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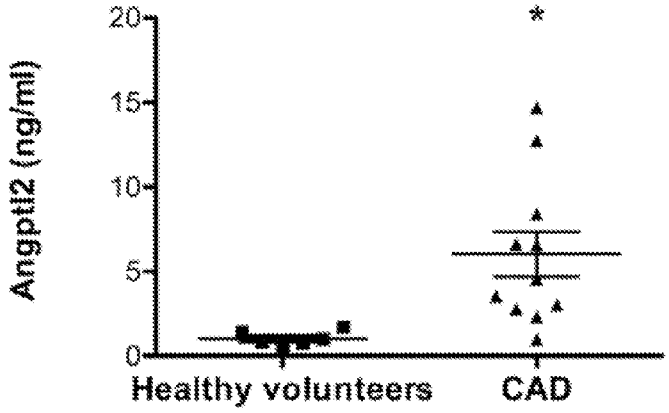
[Continued on next page]

(54) Title: METHODS AND COMPOSITIONS FOR PREVENTING AND TREATING ATHEROSCLEROSIS

Figure 10



(57) Abstract: A method of selecting a subject for treatment with an ANGPTL2 modulator comprising and determining plasma levels of ANGPTL2 and one or more of P-Selectin and ICAM-1 in the sample. The subject is selected for treatment with the ANGPTL2 modulator when ANGPTL2 and P-Selectin; ANGPTL2 and ICAM-1; or ANGPTL2, P-Selectin and ICAM-1 have elevated plasma levels relative to predetermined levels thereof found in healthy controls. Also, related methods of treatments. Subjects with elevated levels of ANGPTL2 and P-selectin, ANGPTL2 and ICAM-1, or ANGPTL2, P-selectin and ICAM-1, are found to have active atherogenesis.

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TITLE OF THE INVENTION

Methods and Compositions for Preventing and Treating Atherosclerosis

FIELD OF THE INVENTION

[001] The present invention relates to compositions and methods for diagnosing, monitoring and prevention of atherosclerosis.

BACKGROUND OF THE INVENTION

[002] Early diagnosis, monitoring and inhibiting atherogenesis to treat atherosclerosis is a critical need in the treatment of cardiovascular disease. Currently treatment strategies focus on reducing the risk factors such as high cholesterol, high glucose, hypertension and obesity. The molecular factors that promote or contribute to atherogenesis, which leads to atherosclerosis and associated catastrophic events, are not well understood. Current therapies, such as statin therapy, for treating and preventing atherosclerosis although widely used are often ineffective including at the earliest stages of the disease and are associated with adverse side effects in particular statin-induced myopathy.

[003] The angiopoietin-like (ANGPTL) family proteins consists of eight members: ANGPTL1, ANGPTL2, ANGPTL3, ANGPTL4, ANGPTL5, ANGPTL6, ANGPTL7, ANGPTL8 (Ando Y, *et al*. J Lipid Res. 2003;44:1216-1223; Kathiresan S, *et al*. Nat Genet. 2008;40:189-197). Of these, ANGPTL3, ANGPTL4 and ANGPTL6 are known to have a role in regulating lipid and energy metabolism and may contribute to the regulation of the cardiovascular functions that influence the progression of cardiovascular disease, including atherosclerosis (MiidaT,

Hirayama S. *et al.*, CurrOpinLipidol. 2010;21:70-75; Kim I, *et al.* J Biol Chem. 1999;274:26523-26528). Angiopoietin-like (ANGPTL) proteins are also known to participate in angiogenesis by affecting the survival and migration of endothelial cells. De-regulated angiogenesis leads to numerous malignant, ischemic, inflammatory, infectious and immune disorders (Carmeliet P. Nature Medicine 2003;9;653-660) and therefore modulation of ANGPTL proteins has many potential therapeutic applications.

[004] ANGPTL2 is a circulating glycoprotein abundantly expressed in the heart, adipose tissue, lung, kidney and skeletal muscle (Kim I, et al. J Biol Chem. 1999;274:26523-26528). ANGPTL2 is a growth factor that stimulates the expansion and the survival of hematopoietic stem cells (Kim I, et al. J Biol Chem. 1999;274:26523-26528; Voghel G, et al. Mech Ageing Dev. 2007;128:662-671). ANGPTL2 expression is stimulated by hypoxia (Oike Y, Tabata M. Circ J. 2009;73:2192-2197; Tabata M, et al. Cell Metab. 2009;10:178-188) and it is known to induce angiogenesis and endothelial cell (EC) migration (Morisada T, et al. Endothelium. 2006;13:71-79; Tabata M, et al. CellMetab. 2009;10:178-188; Oike Y, et al. Blood. 2004;103:3760-3765). ANGPTL2 may play a role in inflammation in various pathologies. A positive correlation between the circulating levels of ANGPTL2 and the concentration of the biomarker C-reactive protein (CRP) has been observed (Tabata M, et al. Cell Metab. 2009;10:178-188). Over-expression of ANGPTL2 is pro-inflammatory in keratinocytes, adipose tissue and EC (Hato T, et al. Trends Cardiovasc. Med. 2008;18:6-14; Tabata M, et al. Cell Metab. 2009;10:178-188). ANGPTL2 activity has been implicated in synovial inflammation in rheumatoid arthritis (Okada T, et al. Am J Pathol. 2010;176:2309-2319), chronic inflammation in dermatomyositis (Ogata A, et al. BiochemBiophysRes Commun. 2012;418:494-499), cancer (Aoi J, et al. Cancer Res. 2011;71:7502-7512; Endo M, et al. Cancer Res. 2012;72:1784-1794) and

abdominal aortic aneurysms (Tazume H, et al. Arteriosclerosis, thrombosis, and vascular biology. 2012;32:1400-1409). Lastly, ANGPTL2 expression by EC isolated from the arteries of active smokers with severe CAD is elevated compared to non-smokers (Farhat N, et al. Can J PhysiolPharmacol. 2008;86:761-769).

[005] Both circulating and aortic levels of ANGPTL2 increase progressively with healthy aging (Tabata M, *et al*. Cell Metab. 2009;10:178-188). A rise in ANGPTL2 blood levels, compared to healthy subjects, has also been observed in both diabetic and obese subjects. Furthermore, increased plasma levels of ANGPTL2 correlate with inflammation, adiposity and insulin resistance (Tabata M, *et al*. CellMetab. 2009;10:178-188; Oike Y, Tabata M. Circ J. 2009;73:2192-2197; Doi Y, *et al*. Diabetes Care. 2013 Jan;36(1):98-100). Plasma levels of ANGPTL2 were found to be higher in Japanese patients with coronary artery disease (CAD) compared to healthy subjects and correlated with the severity of CAD (Oike Y, Tabata M. Circ J. 2009;73:2192-2197; Tabata M, *et al*. Cell Metab. 2009;10:178-188).

[006] ANGPTL2 is currently considered an orphan ligand; the receptors that mediate its activities are largely unknown. However, studies of its interactions have lead to the suggestion that ANGPTL2 signally may involve activation of Mitogen Activated Protein Kinase(MAPK) phosporylation cascades and transactivation of the Epidermal Growth Factor Receptor (EGFR) (Farhat FASEB J April 2009 23 (Meeting Abstract Supplement) 527.4). More recently, it was demonstrated that immune inhibitory receptors such as human leukocyte immunoglobulin-like receptor B2 bind to various ANGPTL proteins including ANGPTL2 (Tabas I, *et al*. Science. 2013;339:166-172).

[007] Studies investigating the physiological and pathophysiological

role of ANGPTL2 are limited and none have demonstrated that ANGPTL2 is pro-atherogenic or participates in the pathology associated with atherosclerosis. Despite strong evidence that ANGPTL2 is positively associated with chronic inflammatory diseases, its role in atherogenesis is unknown.

OBJECTS OF THE INVENTION

[008] An object of the present invention is to provide novel compounds and methods for diagnosing, preventing, reducing or treating atherosclerosis.

SUMMARY OF THE INVENTION

[009] The invention is based on the discovery that over-expression of angiopoietin like-2 (ANGPTL2) by endothelia cells (EC) accelerates atherosclerotic lesion formation by inducing a pro-inflammatory response by EC and leukocyte adhesion to the vascular endothelium. ANGPTL2 (SEQ. ID. NO. 2, SEQ. ID. NO. 3, or SEQ. ID. NO. 4) stimulates the expression of ICAM-1 (SEQ. ID. NO. 16) and P-Selectin (SEQ. ID. NO. 20) by EC and blocking EC expression of ICAM-1 and P-Selectin prevents leukocyte adhesion to the EC. The invention encompasses: methods of using an ANGPTL2 modulator to prevent or reduce atherogenesis; methods for monitoring vascular health, detecting active atherogenesis; and diagnosing atherosclerosis; treatment selection methods and drug screening methods.

[0010] The ANGPTL2 modulators of the invention can be used to block or inhibit the atherogenic effects of ANGPTL2 protein (SEQ. ID. NO. 2, SEQ. ID. NO. 3, or SEQ. ID. NO. 4), thereby preventing the development of atherosclerosis. In one aspect, an ANGPTL2 modulator of the invention is used to inhibit a pro-

inflammatory response or leukocyte adhesion to the vascular endothelium, thereby preventing or reducing atherogenesis or atherosclerosis.

[0011] The invention provides a method of preventing or treating atherogenesis in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of an ANGPTL2 modulator. The invention further provides ANGPTL2 modulators for the prevention or treatment of atherosclerosis. In particular, human subjects at risk of atherosclerosis or having atherosclerosis are selected for treatment with an ANGPTL2 modulator. Subjects considered at risk of atherogenesis or atherosclerosis may include but are not limited to individuals with diabetes or obesity.

[0012] In some embodiments the ANGPTL2 modulator is selected from the group consisting of: an antibody derivative or fragment thereof, a peptide, an agent that binds to ANGPTL2 and any combination thereof. The ANGPTL2 modulator used in such a method can include an antibody or antigen binding fragment thereof that specifically binds to ANGPTL2. In one example, the ANGPTL2 modulator is a monoclonal antibody that binds specifically to ANGPTL2. ANGPTL2 modulators for use in the methods of the invention also include antibody mimetic or peptide-mimetic designed to bind to ANGPTL2. In another embodiment the ANGPTL2 modulator is a molecule that inhibits ANGPTL2 protein expression such as siRNA, shRNA, antisense oligonucleotides, small molecules and ribozymes. The invention further relates to a polynucleotide or polypeptide encoding an ANGPTL2 modulator; methods of preparing such polynucleotides or polypeptides; host cells for expressing an ANGPTL2 modulator and uses of such compositions for the treatment and screening methods described herein.

[0013] ANGPTL2 modulators may be administered in the form of a pharmaceutical composition, as defined herein. Preferably, said modulator is

administered in a therapeutically effective amount. The ANGPTL2 modulator used in the present invention may be administered orally, parenteral, mucosal or topically.

[0014] The invention further provides diagnostic methods and treatment selection methods.

[0015] The invention provides methods of diagnosing atherosclerosis or detecting active atherogenesis in subject comprising assaying ANGPTL2 protein (SEQ. ID. NO. 2, SEQ. ID. NO. 3 or SEQ. ID. NO. 4) and one or more of ICAM-1 protein(SEQ. ID. NO. 15, SEQ. ID. NO. 16 or SEQ. ID. NO. 17) and P-Selectin protein (SEQ. ID. NO. 19, SEQ. ID. NO. 20, SEQ. ID. NO. 21, SEQ. ID. NO. 22 or SEQ. ID. NO. 23) in a plasma sample, for example blood, and more specificallyplasma. The invention provides methods for identifying subjects for treatment with an ANPTL2 modulator comprising analyzing the level of ANGPTL2 and the level of one or more proteins selected from P-Selectin and ICAM-1 in a plasma sample.

[0016] In one embodiment a method of identifying an individual who can benefit from treatment with an ANGPTL2 modulator is provided comprising the steps of: obtaining a plasma sample from the subject; contacting the plasma sample with at least two detection reagents and generating complexes between a respective one of the detection reagents and each protein from a set of proteins selected from the group of sets of proteins consisting of ANGPTL2 and P-Selectin; ANGPTL2 and ICAM-1; and ANGPTL2, P-Selectin and ICAM-1; detecting the complexes and determining plasma levels of ANGPTL2 and one or more of P-Selectin and ICAM-1 in the subject based at least on the detected complexes; and selecting the subject for treatment with the ANGPTL2 modulator when ANGPTL2

and P-Selectin; ANGPTL2 and ICAM-1; or ANGPTL2, P-Selectin and ICAM-1 have elevated plasma levels relative to predetermined levels thereof found in healthy controls. Elevated ANGPTL2 and P-Selectin; ANGPTL2 and ICAM1; or ANGPTL2, P-Selectin and ICAM1, relative to healthy controls, is diagnostic of active atherogenesis, indicating either a risk of atherosclerosis or the presence of atherosclerosis in the subject. Typically the detection reagents d in the methods of the invention are antibodies such as an anti-ANGPTL2 antibody, an anti-P-Selectin antibody and an anti-ICAM-1 antibody. Alternatively, other methods known in the art for the detection of one or more proteins in a plasma sample can be used in the invention including but not limited to mass spectrometry.

[0017] In one embodiment a plasma ANGPTL2 level > 2.5 ng/ml is considered elevated, a plasma soluble P-Selectin level > 40 ng/ml is considered elevated and a protein plasma soluble ICAM-1 level > 300 ng/ml is considered elevated. In one embodiment, a plasma ANGPTL2 level > 2.5 ng/ml and a plasma P-Selectin level > 40 ng/ml indicates the presence of active atherogenesis in a subject. In another embodiment, a plasma ANGPTL2 level > 2.5 ng/ml and a plasma ICAM1 level > 300 ng/ml indicates the presence of active atherogenesis in a subject. In a further embodiment a plasma ANGPTL2 level > 2.5 ng/ml, a plasma P-Selectin level > 40 ng/ml and a plasma ICAM1 level between > 300 ng/ml indicates the presence of active atherogenesis in a subject. In one embodiment a plasma ANGPTL2 level > 2.5 ng/ml indicates the presence of active atherogenesis in a subject.

[0018] In one embodiment the invention provides a method of selecting a subject for treatment with an ANGPTL2 modulator comprising (i) assaying ANGPTL2 protein and one or more of ICAM-1 protein and P-Selectin protein in a plasma sample obtained from the subject; (ii) determining the plasma level of ANGPTL2 protein and one or both of ICAM-1 protein and P-Selectin protein in the

subject; and (iii) selecting the subject for treatment with an ANGPTL2 modulator when plasma ANGPTL2 and one or both of ICAM-1 and P-Selectin are elevated relative to a healthy control.

[0019] In one embodiment, the invention provides a method of selecting a subject for treatment with an ANGPTL2 modulator, the method comprising: obtaining a plasma sample from the subject; contacting the plasma sample with at least two detection reagents and generating complexes between a respective one of the detection reagents and each protein from a set of proteins selected from the group of sets of proteins consisting of ANGPTL2 and P-Selectin; ANGPTL2 and ICAM-1; and ANGPTL2, P-Selectin and ICAM-1; detecting the complexes and determining plasma levels of ANGPTL2 and one or more of P-Selectin and ICAM-1 in the subject based at least on the detected complexes; and selecting the subject for treatment with the ANGPTL2 modulator when ANGPTL2 and P-Selectin; ANGPTL2 and ICAM-1; or ANGPTL2, P-Selectin and ICAM-1 have elevated plasma levels relative to predetermined levels thereof found in healthy controls. In some embodiments, the complexes are detected by an instrument providing a data set associated therewith, the data set being analyzed using a computer to determine the plasma levels.

[0020] In one embodiment the invention provides a method of detecting active atherogenesis in a subject, comprising: obtaining a plasma sample from the subject; contacting the plasma sample with an anti-ANGPTL2 antibody or a fragment thereof and one or more antibodies selected from an anti-ICAM-1 antibody or a fragment thereof and an anti-P-Selectin antibody or a fragment thereof to generate antibody-protein complexes; measuring the level of each type of the antibody-protein complexes generated in step (ii) to determine the levels of ANGPTL2 and ICAM-1, AGPTL2 and P-Selectin, or ANGPTL2, ICAM-1 and P-Selectin in the plasma sample; detecting the presence of active atherogenesis in

the subject if the levels of ANGPTL2 and ICAM-1, or AGPTL2 and P-Selectin, or ANGPTL2, ICAM-1 and P-Selectin are elevated relative to predetermined levels of the same polypeptides in a normal controls.

[0021] In yet a further embodiment, the subject is selected for treatment with an ANGPTL2 modulator when active atherogenesis is detected in the subject. In yet a further embodiment, after the subject is selected for treatment with an ANGPTL2 modulator, the subject is treated with an ANGPTL2 modulator. In yet a further embodiment, the method further comprises diagnosing the subject with atherosclerosis.

[0022] In one embodiment the invention provides a method of detecting active atherogenesis in a subject from a sample obtained from the subject, the method comprising: analyzing the sample using mass spectrometry to determine levels of ANGPTL2 and one or more proteins selected from ICAM-1 and P-Selectin in the sample; and identifying the presence of active atherogenesis in the subject if the levels of ANGPTL2 and the one or more proteins selected are elevated relative to normal levels of the same proteins in a normal control.

[0023] In yet a further embodiment, the subject is selected for treatment with an ANGPTL2 modulator when active atherogenesis is detected in the subject. In yet a further embodiment, after the subject is selected for treatment with an ANGPTL2 modulator the subject is treated with an ANGPTL2 modulator. In yet a further embodiment, the method further comprises diagnosing the subject with atherosclerosis.

[0024] In one embodiment the amount of ANGPTL2 and one or more of ICAM-1 and P-Selectin protein are measured by an immunoassay comprising the steps of: (i) reacting a sample with immobilized antibodies (or a fragment thereof) specific to ANGPTL2 and one or more proteins selected from ICAM-1 and P-

Selectin and (ii) reacting an unlabelled or labelled secondary antibodies (or a fragment thereof) specific to the same two or more proteins with a complexes formed by the immobilized antibodies and proteins present in the sample.

[0025] In a further embodiment the invention relates to a method of determining the levels of a set of proteins present in a biological sample, preferably a plasma sample wherein said set comprises ANGPTL2 and one or two proteins selected from ICM-1 and P-Selectin. The level of a protein in a biological sample can be determined using any method known in the art including but not limited to an ELISA method or mass spectrometry analysis of enriched and digested protein obtained from a biological sample.

[0026] Alternately the level of a protein can be determined by measuring the expression of the mRNA corresponding to the protein of interest. Pathological, abnormal or disease associated levels of a protein can be determined by comparing protein levels detected in a subject with a normal control.

[0027] The invention provides the use of an expression level of a protein set for detecting the presence of active atherogenesis in a subject, the protein set consisting of ANGPTL2 and one or more proteins selected from ICAM-1 and P-Selectin.

[0028] In a further embodiment the invention relates to obtaining a plasma sample from the subject; contacting the plasma sample with anti-ANGPTL2 antibody and anti-ICAM-1 antibody; or anti-ANGPTL2 antibody and anti-P-Selectin antibody; or anti-ANGPTL2 antibody, anti-ICAM-1 antibody and anti-P-Selectin antibody; and determining that the subject has elevated plasma levels of ANGPTL2 and P-Selectin or elevated plasma levels of ANGPTL2 and ICAM-1 or elevated plasma levels of ANGPTL2, P-Selectin and ICAM-1 using the plasma

sample after contact with the antibodies, indicating the presence of active atherogenesis in the subject.

[0029] In some embodiments, the subject is selected for treatment with an ANGPTL2 modulator when active atherogenesis is present in the subject.

[0030] In some embodiments, when active atherogenesis is present in the subject, the subject is selected for treatment with an ANGPTL2 modulator and the ANGPTL2 modulator is administered to the subject.

[0031] In one embodiment when a subject's plasma levels of ANGPTL2 and ICAM-1 or ANGPTL2 and P-Selectin or ANGPTL2, ICAM-1and P-Selectin are elevated relative to a normal control the subject is selected the presence of atherogenesis is detected and the subject is selected for treatment with an ANGPTL2 modulator. In a further embodiment subjects found to have active atherogenesis are diagnosed with atherosclerosis based on the presence of other symptoms or risk factors associated with the disease. In yet a further embodiment subjects found to have active atherogenesis using the methods of the invention are treated with a therapeutic amount of an ANGPTL2 modulator.

[0032] In a further embodiment the invention provides compositions for use in the methods of the invention including a plasma sample or derivative thereof comprising antibody reagents consisting of: (i) an anti-ANGPTL2 antibody or antigen binding fragment thereof and an anti-ICAM-1 antibody or antigen binding fragment thereof, or (ii) an anti-ANGPTL2 antibody or antigen binding fragment thereof and an anti-P-Selectin antibody or antigen binding fragment thereof, or (iii) an anti-ANGPTL2 antibody or antigen binding fragment thereof, an anti-ICAM-1 antibody or antigen binding fragment thereof, and an anti-P-Selectin antibody or antigen binding fragment thereof. Wherein said antibody reagents are monoclonal

or polyclonal and produced by hybridoma cells or isolated from an animal immunized with an antigen of interest and purified. Methods for producing, isolating and purifying antibodies are described in detail in F. Ausubel *et al*, eds., "Current Protocols in Molecular Biology", Wiley Interscience, (2012).

[0033] In one embodiment the invention provides a method of detecting active atherogenesis in a subject comprising: analyzing the sample using mass spectrometry to determine the level of ANGPTL2 and one or more proteins selected from ICAM-1 and P-Selectin in the sample; and identifying the presence of of active atherogenesis in the subject if the levels of ANGPTL2 and the one or more proteins selected are elevated relative to levels of the same proteins in a normal control.

[0034] Prior to mass spectrometry analysis of the protein content of a biological sample, using the methods of the invention, proteins present in the sample, i.e. ANGPTL2, ICAM-1 and P-Selectin can be isolated using high performance liquid chromatography. In one embodiment proteins levels in a plasma sample obtained from a subject are determined using Selective Reaction Monitoring Mass Spectrometry (LC-SRM-MS).

[0035] The invention further relates to methods of screening for therapeutic agents, or drugs, useful in the treatment of prevention of atherosclerosis, comprising the step of analyzing the extent to which a candidate ANGPTL2 modulator inhibits ANGPTL2 activity, expression or function. In one embodiment the screening method comprises the steps of: (i) administering a candidate ANGPTL2 modulator to an animal model of atherosclerosis, (ii) measuring the pro-inflammatory (cytokine and adhesion molecule expression) or pro-atherogenesis (evolution of atherosclerotic plaque size) activities of ANGPTL2 in said animal

model. Pro-inflammatory activities measured as part of the screening methods of the present invention include: measures of cytokines such as TNF-α, IL6, IL1 and measures of adhesion molecules such as P-Selectin, ICAM1. Measures for use in the invention include expression by cells comprising or within arteries or measures circulating levels of cytokines or adhesion molecules in blood, serum or plasma. Pro- atherogenesis activities measured as part of the screening methods of the present invention include: measures of the evolution of the atherosclerotic plaque size through time, measure of the burden of oxidative stress using the measure of 4-HNE, isoprostane, nitrosylated proteins and the like as well as measure of the macrophage load in the atherosclerotic plaque.

[0036] In another embodiment a drug screening method comprises the steps of: (i) bringing a candidate ANGPTL2 modulator in contact with a cell that expresses ANGPTL2 (ii) measuring an amount of an endogenous ANGPTL2 protein or gene contained in the cell or secreted by the cell, and (iii) analyzing a test substance dependent change in the amount thereof. The analyzing step of this method may comprise either measuring ANGPTL2 expression by Western blot (tissue protein) or quantitative PCR (mRNA tissue expression), or circulating ANGPTL2 levels either *in vivo* or in *in vitro* (in cultured cell medium) using an ELISA. In a further embodiment the screening method comprises the steps of: (i) administering a candidate ANGPTL2 modulator to an animal model of atherosclerosis and (ii) measuring circulating plasma levels of ANGPTL2.

[0037] A further aspect of the invention relates a method for screening an ANGPTL2 modulator for the treatment or prevention of atherosclerosis. In one embodiment the screening method measures the binding of a candidate AGPTL2 modulator, or to circulating ANGPTL2, or a fusion protein thereof by means of a label directly or indirectly associated with the candidate compound. Alternatively, a screening method may involve measuring or, qualitatively or quantitatively,

detecting ability of said candidate compound to modulate the pro-inflammatory, pro-atherogenic, pro-leukocyte adhesion effects of ANPTL2, and efficiently treat and prevent atherosclerosis.

