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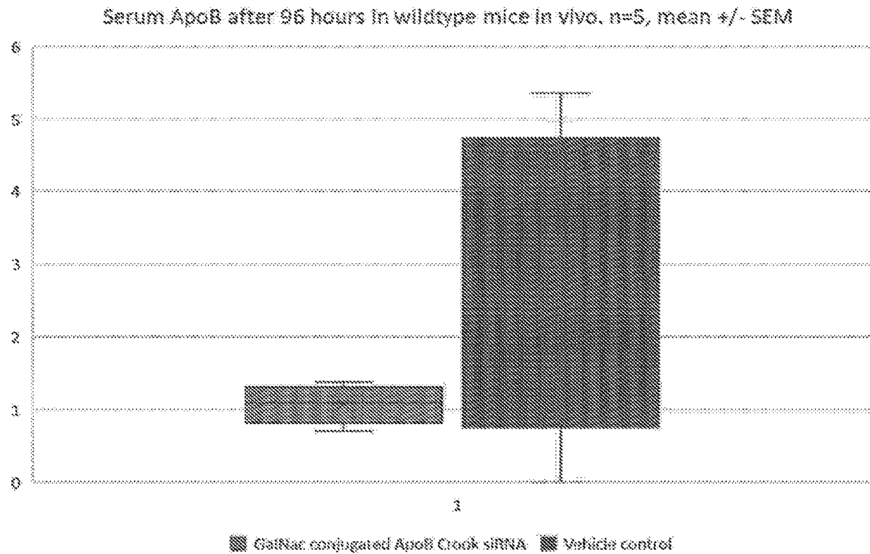

(57) Abrégé/Abstract:
This disclosure relates to a nucleic acid comprising a double stranded RNA molecule comprising sense and antisense strands and further comprising a single stranded DNA molecule covalently linked to the 3' end of either the sense or antisense RNA part of the molecule wherein the double stranded inhibitory RNA targets apolipoprotein B in the treatment hypercholesterolemia.

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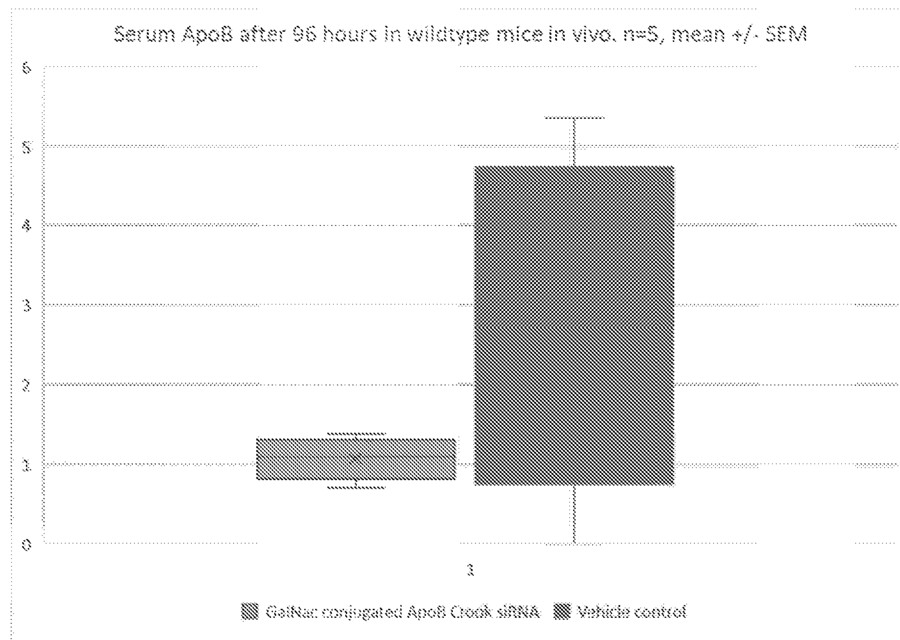
(54) **Title:** APOLIPOPROTEIN B ANTAGONIST

FIGURE 1 a



(57) **Abstract:** This disclosure relates to a nucleic acid comprising a double stranded RNA molecule comprising sense and antisense strands and further comprising a single stranded DNA molecule covalently linked to the 3' end of either the sense or antisense RNA part of the molecule wherein the double stranded inhibitory RNA targets apolipoprotein B in the treatment hypercholesterolemia.

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Apolipoprotein B Antagonist

Field of the Disclosure

5 This disclosure relates to a nucleic acid comprising a double stranded RNA molecule comprising sense and antisense strands and further comprising a single stranded DNA molecule covalently linked to the 3' end of either the sense or antisense RNA part of the molecule wherein the double stranded inhibitory RNA targets apolipoprotein B (ApoB); pharmaceutical compositions comprising said nucleic acid molecule and methods for the 10 treatment of diseases associated with increased levels of ApoB, for example hypercholesterolemia.

Background to the Disclosure

15 Cardiovascular disease associated with hypercholesterolemia is a common condition and results in heart disease and a high incidence of death and morbidity and can be a consequence of poor diet, obesity or an inherited dysfunctional gene. For example, mutations in Low Density Lipoprotein Receptor (LDL-receptor) or apolipoprotein B (ApoB) as in familial hypercholesterolemia. Cholesterol is essential for membrane biogenesis in animal cells. The 20 lack of water solubility means that cholesterol is transported around the body in association with lipoproteins. Apolipoproteins form together with phospholipids, cholesterol and lipids which facilitate the transport of lipids such as cholesterol, through the bloodstream to the different parts of the body. Lipoproteins are classified according to size and can form HDL (High-density lipoprotein), LDL (Low-density lipoprotein), IDL (intermediate-density 25 lipoprotein), VLDL (very low-density lipoprotein) and ULDL (ultra-low-density lipoprotein) lipoproteins.

Lipoproteins change composition throughout their circulation comprising different ratios of apolipoproteins A (ApoA), B (ApoB), C (ApoC), D(ApoD) or E (ApoE), triglycerides, cholesterol 30 and phospholipids. ApoB is the main apolipoprotein of ULDL and LDL and has two isoforms apoB-48 and apoB-100. Both ApoB isoforms are encoded by one single gene and wherein the shorter ApoB-48 gene is produced after RNA editing of the ApoB-100 transcript at residue 2180 resulting in the creation of a stop codon. ApoB-100 is the main structural protein of LDL and serves as a ligand for a cell receptor which allows transport of, for example, cholesterol 35 into a cell.

Familial hypercholesterolemia is an orphan disease and results from elevated levels of LDL cholesterol (LDL-C) in the blood. The disease is an autosomal dominant disorder with both the heterozygous (350-550mg/dL LDL-C) and homozygous (650-1000mg/dL LDL-C) states resulting in elevated LDL-C. The heterozygous form of familial hypercholesterolemia is around 1:500 of the population. The homozygous state is much rarer and is approximately 1:1,000,000. The normal levels of LDL-C are in the region 130mg/dL.

Hypercholesterolemia is particularly acute in paediatric patients which if not diagnosed early can result in accelerated coronary heart disease and premature death. If diagnosed and treated early the child can have a normal life expectancy. In adults, high LDL-C, either because of mutation or other factors, is directly associated with increased risk of atherosclerosis which can lead to coronary artery disease, stroke or kidney problems. Lowering levels of LDL-C is known to reduce the risk of atherosclerosis and associated conditions. LDL-C levels can be lowered initially by administration of statins which block the *de novo* synthesis of cholesterol by inhibiting the HMG–CoA reductase. Some subjects can benefit from combination therapy which combines a statin with other therapeutic agents such as ezetimibe, colestipol or nicotinic acid. However, expression and synthesis of HMG–CoA reductase adapts in response to the statin inhibition and increases over time, thus the beneficial effects are only temporary or limited after statin resistance is established.

There is therefore a desire to identify alternative therapies that can be used alone or in combination with existing therapeutic approaches to control cardiovascular disease because of elevated LDL-C.

A technique to specifically ablate gene function is through the introduction of double stranded inhibitory RNA, also referred to as small inhibitory or interfering RNA (siRNA), into a cell which results in the destruction of mRNA complementary to the sequence included in the siRNA molecule. The siRNA molecule comprises two complementary strands of RNA (a sense strand and an antisense strand) annealed to each other to form a double stranded RNA molecule. The siRNA molecule is typically, but not exclusively, derived from exons of the gene which is to be ablated. Many organisms respond to the presence of double stranded RNA by activating a cascade that leads to the formation of siRNA. The presence of double stranded RNA activates a protein complex comprising RNase III which processes the double stranded RNA into smaller fragments (siRNAs, approximately 21-29 nucleotides in length) which become part of a ribonucleoprotein complex. The siRNA acts as a guide for the RNase complex to cleave mRNA complementary to the antisense strand of the siRNA thereby resulting in destruction of the mRNA.

ApoB is a known target for therapeutic intervention in the regulation of LDL-C. For example, attempts to silence ApoB synthesis by using antisense RNA is known in the art; see WO2006/053430, WO2008/109357, WO2014/076196, WO2010/076248, WO2015/071388, WO2011/000108 and WO2008/118883. A problem with administering RNAi or antisense oligonucleotides is the toxicity caused by modified, non-naturally occurring nucleotides or the length of the RNAi molecules. Moreover, because antisense techniques do not necessarily produce stable transformation the stability of the antisense constructs such as RNAi is variable.

This disclosure relates to a nucleic acid molecule comprising a double stranded inhibitory RNA that is modified by the inclusion of a short DNA part linked to the 3' end of either the sense or antisense inhibitory RNA and which forms a hairpin structure and is designed with reference to the nucleotide sequence encoding ApoB. US8,067,572, discloses examples of said nucleic acid molecules. The double stranded inhibitory RNA uses solely or predominantly natural nucleotides and does not require modified nucleotides or sugars that prior art double stranded RNA molecules typically utilise to improve pharmacodynamics and pharmacokinetics. The disclosed double stranded inhibitory RNAs have activity in silencing ApoB with potentially fewer side effects.

Statements of the Invention

According to an aspect of the invention there is provided a nucleic acid molecule comprising

a first part that comprises a double stranded inhibitory ribonucleic acid (RNA) molecule comprising a sense strand and an antisense strand; and

a second part that comprises a single stranded deoxyribonucleic acid (DNA) molecule, wherein the 5' end of said single stranded DNA molecule is covalently linked to the 3' end of the sense strand of the double stranded inhibitory RNA molecule or wherein the 5' end of the single stranded DNA molecule is covalently linked to the 3' of the antisense strand of the double stranded inhibitory RNA molecule, characterized in that the double stranded inhibitory RNA comprises a sense nucleotide sequence that encodes a part of the human apolipoprotein B protein and wherein said single stranded DNA molecule comprises a nucleotide sequence that is adapted over at least part of its length to anneal by complementary base pairing to a part of said single stranded DNA to form a double stranded DNA structure.

According to an aspect of the invention there is provided a nucleic acid molecule comprising

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a first part that comprises a double stranded inhibitory ribonucleic acid (RNA) molecule comprising a sense strand and an antisense strand; and

a second part that comprises a single stranded deoxyribonucleic acid (DNA) molecule, wherein the 5' end of said single stranded DNA molecule is covalently linked to the 3' end of the sense strand of the double stranded inhibitory RNA molecule or wherein the 5' end of the single stranded DNA molecule is covalently linked to the 3' of the antisense strand of the double stranded inhibitory RNA molecule, characterized in that the double stranded inhibitory RNA comprises a sense nucleotide sequence that encodes a part of the human apolipoprotein B protein, or polymorphic sequence variant thereof, and wherein said single stranded DNA molecule comprises a nucleotide sequence that is adapted over at least part of its length to anneal by complementary base pairing to a part of said single stranded DNA to form a double stranded DNA structure.

A "polymorphic sequence variant" is a sequence that varies by one, two, three or more nucleotides. Apo B polymorphisms are known in the art, some of which are associated with hypercholesterolemia.

In a preferred embodiment of the invention wherein the 5' end of said single stranded DNA molecule is covalently linked to the 3' end of the sense strand of the double stranded inhibitory RNA molecule.

In a preferred embodiment of the invention wherein the 5' end of said single stranded DNA molecule is covalently linked to the 3' end of the antisense strand of the double stranded inhibitory RNA molecule.

In a preferred embodiment of the invention said single stranded DNA molecule comprises the nucleotide sequence TCACCTCATCCCGCGAAGC (SEQ ID NO: 1).

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule is between 10 and 40 nucleotides in length.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule is between 18 and 29 base pairs in length.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule is 21 base pairs in length.

In a preferred embodiment of the invention said double stranded inhibitory RNA is designed with reference to a nucleotide sequence as set forth in SEQ ID NO: 2.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a nucleotide sequence as set forth in SEQ ID NO: 3.

In a preferred embodiment of the invention said a double stranded inhibitory RNA molecule comprises a nucleotide sequence as set forth in SEQ ID NO: 4.

In an alternative embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence selected from the group consisting of: SEQ ID NO: 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56 and 57.

In an alternative embodiment of the invention said double stranded inhibitory RNA molecule comprises an antisense nucleotide sequence selected from the group consisting of: 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94,95, 96, 97, 98, 98, 99, 100, 101, 102, 103. 104, 105, 106, 107, 108, 109 and 110.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 27 and an antisense nucleotide sequence set forth in SEQ ID NO: 47.

In an alternative preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 80 and an antisense nucleotide sequence set forth in SEQ ID NO:100.

In an alternative embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence selected from the group consisting of: 111, 113, 115, 117, 119, 121, 123 and 125.

In an alternative embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence selected from the group consisting of: 112, 114, 116, 118, 120, 122, 124 and 126.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 111 and an antisense nucleotide sequence set forth in SEQ ID NO:112.

