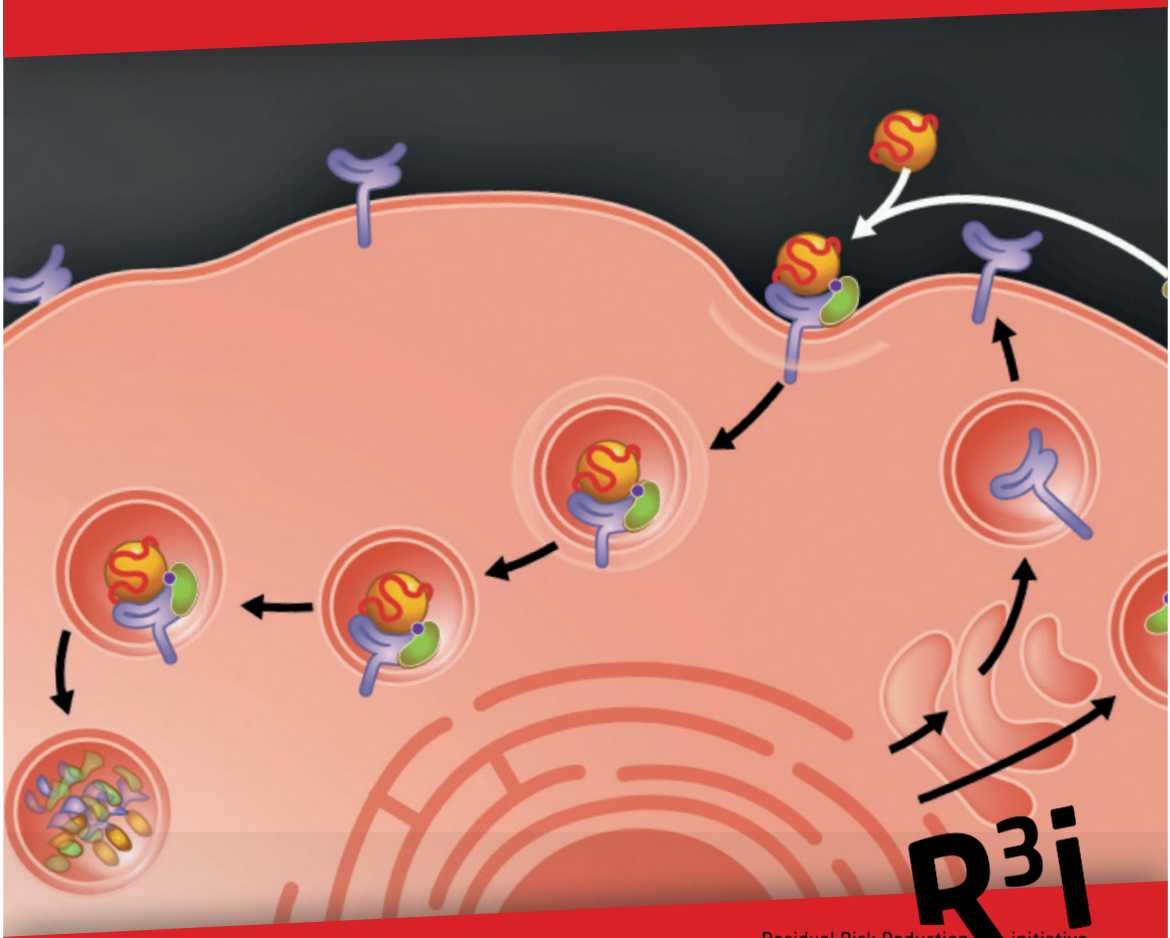
PCSK9 and Atherosclerosis

J Davignon, J-C Fruchart, M Hermans
R³i Foundation



Residual Risk Reduction initiative

Preface

Nabil G. Seidah* and Michel Chrétien**

*Laboratories of *Biochemical Neuroendocrinology and **Functional Endoproteolysis, Clinical Research Institute of Montreal (IRCM) (affiliated to the University of Montreal). 110 Pine Ave West, Montreal, QC, H2W 1R7, Canada.*

Cardiovascular disease (CVD) is the leading cause of death and disability worldwide, with fatalities from CVD projected to exceed 24 million by the year 2030, according to the World Health Organization. Elevated cholesterol levels fuel the atherosclerotic disease process that leads to CVD and premature death. Autosomal dominant hypercholesterolemia (ADH), which affects 1 in 500 subjects worldwide, was known to be caused by heterozygous dominant mutations in either the low-density lipoprotein-receptor (*LDL-R*) or apolipoprotein-B (*APOB*) genes. In 2003, the discovery of PCSK9 – an enzyme encoded by a third gene involved in ADH – revealed an unsuspected regulation of the LDL-R, with major implications for CVD. PCSK9 binds to the LDL-R and directs it to the lysosomal pathway for degradation. This is one of the most exciting developments in CVD research in the last decade.

PCSK9 belongs to a family of serine proteases called the proprotein convertases (PCs), first discovered in 1990. These proteases (encoded by genes designated *PCSK1 to PCSK9*) are related to bacterial subtilisin and yeast kexin,[1] and serve to cleave and activate precursors of hormones, neuropeptides, growth factors, receptors, surface glycoproteins and transcription factors. As expected from the pro-hormone theory, PCs play major roles in many metabolic processes, including glucose homeostasis, diabetes, obesity and, surprisingly, lipid biosynthesis. In 2003 we successfully identified and characterized PCSK9, the last member of the PC-family.[2] Because of its high expression in the liver and intestine and its chromosomal localization (1p32), coinciding with a locus linked to hypercholesterolemia, a relationship between mutations in *PCSK9* and hypercholesterolemia was rapidly established in French families.[3] Gain-of-function (GOF) mutations in *PCSK9* were shown to deplete the LDL-R, with ensuing increased LDL-cholesterol (LDL-C) levels in plasma. In 2005, *PCSK9* loss-of-function (LOF) mutations were shown to result in hypocholesterolemia.[4] Low concentrations of active PCSK9 are also associated with a lower incidence of atherosclerosis, myocardial infarction and stroke. Two women entirely lacking functional PCSK9 are healthy, and have remarkably low levels of LDL-C (~0.4 mM), indicating that PCSK9 inhibition would be safe.

This fundamental knowledge went from bed to bench and back to bedside in less than 9 years, and now pharmaceutical companies are racing to develop PCSK9 inhibitors that may in the future substitute for, or complement, the use of statins. Accordingly, monoclonal antibodies directed against PCSK9 that disrupt the PCSK9≡LDL-R interaction are efficient in reducing LDL-C levels (by more than 50%) and are now in phase-III clinical trials.[5] However, there are still huge gaps in our knowledge and understanding of PCSK9 biology. Furthermore, it is impossible to separate CVD from metabolic diseases when it comes to risk assessment, prevention, and clinical intervention. To address these issues, the present monograph, assembled by world-renowned lipidologists Jean Davignon and colleagues, is not only timely but will surely foster many discussions, hypotheses and collaborations. This book will be a model for all researchers, clinicians, clinician scientists and medical students interested in understanding the fundamentals of dyslipidemia and its associated CVD, and will be essential for those interested in the projected future wave of novel clinical tools and drugs designed to manage these devastating diseases.

References

(1) Seidah NG, Chretien M. Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. *Brain Res* 1999;848:45-62.

(2) Seidah NG, Benjannet S, Wickham L, *et al.* The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. *Proc Natl Acad Sci U.S.A.* 2003;100:928-33.

(3) Abifadel M, Varret M, Rabes JP, *et al.* Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet 2003;34:154-6.

(4) Cohen J, Pertsemlidis A, Kotowski IK, *et al.* Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nat Genet* 2005;37:161-5.

(5) Seidah NG. Proprotein Convertase Subtilisin Kexin 9 (PCSK9) inhibitors in the treatment of hypercholesterolemia and other pathologies. *Curr Pharm Des* 2013;19:3161-72.

Contents

Acknowledgements

The authors would like to thank Pascale Lefèvre and Claire Savourat for creating and illustrating the slides, GK PharmaComm for preparing the slide notes, designing the booklet and managing the project, and Lise St-Germain (Institut de Recherches Cliniques de Montréal) for her contribution in the creation of new slides and the tracking of interactions across the Atlantic.

Cardiovascular diseases (CVDs)

SECTION

1

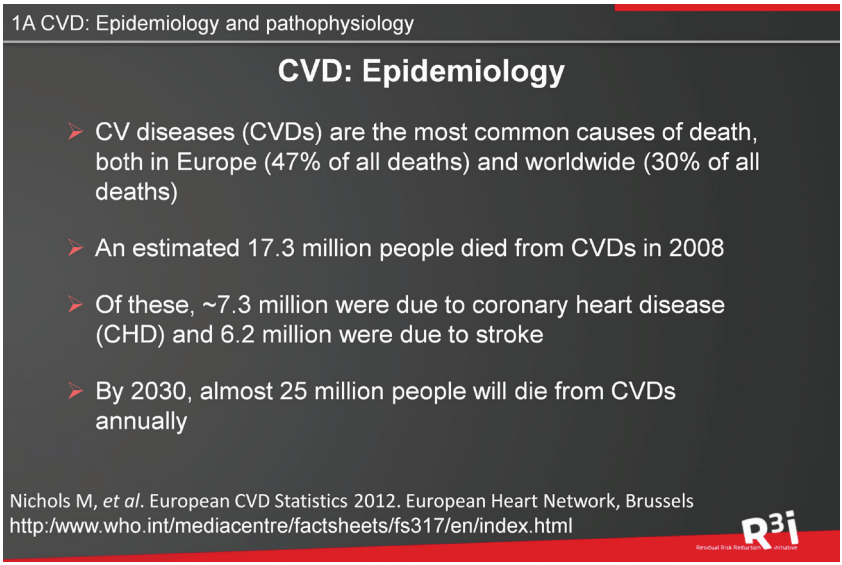
Residual Risk Reduction initiative

CVD: Epidemiology and pathophysiology

SECTION

1A

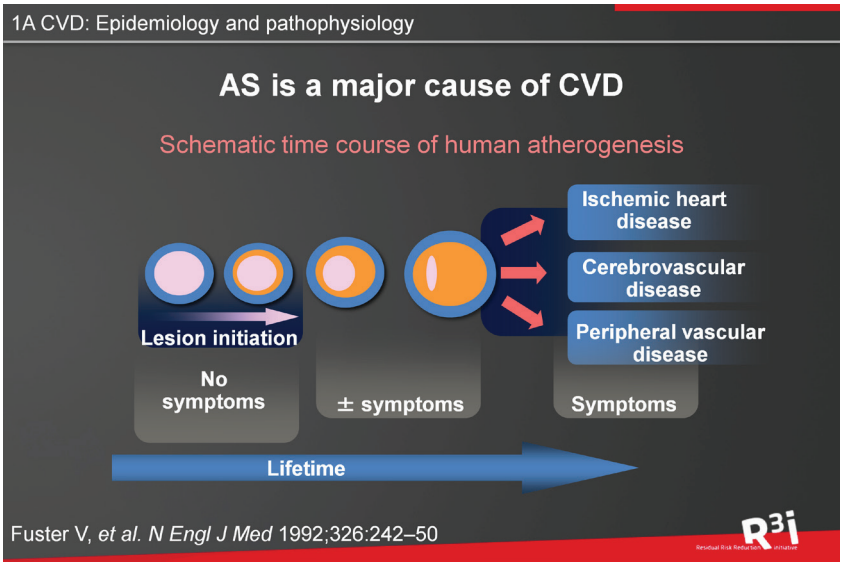
Residual Risk Reduction initiative



CVD: Epidemiology

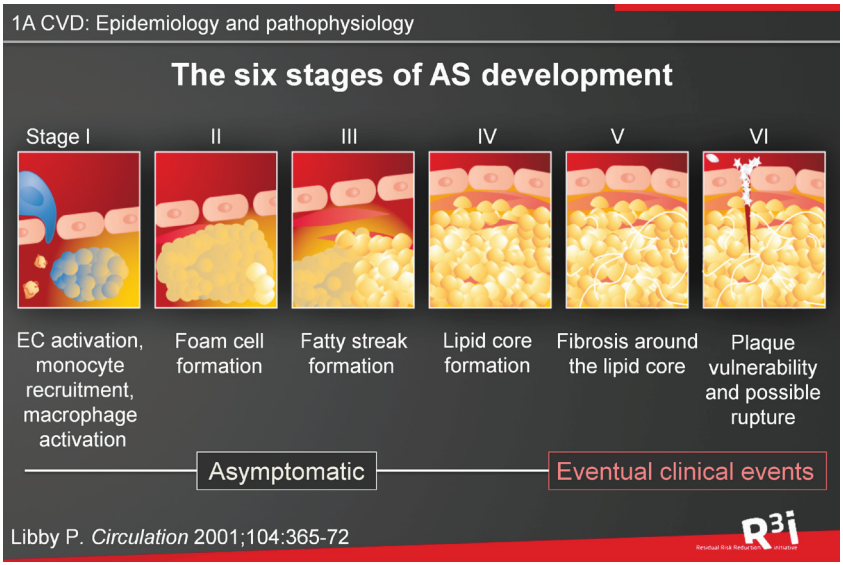
- CVDs are the most common causes of death, both in Europe and worldwide
- An estimated 17.3 million people died from CVDs in 2008 (30% of all deaths, worldwide)
- Of these, ~7.3 million were due to coronary heart disease (CHD) and 6.2 million were due to stroke
- By 2030, almost 25 million people per year will die from CVDs

1A CVD: Epidemiology and pathophysiology



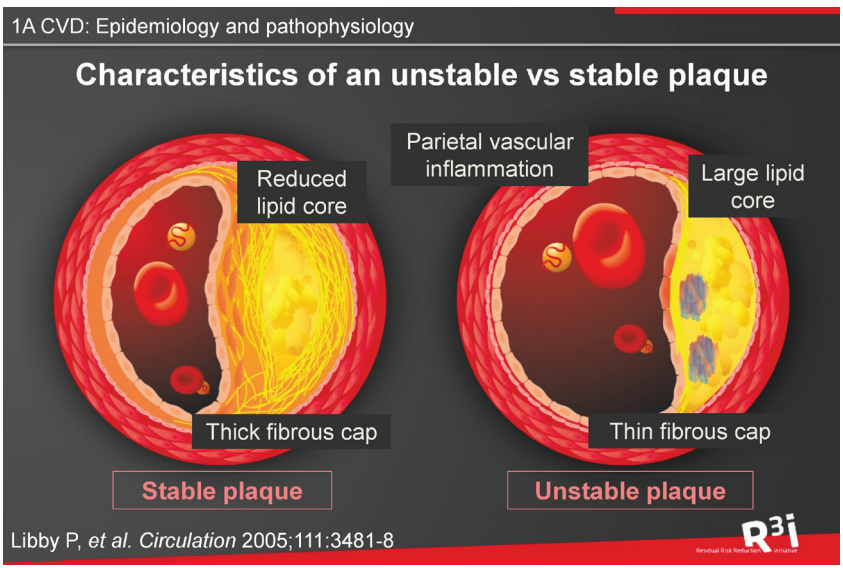
AS is a major cause of CVD

- AS (fatty plaque formation in the intima of an artery followed by thickening and stiffening of the artery wall) is an extensive, progressive, unpredictable and deadly disease affecting the coronary, cerebral and peripheral circulation
- It develops from an early age and may be asymptomatic prior to a first CV event