[0038] In a one embodiment, the screening method of the invention comprises the steps consisting of: (i) providing a plurality of cells expressing ANGPTL2; (ii) incubating said cells with a candidate ANGPTL2 modulator; (iii) determining whether said ANGPTL2 modulator binds to ANGPTL2; and (iv) selecting the candidate compound that binds ANGPTL2. In a particular embodiment, the screening method of the invention may further comprising a step consisting of administering the candidate compound selected at step (iv) to an animal model of atherosclerosis to validate the therapeutic and/or protective effects of said ANGPTL2 modulator on atherosclerosis.

[0039] In one embodiment the screening method of the present invention comprises the steps of: (i) bringing a candidate ANGPTL2 modulator to be tested into contact with a cell, and (ii) measuring an amount of an endogenous ANGPTL2 protein or mRNA expressed by the cell, and analyzing a candidate ANGPTL2 modulator dependent change in the amount thereof, and, if desired, may further comprise the step of: analyzing effects of a candidate, which is selected in the above step (ii) on leukocyte adhesion, expression of inflammatory cytokines or expression of adhesion molecule.

[0040] In one embodiment the screening method of the present invention comprises the steps of: (i) administering an ANGPTL2 modulator to an animal and (ii) measuring an amount of circulating plasma ANGPTL2 protein, and analyzing a candidate ANGPTL2 modulator dependent change in the amount thereof, and, if desired, may further comprise the step of: analyzing effects of a candidate selected in the above step ii) on leukocyte adhesion, expression of inflammatory

cytokines or expression of adhesion molecule on leukocyte adhesion, expression of inflammatory cytokines or expression of adhesion molecules, angiogenesis or the progression of atherosclerosis.

[0041] A further aspect of the invention concerns a kit for detecting active atherogenesis in a mammal, the kit comprising: (a) a first monoclonal antibody or antigen-binding fragment thereof which binds specifically with ANGPTL2; (b) a second antibody or antigen-binding fragment thereof which binds specifically to ICAM-1; and (c) an instructional material describing the use of the first and second monoclonal antibodies or fragments thereof for detecting the active atherogenesis in a mammal. In alternative embodiments, the kit comprises: (a) a first monoclonal antibody or antigen-binding fragment thereof which binds specifically with ANGPTL2; (b) a second antibody or antigen-binding fragment thereof which binds specifically to P-Selectin; and (c) an instructional material describing the use of the first and second antibodies or fragments thereof for detecting the active atherogenesis in a mammal. In other alternative embodiments, the kit comprises: (a) a first monoclonal antibody or antigen-binding fragment thereof which binds specifically with ANGPTL2; (b) a second antibody or antigen-binding fragment thereof which binds specifically to ICAM-1; (c) a third antibody or antigen-binding fragment thereof which binds specifically to P-Selectin; and (d) an instructional material describing the use of the first, second and third antibodies or fragments thereof for detecting the active atherogenesis in a mammal. In all these embodiments, the antibodies may be attached to a solid surface.

[0042] In another broad aspect, the invention provides a method of detecting active atherogenesis in a subject, the method comprising the steps of: determining plasma levels of plasma proteins from a protein set, the protein set consisting of ANGPTL2 and one or more proteins selected from ICAM-1 and P-Selectin; and detecting the presence of active atherogenesis in the subject based

on the plasma levels of the plasma proteins determined in step (i). In some embodiments, the determining step further comprises the step of comparing the expression level of each plasma protein to a normal control.

[0043] In other broad aspects, the invention provides an isolated plasma sample or derivative thereof comprising two antibody reagents consisting of an (i) anti-AGPTL2 antibody or antigen binding fragment thereof, and (ii) an anti-ICAM-1 antibody or antigen fragment thereof; or an isolated plasma sample or derivative thereof comprising two antibody reagents consisting of an (i) anti-AGPTL2 antibody or antigen binding fragment thereof, and (ii) an anti-P-Selectin antibody or antigen fragment thereof; or an isolated plasma sample or derivative thereof comprising three antibody reagents consisting of an (i) anti-AGPTL2 antibody or antigen binding fragment thereof, (ii) an anti-ICAM-1 antibody or antigen fragment thereof and (iii) an anti-P-Selectin antibody or antigen fragment thereof.

[0044] Other aspects, embodiments, advantages and application of the invention will become clear from the further description provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0045] In the appended drawings:

[0046] Figure 1 illustrates thatstimulation of native mouse aortic EC with recombinant ANGPTL2 (100 nmol/L) increases TNF-α and IL-6 gene expression in both WT and ATX mice ($p<0.05$). ANGPTL2 equally stimulates the expression of these inflammatory cytokines in WT and ATX mice. Quantitative RT-PCR analysis

of TNF-α and IL-6 mRNA expression was performed in EC freshly extracted from aortas of 3-mo WT and LDLr-/-; hApoB+/+(ATX) mice stimulated or not (Control) with recombinant ANGPTL2 (100 nmol/L). Results were normalized to CycloA expression and average gene expression level in WT control cells was arbitrarily set at 1. Data are mean ± SEM of n=4 WT and ATX mice, each experiment was performed in triplicates. *: $p< 0.05$ versus Control; †: $p<0.05$ versus condition-matched in WT mice.

[0047] Figure 2 illustrates that ANGPTL2 (100 nM, 30 min) increases expression of pro-inflammatory molecules P-Selectin and ICAM-1 in ATX animals (A). In the absence of exogenous ANGPTL2 stimulation, ICAM-1 is 2X higher in ATX mice compared to WT and P-Selectin is 2X higher in ATX mice compared to WT. After 30 min of stimulation with ANGPTL2, ICAM-1 and P-Selectin increased 2 folds in both groups. Expression of ICAM-1 and P-Selectin mRNA in freshly isolated aortic EC from WT and $LDLr^{-/-}$; $hApoB^{+/+}$ (ATX) mice was quantified by quantitative RT-PCR. Data are mean ± SEM of 6-7 mice. *: $p< 0.05$ versus Control; †: $p<0.05$ versus condition-matched in WT mice. (B) ANGPTL2 stimulates leukocyte adhesion to the native endothelium in ATX, but not WT mice. Leukocytes were labeled with ^{51}Cr and incubated with aortic segments stimulated or not (Control) with thrombin (10 U/ml) or recombinant ANGPTL2 (100 nmol/L). Adherent ^{51}Cr-leukocytes were counted and results were expressed as number of adherent cells / mm2 of endothelium surface area. Data are mean ± SEM of 6-12 mice. *: $p< 0.05$ versus Control; †: $p<0.05$ versus condition-matched in WT mice. (C) Aortic segments were pre-incubated with anti-P-Selectin, anti-ICAM-1 antibodies or the corresponding isotype-matched IgG prior to stimulation with

recombinant ANGPTL2 (100 nmol/L). Adhesion of ^{51}Cr-leukocytes was then quantified. Data are mean ± SEM of 9-16 mice. *: $p < 0.05$ versus Control; †: $p<0.05$ versus + ANGPTL2 condition; φ: $p<0.05$ versus antibody + ANGPTL2 condition.

[0048] Figure 3 illustrates ANGPTL2 mRNA abundance and cell surface expression of cell adhesion molecules in leukocytes from both WT and ATX mice. Basal and ANGPTL2-induced expression of (A) CD18, (B) CD62L and (C) CD162 in leukocytes from 3-mo WT and ATX mice, were quantified by quantitative RT-PCR. Cell surface expression of (D) CD18, (E) CD62L and (F) CD162 was quantified in Control and ANGPTL2-treated WT and ATX leukocytes by flow cytometry. Leukocytes were labeled using monoclonal anti-CD18, anti-CD62 and anti-CD162 antibodies or with corresponding isotype-matched IgG (data not shown). Data are mean ± SEM of 6 mice. *: $p < 0.05$ versus Control condition.

[0049] Figure 4 illustrates that chronic ANGPTL2 infusion for 1 month accelerates atherogenesis in 3-mo ATX mice.(A)Infusion with ANGPTL2 promotes the expression of inflammatory cytokines and adhesion molecules in freshly scraped EC from the aorta evaluated by quantitative RT-PCR and normalized by cyclophilin A, (B) ANGPTL2 accelerates the formation of the atherosclerotic plaque and (C) ANGPTL2 increases total cholesterol and LDL plasma levels. Data are mean ± SEM of n=5 ANGPTL2-infused mice and n=5 TBSE-infused mice. *: $p < 0.05$ *versus* TBSE condition.

[0050] Figure 5 illustrates that ANGPTL2 plasma levels and ANGPTL2 expression in the atherosclerotic plaque increase with age and atherosclerosis. (A) Samples corresponding to equal amounts of total protein were collected from

plasma in wild-type mice (WT) and LDLr-/-;hApoB+/+ (ATX) mice at 3, 6 or 12 months of age. Low abundant proteins in the plasma samples were enriched by depletion of high-abundance proteins. Enriched plasma samples were then subjected to SDS-PAGE and immune-blotting with an antibody against ANGPTL2. Results (arbitrary units, AU) are presented as the mean ± SEM of 4 mice in each group. *: $p < 0.05$ versus 6-mo mice; †: $p<0.05$ versus age-matched WT mice. (B) Photographs of the atherosclerotic lesion in abdominal aorta from 6-, 9- and 12-mo LDLr-/-; hApoB+/+ (ATX) mice. (C) Proteins were specifically extracted from the lesion and Western blot analysis was performed for ANGPTL2 and F4/80, a marker of mature macrophages. (D) Correlation between ANGPTL2 level ($r^2=0.8026$, $p<0.0001$, $n=12$), F4/80 ($r^2=0.9373$, $p<0.0001$, $n=9$), and the surface area of the lesion. Data are mean ± SEM of 9-12 mice.*: $p < 0.05$ versus 6-mo ATX mice; γ: $p<0.05$ versus 9-mo ATX mice.

[0051] Figure 6 illustrates that Aortic ANGPTL2 immuno-fluorescence increases with age and the progression of atherosclerosis with a marked accumulation within the atherosclerotic plaque. Immuno-fluorescence was used to visualize ANGPTL2 in (A) fresh longitudinally opened aortas and (B) frozen aortic sections of WT and $LDLr^{-/-}$;$hApoB^{+/+}$(ATX) mice at 6 and 12 months of age. In Panel A, z-stacks were acquired, deconvoluted, and 3D images rendered. ANGPTL2 levels are shown in red and basal membrane in green. Nuclei are shown in blue. (C) Immuno-fluorescence of ANGPTL2 (red) and CD-31 (green) in frozen aortic sections from 6-mo ATX mice.

[0052] Figure 7 illustrates that Expression and secretion of ANGPTL2 is greater in endothelial cells than in VSMC (A) Western blot analysis of endogenous

ANGPTL2 secreted into the culture medium overnight (16 h) by hIMAEC, HUVEC and VSMC. ANGPTL2 protein expression was also quantified in the cell lysates. (B) Endogenous ANGPTL2 expression in the cells was detected by immuno-fluorescence using a confocal microscope. Scale bar represents 20 µm. (C) Quantitative RT-PCR analysis of ANGPTL2 mRNA expression was performed in the cell lysate from cultured cells. Results were normalized to GAPDH expression and average gene expression level in hIMAEC was arbitrarily set at 1. Data are mean ± SEM of n=3, each experiment was performed in duplicates. *: $p< 0.05$ versus hIMAEC. (D) To assess ANGPTL2 binding, hIMAEC, HUVEC and VSMC were incubated for 10 min with human ANGPTL2-luciferase (100 nmol/L) in phenol-free medium. Cells were then washed and the binding of ANGPTL2-luciferase to cell surface was revealed by adding the luciferase substrate. A selection of confocal time-series images acquired in a single living cell at 0, 50 and 200 seconds is shown (left panel). The average ANGPTL2-luciferase fluorescent signals (AU) recorded in VSCM (n=6 cells), hIMAEC (n=6 cells) and HUVEC (n=4 cells) was derived from the time-series images (right panel). Data are mean ± SEM and the assay was performed 3-4 times.

[0053] Figure 8 illustrates in Panel A that epididymal fat weight is reduced in ANGPTL2 knock-down (KD) mice Data are mean ± SEM of 4-5 wild-type littermates compared to homozygote $^{-/-}$ANGPTL2 KD mice. In panel B, metabolic parameters of 6-month old WT and ANGPTL2 KD mice following a high fat diet of 3 months (n=7-11) are shown. * $P<0.05$, ** $P<0.01$, ** $P<0.001$ vs. a regular diet.

[0054] Figure 9 illustrates LDL-to-HDL cholesterol ratio in diet-treated WT and KD mice. * $P<0.05$ (n=7-11). This lipid profile was accompanied by a lack of

increase in the ratio HDL-to-LDL cholesterol in ANGPTL2 KD mice unlike in WT mice (Figure 9).

[0055] Figure 10 illustrates that plasma ANGPTL2 levels are higher in CAD patients than in healthy volunteers. Circulating ANGPTL2 levels were quantified in CAD patients (n=11) and in age-matched healthy volunteers (n=6) by ELISA.

Data are mean ± SEM. *: $p < 0.05$ versus healthy volunteers.

[0056] Figure 11 illustrates purification of human ANGPTL2 recombinant protein. Coomassie blue (A) and Western blot (B) analysis of the recombinant ANGPTL2-GST protein purified by affinity chromatography on glutathione Sepharose. (A) Lane 1 in each gel contains protein molecular weight markers, lane 2 (in first gel) represents sample before purification, lane 3 represents the flow-through fraction. Fractions 1 to 18 (F1-F18) represent the column wash and product eluted. (B) Western blot detection using a human ANGPTL2 antibody. Coomassie blue staining (grey scale) shows the purity of the protein and Western blot detection confirms the presence of ANGPTL2-GST. (C) Purified ANGPTL2 presents three bands on SDS-PAGE shown in Figure 11.

[0057] Figure 12 illustrates experiments in which HUVEC were infected for 7h with a lentivirus shRNA targeted to ANGPTL2 expression at an MOI = 40, in the presence of polybrene (6 µg/ml), in cell medium without serum. The construct contains the GFP-encoding gene (visible by fluorescence microscopy, see picture) as well as puromycinN-acetyl-transferase. After 48 h, non-infected cells were eliminated with puromycin (8 µg/ml). Efficacy of infection was 85%. Four days after infection, HUVEC were lysed and analyzed by Western blot. ANGPTL2 shRNA efficiently suppresses ANGPTL2 expression (full gel shown).

[0058] Figure 13 liiustrates Angptl2siRNA (human) that was obtained from OriGene Technologies, Inc.Three sequences were tested: A: SR308285A: rUrCrCrUrUrGrUrArArUrGrArCrArCrGrArArUrCrUrGrCAA; B: SR308285B: rArGrArArUrGrUrCrUrArCrArArUrGrCrUrArArUrCrUrCTC; and C: SR308285C:rGrGrArCrArGrGrArCrUrArCrArGrArCrArArCrUrCrUrUTC. In all conditions, the siRNA led to a significant inhibition of ANGPTL2 gene (mRNA) expression in HUVEC. Human Umbilical Vein Endothelial Cells (HUVECs) were seeded one day prior to cell treatment to allow optimal cell conditions. At the time of treatment, cells are grown to approximately 70 to 80% confluency. Oligofectamine from Invitrogen is used as a transfection reagent that forms stable complexes with oligos to permit for efficient transfections in eukaryotic cells. Opti-MEM from Gibco is used as the medium of transfection. Opti-MEM was mixed with Oligofectamine reagent for 5 minutes while Opti-MEM was also mixed with 15nM of siRNA for 5 minutes. After 5 minutes, Opti-MEM with Oligofectamine and Opti-MEM with siRNA were mixed together for another 30 minutes prior to treatment of cells. Cells were treated at 37°C and 7% CO2 for 6 hours. After 6 hours, cells were provided with 5% FBS and allowed to grow at 37°C and 7% CO2 for time-points of interest.

DETAILED DESCRIPTION

[0059] Unless indicated or defined otherwise, all terms used have their usual meaning in the art to which the present invention relates. Reference is for example made to the standard handbooks, such as Sambrook *et al*, "Molecular Cloning: A Laboratory Manual", 4".Ed. Cold Spring Harbor Laboratory Press (2012); F. Ausubel*et al*, eds., "Current protocols in molecular biology", Wiley Interscience, (2012); Lewin, "Genes C", Jones & Bartlett Learning (2011); and Janeway *et al*., "Immunobiology" (7th Ed.), Garland Science (2008). The terminology used herein

is for the purpose of describing particular embodiments only and is not intended to be limiting.

[0060] Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

[0061] Throughout this specification, unless the context requires otherwise, the word “comprise”, or variations such as “comprises” or “comprising”, will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

[0062] As used herein the singular forms “a”, “an” and “the” include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to “a cell” includes a single cell, as well as two or more cells; reference to “an agent” includes one agent, as well as two or more agents; and so forth.

[0063] The term “antibody” is used herein in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multi-specific antibodies (e.g. bi-specific antibodies) formed from at least two intact antibodies, and antibody fragments. “Antibody fragments” comprise only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed usually against a single antigen. The term "antibody" is intended to

encompass "immunoglobulins" and derivatives thereof. Immunoglobulins comprise various broad classes of polypeptides that can be distinguished biochemically. In many examples, immunoglobulins consist of combination heavy chains and light chains. All immunoglobulin classes including IgM, IgA, IgD, IgE, IgG and IgY and where appropriate, their subclasses, are clearly within the scope of the present invention. The following discussion will generally be directed to the IgG class of immunoglobulin molecules. With regard to IgG, a standard immunoglobulin molecule comprises two identical light chain polypeptides of molecular weight approximately 25 kDa, and two identical heavy chain polypeptides of approximate molecular weight 50 kDa. The resulting molecule, which is conventionally referred to as an IgG "monomer" consists of identical halves and the four chains that are typically joined by disulfide bonds in a "Y" configuration wherein the light chains adjoin the heavy chains starting at the mouth of the "Y" and continuing through the variable region or domain. It is well recognized by those skilled in the art that immunoglobulins can be characterized in terms of variable and constant domains. In this regard, it will be appreciated that the variable domains of both the light (VL) and heavy (VH) chain portions determine antigen recognition and specificity. Conversely, the constant domains of the light chain (CL) and the heavy chain (normally consisting of CHI, CH2 or CH3 domains) confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. As indicated above, the variable region allows the antibody to selectively recognize and specifically bind epitopes on antigens. That is, the VL domain and VH domain of an antibody combine to form the variable region that defines a three dimensional antigen binding site, this site is also called the "antigen receptor". This antibody structure forms the antigen binding site or antigen receptor present at the end of each arm of the Y. More specifically, the antigen binding site is defined by three complimentarity determining regions (CD s) on each of the VH and VL chains. Thus within the

amino acid sequence of a variable domain of an antibody there are three CDRs (known as CD 1, CDR2 and CDR3). Since most sequence variation associated with immunoglobulins is found in the CDRs, these regions are sometimes referred to as "hypervariable regions", among these CDRs, CDR3 shows the greatest variability. Since the antigen binding sites are typically composed of two variable domains (on two different polypeptide chains being the heavy and light chain), there are six CDRs for each antigen receptor that can collectively come into contact with the antigen. Thus a single IgG molecule has two antigen receptors, and therefore consists of twelve CDRs. CDRs can also be referred to as "idiotypes". In some instances, for example certain immunoglobulin molecules derived from camelid species or engineered molecules based on camelid-immunoglobulins, a complete immunoglobulin molecule may consist of heavy chains only, with no light chains. See, e.g., Hamers*et al.* Nature 363:446 448 (1993).

[0064] As used herein “modulator” or “inhibitor” means a molecule that completely or partially inhibits the expression, biological activity, binding affinity or binding specificity of ANGPTL2. In alternate embodiments of the invention, the percent inhibition of ANGPTL2 expression, biological activity, binding affinity or binding specificity is 100%, at least 99%, at least 98%, at least 97%, at least 96%, at least 95%, at least 94%, at least 93%, at least 92%, at least 91%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, at least 50%, at least 40%, at least 30%, at least 20%, at least 10% or at least 5%. “Inhibition of ANGPTL2 expression” or ANGPTL2 modulation” as used herein means a decrease or absence in the level of ANGPTL2 protein and/or mRNA product. Specificity refers to the ability to inhibit the target an ANGPTL2 gene product without effecting the expression of gene products corresponding to genes other than ANGPTL2. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism or by biochemical

techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Many such reporter genes are known in the art. Inhibition of the expression ANGPTL2 is characterized by under-production a mRNA transcript of the gene or the polypeptide encoded by the gene, relative to a control. Whereas the differential expression on the mRNA level can be detected by hybridization and amplification assays, the differential expression on the protein level can be determined using agents that specifically bind to the encoded protein product, in e.g., an immunoassay. ANGPTL2 activity, its biological effects on endothelial cells, arteries, skeletal muscle, adipocytes, heart, liver can be determined using the methods described herein as well as by methods known by those skilled in the art.In determining the percent inhibition of ANGPTL2 activity, measurements of said activity made after administration of an ANGPTL2 modulator are compared to measurements made in the same patient before inhibitor administration, or are compared to the appropriate normal range. The ANGPTL2 modulating molecules described herein, bind to ANGPTL2 protein or inhibit the expression of ANGPTL2 mRNA, reducing its pro-inflammatory and pro-atherogenesis activities, reducing the initiation of atherogenesis and leukocyte adhesion onto the vascular endothelium.

[0065] The terms “modulator’, “inhibitor”, “drug”, “composition”, “agent”, “medicament” and “active agent” are used interchangeably herein to refer to a chemical compound, biological molecule or cellular composition that induces a desired pharmacological and/or physiological effect. In particular with respect to the present invention an“AGPTL2 modulator” or “ANPTL2 inhibiting agent” induces

the inhibition, reduction or blocking of ANGPTL2 or its activity when administered to a subject to prevent atherogenesis or atherosclerosis. The terms encompass pharmaceutically acceptable and pharmacologically active ingredients including but not limited to salts, esters, amides, pro-drugs, active metabolites, analogs and the like. The term includes genetic and proteinaceous or lipid molecules or analogs thereof as well as cellular compositions as previously mentioned. The compositions of the instant invention are suitable for the manufacture of a medicament for the prevention of atherogenesis and the treatment and/or prevention of atherosclerosis, as described herein.

[0066] The term “subject” includes, without limitation, humans and non-human primates, livestock animals, companion animals, laboratory test animals, captive wild animals, reptiles and amphibians, fish, birds and any other organism. The most preferred subject of the present invention is a human. A subject, regardless of whether it is a human or non-human organism may be referred to as a patient, individual, subject, animal, host or recipient.

[0067] A “control” is an alternative subject or sample used in an experiment for comparison purpose. A control can be “positive” or “negative”. For example, where the purpose of the experiment is to determine the level of a protein in a biological sample relative to a control, it is generally preferable to use a positive control (a subject or a sample from a subject, carrying such alteration and exhibiting syndromes characteristic of atherosclerosis or atherogenesis), and a negative control (a subject or a sample from a subject lacking the altered expression and syndromes characteristic of atherosclerosis or atherogenesis).

[0068] As used herein, the term "normal" or “normal control” is defined as a defined the plasma level of a protein, the defined level being associated with a disease-free phenotype. It will be appreciated however that in the case of detecting a pathological condition in a patient suffering from a disease, the defined

level of the protein may be associated with a defined stage of disease as opposed to a disease-free phenotype. In an embodiment of the invention, the term "normal" may be the plasma level of a protein evaluated at a first time point. Optionally or additionally, the plasma level of a protein may be evaluated at a second, or subsequent, time point. Further optionally or additionally, the plasma level of a protein may evaluated in a series of more than two subsequent time points. Each or any of the time points may then be used, or referenced as "normal".

[0069] The term "sample" includes any biological sample taken from a patient or individual including a cell, tissue sample or body fluid. For example, a sample may include a skin sample, a cheek cell sample, saliva or blood cells. A sample can include, without limitation, a single cell, multiple cells, fragments of cells, an aliquot of a body fluid, whole blood, platelets, serum, plasma, red blood cells, white blood cells, endothelial cells, tissue biopsies, synovial fluid and lymphatic fluid.

[0070] The term “therapeutically effective amount” refers to an amount of a pharmaceutical composition effective to treat a disease or condition in a subject. A therapeutically effective amount of an ANGPTL2 modulator can be used to effectively treat or to prevent atherosclerosis at a reasonable benefit/risk ratio applicable to any medical treatment. The compositions of the present invention are preferably administered in a therapeutically effective amount. In the case of atherosclerosis, the therapeutically effective amount of the ANGPTL2, may reduce atherosclerotic plaque burden or slow its evolution as well as reduce the inflammatory load of the patient and be associated with an improved cardiopulmonary fitness, i.e. it capacity to physical activity. These measures of efficacy against atherosclerosis can be measured as part of patient care. It will be well within the capabilities of a skilled medical practitioner to determine the appropriate dosage for an individual patient in view of the patent's size, age, sex,

weight, general health, disease progression and previous or current experience of side effects, for example.