5 In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 113 and an antisense nucleotide sequence set forth in SEQ ID NO:114.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule 10 comprises a sense nucleotide sequence set forth in SEQ ID NO: 115 and an antisense nucleotide sequence set forth in SEQ ID NO:116.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 117 and an antisense 15 nucleotide sequence set forth in SEQ ID NO:118.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 119 and an antisense nucleotide sequence set forth in SEQ ID NO:120.

20

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 121 and an antisense nucleotide sequence set forth in SEQ ID NO:122.

25 In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 123 and an antisense nucleotide sequence set forth in SEQ ID NO:124.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule 30 comprises a sense nucleotide sequence set forth in SEQ ID NO: 125 and an antisense nucleotide sequence set forth in SEQ ID NO:126.

In an alternative embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence selected from the group consisting of: SEQ ID NO: 35 7, SEQ ID NO: 36, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115 and SEQ ID NO: 119.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises an antisense nucleotide sequence selected from the group consisting of: SEQ ID NO: 60, SEQ ID NO: 72, SEQ ID NO: 89, SEQ ID NO: 100, SEQ ID NO: 108, SEQ ID NO: 114 and SEQ ID NO: 118.

5

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 7 and an antisense nucleotide sequence set forth in SEQ ID NO:60.

10 In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 111 and an antisense nucleotide sequence set forth in SEQ ID NO:112.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule
15 comprises a sense nucleotide sequence set forth in SEQ ID NO: 117 and an antisense nucleotide sequence set forth in SEQ ID NO:118.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 55 and an antisense
20 nucleotide sequence set forth in SEQ ID NO:108.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 47 and an antisense nucleotide sequence set forth in SEQ ID NO:100.

25

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 36 and an antisense nucleotide sequence set forth in SEQ ID NO:89.

30 In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 19 and an antisense nucleotide sequence set forth in SEQ ID NO:72.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule
35 comprises a sense nucleotide sequence set forth in SEQ ID NO: 115 and an antisense nucleotide sequence set forth in SEQ ID NO:116.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 113 and an antisense nucleotide sequence set forth in SEQ ID NO: 114.

5 In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 119 and an antisense nucleotide sequence set forth in SEQ ID NO: 120.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule
10 comprises a sense nucleotide sequence set forth in SEQ ID NO: 113 and an antisense nucleotide sequence set forth in SEQ ID NO:114.

In a preferred embodiment of the invention said double stranded inhibitory RNA molecule comprises a modified base, sugar, inter-nucleotide linkage, or combinations thereof.

15

In a preferred embodiment of the invention said nucleic acid molecule is covalently linked to a carrier molecule adapted to deliver said nucleic acid molecule to a cell or tissue.

In a preferred embodiment of the invention said nucleic acid molecule is covalently linked to
20 *N*-acetylgalactosamine. Preferably, *N*-acetylgalactosamine is triantennary.

In an alternative preferred embodiment of the invention said nucleic acid molecule is covalently linked to oligomannose, oligofucose, or N-acetylgalactosamine 4-sulfate.

25 According to a further aspect of the invention there is provided a pharmaceutical composition comprising at least one nucleic acid molecule according to the invention.

In a preferred embodiment of the invention said composition further includes a pharmaceutical carrier and/or excipient.

30

When administered the compositions of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers and optionally other therapeutic agents, such as cholesterol lowering
35 agents, which can be administered separately from the nucleic acid molecule according to the invention or in a combined preparation if a combination is compatible.

The combination of a nucleic acid according to the invention and the other, different therapeutic agent is administered as simultaneous, sequential or temporally separate dosages.

5 The therapeutics of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, transdermal or transepithelial.

10 The compositions of the invention are administered in effective amounts. An "effective amount" is that amount of a composition that alone, or together with further doses, produces the desired response. In the case of treating a disease, such as cardiovascular disease, the desired response is inhibiting or reversing the progression of the disease. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves 15 halting the progression of the disease permanently. This can be monitored by routine methods.

Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and 20 weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose of the individual components or combinations thereof be used, that is, the 25 highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

The pharmaceutical compositions used in the foregoing methods preferably are sterile and 30 contain an effective amount of a nucleic acid molecule according to the invention for producing the desired response in a unit of weight or volume suitable for administration to a patient. The response can, for example, be measured by determining regression of cardiovascular disease and decrease of disease symptoms etc.

35 The doses of the nucleic acid molecule according to the invention administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject. Other factors include the desired

period of treatment. If a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. It will be apparent that the method of detection of the nucleic acid according to the invention facilitates the determination of an appropriate dosage for a subject in need of treatment.

In general, doses of the nucleic acid molecules herein disclosed of between 1nM - 1µM generally will be formulated and administered according to standard procedures. Preferably doses can range from 1nM- 500nM, 5nM-200nM, 10nM-100nM. Other protocols for the administration of compositions will be known to one of ordinary skill in the art, in which the dose amount, schedule of injections, sites of injections, mode of administration and the like vary from the foregoing. The administration of compositions to mammals other than humans, (e.g. for testing purposes or veterinary therapeutic purposes), is carried out under substantially the same conditions as described above. A subject, as used herein, is a mammal, preferably a human, and including a nonhuman primate, cow, horse, pig, sheep, goat, dog, cat or rodent.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically acceptable amounts and in pharmaceutically acceptable compositions. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents e.g. statins. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

Compositions may be combined, if desired, with a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "pharmaceutically acceptable carrier" in this context denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate, for example, solubility and/or stability. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the

present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, including acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt. The pharmaceutical compositions also may contain, optionally, suitable preservatives.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product. Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous or non-aqueous preparation of nucleic acid, which is preferably isotonic with the blood of the recipient. This preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example, as a solution in 1, 3-butane diol. Among the acceptable solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

In a further preferred embodiment of the invention said pharmaceutical composition comprises at least one further, different, therapeutic agent.

In a preferred embodiment of the invention said further therapeutic agent is a statin.

Statins are commonly used to control cholesterol levels in subjects that have elevated LDL-C. Statins are effective in preventing and treating those subjects that are susceptible and those

that have cardiovascular disease. The typical dosage of a statin is in the region 5 to 80mg but this is dependent on the statin and the desired level of reduction of LDL-C required for the subject suffering from high LDL-C. However, expression and synthesis of HMG–CoA reductase, the target for statins, adapts in response to statin administration thus the beneficial effects of statin therapy are only temporary or limited after statin resistance is established.

Preferably said statin is selected from the group consisting of atorvastatin, fluvastatin, lovastatin, pitvastatin, pravastatin, rosuvastatin and simvastatin.

In a preferred embodiment of the invention said further therapeutic agent is ezetimibe. Optionally, ezetimibe is combined with at least one statin, for example simvastatin.

In an alternative preferred embodiment of the invention said further therapeutic agent is selected from the group consisting of fibrates, nicotinic acid, cholestyramine.

In a further alternative preferred embodiment of the invention said further therapeutic agent is a therapeutic antibody, for example, evolocumab, bococizumab or alirocumab.

According to a further aspect of the invention there is provided a nucleic acid molecule or a pharmaceutical composition according to the invention for use in the treatment or prevention of a subject that has or is predisposed to hypercholesterolemia.

In a preferred embodiment of the invention said use is the treatment or prevention of diseases associated with hypercholesterolemia.

In a preferred embodiment of the invention said disease associated with hypercholesterolemia is selected from the group consisting of: stroke prevention, hyperlipidaemia, cardiovascular disease, atherosclerosis, coronary heart disease, aortic stenosis, cerebrovascular disease, peripheral arterial disease, hypertension, metabolic syndrome, type II diabetes, non-alcoholic fatty acid liver disease, non-alcoholic steatohepatitis, Buerger's disease, renal artery stenosis, hyperapobetalipoproteinemia, cerebrovascular atherosclerosis, cerebrovascular disease and venous thrombosis.

In a preferred embodiment of the invention said subject is a paediatric subject.

A paediatric subject includes neonates (0-28 days old), infants (1 – 24 months old), young children (2 – 6 years old) and prepubescent [7-14 years old] children.

In an alternative preferred embodiment of the invention said subject is an adult subject.

In a preferred embodiment of the invention the hypercholesterolemia is familial
5 hypercholesterolemia.

In a preferred embodiment of the invention familial hypercholesterolemia is associated with elevated levels of apolipoprotein B expression.

10 In a preferred embodiment of the invention said subject is resistant to statin therapy.

According to a further aspect of the invention there is provided a method to treat a subject that has or is predisposed to hypercholesterolemia comprising administering an effective dose of a nucleic acid or a pharmaceutical composition according to the invention thereby treating or
15 preventing hypercholesterolemia.

In a preferred method of the invention said subject is a paediatric subject.

In an alternative preferred method of the invention said subject is an adult subject.

20

In a preferred method of the invention the hypercholesterolemia is familial hypercholesterolemia.

In a preferred method of the invention familial hypercholesterolemia is associated with
25 elevated levels of ApoB expression.

In a preferred method of the invention said subject is resistant to statin therapy.

According to a further aspect of the invention there is provided a treatment regimen for the
30 diagnosis and treatment of hypercholesterolemia associated with elevated ApoB comprising:

i) obtaining a biological sample from a subject suspected on having or suspected of having hypercholesterolemia;
ii) contacting the sample with an antibody, or antibody fragment, specific for an
35 apolipoprotein polypeptide;
iii) determining the concentration said apolipoprotein B polypeptide and LDL-C in said biological sample; and

iv) administering a nucleic acid molecule or pharmaceutical composition according to the invention if the LDL-C concentration is greater than 350mg/dL.

Typically, in familial hypercholesterolemia disease the levels of LDL-C are 350-550mg/dL in
5 subjects that are heterozygous for a selected mutation in apolipoprotein B and 650-1000mg/dL in those subjects carrying a homozygous mutation in apolipoprotein B. The normal levels of LDL-C are in the region 130mg/dL.

Throughout the description and claims of this specification, the words “comprise” and “contain”
10 and variations of the words, for example “comprising” and “comprises”, means “including but not limited to” and is not intended to (and does not) exclude other moieties, additives, components, integers or steps.

Throughout the description and claims of this specification, the singular encompasses the
15 plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in
20 conjunction with an aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

An embodiment of the invention will now be described by Example only and with reference to
25 the following figures:

FIG.1(a) and 1(b). Graphs illustrating *in vivo* Activity of GalNAc-conjugated Crook anti- mouse ApoB siRNA compared to control siRNA constructs.
(a) Plasma ApoB levels (micrograms/ml) from five adult male wild-type C57BL/6 mice, were
30 measured 96 hours following administration of GalNAc-conjugated ApoB Crook siRNA (one treatment group) and compared with the control treatment group administered with saline. Statistical analysis was applied using the two-tailed paired T test algorithm. Results show a substantive reduction in mean plasma ApoB levels in mice treated with GalNAc-conjugated Crook siRNA, compared to control. However, it just fails significance (p= 0.11), most likely due
35 to small sample size and variation in ApoB levels between control animals;

(b) Plasma ApoB levels (micrograms/ml) from five adult male wild-type C57BL/6 mice, were measured 96 hours following administration of GalNAc-conjugated ApoB Crook siRNA (one treatment group) and compared with the control treatment group, administered with siRNA construct unconjugated (without GalNAc) ApoB Crook siRNA. Statistical analysis was applied using the two-tailed paired T test algorithm. Results show a highly significant reduction in plasma ApoB levels in this GalNAc-conjugated Crook siRNA treatment group when compared to control unconjugated siRNA with Crook (P=0.00435832).

Figure 2 (a-d). *In vitro* screen of 40 custom duplex Crook siRNAs (C1-C40) listed in Table 2. Graphs illustrate relative knock down of ApoB mRNA expression in HepG2 cells by each of the 40 crook siRNAs. Individual graphs present data from each siRNA sense and antisense pair; C1-C20 (sense strand); C21-40 (antisense strand) as shown in **Table 2**. Each of 40 crook siRNA molecules were reverse transfected into HepG2 cells (in quadruplicate) at five doses (100 nM, 25 nM, 6.25 nM, 1.56 nM and 0.39 nM) using the conditions identified in the assay development phase. 72 hours post transfection, cells were lysed and ApoB mRNA levels determined by duplex RT-qPCR. In order to calculate the relative knockdown of ApoB for each siRNA at each concentration, expression was first normalised to housekeeping reference gene GAPDH mRNA expression and then to the average ApoB expression across the five doses of the corresponding NEG control (Sense or Antisense). However, it should be noted that a decrease in ApoB expression was observed for the NEG sense control and so the knockdown of ApoB for siRNAs C1-C20 may be underestimated. ApoB knockdown efficiency of all 40 siRNAs are described in Table 3. C13 and C23 siRNAs show a knock-down efficiency greater than 85% (at 25nM).

Table 3 was compiled from the *in vitro* ApoB mRNA expression data (FIG. 2 (a-d)) and shows ranking of Crook siRNAs (C1-C40) with highest knockdown performers at the top of the table. C13 and C23 siRNAs show a knock-down efficiency greater than 85% (at 25nM).

MATERIALS AND METHODS

In Vivo Activity of GalNAc-conjugated Crook anti-mouse ApoB siRNA

A triantennary GalNAc conjugate was attached to the passenger strand of the Crook-siRNA via phosphoramidate linkage in order to improve selective siRNA delivery to the liver.

Unconjugated (without GalNAc) and conjugated (GalNAc) versions of ApoB Crook-siRNA described below, were administered to adult male wild-type (WT) C57BL/6 mice by

intravenous (IV) and sub-cutaneous (SC) routes, respectively, to investigate the relative plasma and tissue exposure. In addition, control GalNAc-conjugated *unmodified* siRNA (without Crook) consruct was compared.

5 **In vivo ApoB siRNA constructs**

For in vivo silencing of Apo B is a mouse the sequence below was used (corresponds to C10 in table 2 below. This was chosen because a similar sequence had been successful

10 previously in vivo (Soutshek et al Nature 2004; 432:173-178).

