic peptides or polypeptides, i.e., anti-perilipin, anti-HSL, and anti-ADRP can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as affinity chromatography using protein A, protein G, or the neonatal Fc receptor FcRn, which provide primarily the IgG fraction of immune serum. Subsequently, or alternatively, the specific antigen which is the target of the immunoglobulin sought, or an epitope thereof, may be immobilized on a column to purify the immune specific antibody by immunoaffinity chromatography. Purification of immunoglobulins is discussed, for example, by D. Wilkinson (The Scientist, published by The Scientist, Inc., Philadelphia PA, Vol. 14, No. 8 (April 17, 2000), pp. 25-28).

Monoclonal Antibodies

The term "monoclonal antibody" (mAb) or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one molecular species of antibody molecule consisting of a unique light chain gene product and a unique heavy chain gene product. In particular, the complementarity determining regions (CDRs) of the monoclonal antibody are identical in all the molecules of the population. mAbs thus contain an antigen binding site capable of immunoreacting with a particular epitope of the antigen characterized by a unique binding affinity for it.

Monoclonal antibodies can be prepared using hybridoma methods, such as those described by Kohler and Milstein, Nature, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing

antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes can be immunized in vitro. The immunizing agent will typically include the perilipin, HSL, or ADRP antigens, fragments thereof or fusion proteins thereof. Generally, either peripheral blood lymphocytes are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103). Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells can be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Manassas, Virginia. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63).

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the anti- perilipin, anti-HSL, and anti-ADRP monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980), or by non-

linear regression models. It is an objective, especially important in therapeutic applications of monoclonal antibodies, to identify antibodies having a high degree of specificity and a high binding affinity for the target antigen.

After the desired hybridoma cells are identified, the clones can be subcloned by limiting dilution procedures and grown by standard methods (Goding,1986). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells can be grown in vivo as ascites in a mammal, for example but not limited to a mouse. The monoclonal antibodies secreted by the subclones can be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography and the methods disclosed above for purification of polyclonal antibodies.

The monoclonal antibodies can also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA can be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also can be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, Nature 368, 812-13 (1994)) or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

Humanized Antibodies

The antibodies directed against the perilipin, HSL, and ADRP antigens of the invention can further comprise humanized antibodies or human antibodies. These

antibodies are suitable for administration to humans without engendering an immune response by the human against the administered immunoglobulin. Humanized forms of antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) that are principally comprised of the sequence of a human immunoglobulin, and contain minimal sequence derived from a non-human immunoglobulin. Humanization can be performed following the method of Winter and co-workers (Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. (See also U.S. Patent No. 5,225,539.) In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies can also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones et al., 1986; Riechmann et al., 1988; and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)).

Human Antibodies

Fully human antibodies essentially relate to antibody molecules in which the entire sequence of both the light chain and the heavy chain, including the CDRs, arise from human genes. Such antibodies are termed "human antibodies", or "fully human antibodies" herein. Human monoclonal antibodies against perilipin, HSL, and ADRP can be prepared by the trioma technique; the human B-cell hybridoma technique (see Kozbor, et al., 1983 Immunol Today 4: 72) and the EBV hybridoma technique to produce human monoclonal antibodies (see Cole, et al., 1985 In: MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc., pp. 77-96). Human anti-perilipin, anti-HSL, and anti-ADRP monoclonal antibodies may be utilized in the practice of the present invention and may be produced by using human hybridomas (see Cote, et al., 1983. Proc Natl Acad Sci USA 80: 2026-2030) or by transforming human B-cells with Epstein Barr Virus in vitro (see Cole, et al., 1985 In: MONOCLONAL ANTIBODIES AND

CANCER THERAPY, Alan R. Liss, Inc., pp. 77-96).

In addition, human antibodies can also be produced using additional techniques, including phage display libraries (Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)). Similarly, human antibodies can be made by introducing human immunoglobulin loci into transgenic animals, *e.g.*, mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge with perilipin, HSL, or ADRP polypeptides or immunogenic compositions including these, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in Marks et al. (Bio/Technology 10, 779-783 (1992)); Lonberg et al. (Nature 368 856-859 (1994)); Morrison (Nature 368, 812-13 (1994)); Fishwild et al, (Nature Biotechnology 14, 845-51 (1996)); Neuberger (Nature Biotechnology 14, 826 (1996)); and Lonberg and Huszar (Intern. Rev. Immunol. 13 65-93 (1995)).

Human antibodies may additionally be produced using transgenic nonhuman animals which are modified so as to produce fully human antibodies rather than the animal's endogenous antibodies in response to challenge by an antigen. (See PCT publication WO94/02602). The endogenous genes encoding the heavy and light immunoglobulin chains in the nonhuman host have been incapacitated, and active loci encoding human heavy and light chain immunoglobulins are inserted into the host's genome. The human genes are incorporated, for example, using yeast artificial chromosomes containing the requisite human DNA segments. An animal which provides all the desired modifications is then obtained as progeny by crossbreeding intermediate transgenic animals containing fewer than the full complement of the modifications. The preferred embodiment of such a nonhuman animal is a mouse, and is termed the Xenomouse™ as disclosed in PCT publications WO 96/33735 and WO 96/34096. This animal produces B cells which secrete fully human immunoglobulins. The antibodies can be obtained directly from the animal after immunization with an immunogen of interest, as, for example, a preparation of a polyclonal antibody, or alternatively from immortalized B cells derived from the animal, such as hybridomas producing monoclonal antibodies. Additionally, the genes encoding the immunoglobulins with human variable regions can be recovered and expressed to obtain the antibodies directly, or can be further modified to obtain analogs of antibodies such

as, for example, single chain Fv molecules.

An example of a method of producing a nonhuman host, exemplified as a mouse, lacking expression of an endogenous immunoglobulin heavy chain is disclosed in U.S. Patent No. 5,939,598. It can be obtained by a method including deleting the J segment genes from at least one endogenous heavy chain locus in an embryonic stem cell to prevent rearrangement of the locus and to prevent formation of a transcript of a rearranged immunoglobulin heavy chain locus, the deletion being effected by a targeting vector containing a gene encoding a selectable marker; and producing from the embryonic stem cell a transgenic mouse whose somatic and germ cells contain the gene encoding the selectable marker.

A method for producing an antibody of interest, such as a human antibody, is disclosed in U.S. Patent No. 5,916,771. It includes introducing an expression vector that contains a nucleotide sequence encoding a heavy chain into one mammalian host cell in culture, introducing an expression vector containing a nucleotide sequence encoding a light chain into another mammalian host cell, and fusing the two cells to form a hybrid cell. The hybrid cell expresses an antibody containing the heavy chain and the light chain. In a further improvement on this procedure, a method for identifying a clinically relevant epitope on an immunogen, and a correlative method for selecting an antibody that binds immunospecifically to the relevant epitope with high affinity, are disclosed in PCT publication WO 99/53049.

F_{ab} Fragments and Single Chain Antibodies

According to the invention, techniques can be adapted for the production of single-chain antibodies specific to an antigenic protein of the invention, e.g. perilipin, HSL, and ADRP (see *e.g.*, U.S. Patent No. 4,946,778). In addition, methods can be adapted for the construction of F_{ab} expression libraries (see *e.g.*, Huse, et al., 1989 Science 246: 1275-1281) to allow rapid and effective identification of monoclonal F_{ab} fragments with the desired specificity for a protein or derivatives, fragments, analogs or homologs thereof. Antibody fragments that contain the idiotypes to a protein antigen may be produced by techniques known in the art including, but not limited to: (i) an $F_{(ab')2}$ fragment produced by pepsin digestion of an antibody molecule; (ii) an F_{ab} fragment generated by reducing the disulfide bridges of an $F_{(ab')2}$ fragment; (iii) an F_{ab} fragment generated by the treatment of the antibody molecule with papain and a reducing agent and (iv) F_v fragments.

Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens, i.e., perilipin, and HSL, HSL and ADRP, or perilipin and ADRP. Alternatively, one of the binding specificities is for an antigenic protein of the invention (perilipin, HSL orADRP), and the second binding target is any other antigen, and advantageously is a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, Nature, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, 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 co-transfected into a suitable host organism. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

According to another approach described in WO 96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the CH3 region of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created

on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* $F(ab')_2$ bispecific antibodies). Techniques for generating bispecific antibodies from antibody fragments have been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., Science 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Additionally, Fab' fragments can be directly recovered from E. coli and chemically coupled to form bispecific antibodies. Shalaby et al., J. Exp. Med. 175:217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from E. coli and subjected to directed chemical coupling in vitro to form the bispecific antibody. Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers described in Kostelny et al., J. Immunol. 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., Proc. Natl. Acad. Sci. USA 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain.

Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See, Gruber et al., J. Immunol. 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., J. Immunol. 147:60 (1991). Exemplary bispecific antibodies can bind to two different epitopes, at least one of which originates in the protein antigen of the invention. Alternatively, an anti-antigenic arm of an immunoglobulin molecule can be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.* CD2, CD3, CD28, or B7), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the cell expressing the particular antigen. Bispecific antibodies can also be used to direct cytotoxic agents to cells which express a particular antigen. These antibodies possess an antigen-binding arm and an arm which binds a cytotoxic agent or a radionuclide chelator, such as EOTUBE, DPTA, DOTA, or TETA.

Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360; WO 92/200373; EP 03089). It is contemplated that the antibodies can be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins can be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Patent No. 4,676,980.

Effector Function Engineering

It can be desirable to modify the antibody of the invention with respect to effector function, so as to enhance, *e.g.*, the effectiveness of the antibody in treating cancer. For example, cysteine residue(s) can be introduced into the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated can have improved internalization capability and/or increased

complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., J. Exp Med., 176: 1191-1195 (1992) and Shopes, J. Immunol., 148: 2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity can also be prepared using heterobifunctional cross-linkers as described in Wolff et al. Cancer Research, 53: 2560-2565 (1993). Alternatively, an antibody can be engineered that has dual Fc regions and can thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al., Anti-Cancer Drug Design, 3: 219-230 (1989).

Immunoconjugates

The invention also pertains to immunoconjugates comprising an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.*, an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (*i.e.*, a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof that can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include ^{212}Bi, ^{131}I, ^{125}I, ^{90}Y, and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science, 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See

WO94/11026.

In another embodiment, the antibody can be conjugated to a "receptor" (such streptavidin) for utilization in plaque pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.*, avidin) that is in turn conjugated to a cytotoxic agent.

Immunoliposomes

The antibodies disclosed herein can also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., Proc. Natl. Acad. Sci. USA, 82: 3688 (1985); Hwang et al., Proc. Natl Acad. Sci. USA, 77: 4030 (1980); 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. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al .,_J. Biol. Chem., 257: 286-288 (1982) via a disulfide-interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained within the liposome. See Gabizon *et al.*, J. National Cancer Inst., 81(19): 1484 (1989).

Diagnostic Applications of Antibodies of the Invention

In one embodiment, methods for the screening of antibodies that possess the desired specificity include, but are not limited to, enzyme linked immunosorbent assay (ELISA) and other immunologically mediated techniques known within the art. In a specific embodiment, selection of antibodies that are specific to a particular domain of a perilipin, HSL, or ADRP protein is facilitated by generation of hybridomas that bind to the fragment of a perilipin, HSL, and ADRP protein possessing such a domain. Thus, antibodies that are specific for a desired domain within an PERILIPIN, HSL, AND ADRP protein, or derivatives, fragments, analogs or homologs thereof, are also provided herein.

Antibodies directed against a perilipin, HSL, and ADRP protein of the invention may be used in methods known within the art relating to the localization and/or quantitation of a perilipin, HSL, and ADRP protein (*e.g.*, for use in measuring levels of

the perilipin, HSL, and ADRP protein within appropriate physiological samples, for use in diagnostic methods, for use in imaging the protein, and the like). In a given embodiment, antibodies specific to a perilipin, HSL, and ADRP protein, or derivative, fragment, analog or homolog thereof, that contain the antibody derived antigen binding domain, are utilized as pharmacologically active compounds (referred to hereinafter as "Therapeutics").

An antibody specific for a perilipin, HSL, and ADRP protein of the invention (e.g., a monoclonal antibody or a polyclonal antibody) can be used to isolate a perilipin, HSL, and ADRP polypeptide by standard techniques, such as immunoaffinity, chromatography or immunoprecipitation. An antibody to a perilipin, HSL, and ADRP polypeptide can facilitate the purification of a natural perilipin, HSL, and ADRP antigen from cells, or of a recombinantly produced perilipin, HSL, and ADRP antigen expressed in host cells. Moreover, such an anti- perilipin, HSL, and ADRP antibody can be used to detect the antigenic perilipin, HSL, and ADRP protein (*e.g.*, in a plaque, thrombus, cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the antigenic perilipin, HSL, and ADRP protein. Antibodies directed against a perilipin, HSL, and ADRP protein can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, *e.g.*, to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

Antibody Therapeutics

Antibodies of the invention, including polyclonal, monoclonal, humanized and fully human antibodies, may used as therapeutic agents. Such agents will generally be employed to treat or prevent a disease or pathology in a subject, e.g., prevention or

destruction of plaques or thrombi. An antibody preparation, preferably one having high specificity and high affinity for its target antigen, is administered to the subject and will generally have an effect due to its binding with the target. Such an effect may be one of two kinds, depending on the specific nature of the interaction between the given antibody molecule and the target antigen in question. In the first instance, administration of the antibody may abrogate or inhibit the binding of perilipin, HSL, and ADRP with an endogenous ligand to which it naturally binds. In this case, the antibody binds to the perilipin, HSL, and ADRP and masks a binding site of the naturally occurring ligand, wherein the ligand serves as an effector molecule. Thus the receptor mediates a signal transduction pathway for which ligand is responsible.

Alternatively, the effect may be one in which the antibody elicits a physiological result by virtue of binding to an effector binding site on the target perilipin, HSL, and ADRP molecule. In this case the target, a receptor having an endogenous ligand which may be absent or defective in the disease or pathology, binds the antibody as a surrogate effector ligand, initiating a receptor-based signal transduction event by the receptor.

A therapeutically effective amount of an antibody of the invention relates generally to the amount needed to achieve a therapeutic objective. As noted above, this may be a binding interaction between the antibody and its target antigen that, in certain cases, interferes with the functioning of the target, and in other cases, promotes a physiological response. The amount required to be administered will furthermore depend on the binding affinity of the antibody for its specific antigen, and will also depend on the rate at which an administered antibody is depleted from the free volume other subject to which it is administered. Common ranges for therapeutically effective dosing of an antibody or antibody fragment of the invention may be, by way of nonlimiting example, from about 0.1 mg/kg body weight to about 50 mg/kg body weight. Common dosing frequencies may range, for example, from twice daily to once a week.