[0071] The term “polypeptide”, “peptide” and “protein” are used interchangeably herein refer to a polymer of amino acids of any length. The terms not exclude post-translational modifications that include but are not limited to phosphorylation, acetylation, glycosylation and the like. Also encompassed by this definition of “polypeptide” are homologs thereof. Polypeptides of the invention may be produced by any technique known in the art, such as without limitation, any chemical, biological, genetic or enzymatic technique, either alone or in combination(s). Knowing the amino acid sequence of the desired sequence, one skilled in the art can readily produce said polypeptides, by standard techniques for production of polypeptides. For instance, they can be synthesized using well-known solid phase method, preferably using a commercially available peptide synthesis apparatus (such as that made by Applied Biosystems, Foster City, Calif.) and following the manufacturer's instructions. Alternatively, the polypeptides of the invention can be synthesized by recombinant DNA techniques as is now well-known in the art. For example, these fragments can be obtained as DNA expression products after incorporation of DNA sequences encoding the desired polypeptide into expression vectors and introduction of such vectors into suitable eukaryotic or prokaryotic hosts that will express the desired polypeptide, from which they can be later isolated using well-known techniques. The amino acid polymer may be polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms “polypeptide”, “peptide” and “protein” also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a

labeling component. As used herein the term “amino acid” refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics.

[0072] “Angiopoietin-like 2” protein or ANGPTL2 protein as used herein means a protein represented by SEQ ID NO. 2 or 3, or a protein coded for by the ANGPTL2 gene represented by SEQ ID NO. 1 and corresponding to any of the ANGPTL2 mRNAs or cDNA represented by SEQ ID NO. 5 - 14. Synonyms for ANGPTL2 include ARP2; HARP; angiopoietin-like protein 2; angiopoietin-related protein 2; and angiopoietin-like 2.

[0073] ICAM-1 protein as used herein means a protein represented by SEQ ID NO. 16 or 17, or a protein coded for by the ICAM-1 gene represented by SEQ ID NO. 15 and corresponding to any of the ICAM-1 mRNAs or cDNA represented by SEQ ID NO. 18 - 19. Synonyms for ICAM-1 include: BB2; CD54; P3.58; cell surface glycoprotein P3.58; intercellular adhesion molecule 1 (CD54), human rhinovirus receptor; major group rhinovirus receptor; and intercellular adhesion molecule 1.

[0074] P-Selectin protein as used herein means a protein represented by SEQ ID NO. 24 - 28 or a protein coded for by the P-Selectin gene represented by SEQ ID NO. 20 and corresponding to any of the P-Selectin mRNAs or cDNA represented by SEQ ID NO. 21 - 23. Synonyms for P-Selectin include P-selectin glycoprotein ligand 1; CD162; CLA; PSGL-1; PSGL1; cutaneous lymphocyte-associated associated antigen; selectin P ligand and SELPLG.

[0075] The terms “polynucleotide”, “nucleotide”, “nucleotide sequence”, and as used herein refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, analogs or modified forms thereof.

Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component.

[0076] "Oligonucleotides" are a polynucleotide subset generally comprising a length of 200 bases or fewer. Preferably, oligonucleotides are 10 to 60 bases in length and most preferably 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 bases in length. Oligonucleotides can be single stranded, e.g. for probes; although oligonucleotides may be double stranded, e.g. for use in the construction of a gene mutant. The oligonucleotides of the invention include oligonucleotides containing modified backbones or non-natural inter-nucleoside linkages. Oligonucleotides having modified backbones include those retaining a phosphorus atom in the backbone, and those that do not have a phosphorus atom in the backbone. Preferred modified oligonucleotide backbones include phosphorothioates or phosphorodithioate, chiral phosphorothioates, phosphotriesters and alkyl phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including methylphosphonates, 3′-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoroamidates or phosphordiamidates, including 3′-amino phosphoroamidate and aminoalkylphosphoroamidates, and

phosphorodiamidatemorpholino oligomers (PMOs), thiophosphoroamidates, phosphoramidothioates, thioalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3′-5′ linkages, 2′-5′ linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3′-5′ to 5′-3′ or 2′-5′ to 5′-2′. Various salts, mixed salts and free acid forms are also included.

[0077] The term “target polynucleotide” as used herein corresponds to a gene transcript that has served as a template for the synthesis of a polynucleotide detected in a method of the invention for example: a mRNA reverse transcribed to produce a cDNA, cDNA transcribed to produce an RNA molecule, a cDNA amplified to produce a DNA molecule, amplified DNA used to transcribe RNA all may correspond to a target polynucleotide. An ANGPTL2 target polynucleotide may correspond to an ANGPTL2 mRNA or cDNA including but not limited to SEQ. ID. NOs.5, 6, 7, 8, 9, 10, 11, 12, 13 and 14, or a substantially homologous polynucleotide. An ICAM-1 target polynucleotide may correspond to an ICAM-1 mRNA or cDNA including but not limited to SEQ. ID. NOs.18 and 19. A P-Selectin target polynucleotide may correspond to a P-Selectin mRNA or cDNA for example SEQ. ID. NOs.21, 22, and 23.

[0078] A “nucleotide probe” or “probe” refers to an oligonucleotide used for detecting or identifying its corresponding target polynucleotide in a hybridization reaction. The term "probes" includes naturally occurring or recombinant single- or double-stranded nucleic acids or chemically synthesized nucleic acids. They may be labeled by nick translation, Klenow fill-in reaction, PCR or other methods known in the art. Probes of the present invention, their preparation and/or labeling are described in Sambrook et al. (2012) supra. Preferably, for the purpose of the present inventions a probe is a polynucleotide of any length suitable for selective

hybridization to an ANGPTL2 polynucleotide; a P-Selectin target polynucleotide or an ICAM-1 target polynucleotide. The length of the probe used will depend, in part, on the nature of the assay used and the hybridization conditions employed. "ANGPTL2 probe" refers to a probe suitable for hybridization of an ANGPTL2 target polynucleotide. "ICAM-1 probe" refers to a probe suitable for hybridization of an ICAM-1 target polynucleotide. "P-Selectin probe" refers to a probe suitable for hybridization of a P-Selectin target polynucleotide.

[0079] More particularly as used herein "primer" refers a short oligonucleotide, generally with a free 3′-OH group, that binds to a target polynucleotide or "template" potentially present in a sample of interest by hybridizing with the target, and thereafter promoting polymerization of a polynucleotide complementary to the target. "ANGPTL2 primer" refers to a primer suitable for hybridization of an ANGPTL2 target polynucleotide. "ICAM-1 primer" refers to a primer suitable for hybridization of an ICAM-1 target polynucleotide. "P-Selectin primer" refers to a primer suitable for hybridization of a P-Selectin target polynucleotide.

[0080] A "vector" is a nucleic acid molecule, preferably self-replicating, which transfers an inserted nucleic acid molecule into and/or between host cells. The term includes vectors that function primarily for insertion of DNA or RNA into a cell, replication of vectors that function primarily for the replication of DNA or RNA, and expression vectors that function for transcription and/or translation of the DNA

or RNA. Also included are vectors that provide more than one of the above functions. In its broadest sense "vector" as used herein means any vehicle capable of facilitating the transfer of the antisense oligonucleotide siRNA or ribozyme nucleic acid to cells and preferably cells expressing ANGPTL2.

[0081] An "expression vector" is a polynucleotide which, when introduced into an appropriate host cell, can be transcribed and translated into a polypeptide(s). An "expression system" usually connotes a suitable host cell comprised of an expression vector that can function to yield a desired expression product.

[0082] As used herein, the term 'ribozyme' means RNA molecules that contain antisense sequences for specific recognition, and an RNA-cleaving enzymatic activity. Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5′ end of the target mRNA (i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts).

[0083] In general a "substantially homologous sequences", "substantially homologous nucleotide sequences" or "substantially homologous oligonucleotides" are at least about 60% identical with each other, after alignment of the homologous regions. Preferably, the sequences are at least about 80% identical; more preferably, they are at least about 85% identical; more preferably, they are at least about 90% identical; still more preferably, the sequences are 95% identical. Sequence alignment and homology searches can be determined with the aid of computer methods. A variety of software programs are available in the art. Non-limiting examples of these programs are Blast, Fasta (Genetics Computing Group package, Madison, Wis.), DNA Star, MegAlign, Tera-BLAST (Timelogic) and GeneJocky. Any sequence databases that contains DNA sequences corresponding to a target gene or a segment thereof can be used for sequence

analysis. Commonly employed databases include but are not limited to GenBank, EMBL, DDBJ, PDB, SWISS-PROT, EST, STS, GSS, and HTGS. Sequence similarity can be discerned by aligning a small interfering RNA against a target endogenous gene sequence. Common parameters for determining the extent of homology set forth by one or more of the aforementioned alignment programs include p value and percent sequence identity. P value is the probability that the alignment is produced by chance. For a single alignment, the p value can be calculated according to Karlin et al. (1990) Prco.Natl. Acad. Sci 87: 2246. For multiple alignments, the p value can be calculated using a heuristic approach such as the one programmed in Blast. Percent sequence identity is defined by the ratio of the number of nucleotide matches between the query sequence and the known sequence when the two are optimally aligned. To determine that nucleotide sequencesare substantially homologous, it is useful to first establish the lowest temperature at which only homologous hybridization occurs with a particular concentration of salt (e.g., SSC or SSPE). Then, assuming that 1% mismatching results in a 1° C. decrease in the Tm, the temperature of the final wash in the hybridization reaction is reduced accordingly (for example, if sequences having >95% identity are sought, the final wash temperature is decreased by 5° C.). In practice, the change in Tm can be between 0.5° C. and 1.5° C. per 1% mismatch.

[0084] "Stringent hybridization conditions", "high stringency conditions" or "high stringency hybridization" as used herein means hybridizing at 68° C. in 5×SSC/5× Denhardt's solution/1.0% SDS, and washing in 0.2×SSC/0.1% SDS at room temperature, or involve the art-recognized equivalent thereof. Moderately stringent conditions, as defined herein, involve including washing in 3×SSC at 42° C., or the art-recognized equivalent thereof. The parameters of salt concentration and temperature can be varied to achieve the optimal level of identity between the probe and the target nucleic acid. Guidance regarding such conditions is available in the art, for example, by Sambrook et al. "Molecular Cloning: A Laboratory

Manual", 4th Edition, (2012) and F. Ausubel et al, eds., "Current protocols in molecular biology" Chapter 2, Wiley Interscience, (2012).

[0085] "Differentially expressed" as applied to nucleotide sequence or polypeptide sequence in a subject, refers to over-expression or under-expression of that sequence when compared to that detected in a control. Under-expression also encompasses absence of expression of a particular sequence as evidenced by the absence of detectable expression in a test subject when compared to a control.

[0086] "Atherosclerosis" also known as arteriosclerotic vascular disease (ASVD) is characterized by a thickening of an arterial wall as a result of the accumulation of fatty materials such as cholesterol and triglyceride occurring due to atherogenesis. Atherosclerosis is a chronic disease that is asymptomatic for decades. Atherosclerotic plaques can be either stable or unstable (also called vulnerable). Stable plaques are typically asymptomatic. Unstable plaques are prone to rupture leading to intra-luminal thrombi, occluded arteries, coronary occlusion and stroke. The complications of advanced atherosclerosis are chronic, slowly progressive and cumulative. Commonly, vulnerable plaques can suddenly rupture, causing the formation of a thrombus that will rapidly slow or stop blood flow, quickly leading to death of the tissues fed by the blocked artery. This event is called an infarction, such as a myocardial infarction. Atherosclerosis can affect any part of the arterial system, but primarily occurs in larger, high-pressure vessels such as the coronary, renal, femoral, cerebral, and carotid arteries.

[0087] "Atherogenesis" as used herein means the development process of atheromatous plaques characterized by remodeling of arteries leading to sub-endothelial accumulation of fatty substances or plaques containing excess fat, collagen and elastin. This process involves inflammation and the formation of atheromaous plaques in the region of the vessel wall located between the

endothelium and the tunica media. The early stages of atherogenesis are characterized by adhesion of circulating monocytes to the vascular endothelium, migration of these monocytes into the sub-endothelial space and activation of monocyte-derived macrophages. The key driver of this process is oxidized lipoprotein particles such as low-density lipoprotein (LDL) residing within the endothelial wall of the vessel. Active atherogenesis can be present in a subject either at risk of atherosclerosis or with atherosclerosis. When active atherogenesis is detected in a subject, it may indicate either risk of atherosclerosis or with atherosclerosis. Distinguish between risk of atherosclerosis or a diagnosis of atherosclerosis, based on a variety of well known diagnostic measures and atherosclerosis risk factors, is within the current skill in the art of cardiovascular medical care.

[0088] Identifying the presence of active atherogenesis in a subject and can facilitate early diagnosis, prevention or treatment of atherosclerosis.

[0089] ANGPTL2 Modulators

[0090] The present invention relates to ANGPTL2 modulators that can be used in methods for the treating or preventing of atherosclerosis.

[0091] In some embodiments the ANGPTL2 modulators of the invention bind to ANGPTL2 protein (i.e. SEQ. ID NO. 2, 3, 4),prevent interaction of ANGPTL2 with its cognate receptors inhibiting or reducing ANGPTL2 biological activity and thereby reducing or inhibiting the pro-inflammatory and pro-angiogenic activities of ANGPTL2. ANGPTL2 modulators that prevent ANGPTL2 interaction with its cognate receptors include antibodies, peptides, small molecules and aptamers.

[0092] In another embodiment the ANGPTL2 modulators of the invention reduce the expression of ANGPTL2 protein reducing the level of interaction of

ANGPTL2 with its cognate receptors and its biological activity and thereby reducing or inhibiting the pro-inflammatory and pro-angiogenic activities of ANGPTL2. ANGPTL2 modulators that reduce the expression of ANGPTL2 for use in the invention include ribozymes, antisense molecules, siRNA (for example SEQ. ID. NO. 9, SEQ. ID. NO. 10, or SEQ. ID NO. 11), miRNA and shRNA.

[0093] Exemplary ANGPTL2 modulators include the anti-ANGPTL2 miRNA described in U.S patent application US20100010073 (SEQ. ID. NO. 69) and the anti-ANGPTL2 antibodies and antigen binding fragments described in U.S. patent applications: US20120076796, US20100010073 and US20120076796, herein incorporated by reference.

[0094] Antibodies and Antigen-Binding Molecules

[0095] Antibodies directed against ANGPTL2 can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production. Although antibodies useful in practicing the invention can be polyclonal, monoclonal antibodies are preferred. Monoclonal antibodies against ANGPTL2 can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975); the human B-cell hybridoma technique (Cote *et al*., 1983); and the EBV-hybridoma technique (Cole *et al*. 1985). Alternatively, techniques described for the production of single chain antibodies (see, e.g., U.S. Pat. No. 4,946,778) can be adapted to produce anti-ANGPTL2 single chain antibodies. ANGPTL2 modulators also useful in practicing the present invention also include anti-ANGPTL2, antibody fragments including but not limited to F(ab')2 fragments, which can be generated by pepsin digestion of an

intact antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab and/or scFv expression libraries can be constructed to allow rapid identification of fragments having the desired specificity for ANGPTL2.

[0096] Humanized anti-ANGPTL2 antibodies and antibody fragments there from can be prepared using known techniques. Humanized antibodies are forms of non-human (e.g., rodent) chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hyper-variable region (CDRs) of the recipient are replaced by residues from a hyper-variable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. 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 hyper-variable loops correspond to those of a non-human immunoglobulin and all or substantially all FRs correspond to those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Methods for making humanized antibodies are described, for example, by Winter (U.S. Pat. No. 5,225,539) and Boss (Celltech, U.S. Pat. No. 4,816,397).

[0097] Anti-ANGPTL2 antibodies, and variants thereof such as humanized antibodies, including those specifically disclosed herein can be used as an

ANGPTL2 modulator. Furthermore the anti-ANGPTL2 antibodies disclosed herein and in Table 1 can be used as a reagent for the detection of plasma levels of ANGPTL2 protein in a subject.

[0098] Table 1: Examples of Anti-ANGPTL2 antibodies for use in the diagnostic, screening or treatment methods of the present invention

Name	Supplier	Catalogue Number
Human Angiopoietin-like 2 Affinity Purified Polyclonal Ab	R&D Systems	AF2084
Human Angiopoietin-like 2 MAb Clone 239829)	R&D Systems	MAB2084
Angiopoietin like 2 antibody	Biorbyt	orb10092
Anti-ANGPTL2	Atlas Antibodies	HPA041299
Anti-ANGPTL2	Atlas Antibodies	HPA040933
anti-Angiopoietin-Like 2 (ANGPTL2) (C-Term) antibody	antibodies-online	ABIN390460
Anti-ANGPTL2	LifeSpanBioSciences	LS-C81946-50
ANGPTL2	Antibody Novus Biologicals	NBP1-79557
Monoclonal ANGPTL2 Antibody	USCN Life Science	A91919Hu22
ANGPTL2 (H-97)	Santa Cruz Biotech	sc-292811

ANGPTL2 (P-13)	Santa Cruz Biotech	sc-107143
ANGPTL2 (97864)	Santa Cruz Biotech	sc-73711

[0099] Antibodies or immuno-specific fragments thereof for use in the methods of the invention include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized, primatized, or chimeric antibodies, single chain antibodies, epitope-binding fragments, e.g., Fab, Fab' and F(ab')2, Fd, Fvs, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), fragments comprising either a VL or VH domain, fragments produced by a Fab expression library, and anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to binding molecules disclosed herein). ScFv molecules are known in the art and are produced using recombinant DNA technology as described in Winter *et al*. Immunoglobulin or antibody molecules of the invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgGI, IgG2, IgG3, IgG4, IgAI and IgA2) or subclass of immunoglobulin molecule.

[00100] The term "antibody" as used herein is also intended to encompass antibodies, digestion fragments, specified portions and variants thereof, including antibody mimetics or comprising portions of antibodies that mimic the structure and/or function of an antibody or specified fragment or portion thereof, including single chain antibodies and fragments thereof; each containing at least one CDR. See Qiu *et al*.. Nature Biotechnology 25:921 -929 (2007). Functional fragments include antigen binding fragments that bind to the target such as CD63. For example, antibody fragments capable of binding to CD63 or a portion thereof, including, but not limited to Fab (e.g., by papain digestion), facb (e.g., by plasmin digestion), pFc' (e.g., by pepsin or plasmin digestion), Fd (e.g., by pepsin digestion, partial reduction and re-aggregation), Fv or scFv (e.g., by molecular

biology techniques) fragments, are encompassed by the present current invention. Antibody fragments are also intended to include for example, domain deleted antibodies, linear antibodies, single-chain antibody molecules, multi-specific antibodies formed from antibody fragments and diabodies. After producing antigen-binding molecules directed against ANGPTL2 as above described, a person skilled in the art can easily select those that effectively inhibit the biological activities of ANGPTL2 in vivo.

[00101] The AGPTL2 modulator may be an antigen binding molecule. In one embodiment, an antigen binding molecule of the invention comprises at least one heavy or light chain CDR of an antibody molecule. In another embodiment, an antigen binding molecule of the invention comprises at least two CDRs from one or more antibody molecules. In another embodiment, an antigen binding molecule of the invention comprises at least three CDRs from one or more antibody molecules. In another embodiment, an antigen binding molecule of the invention comprises at least four CDRs from one or more antibody molecules. In another embodiment, an antigen binding molecule of the invention comprises at least five CDRs from one or more antibody molecules. In another embodiment, an antigen binding molecule of the invention comprises at least six CDRs from one or more antibody molecules.

[00102] Aptamers

[00103] In another embodiment the ANGPTL2 modulator is an aptamer directed against ANGPTL2. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment

(SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S. D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996). After producing aptamers directed against ANGPTL2 as above described, a person skilled in the art can easily select those that effectively inhibit the biological activities of ANGPTL2 in vivo.

[00104] The efficacy of the polypeptides of the invention, and of compositions comprising the same, can be tested using any suitable in vitro assay, cell based assay or in vivo assay and/or animal model known or in any combination thereof. Exemplary assays include solid phase binding assays, in vivo atherogenesis assay, as well as the in vivo and in vitro assay method described in the methods section included herein.

[00105] Gene Expression Inhibitors

[00106] Another aspect of the invention relates to an inhibitor of ANGPTL2 gene expression for treating or preventing atherosclerosis, in particular an ANGPTL2 modulator that reduces circulating ANGPTL2 by inhibiting gene expression.

[00107] Various synthetic RNA-based compositions can be used to reduce ANGPTL2 expression and circulating ANGPTL2 levels in vivo, RNA-based ANGPTL2 modulators. Molecules for interfering with mRNA expression include short interfering RNA (siRNA), short interfering hairpin RNA (shRNA), double stranded RNA (dsRNA) and the like. Pharmaceutical compositions comprising

siRNA, shRNa, dsRNA can be used in the methods of the present invention as ANGPTL2 modulators. Methods for the selection, design, production and administration and siRNA, shRNA and dsRNA are well known in the art for genes whose sequence is known and described in detail in Carmichael G.C. RNA Silencing Methods and Protocols, Humana Press, 2005; and U.S. Pat. Applications2012/0246747, US 2012/0184598 and 2012/0322855, herein incorporated by reference. siRNA for inhibiting ANGPTL2 expression and methods or preparing such siRNA are known in the art, for example those described in Zhao Y, Ding S ProcNatlAcadSci U S A. 2007 Jun 5;104(23):9673-8. Exemplary siRNA of the invention include SEQ. ID. NOs. 29-33. Exemplary shRNA of the invention include: SEQ. ID. NOs. 34-46. Pharmaceutical compositions comprising a siRNA or shRNA, including those specifically disclosed herein can be used as an ANGPTL2 modulator.

[0100] RNA-basedANGPTL2 modulators of the invention may comprise one or more strands of polymerized ribonucleotide, and the phosphate-sugar backbone or the nucleosides thereof may contain modifications. Phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Bases may be modified to block the activity of adenosine deaminase.

[0101] Preferably greater than 90% sequence identity, or even 100% sequence identity, exists between a RNA-based ANGPTL2 modulator and a portion of an ANGPTL2 gene product such as those corresponding to SEQ ID NO. 5-14.

[0102] Alternatively, an ANGPTL2 RNA-based modulator may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of an ANGPTL2 mRNA sequence under high stringency conditions (e.g.,

400 mMNaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50° C. or 70° C. hybridization for 12-16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 15, 20, 25, 50, 100, bases.

[0103] Preferably, a siRNA ANGPTL2 modulator comprises a nucleic acid sequence of, e.g., at least 9, at least 15, at least 18, or at least 20 contiguous bases in length that is complementary to, or hybridizes under moderately stringent or stringent conditions to a sequence selected from the group consisting of SEQ ID NO. 5-14, and sequences substantially homologous thereto. siRNA may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the in vitro or in vivo introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA such as a plasmid or viral vector. Such a kit may also include instructions to allow a user of the kit to practice the invention.

[0104] shRNA are created by introducing an siRNA into an expression vector that can be sued to gene knockdown by transfection as described in more detail in the examples provided herein. The design and production of shRNA constructs is well known in the art and described in detail in Perrimon *et al*, "In vivo RNAi: Today and Tomorrow, Cold Spring Harbor Perspectives in Biology (2010) and protocols are widely available such as at http://www.protocol-online.org/prot/Molecular_Biology/RNA/RNA_Interference__RNAi_/. shRNA that inhibit ANGPTL2 expression, for example shRNA corresponding to SEQ ID NO. 34-46), and methods of preparing shRNA are known in the art, for example as described in Cleary MA, et al. Nat Methods. 2004 Dec;1(3):241-8; Paddison PJ, et al. Nature. 2004 Mar 25;428(6981):427-31.

[0105] ANGPTL2 modulators for use in the present invention can also be anti-sense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of ANGPTL2 mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of ANGPTL2, and thus activity, in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding ANGPTL2 can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically inhibiting gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

[0106] Suitable injection mixes are known in the art for the direct administration of siRNA or dsRNA. Animals receive an average of 0.5×106 to 1.0×106 molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections are compared with equal masses of RNA (i.e. dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible.

[0107] Exemplary siRNA molecules for use in the present invention include SEQ. ID NO. 29-33. Furthermore, commercially available siRNA can be used as ANGPTL2 modulators in the present invention.