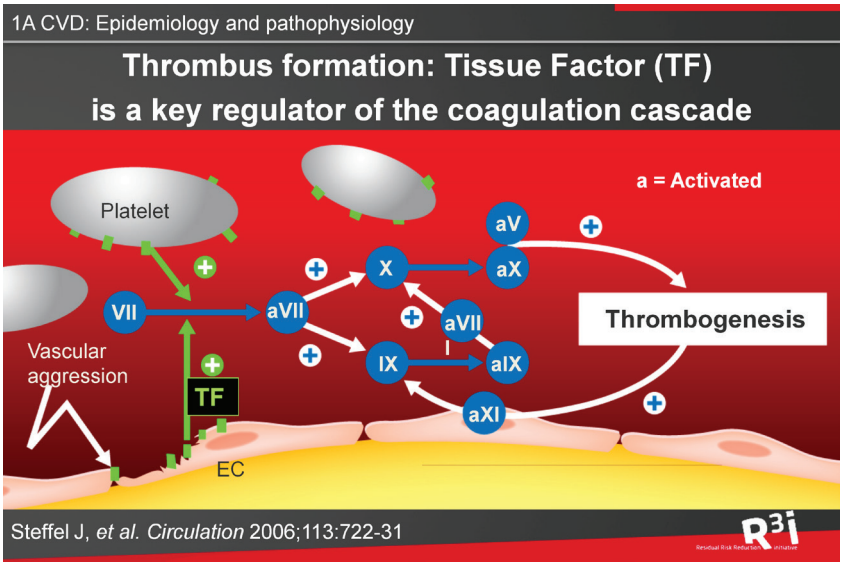
The six stages of AS development

- Stage I: Vascular injury leads to endothelial cell (EC) activation, monocyte recruitment into the intima and macrophage activation
- Stage II: The earliest atherosclerotic lesion—the fatty streak—is an almost purely inflammatory lesion consisting of monocyte-derived, lipid-laden macrophages (foam cells) and T-lymphocytes
- Stage III– IV: The progressive accumulation of lipids (intracellular and then extracellular) forms the lipid core
- Stage V: A fibrous cap develops around the lipid core forming an atherosclerotic plaque
- Stage VI: Activated macrophages secrete enzymes that weaken the fibrous cap leading to plaque rupture, hemorrhage or thrombosis and ischemic attacks/ACS



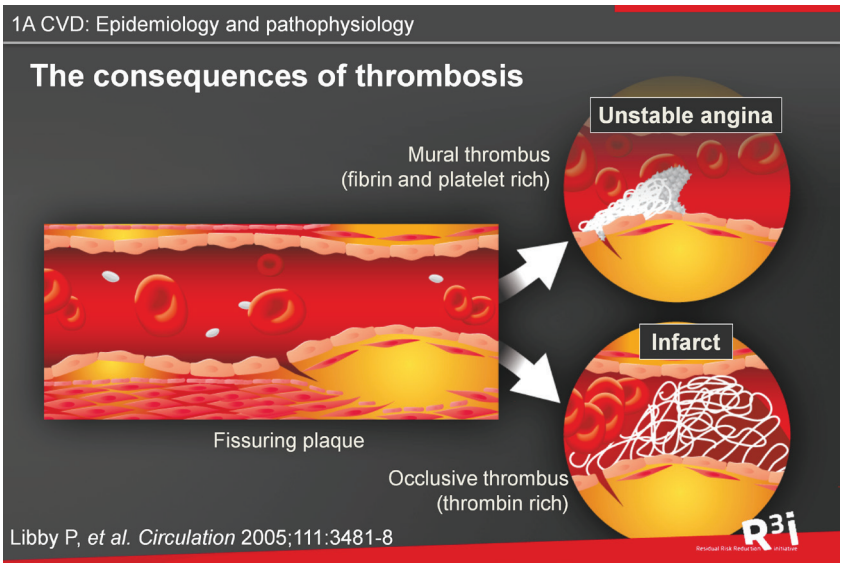
Characteristics of an unstable vs stable plaque

- Stable plaques have a thick fibrous cap and a reduced lipid core
- Unstable plaques have a thin fibrous cap, a large lipid core and inflammation of the vascular wall



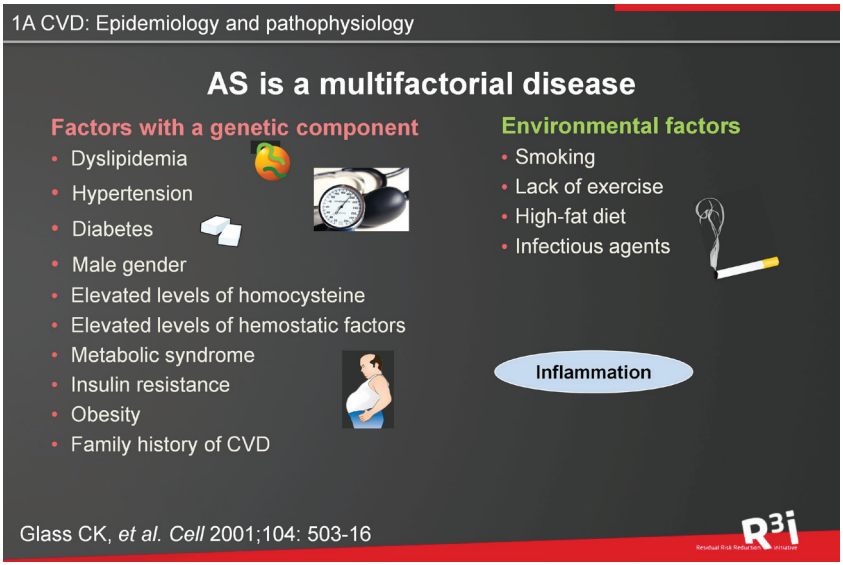
Thrombus formation: Tissue factor (TF) is a key regulator of the coagulation cascade

- Plaque rupture exposes TF to the blood within the arterial lumen, allowing it to form a complex with coagulation factors VII/VIIa
- This process initiates the coagulation cascade that leads to thrombogenesis



The consequences of thrombosis

- Disrupted plaques can lead either to mural thrombosis (the progressive narrowing of the lumen resulting in the partial disruption of blood flow) or occlusive thrombosis (a thrombus that blocks the entire lumen, thereby preventing blood flow)
  - Mural thrombosis causes ischemic symptoms, such as unstable angina
  - Occlusive thrombosis leads to acute coronary events, such as MI and stroke



AS is a multifactorial disease

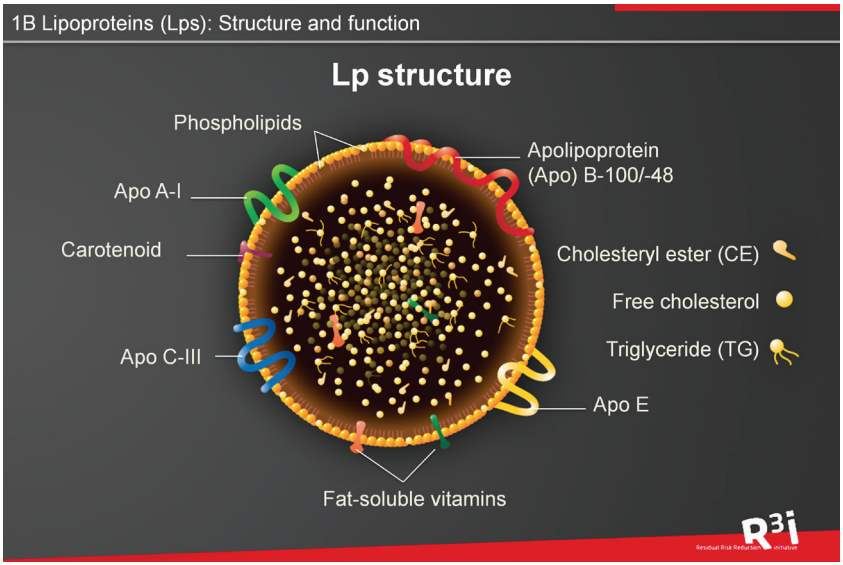
- AS is associated with a number of genetically- and environmentally-determined risk factors
- Many of these risk factors can cause vascular inflammation, a major component of AS

Lipoproteins (Lps): Structure and function

SECTION

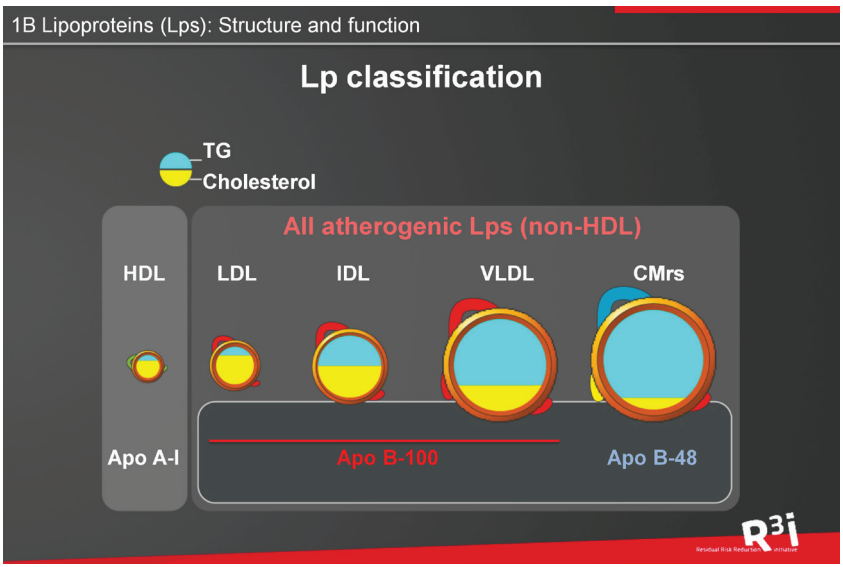
1B

Residual Risk Reduction initiative



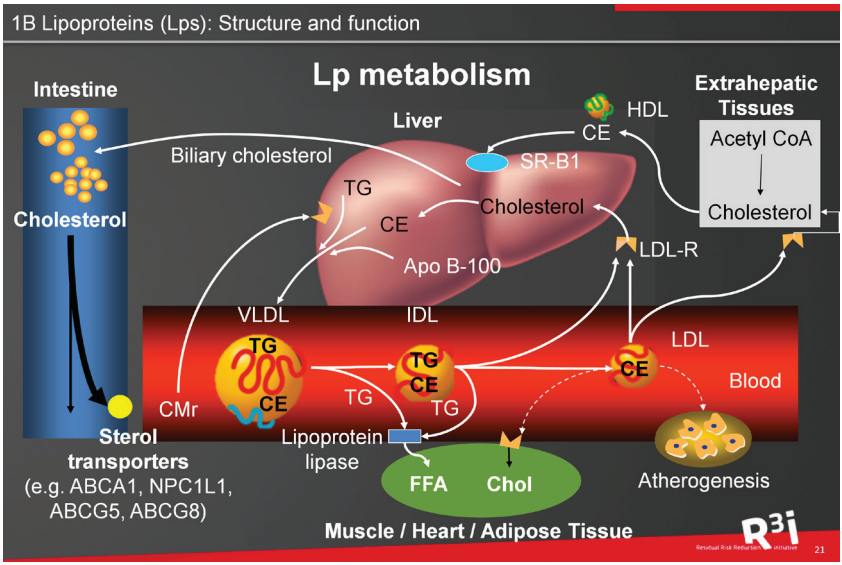
Lp structure

- Lps transport lipids (endogenous and dietary) and fat-soluble vitamins (e.g. vitamin E, carotenoids etc.) around the body in blood
- They comprise a core of hydrophobic lipids (cholesteryl esters [CEs] and triglycerides [TGs]) surrounded by a 'shell' of amphipathic lipids (phospholipids and free cholesterol)
- Lps are stabilized by apolipoproteins (predominantly Apo A-I, B-100/-48, Apo C-III and/or Apo E), which span the region between the central core and the outer envelope



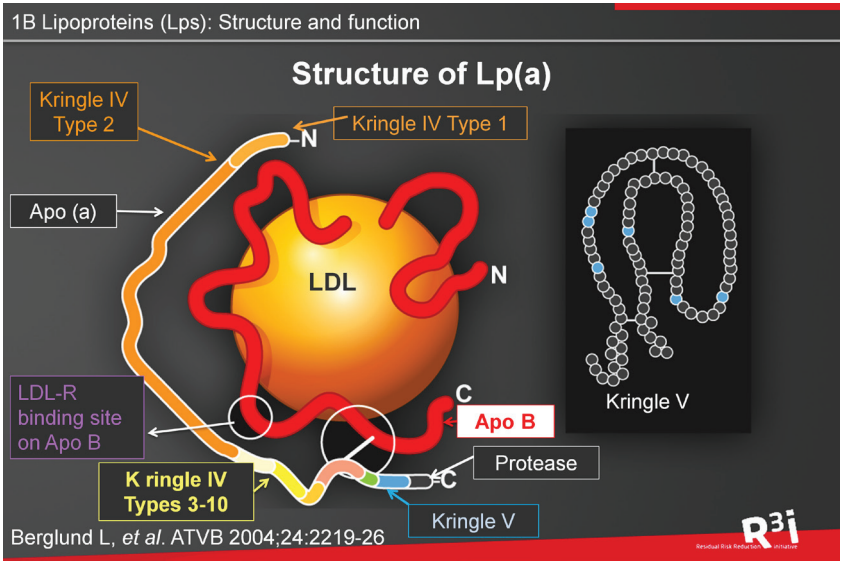
Lp classification

- There are five major classes of Lp, varying in size, density, lipid and Apo composition
- The atherogenic Apo B-containing Lps are low-density lipoproteins (LDLs), intermediate-density lipoproteins (IDLs), very-low density lipoproteins (VLDLs) and chylomicron remnants (CMrs)
- The anti-atherogenic Apo A-I-containing Lps are high-density lipoproteins (HDLs)