Crook-siRNA 21mer-dsRNA construct (1): Anti-mouse ApoB-GalNAc

Sense strand:

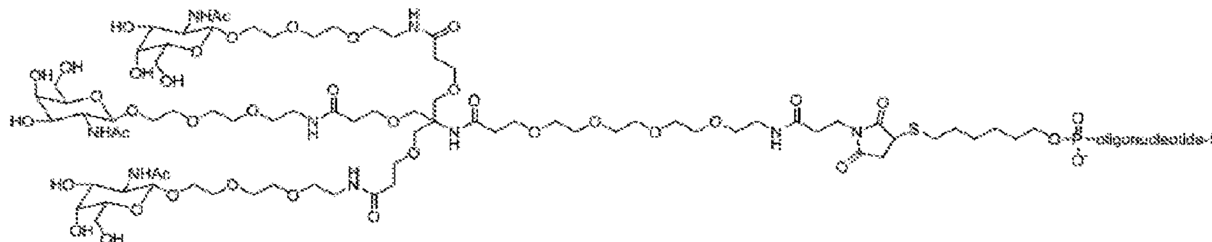
5′-GUCAUCACACUGAAUACCAAU-d(tcacctcatcccgcgaagc)-3′-[Tri-GalNAc]

15 Antisense strand:

5′-AUUGGUAUUCAGUGUGAUGAC-3′

Structure of final GalNAc conjugate:

20



Crook-siRNA 21mer dsRNA construct (2): Unconjugated anti-mouse ApoB

25 Sense strand (Passenger)

5′-GUCAUCACACUGAAUACCAAU-d(tcacctcatcccgcgaagc)-3

Antisense strand (Guide)

30 5′-AUUGGUAUUCAGUGUGAUGAC-3

The rationale for dose selection was based on the following information published in the scientific literature:

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The GalNAc conjugated siRNA is dosed subcutaneously at 5 mg/kg which is expected to produce the required level of gene silencing where the ED_{80} of structurally related siRNAs have been reported as 2.5 mg/kg (Soutschek *et al.*, 2004). These structurally related siRNAs
5 were tolerated up to 25 mg/kg, single administration, in the mouse (Soutschek *et al.*, 2004).

The unconjugated version of the sponsor's siRNA is administered at 50 mg/kg IV. This 10-fold increase in the IV compared to the SC dose is due to the unconjugated siRNA being less effective at targeting the liver. Additionally, it is reported by Soutschek *et al* (2004) that lower
10 levels of RNA are measured in the liver following IV compared to SC administration. It is stated that slower release of the siRNA from the SC depot leads to prolonged exposure increasing the potential for receptor-ligand interactions and greater uptake into the tissue. Similar related siRNA has been well tolerated by mice at up to 50 mg/kg IV administered on 3 consecutive days (Nair *et al.* 2014). As a precaution a 15 minutes observation period is left between dosing
15 the 1st animal IV to determine if the test substance causes any adverse effects before the remaining animals are dosed.

The mouse is the species of choice because it is used as one of the toxicology species in the safety testing of the test substance. The mouse also possesses a very similar metabolic
20 physiology to humans in relation to the therapeutic target of the Crook-siRNA preparations (ApoB). There is a considerable amount of published data available which are acceptable to the regulatory authorities for assessing the significance to man of data generated in this species.

25

Animals

Sufficient C57BL/6 mice were obtained from an approved source to provide 20 healthy male animals (5 mice per treatment group). Animals are in the target weight range of 20 to 30 g at
30 dosing. Mice are uniquely numbered by tail marking. Numbers are allocated randomly. Cages are coded by cards giving information including study number and animal number. The study room is identified by a card giving information including room number and study number. On receipt, all animals were examined for external signs of ill health. Unhealthy animals where be excluded from the study. The animals were acclimatised for a minimum period of 5 days.
35 Where practicable, without jeopardising the scientific integrity of the study, animals were handled as much as possible. A welfare inspection was performed before the start of dosing to ensure their suitability for the study.

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The mice were kept in rooms thermostatically maintained at a temperature of 20 to 24°C, with a relative humidity of between 45 and 65%, and exposed to fluorescent light (nominal 12 hours) each day. Temperature and relative humidity are recorded on a daily basis. The facility is designed to give a minimum of 15 air-changes/hour. Except when in metabolism cages or recovering from surgery, mice were housed up to 5 per cage according to sex, in suitable solid floor cages, containing suitable bedding.

Cages conform to the 'Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes' (Home Office, London, 2014). In order to enrich both the environment and the welfare of the animals, they were provided with wooden Aspen chew blocks and polycarbonate tunnels. The supplier provided certificates of analysis for each batch of blocks used. All animals will be allowed free access to 5LF2 EU Rodent Diet 14%. The diet supplier provided an analysis of the concentration of certain contaminants and some nutrients for each batch used. All animals were allowed free access to mains water from bottles attached to the cages. Periodic analysis of the mains supply is undertaken.

All procedures to be carried out on live animals as part of this study will be subject to provisions of United Kingdom National Law, the Animals (Scientific Procedures) Act 1986.

All animals were examined at the beginning and the end of the working day, to ensure that they are in good health. Any animal, which shows marked signs of ill health, were isolated. Moribund animals or those in danger of exceeding the severity limits imposed by the relevant Home Office Licence were killed.

MATERIALS AND METHODS FOR IN VIVO EXPERIMENTS

Preparation of Formulations

Test substances were diluted in 0.9% saline to provided concentrations of 25 mg/mL and 0.6 mg/mL for the IV and SC doses of ApoB Crook-siRNA GalNAc-unconjugated and conjugate, respectively. The formulations were gently vortexed as appropriate until the test substances are fully dissolved. The resulting formulation(s) were assessed by visual inspection only and categorised accordingly:

(1) Clear solution
(2) Cloudy suspension, no particles visible
(3) Visible particles

After use, formulations were stored refrigerated nominally at 2-8°C.

Dosing Details

5 Each animal received either a single IV dose of the ApoB Crook-siRNA - unconjugated or a single SC dose of the ApoB Crook-siRNA GalNAc- conjugate. The IV dose was administered as a bolus into the lateral tail vein at a volume of 2 mL/kg. The SC dose was administered into the subcutaneous space at a volume of 5 mL/kg.

10 Group1: GalNAc-conjugated ApoB Crook siRNA 5mg/kg dose

Group 2: Unconjugated (without GalNAc) ApoB Crook siRNA 50mg/kg dose

Group 3: Saline control group

15

Body Weights

As a minimum, body weights were recorded the day after arrival and before dose administration. Additional determinations were made, if required.

20 **Sample Storage**

Samples were uniquely labelled with information including, where appropriate: study number; sample type; dose group; animal number/ Debra code; (nominal) sampling time; storage conditions. Samples were stored at <-50°C.

25 **PHARMACOKINETIC INVESTIGATION**

Designation of Dose Groups

Animals were assigned to dose groups as follows:

30

Dose Group	Dose route	Test Substance	Dose level mg/kg	Number of animals Male

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A	subcutaneous	ApoB Crook-siRNA GalNAc-conjugate	*5*	*5*
B	subcutaneous	Saline control	*0*	*5*
C	Intravenous (bolus)	ApoB Crook-siRNA unconjugated	*50*	*5*
D	Intravenous (bolus)	Saline control	*0*	*5*

Blood Sampling

Serial blood samples of (nominally 100 µL, dependent on bodyweight) were collected by tail nick at the following times: 0, 48- and 96-hours post dose. Animals were terminally anaesthetised using sodium pentobarbitone and a final sample (nominally 0.5 mL) was collected by cardiac puncture.

Blood samples were collected in to a K2EDTA microcapillary tube (tail nick) or a K2EDTA blood tube (cardiac puncture) and placed on ice until processed. Blood was centrifuged (1500 g, 10 min, 4°C) to produce plasma for analysis. The bulk plasma was divided into two aliquots of equal volume. The residual blood cells were discarded.

Scheduled Collection Time	Acceptable Time Range
0 – 15 minutes	± 1 minute
16 – 30 minutes	± 2 minutes
31 – 45 minutes	± 3 minutes
46 – 60 minutes	± 4 minutes
61 minutes – 2 hours	± 5 minutes
2 hours 1 minute – 8 hours	± 10 minutes
8 hours 1 minute – 12 hours	± 15 minutes
12 hours onwards	± 30 minutes

Where a scheduled collection time is outside the acceptable range, the actual blood collection time was reported for inclusion in any subsequent PK analysis

Animal fate

Animals were anaesthetised via an intraperitoneal injection of Sodium Pentobarbitone prior to terminal blood sampling and sacrificed by perfusion and exsanguination.

5 A full body perfusion was performed, all animals were flushed with Heparinised Saline Solution at a rate 4ml/min for 5 minutes (approximately 20mL total flush). Death was confirmed by the absence of breathing, heartbeat and blood flow. Animal carcasses were retained for tissue collection.

10 **Tissue collection**

The liver was removed from all animals (Groups A-D) and placed into a pre-weighed tube. The tissue samples were homogenised with 5 parts RNAlater to 1 part tissue using the UltraTurrax homogenisation probe. The following tissues were excised from animals in ApoB
15 treated groups (Groups A & C) and placed into a pre-weighed pot:

- Spleen
- Brain
- Heart
20 - Lung Lobes
- Skin (Inguinal region *ca*. 25 mm^2)

Following collection, the external surface of the tissues is rinsed with PBS and gently patted dry using a tissue. Tissues are initially placed on wet ice until weighed and then tissues were
25 snap frozen on dry ice prior to storage. Tissues are stored at <-50°C (nominally -80°C).

Immunoassay for APOB

Plasma ApoB levels were measured via enzyme-linked immunosorbent assay (ELISA) using
30 the commercial mouse ApoB detection kit from Elabscience Biotechnology Inc. (catalogue number E-EL-M0132). Plasma samples were stored at -80°C prior to analysis, thawed on ice and centrifuged at 13,000 rpm for 5 minutes prior to aliquots being diluted in Assay Buffer and applied to the ELISA plate. The ApoB assay kit uses a sandwich ELISA yielding a colorimetric readout, measured at OD450. Samples from each animal at specific time points (0 hours and
35 96 hours) were assayed in duplicate and measurements were recorded as micrograms ApoB

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per ml of plasma based on the standard curve reagents supplied with the kit. All data points were measured with a coefficient of variation <20%.

In vitro Screening of ApoB Crook siRNA

5

HepG2 reverse transfection

A description of the custom library evaluated in this study is provided in Table 2. Custom duplex siRNAs synthesized by Horizon Discovery were resuspended in
10 UltraPure DNase and RNase free water to generate a stock solution of 10 μM.

- Stock siRNAs were dispensed into 4 x 384-well assay plates. On each assay plate, 10 Custom siRNAs and 3 controls (POS ApoB, NEG sense and NEG antisense) were dispensed to generate five-point four-fold dilution series from a top final concentration in the assay plate of 100 nM. ON-TARGETplus Non-Targeting and ApoB siRNAs
15 controls were dispensed to give a final concentration of 25 nM.
- Lipofectamine RNAiMAX (ThermoFisher #13778075) was diluted in OptiMEM media before 10 μL of the Lipfectamine RNAiMAX:OptiMEM solution was added per well to the assay plate. The final volume of RNAiMAX per well was 0.08 μL.
- The lipid-siRNA mix was incubated 30 min at room temperature before being added
20 to the cells.
- HepG2 cells were diluted in assay media (MEM GlutaMAX (GIBCO) 10% FBS 1% Pen/Strep) before 4,000 HepG2 cells were seeded into each well of the assay plate in 40 μL volume. Quadruplicate technical replicates were seeded per assay condition.
- The plates were incubated 72 h at 37°C, 5% CO_2 in a humidified atmosphere, prior
25 to assessment of the cells.

ApoB/GAPDH duplex RT-qPCR

- 72h post-transfection, cells were processed for RT-qPCR read-out using the Cells-
30 to-CT 1-step TaqMan Kit (Invitrogen, 4391851C). Briefly, cells were washed with 50 μl cold PBS and then lysed in 20 μl Lysis solution containing DNase I. After 5 min, lysis was stopped by addition of 2 μl STOP Solution for 2 min.
- For the RT-qPCR analysis, 3 μl of lysate was dispensed per well into 384-well PCR plate as template in an 11 μl RT-qPCR reaction volume.
35
- RT-qPCR was performed using the ThermoFisher TaqMan Fast Virus1-Step Master Mix (#4444434) with TaqMan probes for GAPDH (VIC #4448486) and ApoB (FAM #4351368).

- RT-qPCR was performed using a QuantStudio 6 thermocycling instrument (Applied BioSystems).
- Relative quantification (RQ) was determined using the $\Delta\Delta$CT method, where GAPDH was used as internal control and expression changes normalized to the reference
5 sample (either NEG sense or NEG antisense siRNA treated cells).

Statistics

- For all assays in this project, four technical replicates were obtained for each data point.
10 - Mean and Standard Error of the Mean (SEM) were calculated using Excel or Graphpad Prism.
- All graphs were generated using Graphpad Prism.

Table 1: ApoB crook siRNA sequences and corresponding SEQ ID NOs.