Pharmaceutical Compositions of Antibodies

Antibodies of the invention specifically binding a perilipin, an HSL, or an ADRP protein, as well as other molecules identified by the screening assays disclosed herein, can be administered for the treatment of various disorders in the form of pharmaceutical compositions. Principles and considerations involved in preparing such compositions, as well as guidance in the choice of components are provided, for example, in Remington : The Science And Practice Of Pharmacy 19th ed. (Alfonso R. Gennaro, et al., editors) Mack Pub. Co., Easton, Pa. : 1995; Drug Absorption Enhancement : Concepts,

Possibilities, Limitations, And Trends, Harwood Academic Publishers, Langhorne, Pa., 1994; and Peptide And Protein Drug Delivery (Advances In Parenteral Sciences, Vol. 4), 1991, M. Dekker, New York.

If the antigenic protein is intracellular and whole antibodies are used as inhibitors, internalizing antibodies are preferred. However, liposomes can also be used to deliver the antibody, or an antibody fragment, into cells. Where antibody fragments are used, the smallest inhibitory fragment that specifically binds to the binding domain of the target protein is preferred. For example, based upon the variable-region sequences of an antibody, peptide molecules can be designed that retain the ability to bind the target protein sequence. Such peptides can be synthesized chemically and/or produced by recombinant DNA technology. See, *e.g.*, Marasco et al., Proc. Natl. Acad. Sci. USA, 90: 7889-7893 (1993). The formulation herein can also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Alternatively, or in addition, the composition can comprise an agent that enhances its function, such as, for example, a cytotoxic agent, cytokine, chemotherapeutic agent, or growth-inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients can 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.

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations can 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 γ 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), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods.

ELISA Assay

An agent for detecting an analyte perilipin, HSL, and ADRP protein is an antibody capable of binding to an analyte protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, F_{ab} or $F_{(ab)2}$) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently-labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently-labeled streptavidin. The term "biological sample" is intended to include tissues such as those obtained during surgery, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. Included within the usage of the term "biological sample", therefore, is blood and a fraction or component of blood including blood serum, blood plasma, or lymph. That is, the detection method of the invention can be used to detect an analyte mRNA, protein, or genomic DNA in a biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of an analyte mRNA include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of an analyte protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations, and immunofluorescence. *In vitro* techniques for detection of an analyte genomic DNA include Southern hybridizations. Procedures for conducting immunoassays are described, for example in "ELISA: Theory and Practice: Methods in Molecular Biology", Vol. 42, J. R. Crowther (Ed.) Human Press, Totowa, NJ, 1995; "Immunoassay", E. Diamandis and T. Christopoulus, Academic Press, Inc., San Diego, CA, 1996; and "Practice and Thory of Enzyme Immunoassays", P. Tijssen, Elsevier Science Publishers, Amsterdam, 1985. Furthermore, *in vivo* techniques for detection of an analyte protein include introducing into a subject a labeled anti-an analyte protein antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be

detected by standard imaging techniques.

Pharmaceutical Compositions

The perilipin, HSL, and ADRP nucleic acid molecules, perilipin, HSL, and ADRP proteins, and anti- perilipin, HSL, and ADRP antibodies (also referred to herein as "active compounds") of the invention, and derivatives, fragments, analogs and homologs thereof, can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, finger's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (*i.e.*, topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a perilipin, HSL, and ADRP protein or anti- perilipin, HSL, and ADRP antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared

using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer. Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery. In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in

dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (*see, e.g.,* U.S. Patent No. 5,328,470) or by stereotactic injection (*see, e.g.*, Chen, *et al.*, 1994. *Proc. Natl. Acad. Sci. USA* 91: 3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells that produce the gene delivery system. The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

Nucleic Acids

As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (e.g., cDNA or genomic DNA), RNA molecules (e.g., mRNA), and analogs of DNA, such as peptide nucleic acid molecules (PNA), phosphorothioate DNA, and DNA based on a peptide analog backbone such as a trans-olefin peptidomimetics and phosphonate peptidomimetics. Peptide nucleic acid (PNA) is an oligomer in which the charged phosphateribose backbone has been eliminated and replaced with an uncharged polyamide backbone (Egholm M., Buchardt O., Nielsen P E., Berg R H., J. Am. Chem. Soc., 114: 1895-1897; 1992). These oligomers have been reported to resist nuclease and protease degradation (Egholm M., Buchardt O., Christensen L., Behrens C., Freier S M., Driver D A., Berg R H., Kim S K., Norden B., Nielsen P E., Nature, 365: 566-568; 1993.). Furthermore, the binding affinities of PNA for its complementary single-stranded PNA has been shown to exceed that of comparable DNAs (Egholm M., Buchardt O., Nielsen P E., Berg R H., J. Am. Chem. Soc., 114: 1895-1897; 1992).

A nucleic acid molecule may be single-stranded or double-stranded, for example,

it is single stranded. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature, whether it is prepared by isolation from an organism or is chemically synthesized. A nucleic acid may be chemically synthesized using a commercially available automated synthesizer and reagents, or custom made by a commercial supplier (for example, PerSeptive Biosystems, Framingham, Mass.). An "isolated" nucleic acid molecule is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments an isolated tumor-specific nucleic acid molecule may contain less than about 5 kb, about 1 kb, about 0.5 kb, about 100 bases or about 50 bases of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived (e.g. a human brain tumor). Moreover, an "isolated" nucleic acid molecule, such as an RNA molecule, may be free of other cellular material. The nucleic acid molecule may comprise only a portion of a coding region of a naturally occurring sequence.

It is also possible to modify the structure of the oligonucleotides and polynucleotides for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., ex vivo shelf life and resistance to nucleolytic degradation in vivo). Exemplary modified nucleic acids and nucleic acid analogs include PNA and phosphorothioate-linked nucleic acids. Such modified nucleotides are considered functional equivalents of the compositions described in more detail herein.

A nucleic acid molecule having a known nucleotide sequence can be isolated using standard molecular biology techniques (e.g., as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). For example, mRNA can be isolated from cells (e.g., by the guanidinium-thiocyanate extraction procedure of Chirgwin et al. (1979) Biochemistry 18: 5294-5299) and cDNA can be prepared using reverse transcriptase (e.g., Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, Md.; or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, Fla.). Synthetic oligonucleotide primers for PCR amplification can be designed based upon knowledge of the nucleotide sequence or fragments of the sequence, as will be appreciated by those with skill in the art.

In certain embodiments, an isolated nucleic acid molecule useful in the compositions and methods of the invention is at least about 12 nucleotides in length and

hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence of interest. In other embodiments, a nucleic acid is at least about 15, about 20, about 30, about 50, or about 100 nucleotides in length. As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences that are at least about 60% homologous to each other typically remain hybridized to each other. In various embodiments, the conditions are such that at least sequences at least about 65%, for example, at least about 70%, and at least about 75% homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found, e.g.,in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A non-limiting example of stringent hybridization conditions is hybridization in about 6X sodium chloride/sodium citrate (SSC) at about 45°C., followed by one or more washes in a buffer containing about 0.2X SSC, and about 0.1% SDS at a temperature of about 50 ° C to about 65° C. A naturally occurring nucleic acid molecule comprises any isolated nucleic acid molecule that hybridizes under stringent conditions to a sequence of the invention corresponds to.

Another aspect of the invention provides a nucleic acid which hybridizes under high stringency conditions to a "probe", which is a nucleic acid which encodes a portion of any of the nucleotides sequences as shown in SEQ ID Nos: 1-13. A suitable probe is at least 12 nucleotides in length, is single-stranded, and is labeled, for example, radiolabeled or fluorescently labeled. Appropriate moderate conditions of stringency of conditions of formation of double-strandedness which promote DNA hybridization, for example, about 6X sodium chloride/sodium citrate (SSC) at about 45°C, are followed by successive washes of greater stringency, e.g., about 2X SSC at 50°C, and are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Other suitable stringency conditions include selecting the salt concentration in the wash step from a stringency of about 2X SSC at about 50°C, and then using a wash of a high stringency condition, of about 0.2X SSC at about 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions, e.g., at room temperature, about 22°C, to very high stringency conditions at about 65°C. A perilipin protein which is an embodiment of the invention is encoded by a gene that hybridizes to a nucleotide sequence from the normal human perilipin gene under stringent conditions.

Conditions for hybridizations are largely dependent on the melting temperature

for half of the molecules of a substantially pure population of a double-stranded nucleic acid, a parameter known as the T_m. For nucleic acids of sequence about 11 to about 23 bases, the approximate T_m can be calculated in °C as 2(number of A+T residues) + 4(number of C+G residues). Hybridization or annealing of the probe to the nucleic acid being probed should be conducted at a temperature lower than the T_m, e.g., about 15°C, about 20EC, about 25°C or about 30°C lower than the T_m. The effect of salt concentration (in M of NaCl) can also be calculated, see for example, Brown, A., "Hybridization" pp. 503-506, in *The Encyclopedia of Molec. Biol.*, J. Kendrew, Ed., Blackwell, Oxford (1994).

In an embodiment of the invention, a mouse mutant that is an animal model of human atherosclerosis is provided. An "animal model" for a disease is an animal treated with a chemical composition, or a mutant animal, or a transgenic animal such as an animal that carries a knock-out mutation in a gene of interest, that displays symptoms identical or similar to a human subject having the disease. Animal models for human atherosclerosis can be formed using the animal mutants that are embodiments of the present invention, for example, mouse mutants that are transgenic animals that carry a human perilipin gene, and have a knock-out of the animal's endogenous gene that encodes the animal version of this protein.

A knock-out animal can be constructed by methods known in the art, for example, see Snouwaert et al. 1992 *Science* Vol 257:1083; and Lowell et al. 1993 *Nature* Vol 366: 740. Further, the animal can be heterozygous for the knock-out mutation, or can even be homozygous for a knock-out mutation in a gene that is not essential. Mice carrying knock-out mutations for the murine perilipin protein are known to one of skill in the art, and homozygote mice of this genotype, i.e., mice carrying two copies of this mutation and having no gene encoding normal murine perilipin, have been constructed, and are found to be viable and non-obese in phenotype.

Murine perilipin-knockout mice can be further genetically manipulated to carry a gene for human perilipin, by techniques well-known in the art of transgenic mice. Further, the gene for perilipin can be regulated, for example, can be provided with a promoter that is capable of up-regulation by addition of a chemical inducer, such as with a tetracycline-inducible promoter. See, for example, U.S. patent number 6,271,348 issued Aug. 7, 2001, and 6,271,341, issued Aug. 7, 2001.

In addition to nucleic acid molecules with sequences of nucleotides, for example, encoding the perilipin protein, the invention encompasses use of nucleic acid molecules

which are antisense thereto. An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid, e.g., complementary to the coding strand of a double-stranded cDNAmolecule or complementary to an mRNA sequence. Antisense constructs of the present invention, by antagonizing the normal biological activity of tumor-specific or infectious DNA can be used in the therapeutic context, both to deliver a therapeutic dose of a covalently bound atom or molecule such as a radionuclide, and to inhibit expression of plaque-specific genetic information. In an embodiment, antisense nucleic acid can deliver a radionuclide complexed to a chelator covalently linked to the nucleic acid, to the target cells, tissue or sample.

Antisense nucleic acids can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid can be complementary to an entire coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues. In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence of interest. The term "noncoding region" refers to 5' and 3' sequences which flank a coding region and are not translated into amino acids (i.e., also referred to as 5' and 3' untranslated regions). Antisense nucleic acid may be complementary to either or both of a coding region and an adjacent noncoding region.

The antisense nucleic acid molecule may be complementary to an entire coding region, for example, an antisense nucleic acid is an oligonucleotide or a polynucleotide which is antisense to only a portion of a coding or noncoding region. For example, an antisense oligonucleotide may be complementary to the region surrounding the translation start site of an mRNA. An antisense polynucleotide can be, for example, about 15, about 20, about 25, about 30, about 35, about 40, about 45 or about 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and/or enzymatic reactions using procedures known in the art.

Further, an antisense nucleic acid (e.g., an antisense polynucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., peptide nucleic acid or phosphorothioate nucleotides can be used. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into

which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid).

Further, an antisense polynucleotide can have added to it one or more sequences known as ribozymes, which are capable of acting as an endonuclease and causing cleavage of a double-stranded region formed by complementarity and base-specific binding of the antisense polynucleotide to mRNA in a cell. The ribozyme, which is well-known in the art (see, for example, U.S. patent number 6,103,890, issued Aug. 15, 2000, Jarvis et al.), forms a "hammerhead" configuration which is a hairpin loop, to act as a ribonuclease for a double-stranded RNA in proximity to the hammerhead (see, U.S. patent number 5,525,468, issued June 11, 1996.)

The antibodies provided herein e.g., specific for perilipin in ruptured atherosclerotic plaque, can be combined with magnetic materials for use as imaging agents for clinical applications, which are well-known to practitioners of ordinary skill in the art of whole body imaging for diagnostic purposes. See U.S. patent numbers 4,770,183 issued Sept. 13, 1988, and 4,827,945 issued May 9, 1989. Magnetically active metals include iron, cobalt, and manganese. Metal particles of about 50 Å to about 300 Å in diameter, or about 10 Å to about 500 Å in diameter, can be coated with a protein such as the anti-perilipin antibody or antibody fragments described herein.

The magnetic materials are used as contrast-enhancing agents for in vivo magnetic resonance (MR) imaging. In one embodiment, the particles are dispersed in a suitable injection medium, such as distilled water or normal saline, or any other physiologically acceptable carrier known in the art, to form a dispersoid which is introduced into the subject's vascular system by intravenous injection. The particles are then carried through the vascular system to the target organ where they are taken up. Regardless of the route, once administered, the particles distribute to and collect rapidly in the target organs, generally in 30 minutes to an hour. In the organ, these superparamagnetic particles will alter the magnetic fields produced by the MR imager.

The antibodies provided herein can be conjugated to fluorescent dyes, described for example, in U.S. patent number 6,146,837, issued Nov. 14, 2000. Methods for conjugating an antibody to the dye can be found, for example, see Harlow, E. et al. "Antibodies, a Laboratory Manual", 1988, Cold Spring Harbor Press, pp. 353-357, and Table 10.3C p. 409). Commercial services are available for performing custom chemical coupling of an antibody as provided, and a fluorescent dye (Molecular Probes, Eugene,

OR). Immunoprecipitations can be performed in "Using Antibodies, a Laboratory Manual", 1999, Cold Spring Harbor Press, pp. 223-265.