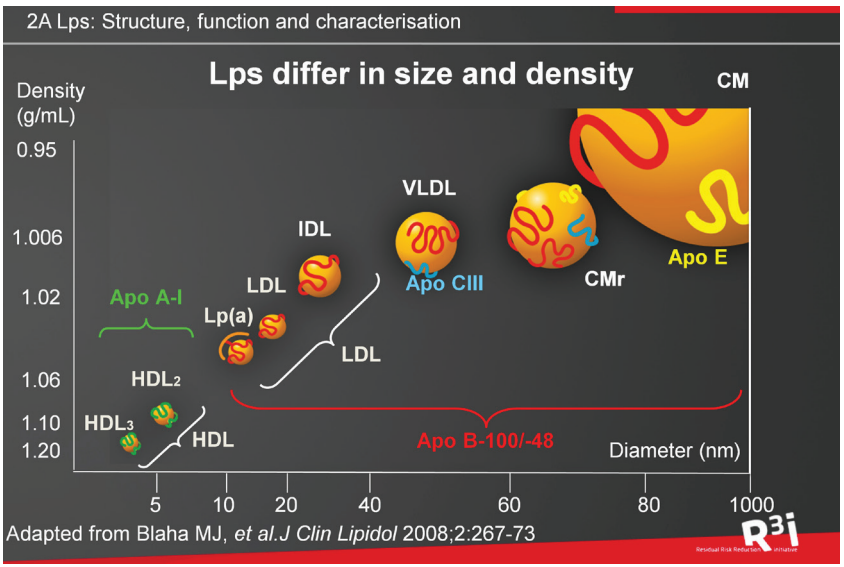
Lp metabolism

- Sterol transporters transfer sterols and bile acids between the intestinal wall lumen and the blood
- Chylomicrons (CMs) carry lipids from the intestine to the tissues; the TG component is hydrolyzed to form CMr, which are taken up by the liver and converted into VLDL
- VLDLs incorporate cholesterol in the liver and are secreted into the circulation, where they are catabolized to free fatty acids (FFAs), glycerol and VLDL remnants/IDL. FFAs are taken up by the surrounding tissues for energy/storage; IDL is either taken up by the liver or is further catabolized to form LDL
- LDLs are taken up via LDL-Rs in the liver, adrenal and peripheral cells
- HDLs facilitate cholesterol homeostasis in peripheral cells and transport excess cholesterol from extra-hepatic tissues to the liver for excretion into the bile (reverse cholesterol transport)



Structure of Lp(a)

- A highly atherogenic Lp comprising an LDL-like lipid core of CEs and TGs surrounded by a surface layer of phospholipid and free cholesterol
- Contains one molecule of Apo B, which is linked to Apo (a) via a single disulfide bond
- The Apo (a) moiety consists of a single copy of kringles IV, types 1 and 3 to 10, kringle V, and a protease domain analogous to plasminogen. In addition, it contains multiple copies of kringle IV, type 2
- Lp(a) inhibits fibrinolysis, promotes cholesterol accumulation, foam-cell development, and the propagation of AS plaques



Lps differ in size and density

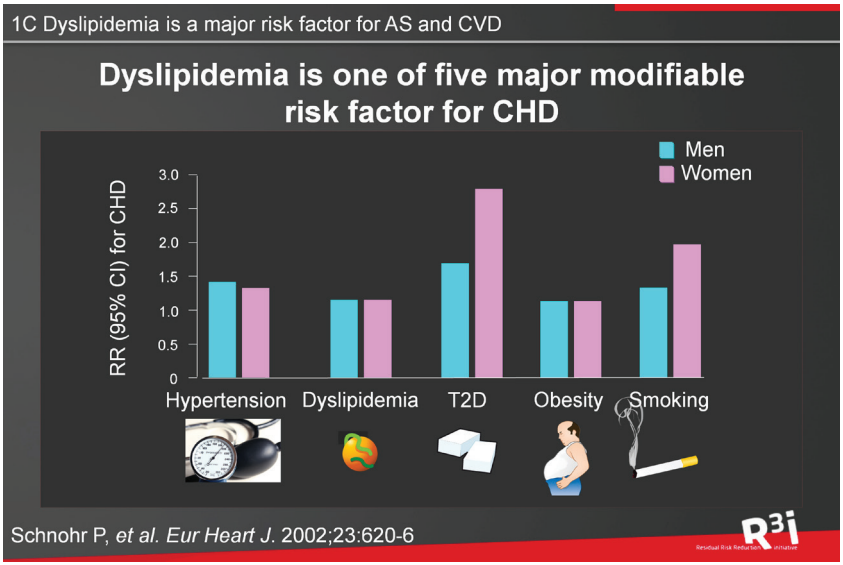
- As the Apo B-containing Lps are metabolized, their TG content decreases and their cholesterol and phospholipid content increases, causing them to become smaller and more dense
- Lp particles can therefore be separated by buoyancy

Dyslipidemia is a major risk factor for AS and CVD

SECTION

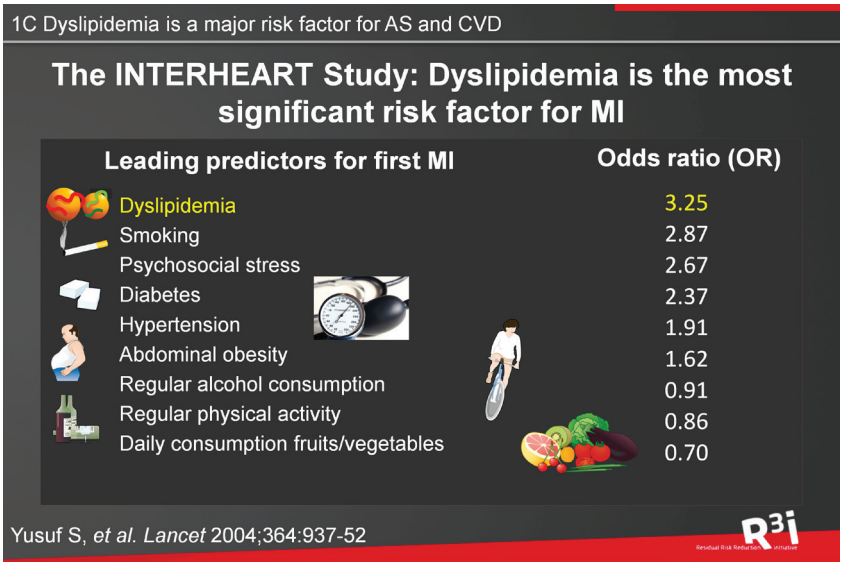
1C

Residual Risk Reduction initiative



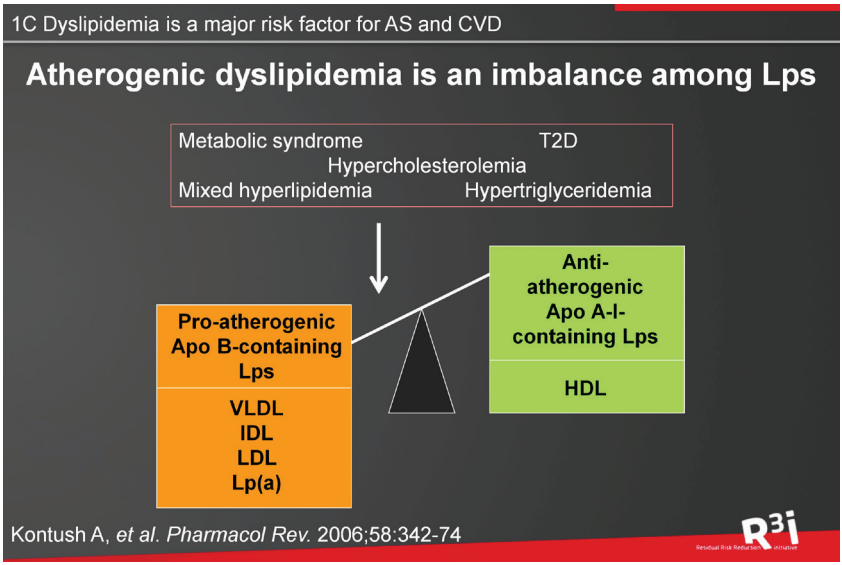
Dyslipidemia is one of five major modifiable risk factors for CHD

- Other risk factors include hypertension, Type 2 diabetes (T2D), obesity and smoking



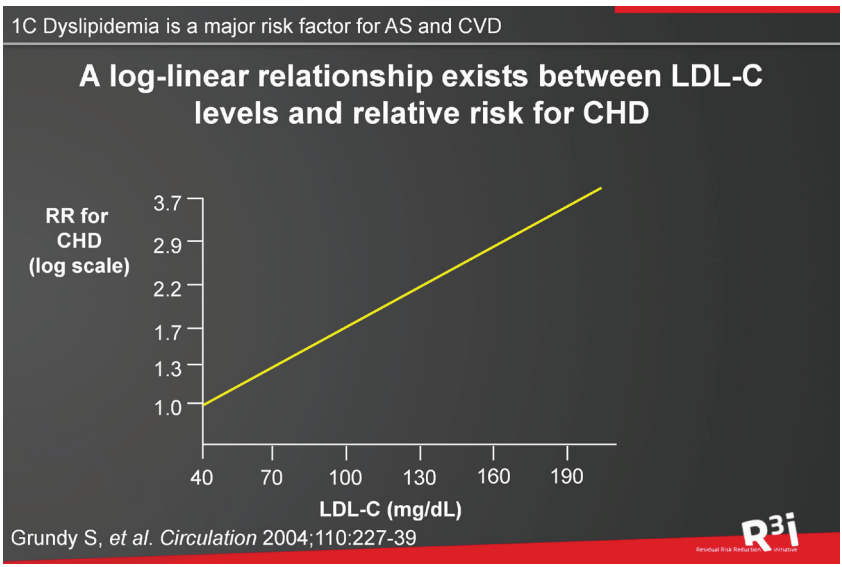
The INTERHEART Study: Dyslipidemia is the most significant risk factor for MI

- The multinational INTERHEART study showed that the 9 risk factors listed above accounted for >90% of the population-attributable risk
- Of these, dyslipidemia (defined as an elevated Apo B: Apo A-I ratio) was the most significant risk factor for MI, accounting for 50% of the population-attributable risk



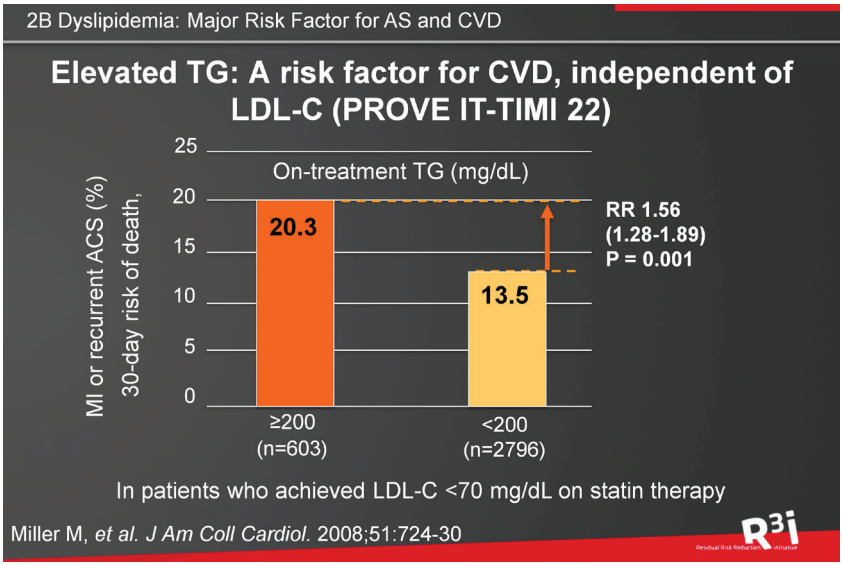
Atherogenic dyslipidemia is an imbalance among Lps

- Atherogenic dyslipidemia is defined as an imbalance between pro-atherogenic Apo B-containing Lps and anti-atherogenic Apo A-I-containing Lps contributing to the development of AS



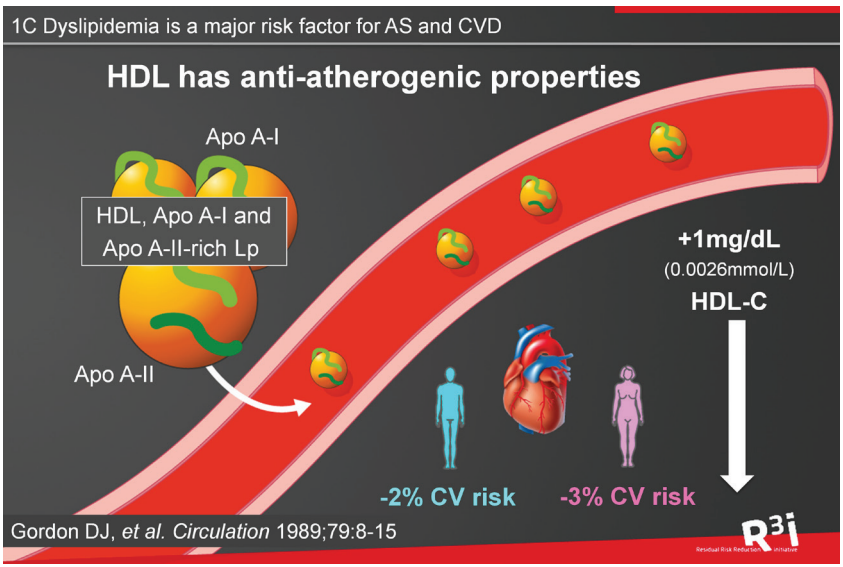
A log-linear relationship exists between LDL-C levels and relative risk for CHD

- There is a strong, continuous relationship between levels of LDL-C and CHD risk



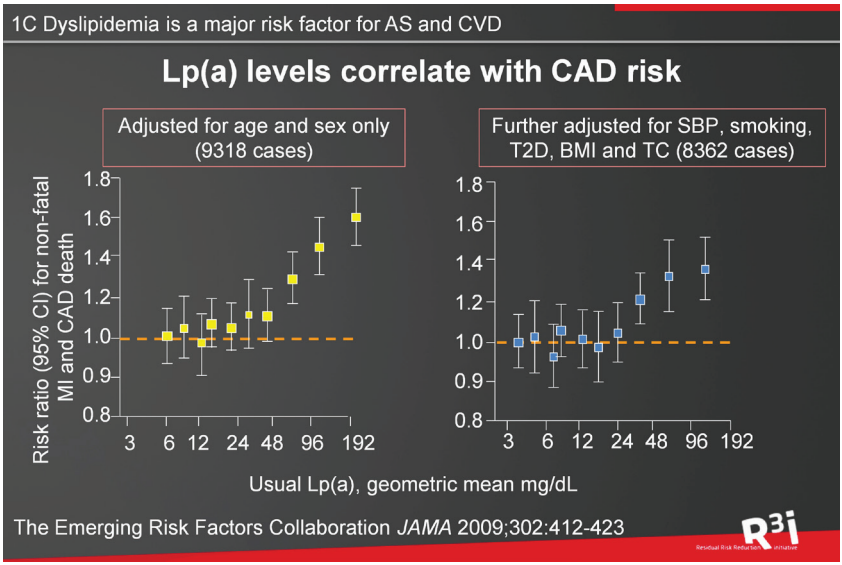
Elevated TG: A risk factor for CVD, independent of LDL-C

- The PROVE IT-TIMI (Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis In Myocardial Infarction) 22 trial showed a 56% increase in the risk of death, MI or acute coronary syndrome (ACS) among men and women hospitalized for ACS with on-treatment TG levels ≥ vs. <200 mg/dL and 'normal' levels of LDL-C