SEQ ID NO	Sense strand base sequence	SEQ ID NO	Antisense strand base sequence	**Start NM_009693.2**
5	GAGGUGUAUGGCUUCAACCCU	58	AGGGUUGAAGCCAUACACCUC	403
6	AGGUGUAUGGCUUCAACCCUG	59	CAGGGUUGAAGCCAUACACCU	404
7	GGUGUAUGGCUUCAACCCUGA	60	UCAGGGUUGAAGCCAUACACC	405
8	GUGUAUGGCUUCAACCCUGAG	61	CUCAGGGUUGAAGCCAUACAC	406
9	GUAUGGCUUCAACCCUGAGGG	62	CCCUCAGGGUUGAAGCCAUAC	408
10	AUGGCUUCAACCCUGAGGGCA	63	UGCCCUCAGGGUUGAAGCCAU	410
11	UGGCUUCAACCCUGAGGGCAA	64	UUGCCCUCAGGGUUGAAGCCA	411
12	CUGAACAUCAAGAGGGGCAUC	65	GAUGCCCCUCUUGAUGUUCAG	562
13	UGAACAUCAAGAGGGGCAUCA	66	UGAUGCCCCUCUUGAUGUUCA	563
14	GAUACCGUGUAUGGAAACUGC	67	GCAGUUUCCAUACACGGUAUC	640
15	UACCGUGUAUGGAAACUGCUC	68	GAGCAGUUUCCAUACACGGUA	642
16	GUCCAGCCCCAUCACUUUACA	69	UGUAAAGUGAUGGGGCUGGAC	1221
17	CAGCCCCAUCACUUUACAAGC	70	GCUUGUAAAGUGAUGGGGCUG	1224
18	AGCCCCAUCACUUUACAAGCC	71	GGCUUGUAAAGUGAUGGGGCU	1225
19	GCCCCAUCACUUUACAAGCCU	72	AGGCUUGUAAAGUGAUGGGGC	1226
20	CUUUACAAGCCUUGGUUCAGU	73	ACUGAACCAAGGCUUGUAAAG	1235
21	ACAAGCCUUGGUUCAGUGUGG	74	CCACACUGAACCAAGGCUUGU	1239
22	AAGCCUUGGUUCAGUGUGGAC	75	GUCCACACUGAACCAAGGCUU	1241
23	UCACAUCCUCCAGUGGCUGAA	76	UUCAGCCACUGGAGGAUGUGA	1278
24	AAAUAGAAGGGAAUCUUAUAU	77	AUAUAAGAUUCCCUUCUAUUU	2063
25	UAGAAGGGAAUCUUAUAUUUG	78	CAAAUAUAAGAUUCCCUUCUA	2066
26	GAAGGGAAUCUUAUAUUUGAU	79	AUCAAAUAUAAGAUUCCCUUC	2068

27	GGAAUCUUAUAUUUGAUCCAA	80	UUGGAUCAAAUAUAAGAUUCC	2072
28	GAGUUUGUGACAAAUAUGGGC	81	GCCCAUAUUUGUCACAAACUC	2746
29	GUUUGUGACAAAUAUGGGCAU	82	AUGCCCAUAUUUGUCACAAAC	2748
30	GUGACAAAUAUGGGCAUCAUC	83	GAUGAUGCCCAUAUUUGUCAC	2752
31	AGAUGAACACCAACUUCUUCC	84	GGAAGAAGUUGGUGUUCAUCU	2801
32	GAUGAACACCAACUUCUUCCA	85	UGGAAGAAGUUGGUGUUCAUC	2802
33	UGAACACCAACUUCUUCCACG	86	CGUGGAAGAAGUUGGUGUUCA	2804
34	GAACACCAACUUCUUCCACGA	87	UCGUGGAAGAAGUUGGUGUUC	2805
35	ACACCAACUUCUUCCACGAGU	88	ACUCGUGGAAGAAGUUGGUGU	2807
36	CACCAACUUCUUCCACGAGUC	89	GACUCGUGGAAGAAGUUGGUG	2808
37	CAAAUGGACUCAUCUGCUACA	90	UGUAGCAGAUGAGUCCAUUUG	3547
38	GGACUCAUCUGCUACAGCUUA	91	UAAGCUGUAGCAGAUGAGUCC	3552
39	UCUGUGGGAUUCCAUCUGCCA	92	UGGCAGAUGGAAUCCCACAGA	4075
40	AUUCCAUCUGCCAUCUCGAGA	93	UCUCGAGAUGGCAGAUGGAAU	4083
41	ACAAUUUGAUCAGUAUAUUAA	94	UUAAUAUACUGAUCAAAUUGU	6636
42	CAAUUUGAUCAGUAUAUUAAA	95	UUUAAUAUACUGAUCAAAUUG	6637
43	UAAAUCAAGUGUCAUCACACU	96	AGUGUGAUGACACUUGAUUUA	10116
44	AUCAAGUGUCAUCACACUGAA	97	UUCAGUGUGAUGACACUUGAU	10119
45	UCAAGUGUCAUCACACUGAAU	98	AUUCAGUGUGAUGACACUUGA	10120
46	CAAGUGUCAUCACACUGAAUU	99	AAUUCAGUGUGAUGACACUUG	10121
47	GUCAUCACACUGAAUACCAAU	100	AUUGGUAUUCAGUGUGAUGAC	10126
48	UCAUCACACUGAAUACCAAUG	101	CAUUGGUAUUCAGUGUGAUGA	10127
49	CAUCACACUGAAUACCAAUGC	102	GCAUUGGUAUUCAGUGUGAUG	10128
50	UAACACUAAGAACCAGAAGAU	103	AUCUUCUGGUUCUUAGUGUUA	10959
51	AUUGGGAAGAAGAGGCAGCUU	104	AAGCUGCCUCUUCUUCCCAAU	12167
52	GAUUGAUUGACCUGUCCAUUC	105	GAAUGGACAGGUCAAUCAAUC	13478
53	UGAUUGACCUGUCCAUUCAAA	106	UUUGAAUGGACAGGUCAAUCA	13481
54	GAUUGACCUGUCCAUUCAAAA	107	UUUUGAAUGGACAGGUCAAUC	13482
55	GACCUGUCCAUUCAAAACUAC	108	GUAGUUUUGAAUGGACAGGUC	13486
56	ACCUGUCCAUUCAAAACUACC	109	GGUAGUUUUGAAUGGACAGGU	13487
57	CUGUCCAUUCAAAACUACCAC	110	GUGGUAGUUUUGAAUGGACAG	13489
111	CAGCACCUAGCUGGAAAGUUA	112	UAACUUUCCAGCUAGGUGCUG	
113	CUCCAUGGAAUUUAAGUAUGA	114	UCAUACUUAAAUUCCAUGGAG	
115	UUCCCUGAAGUUGAUGUGUUA	116	UAACACAUCAACUUCAGGGAA	
117	GUCCAAUAAGAUCAAUAGCAA	118	UUGCUAUUGAUCUUAUUGGAC	
119	AACUCUCAAACCCUAAGAUUA	120	UAAUCUUAGGGUUUGAGAGUU	
121	UCGGAACAAUCCUCAGAGUUA	122	UAACUCUGAGGAUUGUUCCGA	
123	AAGCAAGAACUUAAUGGAAAU	124	AUUUCCAUUAAGUUCUUGCUU	
125	GGCCAUUAGGCAAAUUGAUGA	126	UCAUCAAUUUGCCUAAUGGCC	

Table 2. A library of 40 duplex siRNAs was synthesized by Horizon Discovery. The table shows the sequences of both strands of RNA for each siRNA. The following DNA sequence (dTdCdAdCdCdTdCdAdTdCdCdCdGdCdGdAdAdGdC) was appended to the 3' end of either the sense strand (siRNAs C1 to C20, thereafter referred to as sense siRNAs) or the antisense
5 strand (siRNAs C21 to C40, thereafter referred to as antisense siRNAs).

siRNA ID		Sense sequence (5' – 3')	Antisense sequence (5' – 3')
Sense	Antisense		
C1	C21	UAGAAGGGAAUCUUAUAUUUG	CAAAUAUAAGAUUCCCUUCUA
C2	C22	CACCAACUUCUUCCACGAGUC	GACUCGUGGAAGAAGUUGGUG
C3	C23	GGUGUAUGGCUUCAACCCUGA	UCAGGGUUGAAGCCAUACACC
C4	C24	GACCUGUCCAUUCAAAACUAC	GUAGUUUUGAAUGGACAGGUC
C5	C25	UACCGUGUAUGGAAACUGCUC	GAGCAGUUUCCAUACACGGUA
C6	C26	GCCCCAUCACUUUACAAGCCU	AGGCUUGUAAAGUGAUGGGGC
C7	C27	GAUUGAUUGACCUGUCCAUUC	GAAUGGACAGGUCAAUCAAUC
C8	C28	GAGGUGUAUGGCUUCAACCCU	AGGGUUGAAGCCAUACACCUC
C9	C29	UCUGUGGGAUUCCAUCUGCCA	UGGCAGAUGGAAUCCCACAGA
C10	C30	GUCAUCACACUGAAUACCAAU	AUUGGUAUUCAGUGUGAUGAC
C11	C31	GUGACAAAUAUGGGCAUCAUC	GAUGAUGCCCAUAUUUGUCAC
C12	C32	ACCUGUCCAUUCAAAACUACC	GGUAGUUUUGAAUGGACAGGU
C13	C33	CAGCACCUAGCUGGAAAGUUA	UAACUUUCCAGCUAGGUGCUG
C14	C34	CUCCAUGGAAUUUAAGUAUGA	UCAUACUUAAAUUCCAUGGAG
C15	C35	UUCCCUGAAGUUGAUGUGUUA	UAACACAUCAACUUCAGGGAA
C16	C36	GUCCAAUAAGAUCAAUAGCAA	UUGCUAUUGAUCUUAUUGGAC
C17	C37	AACUCUCAAACCCUAAGAUUA	UAAUCUUAGGGUUUGAGAGUU
C18	C38	UCGGAACAAUCCUCAGAGUUA	UAACUCUGAGGAUUGUUCCGA
C19	C39	AAGCAAGAACUUAAUGGAAAU	AUUUCCAUUAAGUUCUUGCUU
C20	C40	GGCCAUUAGGCAAAUUGAUGA	UCAUCAAUUUGCCUAAUGGCC

EXAMPLE 1

10

A pilot in vivo mouse experiment was performed to assess activity of GalNAc-conjugated Crook anti- mouse ApoB siRNA compared to control siRNA constructs. Conjugated (GalNAc) and **u**nconjugated (without GalNAc) versions of ApoB Crook siRNA (sequence C10 in Table 2; Covance) were administered to adult male wild-type (WT) C57BL/6 mice by sub-cutaneous
15 (SC) and intravenous (IV) routes, respectively described previously in Material & Methods section.

Blood plasma ApoB was measured by ELISA (described earlier) at time 0 (prior to administration of siRNA construct) and at 96 hours following siRNA construct administration,
20 as indicated in the four Treatment groups (5 mice per group) as detailed above under Dosing Details.

Plasma ApoB levels (micrograms/ml) from 5 mice in each treatment group, were used to calculate a mean ApoB value +/- standard error of the mean (SEM). Change in plasma ApoB level after 96 hours following SC administration of GalNAc-conjugated Crook siRNA was compared to levels in mice receiving either control (i) vehicle saline, or (ii) unconjugated siRNA with Crook. Statistical analysis was applied using the two-tailed paired T test algorithm.

With reference to FIG.1 (a), plasma ApoB levels (micrograms/ml) of mice 96 hours following treatment with GalNAc-conjugated ApoB Crook siRNA were compared with the control treatment group administered with saline. Statistical analysis was applied using the two-tailed paired T test algorithm. Results show a substantive reduction in mean plasma ApoB levels in mice treated with GalNAc-conjugated Crook siRNA, compared to control. However, it just fails significance (p= 0.11), most likely due to small sample size and variation in ApoB levels between control animals.

With reference to FIG.1 (b), plasma ApoB levels (micrograms/ml) measured 96 hours following administration of GalNAc-conjugated ApoB Crook siRNA were compared to the control group, treated with siRNA construct unconjugated (without GalNAc) ApoB Crook siRNA. Statistical analysis was applied using the two-tailed paired T test algorithm.
Results show a highly significant reduction in plasma ApoB levels in this GalNAc-conjugated Crook siRNA treatment group when compared to control unconjugated siRNA with Crook (P=0.00435832).

Importantly, the selected ApoB Crook siRNA sequence (C10 in Table 2; Covance) used in this pilot *in vivo* experiment was performed prior to the *in vitro* ApoB Crook siRNA screen. Our subsequent *in vitro* data (Example 2) shows that there are other siRNA sequences with greater ApoB mRNA knockdown (KD) efficiency (Table 3) eg. C23 gives 89% KD at 25nM when compared to C10 (74%).

EXAMPLE 2

With reference to FIG. 2 (a-d), an arrayed RNAi screen in HepG2 cells was performed to evaluate a custom library of 40 “crook” siRNAs targeting the human ApoB gene (Table 2). All siRNAs in this library possess a DNA extension (or “crook”) appended to the sense RNA strand (sense siRNA) or to the antisense RNA strand (antisense siRNA).

Prior to performing the screen, suitable conditions for HepG2 reverse transfection were identified. First, siRNAs targeting essential genes were used to evaluate a number of

transfection conditions before the selected condition was taken forward to knockdown expression of ApoB using an ON-TARGETplus siRNA and assess the molecular detection tools for analysis of ApoB gene and protein expression. Homogeneous Time-Resolved Fluorescence (HTRF) and Duplex Real-Time quantitative PCR (RT-qPCR) assays were
5 developed to quantify ApoB expression change at the protein and mRNA levels, respectively. In the Screening phase, the 40 custom crook siRNAs were assessed over a five-point dose range. 72h post transfection, ApoB expression was evaluated by Duplex RT-qPCR. With a few notable exceptions, the knockdown of ApoB expression was similar between the sense and antisense siRNAs sharing the same RNA sequence, when the data is normalised to its
10 relevant negative control (NEG sense for sense siRNAs and NEG antisense for the antisense siRNAs). However, it should be noted that a decrease in ApoB expression was observed for the NEG sense control and so the knockdown of ApoB for siRNAs C1-C20 may be underestimated.

15 HepG2 cells were reverse transfected with a library of 40 custom crook siRNAs (20 sense siRNAs and 20 antisense siRNAs) alongside the siRNA controls using conditions identified in the assay development phase. 72h post transfection, ApoB mRNA levels in transfected cells were quantified by duplex RT-qPCR, normalizing the ApoB mRNA levels to the levels of the housekeeping reference gene GAPDH mRNA (FIG. 2 (a-d)).