An antibody can be covalently attached to a Green Fluorescent Protein (GFP), or an antibody-GFP fusion protein can be engineered, for use as an imaging agent. Whole body imaging of GFP expressed in tumors in animals is shown in Yang, M. et al., Proc Nat Acad Sci US 97(3): 1206-1211. GFP and other fluorescent proteins having different emission spectra, and vectors encoding these proteins, are available from BD Clontech, Palo Alto, CA. Custom cloning services to produce fusions of genes encoding antibody specificity determinants to fluorescent proteins are also available.

The term "chelator", as used herein, refers to a chemical compound which is capable of binding an atom of a radionuclide, for example, through non-covalent interactions, e.g., through ionic interactions. In most embodiments, the radionuclide is a radioactive isotope atom of an element.

Chelators which bind to radionuclides are known in the art, see, e.g., M. Nicolini et al., eds., "Technetium and Rhenium in Chemistry and Nuclear Medicine," SG Editoriali, Padova (1995). In general, exemplary chelators are capable of binding to radionuclides such as $Tc(O^{3+})$. Radioactive compounds such as Tc-99m-labeled reagents for imaging various tissues and organs are known in the art (see, Fritzberg et al. 1988 *Proc. Matl. Acad. Sci.* Vol 85:4025-4029; Hnatowich et al. U.S. patent number 5,980,861 issued Nov. 9, 1999; Lister-James et al. U.S. patent number 6,248,304 issued June 19, 2001). Hnatowich et al. provides methods of forming a polymer-chelator-radionuclide complex under mild conditions, for example such that a polymer which is an antibody can be radiolabeled for use in whole body imaging. The antibody or antibody fragments with affinity for perilipin as described herein can be covalently linked to the chelator composisitons of Hnatowich et al., for non-radioactive storage and handling by clinicians.

The radionuclide can be provided prior to administration to the subject, for example a subject having atherosclerotic plaque. The anti-perilipin antibody moiety preferentially directs this composition to unstable and ruptured plaque. While technetium-99m is commonly used, other radionuclides suitable for complexation with the chelator-anti-perilipin antibody include technetium-93m, technetium-95m, rhenium-186, rhenium-188, and rhenium-189.

Combinatorial libraries can be screened to obtain members of the library with a desired binding activity, for example, down-regulation of the gene for human perilipin,

and to identify the active species, by methods that have been described (see, e.g., Gordon et al. 1994 *J. Med. Chem.* 37:1385-1401). These include affinity chromatography with an appropriate biological "receptor" to isolate ligands for this receptor, followed by identification of the isolated ligands by conventional techniques (e.g., with cultured tissue as described herein, or by mass spectrometry and NMR).

The synthesis of combinatorial libraries is well known in the art and has been reviewed (see, e.g., E. M. Gordon et al., *supra*). The subject invention contemplates methods for synthesis of combinatorial libraries of transcriptional effector molecules, or molecules that bind to perilipin in site. Such libraries can be synthesized according to a variety of methods. For example, a "split-pool" strategy can be implemented in the following way: beads of a functionalized polymeric support (e.g., a resin for peptide synthesis, e.g., Merrifield resin) are placed in a plurality of reaction vessels. To each sample of beads is added a solution of a different chelator (e.g., different tripeptides), and the reactions proceed to yield a plurality of solid-supported chelator compounds. The aliquots of derivatized beads are then washed, "pooled" (i.e., recombined), and the pool of beads is again divided, with each aliquot being placed in a separate reaction vessel. To each reaction vessel is added a solution of a different S-protected thioglycolic acid (i.e., differently S-protected), and reaction occurs to yield a plurality of reaction vessels, each containing a plurality of S-protected mercaptoacetylchelator compounds, which can then be released from the solid support and screened, e.g., for lipophilicity, ease of removal of the protecting group, and the like.

Other synthesis methods, including the "diversomer library" synthesis of Hobbs DeWitt et al. 1993 (*Proc. Natl. Acad. Sci.* U.S.A. 90:6909) or the "tea-bag" technique of Houghten 1993 (see, e.g., Houghten et al. Nature 354:84-86) can also be used to synthesize libraries of compounds according to the subject invention.

The methods of the present invention include use of, in one embodiment, an antibody, and in another embodiment, an anti-sense nucleic acid as a pharmaceutical composition suitable for administration to a subject. In one embodiment, the antibody includes specificity determinants for perilipin. In the other embodiment, the anti-sense nucleic acid includes a nucleotide sequence complementary to a naturally occurring transcript of the gene encoding human perlipin.

A composition of the present invention can be administered by a variety of methods known in the art as is appreciated by the skilled artisan. The active compound can be prepared with an excipient or a carrier that will protect it against rapid release,

such as a controlled release formulation, including implants, transdermal patches, microencapsulated delivery systems. Many methods for the preparation of such formulations are patented and are generally known to those skilled in the art. See, e.g., *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, Ed., Marcel Dekker, Inc., NY, 1978. Therapeutic compositions for delivery in a pharmaceutically acceptable excipient are sterile, and are stable under the conditions of manufacture, sterilization, and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration.

Dosage regimens can be adjusted to provide the optimum desired response (e.g., a therapeutic response such as inhibition of rupture of plaque). For example, a single bolus can be administered, several divided doses can be administered over time, or the dose can be proportionally reduced or increased as indicated by the exigencies of the extent of atherosclerosis. Determination of an effective dose and a protocol for administration can be determined by one of skill in the art.

In general, an exemplary embodiment of the invention is to administer a suitable daily dose of a therapeutic composition that will be the lowest effective dose to produce a therapeutic effect, for example, mitigation of symptoms, for example, a change in the appearance of atherosclerotic plaque. The therapeutic compounds of the invention are administered at a dose per subject per day of at least about 2 mg, at least about 5 mg, at least about 10 mg or at least about 20 mg as appropriate minimal starting dosages. In general, the compound of the effective dose of the composition of the invention can be administered in the range of about 50 micrograms to about 400 micrograms of the compound per kilogram of the subject per day.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective dose of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compound of the invention employed in the pharmaceutical composition at a level lower than that required in order to achieve the desired therapeutic effect, and increase the dosage with time until the desired effect is achieved.

In another embodiment, the pharmaceutical composition includes also an additional therapeutic agent. Thus in a method of the invention the pharmaceutical composition can be administered as part of a combination therapy, i.e. in combination with an additional agent or agents. Examples of materials that can be used as combination therapeutics with the antibodies or antisense molecules for treatment of

atherosclerotic disease as additional therapeutic agents include: an anti-clot agent, such as heparin or aspirin; a heart stabilizer such as digitonin; an enzyme inhibitor which can be a protein, such as α_1-antitrypsin, or aprotinin; an enzyme inhibitor which can be a cyclooxygenase inhibitor; an engineered binding polypeptide, for example, an engineered polypeptide that is a protease inhibitor such an engineered inhibitor of kallikrein; an antibacterial agent, which can be an antibiotic such as amoxicillin, rifampicin, erythromycin; an antiviral agent, which can be a low molecular weight chemical, such as acyclovir; a steroid, for example a corticosteroid, or a sex steroid such as progesterone; a non-steroidal anti-inflammatory agent such as aspirin, ibuprofen, or acetaminophen; an anti-cancer agent such as methotrexate or adriamycin; or a cytokine.

A therapeutic agent to be used with the composition of the invention can be an engineered binding polypeptide, known to one of skill in the art of remodeling a protein that is covalently attached to a virion coat protein by virtue of genetic fusion (U.S. Patent 5,233,409; U.S. Patent 5,403,484), and can be made according to methods known in the art. A protein that binds any of a variety of other targets can be engineered and used in the present invention as a therapeutic agent in combination with an antibody or anti-sense nucleic acid of the invention.

An improvement in the symptoms as a result of such administration is noted by a reduction in formation or appearance of ruptured plaque, or rupture of stable plaque, by at least about 10%, for example, by at least about 20%, by at least about 30%, or by at least about 40%, relative to untreated subjects.

While differential gene expression between activated and non-activated human umbilical vein cells and vascular smooth muscle cells (Lu K. et al. 1998 *Biochem. Biophys. Res. Commun.* Vol. 253 pp. 828-833; de Vries C. et al. 2000 *J. Biol. Chem.* Vol. 275 pp. 23939-23947) has been analyzed, however, it remains to be determined whether gene expression in those tissues *in vitro* mimics gene expression *in vivo.*

Antibodies having affinity for oxidation-specific epitopes on lipoproteins, including atherosclerotic plaque in arterial tissue are described (U.S. patent number 6,225,070 issued May 1, 2001). The lipoproteins include LDL, and apo B, but not perilipin, hormone sensitive lipase, nor adipocyte differentiation related differentiation protein. Further, antibodies are used to detect atheroma formation *in vivo*, and do not distinguish stable and ruptured plaque.

As shown in the Examples herein, genes that are differentially expressed in a larger panel of whole mount stable or ruptured human atherosclerotic plaques have been

identified, and several candidates that may be involved in destabilization of atherosclerotic plaques have been found.

Embodiments of the invention are illustrated by the following examples, which should not be construed as further limiting. The contents of all references, patents, and published patent applications, cited throughout this application, are hereby expressly incorporated herein by reference.

EXAMPLES

The following Materials and Methods are used throughout the Examples herein.

Materials and Methods

Suppressive subtractive hybridization (SSH) was performed on RNA pools of three ruptured and three stable human atherosclerotic plaques. Two libraries were constructed, one containing clones up regulated in ruptured plaques and the other containing clones down regulated in ruptured plaques. Differential expression was confirmed by macro array analysis. Clones showing an at least 2-fold difference in expression were sequenced. To validate the reproducibility of expression of these clones, reverse transcriptase PCR (RT-PCR) analysis was performed on a larger series of ruptured (n=10) and stable (n=10) plaques. Cellular distribution of the two clones with the most reproducible expression pattern was assessed by RNA *in situ* hybridization, and expression and localization of the protein of one of the two clones was determined by immunohistochemistry. Figure 1 schematically depicts the experimental design and the identification of clones by reduction in the number of clones carrying candidate genes at each of the various screening steps.

Tissue sampling and RNA isolation

Plaques were obtained from patients undergoing vascular surgery (Departments of General Surgery and Pathology, Academic Hospital Maastricht). Patient characteristics are summarized in Tables 1 and 2. Immediately after resection, the atherosclerotic specimen was divided into parallel portions of 5 mm for RNA isolation and histological analysis. Tissue for RNA isolation was immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated using the guanidine isothiocyanate/CsCl method (Chomczynski P. et al. 1987 *Anal. Biochem.* Vol. 162 pp. 156-159). Specimens for histological analysis were fixed in 10% phosphate buffered formalin (pH 7.4), routinely processed and embedded in paraffin. Sections were cut, stained with hematoxylin and eosin and classified according to the morphological criteria of the American Heart Association (Stary H. et al. 1995 *Arterioscler.Thromb. Vasc.Biol.*

Vol.15 pp.1512-1531). Stable lesions (type IV and V) are characterized by an intact fibrous cap that contains smooth muscle cells, fibroblasts and connective tissue. These plaques contain either a large lipid core (type Va), calcification (type Vb) or fibrous tissue (type Vc). A disrupted fibrous cap and presence of a thrombus characterize the ruptured lesion (type VI). Vascular specimens were processed as described above and classified according to Virmani et al. Expression profiles did not differ between arteries and between plaques originating from either surgery or autopsy.

Suppression subtractive hybridization (SSH)

The SSH procedure was performed using the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) essentially according to the protocol of the manufacturer, with the following minor adjustments. Total RNA was isolated from whole mount plaques obtained from six age matched male patients undergoing vascular surgery (Table 1). To correct for patient based differences in gene expression, two pools of total RNA were generated. Pool 1 contained 1 μg of total RNA derived from three ruptured plaques of the abdominal artery of three individual patients. Pool two contained 1 μg of total RNA derived from three stable plaques obtained respectively from the iliac artery, the femoral artery and the carotid artery of three individual patients. The SMART™ PCR cDNA Synthesis Kit (Clontech) was used for the preparation and amplification of double stranded cDNA. In the forward reaction, genes upregulated in ruptured plaques were isolated, while the reverse reaction resulted in the isolation of genes downregulated in ruptured plaques. Endonuclease RsaI digested tester cDNA was ligated to two different adaptors and hybridized twice to a four-fold excess of driver cDNA to enrich for differentially expressed genes. Differentially expressed genes were amplified by two rounds of PCR. The resulting fragments were gel purified, cloned into the pGEMT-easy vector (Promega, Madison, WI) and were subsequently transformed into highly competent *E.coli* JM109 cells (Promega).

Analysis of subtracted cDNA libraries

Macro array analysis.

Clones derived by SSH were tested by macro array analysis. Inserts were amplified by PCR using the T7 (5' TAATACGACTCACTATAGGG 3'; SEQ ID NO: 1) and SP6 (5' ATTTAGGTGACACTATA 3'; SEQ ID NO: 2) primers under standard conditions. Briefly, 10 μl of PCR product was diluted in 190 μl 6XSSC, heated to 95°C and quenched on ice. Two identical macro arrays were made by transferring 100 μl of

the sample to a nylon membrane (Nytran; Schleicher & Schuell, Keene, NH) using a 96-well BioRad Dot Blot apparatus and the DNA was subsequently crosslinked by UV irradiation. The filters were hybridized under conditions of high stringency with ^{32}P-labeled (High Prime, Boehringer Mannheim, Roche Diagnostics, Basel, Switzerland) SMART™ cDNA of either stable or ruptured plaques using standard procedures. Hybridization signals were normalized using RNA-polymerase II and genomic DNA signals. Quantitative analysis was performed by phosphor image analysis (Quantity One, BioRad, Hercules, CA).

Sequencing.

Differentially expressed clones were sequenced using the Thermo Sequenase fluorescent labeled primer (M13 reverse 5' TTTCACACAGGAAACAGGAAACAGCTATGAC 3'; M13 forward 5' CGCCAGGGTTTTCCCAGTCACGAC 3'; SEQ ID NOs: 3 and 4, respectively) cycle sequencing kit (Amersham Pharmacia Biotech, Bucks., UK) and analyzed on an ALF-express automatic sequencer. Homology searches were performed using the advanced Blast Program on the combined GenBank/EMBL nonredundant (nr), Expressed Sequence Tag (dEST), mouse EST, human EST, rat EST, Swissprotein and human tagged genomic sequence (htgs) databases (National Centre for Biotechnology Information: www.ncbi.nlm.nih.gov/).