[0108] Table 2: Commercially available siRNA ANGPTL2 modulators

Name	Supplier	Catalogue Number
ANGPTL2 siRNA (m)	Santa Cruz Biotechnology	sc-72352
ANGPTL2 siRNA (human)	OriGene Technologies, Inc	SR308285A
ANGPTL2 siRNA (human)	OriGene Technologies, Inc	SR308285B
ANGPTL2 siRNA (human)	OriGene Technologies, Inc	SR308285C

[0109] Both antisense oligonucleotides and ribozymes are useful for inhibiting ANGPTL2 gene expression, can be prepared using known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramadite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and

half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5′ and/or 3′ ends of the molecule, or the use of phosphorothioate or 2′-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone. Antisense molecules for use as an ANGPTL2 modulator in the present invention comprise at least 10, 15, 20 or 25 consecutive nucleotides complementary to one or more ANGPLT target polynucleotides and hybridize under stringent or highly stringency conditions one of more ANGPTL2 target polynucleotides (SEQ ID NO. 5 – 14).Antisense molecules preferably comprise at least 20, or at least 25, and preferably less than about 35 consecutive complementary nucleotides. In one embodiment the antisense molecules are phosphorodiamidatemorpholino oligomers (PMO) molecules. Particular embodiments provide an isolated polynucleotide with a sequence comprising a transcriptional initiation region and a sequence encoding an ANGPTL2 expression modulating antisense oligonucleotide at least 15, 20 or 25 nucleotides in length, and a recombinant vector comprising this polynucleotide (e.g., expression vector). Preferably, the transcriptional initiation region is a strong constitutively expressed mammalian pol III- or pol II-specific promoter, or a viral promoter. Chimeric antisense oligonucleotides are also within the scope of the invention, and can be prepared from the present inventive oligonucleotides using the methods described in, for example, U.S. Pat. 6,677,445, 6,846,921, 5,700,922, 7,259,150 and 5,958,773 and U.S. Pat. Application 2001/0044528 A1.

[0110] Ribozymes can also function to inhibit ANGPTL2 gene expression and modulate ANGPTL2 activity in vivo. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic

cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage ANGPTL2 mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. Ribozymes can be targeted to any RNA transcript and can catalytically cleave such transcripts (see, e.g., U.S. Pat. Nos. 5,272,262; 5,144,019; 5,168,053, 5,180,818, 5,116,742 and 5,093,246). According to certain embodiments of the invention, any such ANGPTL2 modulating gene ribozyme, or a nucleic acid encoding such a ribozyme, may be delivered to a host cell to effect inhibition ANGPTL2 gene expression. Ribozymes and the like may therefore be delivered to the host cells by DNA encoding the ribozyme linked to a eukaryotic promoter (e.g., a strong constitutively expressed pol III- or pol II-specific promoter), or a eukaryotic viral promoter, such that upon introduction into the nucleus, the ribozyme will be directly transcribed. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays. Ribozymes of the present invention typically consist of RNA, but may also be composed of DNA, nucleic acid analogs (e.g., phosphorothioates), or chimerics thereof (e.g., DNA/RNA/RNA). A wide variety of ribozymes may be utilized within the context of the present invention, including for example: the hammerhead ribozyme (for example, as described in U.S. Pat. Appl. US 2006/0121466 A1 and U.S. Patent 6,307,041); the hairpin ribozyme (for example, as described in U.S. Patents 6,022,962, and 5,837,855, and U.S. Pat.

Appl. 2005/0260163); and Tetrahymena ribosomal RNA-based ribozymes (for example, as described in U.S. No. 4,987,071).

[0111] Antisense oligonucleotides, shRNA and ribozymes of the invention may be delivered in association with a vector. Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide siRNA or ribozyme nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

[0112] Pharmaceutical Compositions

[0113] The ANGPTL2 modulators of the present invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions. The pharmaceutical compositions of the present invention contain an active agent, an ANGPTL2 modulator, alone or in combination with another active agent. The therapeutic compositions of the invention can be administered

in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intra-peritoneal, intra-muscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

[0114] Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions. Pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability 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.

[0115] Solutions comprising ANGPTL2 modulators as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative

to prevent the growth of microorganisms.

[0116] The ANGPTL2 modulators of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. 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. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium-monostearate and gelatin.

[0117] Sterile injectable solutions are prepared by incorporating the active polypeptides in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the 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, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0118] ANGPTL2 modulators of the invention maybe formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; liposomal formulations; time release capsules; and any other form currently used. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

[0119] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed. The ANGPTL2 modulators of the

invention may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered.

[0120] The ANGPTL2 modulators of the invention may also be used in combination with other therapeutic agents, for instance. HMG-CoA reductase inhibitors such as statins; niacin; cholesterol absorption-inhibiting supplements such as ezetimibe and fibrates; CETP inhibitors such as evacetrapib, anacetrapib, dalcetrapib; HDL-mimetics, angiotensin-converting enzyme inhibitors such as perindopril, captopril, enalapril, lisinopril, and ramipril;angiotensin receptor antagonists such aslosartan, candesartan, telmisartan, valsartan; beta-blocker drugs such as bisoprolol, carvedilol and sustained-release metoprolol; cardio tonic agents such as ivabradine; calcium channel blockers such as amlodipine, aranidipine, azelnidipine, barnidipine, benidipine, cilnidipin, clevidipine, isradipine, efonidipine; folic acid, aspirin, anti-inflammatory drugs or other drugs commonly used in standard cardiovascular care arelikely to be co-administered in the treatment of patients with cardiovascular or coronary artery disease. Moreover, if they are contained in different pharmaceutical compositions, said compositions may be administered to the patient at the same time or successively. The foregoing therapeutically active agents are listed by way of example and are not meant to be limiting. Other therapeutically active agents which are currently available or that may be developed in the future are equally applicable to the methods of the present invention.

[0121] Diagnostic and Therapeutic Screening Methods

[0122] The present invention provides methods of diagnosing

atherosclerosis or detecting active atherogenesis in a subject comprising:(i) obtaining a biological sample from the subject, (ii) analyzing the sample to determine gene expression or protein levels corresponding to ANGPTL2 and P-Selectin or ANGPTL2 and ICAM-1; or ANGPTL2, P-Selectin and ICAM-1, wherein an elevation in ANGPTL2 and P-Selectin or ANGPTL2 and ICAM-1 or ANGPTL2, P-Selectin and ICAM-1 relative to a healthy control indicates the presence of atherosclerosis or active atherogenesis in the subject.

[0123] The present invention provides methods for screening subjects and selecting subjects for treatment with an ANGPTL2 modulator. In some embodiments the treatment selection method comprises(i) obtaining a biological sample from the subject, (ii) analyzing the sample to determine gene expression or protein levels corresponding to ANGPTL2 and P-Selectin or ANGPTL2 and ICAM-1 or ANGPTL2, P-Selectin and ICAM-1 and (iii) selecting the subject for treatment with an ANGPTL2 modulator when plasma levels of ANGPTL2 and P-Selectin or ANGPTL2 and ICAM-1 or ANGPTL2, P-Selectin and ICAM-1 are significantly elevated in the subject relative to a control or 'healthy level. In a further embodiment the method further comprises administering an ANGPTL2 modulator to a subject when levels plasma levels of ANGPTL2 and P-Selectin or ANGPTL2 and ICAM-1 or ANGPTL2, P-Selectin and ICAM-1 are found to be elevated in the subject.

[0124] The *in vivo* level of a protein can be determined either by assaying the level of the protein itself, in a biological fluid, or by measuring the level of gene expression in a cell that produces the protein, preferably vascular endothelial cells, adipocytes, hepatocytes, macrophages, neuronal cells, which are among the cell types known to produce ANGPTL2. As such plasma levels of ANGPTL2 can be estimated based on gene expression data and gene expression

analysis can be used to detect an elevated levels of ANGPTL2 and ICAM-1, or ANGPL2 and P-Selectin, or ANGPLT2, P-Selectin and ICAM-1 in an subject, to detect active atherogenesis and determine if the subject is either at risk of atherosclerosis or has atherosclerosis. Where over-expression of ANGPTL2 and ICAM-1, or ANGPL2 and P-Selectin, or ANGPLT2, P-Selectin and ICAM-1 indicates elevated plasma levels of the same. Gene expression analysis can be performed using a variety of methods such as but not limited to those described herein.

[0125] The determination of the level of a protein or levels of proteins in a biological sample sample can be performed using a computer by querying a pre-existing dataset comprising data corresponding to levels of proteins i.e. ANGPTL2, ICAM-1 and P-Selectin, in a biological sample, preferably a plasma sample. Accordingly, the present invention provides a computer-implemented methods and systems for detecting active atherogenesis or atherosclerosis in a subject. Such system comprises: (a) a computer; (b) a database coupled to the computer; (c) a database coupled to a database server having data stored thereon, the data comprising records of data captured from an instrument that detects proteins or reagent-protein complexes in a sample and can be used to determine the level of a protein or reagent-protein complex in a sample.

[0126] Gene Expression Analysis

[0127] Oligonucleotides, including probes, the bind specifically to a target oligonucleotide corresponding to ANGPTL2, P-Selectin or ICAM-1 can be used as reagents to detect ANPTL2, P-Selectin or ICAM-1 expression. Specific oligonucleotides are capable of hybridizing to a sequence, and under suitable

conditions will not bind to a sequence differing by a single nucleotide. Oligonucleotides of the invention, used as probes, can be detectably labeled. Labels can be detected either directly, for example for fluorescent labels, or indirectly. Indirect detection can include any detection method known to one of skill in the art, including biotin-avidin interactions, antibody binding and the like. Fluorescently labeled oligonucleotides also can contain a quenching molecule. Oligonucleotides can be bound to a surface. In some embodiments, the surface is silica or glass. In some embodiments, the surface is a metal electrode.

[0128] The determination of the level of a protein or levels of proteins in a biological sample sample can be performed using a computer by querying a pre-existing dataset comprising data corresponding to levels of proteins i.e. ANGPTL2, ICAM-1 and P-Selectin, in a biological sample, preferably a plasma sample. Accordingly, the present invention provides a computer-implemented methods and systems for detecting active atherogenesis or atherosclerosis in a subject. Such system comprises: (a) a computer; (b) a database coupled to the computer; (c) a database coupled to a database server having data stored thereon, the data comprising records of data captured from an instrument that detects proteins or reagent-protein complexes in a sample and can be used to determine the level of a protein or reagent-protein complex in a sample.

[0129] Oligonucleotides can be detected and/or isolated by specific hybridization, under high stringency conditions. “High stringency conditions” are known in the art and permit specific hybridization of a first oligonucleotide to a second oligonucleotide where there is a high degree of complimentarity between the first and second oligonucleotide. For the expression profiling methods disclosed herein this degree of complimentarity is between 80% and 100% and

preferably between 90% and 100%. Preferably, a probe useful for detecting a mRNA or a corresponding polynucleotide is substantially homologous to a ANGPTL2 target polynucleotide (i.e. SEQ ID NO. 5-14), at least about 80% identical to the homologous region of comparable size contained in a ANGPTL2 target polynucleotide. More preferably, an ANGPTL2 probe exhibits 85% identity, and even more preferably the probe exhibits 90% identity. Similarly an ICAM-1 probe for detecting an ICAM-1 target polynucleotide (i.e. SEQ ID. NO. 18-19) is substantially homologous to an ICAM-1 target polynucleotide and exhibits at least 80% identity over the length of the probe, preferably at least 85% identity over the length of the probe and more preferably at least 90% identity over the length of the probe. Similarly a P-Selectin probe for detecting a P-Selectin target polynucleotide (SEQ. ID. NO. 21-23) is substantially homologous to a P-Selectin target polynucleotide and exhibits at least 80% identity over the length of the probe, preferably at least 85% identity over the length of the probe and more preferably at least 90% identity over the length of the probe.

[0130] Gene expression analysis, profiling a target polynucleotide obtained from a test sample and the control sample to analyze differential expression, can be performed by hybridization techniques well established in the art. Representative procedures include but are not limited to cDNA subtraction and differential display (Liang et al. (1992) Science 257:967-971), Serial Analysis of Gene Expression or "SAGE" (Velculescu, *et al.* (1995) Science 270:484-487 and U.S. Pat. No. 5,695,937), and array-based methodology (see, e.g., U.S. Pat. No. 5,445,934). Array-based analysis is in particular preferred for gene expression profiling. Array-based technology involves hybridization of a pool of target polynucleotides corresponding to gene transcripts of a test sample to an array of tens and thousands of probe sequences immobilized on the array

substrate. The technique allows simultaneous detection of multiple gene transcripts and yields quantitative information on the relative abundance of each gene transcript expressed in a test subject. By comparing the hybridization patterns generated by hybridizing different pools of target polynucleotides to the arrays, one can readily obtain the relative transcript abundance in two pools of target samples.

[0131] Microarray technology, e.g., DNA chip devices and high-density microarrays for high-throughput screening applications and lower-density microarrays, may be used to assay mRNA levels in the methods of the invention. Methods for microarray fabrication are known in the art and include various inkjet and microjet deposition or spotting technologies and processes, in situ or on-chip photolithographic oligonucleotide synthesis processes, and electronic probe addressing processes. Additional methods include interference RNA microarrays and combinations of microarrays and other methods such as laser capture micro-dissection (LCM. See, e.g., He et al. (2007) Adv. Exp. Med. Biol. 593: 117-133 and Heller (2002) Annu. Rev. Biomed. Eng. 4: 129-153. Other methods include PCR, xMAP, invader assay, mass spectrometry, and pyrosequencing (Wang et al. (2007) Microarray Technology and Cancer Gene Profiling Vol 593 of book series Advances in Experimental Medicine and Biology, pub. Springer New York). DNA probes for microarray analysis can be used as reagents for detection in the screening and treatment methods of the invention. The levels determined can be compared with control, normal or healthy levels to identify over-expression, elevated mRNA or protein levels.

[0132] Probes can be affixed to surfaces for use as "gene chips." Such gene chips can be used to detect genetic variations by a number of techniques known to one of skill in the art. In one technique, oligonucleotides are arrayed on a

gene chip for determining the DNA sequence of a by the sequencing by hybridization approach, such as that outlined in U.S. Pat. Nos. 6,025,136 and 6,018,041. The probes of the invention also can be used for fluorescent detection of a genetic sequence. The probes of the invention also can be used for fluorescent detection of a genetic sequence. Such techniques have been described, for example, in U.S. Pat. Nos. 5,968,740 and 5,858,659. A probe also can be affixed to an electrode surface for the electrochemical detection of nucleic acid sequences such as described in U.S. Pat. No. 5,952,172 and by Kelley, S. O. *et al*. (1999) Nucl. Acids Res. 27:4830-4837 .One or more probes for detecting expressed oligonucleotides corresponding to ANGPTL2, P-Selectin or ICAM-1 can be affixed to a chip and such a device used to predict therapeutic response to an ANGPTL2 modulator, and select an effective treatment for an individual with atherosclerosis or at risk of atherosclerosis. It is conceivable that detection probes (reagents) for detecting an expressed oligonucleotides corresponding to ANGPTL2, P-Selectin or ICAM-1 can included on a chip with a variety of other probes for uses other than those of the presently claimed screening and selection methods.

[0133] In assaying for the differential expression of ANGPTL2, probes are allowed to form stable complexes with the target polynucleotides contained within the biological sample derived from the test subject in a hybridization reaction. Where the nucleotide probe is a sense nucleic acid, the target polynucleotide is selected to be complementary to sequences of the sense nucleic acid. Conversely, where antisense is used as the probe nucleic acid, the target polynucleotides provided in the sample are chosen to be complementary to sequences of the antisense nucleic acids.

[0134] Synthetic oligonucleotides used as probes may be modified to

be more stable. Exemplary nucleic acid molecules which are modified include uncharged linkages such as phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Pat. Nos. 5,176,996; 5,264,564 and 5,256,775). Probes of the invention can include for example, labelling methylation, inter-nucleotide modification such as pendent moieties (e.g. polypeptides), intercalators (e.g., acridine, psoralen), chelators, alkylators, and modified linkages (e.g., alpha anomeric nucleic acids). Also included are synthetic molecules that mimic nucleotide acid molecules in the ability to bind to a designated sequence by hydrogen bonding and other chemical interactions, including peptide linkages that substitute for phosphate linkages in the nucleotide backbone.

[0135] The nucleotide probes of the present invention can also be used as primers for determining the expression of target polynucleotide. An ANGPTL2 primer is one comprising a sequence of between 10 to 30 residues in length and complimentary to one more ANGPTL2 target polynucleotides (i.e. SEQ ID NO. 5-14), or its respective complement. An ICAM-1 primer is one comprising a sequence of between 10 to 30 residues in length and complimentary to one more ICAM-1 target polynucleotides (i.e. SEQ ID NO. 18-19), or its respective complement. A P-Selectin primer is one comprising a sequence of between 10 to 30 residues in length and complimentary to one more P-Selectin target polynucleotides (i.e. SEQ ID NO. 21-23), or its respective complement.

[0136] Numerous nucleotide probes and gene expression analysis kits are commercially available for analysis of ANGPTL2 and can be used in the methods of the present invention for example: RT² qPCR Primer Assay for Human ANGPTL2: PPH07579A (SABiosciences) and HP210100 qSTARqPCR primer

pairs against Homo sapiens gene ANGPTL2 (Origene) for quantitative real-time PCR assays; TaqManprobes , Hs00765776_m1, Hs00765775_m, Hs00765775_m1, Hs00765773_m, Hs00171912_m1, Hs00171912_m1, Hs00765776_m1, Hs00765775_m1, and Hs00765773_m1 (LifeTechnologies). Other probes for use in the methods of the present invention include the microarrary primers represented by SEQ ID NO. 47-50 and the primer probes represented by SEQ ID NO. 51-68.

[0137] For the purpose of this invention, amplification means any method employing a primer and a polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA-polymerases such as T7 DNA polymerase, Klenow fragment of E. coli DNA polymerase, and reverse transcriptase. A preferred amplification method is PCR. General procedures for PCR are taught in MacPherson M. et al., PCR, Taylor & Francis (2007). However, PCR conditions used for each application reaction are empirically determined. A number of parameters influence the success of a reaction. Among them are annealing temperature and time, extension time, Mg2+ ATP concentration, pH, and the relative concentration of primers, templates, and deoxyribonucleotides.

[0138] In one aspect differentially expressed genes are selected based on the following criteria: (a) an expression ratio of at least 1.2× higher than the corresponding control measure (over-expression); and (b) a 99% confidence that the difference between the control and the test samples does not occur by chance ($p<0.01$). In another aspect, the expression ratio is 1x, preferably 5x, more preferably 10x, and even more preferably 50x higher than the expression level of the same polynucleotide in the control sample. In another aspect, the target

polynucleotide is under-expressed and the expression ratio is 1x, preferably 5x and more preferably 10x less than the expression level of the same polynucleotide in the control sample.

[0139] The determination of differential expression of a target polynucleotide in a test sample can be performed using a computer. Accordingly, the present invention provides a computer-based system designed to detect differential expression of a target polynucleotide in the test subject. Such system comprises: (a) a computer; (b) a database coupled to the computer; (c) a database coupled to a database server having data stored thereon, the data comprising records of polynucleotides encoding a polypeptide that comprises a linear peptide sequence of at least 8 amino acids, whereas such linear peptide is essentially identical to a contiguous fragment of 8 amino acids contained the target polynucleotide of interest. The nucleotide probes of the present invention can also be used as primers for determining the expression of an ANGPTL2 target polynucleotide, ICAM-1 target polynucleotide or a P-Selectin target polynucleotide. A preferred primer is one comprising a sequence of between 10 to 30 residues in length and complimentary to a target polynucleotide, or its respective complement; and (d) a code mechanism for applying queries based upon a desired selection criterion to a data file in the database to produce reports of polynucleotide records which matches the desired selection criterion.

[0140] Drug Screening Methods

[0141] In general, such screening methods involve providing appropriate cells which express and secrete ANGPTL2. In particular, a nucleic acid encoding ANGPTL2 may be employed to transfect cells to thereby express

ANGPTL2. Such a transfection may be accomplished by methods well known in the art. In a particular embodiment; said cells may be selected from the group consisting of lentiviral-based infection or adenoviral-based transfection.

[0142] According to the ANGPTL2-type screening method of the present invention, a modulator that reduces the expression of ANGPTL2 or plasma levels of ANGPTL2 in vivo can be obtained. An ANGPTL2 modulator obtained with the screening method of the present invention can be used as an active ingredient of a pharmaceutical composition for treating or preventing atherosclerosis.

[0143] A cell which may be used in the screening methods of the present invention is not limited, so long as an endogenous ANGPTL2 is being expressed or can be expressed. Exemplary cells for use in the screening methods of the present invention include: endothelial cells, adipocytes, macrophages, skeletal muscle cells. The cell used in the screening method is not limited to culture cells, and an embodiment in which a test candidate ANGPTL2 modulator is brought into contact with animal cells by administering the test candidate ANGPTL2 modulator to an animal is included in the screening method of the invention. After the contact of a test candidate ANGPTL2 modulator with cells, a culture supernatant or a cell lysate, or a sample (such as a serum or tissue-derived cells) collected from the animal may be used as a sample, to measure an amount of an endogenous ANGPTL2 protein or gene contained in the cells, and to analyze a test candidate ANGPTL2 modulator dependent change in the amount thereof.

[0144] A level of ANGPTL2 protein may be measured by known

methods, such as an immunoassay or Western blotting, preferably an immunoassay, more preferably an ELISA as described above.

[0145] Pro-inflammatory activities measured as part of the screening methods of the present invention include: measures of cytokines such as TNF-a, IL6, IL1 and measures of adhesion molecules such as P-Selectin, ICAM1. Measures for use in the invention include expression by cells comprising or within arteries or measures circulating levels of cytokines or adhesion molecules in blood, serum or plasma. Pro- atherogenesis activities measured as part of the screening methods of the present invention include: measures of the evolution of the atherosclerotic plaque size through time, measure of the burden of oxidative stress using the measure of 4-HNE, isoprostane, nitrosylated proteins and the like as well as measure of the macrophage load in the atherosclerotic plaque, as further described herein. Methods for measuring isoprostane are known in the art and described in Leblond F, *et al* (2013). Pflugers Arch. 465:197-208, herein incorporated by reference. Methods for measuring 4-HNE are known in the art and described in Voghel G*, *et al*. E (2008). Mech Ageing Dev. 129:261-270, herein incorporated by reference..Methods for measuring nitrosylated proteins are known in the art and described in Qin Y, *et al*. Methods Enzymol. 2013;522:409-25, herein incorporated by reference.

[0146] Protein Detection Assays

[0147] Protein levels can be assayed in a biological sample using an Enzyme-linked immunosorbent assay (ELISA) or using a mass spectrometry based assay. The methods and technologies for Indirect ELISA (Biochemistry. 7th edition. Berg JM, Tymoczko JL, Stryer L. New York: W H Freeman; 2012),

Sandwich ELISA, Competitive ELISA as well as Multiple and Portable ELISA assays (U.S. Patent 7,510,687; European Patent EP 1 499 894) are well known in the art and widely used. Detection antibodies against ANGPTL2 protein (i.e. SEQ. ID. NO. 2-4), P-Selectin protein (SEQ. ID. NO. 24-28) or ICAM-1 protein (SEQ. ID. NO. 16-17)can be used as reagents in the ELISA-based assays of the invention.

[0148] Determining a protein level typically involves a) contacting the polypeptides contained in the biological sample with an agent that specifically binds an ANGPTL2 polypeptide; and (b) detecting any agent:polypeptide complex formed. In one aspect of the invention, the agent that specifically binds an ANGPLT2 polypeptide, ICAM-1 polypeptide or P-Selectin polypeptide is an antibody, preferably a monoclonal antibody. The formation of an agent:polypeptide complex can be detected directly or indirectly according to standard procedures known in the art. In the direct detection method, the agents are supplied with a detectable label and unreacted agents may be removed from the complex; the amount of remaining label thereby indicating the amount of complex formed. In the alternative, an indirect detection procedure requires the agent to contain a label introduced either chemically or enzymatically, that can be detected by affinity cytochemistry. A desirable label generally does not interfere with binding or the stability of the resulting agent:polypeptide complex. However, the label is typically designed to be accessible to an antibody for an effective binding and hence generating a detectable signal. A wide variety of labels are known in the art. Non-limiting examples of the types of labels that can be used in the present invention include radioisotopes, enzymes, colloidal metals, fluorescent compounds, bioluminescent compounds, and chemiluminescent compounds.

[0149] A variety of antibodies for use in the methods of the present invention are commercially available or could be generated by a person skilled in

the art using standard methods. For example the anti-ANGPTL2 antibodies listed in Table 2 herein. Anti-ICAM-1 antibodies are available from a variety of sources including but not limited to [MEM-111] (ab2213) or [EP1442Y] (ab53013) provided by Abcam(insert registered symbol); anti-ICAM-1 antibody clone P2A4 provided by EMD Millipore; ICAM-1 antibody HPA002126 provided by Sigma-Aldrich. Similarly a variety of P-Selectin antibodies suitable for use in the present invention are commercially available.

[0150] For the purpose of the methods of the invention the antibody reagents used need not target a specific epitope of the target protein but only be selective for the target protein such that the quantity, level, of the target protein in the sample can be accurately detected and quantified. Either polyclonal or monoclonal antibodies can be used in the methods of the invention, preferably in ELISA type assays. Typically a capture antibody and a detection antibody are used in an ELISA assay. To maximize the specificity of detection the capture antibody is preferably a monoclonal antibody. Either a monoclonal or polyclonal antibody can be used as a detection antibody. Alternatively a monoclonal antibody can be used as both the capture antibody and the detection antibody.