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As the assessment of the 40 custom crook siRNA molecules covered a number of assay plates, in order to be able to perform an assay QC step, each plate contained a number of controls. These included the ON-TARGETplus (OT+) siRNAs targeting ApoB and a matched non-targeting control assessed at 25 nM as well as the Negative controls for the sense and
25 antisense siRNAs (NEG sense and NEG antisense, respectively) and the Argonaute control ApoB siRNA (POS ApoB).

With reference to FIG. 2 (a-d), a dose dependent decrease in ApoB expression with increasing
30 siRNA concentration was observed for the majority of the siRNA tested, however the level of knockdown observed differed between the siRNAs, as anticipated. Knockdown of ApoB by the targeting siRNAs was greater than for the NEG controls for all siRNAs tested, with the exception of siRNAs C28 and C29, where limited knockdown was observed.

35 With reference to Table 3, when sense and antisense siRNAs targeting the same RNA sequence are compared the knockdown was similar. Four siRNA pairs appeared to show a differential in knockdown efficiency between the sense and antisense siRNA. These were C3-

C23, C8-C28, C9-C29, and C13-C33. For all of these, except C3-C23, the sense siRNA appeared to be more efficient than the antisense siRNA, however for C8-C28 and C9-C29, the antisense siRNA did not appear to knockdown ApoB expression.

5 Overall based on the ApoB mRNA level following treatment with 25 nM siRNA, the following siRNAs display the best knock-down efficiency: the sense crook siRNAs C3 and C13 and the antisense crook siRNAs C23, C24, C30 and C36. C13 and C23 are the only two siRNAs showing a knock-down efficiency greater than 85% at this dose; see Table 3.

10 **Table 3**. *In vitro* activity of ApoB Crook siRNAs (C1-C40) in HepG2 cells. Each siRNA is ranked according to ApoB mRNA knockdown (KD) performance, with highest KD at the top of the table.

siRNA	ApoB mRNA Knockdown		Crook position:	NM_000384
Construct Code	6.25nM	25nM	Sense (S) Anti-sense (A)	Start position
C23	0.18 (82%)	0.11 (89%)	A	423
C13	0.20 (80%)	0.15 (85%)	S	6964
C36	0.18 (82%)	0.20 (80%)	A	9006
C24	0.24 (76%)	0.20 (80%)	A	13693
C30	0.25 (75%)	0.20 (80%)	A	10168
C2	0.25 (75%)	0.22 (78%)	S	2835
C22	0.27 (73%)	0.22 (78%)	A	2835
C3	0.36 (64%)	0.22 (78%)	S	423
C26	0.31 (69%)	0.23 (77%)	A	1253
C15	0.29 (71%)	0.24 (76%)	S	11500
C14	0.24 (76%)	0.25 (75%)	S	10497
C17	0.29 (71%)	0.25 (75%)	S	8567
C35	0.26 (74%)	0.36 (64%)	A	11500
C10	0.32 (68%)	0.26 (74%)	S	10168
C8	0.32 (68%)	0.26 (74%)	S	430
C27	0.34 (66%)	0.26 (74%)	A	13685
C16	0.30 (70%)	0.28 (72%)	S	9006
C18	0.30 (70%)	0.28 (72%)	S	3347
C5	0.49 (51%)	0.28 (72%)	S	669
C7	0.52 (48%)	0.28 (72%)	S	13685
C9	0.54 (46%)	0.30 (70%)	S	4102
C4	0.26 (74%)	0.31 (69%)	S	13693
C25	0.40 (60%)	0.32 (68%)	A	669
C20	0.44 (56%)	0.33 (67%)	S	4102
C1	0.75 (25%)	0.32 (68%)	S	2093
C34	0.25 (75%)	0.33 (67%)	A	10497
C33	0.31 (69%)	0.33 (67%)	A	6964

C12	0.33 (67%)	0.33 (67%)	S	13694
C37	0.51 (49%)	0.36 (64%)	A	8567
C6	0.40 (60%)	0.37 (63%)	S	1253
C21	0.62 (38%)	0.36 (64%)	A	2093
C38	0.36 (64%)	0.39 (61%)	A	3347
C39	0.53 (47%)	0.41 (59%)	A	10450
C31	0.57 (43%)	0.43 (57%)	A	2778
C19	0.67 (33%)	0.42 (58%)	S	10450
C32	0.43 (57%)	0.48 (52%)	A	13694
C11	0.55 (45%)	0.52 (48%)	S	2778
C40	0.51 (49%)	0.58 (42%)	A	4102
C28	0.60 (40%)	0.63 (37%)	A	430
C29	1.25 (-25%)	0.69 (31%)	A	4102

References

Nair, J.K., Willoughby, J.L., Chan, A., Charisse, K., Alam, M.R., Wang, Q., Hoekstra, M., Kandasamy, P., Kel'in, A.V., Milstein, S. and Taneja, N., 2014. Multivalent N-
5 acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. *Journal of the American Chemical Society*, *136*(49), pp.16958-16961.

Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J. and John, M., 2004. Therapeutic silencing of an
10 endogenous gene by systemic administration of modified siRNAs. *Nature*, *432*(7014), p.173.

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CLAIMS:

1. A nucleic acid molecule comprising

a first part that comprises a double stranded inhibitory ribonucleic acid (RNA) molecule comprising a sense strand and an antisense strand; and

a second part that comprises a single stranded deoxyribonucleic acid (DNA) molecule, wherein the 5' end of said single stranded DNA molecule is covalently linked to the 3' end of the sense strand of the double stranded inhibitory RNA molecule or wherein the 5' end of the single stranded DNA molecule is covalently linked to the 3' of the antisense strand of the double stranded inhibitory RNA molecule, characterized in that the double stranded inhibitory RNA comprises a sense nucleotide sequence that encodes a part of the human apolipoprotein B protein and wherein said single stranded DNA molecule comprises a nucleotide sequence that is adapted over at least part of its length to anneal by complementary base pairing to a part of said single stranded DNA to form a double stranded DNA structure, wherein the double stranded inhibitory RNA consists of natural nucleotides and inhibits expression of apolipoprotein B.

2. A nucleic acid molecule comprising

a first part that comprises a double stranded inhibitory ribonucleic acid (RNA) molecule comprising a sense strand and an antisense strand; and

a second part that comprises a single stranded deoxyribonucleic acid (DNA) molecule, wherein the 5' end of said single stranded DNA molecule is covalently linked to the 3' end of the sense strand of the double stranded inhibitory RNA molecule or wherein the 5' end of the single stranded DNA molecule is covalently linked to the 3' of the antisense strand of the double stranded inhibitory RNA molecule, characterized in that the double stranded inhibitory RNA comprises a sense nucleotide sequence that encodes a part of the human apolipoprotein B protein, or polymorphic sequence variant thereof, and wherein said single stranded DNA molecule comprises a nucleotide sequence that is adapted over at least part of its length to anneal by complementary base pairing to a part of said single stranded DNA to form a double stranded DNA structure wherein the double stranded inhibitory RNA consists of natural nucleotides and inhibits expression of apolipoprotein B.

3. The nucleic acid molecule according to claim 1 or 2 wherein the 5' end of said single stranded DNA molecule is covalently linked to the 3' end of the sense strand of the double stranded inhibitory RNA molecule.

4. The nucleic acid molecule according to claim 1 or 2 wherein the 5' end of said single stranded DNA molecule is covalently linked to the 3' end of the antisense strand of the double stranded inhibitory RNA molecule.

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5. The nucleic acid molecule according to any one of claims 1 to 4 wherein said single stranded DNA molecule comprises the nucleotide sequence TCACCTCATCCCGCGAAGC (SEQ ID NO: 1).

6. The nucleic acid molecule according to any one of claims 1 to 5 wherein said double stranded inhibitory RNA molecule is between 18 and 29 nucleotides in length.

7. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence selected from the group consisting of SEQ ID NOs: 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56 and 57.

8. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises an antisense nucleotide sequence selected from the group consisting of SEQ ID NOs: 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94,95, 96, 97, 98, 98, 99, 100, 101, 102, 103. 104, 105, 106, 107, 108, 109 and 110.

9. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence selected from the group consisting of SEQ ID NOs: 111, 113, 115, 117, 119, 121, 123 and 125.

10. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises an antisense nucleotide sequence selected from the group consisting of SEQ ID NOs: 112, 114, 116, 118, 120, 122, 124 and 126.

11. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence selected from the group consisting of: SEQ ID NO: 7, SEQ ID NO: 36, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115 and SEQ ID NO: 119.

12. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises an antisense nucleotide sequence selected from the group consisting of: SEQ ID NO: 60, SEQ ID NO: 72, SEQ ID NO: 89, SEQ ID NO: 100, SEQ ID NO: 108, SEQ ID NO: 114 and SEQ ID NO: 118.

13. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 7 and an antisense nucleotide sequence set forth in SEQ ID NO:60.

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14. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 111 and an antisense nucleotide sequence set forth in SEQ ID NO:112.

15. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 117 and an antisense nucleotide sequence set forth in SEQ ID NO:118.

16. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 55 and an antisense nucleotide sequence set forth in SEQ ID NO:108.

17. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 47 and an antisense nucleotide sequence set forth in SEQ ID NO:100.

18. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 36 and an antisense nucleotide sequence set forth in SEQ ID NO:89.

19. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 19 and an antisense nucleotide sequence set forth in SEQ ID NO:72.

20. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 115 and an antisense nucleotide sequence set forth in SEQ ID NO:116.

21. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 113 and an antisense nucleotide sequence set forth in SEQ ID NO: 114.

22. The nucleic acid molecule according to any one of claims 1 to 6 wherein said double stranded inhibitory RNA molecule comprises a sense nucleotide sequence set forth in SEQ ID NO: 119 and an antisense nucleotide sequence set forth in SEQ ID NO: 120.

23. The nucleic acid molecule according to any one of claims 1 to 22 wherein said nucleic acid molecule is covalently linked to *N*-acetylgalactosamine.

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24. The nucleic acid molecule according to claim 23 wherein said N-acetylgalactosamine is linked to the second part of said nucleic acid molecule.

25. The nucleic acid molecule according to claim 24 wherein said N-acetylgalactosamine is linked to the 3' end of the second part of said nucleic acid molecule.

26. A pharmaceutical composition comprising at least one nucleic acid molecule according to any one of claims 1 to 25 and further comprising a pharmaceutical carrier and/or excipient.

27. The nucleic acid molecule according to any one of claims 1 to 25 or the pharmaceutical composition according to claim 26 for use in treating a subject that has or is predisposed to hypercholesterolemia.

28. The nucleic acid or pharmaceutical composition for use according to claim 27, wherein the treating comprises treatment or prevention of a disease associated with hypercholesterolemia.

29. The nucleic acid or pharmaceutical composition for use according to claim 28 wherein said disease associated with hypercholesterolemia is selected from the group consisting of: familial hypercholesterolemia, stroke prevention, hyperlipidaemia, cardiovascular disease, atherosclerosis, coronary heart disease, aortic stenosis, cerebrovascular disease, peripheral arterial disease, hypertension, metabolic syndrome, type II diabetes, non-alcoholic fatty acid liver disease, non-alcoholic steatohepatitis, Buerger's disease, renal artery stenosis, hyperapobetalipoproteinemia, cerebrovascular atherosclerosis, cerebrovascular disease and venous thrombosis.