RT-PCR.

Isolation of total RNA was carried out as described above. The SMART™ PCR cDNA Synthesis Kit (Clontech) was used for the preparation of double stranded cDNA from 0.5 μg template RNA. cDNA was diluted to a total volume of 50 μl. PCR amplification of SSH6 sense: 5'-GGCTAATTCGGGAGATAGCC-3' (SEQ ID NO: 5) + antisense: 5'-CAACACCTCATGGCAAGTCC-3' (SEQ ID NO: 6); perilipin (SSH1/SSH11) sense: 5'-CTTTAACCAAACTTGTGGCC-3' (SEQ ID NO: 7) + antisense: 5'-TACTCAGAAAGTGACACTAG-3' (SEQ ID NO: 8); SSH42 sense: 5'-TTAGGGCTACACATGTTGCC-3' (SEQ ID NO: 9) + antisense: 5'-AAAGTGGCACTGTTGTGAC- 3' (SEQ ID NO: 10); and GAPDH sense: 5'-GGGAAGCTTGTCATCAATGG-3' (SEQ ID NO: 11) + antisense: 5' CATGGTTCACACCCATGACG-3' (SEQ ID NO: 12) was performed on 1 μl of first strand cDNA using standard conditions (30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C and extension for 1 min at 72 °C, reaction volume 25 μl).

Resulting PCR products of approximately 300 bp were analyzed on a 1% agarose gel. The following primer sets were used for spatio-temporal analysis of gene expression in various tissues: perilipin: sense 5'-CTTTAACCAAACTTGTGGCC-3' (SEQ ID NO: 7) + antisense (as) 5'-TACTCAGAAAGTGACAC TAG-3' (SEQ ID NO: 8). HSL: sense 5'-GCTGGTGCGGCGGGACAC-3' (SEQ ID NO: 18) + antisense 5'-GAAGGCGGCACGGACGCC-3' (SEQ ID NO: 19). ADRP: sense 5'-TGTGAGATGGCAG AGAACGG-3' (SEQ ID NO: 20) + antisense 5'-CTCAGTGAGAGGGAGGTAC-3' (SEQ ID NO: 21); and for normalization GAPDH: sense 5'-GGGAAGCTTGTCATCAATGG-3' (SEQ ID NO: 11) + antisense 5' CATGGT TCACACCCATGACG-3' (SEQ ID NO: 12).

RNA in situ hybridization

Sense and antisense digoxygenin (DIG) labeled RNA probes of perilipin (SSH1) and SSH6 were generated from the EcoRI-EcoRI cDNA fragments, which were first re-cloned using vector pTZ18 (Pharmacia, Uppsala, Sweden) in both orientations. After linearization with BamH1, DIG labeled RNA was transcribed from the T7 promoter. Paraffin embedded sections of 4 μm were hybridized, and visualization with alkaline phosphatase coupled anti-DIG antibodies and indoxil-nitroblue tetrazolium (NBT/BCIP) substrate was performed as described previously (De Block M. et al.1993 *Anal. Biochem.* Vol. 215, pp. 86-89). Sections were counterstained with nuclear red.

Immunohistochemistry

Rabbit polyclonal antibody was prepared by immunizing animals with peptides comprising amino acid sequences from human perilipin, mouse perilipin, and rat perilipin. In certain examples, animals were administered a polypeptide having the amino acid sequence MSMNKGPTLLGDLPEQENV (SEQ ID NO: 13). In other examples, the following sequences were each used: CLLGDLPEQENVL (SEQ ID NO: 14); PRELPARRVSDSFFRPSV (SEQ ID NO: 15); PREKPARRVSDSFFPSV (SEQ ID NO: 16); and RLAQPRRSLRSAQSPGC (SEQ ID NO: 17). Design of the immunizing peptide included in some examples incorporating at least a cysteine residue at one end of the peptide. Further, an acetyl group was added to the amino terminus residue, and an amide to the carboxy terminus residue for a peptide used for immunization of animals in some examples. These chemical adducts were used to attach at least one of the immunizing peptides to a column material such as a resin, a cellulose, or a bead, for the purpose of affinity purification of the antibody.

Paraffin sections (4 μm) of plaque were deparaffinized, hydrated and pre-treated by boiling in 0.01 M citrate buffer (pH 6.0) for 10 min in a microwave oven (750 Watt). Sections were incubated with the polyclonal perilipin antibody (1:1000) for 30 min, and were further incubated with biotinylated swine anti rabbit antibody (1:1000, Dako, Carpinteria, CA) for 30 min followed by a 30 min incubation with an alkaline phosphatase coupled ABC reagent (1:200, Dako). Alkaline phosphatase activity was visualized using the Alkaline Phosphatase Kit I (Vector, Burlingame, CA), resulting in a red precipitate. The sections were counterstained with hematoxylin.

Combined immunohistochemical staining was performed to identify the cell types expressing perilipin. Perilipin first was localized using the above protocol, with the following minor adjustments. The ABC reagent was coupled to horseradish peroxidase (1:500, Dako), and the peroxidase activity was visualized using diamine benzidine, which resulted in a brown precipitate. Subsequently, the sections were treated with 0.1% pepsin (Boehringer) in 0.1N HCl for 30 min and incubated with either monoclonal anti-CD68 (1:100, Dako) or polyclonal anti-Factor VIII (1:2000, Biomakor) for 30 min. The sections were then incubated with, respectively, a biotinylated swine anti mouse antibody (1:250, Amersham Life Science), or biotinylated swine anti-rabbit antibody (1:1000, Dako) for 30 min followed by a 30 min, incubation with an alkaline phosphatase coupled ABC reagent (1:200, Dako). Alkaline phosphatase activity was visualized using the Alkaline Phosphatase Kit III (Vector), which yielded a blue precipitate.

Serial sections were used to assess co-localization of perilipin, ADRP and HSL. Paraffin sections were immunolabeled with either perilipin (1:1000; raised against the peptide MSMNKGPTLLDGDLPEQENVC (SEQ ID NO: 22), recognizing all perilipin isotypes), HSL (1:500), or ADRP (1:1000; raised against the peptide CAQDQGAEMDKSSQETQR; SEQ ID NO: 23) polyclonal antibodies. To determine cell types expressing perilipin, HSL and ADRP double immunohistochemical staining was performed. Sections labeled for either perilipin, HSL or ADRP were subsequently labeled for either CD68 (1:100, Dako; staining macrophages) or Factor VIII (1:2000, Biomakor; staining endothelial cells). All stainings were performed as described above. Negative controls, sections incubated without the primary antibody, did not show any staining.

Western blot analysis

Proteins and polypeptide fragments thereof were extracted essentially by homogenizing vascular tissue at 4°C in 1ml 20mM TrisHCl (pH7.4), 255mM sucrose, 10mM NaF, 200μM sodium orthovanadate, 1mM EDTA, 10μl/ml leupeptin, 1mM benzamidine and 0.1μM phenylmethylsulfonyl fluoride, and centrifuging for 5 minutes at 4000 rpm. The supernatant including floating fat, was mixed with 1ml of 1% SDS, 1mM EDTA, 20μM leupeptin 1mM benzamidine and 20mM NaF at 4°C. The mixture was sonified and stored at -20°C. Polypeptides (20μg/sample) were separated on a 10% SDS-PAGE gel and transferred to a nitro-cellulose membrane (Protran, Schleicher & Schuell). Blots were incubated with polyclonal antibodies directed towards either perilipin (1:1,000; recognizing all perilipin isoforms), HSL (1:10,000) or ADRP (1:750). Anti rabbit horseradish peroxidase (1:2,000, Dako) was used as the secondary antibody. Specific antibody binding was visualized using enhanced chemiluminiscence (Amersham Pharmacia Biotech). Mouse adipocyte lysate, a source rich in perilipin and HSL, was used as a positive control.

Example 1. Macro array and sequencing analysis.

Suppression subtractive hybridization was used to produce both a forward library containing 3000 clones upregulated in ruptured atherosclerotic plaques, and a reverse library containing 2000 clones downregulated in ruptured plaques. The differential expression of genes of a sample of the clones obtained from analysis of both SSH libraries was verified by an independent method, the macro array analysis. Clones of both libraries, 300 from the forward subtracted library and 200 from the reverse library (10% of each total), were randomly chosen and screened for expression in ruptured and stable plaques, and macro arrays were performed in triplicate.

Representative macro array data of clones selected by SSH, hybridized to the pool of stable plaque cDNA (Figure 2 panel A), and to the pool of ruptured plaque cDNA (Figure 2 panel B) are shown in Figure 2. Hybridization signals were normalized for the signals of RNA-polymerase II and human genomic DNA. Of the 500 total library clones tested, 45 showed a differential expression pattern by macro array analysis. Three clones contained genetic fragments that were uniquely expressed in ruptured plaques, and four clones contained genetic fragments that were uniquely expressed in stable plaques. Further, eight of the 45 clones showed an at least two-fold difference in expression between ruptured and stable plaques. The genetic material in the 25 clones was sequenced and compared to sequence data available in Genbank.

Of the 11 clones upregulated in ruptured plaques, all carried different sequences, of which two clones carried sequences homologous to distinct parts of the gene encoding perilipin (clones SSH1 and SSH11). Furthermore, one clone encoded a peptide that was homologous to a SRProtein involved in RNA splicing. Additional searches in the EST (human, mouse and rat expressed sequence tags) and htgs databases revealed homology to human ESTs for four clones, and two clones showed homology to distinct parts of the human chromosome 5. One clone (SSH6) shared high homology with a sequence encoding a putative protein; however, no information regarding expression of this protein is available. Finally, one clone contained a previously unknown sequence. Homology data of the clones upregulated in ruptured plaques are shown in Table 2.

Out of 500 randomly chosen clones, 45 (9%) showed differential expression between ruptured plaques and stable plaques by the technique of macro array analysis. Differential expression of 9% of the clones obtained by the SSH procedure is in close agreement with previous studies investigating differential gene expression in other tissues and cell types by this technique (von Stein O. et al. 1997 *Nucleic Acids Res.* Vol. 25 pp. 2598-2602; Hufton S. et al. 1999 *FEBS Lett.* Vol. 463 pp. 77-82). The number of differentially expressed genes might even be higher, because this technique may not detect differential expression of some low abundant genes, if the expression level was below the detection level of the macro array.

Alignment of the 14 clones downregulated in ruptured plaques revealed high homology of three clones to the known genes for β-actin, fibronectin and an immunoglobulin λ light chain. Additional searches in the EST (human, mouse and rat) and htgs database showed homology to human ESTs for eight clones, and two clones were homologous to parts of chromosome 5 and 17. Finally, one clone was homologous to a human genomic clone of a thus far unknown chromosomal localization. The results of the homology searches are shown in Table 3.

Sequence analysis of the 25 clones which showed an at least two-fold difference in expression, revealed: homology of six of these clones (24%) to known genes, homology of 12 clones to ESTs (48%), and homology of six clones (24%) to parts of genomic sequences without any functional annotation. One of the 25 clones contained a completely novel sequence.

To circumvent patient based differences, initial SSH is here performed on two pools, each containing three atherosclerotic lesions. Data indicate that the methods

herein can select for differences in gene expression, rather than differences in cellular composition. Only one inflammatory cell related gene was selected (human immunoglobulin λ chain), which was observed to be downregulated in ruptured plaques.

Example 2. RT-PCR analysis of plaques.

RT-PCR analysis on ten ruptured and ten stable plaques was performed to further validate the expression profile found in the macro array. Analysis was focused on those genes that showed unique expression in ruptured plaques (SSH1/11 and SSH6), i.e., genes that were upregulated in ruptured plaques, and on one (SSH42) of the four clones that were uniquely expressed in stable plaques. To exclude patient- and artery-biased expression, plaques that originated from different arteries of different patients were obtained (Table 1). Expression was normalized to the expression level of GAPDH, which was observed to be comparable among the samples (Figure 3).

Expression of clone SSH6 was found in eight of ten ruptured plaques, while only two of ten stable plaques tested positive. Perilipin (clones SSH1 and SSH11) was also expressed in eight out of ten ruptured plaques, and was completely absent in all stable plaques tested (Figure 3). Although macro array analysis showed absence of expression of clone SSH42 in ruptured plaques, RT-PCR analysis showed no qualitative difference in expression of this sequence between stable and ruptured plaques (Figure 3).

Differential expression of two genes (SSH6, and perilipin [SSH1/SSH11]) as analyzed by RT-PCR was observed in ten different clones from ruptured plaques, when compared to ten different stable plaques, including some stable plaques that contained many inflammatory cells. Although heterogeneous samples with respect to types of arteries used for the initial SSH procedure were used, the RT-PCR analysis shows that selection for plaque type specific genes rather than artery specific genes was obtained. This is illustrated by the expression profile for perilipin and SSH6, present in ruptured plaques derived from each of abdominal aorta femoral artery and carotid artery SSH6 was expressed in stable plaques of peripheral and carotid artery, while SSH42 was expressed at comparable levels in all specimens tested.

Differences in gene expression found in small pools (n=3) of stable or ruptured plaques are here further extended to a larger panel (n=10 for both stable and ruptured plaques) of plaques derived from individual patients. RT-PCR analysis revealed mRNA expression of clone SSH6 in 80% of the ruptured plaques and in 20% of the stable plaques. Expression of SSH6 in one apparent stable plaque (patient number 6, see Table 1) can be correlated with the presence in that plaque of a very thin fibrous cap. Such a

phenotypic characteristic is a known risk factor for plaque rupture.

Differential expression of perilipin (SSH1/SSH11) was completely absent in all 10 stable plaques, while 80% of the ruptured plaques tested positive. Although perilipin expression has previously been associated with lipid droplets (Nishiu J. et al. 1998 *Genomics* Vol. 48 pp. 254-257; Greenberg A. et al. 1991 *J. Biol. Chem.* Vol. 266 pp. 11341-11346; Blanchette-Mackie E. et al.1995 *J. Lipid Res.* Vol. 36 pp. 1211-1226), mRNA expression observed here was not associated either with the presence of a lipid core, or with large amounts of foam cells, as several of the stable plaques also shared these features. In fact, plaques 3 and 5 that showed high levels of expression of perilipin contained a very small lipid core.