[0151] ELISA kits for ANGPTL-2, ICAM-1 and P-Selectin suitable for use in the present invention are widely available to a person skilled in the art. For example Human Angiopoietin-like protein 2, ANGPTL2 ELISA Kit, MyBioSource; Human ICAM-1 ELISA Kit, Boster Immunoleader; LEGEND MAX™ Human sICAM-1/CD54 ELISA Kit with Pre-coated Plates, BioLegend; Human Intercellular Adhesion Molecule 1/CD54 Matched Antibody Pair for ELISA, Cell Sciences; Human Intercellular Adhesion Molecule 1/CD54 Matched Antibody Pair for ELISA, Cell Sciences; ICAM-1 (soluble) Human ELISA Kit, Life Technologies Corporation; Human sICAM-1 ELISA, EMD Millipore; Human ICAM-1/CD54 DuoSet, R and D

Systems; Human sICAM-1/CD54 Quantikine ELISA Kit, R and D Systems; Human ICAM-1/CD54 DuoSet Economy Pack, 45 Plate, R and D Systems; SELPLG (Human) ELISA Kit, Abnova; sPSGL-1 (Human) ELISA, Immuno-Biological Laboratories; and Human sPSGL-1 Platinum ELISA 96, eBioscience.

[0152] A variety of techniques for protein analysis using the basic principles outlined above are available in the art. They include but are not limited to radioimmunoassays, ELISA (enzyme linked immunoradiometric assays), "sandwich" immunoassays, immuno-radiometric assays, in situ immunoassays (using e.g., colloidal gold, enzyme or radioisotope labels), western blot analysis, immuno-precipitation assays, immuno-fluorescent assays, and SDS-PAGE and mass spectrometry.

[0153] Mass spectrometry methods appropriated for both the identification and quantitative analysis of proteins can be used in the present invention including peptide mass fingerprinting or tandem mass spectrometry. Such methods are well known in the art and described in more detail in (Snijders AP, de Vos MG, Wright PC (2005). J. Proteome Res; M. Miyagi and K. C. S. Rao (2007). Mass Spectrometry Reviews; Haqqani AS, Kelly JF, Stanimirovic DB (2008). Methods Mol. Biol.). Further Liquid Chromatography Selected Reaction Monitoring Mass Spectrometry (LC-SRM-MS) may be used for the detection and quantification of sets of proteins in a biological sample. Such methods are known in the art and described in more detail in the patent application WO2013/151726.

[0154] Kits

[0155] The present invention also encompasses kits containing antibodies, antigen-binding fragments, or reference polypeptides for carrying out the methods of the invention, in suitable packaging. The present invention further comprises kits containing probes, primers or reference polynucleotides for analyzing the expression of ANGPTL2 and one or more of ICAM-1 and P-Selectin for carrying out the methods, in suitable packaging. In one embodiment the kit enables a person skilled in the art to detect the presence or quantify the level of ANGPTL2 polynucleotide or polypeptide that is suspected to be present in a biological sample. The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits, comprising reagents, such as a antibodies for detecting protein levels; probes or primers for detecting gene expression, which may be conveniently used according to methods well known in the art to determine the protein or mRNA levels respectively corresponding to ANGPTL2 and one or more of P-Selectin and ICAM-1 e.g., for selecting subjects with increased likelihood of benefiting from treatment with an ANGPTL2 modulator or identifying subjects having active atherogenesis or having atherosclerosis. The sample is optionally pre-treated for enrichment of the polypeptides or polynucleotides being tested for. The user then applies a reagent contained in the kit, according to instructions provided in the kit, to detect an increase in the level of ANGPTL2 and one or more of ICAM-1 and P-Selectin.

[0156] Each kit necessarily comprises reagents which render the method specific for the analyses of interest i.e. ANGPTL2 protein and one or more proteins selected from ICAM-1 and P-Selectin. Each reagent can be supplied in a solid form or dissolved/suspended in a liquid buffer suitable for inventory storage, and later for exchange or addition into the reaction medium when the test is performed. Alternately a reagent such as an antibody or oligonucleotide probe or a set of reagents may be covalently coupled to a solid support. Suitable packaging is

provided. The kit can optionally provide additional components that are useful in the method. These optional components include, but are not limited to, buffers, capture reagents, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information. Diagnostic or treatment selection methods of the invention using a protein or mRNA selective reagent can be performed by diagnostic laboratories, experimental laboratories, practitioners, or private individuals.

[0157] In some embodiments of the invention subjects at risk of atherosclerosis or having atherosclerosis are treated with an ANGPTL2 modulator. Risk factors for atherosclerosis include: unhealthy blood cholesterol levels, high LDL or low HDL; high blood triglyceride levels; high blood pressure; Smoking; insulin resistance; diabetes; overweight or obesity; family history of early coronary artery disease; lack of physical activity; high levels of C-reactive protein (CRP) in blood; heart attack; chronic inflammation and diseases associated with chronic inflammation; sleep apnea; stress and alcoholism or heavy drinking. These risk factors can be used in combination with the diagnostic and treatment selection methods of the invention to identify subjects at risk of atherosclerosis.

[0158] The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

[0159] EXAMPLES

[0160] Mouse model of atherogenesis

[0161] To study the role of ANGPTL2 in atherogenesis, we used $LDLr^{-/-}$; $hApoB100^{+/+}$ mice characterized by severe dyslipidemia, i.e. abnormally high circulating levels of total cholesterol (8-fold), LDL-cholesterol (20-fold) and triglycerides (9-fold) [20, 21, 25]. Three month old ATX mice exhibit premature endothelial dysfunction, oxidative stress, inflammation and these mice develop aortic atherosclerotic plaques by the age of 6 months (Libby P. *et al.* 2012;32:2045-2051; Bolduc V, *et al.* Am J Physiol Heart Circ Physiol. 2011;301:H2081-2092; Kanda A, *et al.* Lab Invest. 2012;92:1553-1563). At the age of 3 months ATX mice are therefore prone to react to a pro-atherosclerotic factor.

[0162] Effect of IV ANGPTL2 administration on atherogenesis in ATX mouse model

[0163] Chronic intravenous (IV) administration of ANGPTL2 for 1 month in young pre-atherosclerotic ATX mice induced a potent inflammatory response, potentiated the expression of endothelial adhesion molecules (Figure 4 A) and strongly accelerated the formation of atherosclerotic lesions (Figure 4 B). IV administration of ANGPTL2 for 1 month in pre-atherosclerotic 3-mo ATX mice increased ($P<0.05$) total- and LDL-cholesterol levels, strongly induced ($P<0.05$) the expression of endothelial pro-inflammatory cytokines and adhesion molecules and accelerated atherosclerotic lesion formation by 10 fold ($P<0.05$). The increase in plasma levels of total-cholesterol and LDL-cholesterol observed with chronic ANGPTL2 administration was not accompanied by an increase in HDL-cholesterol or triglycerides (Figure 4C). One month of ANGPTL2 administration increased total cholesterol (19.1±1.6 mM) and LDL-cholesterol (12.9±1.1 mM) to values similar to those in 12-mo ATX mice (23.3±2.6 mM and 14.7±0.5 mM, respectively).

[0164] Chronic administration of ANGPTL2 to pre-atherosclerotic ATX mice led to a 10-fold increase in atherosclerotic lesion size compared to ATX mice infused with vehicle (TBSE) (Figure 4B). While non-significant lesions were observed in vehicle-treated ATX mice (1.5±0.2% of total thoracic aorta area), ANGPTL2 administration increased the area of the lesions (14±2%) to values comparable to 6-mo ATX mice (10±1%) (Figure 5B). By comparison, in untreated 9-mo and 12-mo ATX mice, aortic lesions covered 33±4% and 74±4%, respectively, of the internal surface (Figure 5B).

[0165] A positive correlation between the area of the lesion and the circulating levels of total cholesterol ($p=0.0009$, $r2=0.765$, $n=10$) and LDL-cholesterol ($p=0.0002$, $r2=0.836$, $n=10$) in 4-mo ATX mice treated with ANGPTL2 or its vehicle was observed.

[0166] With age, plasma and aortic tissue levels of ANGPTL2 increased in both WT and ATX mice ($P<0.05$) and were higher in 6- and 12-mo ATX mice than in age-matched WT mice. While below the limits of detection in 3-mo WT and ATX mice, circulating levels of ANGPTL2 increased significantly from 6- to 12-mo in WT and even further in ATX mice ($P<0.05$)(Figure 5A).

[0167] High levels of ANGPTL2 accumulated in the atherosclerotic lesion ($P<0.05$). Endogenous aortic ANGPTL2 expression increases with age and progression of atherosclerosis In ATX mice, ANGPTL2 protein expression was quantified specifically in the aortic lesion: its pattern of expression positively correlates with the lesion area and followed the expression of F4/80, a specific

marker of macrophages (Figure 5C,D). WT mice did not develop lesions (Figure 6), but endogenous ANGPTL2 aortic mRNA(ΔΔCT) and protein expression (normalized to α-actin; arbitrary units) increased with age from 1.2±0.4 and 0.51±0.03, respectively, at the age of 3-mo, to 1.4±0.27 and 0.62±0.03 at 6-moand 2.53±0.23 ($P<0.05$ vs. 3-mo) and 6.90±1.36 ($P<0.05$ vs. 3- and 6-mo) at12-mo in WT mice. The increase in endogenous ANGPTL2 aortic mRNA and protein expression was delayed in WT compared to ATX mice. Indeed, in ATX mice, mRNA and protein expression increased from 1.76±0.25 and 0.88±0.20, respectively, at the age of 3-mo, to 2.81±0.34 ($P<0.05$ vs. age-matched WT) and 1.88±0.23 ($P<0.05$ vs. age-matched WT) at 6-moand 3.70±0.32 ($P<0.05$ vs. 3-mo) and 9.60±1.01 ($P<0.05$ vs. 3-mo) at 12-mo.

[0168] Effect on leukocyte adhesion to isolated EC from ATX and WT animals

[0169] ANGPTL2 accelerates leukocyte adhesion onto the native endothelium in pre-atherosclerotic young ATX mice, but not in healthy young mice (Figure Figure 2B). This was associated with increased expression of ICAM-1 and P-Selectin in EC (Figure 2A). Antibodies against ICAM-1 and P-selected further prevented leucocyte adhesion induced by ANGPTL2 (Figure 2C).

[0170] Acute stimulation with exogenous ANGPTL2 induces adhesion of leukocytes onto the native endothelium in ATX, but not WT mice. Expression of adhesion molecule promotes leukocyte adhesion. The adhesion of 51Cr-

leukocytes to native aortic endothelium was similar in 3-mo WT and ATX mice (Figure 2B). Pre-incubation of the endothelium with 10 U/ml of thrombin stimulated leukocytes adhesion in WT mice and this response was potentiated in ATX mice (Figure 2B). In contrast, stimulation with recombinant ANGPTL2 (100 nmol/L) did not induce leukocyte/endothelium interactions in WT mice, while it stimulated the adhesion of leukocytes in ATX mice as efficiently as did thrombin (Figure 2B). Pre-incubation of the aortic segments with anti-P-Selectin or anti-ICAM-1 antibodies prior to stimulation with recombinant ANGPTL2 prevented the adhesion of leukocytes on the endothelium in ATX mice (Figure 2C). Incubation with the isotype-matched IgG did not affect ANGPTL2-mediated leukocytes adhesion (Figure 2C).

[0171] Effect of ANGPTL2 administration on the expression of inflammatory cytokines by aortic endothelial cells (EC)

[0172] 1 month exposure to ANGPTL2 (plasma concentration of ~150 ng/ml) increased the expression of inflammatory TNF-α and IL-6 mRNA in fresh aortic EC by 9- and 4-fold, respectively (Figure 4A).

[0173] Acute stimulation with exogenous ANGPTL2 promotes inflammation in EC. The pro-inflammatory effect of ANGPTL2 was evaluated ex vivo on EC freshly isolated from aortas of 3-mo WT and ATX mice. Baseline mRNA levels of TNF-α and IL-6 were significantly higher in EC from ATX mice (Figure 1). Stimulation of EC with recombinant ANGPTL2 (100 nmol/L) further increased ($p<0.05$) TNF-α and IL-6 gene expression in EC, equally in EC from both WT and ATX mice (Figure 1). In EC freshly isolated from the aorta, basal

expression of TNF-α and IL-6 mRNA was higher in 3-month old (mo) severely dyslipidemic mice ($LDLr^{-/-}$; $hApoB100^{+/+}$; ATX) compared to healthy wild type (WT) mice ($P<0.05$). TNF-α and IL-6 mRNA expression was increased in both WT and ATX mice in response to exogenous ANGPTL2 (100 nmol/L).

[0174] In both WT and ATX mice, expression of inflammatory cytokines (TNF-α, IL-6) by EC obtained from freshly isolated aortas was increased following administration of ANGPTL2 (Figure 1). Although baseline levels of inflammatory cytokines were lower in the WT model, EC obtained from a WT and ATX mice responded similarly to stimulation with ANGPTL2 (Table 3).

[0175] Table 3: Cytokine measures from EC from WT or ATX mice following AGPTL2 stimulation

Cytokine	Fold Increase - WT Model	Fold Increase – ATX Model
TNF-α	2X	2X
IL-6	2X	2X

[0176] These findings are consistent with the known relationship between inflammation and the progression of atherosclerosis (Drouin A, *et al*. Am J Physiol Heart Circ Physiol. 2011;300:H1032-1043).

[0177] Effect of ANGPTL2 administration on the expression of adhesion molecules by leukocytes

[0178] One month exposure to ANGPTL2 (plasma concentration of 150 ng/ml) of WT and ATX animals was associated with increased the expression the expression of ICAM-1 and P-Selectin mRNA was increased by 7- and 4- fold, respectively (Figure 4A). Expression of P-Selection and ICAM at the surface of leukocytes was similar in WT and ATX animals (Figure 2). Since ANGPTL2 increases the adhesion of leukocytes to endothelial cells in ATX animals and not WT, this finding suggests that the expression of adhesion molecules alone by leukocytes does not account for to the pro-adhesive properties of ANGPTL2 in ATX mice. Factors or conditions present in the ATX mice and not in WT mice may contribute to the pro-adhesive effects of increased expression of adhesion molecules. Furthermore ANGPTL2 may participate in the initiating stages of leukocyte adhesion onto the inflamed vascular endothelium, promoting leukocyte adhesion, atherogenesis and atherosclerosis.

[0179] Acute stimulation with exogenous ANGPTL2 increases P-Selectin and ICAM-1 expression in EC. Inflammation is associated with an up-regulation of endothelial adhesion molecules. Basal expression of ICAM-1 and P-Selectin mRNA was higher in aortic EC freshly isolated from ATX mice than WT mice (Figure 2A). ANGPTL2 stimulated the expression of ICAM-1 in ATX mice but not in WT mice (Figure 2A) and increased the expression of P-Selectin mRNA in WT and even further in ATX mice (Figure 2A).

[0180] Acute stimulation with exogenous ANGPTL2 increases adhesion molecule expression at the surface of the leukocytes. Basal expression of CD18,

CD62L and CD162 mRNA were similar in leukocytes from WT and ATX mice (Figure 3A-C). Incubation with ANGPTL2 increased the expression of these genes similarly in leukocytes isolated from WT and ATX mice (Figure 3A-C). Flow cytometry revealed that expression of adhesion molecule proteins was similar in leukocytes from both groups of mice (Figure 3D-F). ANGPTL2 enhanced the fluorescence intensity of CD18 similarly in WT and ATX mice (Figure 3D). Due to proteolysis of cell surface molecules, CD162 levels decreased after stimulation with ANGPTL2 similarly in both groups (Figure 3F). ANGPTL2 only tended to induce CD62L shedding (Figure 3E). ANGPTL2 mRNA and protein were not detected in leukocytes isolated from 3-mo WT or ATX mice (data not shown). These data suggest that the adhesion-promoting properties of ANGPTL2 in ATX mice are not dependent on the expression of adhesion molecules on the surface of leukocytes.

[0181] Effect of ANGPTL2 on circulating cholesterol and LDL-cholesterol levels

[0182] Unexpectedly, ANGPTL2 significantly increased both total cholesterol (chol) and LDL-cholesterol level (Figure 5). This is the first report of such an effect of ANGPTL2 on circulating cholesterol levels. Although, ANGPTL3, ANGPTL4 and ANGPTL8 are known to modulate lipid metabolism (see background section herein) the same is not true for ANGPTL2. However it has been shown that levels of ANGPTL2 correlate with abdominal adiposity (Ley K, *et al*. Nature. 2007;7:678-689).

[0183] Effect of siRNA Transfection of ANGPTL2 expression

[0184] ANGPTL2 siRNA (human) were obtained from OriGene Technologies, Inc. Three sequences were tested:

[0185] A: SR308285A: rUrCrCrUrUrGrUrArArUrGrArCrArCrGrArArUrCrUrGrCAA

[0186] B: SR308285B: rArGrArArUrGrUrCrUrArCrArArUrGrCrUrArArUrCrUrCTC

[0187] C: SR308285C:rGrGrArCrArGrGrArCrUrArCrArGrArCrArArCrUrCrUrUTC

[0188] In all conditions, the siRNA led to a significant inhibition of ANGPTL2 gene (mRNA) expression in HUVEC as shown in Figure 13.

[0189] Effect of ANGPTL2 by pan-genomic knock-down on epidermal fat and LDL-cholesterol

[0190] Inactivation of ANGPTL2 by pan-genomic knock-down (KD) led to viable and healthy mice. ANGPTL2 knock-down (KD) mice did not present any macroscopic abnormal phenotype but were observed to have 45% less epididymal fat ($P<0.005$) (Figure 8).

[0191] A high fat diet (HFD) provided from the age of 3 to 6 months, significantly increased LDL-cholesterol in WT mice but not in ANGPTL2 KD mice (Table 4), compared to a regular diet (RD). This diet did not increase LDL-cholesterol in the ANGPTL2 KD mice and a constant LDL/HDL cholesterol ratio

was maintained compared to wild-type littermates (Figure 9). These data indicate that the lack of expression of ANGPTL2 is protective to the vasculature.

[0192] Table 4: Metabolic parameters measured in 6-month old WT and ANGPTL2 KD mice (ANGPTL2 $^{-/-}$) following a high fat diet (HFD) (n=7-11). * P<0.05, ** P<0.01, ** P<0.001 vs. regular diet (RD).

	WT		KD, ANGPTL2 $^{-/-}$	
Parameters	RD	HFD	RD	HFD
Weight (g)	26.3 ± 0.9 (10)	33.1 ± 1.4 (9)	24.2 ± 0.5 (13)	29.0 ± 1.6 (11)
Systolic pressure (mmHg)	145 ± 6 (10)	146 ± 5 (6)	146 ± 2 (7)	153 ± 5 (8)
Glucose (mM)	17.0 ± 1.1 (10)	17.4 ± 1.7 (9)	13.5 ± 1.1 (12)	17.1 ± 1.5 (11)
Triglyceride (mM)	0.45 ± 0.04 (10)	0.49 ± 0.05 (9)	0.41 ± 0.03 (12)	0.49 ± 0.03 (11)
Cholesterol (Chol) – total (mM)	2.7 ± 0.1 (10)	3.9 ± 0.2 (9)	2.7 ± 0.2 (12)	3.9 ± 0.3 (11)

Chol – HDL (mM)	2.5 ± 0.1 (10)	3.3 ± 0.1 (9)	2.4 ± 0.2 (12)	3.5 ± 0.2 (11)
Chol – LDL (mM)	0.15 ± 0.02 (9)	0.41 ± 0.11 (8)	0.13 ± 0.01 (12)	0.25 ± 0.05 (11)
Chol-total/HDL	1.07 ± 0.02 (10)	1.17 ± 0.03 (9)*	1.12 ± 0.02 (12)	1.12 ± 0.01 (11)
LDL/HDL	0.06 ± 0.01 (9)	0.12 ± 0.03 (8)*	0.06 ± 0.01 (12)	0.08 ± 0.02 (11)
Insulin (ng/ml)	0.10 ± 0.02 (9)	0.27 ± 0.03 (9)*	0.14 ± 0.02 (12)	0.30 ± 0.08 (10)
FFA (mM)	0.39 ± 0.05 (7)	0.50 ± 0.05 (5)	0.39 ± 0.03 (7)	0.44 ± 0.06 (7)

[0193] Immuno-fluorescent imaging of ANGPTL2 in atherosclerotic plaques

[0194] Confocal immune-fluorescent images show that the endogenous levels of ANGPTL2 increased with age and the progression of atherosclerosis (Figure 6A-B). This was observed in both freshly isolated, longitudinal aortic

sections (Figure 6A and Figure S2) and in frozen sections (Figure 6B). ANGPTL2 was particularly abundant in the atherosclerotic lesion, but also present in EC as demonstrated by the co-localization of ANGPTL2 with the endothelial marker CD-31 (Figure 6C). ANGPTL2-fluorescence was also observed throughout the media, implicating VSMC as a potential source of ANGPTL2.

[0195] Confocal immuno-fluorescent images showed high levels of ANGPTL2 in the plaque, but also in the endothelium and throughout the media (Figure 6), however it is important to note that this method is not quantitative and only indicates the presence of ANGPTL2. This pattern does not necessarily mean that ANGPTL2 is expressed in all vascular cell types, as ANGPTL2 may also bind to EC, VSMC and cells in the plaque.

[0196] Immuno-blotting analysis of cultured human EC

[0197] In order to determine the cellular specificity of ANGPTL2 expression, we compared its production by cultured human EC isolated in coronary patients (hIMAEC) [22], HUVEC and human VSMC. Immuno-blotting analysis (Figure 7A) showed that ANGPTL2 protein was abundantly expressed and secreted by hIMAEC, less detectable in HUVEC, and undetectable in VSMC. Similar results were observed by immuno-fluorescence (Figure 7B) and at the mRNA level (Figure 7C). These results indicate that ANGPTL2 is secreted by EC, particularly in a pro-atherosclerotic environment, but not by VSMC.

[0198] The intensity of ANGPTL2-luciferase binding was found to be stronger in VSMC than in EC. The binding of ANGPTL2-luciferase to the cell surface of living hIMAEC, HUVEC and VSMC was examined (Figure 7C). The

binding of luciferase alone (no ANGPTL2 in the construct) was negligible (data not shown). We observed a marked accumulation of ANGPTL2-luciferase on the surface of living VSMC over time; the signal remained stable for up to 15 min (Figure 7C). The signal was less intense on the surface of HUVEC and hIMAEC (Figure 7C). These results suggest that ANGPTL2-luciferase is more efficiently captured by VSMC than by EC, implying that ANGPTL2 receptors are in greater abundance and/or affinity on VSMC than EC.

[0199] In studying the cellular origin of ANGPTL2 we found that ANGPTL2 was secreted by EC isolated from coronary patients and to a lesser extent by HUVEC, but not by VSMC (Figure 7C).

[0200] Circulating levels of ANGPTL2 in patients with coronary artery disease (CAD)

[0201] In addition, ANGPTL2 was found to be highly expressed ($P<0.05$) in EC cultured from CAD patients and circulating ANGPTL2 levels were 6-fold higher in CAD patients compared to age-matched healthy volunteers (Figure 10). Circulating levels of ANGPTL2 increased in CAD patients compared to age-matched male healthy volunteers (Table 5), plasma ANGPTL2 levels were significantly higher in CAD patients (Figure 10). The presence of CAD was documented by the history of angina (5/11 patients), infarct (6/11), previous dilatation (3/11) or coronary bypass (1/11). Almost all CAD patients were dyslipidemic and treated with statins (10/11), explaining their lower total cholesterol and LDL-cholesterol levels when compared to healthy volunteers (Table 5).

[0202] Table 5: Clinical profiles of male subjects

	Healthy volunteers (n=6)	CAD (n=11)
Age (years)	60 ± 3	60 ± 4
Glucose (mM)	5.2 ± 0.1	5.6 ± 0.2
Triglycerides (mM)	1.4 ± 0.2	1.1 ± 0.2
AGPTL2 (ng/ml)	1.00 ± 0.18	6.02±1.33
Total cholesterol (mM)	4.8 ± 0.2	3.8 ± 0.2 *
HDL cholesterol (mM)	1.5 ± 0.1	1.3 ± 0.1
LDL cholesterol (mM)	2.9 ± 0.2	2.0 ± 0.2 *
BMI (kg/m^2)	23.9 ± 0.9	28.2 ± 1.1 *
SAP (mm Hg)	118 ± 5	132 ± 5
DAP (mm Hg)	72 ± 3	80 ± 2 *

[0203] Data are mean ±SEM of n=6 healthy individuals and n=11 CAD patients. *: $p<0.05$ *versus* healthy volunteers.