HDL has anti-atherogenic properties

- HDL is central to the 'reverse cholesterol transport' pathway, a process that facilitates cholesterol homeostasis in peripheral cells and transports excess cholesterol from extra-hepatic tissues to the liver for excretion into the bile
- For each 1 mg/dL increase in HDL-C, CV risk decreases by 2% in men and by 3% in women



Lp(a) levels correlate with CAD risk

- A meta-analysis of 126,634 subjects in 36 prospective studies showed that Lp(a) concentrations correlated with coronary artery disease (CAD) risk when adjusted for age and sex only
- These associations were only slightly reduced after adjustment for other established risk factors suggesting that Lp(a) is an independent risk factor for CAD

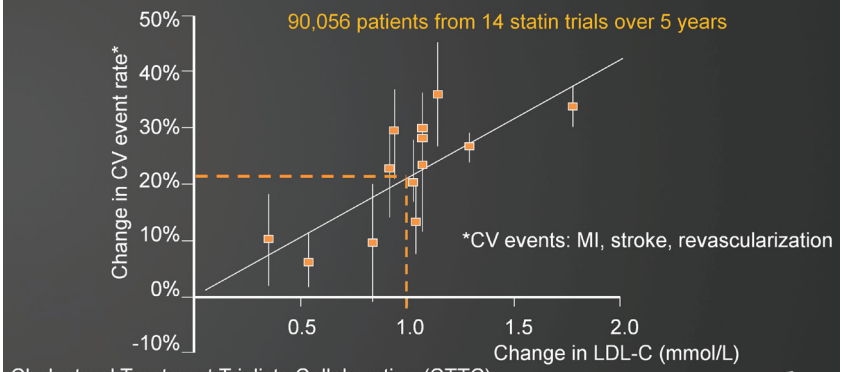
Reducing CV risk in people with AS

SECTION

1D

Residual Risk Reduction initiative

A relatively small decrease in LDL-C can lead to a large reduction in CV events



Cholesterol Treatment Trialists Collaboration (CTTC)
Lancet 2005;366:1267-1278

A relatively small decrease in LDL-C can lead to a large reduction in CV events

- A meta-analysis by the Cholesterol Treatment Trialists Collaboration (CTTC) showed that a 1 mmol/L reduction in LDL-C results in a 21% reduction in major vascular events and a 12% reduction in all-cause mortality

Lipid guidelines in the USA, Europe and Canada: Recommended lipid targets

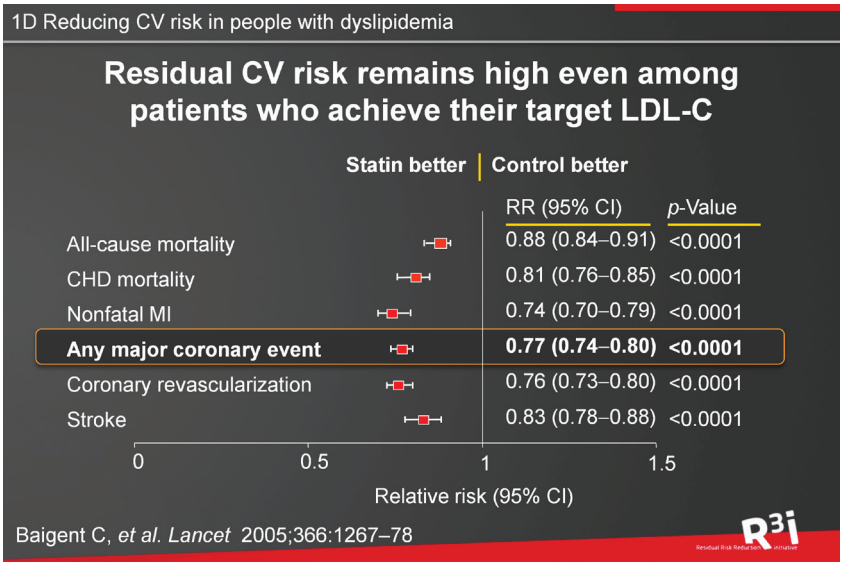
Year	2004	2009	2011
Guideline	NCEP ATP III Guidelines[1]	Canadian CV Society[2]	ESC/EAS Guidelines[3]
Patient groups	Secondary and high-risk primary prevention	High- to moderate- risk	High CV risk
LDL-C	<2.6 mmol/L <1.8 mmol/L reasonable for high risk patients	<2 mmol/L or >/= 50% decrease in LDL-C	<2.5 mmol/L with an option of <2 mmol/L if feasible
Non HDL-C			<3.3 mmol/L

N.B. 1 mmol/L = 39 mg/dL

1.Grundy S, *et al. Circulation* 2004;110:227–39
2.Genest J, *et al. Can J Cardiol.* 2009;25:567–79
3.Reiner Z, *et al. Eur Heart J. 2011*;32:1769–1818

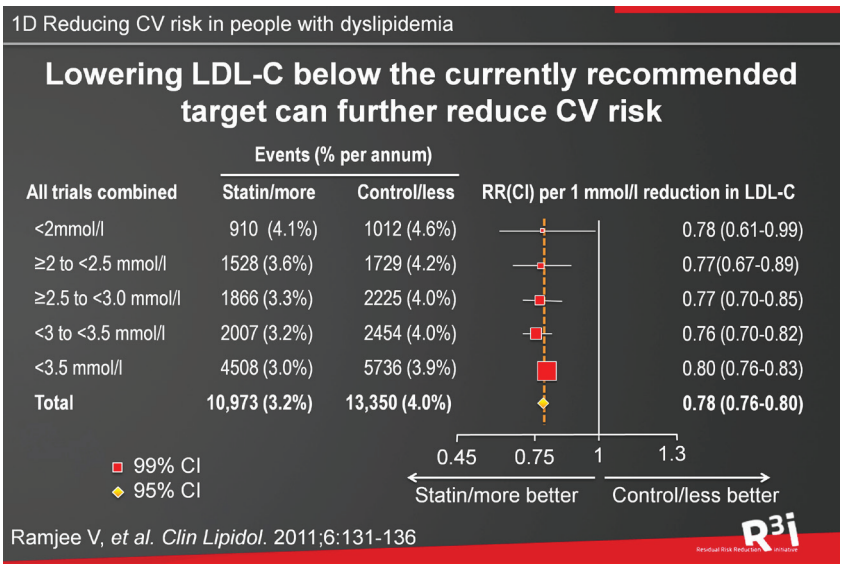
Lipid guidelines in the USA, Europe and Canada: Recommended lipid targets

- International guidelines for the treatment of dyslipidemia recommend lowering LDL-C to <2.5-2.6 mmol/L in most patients (<1.8-2 mmol/L in high-risk patients)



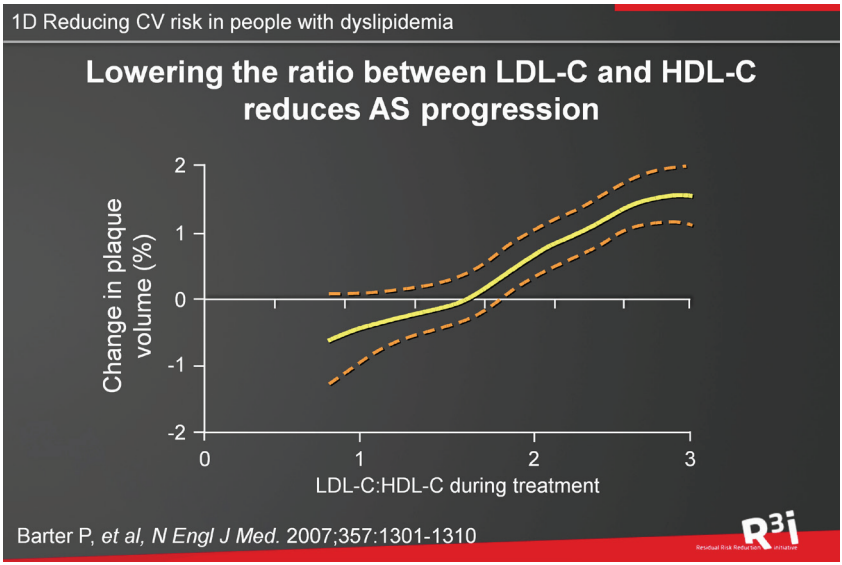
Residual CV risk remains high even among patients who achieve their target LDL-C

- Reducing LDL-C levels to the recommended target only reduces residual risk by ~1/3, leaving substantial residual risk



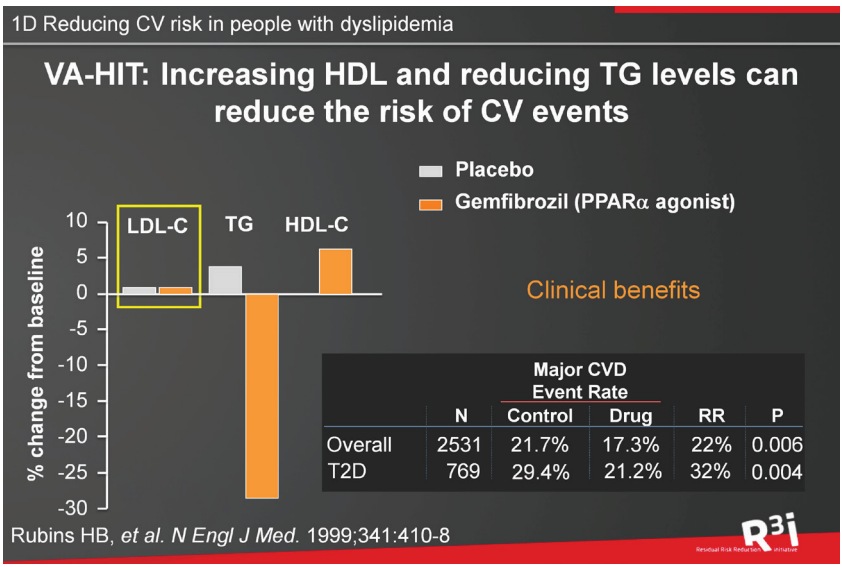
Lowering LDL-C below the currently recommended target can further reduce CV risk

- Results from a recent CTTC meta-analysis suggest that future guidelines may need to include lower cut-off points for LDL-C targets
- Physicians should intensify LDL-lowering therapy to achieve the greatest absolute reductions in LDL-C without increasing the known side-effects of statins



Lowering the ratio between LDL-C and HDL-C reduces AS progression

- Multivariate analysis of data from 1,455 patients in 4 coronary intravascular ultrasound imaging trials showed that both the reduction in LDL-C and the increase in HDL-C achieved during statin treatment were significant independent predictors of coronary AS progression



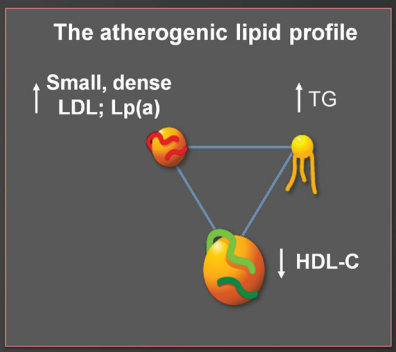
VA-HIT: Increasing HDL and reducing TG levels can reduce the risk of CV events

- Compared to placebo, gemfibrozil was associated with significant improvements in TG and HDL levels and significant reductions in the risk of major CV events in patients with CHD whose primary lipid abnormality was low HDL-C (HDL-C ≤40 mg/dL, LDL-C ≤140 mg/dL and TG ≤300 mg/dL)
- The rate of CV events was reduced by raising HDL-C levels and lowering TG levels without lowering LDL-C, especially in patients with T2D

1D Reducing CV risk in people with dyslipidemia

Reducing residual risk in people with T2D or Metabolic Syndrome

- Although effective for reducing LDL-C, statins have little or no effect on other atherogenic lipid parameters
- An atherogenic lipid profile, characterized by low levels of HDL and high levels of small, dense LDL, TGs and Lp(a), is commonly found in patients with Metabolic Syndrome and T2D



Reducing residual risk in people with T2D or Metabolic Syndrome

- Statins can reduce LDL-related CV risk but have little or no effect on other lipid-related risk factors, e.g. low HDL, elevated TG, Lp(a) and increased numbers of small, dense LDLs
- Thus, people with atherogenic lipid profiles, such as those with T2D and Metabolic Syndrome, often require additional lipid-lowering therapies, such as fibrates, to reduce residual CV risk

1D Reducing CV risk in people with dyslipidemia

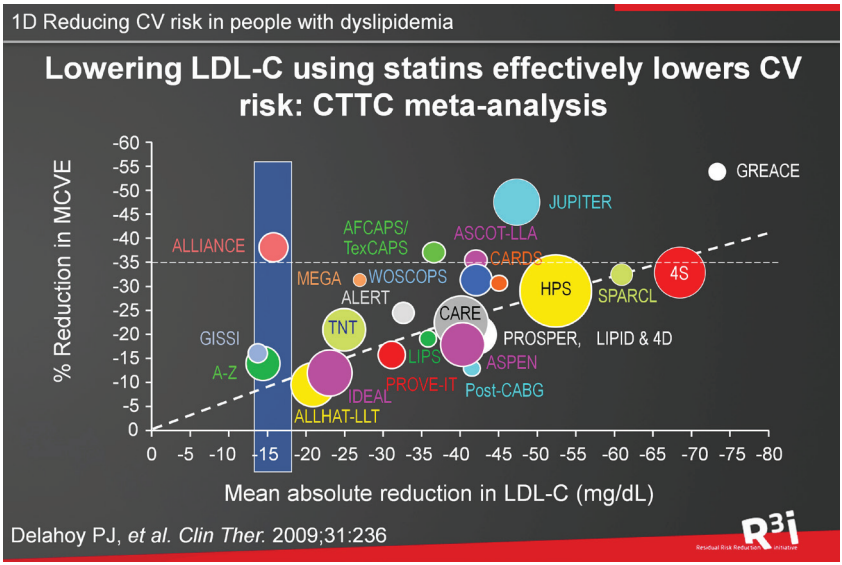
Lipid-lowering treatment options: Effects on lipid profile

	LDL-C	HDL-C	TGs	VLDL-C
Statins	↓18-55%	↑5-15%	↓7-30%	↓7-30%
Fibrates	↓5-20%	↑10-20%	↓20-50%	↓20-50%
Bile acid sequestrants	↓15-30%	↑3-5%	↔ or ↑	↔ or ↑
Niacin	↓5-25%	↑15-35%	↓20-50%	↓20-50%
Ezetimibe	↓18%	↑1%	↓8%	↓7-9%