The differential expression pattern of SSH6 and perilipin (SSH1/11) indicates a potential functional role of these genes in plaque rupture. SSH6 is highly homologous to clone AL161991, expressed in amygdala. However the clone isolated herein contains an insertion of 122 nucleotides at position 1116 of the previously identified sequence in the public domain. Further, clone AL161991 encodes a putative polypeptide of 493 amino acids, however.

The probes and methods provided in this example can be used for in vivo detection of mRNA specific for perilipin, or for other polypeptides differentially expressed in ruptured plaque, for example, hormone sensitive lipase (HSL) as shown below.

Example 3. RNA *in situ* hybridization.

Since perilipin encoded by clones (SSH1 and SSH11) showed a unique expression pattern in ruptured plaques, RNA *in situ* hybridization (ISH) was performed to localize expression of the perilipin gene within different lesion types.

Specific expression of perilipin within ruptured plaques (n=2) was observed in the cytoplasm of several cells surrounding cholesterol clefts (indicated with an arrow) and in cells (indicated with an arrowhead) most resembling foam cells (Figure 4 panels A, B). Furthermore, positive staining was observed in endothelial cells of newly formed vessels. No detectable signal was observed in non-diseased arteries (n=2) and in stable plaques (n=2; Figure 4 panels E, F). The sense perilipin riboprobe did not show any hybridization signal (Figure 4C).

Example 4. Immunohistochemistry within plaque.

To extend analysis of the perilipin mRNA expression data, immunohistochemistry was performed. Perilipin immunoreactivity in ruptured human atherosclerotic plaques (n=6) was

observed in several cells surrounding cholesterol clefts (indicated with an arrow), foam cells (indicated with an arrowhead; Figure 5 panel A) and endothelial cells of newly formed small vessels (indicated with an arrow; Figure 5 panel B). Combined staining for perilipin and with antibodies directed towards CD68 or factor VIII confirmed that perilipin was expressed in foam cells (Figure 5 panel C) and in endothelial cells (Figure 5 panel D) of ruptured plaques. No staining was observed in non diseased arteries (n=3; Figure 5 panel E), and only few cells in the shoulder region of stable plaques (indicated with an arrow; n=6) tested positive for perilipin (Figure 5, panel F).

The perilipin gene is here shown to be differentially expressed in ruptured atherosclerotic lesions, demonstrating association of perilipin expression with atherosclerosis and providing a basis for diagnosis of progression of this condition.

Example 5. Expression in veins

Accumulation of lipids in atherosclerotic lesions is a risk factor for plaque rupture. An inventory of perilipin, ADRP and HSL mRNA and protein in various stages of human atherosclerosis was performed. Saphenous vein segments (n=5) were included to assess whether perilipin, HSL and ADRP mRNA and protein expression were present in veins.

With respect to mRNA, perilipin mRNA was detected in only 1 vein, while all veins expressed HSL mRNA and 4 out of 5 veins tested positive for ADRP mRNA, as analyzed by RT-PCR analysis (Figure 6).

None of perilipin, HSL nor ADRP protein expression was detected in any of the veins tested as analyzed by immunohistochemistry.

Example 6. Expression in non-diseased artery

Perilipin mRNA was not detected in non-diseased artery (n=4). Only 1 non-diseased artery expressed HSL mRNA, while 3 out of 4 specimens tested positive for ADRP mRNA (Figure 6).

Neither HSL nor ADRP protein expression was observed in non-diseased artery (n=3). The intima and media of the non-diseased artery did not express perilipin. In contrast, adipocytes and endothelial cells (as determined by double immunohistochemical staining using FVIII) of small vessels in the adventitial layer showed intense immunoreactivity for perilipin.

Example 7. Expression in intimal xanthomas.

Intimal xanthomas are early lesions that can develop into plaque (Virmani R. et al. 2000 *Arterioscler. Thromb. Vasc. Biol.* Vol. 20 pp.1262-1275), and are characterized

by the presence of foam cells in the intimal layer of the vessel wall.

Perilipin and HSL mRNAs were observed in only 1 out of 5 intimal xanthomas. In contrast, ADRP mRNA was present in the majority of the specimens tested (four out of five; Figure 6).

In intimal xanthomas (n=4), lipid loaded macrophages (CD68 positive) just underneath the endothelial layer stained positive for ADRP. These cells expressed neither perilipin nor HSL polypeptide (Figure 7A, B and C). Surprisingly, macrophage derived foam cells deeper in the intima expressed either perilipin or ADRP, and only a small subset of foam cells in the intima expressed both proteins.

In general, expression of ADRP was more abundant than perilipin expression. HSL immunostaining was faint and present in only some intimal foam cells.

Example 8. Expression in stable lesions

Stable lesions include fibrous plaques, fibrocalcific plaques and fibrous cap atheromas.

As described herein, perilipin mRNA was completely absent in 10 individual stable plaques, representing all three of these plaque subtypes. In contrast, HSL mRNA was detected in four out of 10 stable plaques, and ADRP mRNA was highly expressed in all stable plaque tested (n=10; Figure 6).

Fibrous plaques (n=4) expressed low levels of perilipin polypeptide mainly restricted to macrophages in the shoulder of the plaque (Figure 7D). Calcified areas did not show perilipin expression (n=3). In fibrous cap atheromas (n=3), perilipin was expressed in foam cells in the outer rim of the lipid core and in foam cells in the shoulder of the plaque. Surprisingly, foam cells in areas of intense inflammatory infiltration showed high levels of perilipin protein expression (Figure 7G). Perilipin immunoreactivity was also observed in endothelial cells of small vessels in advanced plaques.

Fibrous cap atheromas showed low levels of HSL protein expression (Figure 2E and H), which was restricted mainly to a small subset of perilipin expressing foam cells (Figure 8A, B, C and E). Only a few foam cells demonstrated both HSL and ADRP protein expression (Figure 7H and I).

ADRP was found to be expressed in foam cells in the shoulder of fibrous plaques, whereas no expression was observed in areas of calcification (Figure 7F). In fibrous cap atheromas, ADRP was abundantly expressed in foam cells in the shoulder and in the outer rim of the lipid core (Figures 7I and 8F).

Foam cells expressing both ADRP and perilipin were scarce (Figure 8A, C, D and F). The series of fibrous cap atheromas included two samples with lateral xanthomas. Macrophages just underneath the endothelial layer were ADRP negative, whereas macrophages deeper into the lateral xanthomas showed abundant ADRP expression (Figure. 7O). The lateral xanthomas showed a very low level of perilipin protein, and no HSL protein was expressed (Figures 7M and N).

Example 9. Expression in lesions with thrombi.

Lesions with thrombi are associated with increased risk of heart attach and stroke. These lesions are characterized by either the presence of a ruptured fibrous cap and a related thrombus, or an intraplaque hemorrhage.

Perilipin mRNA was found to be expressed in eight out of 10 plaques with a thrombus. Further, seven out of 10 plaques expressed HSL mRNA, and ADRP mRNA was observed in all 10 plaques (Figure 6).

In lesions with a ruptured cap (n=4), perilipin protein expression was localized in foam cells at the actual site of cap rupture. Furthermore, foam cells surrounding the thrombus showed perilipin protein expression (Figure 7J). In lesions containing intraplaque hemorrhage (n=3), foam cells near the luminal site of the plaque expressed perilipin. As described above, foam cells in highly inflamed regions showed extensive perilipin protein expression.

The majority of the ruptured plaques showed HSL protein expression, predominantly in foam cells in the ruptured cap (Figure 7K). In lesions with thrombi, a low level of HSL expression was observed in a subset of perilipin positive cells.

ADRP protein was expressed at a low level in foam cells of the ruptured cap. In contrast, lipid loaded macrophages within the thrombus or foam cells adjacent to the thrombus showed high levels of ADRP expression (Figure 7L). As described above, foam cells at the rim of the lipid core were ADRP positive. Foam cells migrating several cell layers into the intima of lesions containing an intraplaque hemorrhage expressed ADRP. Furthermore, ADRP expression near an intraplaque hemorrhage was comparable to expression near the thrombus after plaque rupture.

A summary of patterns of mRNA and protein expression during human atherogenesis is shown in Table 1. Expression of perilipin mRNA was here found to be very low during the early phase of atherogenesis, and was absent in stable plaques, while perilipin mRNA expression was upregulated in plaques containing a thrombus. Perilipin polypeptide expression was observed in macrophage derived foam cells, and expression

increased during plaque progression.

HSL mRNA expression increased during plaque progression. HSL polypeptide, although expressed at a low level, was also increased during plaque progression and co-localized mainly with perilipin protein expression.

ADRP mRNA was abundantly expressed during all stages of human atherogenesis. ADRP protein expression, observed in macrophage derived foam cells, increased during plaque progression in advanced but stable lesions. In lesions containing a thrombus, ADRP polypeptide was expressed by foam cells near the thrombus.

Example 10. Western blot analysis of perilipin, HSL and ADRP expression.

In order to assess perilipin isoform distribution during human atherogenesis, western blotting of lysates of advanced stable lesions and plaques containing a thrombus was performed.

Perilipin A (65kDa) was the only perilipin isoform detected in human atherosclerotic lesions. Comparison of stable and ruptured lesions revealed no significant differences in expression levels and phosphorylation status as judged by perilipin A mobility in SDS-PAGE (Figure 9). Expression of the 84kDa HSL isoform was observed in all human plaque lysates (Figure 9). The doublet in two stable plaque lysates might be indicative of two separate HSL-isoforms, or a post-translational modification such as phosphorylation. ADRP polypeptide (53kDa) was expressed in three out of four stable plaque lysates and only in one lysate of a lesion containing a thrombus (Figure 9).

Examples herein describe perilipin and the functionally closely related proteins HSL and ADRP, which are differentially expressed during the initiation and progression of human atherosclerosis. Perilipin, HSL, and ADRP polypeptide expression was observed in macrophage derived foam cells in the intima. Low levels of perilipin polypeptide expression were found in the early stages of atherosclerosis and in very fibrous plaques.

In contrast to foam cells in the intima, perilipin protein was expressed at high levels in foam cells of fibrous cap atheromas and in the ruptured caps of thrombus containing plaques. Furthermore, perilipin expression was frequently co-localized with areas of inflammation. Since large lipid cores, thin fibrous caps and areas of inflammation are risk factors for plaque instability, perilipin expression herein is found to be associated with plaque vulnerability, i.e., a prognosis associated with heart attack and stroke.

In contrast to perilipin polypeptide, perilipin mRNA was not detectable in stable plaques. Lipid loading of adrenal cortical cells resulted in a 6-fold increase of perilipin mRNA while perilipin protein was enhanced up to 140-fold (Brasaemle et al. 1997 J Biol Chem 272 :9378-9387)). This difference is due to high stability of perilipin protein in the presence of lipids, compared to levels of perilipin mRNA that are below the detection limit of the RT-PCR.

Low level of HSL mRNA and polypeptide found herein in foam cells of advanced lesions was found to be mainly restricted to perilipin positive foam cells. Further, co-localization of HSL and ADRP was only rarely observed.

Lipolytic stimuli result in phosphorylation and activation of HSL, and subsequent translocation to the lipid surface (Egan, et al. 1992. Proc Natl Acad Sci U S A 89:8537-8541). Perilipin present on the surface of the lipid droplet can act as barrier for HSL, resulting in inhibition of lipolysis. Hyperphosphorylation of perilipin facilitates access of the lipid droplet for the lipolytic enzyme HSL. An interplay of these two proteins in human atherosclerotic plaques occurs as a result of co-expression of perilipin and HSL in the same foam cells.

Without being bound by any particular theory, presence of non-hyperphosphorylated perilipin may result in a reduction of lipolysis that leads to accumulation of lipids in the plaque, and possibly increased plaque vulnerability. In contract, hyperphosphorylated perilipin increases access of the lipid droplet to HSL, and leads to increased lipolysis. Increased lipolysis however, does not necessarily result in smaller atherosclerotic lesions. HSL overexpression in macrophages increases lipolysis, but results in more advanced atherosclerotic lesions in mice (Escary et al. 1999 J Lip Res 40:397-404). An increase in free cholesterol may lead to an induction of a pro-atherogenic inflammatory response. Because perilipin polypeptide expression is increased in areas of inflammation, perilipin may act as a negative feedback mechanism on the pro-atherogenic inflammatory response that results from the increase in local free cholesterol.

Expression of ADRP in infiltrating macrophages underneath the endothelial layer at an early stage of atherogenesis is shown herein, in foam cells surrounding the lipid core of advanced lesions, and in macrophages that infiltrate thrombi in ruptured lesions. Without being bound by any particular theory, ADRP might serve either as a shuttling protein of the lipid substrate to the lipid droplet, or as a protein that stabilizes the lipid droplet. Furthermore, ADRP mRNA is highly upregulated in macrophages soon after

the onset of cholesterol loading. A role for ADRP in the formation or stabilization of lipid droplets in human atherosclerotic plaques is thus shown herein. Surprisingly, the prevalence of ADRP polypeptide expression was found to be decreased after plaque rupture. Replacement of ADRP by perilipin in plaques containing a thrombus may be responsible.

Perilipin and ADRP were found herein mainly expressed in separate sets of foam cells. Only a small subset of foam cells were found that showed co-localization of both proteins. Further, in these cells only one of these two proteins was expressed abundantly. Foam cells in lateral xanthomas herein showed a gradient (low levels at the luminal site) of ADRP polypeptide, while no perilipin was detected. ADRP in adipocytes was expressed during the early phase of lipid uptake, while expression decreased after the onset of perilipin expression. Abundant ADRP mRNA expression and absence of ADRP polypeptide was found in non-diseased artery and vein. The absence of lipid depositions in veins and normal arteries may explain rapid degradation of ADRP protein in these vessels.

Perilipin, HSL and ADRP expression patterns during progression of human atherosclerosis are summarized in Table 4, for tissues that include human vein, non-diseased artery, intimal xanthoma, stable lesions and lesions with a thrombus. Expression patterns of these proteins observed herein indicate that ADRP is expressed in infiltrating macrophages, in developing lesions, and in macrophages that engulf debris at the border of the lipid core or within a thrombus. If the macrophage derived foam cells take up more lipids, perilipin is expressed, and expression of ADRP diminishes. Perilipin regulates the accessibility of the lipid droplet for HSL and might therefore influence lipolysis within an atherosclerotic lesion. The expression patterns of perilipin, HSL and ADRP during lesion progression indicate that these proteins are regulators of neutral lipid metabolism during human atherogenesis, and provide targets for intervention in this process.

Example 11. Inhibition of destabilization of plaques.