[0204] These data are the first to demonstrate that Caucasian CAD patients have higher ANGPTL2 plasma concentrations than healthy volunteers. Although ANGPTL2 levels are similar in healthy Caucasian and Japanese subjects, circulating levels of ANGPTL2 have been found to be higher in North American than in Asians (Oike Y, Tabata M. Circ J. 2009;73:2192-2197; Tabata M, *et al*. Cell Metab. 2009;10:178-188).A strong increase in endogenous ANGPTL2 expression in human EC isolated from patients undergoing coronary artery by-pass surgery has also been observed and characterized by a highly pro-atherogenic (Farhat N, *et al*. Can J PhysiolPharmacol. 2008;86:761-769; Gendron ME, *et al*. Am J Physiol Heart Circ Physiol. 2010;298:H2062-2070).

[0205] Together, the data presented in the examples provided show that ANGPTL2 causally accelerates atherogenesis and that reducing or blocking circulating ANGPTL2 is a viable therapeutic approach for slowing or preventing atherogenesis. ANGPTL2 promotes the adhesion of leukocytes to the native inflamed endothelium of pre-atherosclerotic young ATX mice, but not WT mice, via a robust activation of adhesion factors P-Selectin and ICAM-1. Chronic administration of ANGPTL2 to pre-atherosclerotic young ATX mice strongly accelerates the formation of atherosclerotic lesions while increasing circulating cholesterol levels, endothelial pro-inflammatory cytokines and adhesion

molecules. ANGPTL2 concentrates in the atherosclerotic lesion of aging ATX mice and is secreted by EC but not by VSMC. We also show for the first time that circulating levels of ANGPTL2 are significantly higher in CAD patients than in healthy volunteers.

[0206] Endothelium-derived ANGPTL2 contributes to the pathogenesis of atherosclerosis by promoting inflammation, leukocyte adhesion and LDL cholesterol increase. The elevation in circulating ANGPTL2 associated with the progression of atherosclerosis in ATX mice and its accumulation in the plaque indicates that ANGPTL2 contributes to the pathology. The results show that a pro-inflammatory environment favours ANGPTL2 production and furthermore that elevated ANGPTL2 increases circulating levels of cholesterol; contributing to the deleterious vascular effect, increasing endothelial inflammation, and accelerating the rate of plaque formation. We have shown that ANGPTL2 is a key inflammatory factor activating adhesion molecule expression, increasing LDL-cholesterol levels and thereby accelerating atherosclerotic plaque development. ANGPTL2 contributes directly to leukocyte adhesion and to atherogenesis and the progression of atherosclerosis. ANGPTL2 requires a primed pro-inflammatory environment as is triggered by severe dyslipidemia. Reduction of ANGPTL2 activity is a promising therapeutic approach for treating chronic inflammatory disease including atherosclerosis.

[0207] EXPERIMENTAL METHODS

[0208] ANGPTL2-GST construct

[0209] To generate a recombinant protein, ANGPTL2 was fused to

glutathione S-transferase (GST) at its C-terminus. ANGPTL2 was cloned into pSPORT1 vector (clone ID LIFESEQ2268890) (Open Biosystems, Thermo Fisher Scientific, Waltham, MA). The pSPORT1 insert was subcloned in pcDNA3.1 (Life Technologies, Burlington, ON, Canada) and ANGPTL2 cDNA insert was confirmed by sequencing. To clone GST in phase 3' to ANGPTL2, the ANGPTL2stop codon was first removed from the pcDNA3.1 construct by PCR and the latter was ligated and amplified. GST cDNA was generated by PCR using pGEX6P2vector (GE Health Care Lifescience, Baied'Urfe, QC, Canada) as template and ligated in phase with the 3' end of ANGPTL2. The final clone was confirmed by sequencing.

[0210] Purification of the ANGPTL2-GST fusion protein

[0211] HEK-293 cells were transfected with the pGEX6P2 plasmid vector containing ANGPTL2-GST cDNA using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. To obtain stable cell lines, transfected HEK-293 cells were cultured in the presence of 1 mg/ml of Geneticin (G418, Life Technologies). Culture medium from transfected HEK-293 cells was collected twice a week, centrifuged for 2 h at 100 000 g (4ºC) and then loaded (2 liters of medium) onto a GSTrap HP column (GE Health Care). After extensive washing of the column with Tris-Buffered Saline-EDTA (TBSE, pH 7.5) to remove unbound proteins, the recombinant protein was eluted with 10 ml of the washing buffer containing 10 mmol/L of glutathione (pH 8.0, Roche). The wash and eluted products were collected (20 fractions; 0.5 ml/tube).To assess the purity of the eluted recombinant protein, an aliquot of each fraction was resolved on SDS-PAGE (15% acrylamide) and proteins revealed using Coomassie Brilliant blue R250 (Figure S1, Panel A). The elution of ANGPTL2-GST was confirmed by Western blot (Figure S1, Panel B).In addition, the Coomassie-stained band eluting in fractions F16-F18 was excised and confirmed to be ANGPTL2-GST by

sequencing on LC/MS-MS. The fractions containing ANGPTL2-GST were pooled, concentrated using a centrifugal concentrator (Centricon plus 20, Millipore, Billerica, MA), dialyzed against TBSE at 4°C and quantified using BSA as a standard. For long-term storage, purified ANGPTL2-GST was aliquoted and frozen at -80°C. From the 2 liters of medium loaded on the column, at least 50 µg of purified ANGPTL2-GST protein were recovered.

[0212] Animal models

[0213] All experiments were performed in wild-type (WT) C57Bl/6 and in our colony of knockout/transgenic atherosclerotic $LDLr^{-/-}$; $hApoB^{+/+}$ (ATX) male mice fed a normal diet. Mice were anaesthetized with 44 mg/kg ketamine and 2.2 mg/kg xylazine and ventilated.

[0214] Quantification of circulating ANGPTL2 levels in mice

[0215] High-abundance plasma proteins masking ANGPTL2 levels were removed using the ProteoMiner protein enrichment kit optimized for plasma samples (BioRad, Mississauga, ON, Canada) following the manufacturer's recommendations. To quantify plasma ANGPTL2, 1 µl of the ProteoMinereluate was subjected to analysis by Western blot (see below).

[0216] Quantification of ANGPTL2 levels in aorta tissues

[0217] Aortas extracted from WT and ATX mice were powdered under liquid nitrogen and solubilized in a lysis buffer (50 mmol/LTris-HCl pH 7.5 at 4°C, 20 mmol/L β-glycerophosphate, 20 mmol/LNaF, 5 mmol/L EDTA, 10 mmol/L

EGTA, 1 mmol/L Na_3VO_4, 10 mmol/L benzamidine, 0.5 mmol/L PMSF, 10 µg/ml leupeptin, 5 mmol/L DTT, 1 µmol/L microcystin and 1% (v/v) reduced Triton X-100). The whole-tissue lysate was centrifuged for 15 min at 10 000 g and 4ºC. The protein concentration was determined in the supernatant using γ-globulin as a standard.

[0218] Quantification of ANGPTL2 in the atherosclerotic lesion

[0219] Atherosclerotic plaques were dissected from the aorta of ATX mice, homogenized in liquid nitrogen and cells were disrupted for 10 min with 1ml of QIAzolLysis Reagent (Qiagen, Toronto, ON, Canada) 1/5 volume of chloroform was added to the cell lysate, incubated for 15 min at room temperature and centrifuged for 15 min (12, 000 x g at 4°C). After removal of the upper aqueous phase, DNA was precipitated from the organic phase with 1 volume of 100% ethanol (5 min, room temperature). Samples were then centrifuged to sediment DNA. Protein in the phenol/ethanol supernatant was precipitated with 1 volume of isopropanol. After incubation for 20 min at room temperature, samples were centrifuged to remove supernatant and the pellet was precipitated with 2 ml of guanidine/ethanol for 20 min at room temperature. The pellet was washed with 2 ml of 100 % ethanol for 20 min at room temperature and then centrifuged at 7500 x g for 5 min. This step was repeated three times. After air drying, the pellet was dissolved in denaturing solution (10 mol/L urea / 50 mmol/L DTT) and incubated 1h at room temperature. During the last incubation, samples were sonicated 10 times on ice and then centrifuged 10 min at 10,000 x g. The samples were then analyzed by SDS-PAGE.

[0220] Western blotting

[0221] To detect ANGPTL2 expression, 1 µl from the enrichment plasma sample, 50 µg of tissue-lysate protein, 50 µg of the lesion protein or 0.5 µl from the purified recombinant ANGPTL2 protein were denatured by heating (80°C) in Laemmli sample buffer, resolved by electrophoresis on 12.5% SDS-PAGE gels and transferred to nitrocellulose (0.45µm) at 100 V and 4ºC for 90 min. After blocking, membranes were incubated for 3 h with a goat anti-ANGPTL2 antibody (dilution 1:200, R&D Systems, Minneapolis, MN, #AF2084) at room temperature in 5% milk, then washed three times with TBST (25 mm Tris-HCl pH 7.5, 150 mMNaCl and 0.05 % (v/v) Tween-20) and re-incubated with an anti-goat horseradish peroxidase-conjugated secondary antibody (dilution 1:10,000 in 5% [w/v] milk, Jackson ImmunoResearch Laboratories, West Grove, PA) for 2h at room temperature. Immunoreactive bands were revealed with Enhanced Chemiluminescence Substrate using BioMax BML Kodak films. The protein loading was normalized to α-actin immunoreactivity (dilution 1: 10^6, Ambion Life technologies, Burlington, ON, Canada) or to Ponceau red (for plasma samples).

[0222] Quantification of plaque area

[0223] Thoracic aortas still attached to the heart were removed and dissected from surrounding tissues. The aortic arch and the aorta were opened longitudinally from the aortic valve through the end of the thoracic aorta and pinned on a Petri dish. Unstained aortas were directly photographed and digitalized. Plaque areas were quantified using Gimp 2.6 software (www.gimp.org) and expressed in percentage of total aorta area.

[0224] Methods for siRNA Transfection

[0225] For siRNA-transfection, Human Umbilical Vein Endothelial Cells (HUVECs) are seeded one day prior to cell treatment to allow optimal cell conditions. At the time of treatment, cells are grown to approximately 70 to 80% confluency. Oligofectamine from Invitrogen is used as a transfection reagent that forms stable complexes with oligos to permit for efficient transfections in eukaryotic cells. Opti-MEM from Gibco is used as the medium of transfection. Opti-MEM was mixed with Oligofectamine reagent for 5 minutes while Opti-MEM was also mixed with 15nM of siRNA for 5 minutes. After 5 minutes, Opti-MEM with Oligofectamine and Opti-MEM with siRNA were mixed together for another 30 minutes prior to treatment of cells. Cells were treated at 37°C and 7% CO2 for 6 hours. After 6 hours, cells were provided with 5% FBS and allowed to grow at 37°C and 7% CO2 for time-points of interest.

[0226] Methods for shRNA transfection using lentiviral particle generation containing ANGPTL2 shRNA

[0227] Individual clone purchased from OpenBiosystems, Clone ID : V2LHS_28517 - Mature antisense : TTTAAAGAAAGAGTTGTCT - Target : 3'UTR Human ANGPTL2 Gene Bank accession : NM_012098 - Packaging constructs purchased from Addgene - psPax2 : plasmid # 12260 - pMD2.G : plasmid # 12259

[0228] The three constructs were co-transfected in HEK293T (ATCC) using a Ca-PO4 protocol. Cells were incubated at 37ºC. Viral particles were collected in cell media 48 hours after transfection, centrifuged at low speed (5 min, 1500rpm) to get rid of cellular debris. The supernatant was filtered on 0.45µm filter

and ultra-centrifuged at 24 000rpm at 4ºC to concentrate the particles. Pellet was re-suspended in PBS+4%BSA and kept in -80ºC in 10µl aliquots. Viral particles were titrated using HEK293 cells.

[0229] HUVEC cells were then transduced using a multiplicity of infection of 50 by adding directly virus suspension to cells in the presence of polybrene.

[0230] RNA extraction and RT reaction

[0231] Total RNA was extracted from leukocytes and from freshly isolated EC, scraped with a blade from longitudinally opened segments of the thoracic aorta, using the RNeasy Mini Kit (Qiagen). Total RNA was also extracted from whole aortic tissue using the RNeasy Fibrous Tissue Kit (Qiagen) to quantify mRNA ANGPTL2 expression in aortas. Contamination with DNA was prevented by a digestion with a DNase I mix (Qiagen) according to the manufacturer's guidelines and total RNA was quantified using a NanoDrop ND-100 spectrophotometer. Total RNA was reverse transcribed to generate a cDNA library, following the manufacturer's protocol. Briefly, 1 µg of total RNA was mixed with 100 ng of random primers and 1 µl of 10 mmol/L dNTP mix in a total volume of 12 µl. Samples were incubated at 65ºC for 5 min. Then, 4 µl of 5X First-Standard buffer, 2 µl of 0.1mol/L of DTT, 1 µl of RNase out and 1 µl of Moloney murine leukemia virus (M-MLV) reverse transcriptase were added and incubated 50 min at 37ºC. The RT reaction was stopped by heating at 70ºC for 15 min.

[0232] Real time quantitative PCR

[0233] 25 µl of qPCR reaction mix contained 12.5 µl of Platinum SYBR Green qPCRSuperMix-UDG (Life Technologies), a final concentration of 300 nmol/L of forward and reverse primers (2 µl; Table S1), 0.5 µl of reference dye ROX (Life Technologies) and 1 ng (10 µl) of cDNA sample. The qPCR reactions were performed using a MxPro3000 platform (Agilent, Mississauga, ON, Canada) with the following profile: initial step of enzyme activation (10 min at 95ºC) then 40 cycles of denaturing (30 s at 95ºC), annealing (1 min at 55ºC) and extension (1 min at 72ºC). The primers of target genes were designed using the Clone Manager software (Table S1). The efficiency of real time qPCR was calculated using a standard curve. The presence of a valid product was confirmed by both the appearance of a single peak in the dissociation curve and by sequencing. All samples were run in duplicate and the fold changes in gene expression were calculated by a $\Delta\Delta C_T$ method using cyclophilin A or GAPDH as the housekeeping gene.

[0234] Immuno-fluorescence imaging

[0235] Freshly dissected thoracic aortas were cut longitudinally and fixed in a Petri dish with the endothelium faced up. Aorta sections were washed with PBS, fixed with 4% paraformaldehyde (in PBS, pH 7.45) for 30 min at room temperature, washed again with PBS and then blocked with 2% normal donkey serum in PSB for 1 h at room temperature. Fixed tissue segments were then incubated with goat anti-ANGPTL2 (1:50, R&D Systems) or anti-CD31 (1: 50, Abcam, #ab7388-50) in the blocking buffer for 2 h at room temperature. Aorta sections were washed with PBS and incubated for 2 h at room temperature with the appropriate secondary antibody (Alexa fluor-555 donkey anti-goat, #A21433, Molecular Probes, Burlington, ON, Canada) diluted 1:500 in the blocking buffer. Nuclei were stained with TO-PRO3 (1:1000, Life Technologies). After washing,

sections were mounted between coverslips and glass slides and fluorescence was visualized using a confocal microscope (Zeiss, Toronto, ON, Canada). Negative controls were performed by omitting the primary antibodies during the staining procedure.

[0236] hIMAEC, HUVEC and VSMC were cultured for 24 h on coverslips, washed with PBS, fixed with 2% paraformaldehyde (in PBS, pH 7.45) for 20 min at room temperature, washed again with PBS and then blocked with 2% normal donkey serum and 0.1% Triton X-100 in PSB for 1 h at room temperature. The cells were then incubated with an ANGPTL2 specific antibody and then with the Alexa Fluor 488-conjugated donkey anti-goat secondary antibody (#A11055, Molecular Probes) as described above. Fluorescence was visualized using a confocal microscope. Negative controls were performed in the absence of the primary antibody.

[0237] Preparation of leukocytes in suspension

[0238] Spleens were dissected from WT and ATX mice, gently crushed and squeezed into single cells in 5 ml RPMI 1640 medium (Life Technologies). The cell suspension was passed through a 200-µm mesh and then centrifuged for 20 min at 1200 rpm. Contaminating erythrocytes were lysed by agitating with 4 ml of water (for 15 s) followed by the addition of 4 ml of 1.8% NaCl. Leukocytes were filtered, washed twice and resuspended with the appropriate volume of RPMI 1640 medium as described previously [1]. Briefly, the leukocytes were suspended in 2 ml of RPMI 1640 medium and labeled with ^{51}Cr (activity of 100 µCi, Perkin-Elmer, Waltham, MA) for 1 h at 37°C, with gentle agitation every 15 min. Leukocytes were then centrifuged and washed three times. The number of leukocytes was counted

and the cell density adjusted to $1x10^6$ cells/ml with DMEM.

[0239] Splenocyte adhesion to native endothelium

[0240] Aorta was carefully dissected, cut into sections and pinned out with EC facing up onto silicone-coated Petri dishes. The aorta sections were treated for 30 min with or without 100 nmol/L of recombinant ANGPTL2, 10 U/ml of thrombin (Sigma-Aldrich, Oakville, ON, Canada), anti-P-Selectin (1:50, Santa Cruz Biotech, Santa Cruz, CA, #sc6943), anti-ICAM-1 (1:50, Santa Cruz Biotech, #sc-1511), goat-IgG (1:50, Santa Cruz Biotech, #sc-2028) or rat-IgG (1:50, Santa Cruz Biotech, #sc-2026)[1]. After 30 min of stimulation, aortic segments were washed and incubated with 20 µl of labeled leukocytes for an additional 30 min. The segments were then washed and the radiation was detected using a gamma counter. The number of leukocytes adhering to the endothelium was expressed per surface area of the segment (adherent cells/mm^2 of endothelium surface area).

[0241] Expression of adhesion molecules on the leukocytes

[0242] The expression of CD18 (#553293), CD62L (#553151) and CD162 (#555306) (BD Biosciences, Mississauga, ON, Canada) was analyzed by imaging flow cytometry (AMNIS, Markham, ON, Canada). Leukocytes in suspension (5 x 10^6 cell/ml) were stimulated with or without 100 nmol/L of ANGPTL2 (for 10 min at 37°C) and then fixed in 2% paraformaldehyde for 20 min at room temperature. After three wash steps with ice-cold PBS, leukocytes were labeled with monoclonal antibodies conjugated with phycoerythrin (PE, 5:100, BD Biosciences) or with their isotype control IgG (5:100, PE-rat $IgG_{2a,k}$isotype #553930 and PE-rat $IgG_{1,k}$isotype #553925, BD Biosciences) in the dark, for 30 min at 4°C.

Nuclei were stained by incubating the leukocytes with DRAQ5 dye (1:10 000, Cell Signaling Technology, Danvers, MA, #4084) for 10 min at 4°C. Fluorescence was assessed on an AMNIS ImageStream system using 488 nm and 678 nm lasers to excite DRAQ5 and PE, respectively. A total of 5000 events were acquired for each sample. All data were collected with INSPIRE software (AmnisMilipore, Seattle, WA) and analyzed with IDEAS analytical software (AmnisMilipore, Seattle, WA).

[0243] Cell culture

[0244] Experiments were performed with (i) human internal mammary arteries endothelial cells (hIMAEC), (ii) HUVEC (Lonza, Mississauga, MA) and (iii) VSMC (Lonza). hIMAEC were isolated from discarded segments of IMA from patients undergoing coronary artery bypass graft and were cultured as previously described in Morisada T, et al. Endothelium. 2006;13:71-79 and in Voghel G, et al. Mech Ageing Dev. 2007;128:662-671.

[0245] ANGPTL2-Gaussia luciferase-GST constructs

[0246] To visualize ANGPTL2 binding to receptors on the cell surface, EC and VSMC were incubated with recombinant ANGPTL2-luciferase. The vector pCMVGaussialuciferase was a gift from Dr. Stéphane Angers (University of Toronto, ON, Canada). Briefly, the stop codon was removed by PCR from pSPORT1 ANGPTL2 (Open Biosystems) and the coding sequence was fused in phase to the 5' end of Gaussia luciferase by overlapping PCR. The validation of the construct was confirmed by sequencing.HEK-293 cells were transfected with ANGPTL2-luciferase-pcDNA 3.1 or with luciferase-pcDNA 3.1 (as a negative control). Cells were plated into 175 cm^2 flasks and allowed to grow to 90%

confluence in the presence of Geneticin. The medium was then replaced by starving phenol red-free DMEM and the cells were cultured for 48 h at 37ºC. The medium was then collected, centrifuged 1 h at 100 000xg and the ANGPTL2-luciferase concentrated 1,000-fold using an Amicon Stirred-Cell system (membrane nominal molecular weight limit 10 kDa) .The concentration of ANGPTL2-luciferase protein was estimated by Western blot and the protein was used at ~100 nmol/L.

[0247] For live cell imaging, VSMC or EC (HUVEC and hIMAEC) were cultured overnight on coverslips inserted in a 35 mm petri dish, in their respective medium. The cells were washed two times with phenol-free medium and incubated for 10 min with HEK-293-ANGPTL2-luciferase or HEK-293-luciferase transfected medium. The fraction of ANGPTL2-luc binding to the cell surface was monitored by the activity of luciferase, using Gaussia Luciferase assay kit (New England Biolabs, Whitby, ON, Canada). Live cells were monitored using a confocal microscope (Zeiss, Toronto, ON, Canada) before and immediately after addition of the luciferase substrate. During image acquisition, petri dishes were maintained at 37ºC for the duration of the video and supplied with 5% CO_2. A 450 nm laser was used and images (512x512), acquired at 0.5 sec intervals, were collected with a 63x/1.4 plan apochromat Oil DIC objective.

[0248] Western blot

[0249] Cells were homogenized in a Triton based lysis buffer (50 mmol/LTris-HCl pH 7.5 at 5°C, 20 mmol/L β-glycerophosphate, 20 mmol/LNaF, 5 mmol/L EDTA, 10 mmol/LEGTA, 1 mmol/L Na_3VO_4, 10 mmol/L benzamidine, 0.5 mmol/L PMSF, 10 µg/ml leupeptin, 5 mmol/L DTT, 1 µmol/L

microcystin and 1% (v/v) reduced Triton X-100). Cell homogenates were centrifuged for 15 min at 10 000xg and 4ºC. Protein concentrations in the supernatant were determined by the Bradford method using γ-globulin as a standard.

[0250] To measure the secretion of endogenous ANGPTL2 into the culture medium, hIMAEC, HUVEC or VSMC were cultured in serum free medium for 16 h and 1 ml of this medium was concentrated to 20 µl using Microcon centrifugal filters (with a cutoff of 10 kDa, Millipore) and combined to 10 µl of Laemmli buffer (containing 1% SDS).For Western blot analysis, the proteins (50 µg of protein from the cell lysate, 20 µl from the concentrated culture medium or 2 µl from the purified recombinant protein) were resolved by electrophoresis as described above.

[0251] Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

[0252] All references cited and/or discussed in this specification are incorporated herein by reference in their entirety and to the same extent as if each reference was individually incorporated by reference.

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WHAT IS CLAIMED IS:

1. A method of selecting a subject for treatment with an ANGPTL2 modulator, said method comprising:
   (i) obtaining a plasma sample from the subject;
   (ii) contacting the plasma sample with at least two detection reagents and generating complexes between a respective one of the detection reagents and each protein from a set of proteins selected from the group of sets of proteins consisting of ANGPTL2 and P-Selectin; ANGPTL2 and ICAM-1; and ANGPTL2, P-Selectin and ICAM-1;
   (iii) detecting the complexes and determining plasma levels of ANGPTL2 and one or more of P-Selectin and ICAM-1 in the subject based at least on the detected complexes; and
   (iv) selecting the subject for treatment with the ANGPTL2 modulator when ANGPTL2 and P-Selectin; ANGPTL2 and ICAM-1; or ANGPTL2, P-Selectin and ICAM-1 have elevated plasma levels relative to predetermined levels thereof found in healthy controls.

2. The method of claim 1 wherein the plasma level of ANGPTL2 is >2.5 ng/ml.

3. The method of claim 1 wherein complexes including ANGPTL2 and complexes including P-Selectin are detected in step (iii) and the plasma level of ANGPTL2 is > 2.5ng/ml and the plasma level of P-Selectin is > 40ng/ml.

4. The method of claim 1 wherein complexes including ANGPTL2 and complexes including ICAM-1 are detected in step (iii) and the level of ANGPTL2 is > 2.5ng/ml and the plasma level of ICAM-1 is > 300 ng/ml.

5. The method of claim 1 wherein complexes including ANGPTL2, complexes including P-Selectin and complexes including ICAM-1 are detected in step (iii) and the level of ANGPTL2 is > 2.5ng/ml, the plasma level of P-Selectin is > 40ng/ml and the level of ICAM-1 is > 300.