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FIGURE 1 a

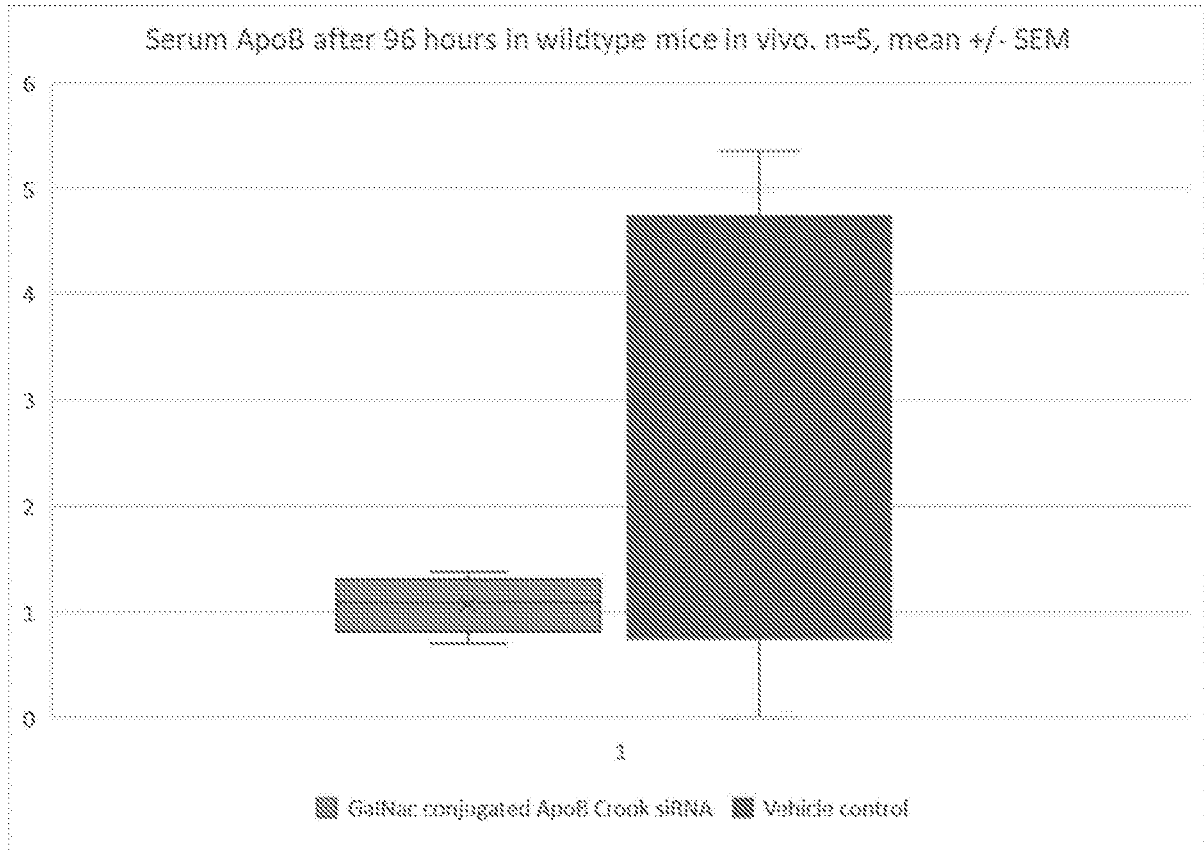

Figure 1 b

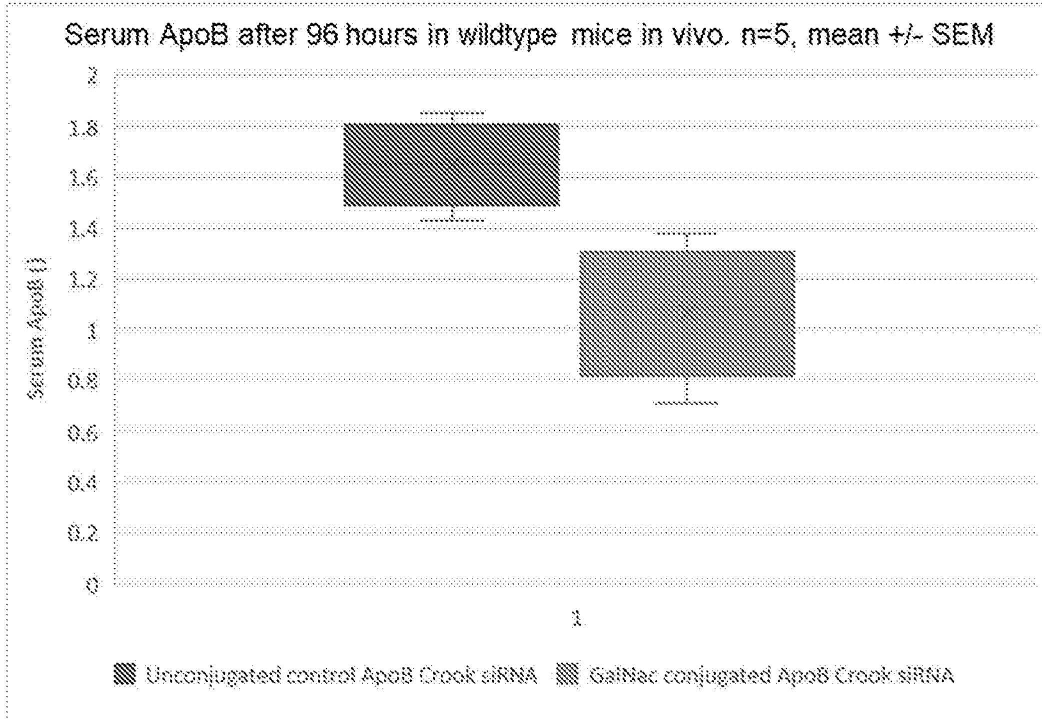

Figure 2a

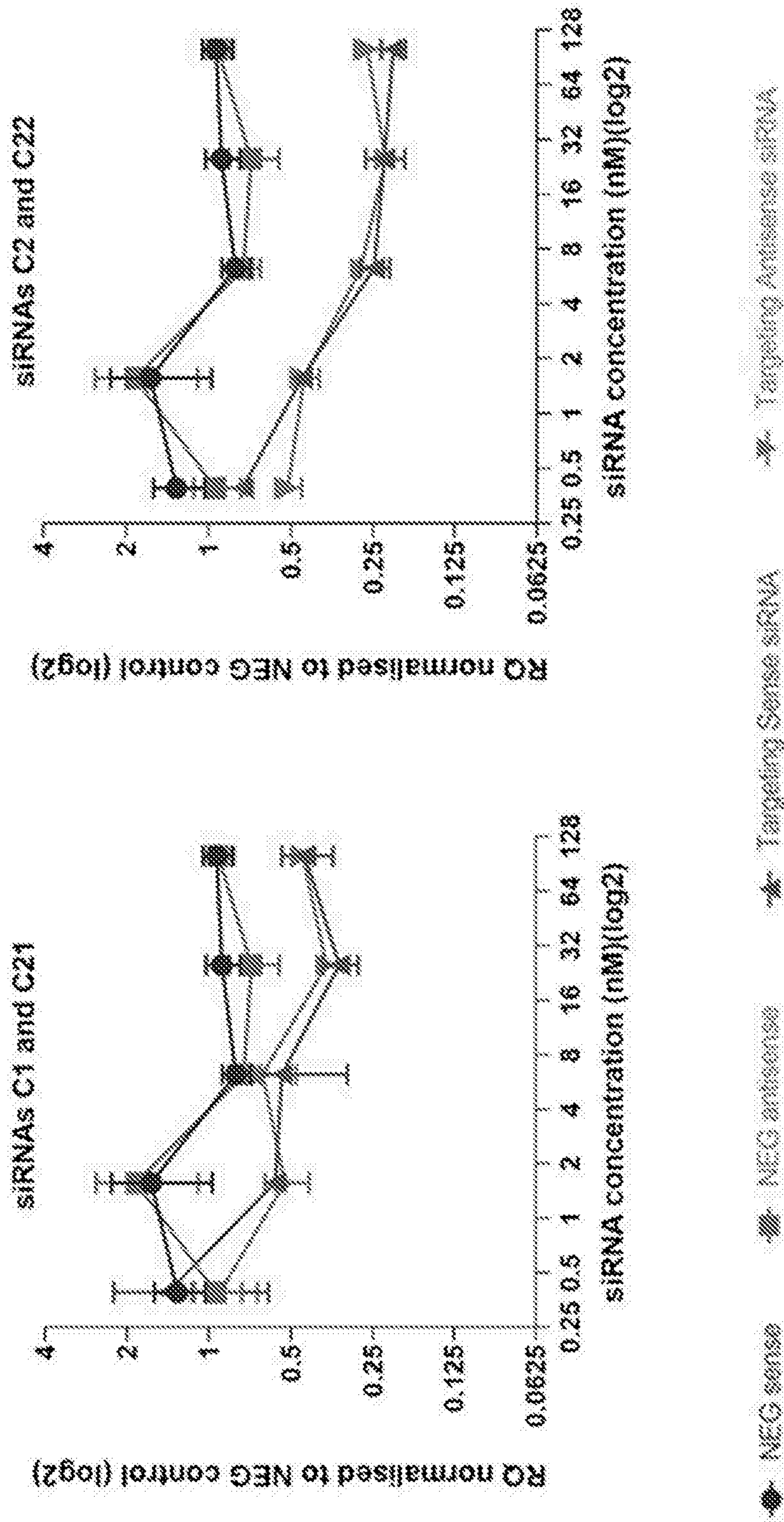

Figure 2a continued

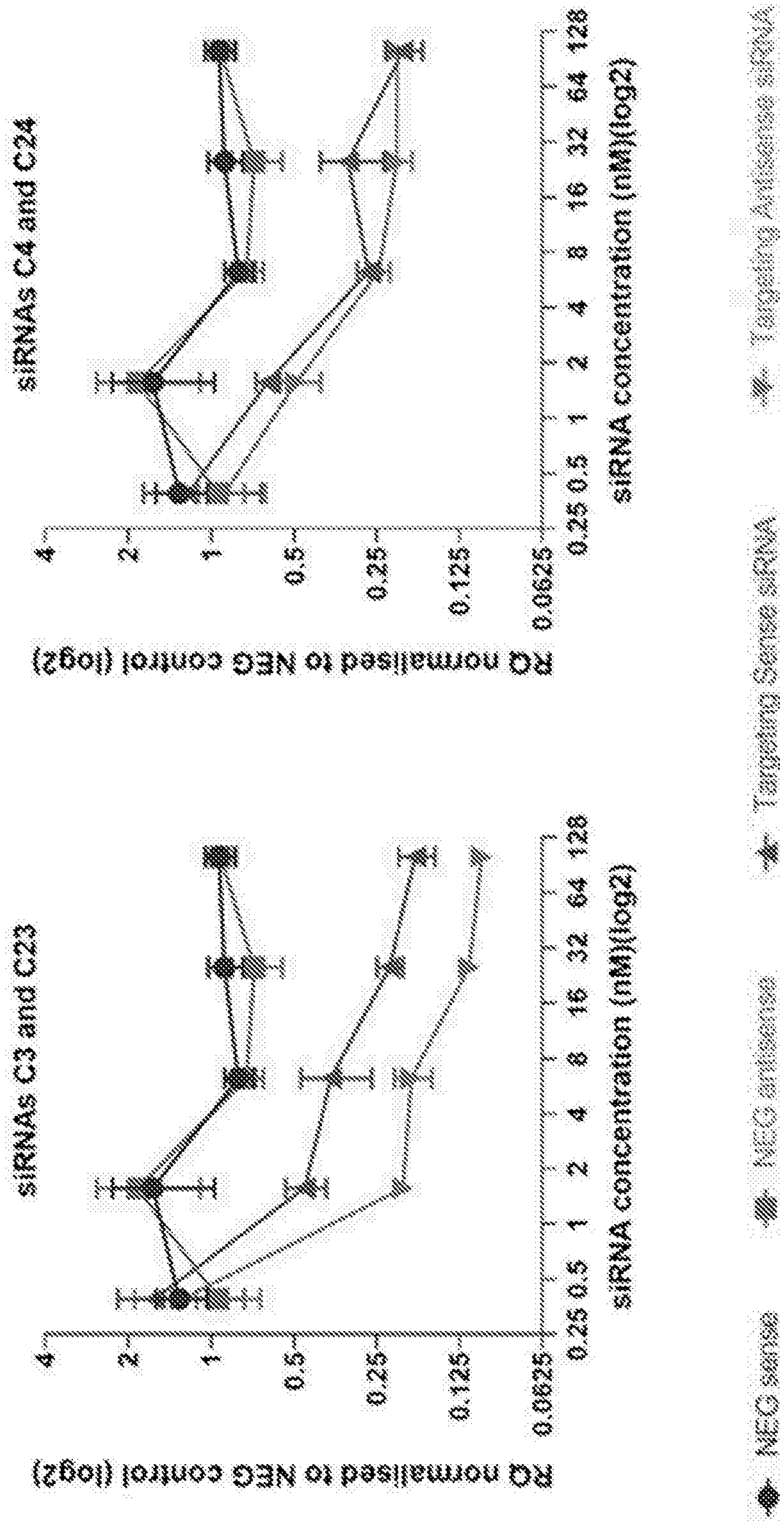

Figure 2 a continued

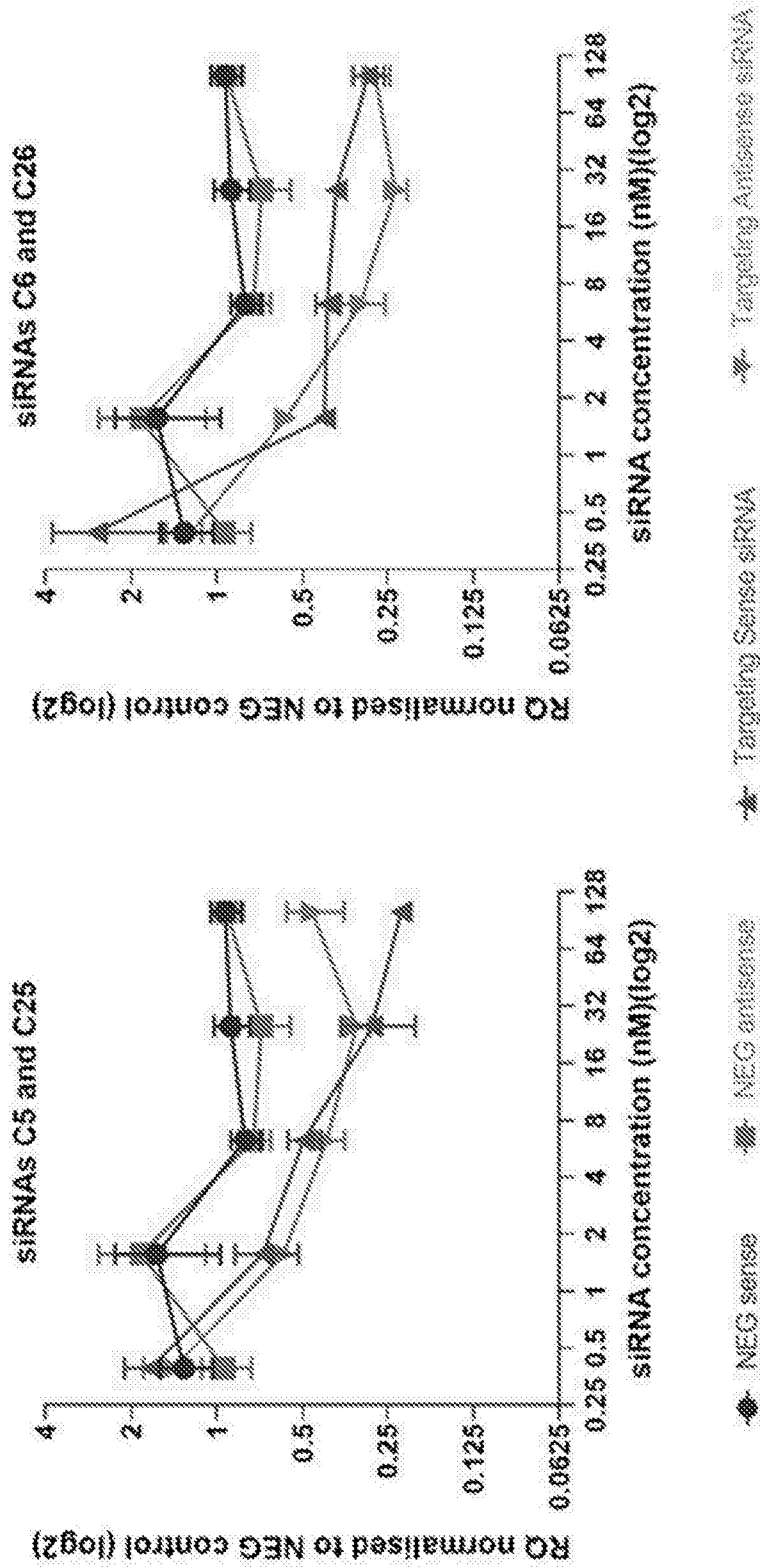