Adapted from Lin Y, *et al. Vasc Health Risk Man.* 2010;6:73-85

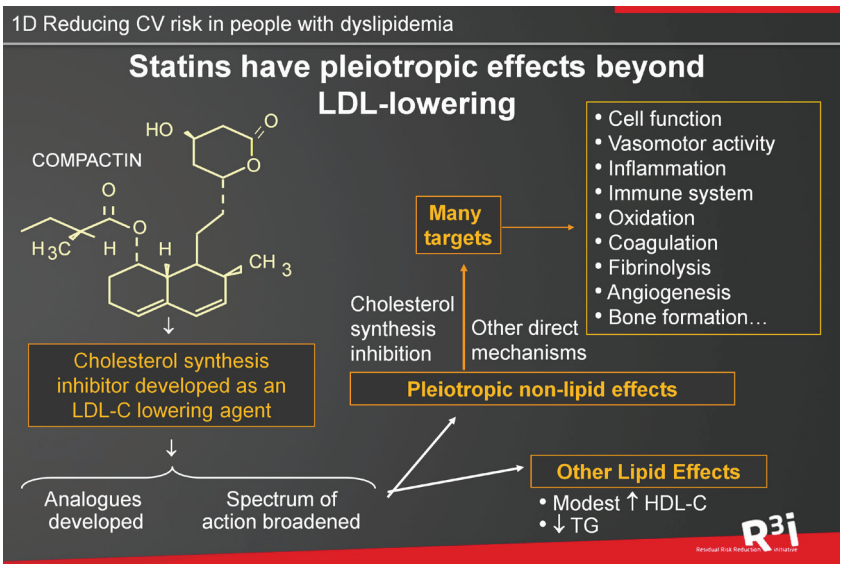
Lipid-lowering treatment options: Effects on lipid profile

- Statins are the recommended first-line drugs for lowering LDL-C levels
- Bile acid sequestrants and niacin are also effective for reducing LDL-C levels; however they are associated with an increased incidence of AEs compared to statins
- Fibrates are more effective than statins for reducing TG levels and increasing HDL but have less effect on LDL-C



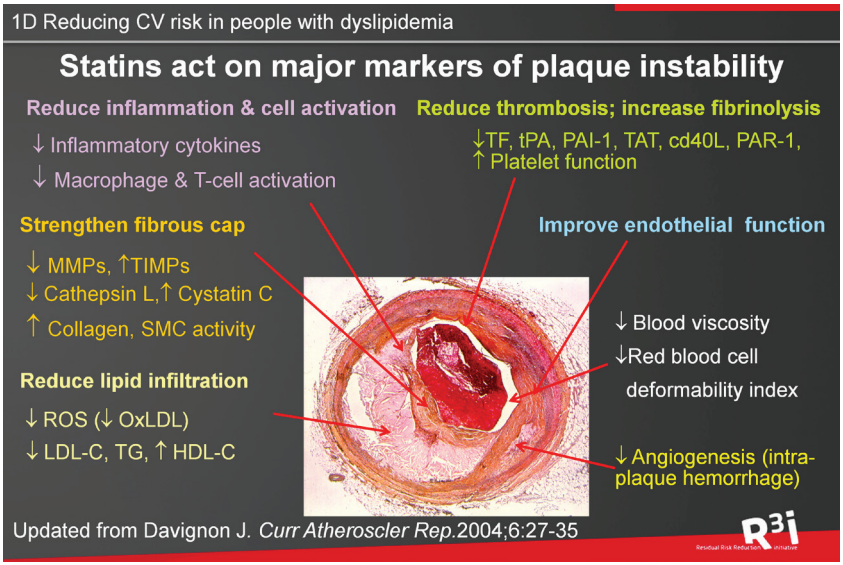
Lowering LDL-C using statins effectively lowers CV risk: CTTC meta-analysis

- A meta-regression analysis of 25 trials, involving 155,613 subjects showed that LDL-C-lowering using statins effectively lowers the risk for major coronary and vascular events (MCVE) in proportion to LDL reduction. Deviation of some trials from the regression line illustrate the context dependency of such studies
- This study reported a total of 6321 vascular deaths, 23,791 major vascular events, 11,357 MCVE (nonfatal MI- or CAD-related death) and 4717 strokes
- The size of the colored circle is proportional to the number of subjects in the trial



Statins have pleiotropic effects beyond LDL-lowering

- The first statin, compactin, was discovered in 1977 as an inhibitor of cholesterol synthesis
- Over time, other statins (including atorvastatin, simvastatin, lovastatin, fluvastatin, pravastatin, cerivastatin, and pitavastatin) have been developed
- In addition to their LDL-C-lowering effects, some of these statins have beneficial effects on other lipid parameters, including HDL-C and TG
- Many statins have non-lipid pleiotropic effects that are thought to contribute to the reduction in CV risk



Statins act on major markers of plaque instability

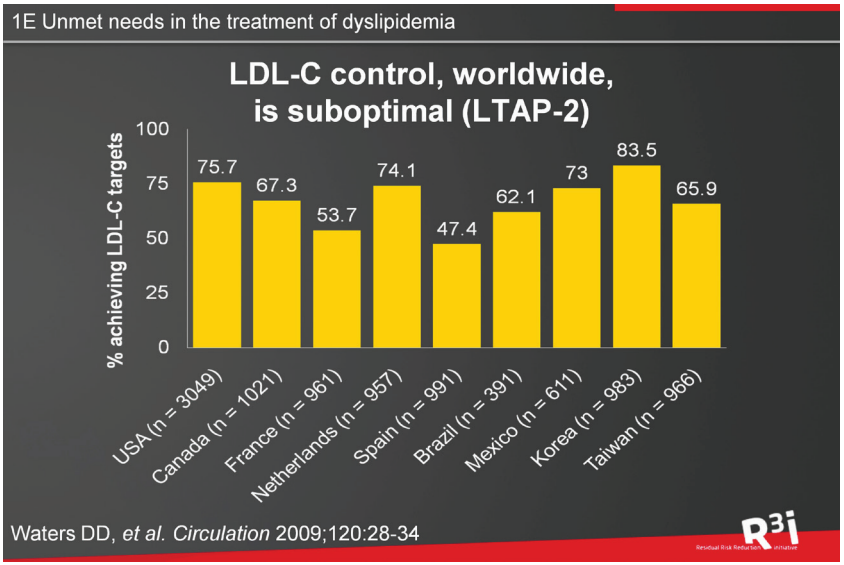
- Some of the pleiotropic effects associated with statins are thought to improve plaque stability, thereby leading to a reduction in CV risk

Unmet needs in the treatment of dyslipidemia

SECTION

1E

Residual Risk Reduction initiative

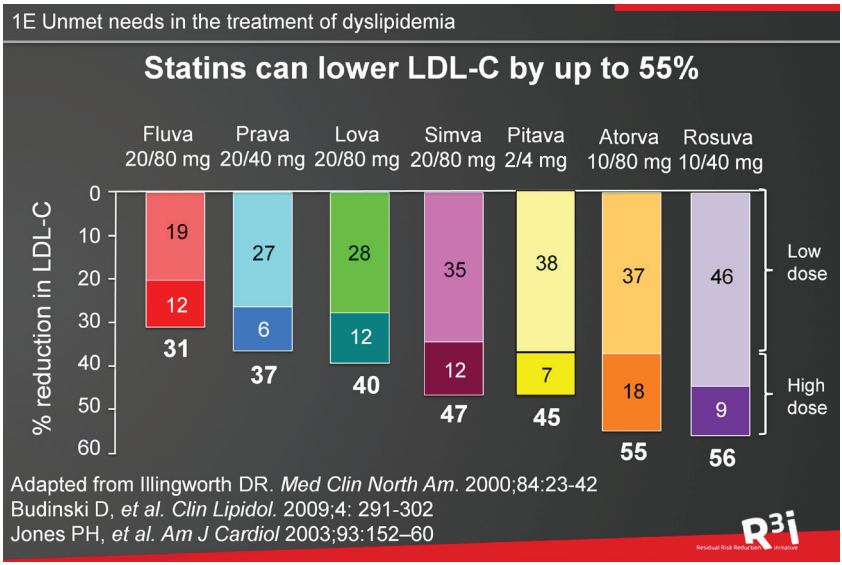


LDL-C control, worldwide, is suboptimal (LTAP-2)

- The Lipid Treatment Assessment Project 2 (L-TAP 2) followed 9955 dyslipidemic adult patients on stable lipid-lowering therapy in 9 countries between 2006 and 2007
- The proportion of patients achieving LDL-C goals according to relevant national guidelines ranged from 47% to 84%

Potential reasons for suboptimal CV risk management in clinical practice

- Under-diagnosis
- Failure to tailor therapy for individual patients
- Failure to treat residual CV risk factors (e.g. low HDL, high TG, Lp(a)) in addition to LDL-C
- Inadequate dosing/failure to use combination therapy
- Poor adherence to therapy due to AEs, drug interactions, perceived lack of effect, cost, etc.
- Lack of suitable treatments for refractory/difficult-to-treat patients (those with FH or primary hypercholesterolemia; patients refractory/intolerant to statins; high-risk populations e.g. elderly, T2D, chronic kidney disease)



Statins can lower LDL-C by up to 55%

- Statins have a maximum LDL-lowering efficacy of ~55%
- Doubling a statin dose generally achieves a modest, incremental reduction in LDL-C of 5% to 6%. This phenomenon is known as "the rule of 6"

1E Unmet needs in the treatment of dyslipidemia

Patients with high CV risk and/or very high levels of LDL-C may need combinations of lipid-lowering agents to achieve their targets

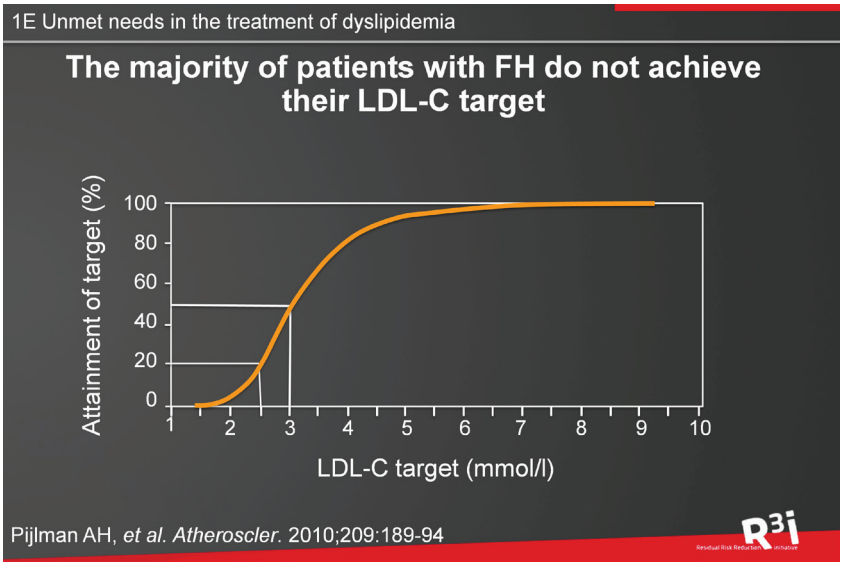
Lipid-lowering combination	Pros	Cons
Statins + bile acid sequestrants or ezetimibe	Further decreases in LDL-C levels	Bile acids can decrease the absorption of statins Ezetimibe can cause myopathy
Statins + fibrates	Additional decrease in TGs and further increases in HDL-C	Increased incidence of myopathy and liver toxicity
Statins + niacin	Patients with *FH; further increases in HDL-C	Can cause flushing

*FH = familial hypercholesterolemia

Grundy SM, *et al. Circulation* 2004;110:227-39; Lin Y, *et al. Vasc Health Risk Man.* 2010;6:73-85; Jacobson TA, *et al. Am J Cardiol* 2007;99:S47-55

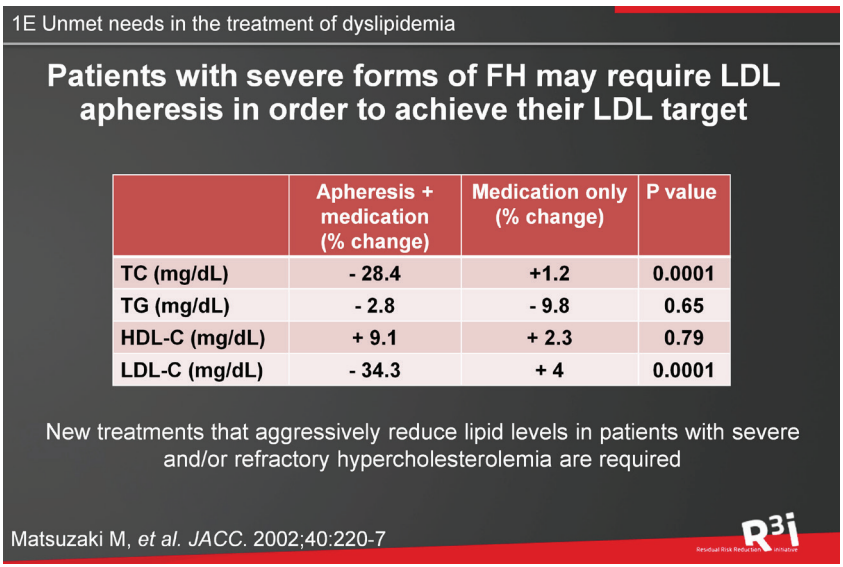
Patients with high CV risk and/or very high levels of LDL-C may need combinations of lipid-lowering agents to achieve their targets

- Recommended combinations include drugs that lower lipid levels via different pathways, e.g. statins + bile acid sequestrants or ezetimibe, statins + fibrates and statins + niacin



The majority of patients with FH do not achieve their LDL-C target

- Up to 80% of patients with severe forms of hypercholesterolemia, such as FH, are unable to achieve their target LDL-C using conventional therapies
- For these patients, more expensive, invasive treatments such as extracorporeal apheresis may be necessary
- New treatments that aggressively reduce lipid levels in patients with severe and/or refractory hypercholesterolemia are therefore required



Patients with severe forms of FH may require LDL apheresis in order to achieve their LDL target

- Rare patients with severe hyperlipidemias, especially homozygous and severe heterozygous FH, require specialist evaluation and possible LDL apheresis
- During this process, LDL and Lp(a) are removed from plasma during extracorporeal circulation weekly or every other week
- This procedure is expensive, invasive and can only be performed at highly specialized centres

Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9) biology

SECTION

2

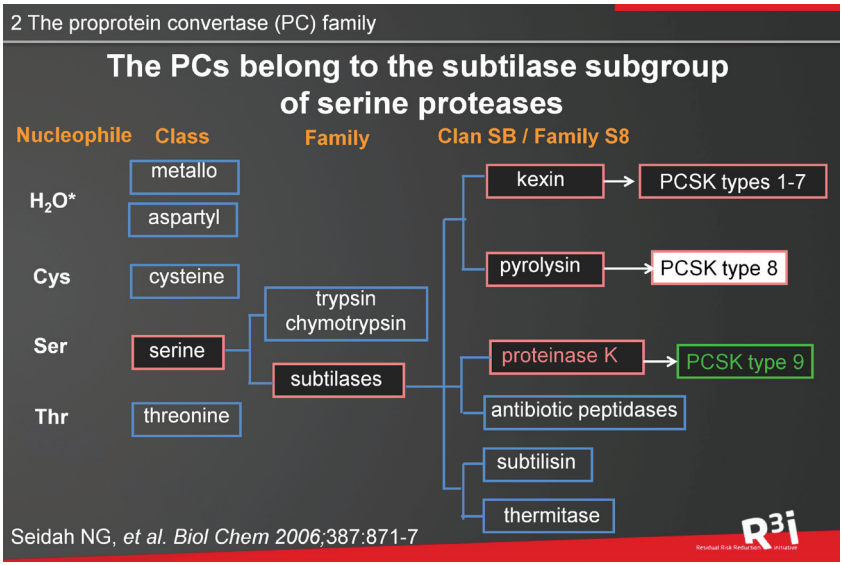
Residual Risk Reduction initiative

The proprotein convertase (PC) family

SECTION

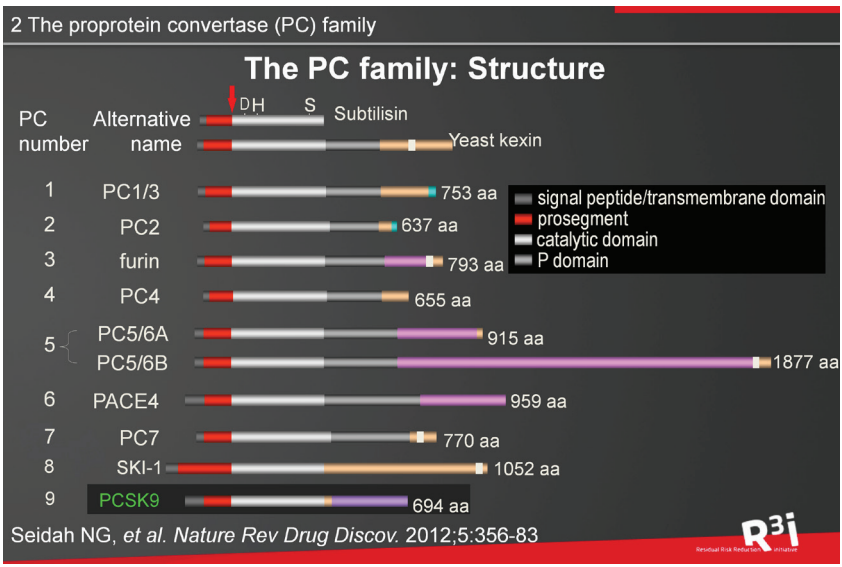
2A

Residual Risk Reduction initiative



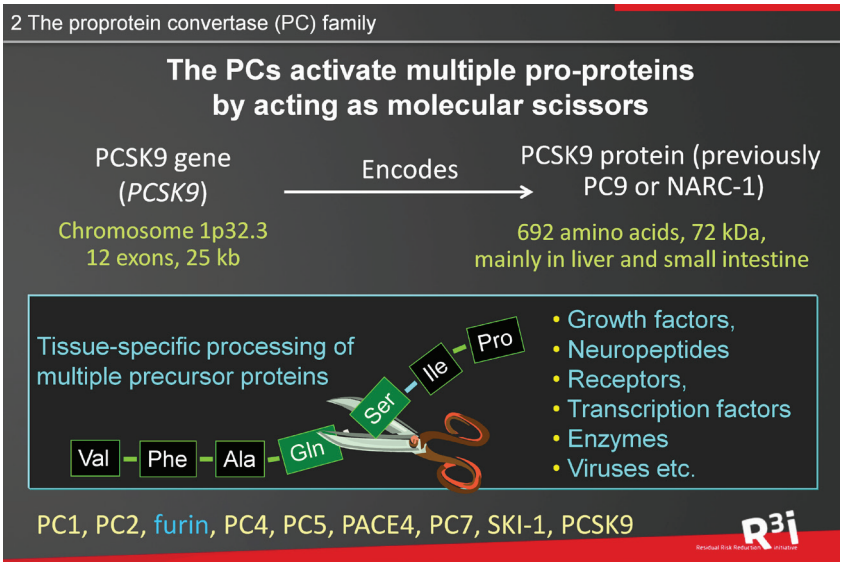
The PCs belong to the subtilase subgroup of serine proteases

- ~600 distinct proteases have been identified in the human and mouse genomes, belonging to five major classes of proteolytic enzymes (metallo, aspartyl, cysteine, serine and threonine)
- Among these, the serine proteases can be further divided into two subgroups (trypsin chymotrypsins and subtilases)
- Subtilases have been subdivided into six clans (kexin, pyrolysin, proteinase K, antibiotic peptidases, subtilisin and thermitase)
- PCSK9 is a member of the proteinase K clan of subtilases



The PC family: Structure

- PCSK9 is the last-discovered member of a 9-member family of subtilisin-like calcium-dependent serine proteases, called PCs
- The others eight are furin, PC1, PC2, furin (PC3), PC4, PC5, paired basic amino acid cleaving enzyme 4 (PACE4 or PC6), PC7, and subtilisin kexin isozyme 1 (SKI-1 or PC8)
- The first seven are related to the yeast Kexin protease; SKI-1 is related to pyrolysin and PCSK9 is related to proteinase K
- PCs are found in various combinations in the secretory pathway compartments of all nucleated mammalian cells



The PCs activate multiple pro-proteins by acting as molecular scissors

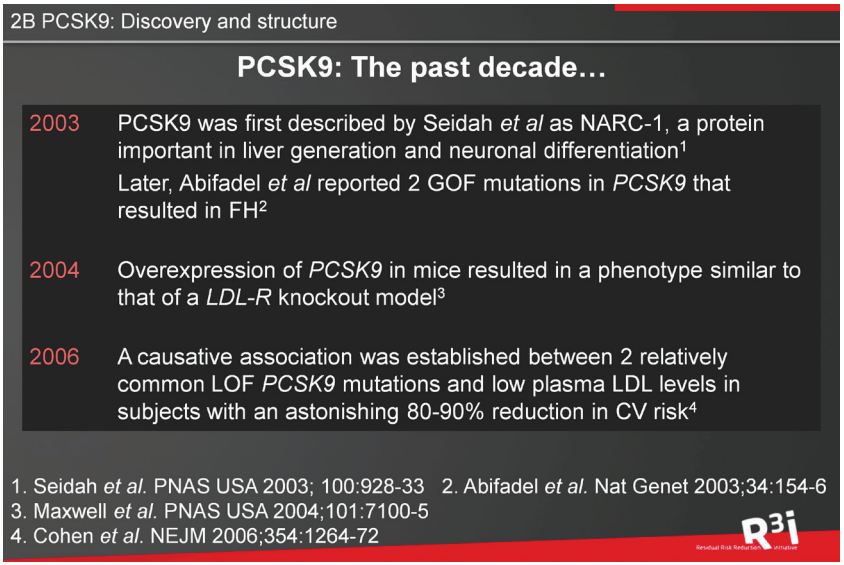
- The PCs acts as 'molecular scissors' for the tissue-specific processing of multiple precursor proteins such as growth factors, neuropeptides, receptors, enzymes, transcription factors, and viruses
- Seven of the PCs (PC1, PC2, furin, PC4, PC5, PACE4 and PC7) activate cellular and pathogenic PCs by tissue-specific cleavage at single or paired basic residues (R/K $[X]_{0,2,4,6}$ R/K)
- SKI-1 activates multiple pro-proteins by cleaving at non-basic residues
- PCSK9 cleaves at non-basic residues (as illustrated in the slide) but only activates itself; PCSK9 is further cleaved by furin

PCSK9: Discovery and structure

SECTION

2B

Residual Risk Reduction initiative

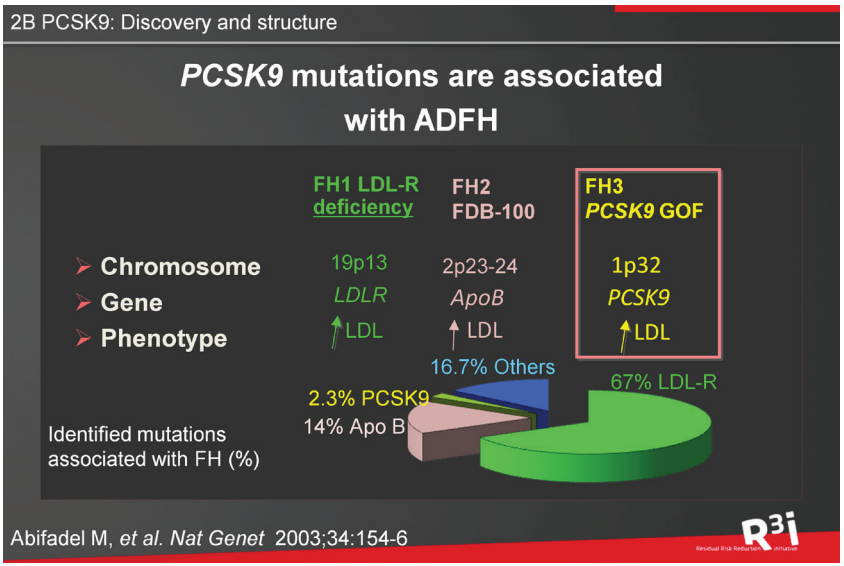


PCSK9: The past decade...

2003: PCSK9 was first described as NARC-1, a protein important in liver generation and neuronal differentiation
Two *PCSK9* gain-of-function (GOF) mutations were later reported that resulted in FH

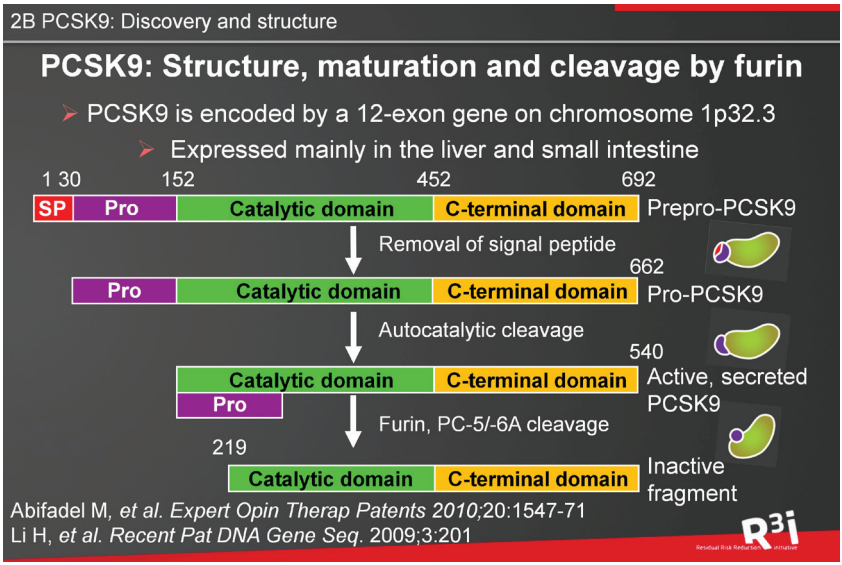
2004: Overexpression of *PCSK9* in mice resulted in a phenotype similar to that found in *LDL-R* knockout models

2006: An association was established between 2 loss-of-function (LOF) *PCSK9* mutations and low LDL-C levels in subjects with an 80-90% reduction in CV risk. PCSK9 therefore became an attractive drug target and the subject of intensive research



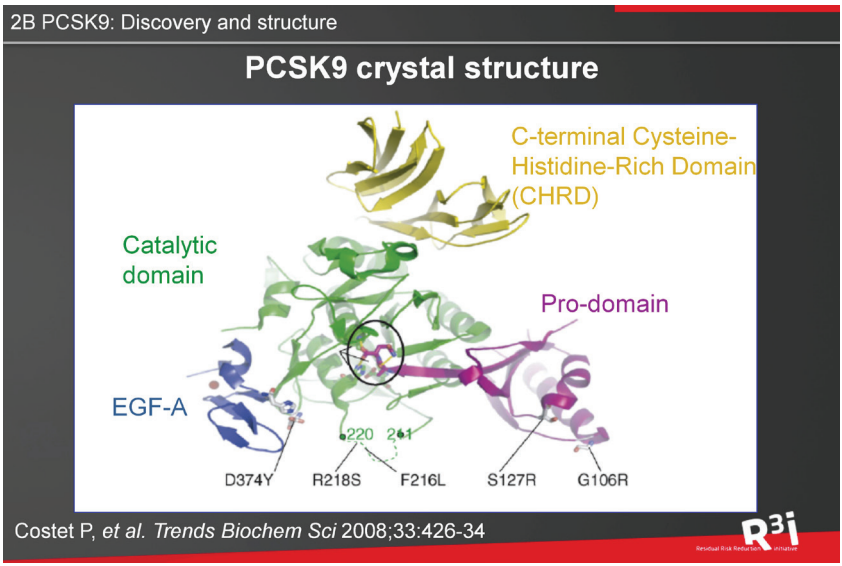
***PCSK9* mutations are associated with ADFH**

- FH is a co-dominant condition (i.e. has a gene dosage effect on the LDL phenotype) and a risk factor for CHD. It is characterized by raised levels of LDL-C caused by:
  - A LOF mutation in the *LDL-R* gene (accounts for 67% of FH cases; referred to as FH1)
  - A LOF mutation in the *Apo-B* gene (14% of FH cases; referred to as FH2 or familial defective Apo B-100 [FDB-100])
  - A gain-of-function (GOF) mutation in the *PCSK9* gene (2.3% of cases; FH3)



PCSK9: Structure, maturation and cleavage by furin

- PCSK9 is encoded by a gene comprising 12 exons on chromosome 1p32.3; it is expressed mainly in the liver and small intestine
- PCSK9 is synthesized as a 692-amino acid (~75 kDa) precursor (prepro-PCSK9) comprising a signal peptide (SP), a pro-domain (Pro), a catalytic domain and a C-terminal domain
- Cleavage of the SP forms pro-PCSK9, which undergoes autocatalytic intra-molecular processing between residues 152-153 in the ER to form the active 62kDa PCSK9
- PCSK9 is either secreted in its active form or undergoes furin- or PC5/6A-mediated cleavage of the pro-domain leading to the secretion of less active/inactive PCSK9 fragments (constitutes 15-40% of total human plasma PCSK9)



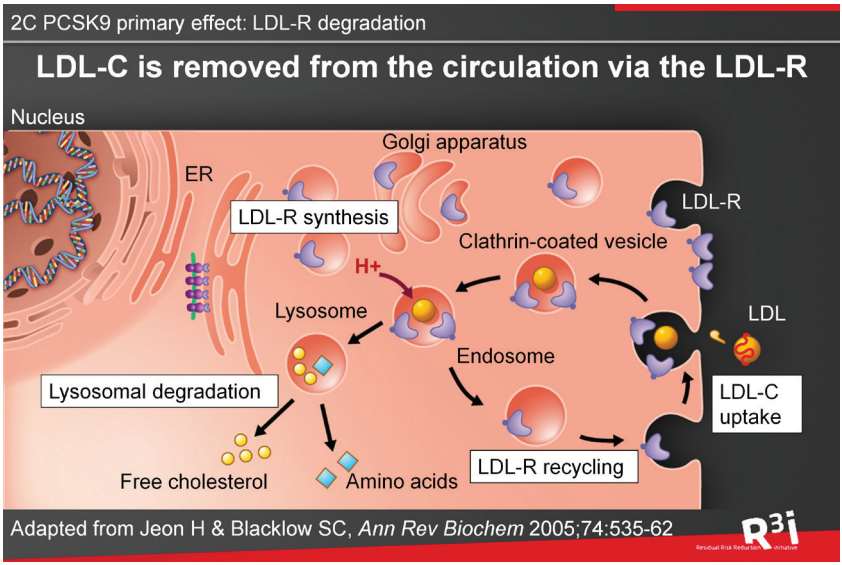
PCSK9 crystal structure

- The crystal structure of purified, secreted PCSK9 shows that, within the plasma, the pro-domain remains associated with the protein, thereby blocking substrate access to the catalytic triad (circled)
- PCSK9 binds the LDL-R via its extra-cellular epidermal growth factor-like A (EGF-A) domain (blue)
- Intensely studied PCSK9 variants are indicated