6. The method of claim 1 wherein the detection reagents are antibodies,

7. The method of claim 1 wherein the detection reagents are an anti-ANGPTL2 antibody and an anti-P-Selectin antibody.

8. The method of claim 1 wherein the detection reagents are an anti-ANGPTL2 antibody and an anti-ICAM-1 antibody.

9. The method of claim 1 wherein the detection reagents are an anti-ANGPTL2 antibody, an anti-P-Selectin antibody and an anti-ICAM-1 antibody.

10. A method of detecting active atherogenesis in a subject comprising:
    (i) obtaining a plasma sample from the subject;
    (ii) contacting the plasma sample with anti-ANGPTL2 antibody and anti-ICAM-1 antibody; or anti-ANGPTL2 antibody and anti-P-Selectin antibody; or anti-ANGPTL2 antibody, anti-ICAM-1 antibody and anti-P-Selectin antibody; and
    (iii) determining that the subject has elevated plasma levels of ANGPTL2 and P-Selectin or elevated plasma levels of ANGPTL2 and ICAM-1 or elevated plasma levels of ANGPTL2, P-Selectin and ICAM-1 using the plasma sample after contact with the antibodies, indicating the presence of active atherogenesis in the subject.

11. The method claim 10 wherein after step (iii) the subject is selected for treatment with an ANGPTL2 modulator when active atherogenesis is present in the subject.

12. The method claim 10 wherein after step (iii), when active atherogenesis is present in the subject, the subject is selected for treatment with an ANGPTL2 modulator and the ANGPTL2 modulator is administered to the subject.

13. The method of claim 10, 11 or 12 wherein the plasma level of ANGPTL2 is >2.5 ng/ml.

14. The method of claims 10, 11 or 12 wherein the plasma levels of ANGPTL2 and P-Selectin are determined in step (iii) and the plasma level of ANGPTL2 is > 2.5ng/ml and the plasma level of P-Selectin is > 40ng/ml.

15. The method of claim 10, 11 or 12 wherein the plasma levels of ANGPTL2 and ICAM-1 are determined in step (iii) and the plasma level of ANGPTL2 is > 2.5ng/ml and the plasma level of ICAM-1 is > 300 ng/ml.

16. The method of claim 10, 11 or 12 wherein the plasma levels of ANGPTL2, P-Selectin and ICAM-1 are determined in step (ii) and the plasma level of ANGPTL2 is > 2.5ng/ml, the plasma level of P-Selectin is > 40ng/ml and the plasma level of ICAM-1 is > 300.

17. A method of preventing or treating atherogenesis in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of an ANGPTL2 modulator.

18. The method of claim 17 wherein the ANGPTL2 modulator is a peptide.

19. The method of claim 17 wherein the ANGPTL2 modulator is an antibody.

20. The method of claim 17 wherein the ANGPTL2 modulator is an antibody fragment.

21. The method of claim 17 wherein the ANGPTL2 modulator is an aptamer.

22. The method of claim 17 wherein the ANGPTL2 modulator is a siRNA.

23. The method of claim 17 wherein the ANGPTL2 modulator is a shRNA.

24. A pharmaceutical composition for preventing or treating atherogenesis comprising an ANGPTL2 modulator.

25. A method of drug screening comprising:
    (i) administering a candidate ANGPTL2 modulator to an animal model of atherosclerosis, and
    (ii) detecting a pro-inflammatory or pro-atherogenesis response in said animal model.

26. The method of claim 25 wherein the pro-inflammatory response is detected and cytokine or adhesion molecule expression is determined.

27. The method of claim 26 wherein the cytokine is selected from TNF-α, interleukin-6 and interleukin-1.

28. The method of claim 25 wherein the pro-atherogenesis response is detected and atherosclerotic plaque size is determined.

29. The method of claim 1 wherein in step (iii), the complexes are detected by an instrument providing a data set associated therewith, the data set being analyzed using a computer in step (iv) to determine the plasma levels.

30. A kit for detecting active atherogenesis in a mammal, said kit comprising: (a) a first monoclonal antibody or antigen-binding fragment thereof which binds specifically with ANGPTL2; (b) a second antibody or antigen-binding fragment thereof which binds specifically to ICAM-1; and (c) an instructional material describing the use of said first and second monoclonal antibodies or fragments thereof for detecting the active atherogenesis in a mammal.

31. A kit for detecting active atherogenesis in a mammal, said kit comprising: (a) a first monoclonal antibody or antigen-binding fragment thereof which binds specifically with ANGPTL2; (b) a second antibody or antigen-binding fragment thereof which binds specifically to P-Selectin; and (c) an instructional material describing the use of said first and second antibodies or fragments thereof for detecting the active atherogenesis in a mammal.

32. A kit for detecting active atherogenesis in a mammal, said kit comprising: (a) a first monoclonal antibody or antigen-binding fragment thereof which binds specifically with ANGPTL2; (b) a second antibody or antigen-binding fragment thereof which binds specifically to ICAM-1; (c) a third antibody or antigen-binding fragment thereof which binds specifically to P-Selectin; and (d) an instructional material describing the use of said first, second and third antibodies or fragments thereof for detecting the active atherogenesis in a mammal.

33. A kit of claim 30 or 31 wherein said first and second antibodies are attached to a solid surface.

34. A kit of claims 32 wherein said first, second and third antibodies are attached to a solid surface.

35. A method of detecting active atherogenesis in a subject, comprising:
    (i) obtaining a plasma sample from the subject;
    (ii) contacting the plasma sample with an anti-ANGPTL2 antibody or a fragment thereof and one or more antibodies selected from an anti-ICAM-1 antibody or a fragment thereof and an anti-P-Selectin antibody or a fragment thereof to generate antibody-protein complexes;
    (iii) measuring the level of each type of the antibody-protein complexes generated in step (ii) to determine the levels of ANGPTL2 and ICAM-1, AGPTL2 and P-Selectin, or ANGPTL2, ICAM-1 and P-Selectin in said plasma sample;
    (iv) detecting the presence of active atherogenesis in said subject if the levels of ANGPTL2 and ICAM-1, or AGPTL2 and P-Selectin, or ANGPTL2, ICAM-1 and P-Selectin are elevated relative to predetermined levels of the same polypeptides in a normal controls.

36. The method of claim 35 further comprising selecting the subject for treatment with an ANGPTL2 modulator when the presence of the active atherogenesis is detected.

37. The method of claim 35 further comprising treating the subject with an ANGPTL2 modulator when the presence of the active atherogenesis is detected.

38. The method of claim 35 further comprising diagnosing the subject with atherosclerosis.

39. A method of detecting active atherogenesis in a subject from a sample obtained from the subject, the method comprising:
    (i) analyzing the sample using mass spectrometry to determine levels of ANGPTL2 and one or more proteins selected from ICAM-1 and P-Selectin in the sample; and
    (ii) identifying the presence of active atherogenesis in the subject if the levels of ANGPTL2 and the one or more proteins selected are elevated relative to normal levels of the same proteins in a normal control.

40. The method of claim 39 wherein prior to step (ii) the proteins analyzed are enriched using high performance liquid chromatography.

41. The method of claim 39 wherein the one or more proteins are analyzed using liquid chromatography selected reaction monitoring mass spectrometry (LC-SRM-MS).

42. The method of claim 39 further comprising selecting the subject for treatment with an ANGPTL2 modulator when the presence of the active atherogenesis is detected.

43. The method of claim 39 further comprising treating the subject with an ANGPTL2 modulator when the presence of the active atherogenesis is detected.

44. The method of claim 39 further comprising diagnosing the subject with atherosclerosis.

45. Use of an expression level of a protein set for detecting the presence of active atherogenesis in a subject, the protein set consisting of ANGPTL2 and one or more proteins selected from ICAM-1 and P-Selectin.

46. A method of detecting active atherogenesis in a subject, the method comprising the steps of:
    (i) determining plasma levels of plasma proteins from a protein set, the protein set consisting of ANGPTL2 and one or more proteins selected from ICAM-1 and P-Selectin; and
    (ii) detecting the presence of active atherogenesis in the subject based on the plasma levels of the plasma proteins determined in step (i).

47. A method according to claim 46, wherein the determining step further comprises the step of comparing the expression level of each plasma protein to a normal control.

48. An isolated plasma sample or derivative thereof comprising two antibody reagents consisting of an (i) anti-AGPTL2 antibody or antigen binding fragment thereof, and (ii) an anti-ICAM-1 antibody or antigen fragment thereof.

49. An isolated plasma sample or derivative thereof comprising two antibody reagents consisting of an (i) anti-AGPTL2 antibody or antigen binding fragment thereof, and (ii) an anti-P-Selectin antibody or antigen fragment thereof.

50. An isolated plasma sample or derivative thereof comprising three antibody reagents consisting of an (i) anti-AGPTL2 antibody or antigen binding fragment thereof, (ii) an anti-ICAM-1 antibody or antigen

fragment thereof and (iii) an anti-P-Selectin antibody or antigen fragment thereof.

Figure 1

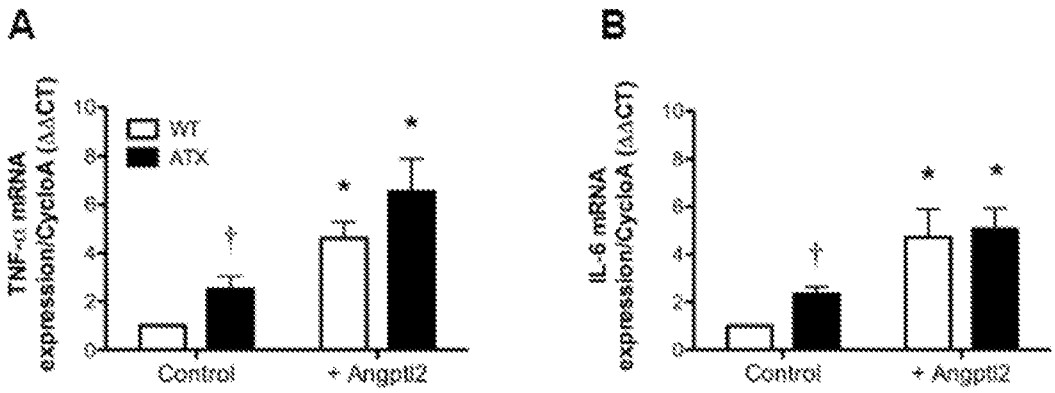

A
TNF-α mRNA expression/CycloA (ΔΔCT)
WT
ATX
0
2
4
6
8
10
†
*
*
Control
+ Angptl2
B
IL-6 mRNA expression/CycloA (ΔΔCT)
0
2
4
6
8
10
†
*
*
Control
+ Angptl2

Figure 2

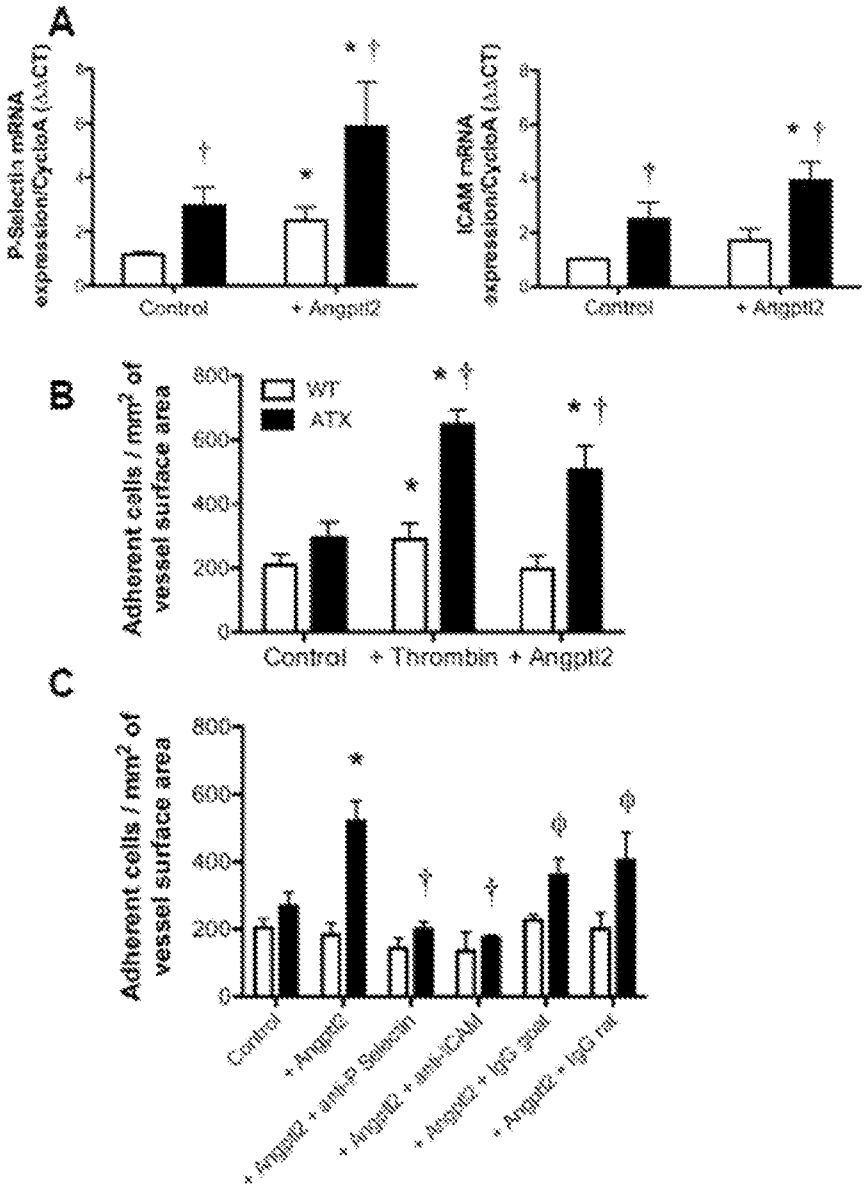

A
P-Selectin mRNA expression/CycloA (ΔΔCT)
ICAM mRNA expression/CycloA (ΔΔCT)
Control
+ Angptl2
B
Adherent cells / mm² of vessel surface area
WT
ATX
Control
+ Thrombin
+ Angptl2
C
Adherent cells / mm² of vessel surface area
Control
+ Angptl2
+ Angptl2 + anti-P Selectin
+ Angptl2 + anti-ICAM
+ Angptl2 + IgG goat
+ Angptl2 + IgG rat

Figure 3

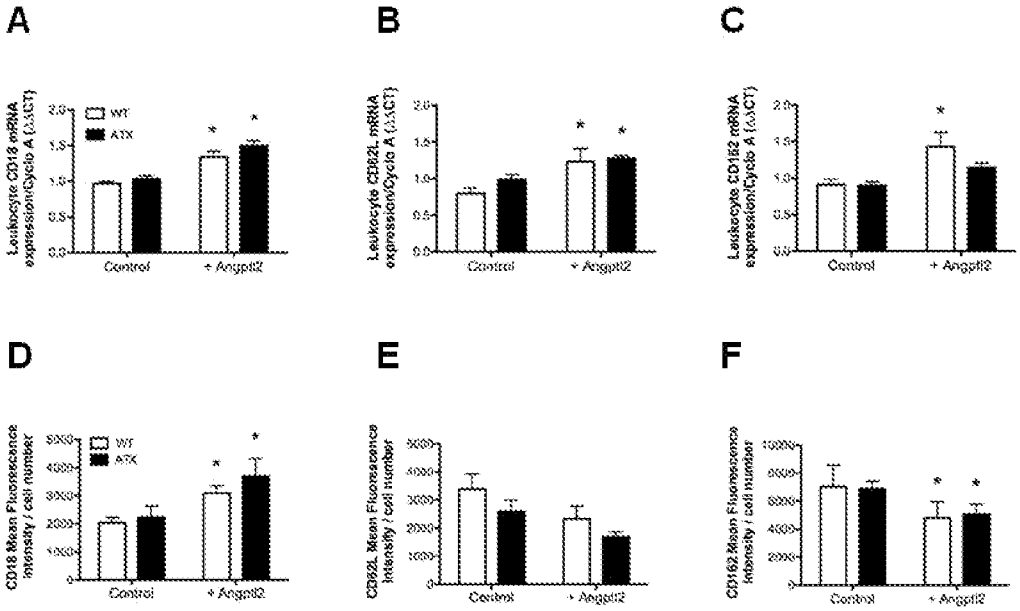


Figure 4

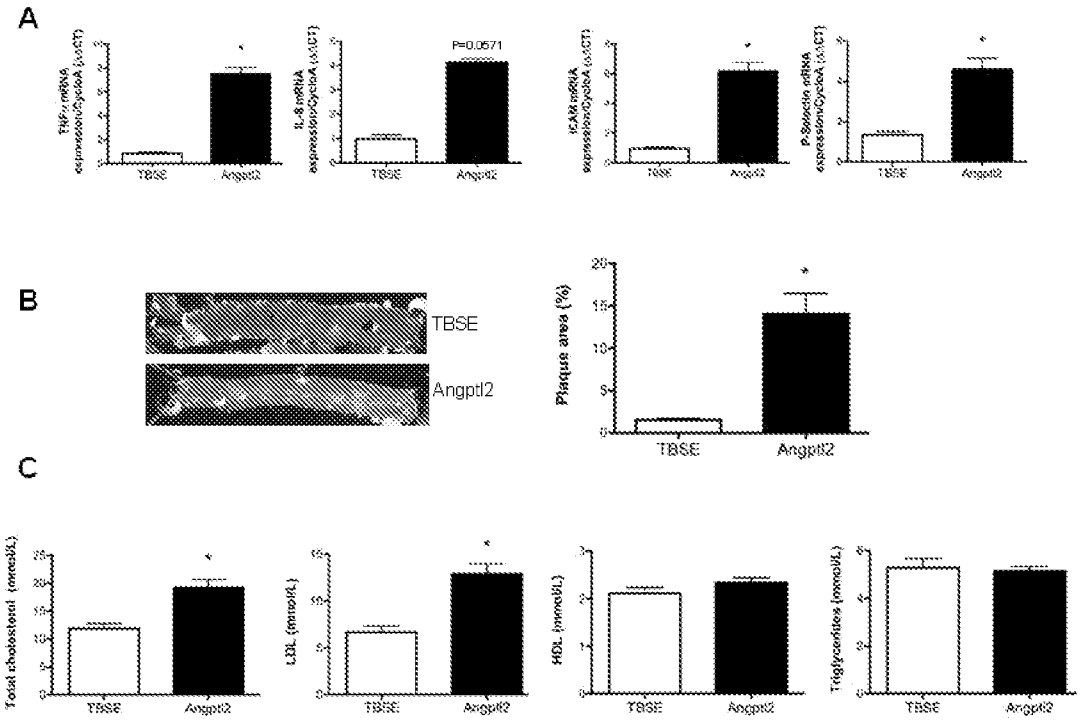

A
P=0.0571
TBSE
Angptl2
B
TBSE
Angptl2
Plaque area (%)
C
Total cholesterol (mmol/L)
LDL (mmol/L)
HDL (mmol/L)
Triglycerides (mmol/L)

Figure 5

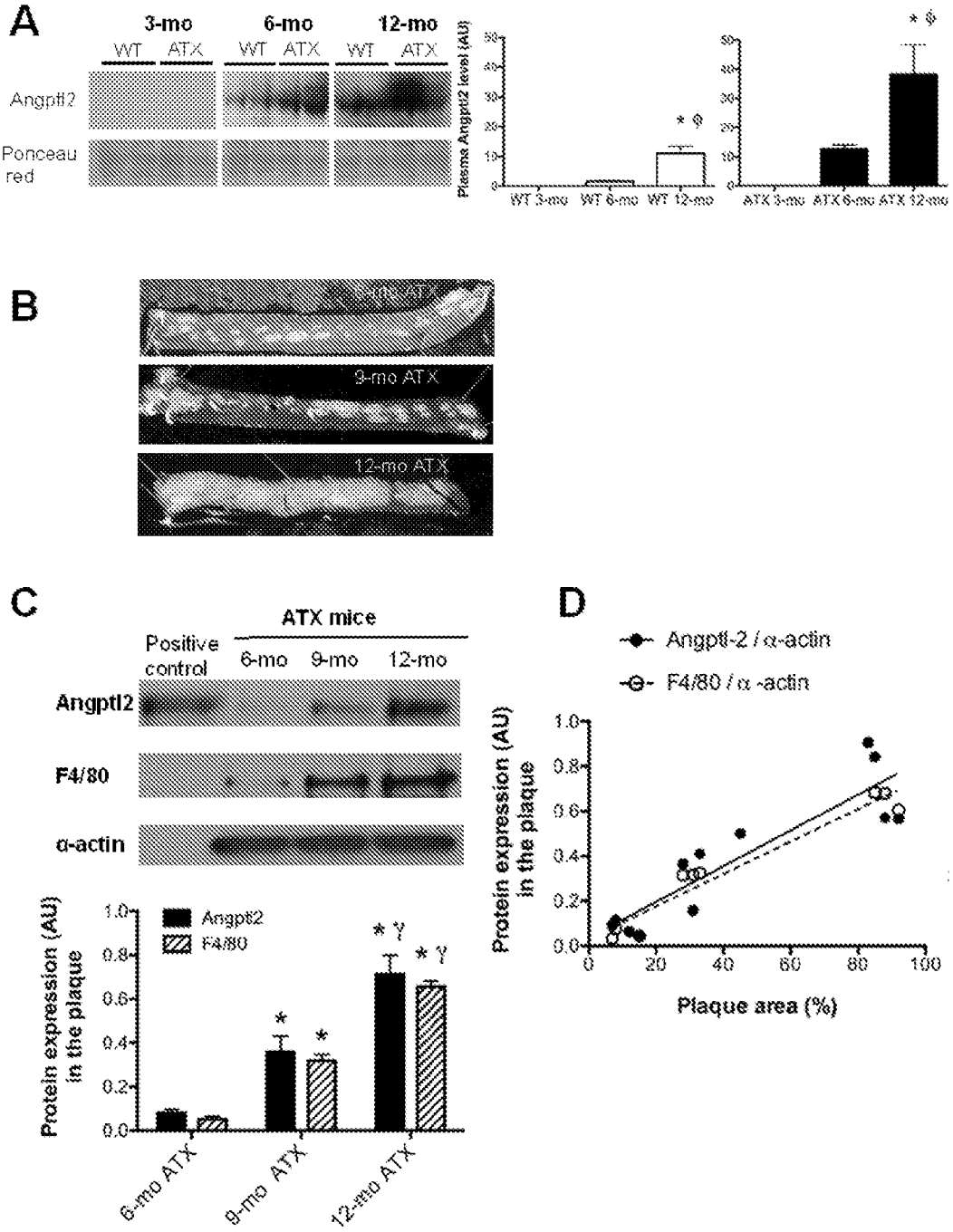

A
3-mo
6-mo
12-mo
WT
ATX
Angptl2
Ponceau red
Plasma Angptl2 level (AU)
WT 3-mo
WT 6-mo
WT 12-mo
ATX 3-mo
ATX 6-mo
ATX 12-mo
B
6-mo ATX
9-mo ATX
12-mo ATX
C
ATX mice
Positive control
6-mo
9-mo
12-mo
Angptl2
F4/80
α-actin
Protein expression (AU) in the plaque
Angpt82
F4/80
6-mo ATX
9-mo ATX
12-mo ATX
D
Angptl-2 / α-actin
F4/80 / α -actin
Protein expression (AU) in the plaque
Plaque area (%)