Figure 2b

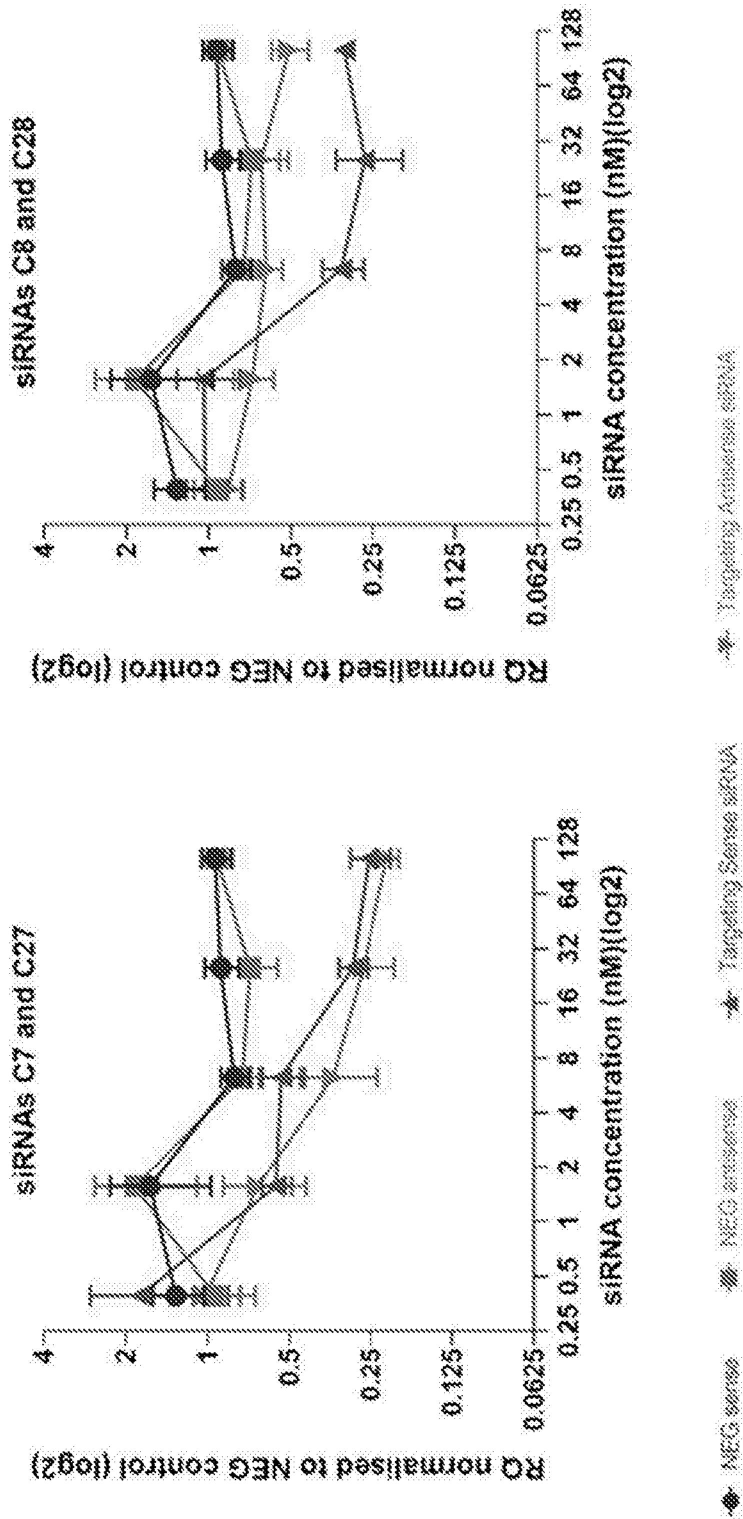

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Figure 2b

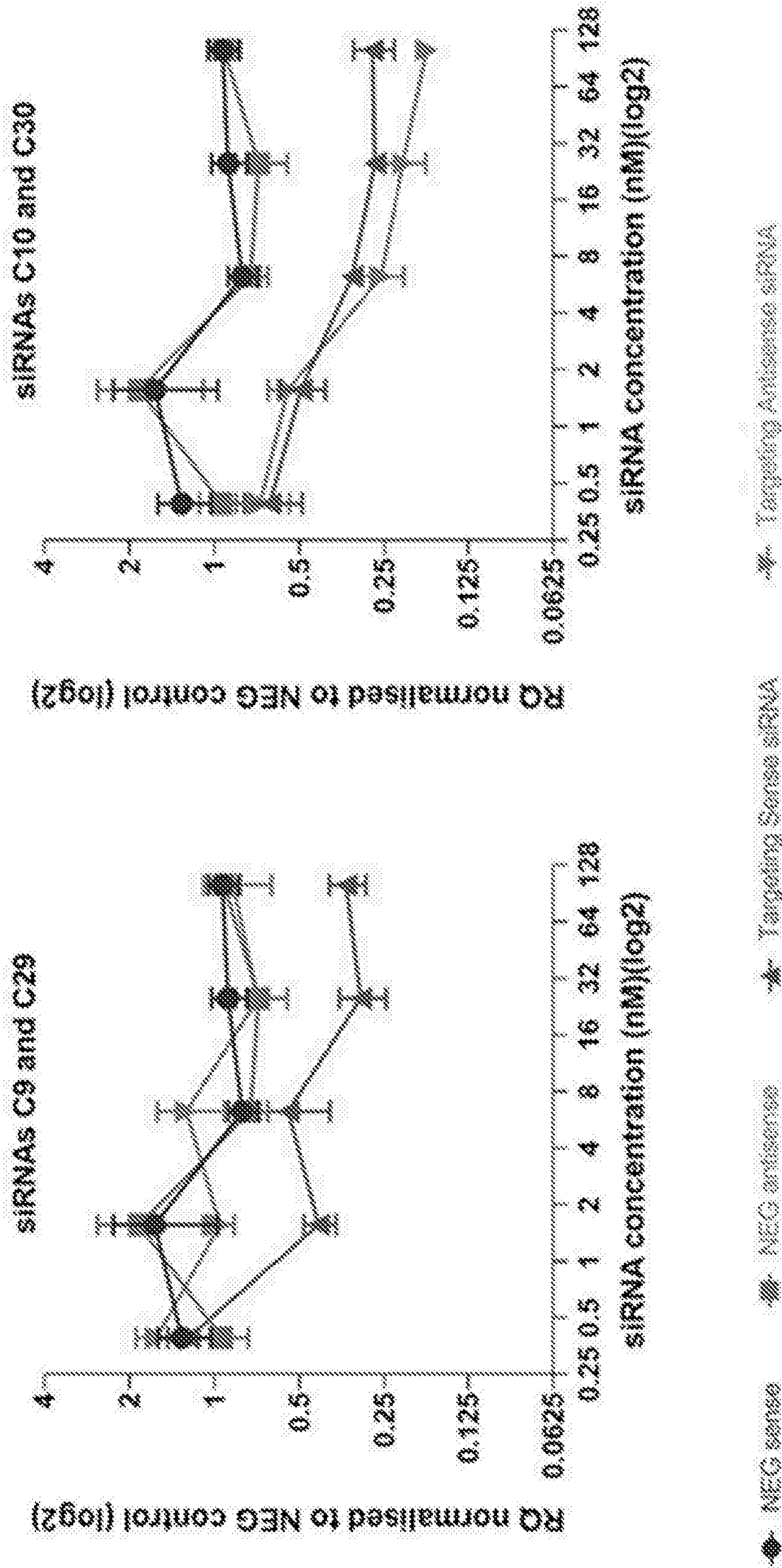

Figure 2b

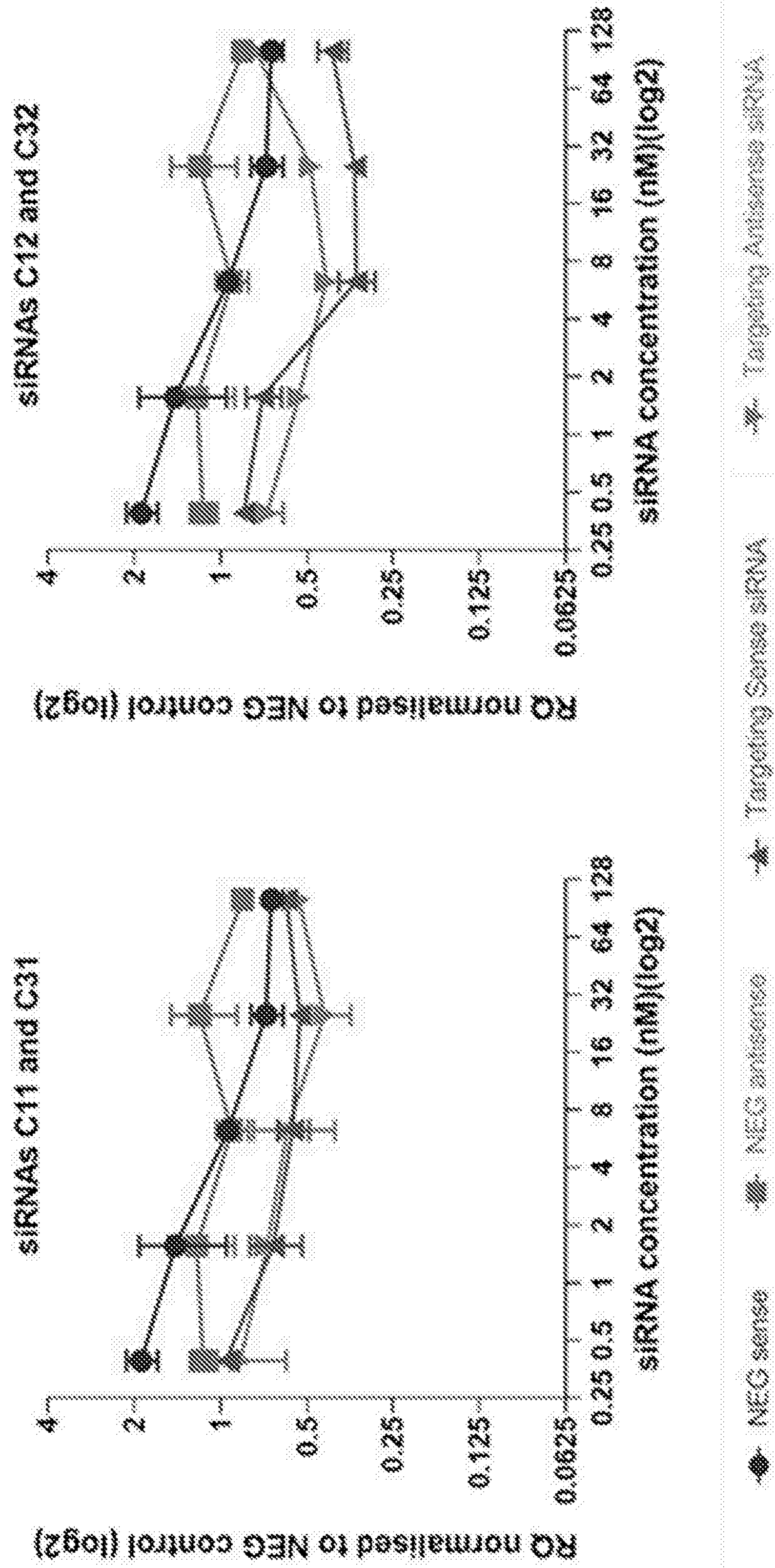

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Figure 2c

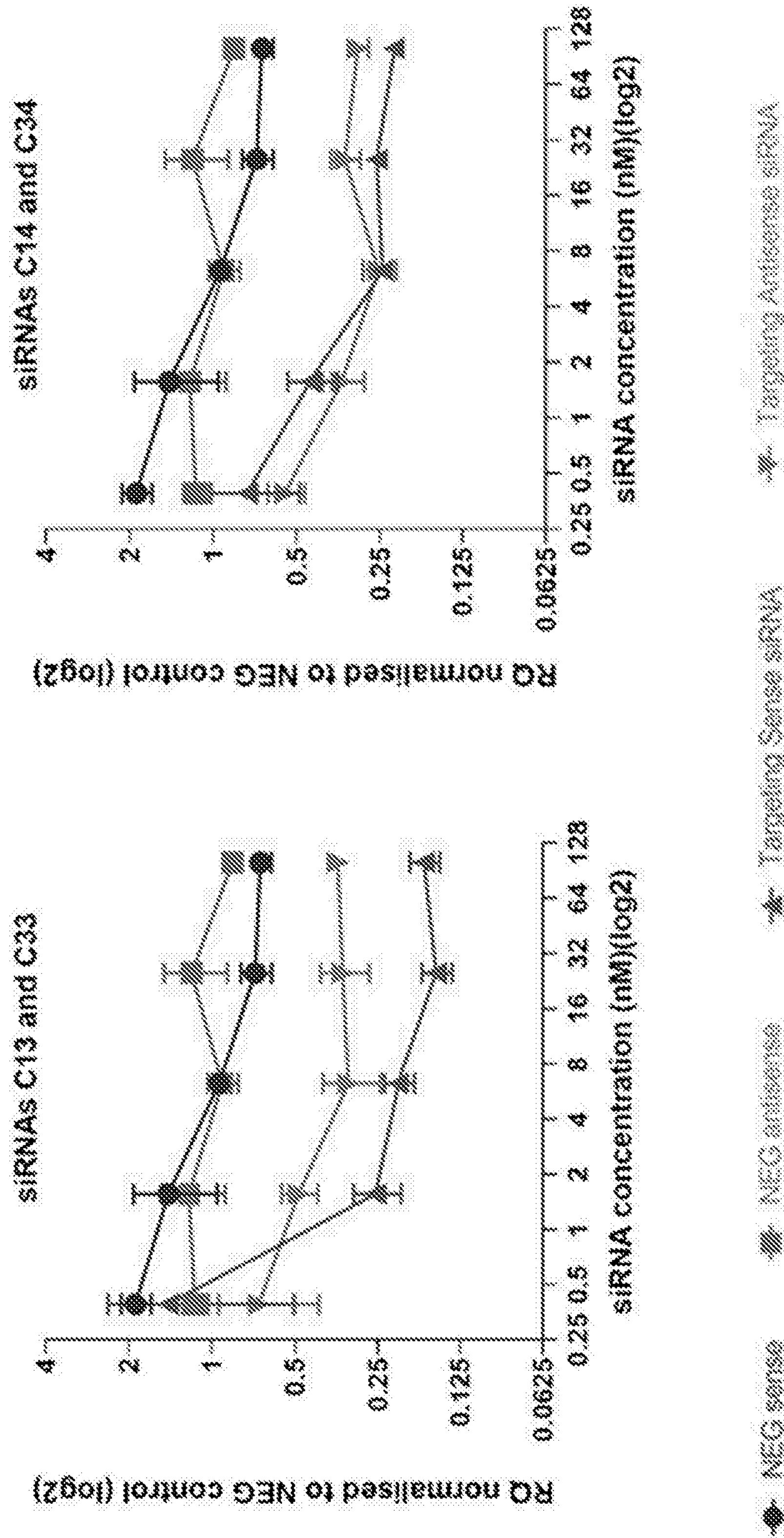


Figure 2c continued