PCSK9 primary effect: LDL-R degradation

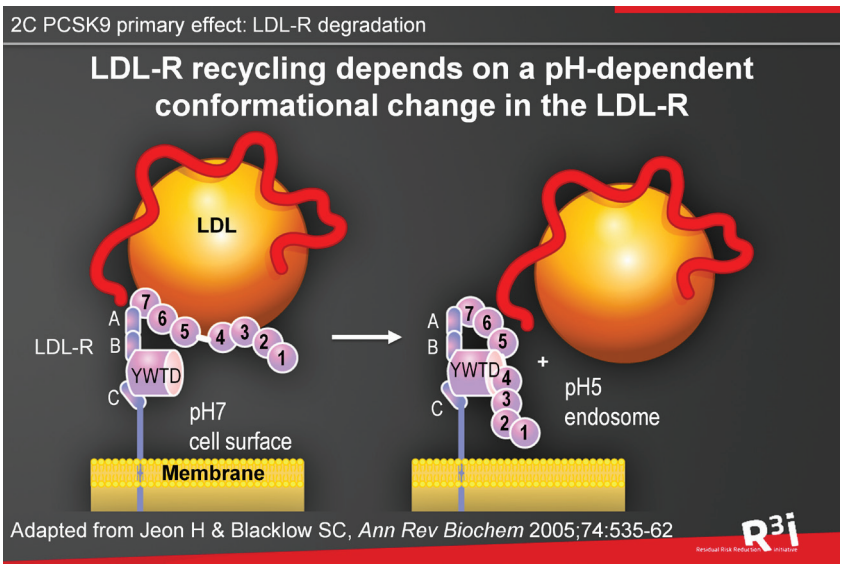
SECTION 2C

Residual Risk Reduction initiative



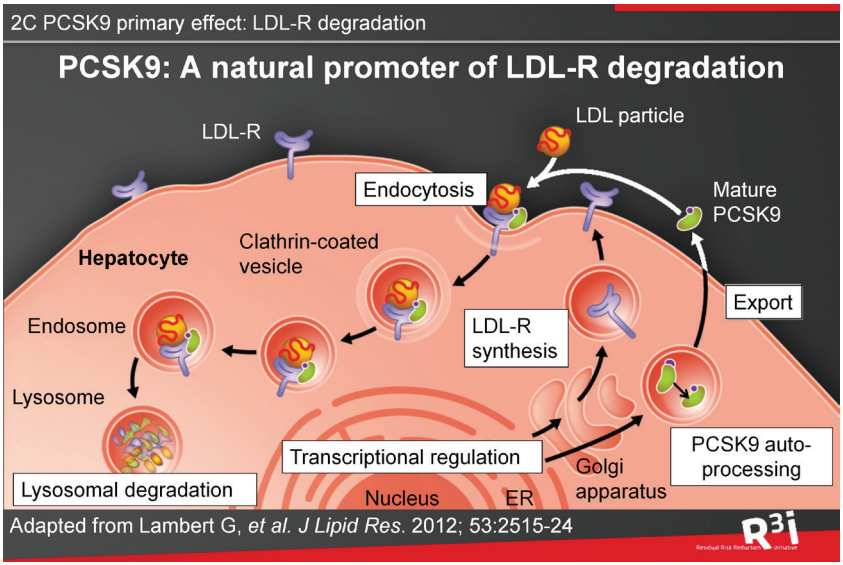
LDL-C is removed from the circulation via the LDL-R

- LDL binds to the LDL-R and enters hepatocytes via clathrin-coated pit-mediated endocytosis
- Under normal conditions, the LDL/LDL-R complex dissociates in the endosome and the LDL-R is returned to the cell surface (LDL-R recycling)
- Free LDLs then enter the lysosome for further degradation



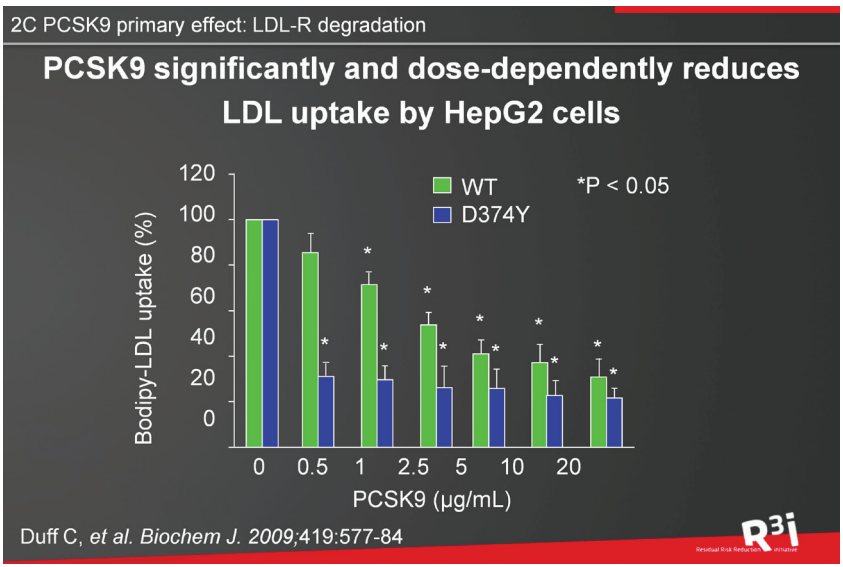
LDL-R re-cycling depends on a pH-dependent conformational change in the LDL-R

- The LDL-R is thought to open and close as a function of pH:
- At neutral pH (cell surface), the LDL-R is elongated, with ligand-binding epitopes open to solution
- At acidic pH (<6.0; endosomes), the LDL-R undergoes a conformational change, whereby the β-propeller (YWTD) of the EGF-precursor homology domain binds to the ligand-binding domain. The closed conformation displaces the ligand, freeing the LDL-R for re-cycling



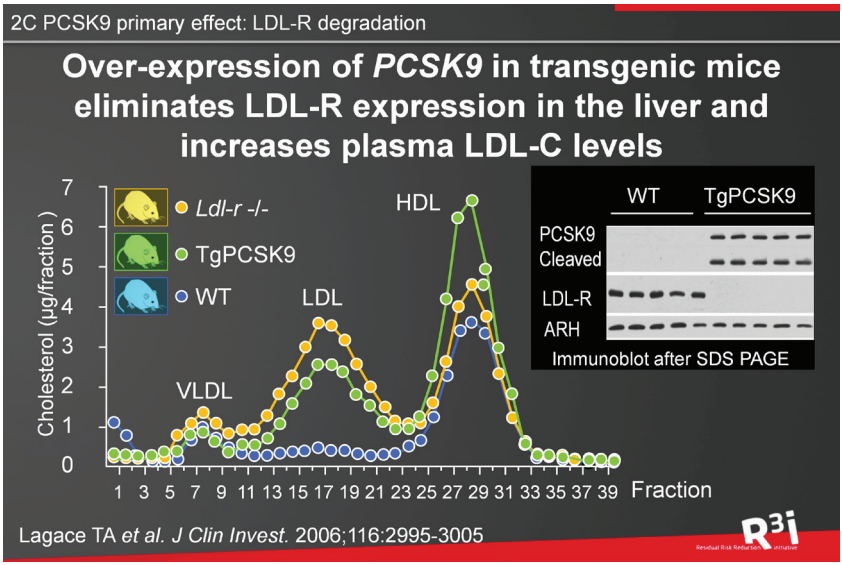
PCSK9: A natural promoter of LDL-R degradation

- PCSK9 binds to the LDL-R/LDL complex, 'locking' the LDL-R in an open conformation and preventing it from releasing the LDL particle
- The entire PCSK9/LDL-R/LDL complex is directed to the lysosome, where LDL-R is degraded
- The net result is reduced cell-surface concentrations of LDL-R leading to decreased LDL plasma clearance and increased plasma LDL-C levels



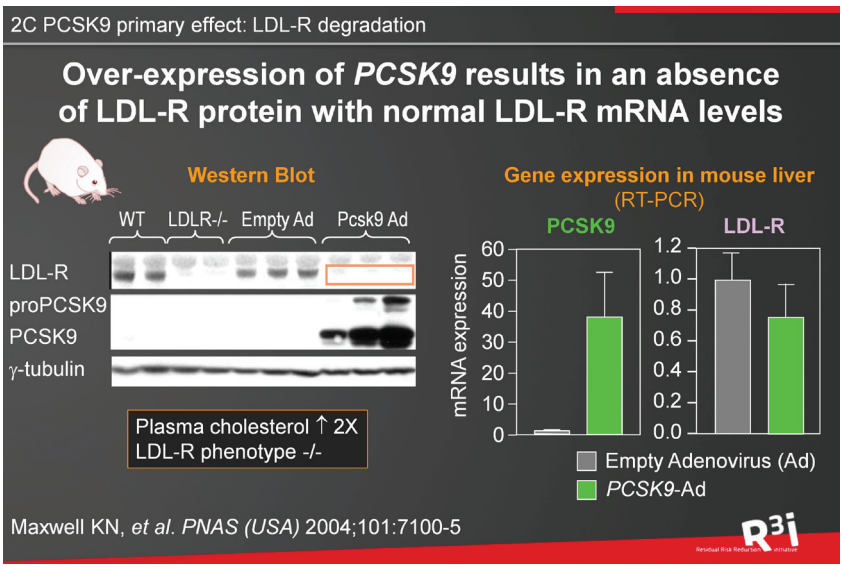
PCSK9 significantly and dose-dependently reduces LDL uptake by HepG2 cells

- HepG2 cells were incubated for 6 h with various concentrations of purified PCSK9 (WT or the GOF variant, D374Y) in the presence of BODIPY®–LDL, and fluorescence was measured. Results are a percentage of LDL taken up by cells in the absence of PCSK9
- There was a dose-dependent reduction in LDL uptake after treatment with purified PCSK9; the increased binding affinity of the GOF mutant was clearly evident



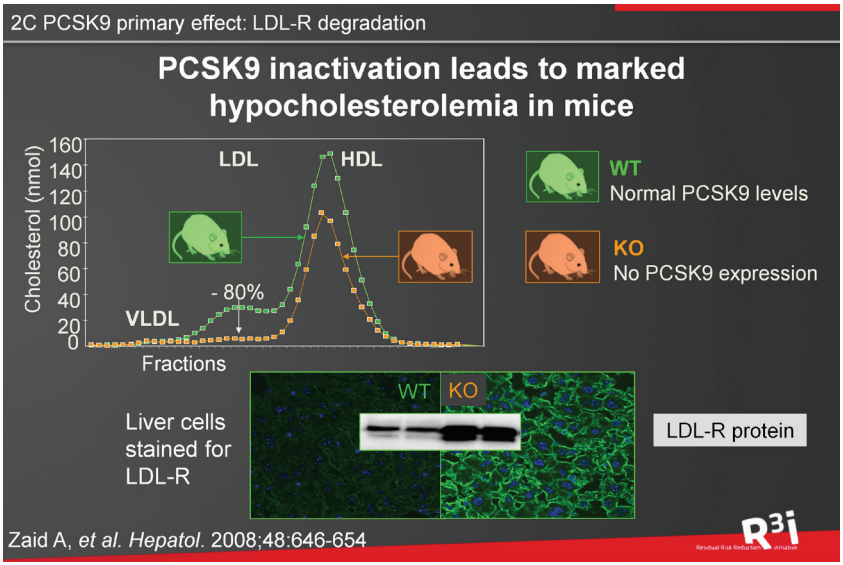
Over-expression of *PCSK9* in transgenic mice eliminates LDL-R expression in the liver and increases plasma LDL-C levels

- Over-expression of human *PCSK9* in the livers of transgenic mice (TgPCSK9) eliminated LDL-R protein expression in liver and caused a marked increase in plasma LDL-C vs WT
- The increase in plasma LDL-C was similar to that found in *Ldl-r*$^{-/-}$ mice that lack LDL-Rs in all tissues



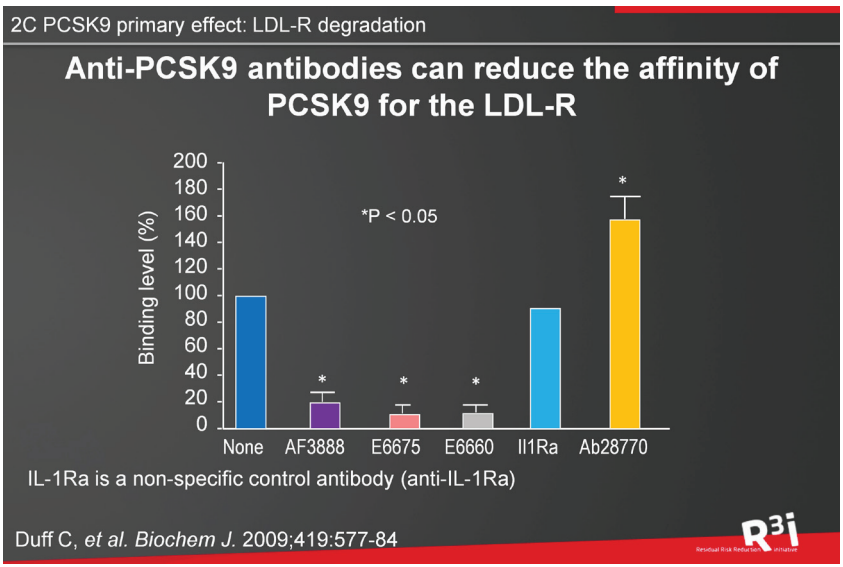
Over-expression of *PCSK9* results in an absence of LDL-R protein with normal LDL-R mRNA levels

- Western blotting of protein from mice injected with either adenovirus (Ad) empty or *PCSK9*-Ad showed that Ad empty mice had normal levels of LDL-R protein, whereas mice injected with *PCSK9*-Ad had a complete absence of LDL-R protein
- The levels of LDL-R mRNA in the livers of mice injected with Ad empty and *PCSK9*-Ad were measured by quantitative RT-PCR
- Over-expression of *PCSK9* did not change the levels of LDL-R mRNA