Figure 6

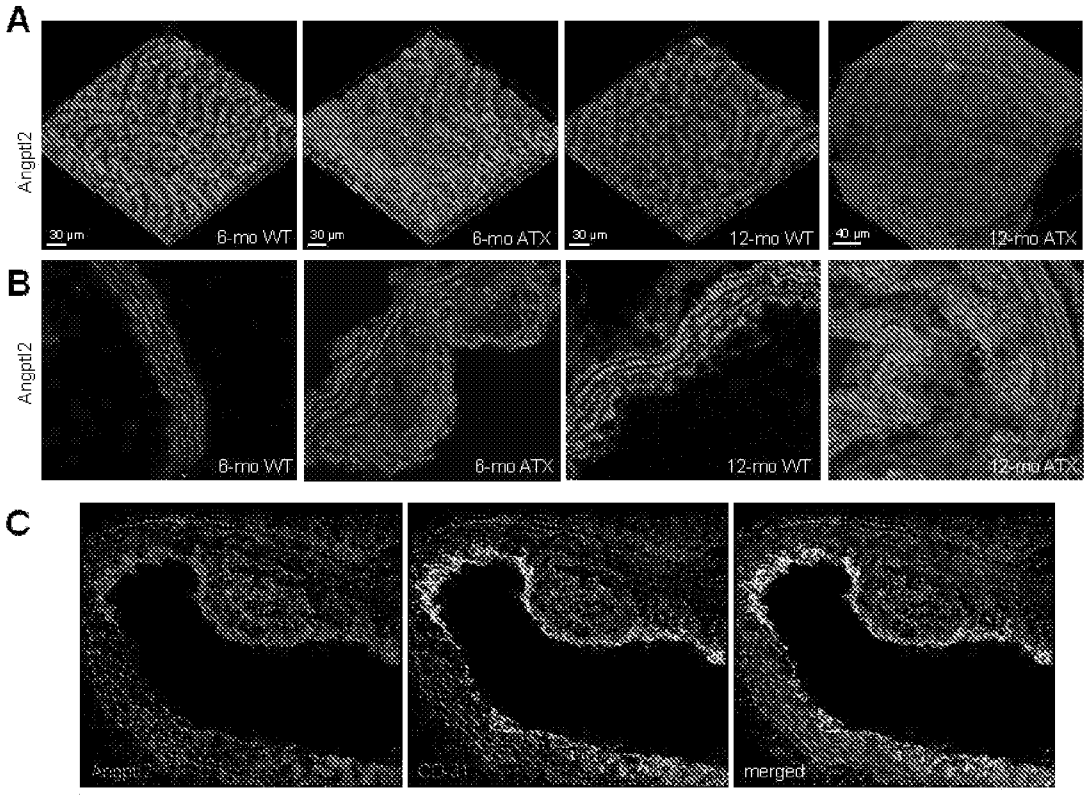

A
Angptl2
30 µm
6-mo WT
30 µm
6-mo ATX
30 µm
12-mo WT
40 µm
12-mo ATX
B
Angptl2
6-mo WT
6-mo ATX
12-mo WT
12-mo ATX
C
merged

Figure 7

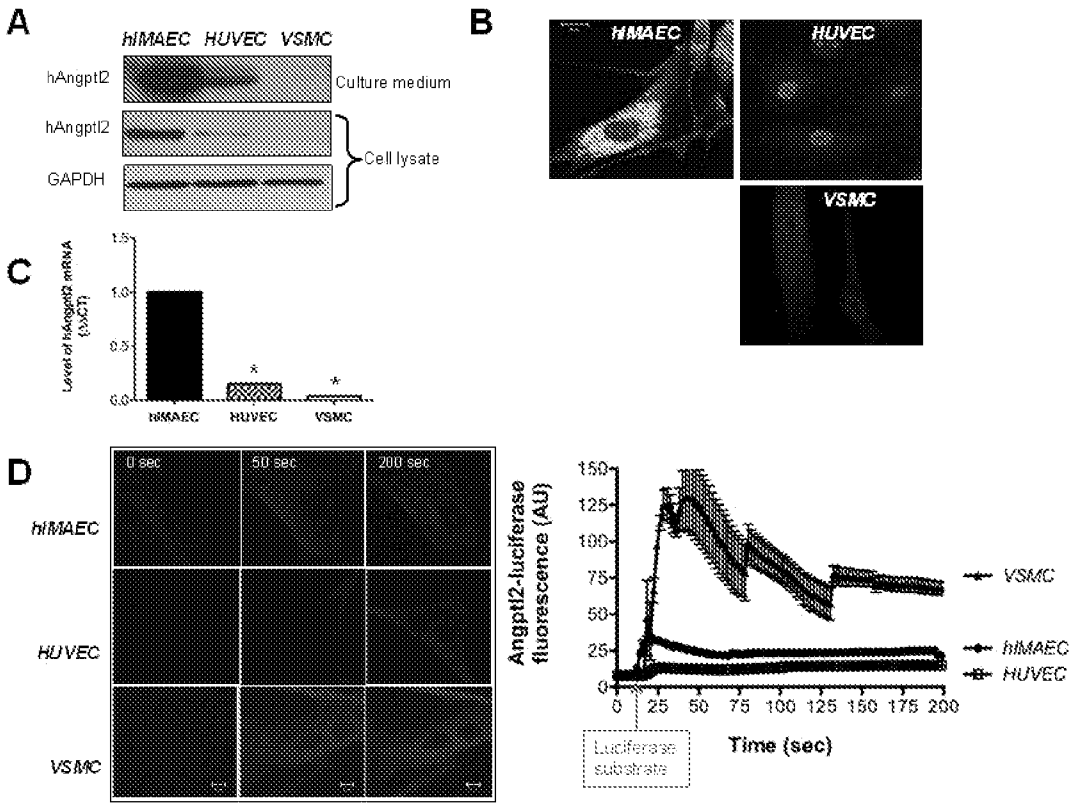

A
hlMAEC HUVEC VSMC
hAngptl2
Culture medium
hAngptl2
GAPDH
Cell lysate
B
HMAEC
HUVEC
VSMC
C
Level of hAngptl2 mRNA (ΔΔCT)
1.5
1.0
0.5
0.0
*
*
HMAEC
HUVEC
VSMC
D
0 sec
50 sec
200 sec
hlMAEC
HUVEC
VSMC
Angptl2-luciferase fluorescence (AU)
150
125
100
75
50
25
0
0 25 50 75 100 125 150 175 200
Time (sec)
Luciferase substrate
VSMC
hlMAEC
HUVEC

Figure 8

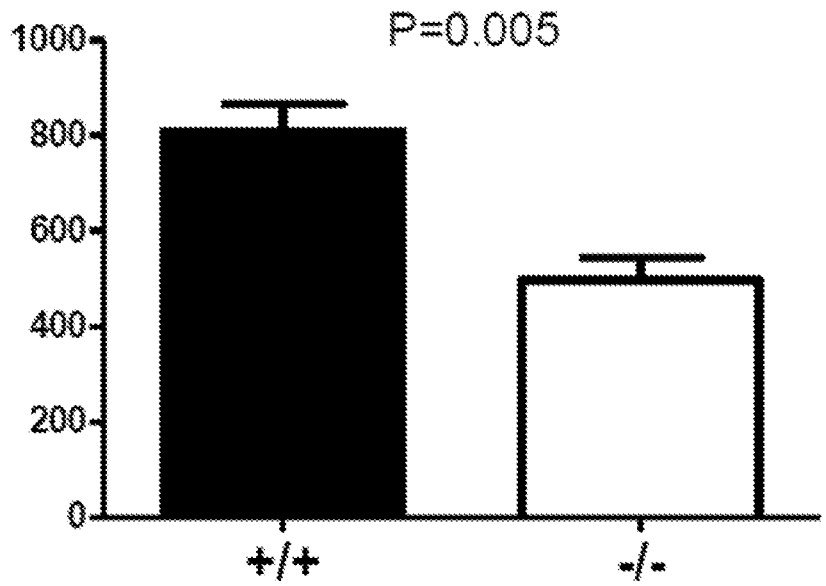


Figure 9

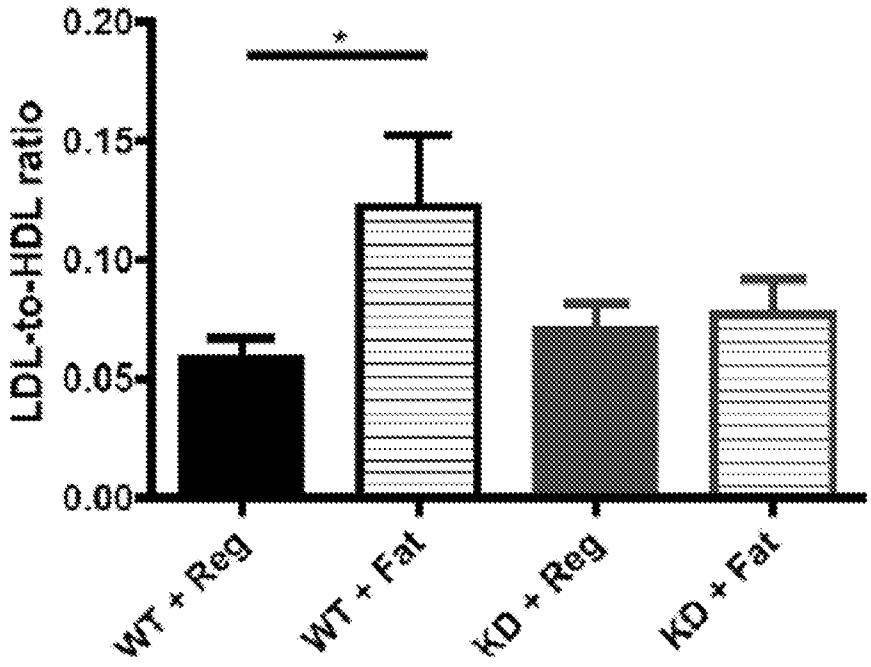


Figure 10

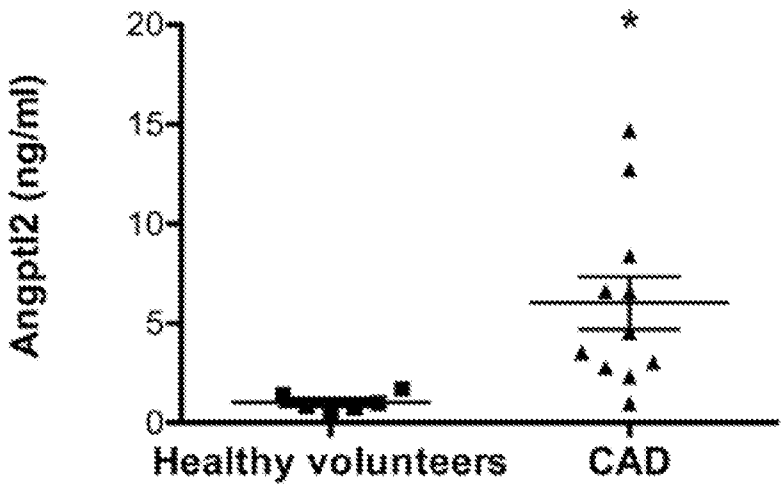


Figure 11

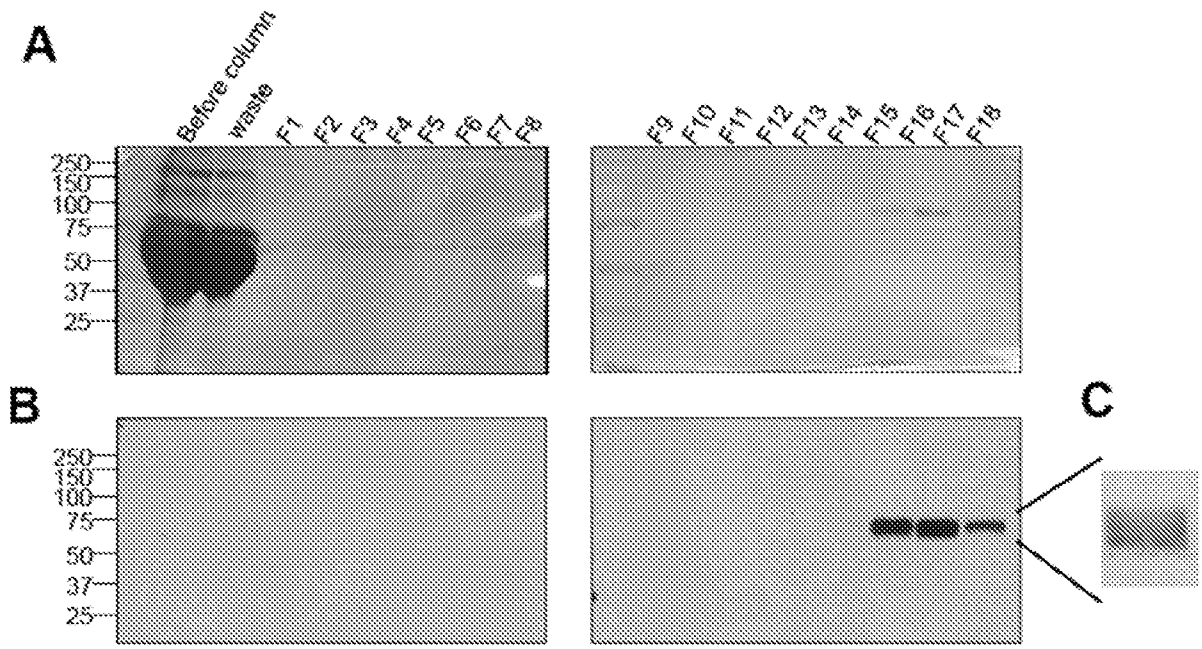

A
Before column
waste
F1
F2
F3
F4
F5
F6
F7
F8
F9
F10
F11
F12
F13
F14
F15
F16
F17
F18
250
150
100
75
50
37
25
B
C

Figure 12

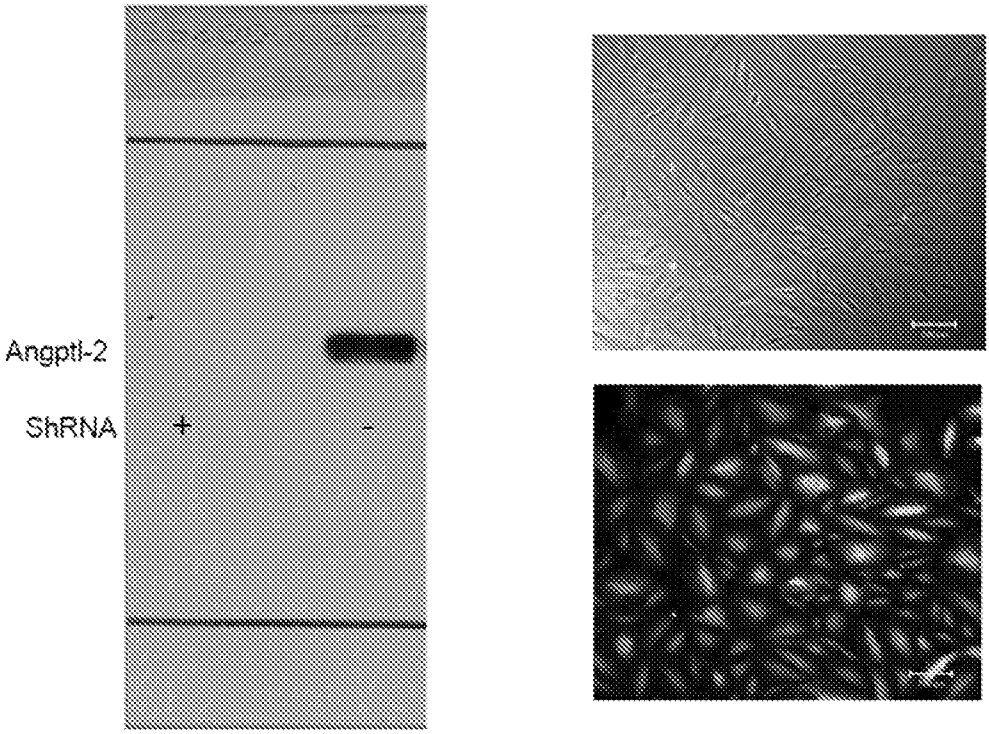
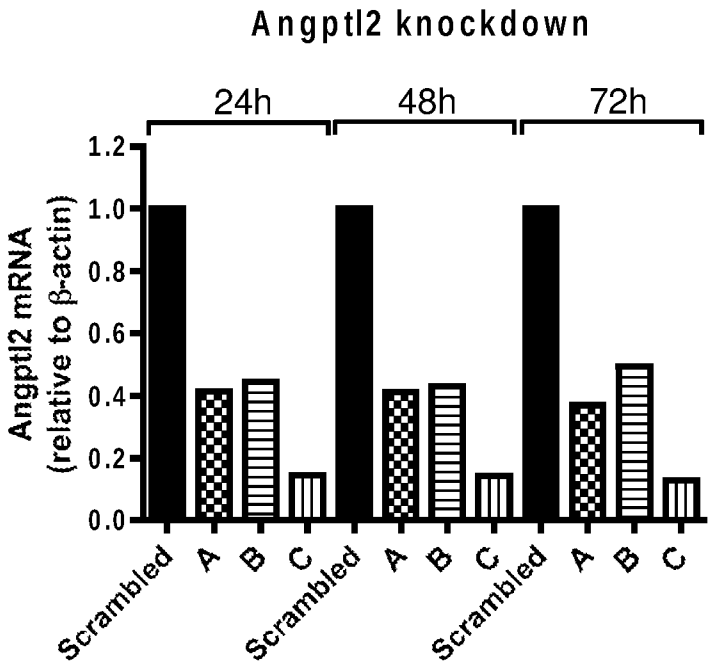


Figure 13: ANGPTL2 Knockdown



INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2014/060632

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***G01N 33/53*** (2006.01), ***A61K 31/713*** (2006.01), ***A61K 39/395*** (2006.01), ***A61P 9/10*** (2006.01), ***C12Q 1/00*** (2006.01), ***G01N 33/68*** (2006.01)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
(see extra sheet)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
(see extra sheet)

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
(see extra sheet)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	FARHAT, N. *et al.* 'Angiopoietin-Like 2 Promotes Atherogenesis in Mice'. *Journal of the American Heart Association*, 10 May 2013 (10.05.2013), Vol. 2, No. 3, e000201, 13 pages.	
X, P	HORIO, E. *et al.* 'Role of Endothelial Cell–Derived Angptl2 in Vascular Inflammation Leading to Endothelial Dysfunction and Atherosclerosis Progression'. *Arteriosclerosis, thrombosis, and vascular biology*, 2014, Vol. 34, No. 4, pages 790 to 800.	
X, P	FARHAT, N. 'Angiopoietine-like 2: un facteur circulant pro-oxydant et pro-inflammatoire qui contribue au développement de l'athérosclérose'. (01.05.2014). Theses at the Université de Montréal, Canada, April 2013 (04.2013). http://hdl.handle.net/1866/10886	
X	FARHAT, N. *et al.* 'Angiopoietin like-2 stimulates leukocytes adhesion to the native aortic endothelium in LDLr–/–; hApoB100+/+ mice'. *The FASEB Journal*, 2012, No. 26, abstract 841.4.	1 to 11, 13 to 16, 24 to 36, 38 to 42, 44 to 50

☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report 08 August 2014 (08-08-2014)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Christian Barrette (819) 994-0477

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HORIO, E. *et al.* 'Angiopoietin-Like Protein2 (angptl2) Promotes Coronary Endothlial Dysfunction and Atherosclerosis'. *Circulation*, November 2010 (11.2010), Vol. 122, No. 21.	24, 25, 28
X	HORIO, E. *et al.* 'Angiopoietin-Like Protein2 (angptl2)-Induced Vascular Inflammation Causes the Progression of Endothelial Dysfunction and Atherosclerosis'. *Circulation*, 2011, Vol. 124, No. 21 Supplement, Abstract 12277.	24, 25, 28
A	CA2699440 A1 (SWAGEMAKERS, SMA *et al.*) 5 February 2009 (05.02.2009)	
A	CA2592372 A1 (THORIN E) 20 December 2007 (20.12.2007)	
A	OIKE Y. [Roles of angiopoietin-like protein 2 (Angptl2) in chronic inflammation and atherosclerotic diseases]. Nihon rinsho. *Japanese journal of clinical medicine*, 2011, Vol. 69, No. 1, pages 163 to 167.	
A	FARHAT, N. *et al.* 'Angiopoietin like-2 increases with aging and atherosclerosis'. *The FASEB Journal*, 2011, No. 25, Abstract 637.5.	
A, P	ERKAN, A. *et al.* 'Serum Angiopoietin Like Protein-2 Levels are Positively Correlated to the Angiographic Severity and Extent of Coronary Artery Disease'. *Journal of the American College of Cardiology*, October 2013 (10.2013), Vol. 62, No.18_S2, page C72, Abstract OP-162.	
A	HATO T. *et al.* 'The role of angiopoietin-like proteins in angiogenesis and metabolism'. *Trends in cardiovascular medicine*, 2008, Vol. 18, No. 1, pages 6 to 14.	
A	ZHAO, W. W. *et al.* 'Research progress in association between angiopoietin-like protein 2 and inflammation-related diseases.' *Chinese Journal of Pathophysiology*, 2012, Vol. 28, No. 11, pages 1986 to 1990.	
A	OIKE, Y. *et al.* 'Angiopoietin-like proteins--potential therapeutic targets for metabolic syndrome and cardiovascular disease'. *Circulation journal: official journal of the Japanese Circulation Society*, 2009, Vol. 73, No. 12, pages 2192 to 2197.	

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

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

1. ☑ Claim Nos.: 1 to 9 (partly), 12 (entirely), 17 to 23 (entirely), 37 (entirely) and 43 (entirely),
because they relate to subject matter not required to be searched by this Authority, namely:

claims 1 to 9 comprise embodiments wherein a medical treatment is decided for said subject, which decision is considered as a form of medical treatment; claim 12 is directed to a method of medical treatment by the step of administering a composition to said subject; and claims 17 to 23 are directed to a method of medical treatment.

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

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

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

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

- Claims 1 to 11, 13 to 16, 30 to 36, 38 to 42, and 44 to 47 are directed to methods, kits and a use related to active atherogenesis through ANGPTL-2, ICAM-1 and P-Selectin detection and modulation.
- Claims 24 to 29 are directed to a pharmaceutical composition and methods of drug screening related to the modulation of ANGPTL2 in atherogenesis.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☑ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

Continuation of second sheet, "B. FIELDS SEARCHED"

Type	Engine	Dbase or tool	Search string or citing(ed) document
Keyword	Intellect	Canadian Patent Dbase	(ANGPTL*2 <or> angiopoietin?like*2 <or> ARP*2 <or> HARP <or> angiopoietin?like?protein*2 <or> angiopoietin-related?protein*2) <and> (atherogenesis <or> atherosclerosis <or> arteriosclerotic vascular disease <or> ASVD) <and> (ICAM*1 <or> P*Selectin <or> BB2 <or> CD54 <or> P3.58 <or> intercellular adhesion molecule*1 <or> human rhinovirus receptor <or> major group rhinovirus receptor <or> P-selectin glycoprotein ligand*1 <or> CD162 <or> "CLA" <or> PSGL*1 <or> cutaneous lymphocyte-associated antigen <or> selectin*P ligand <or> SELPLG)
Keyword	Questel	PlusPat FamPat Fulltext	((ANGPTL?2 OR angiopoietin?like?2 OR ARP?2 OR HARP OR angiopoietin?like?protein?2 OR angiopoietin-related?protein?2)/TI/BI/TX AND (atherogenesis OR atherosclerosis OR (arteriosclerotic vascular disease) OR ASVD)/BI/OBJ/ADB/ICLM/TX AND (ICAM?1 OR P?Selectin OR BB2 OR CD54 OR (P3.58) OR (intercellular adhesion molecule 1) OR (human rhinovirus receptor) OR (major group rhinovirus receptor) OR (P?selectin glycoprotein ligand 1) OR CD162 OR CLA OR PSGL?1 OR (cutaneous lymphocyte?associated) OR selectin?P?ligand OR SELPLG)/BI/OBJ/ADB/ICLM)
Keyword	Questel	PlusPat FamPat Fulltext	((angptl2)/TI/BI/TX AND (athero+)/BI/OBJ/ADB/ICLM/TX)
Citing	Questel	Citation	CA2592372
Keyword	Google scholar	Excl. patents; incl. citations	angptl2 atherogenesis
Cited by	-	-	PCT request (i.e. applicant's priority documents)
Cited by	-	-	Kadomatsu, T., Tabata, M., & Oike, Y. (2011). Angiopoietin□like proteins: emerging targets for treatment of obesity and related metabolic diseases. FEBS journal, 278(4), 559-564.
Cited by	-	-	Examined application.
Assignee	Questel	FamPat PlusPat Fulltext	(ANGPTL?2 OR angiopoietin?like?2 OR ARP?2 OR HARP OR angiopoietin?like?protein?2 OR angiopoietin-related?protein?2)/BI/CLMS/TX AND ((INSTITUT W DE W CARDIOLOGIE W DE W MONTREAL)/PA/OPA/PAH/OWR/REAS)
Inventor	Questel	FamPat PlusPat Fulltext	(ANGPTL?2 OR angiopoietin?like?2 OR ARP?2 OR HARP OR angiopoietin?like?protein?2 OR angiopoietin-related?protein?2)/BI/CLMS/TX AND ((Thorin 1D Eric)/IN/OIN/INH/INV OR (Farhat 1D Nada)/IN/OIN/INH/INV OR (Trescases 1D Nathalie)/IN/OIN/INH/INV OR (Yu 1D Carol)/IN/OIN/INH/INV)
Author	Google scholar	Excluding patents	angptl author:"Eric Thorin" OR author:"Nada Farhat" OR author:"Nathalie Trescases" OR author:"Carol Yu"
Author	Google scholar	Excluding patents	angiopoietin author:"Eric Thorin" OR author:"Nada Farhat" OR author:"Nathalie Trescases" OR author:"Carol Yu"