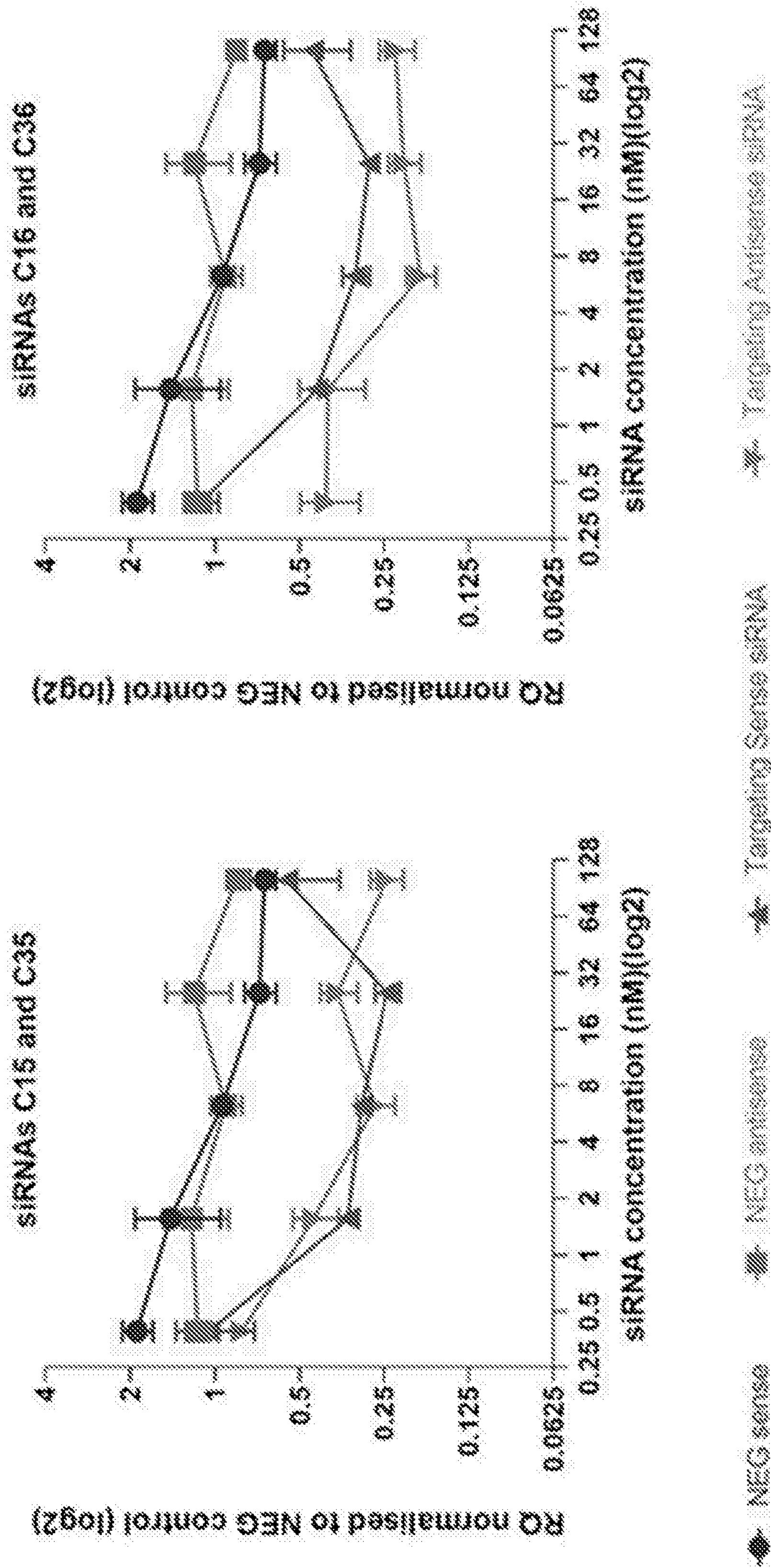


Figure 2c continued

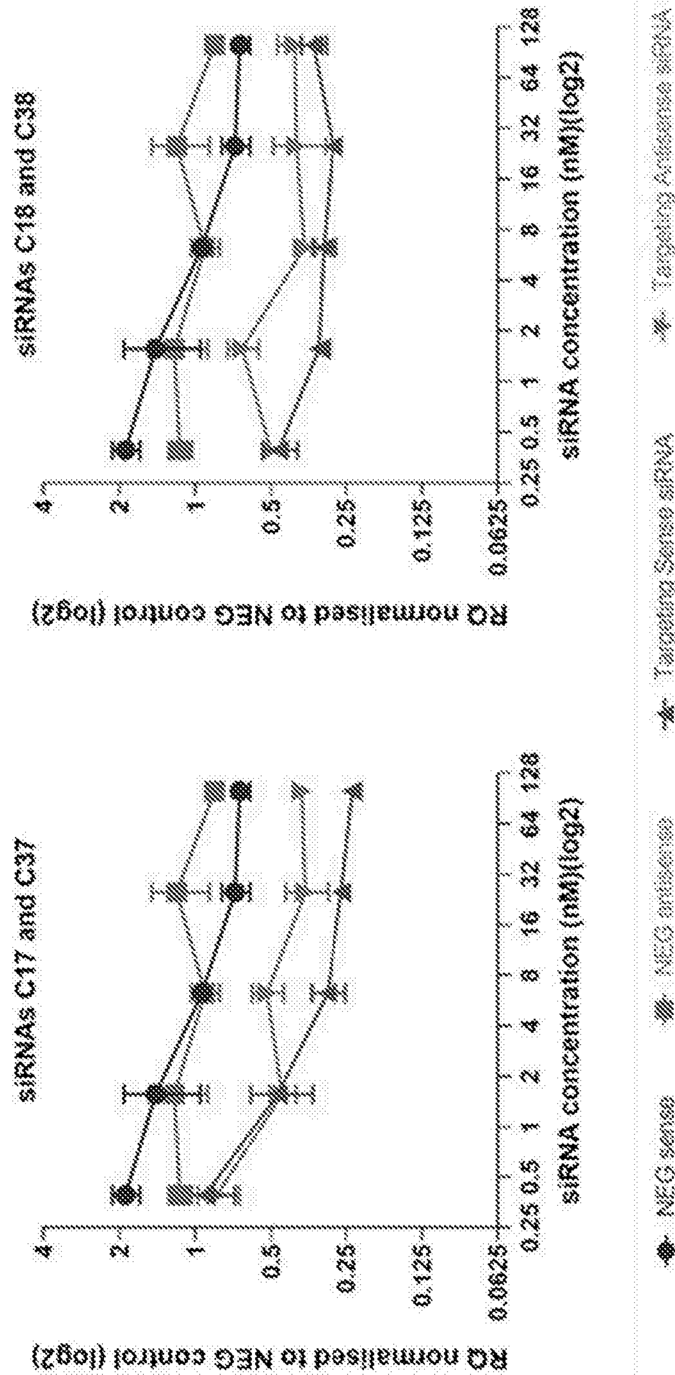
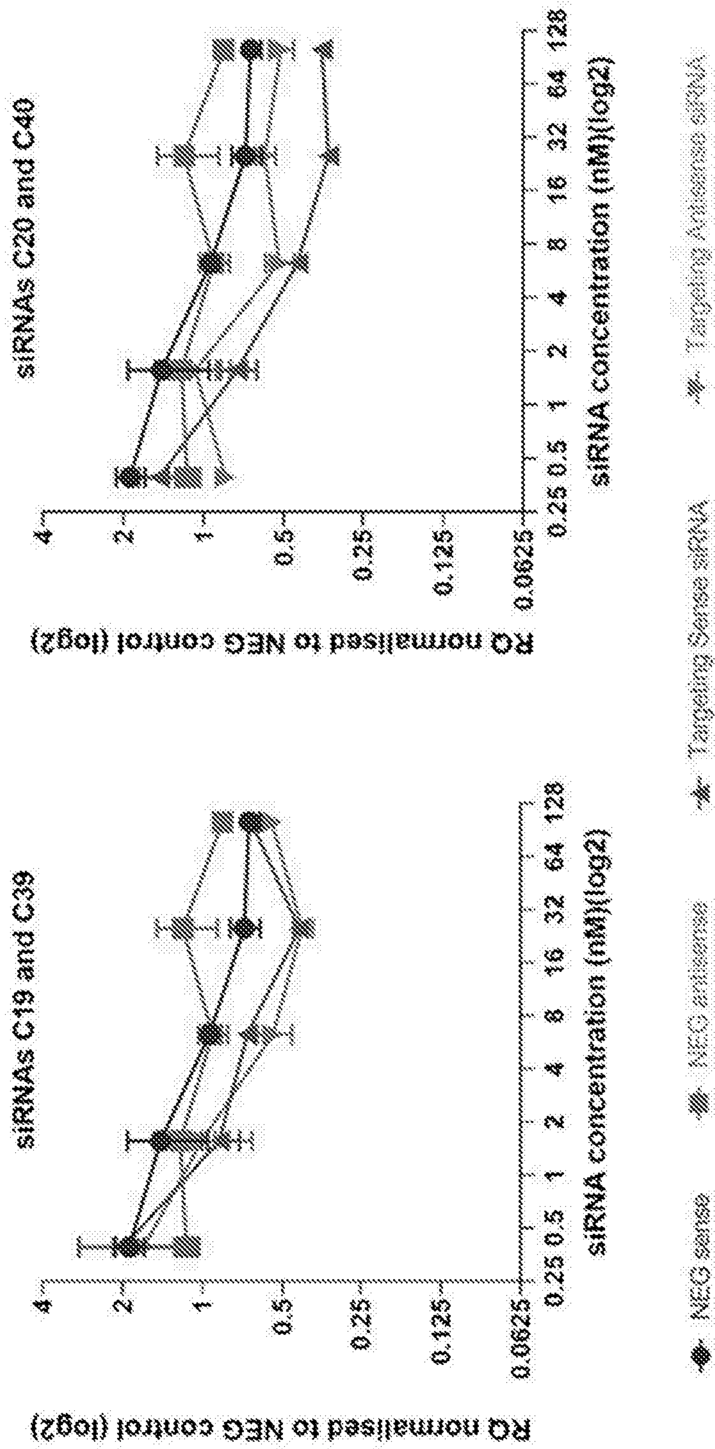
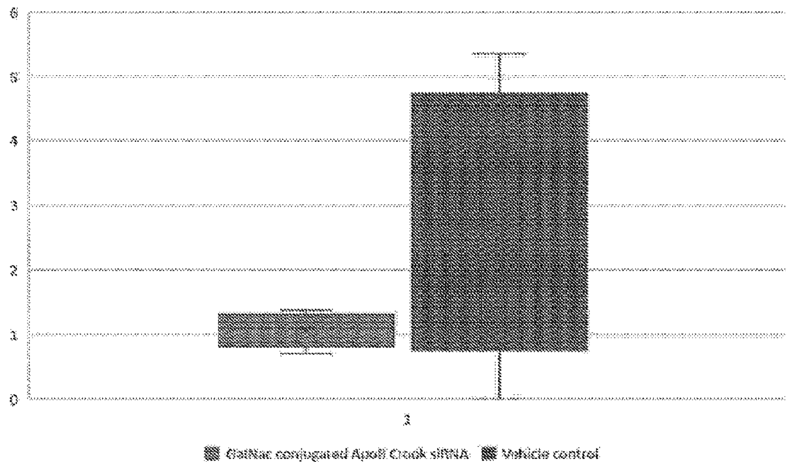


Figure 2d




Serum ApoB after 96 hours in wildtype mice in vivo. n=5, mean +/- SEM
6
5
4
3
2
1
0
1
GalNac conjugated ApoB Crook siRNA
Vehicle control