Phosphorylation of perilipin causes an alteration in the lipid droplet surface, which in turn may facilitate the actions of hormone sensitive lipase (HSL) in catalyzing the process of lipolysis (Clifford G. et al. 2000 *J. Biol. Chem.* Vol. 275 pp. 5011-5015). Furthermore, overexpression of perilipin in 3T3-L1 pre-adipocytes in culture increases triacylglycerol storage by a reduction of triacylglycerol hydrolysis (Souza S. et al. 1998 *J. Biol. Chem.* Vol. 273,. pp. 24665-24669; Brasaemle D. et al. 2000 *J. Biol. Chem.* Vol. 291 pp. 1304-1351). Without being bound by any

particular theory or mechanism, the association of perilipin with atherosclerois shown herein indicates that non-phosphorylated perilipin forms a barrier for the enzymes involved in lipolysis.

Mice carrying a defective perilipin gene have a phenotype of smaller white adipocytes, and elevated basal lipolysis due to an increased activity of HSL, compared to normal mice (Martinez-Botas J. et al.2000 *Nat. Genet.* Vol. 26 pp. 474-479). Expression of perilipin specifically in ruptured plaques, and localization of perilipin mRNA and polypeptide to cells surrounding cholesterol crystals and in foam cells as shown in Examples above, indicate reduced lipolysis in these plaques, that results in increased lipid retention and plaque destabilization.

In order to inhibit destabilization of plaques and induce increased lipolysis of plaques, useful agents are those that negatively impact expression of the gene encoding perilipin, and that interfere with perilipin localization. Candidate agents in combinatorial libraries of chemical compounds can be identified by use of an *in vivo* screen provided herein, using the methods of analysis in the Examples supra.

Further, an "antisense" perilipin nucleic acid capable of stable base-pair complementary hybridization with a naturally-occuring transcript of at least a portion of the gene encoding perilipin, can function as a negative regulator of perilipin expression, as a potential therapeutic agent, and as a positive control for use in a method for obtaining additional agents in such a screen.

Example 12. Delivery of a therapeutic agent to a specific type of atherosclerotic plaque by use of anti-perilipin antibodies.

A therapeutic agent is localized to an atherosclerotic plaque by one of a variety of delivery technologies, for the purpose of bringing the therapeutic agent into proximity with a plaque in need of suppression of perilipin expression. These technologies can exploit the binding specifities and affinities of the antibodies described herein. Technologies include liposomes coated with the antibodies described herein, and further conjugation of antibodies to cytotoxic agents such as to toxin molecules, and conjugation of antibodies to phage or viruses such as by expression of the gene encoding the antibody within the viral genome.

By these methods, therapeutic agents are administered systemically, for example by intravenous injection, and then delivered specifically to a set of stable or unstable plaques.

Example 13. Delivery of an imaging agent to a specific type of atherosclerotic plaque by use of anti-perilipin antibodies.

The antibodies described herein are covalently complexed with chelating agents and a chelating labeled metal such as and Tc^{99}, or equivalent radionuclides, or with ferromagnetic particles. The binding of the antibodies to the radionuclides or to the particles enables localization of the agent to stable plaque, or to ruptured plaque. Subsequent magnetic resonance image analysis

can be used for diagnostic or pre-surgical visualization purposes.

Example 14. Diagnosis of plaque type in biopsy material.

The antibodies described herein are conjugated to adducts that are standard imaging agents, such as radioactive, fluorescent, colorimetric, or enzymatic adducts, to prepare reagents used to identify whether plaque in a tissue sample taken from a subject, or present in vivo and detectable from whole body scans, are stable or ruptured. Standard pathology or histology techniques are used on whole mount tissue or sections of tissue, respectively, to identify the type of plaques present in the biopsied tissue. Whole body scanning uses medical scanning technologies such as MRI, PET, CAT and the like.

TABLE 1. Patient characteristics

plaque type	No	sex*	Age	Artery	used for
Ruptured	1	m	60	Abdominal aorta	RT-PCR†
	2	f	66	Femoral artery	RT-PCR
	3	m	72	Abdominal aorta	SSH‡/ array§/ RT-PCR/ ISH‖/ IHC¶
	4	m	74	Abdominal aorta	RT-PCR
	5	m	73	Abdominal aorta	SSH/ array/ RT-PCR
	6	m	55	Femoral artery	RT-PCR
	7	m	75	Abdominal aorta	SSH/ array/ RT-PCR
	8	m	73	Femoral artery	RT-PCR
	9	m	63	Abdominal aorta	RT-PCR
	10	m	58	Carotid artery	RT-PCR
Stable	11	m	72	Carotid artery	RT-PCR
	12	f	67	Carotid artery	RT-PCR
	13	m	57	Carotid artery	RT-PCR
	14	f	71	Femoral artery	RT-PCR
	15	m	78	Common femoral artery	SSH/ array/ RT-PCR
	16	m	78	Common iliac artery	SSH/ array/ RT-PCR
	17	m	60	Abdominal aorta	RT-PCR
	18	m	68	Carotid artery	RT-PCR
	19	m	67	Carotid artery	SSH/ array/ RT-PCR
	20	m	70	Carotid artery	RT-PCR

*f: female, m: male

†RT-PCR: reverse transcriptase PCR

‡SSH: suppression subtractive hybridization

§ Array: custom made macro array

‖ ISH: RNA *in situ* hybridization

¶ IHC: Immunohistochemistry

TABLE 2. Summary of characteristics of genes upregulated in ruptured plaques

cDNA fragment	Ratio R/S*	Fragment size(bp)	GenBank match†	Accession no. ‡	Homology %
SSH16	2	337	SRProtein member	EmbZ85986.1	100
SSH1	B/W§	447	Perilipin	AB005293	100
SSH11	B/W	730	Perilipin	AB005293	97
SSH6	B/W	548	cDNA	AL161991	99
SSH27	2	579	EST‖	AA359550	99
SSH15	2	276	Chromosome 5	AC016606	97
SSH19	3	1050	EST	BE142819	96
SSH18	2	595	EST	W95753	100
SSH28	2	473	EST	AI802383	98
SSH22	2	483	Unknown		
SSH24	2	700	Chromosome 5	AC026775	97

* Ratio of hybridization signal between ruptured human atherosclerotic plaques and stable human atherosclerotic plaques as determined by macro array analysis.

† Homology of the cDNA clone to an annotation in the public database (genbank, human-/ mouse-/ rat-EST/htgs)

‡ Accession number in the public data base

§ B/W: Black and white difference, i.e., a significant signal obtained with probe either for stable or ruptured plaque, with only background levels observed of the other probe

‖ EST: Expressed sequence tag

The vertical line indicates the clones obtained for this perilipin gene.

TABLE 3. Summary of characteristics of genes downregulated in ruptured plaques

cDNA fragment	Ratio S/R *	Fragment size (bp)	GenBank match†	Accession no.‡	Homology %
SSH38	B/W§	520	β-actin	NM001101.1	100
SSH36	4	400	Fibronectin	K00799	100
SSH55	4	600	Human Immunoglobulin λ gene	D87018	99
SSH33	B/W	380	EST‖	AA364833	99
SSH39	3	320	EST	AI221289	98
SSH41	3	600	EST	AA988599	100
SSH48	3	832	Chromosome 17	AC004797	95
SSH43	2	297	EST	AA66273	100
SSH57	4	480	EST	AI30636	99
SSH59	3	510	EST	AA724619	98
SSH32	B/W	409	Genomic sequence	AL121997	97
SSH42	B/W	1127	Chromosome 5	AC016636	93
SSH58	3	316	EST	AI695804	95
SSH54	4	505	EST	AI050729	97

* Ratio of hybridization signal between stable human atherosclerotic plaques and ruptured human atherosclerotic plaques as determined by macro array analysis.

† Homology of the cDNA clone to an annotation in the public database (genbank, human-/ mouse-/ rat-EST/htgs)

‡ Accession number in the public data base

§ B/W: Black and white difference

‖ EST: Expressed sequence tag

TABLE 4. Summary of mRNA and protein expression patterns of perilipin, HSL and ADRP in human vein, non-diseased arter, intimal xanthoma, stable lesions, and a thrombus.

		Vein	Non diseased artery	Intimal xanthoma	Stable lesions	Lesions with a thrombus
mRNA	Perilipin					
	HSL					
	ADRP					
Protein	Perilipin					
	HSL					
	ADRP					

What is claimed is:

1. A method of identifying an unstable atherosclerotic plaque, the method comprising introducing into the vascular lumen an agent, wherein the agent binds a polypeptide which is upregulated in an atherosclerotic plaque, and determining the presence of the unstable plaque by imaging a location of the agent contacting the plaque in the lumen, wherein contacting the plaque by the agent identifies the unstable plaque having the upregulated protein.

2. A method according to claim 1, wherein the agent comprises an antibody.

3. A method according to claim 1, wherein the protein is perilipin or hormone sensitive lipase.

4. A method according to claim 2, wherein the antibody further comprises a covalently linked adduct.

5. A method according to claim 4, wherein the adduct is an imaging agent.

6. A method according to claim 5, wherein the imaging agent is selected from the group of molecules that are magnetic, chemiluminescent, bioluminescent, radioactive, and fluorogenic.

7. A method according to claim 6, wherein the magnetic molecule is paramagnetic iron.

8. A method according to claim 6, wherein the radioactive molecule is selected from the group consisting of: ^{18}fluorine, ^{76}bromine, ^{77}bromine, ^{124}iodine, ^{125}iodine, ^{94}technetium, and ^{99}technetium.

9. A method according to claim 8, wherein the imaging agent is a chelator molecule complexed with ^{99}technetium.

10. A method according to claim 6, wherein the bioluminescent molecule is a green fluorescent protein.

11. A method according to claim 6, wherein determining presence of the unstable plaque further comprises imaging the vascular lumen with a device selected from the group of PET, MRI, CAT, and micro-photography.

12. A method for identifying a perilipin binding composition, comprising:

providing a sample containing a potential binding composition;

adding an antibody specific for all or a portion of perilipin; and

analyzing components of a resulting complex, thereby identifying the perilipin binding composition.

13. A method according to claim 12, wherein providing a sample is providing a biological fluid.

14. A method according to claim 12, wherein providing a sample is providing a chemical composition.

15. A method according to claim 13, wherein the biological fluid is selected from a cell lysate, a spent culture medium, and a bodily fluid.

16. A method according to claim 34, wherein the bodily fluid is selected from the group consisting of blood, serum, plasma, urine, saliva, cerebrospinal fluid, pleural fluid, perspiration, lachrymal fluid, lymph, bile, and gastric fluid.

17. A method according to claim 14, wherein the chemical composition is a component of a library of chemical compounds.

18. An antibody composition having affinity for an epitope of a protein in a human ruptured atherosclerotic plaque.

19. A composition according to claim 18 wherein the antibody binds to an epitope selected from the group of perilipin, hormone sensitive lipase, and adipocyte related differentiation protein.

20. An antibody according to claim 18, wherein the protein is perilipin.

21. An antibody according to claim 20, wherein the antibody is obtained by immunizing an animal with a peptide selected from all or a portion of amino acid sequences consisting of: MSMNKGPTLLDGDLPEQENVC (SEQ ID NO: 13); CLLGDLPEQENVL (SEQ ID NO: 14); PRELPARRVSDSFFRPSV (SEQ ID NO: 15); PREKPARRVSDSFFPSV (SEQ ID NO: 16); and RLAQPRRSLRSAQSPGC (SEQ ID NO: 17); and MSMNKGPTLLDGDLPEQENVC (SEQ ID NO: 22), wherein the resulting antibody binds to human perilipin in a ruptured plaque.

22. An antibody according to claim 20, wherein the antibody is polyclonal.

23. An antibody according to claim 20, wherein the antibody is selected from the group consisting of IgA, IgG, and IgM.

24. An antibody according to claim 20, wherein antibody is a fragment selected from the group consisting of a Fab, an Fab', an Fv, and a single-chain antibody.

25. An antibody according to claim 20, wherein binding to ruptured plaques is at least two-fold greater than to stable plaques.

26. An antibody according to claim 25, wherein the binding to ruptured plaques is at least five-fold greater than to stable plaques.

27. An antibody according to claim 20, wherein the antibody is monoclonal.

28. An antibody according to claim 20, which is substantially purified.

29. A perilipin fragment comprising the amino acid sequence MSMNKGPTLLDGDLPEQENVC (SEQ ID NO: 13).

30. An adipocyte differentiation related protein (ADRP) fragment comprising the amino acid sequence CAQDQGAEMDKSSQETQR (SEQ ID NO: 23).

31. An antibody obtained by immunizing an animal with the peptide of claim 30, wherein the resulting antibody binds to human ADRP in a plaque.

32. An antibody according to claim 31, wherein the antibody is monoclonal.

33. A cell producing an antibody according to either of claims 27 or 32.

34. A cell according to claim 33, wherein the cell is a hybridoma.

35. A nucleotide sequence encoding the antibody according to either of claims 27 or 32.

36. A transgenic animal carrying the nucleotide sequence according to claim 35.

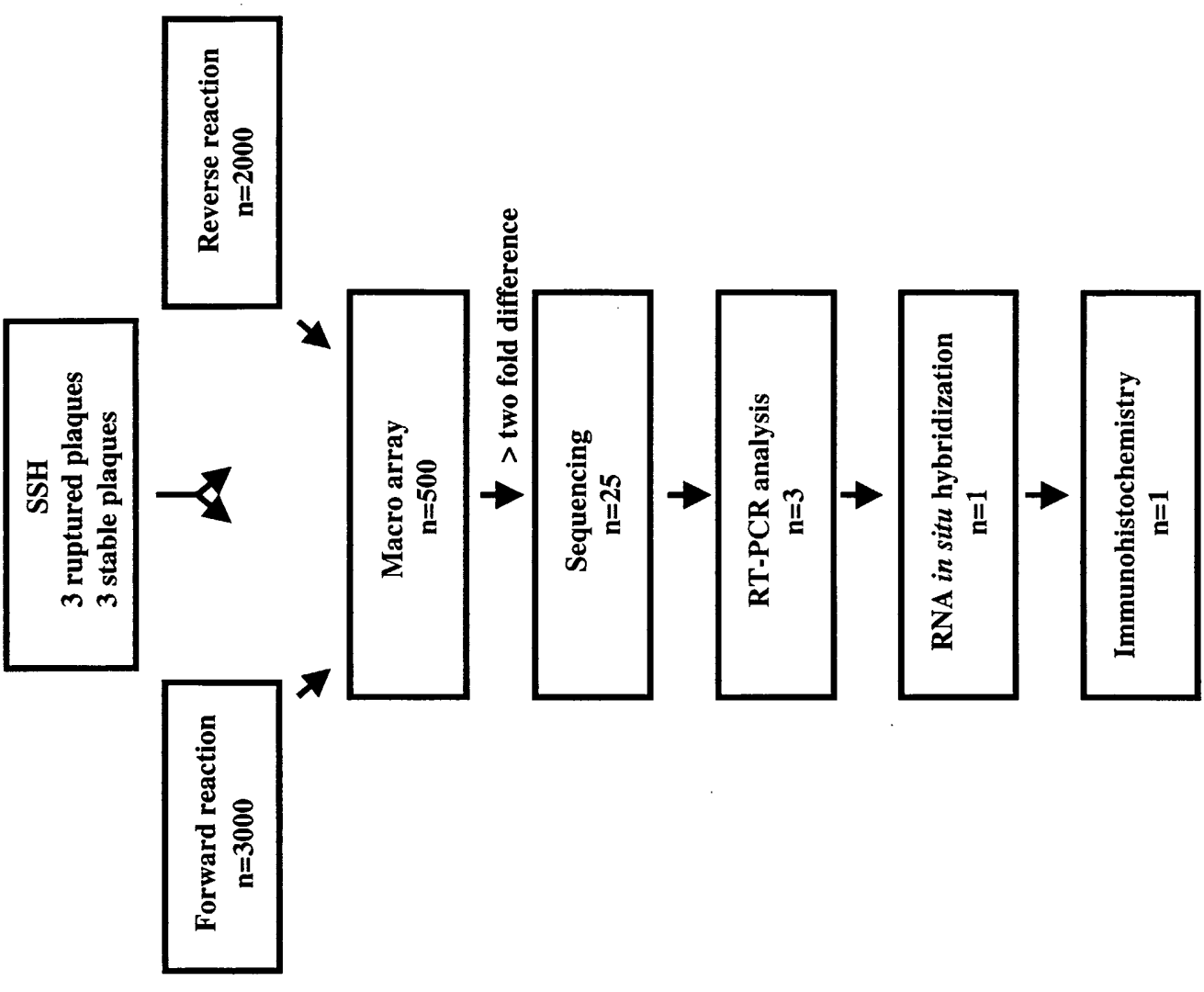

SSH
3 ruptured plaques
3 stable plaques
Forward reaction
n=3000
Reverse reaction
n=2000
Macro array
n=500
> two fold difference
Sequencing
n=25
RT-PCR analysis
n=3
RNA in situ hybridization
n=1
Immunohistochemistry
n=1

Figure 1

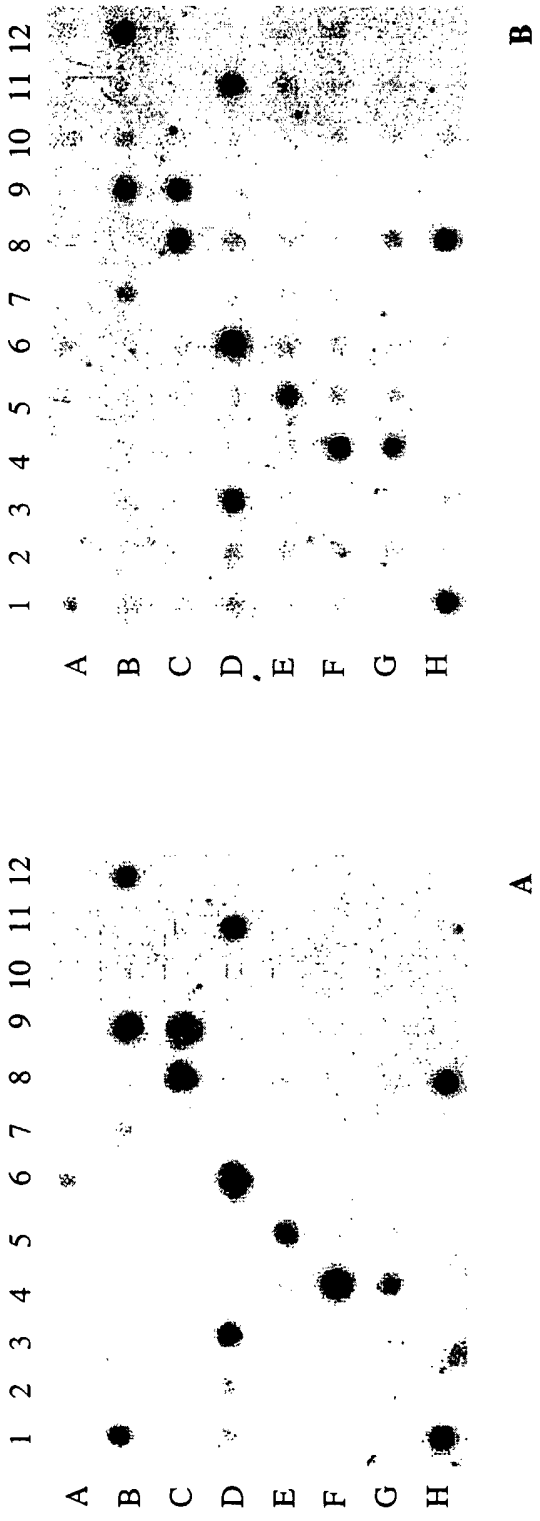


Figure 2

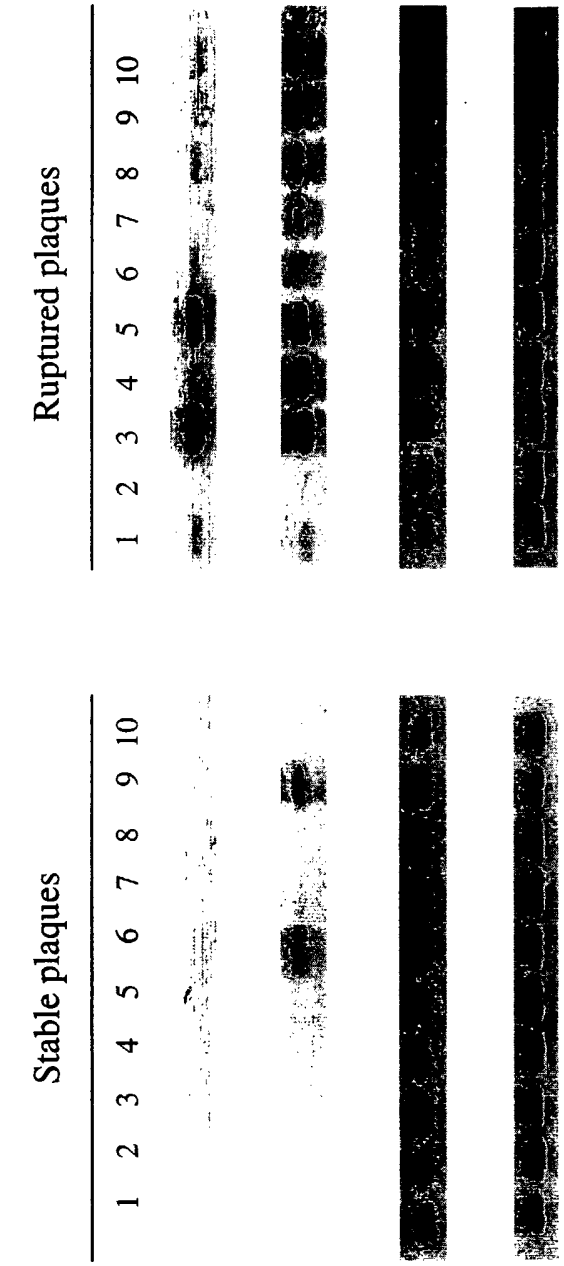

Stable plaques
1 2 3 4 5 6 7 8 9 10
Ruptured plaques
1 2 3 4 5 6 7 8 9 10
Perilipin
SSH 6
SSH42
GAPDH

Figure 3

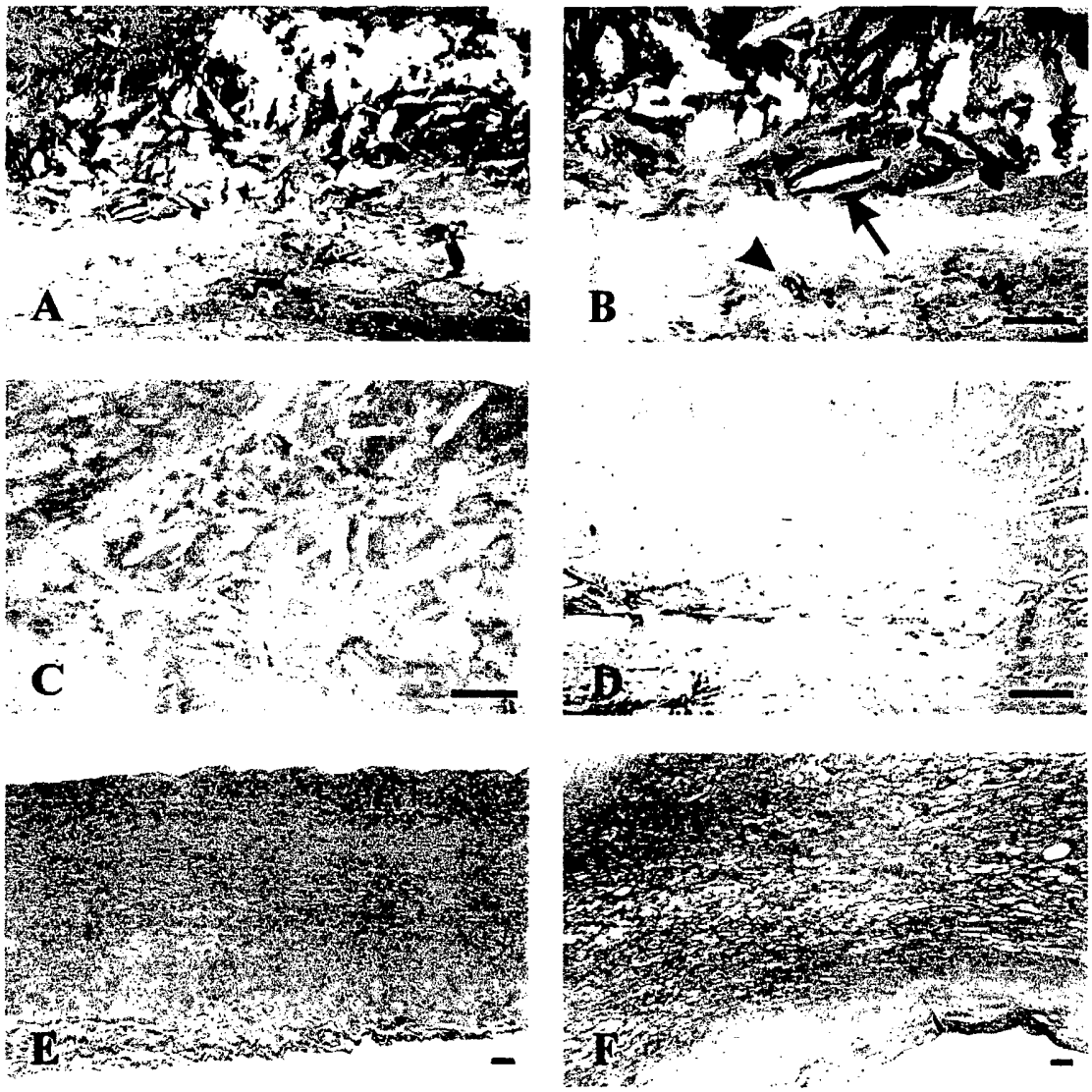


Figure 4

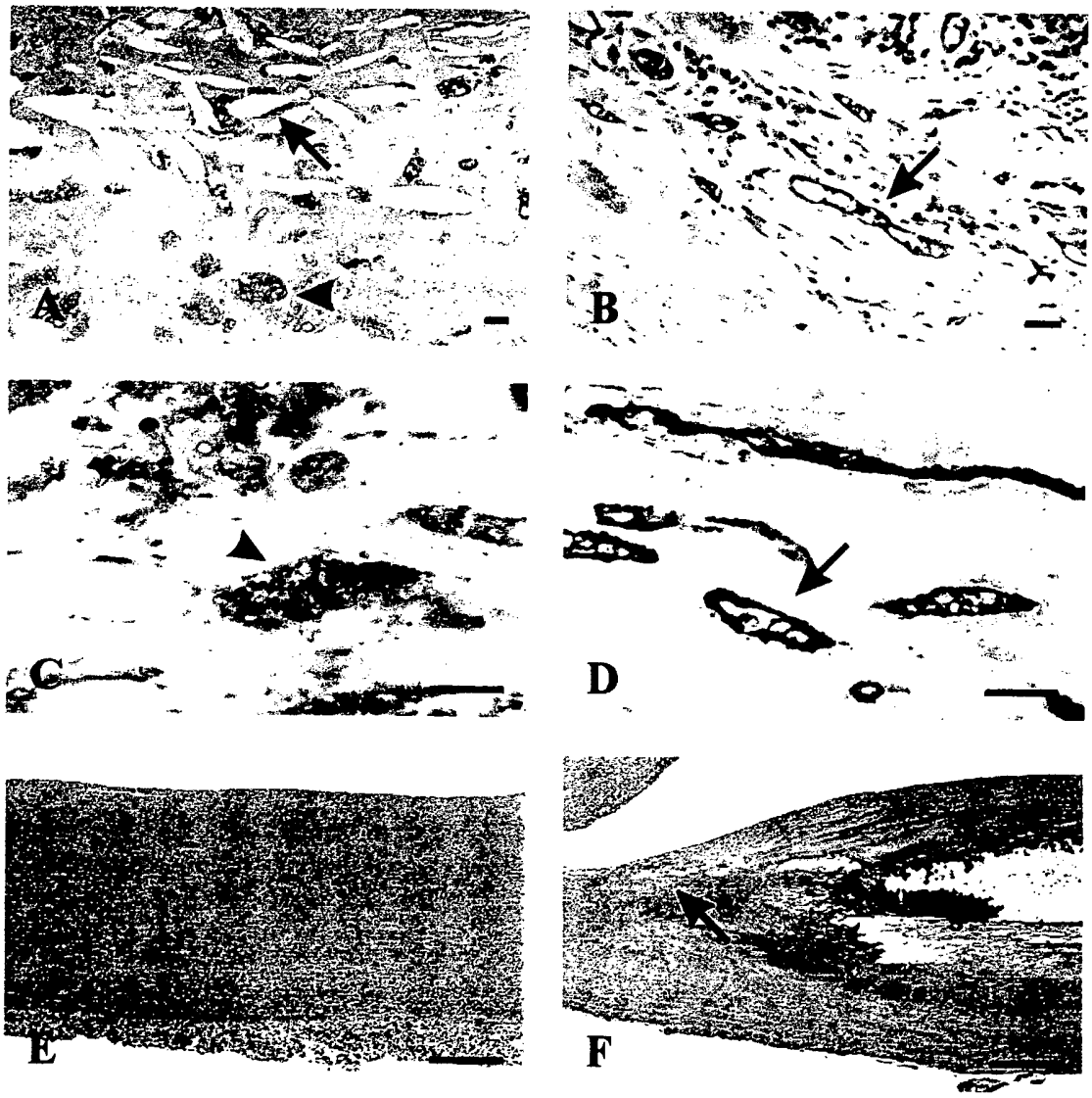


Figure 5

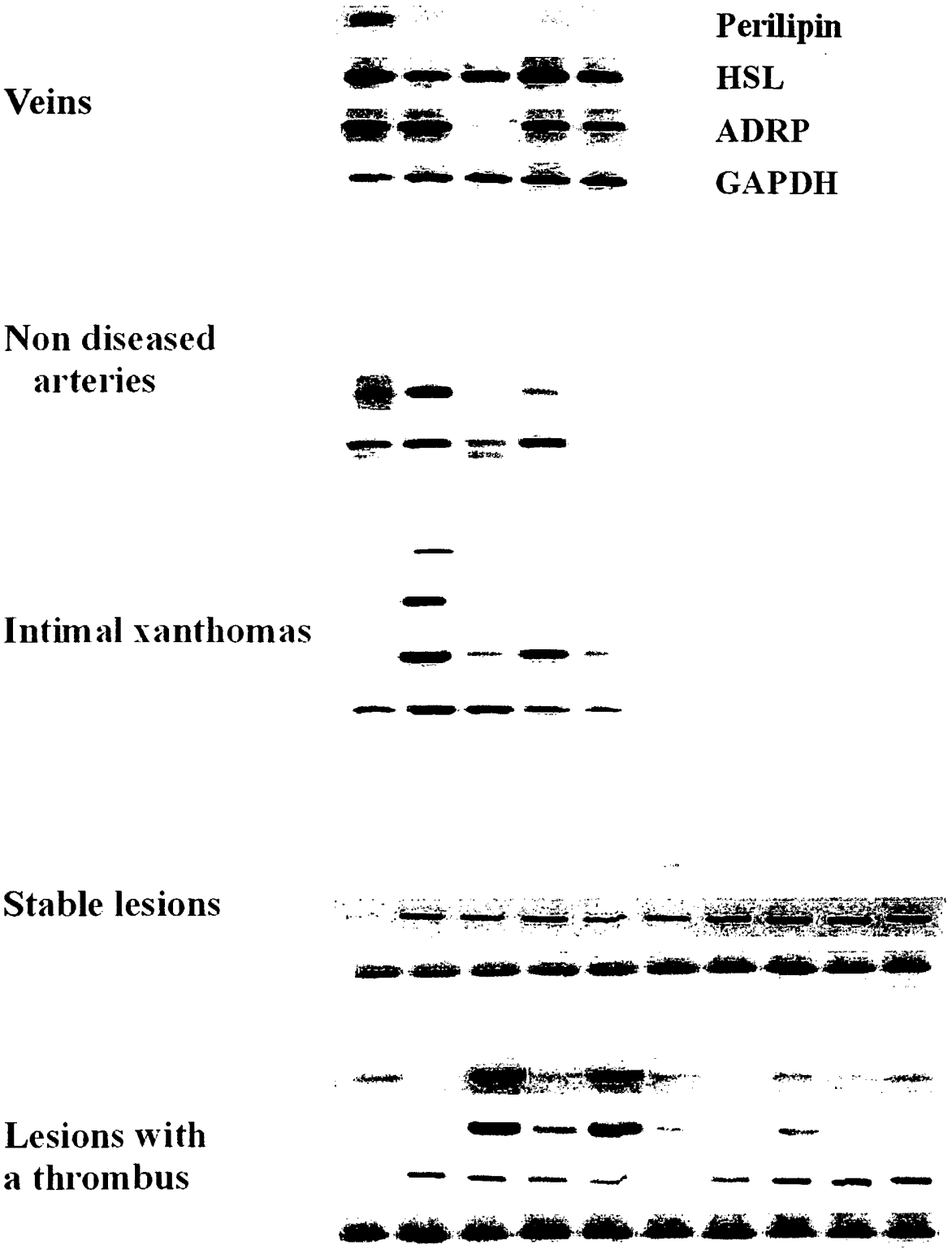

Perilipin
HSL
ADRP
GAPDH
Veins
Non diseased arteries
Intimal xanthomas
Stable lesions
Lesions with a thrombus

FIGURE 6

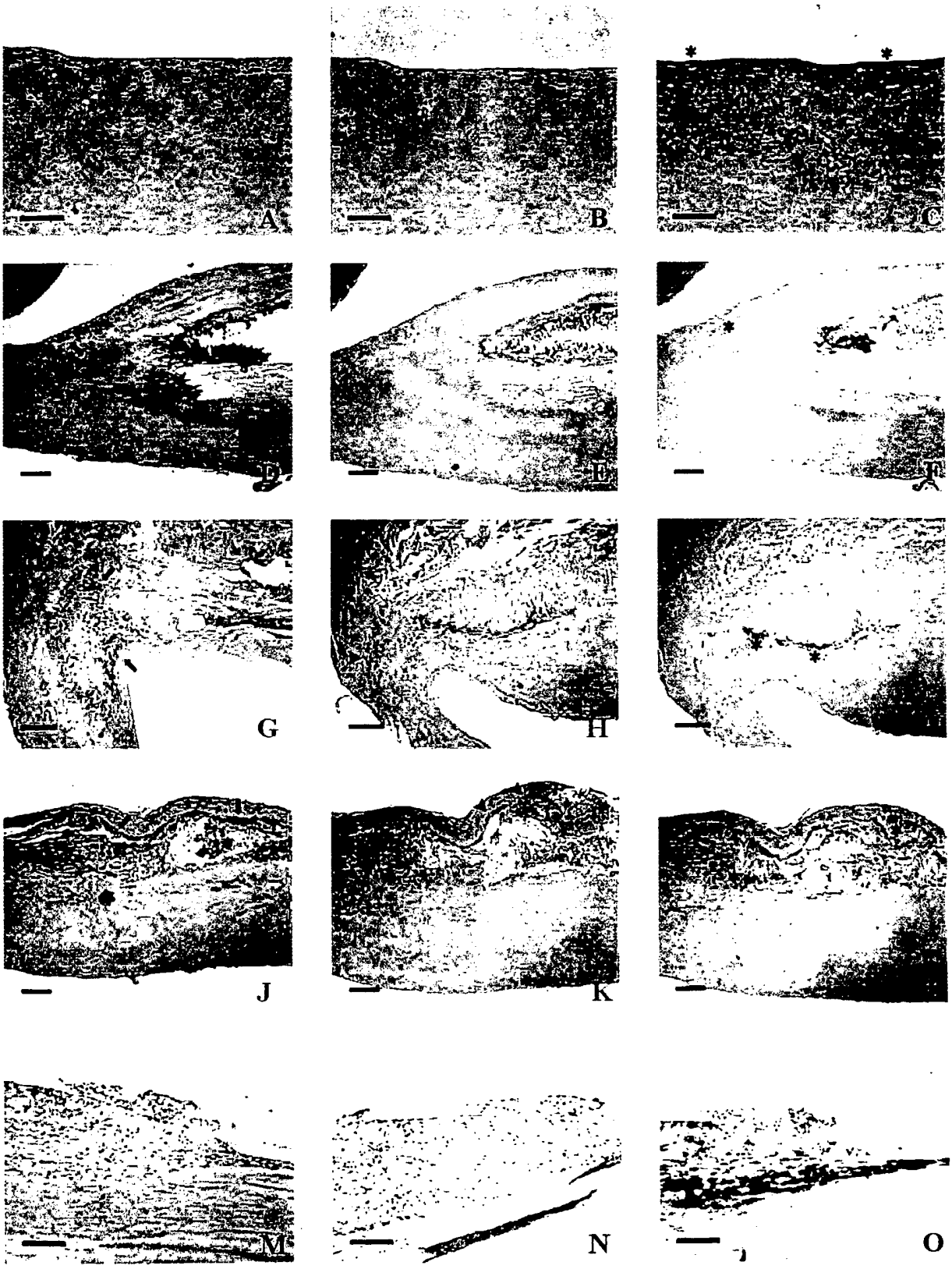


FIGURE 7

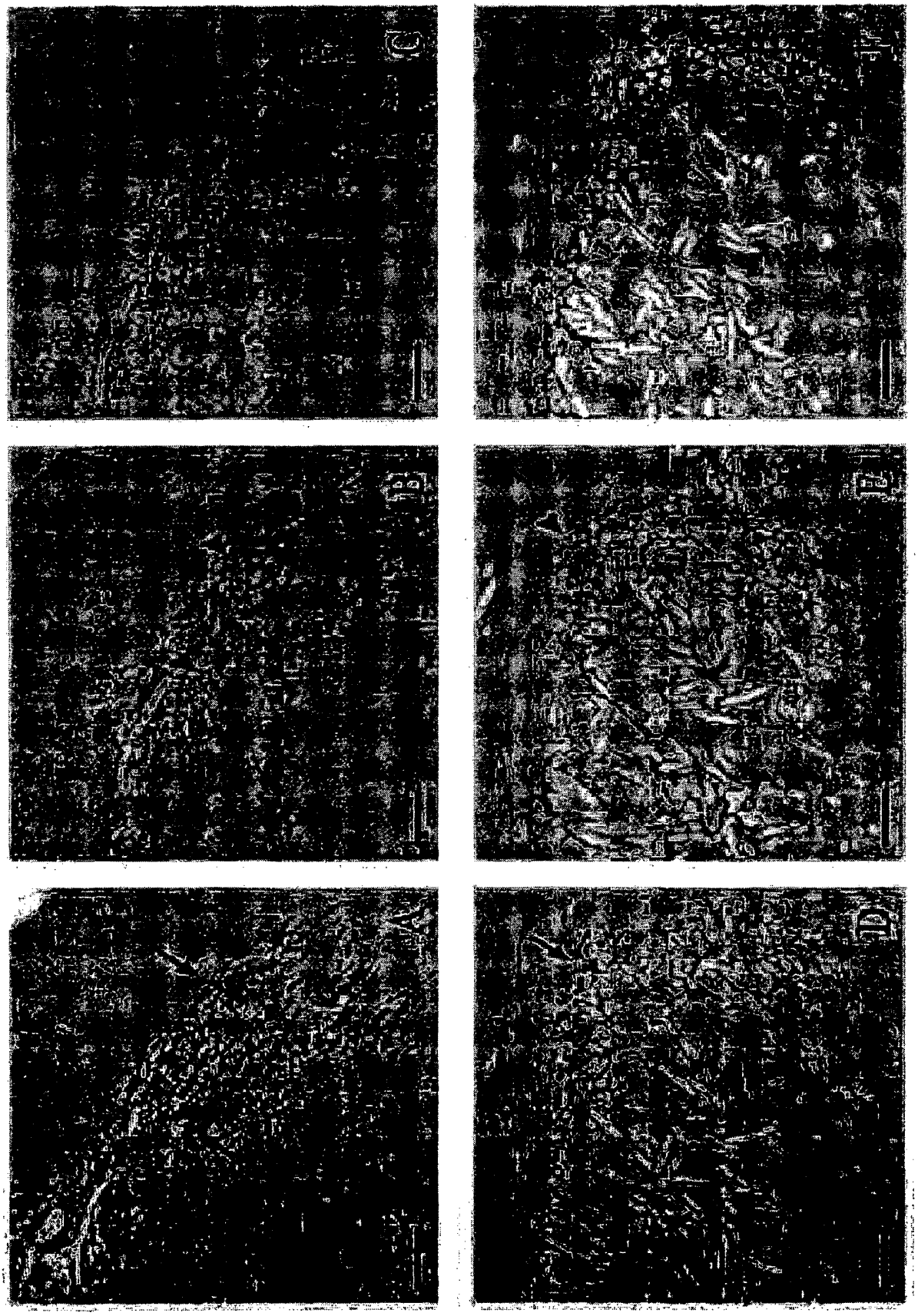


FIGURE 8

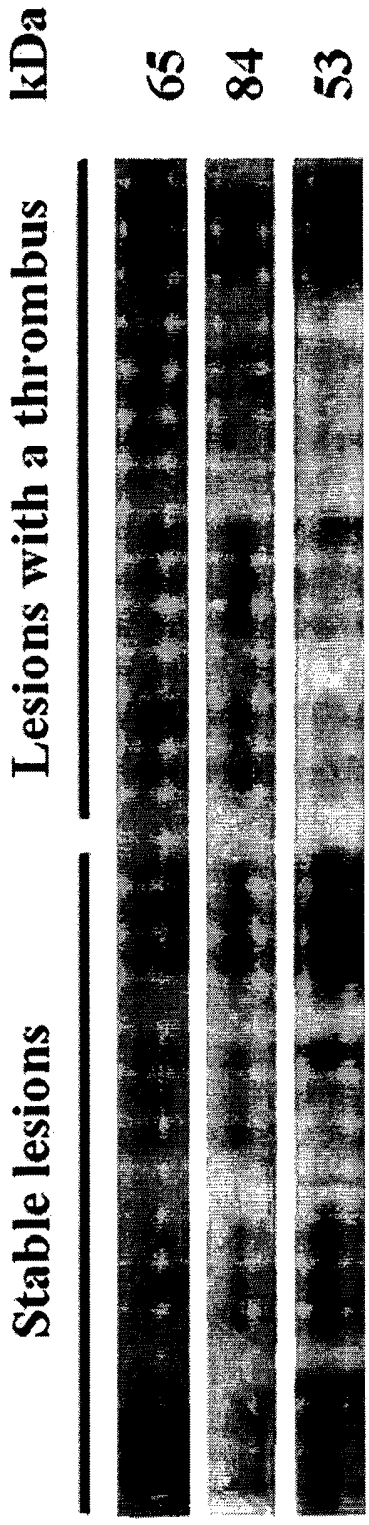

Stable lesions
Lesions with a thrombus
kDa
65
84
53
Perilipin
HSL
ADRP

Figure 9