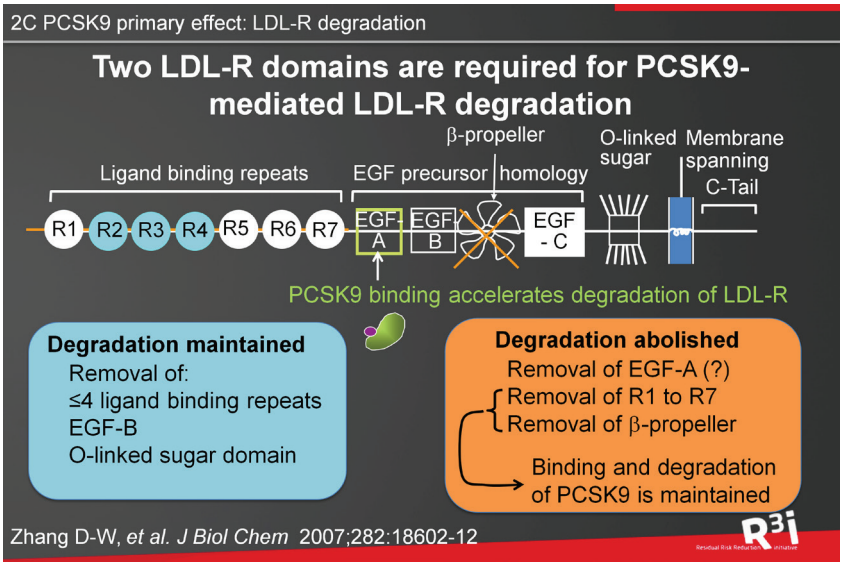
PCSK9 inactivation leads to marked hypocholesterolemia in mice

- FPLC analysis of plasma pools revealed that, compared to WT mice, LDL-C and HDL-C in *PCSK9* KO mice were reduced by 80% and ~40%, respectively
- Western blot analysis of the liver extracts using LDL-R showed that *PCSK9* KO mice had 2- to 3-fold higher levels of LDL-R than WT mice
- Immunohistochemistry confirmed these results and demonstrated that PCSK9 deficiency led to higher levels of cell surface LDL-R in hepatocytes



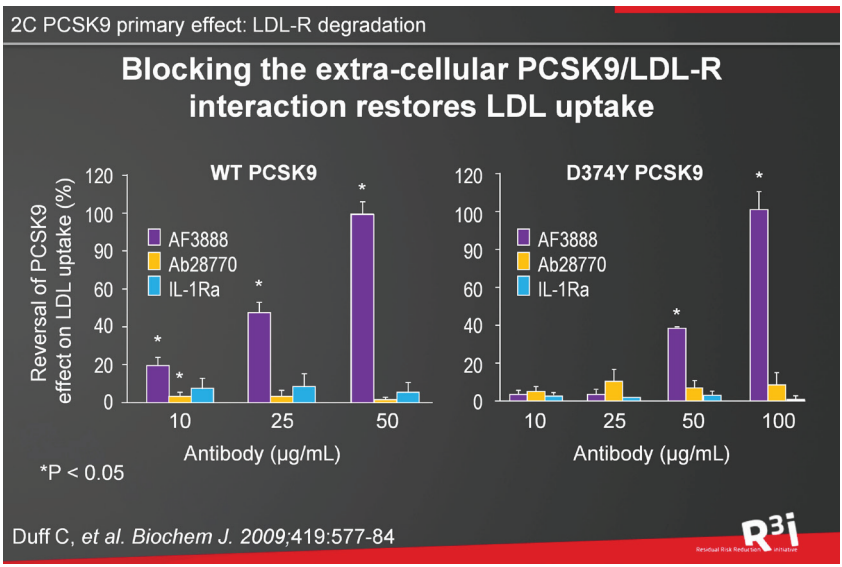
Anti-PCSK9 antibodies can reduce the affinity of PCSK9 for the LDL-R

- PCSK9 (50 nM) was pre-incubated with one of four anti-PCSK9 pAbs (AF3888, E6675, E6660 or Ab28770) or a non-specific control antibody (anti-IL-1Ra) and passed over a CM5 sensorchip surface coupled to LDL-R
- Antibodies AF3888, E6676 and E6660 significantly reduced PCSK9/LDL-R binding, whereas AB28770 significantly increased PCSK9/LDL-R binding



Two LDL-R domains are required for PCSK9-mediated LDL-R degradation

- To locate the LDL-R regions required for PCSK9-mediated degradation, a series of LDL-R mutant proteins were produced and their levels of degradation in mouse hepatocytes (Hepa1c1c7 cells) determined. Results suggest that:
  - ≥3 ligand-binding repeats are required for efficient PCSK9-mediated LDL-R degradation but it does not matter which 3
  - The EGF-B repeat and the clustered O-linked sugar region are not required for degradation
  - The β-propeller domain (YWTD repeats) is required
  - Deletion of EGF-C alone resulted in retention of the protein in the ER



Blocking the extra-cellular PCSK9/LDL-R interaction restores LDL uptake

- PCSK9 (WT or GOF variant, D374Y) (2.5 μg/ml) pre-incubated with increasing concentrations of anti-PCSK9 antibody (AF3888) or control antibodies (IL-1Ra and Ab28770) were added to HepG2 cells and LDL uptake was determined
- Anti-PCSK9 antibodies (but not control antibodies) blocking the interaction between PCSK9 (both WT and the D374Y variant) and LDL-R dose-dependently restored LDL uptake to normal levels

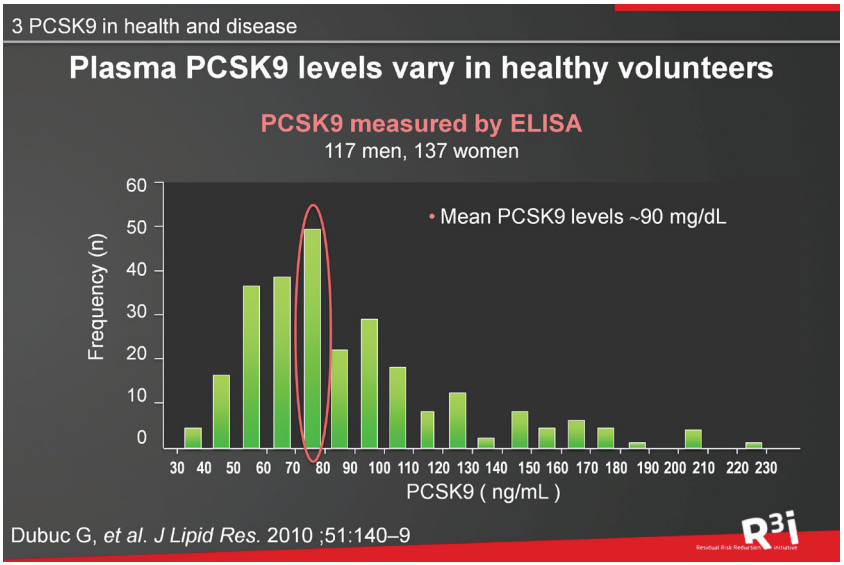
PCSK9 primary effect: LDL-R degradation

PCSK9 in health and disease

SECTION

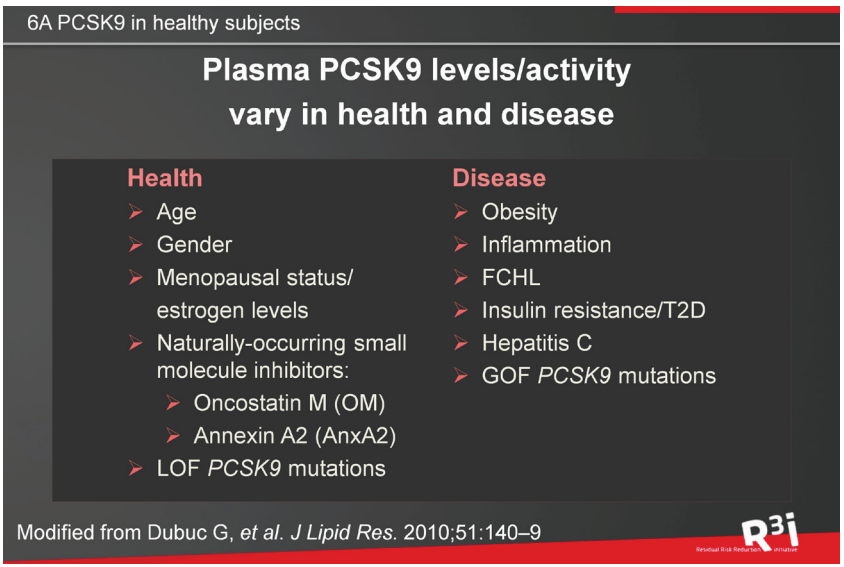
3

Residual Risk Reduction initiative



Plasma PCSK9 levels vary in healthy volunteers

- PCSK9 levels in 254 healthy volunteers were measured using enzyme-linked immunosorbant assay (ELISA)
- The mean PCSK9 level was ~90 mg/dL; levels varied considerably among subjects



Plasma PCSK9 levels/activity vary in health and disease

- Factors that affect PCSK9 levels in healthy individuals include age, gender, menopausal status/estrogen levels, oncostatin M (OM) treatment, annexin A2 (AnxA2) and LOF *PCSK9* mutations
- Clinical conditions that affect PCSK9 levels include obesity, inflammation, FCHL, insulin resistance/T2D, hepatitis C, and GOF *PCSK9* mutations

PCSK9 in health

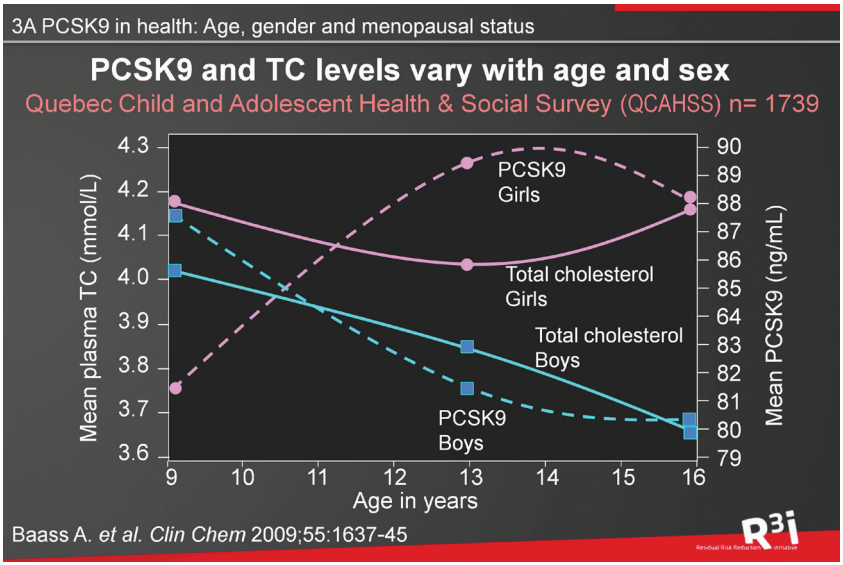
SECTION

3A

Residual Risk Reduction initiative

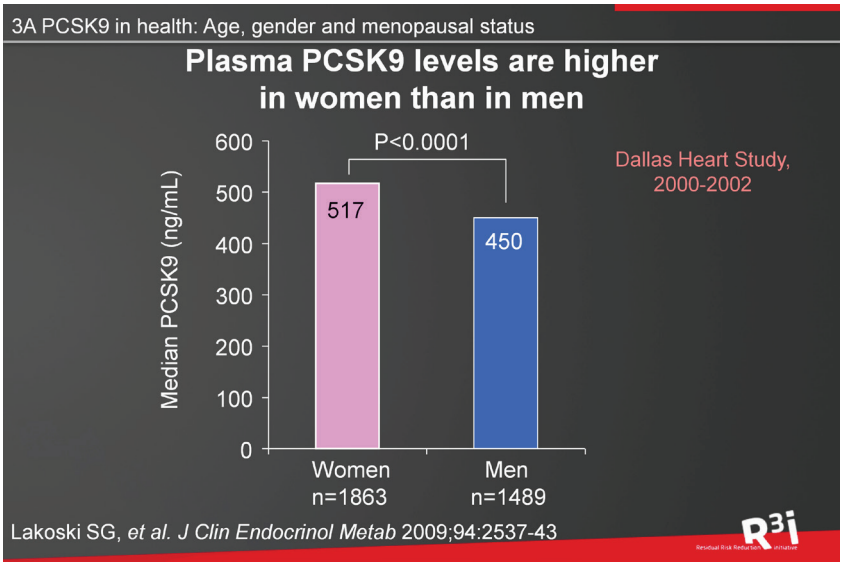
Age, gender and menopausal status/estrogen levels

DEFINING TOMORROW'S VASCULAR STRATEGIES



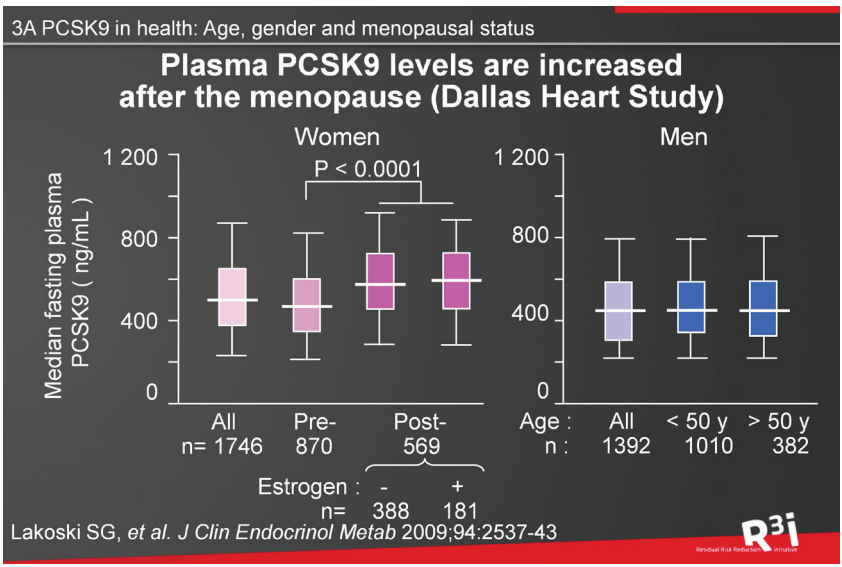
PCSK9 and TC levels vary with age and sex

- In boys aged 9- to 16-years old, mean TC and PCSK9 levels decrease steadily with age
- In girls of the same age, mean TC levels are higher in 9- and 16-year olds than in 13-year olds and there is an inverse effect on mean plasma PCSK9 concentrations



Plasma PCSK9 levels are higher in women than in men

- In the multi-ethnic Dallas Heart Study carried out in 3138 patients between 2000 and 2002, plasma PCSK9 levels were significantly higher in women (n = 1863) than in men (n = 1489)
- This difference persisted after adjusting for age, ethnicity, BMI, SBP, menopausal status, fasting glucose, LDL-C, HDL-C, TG and CRP levels



Plasma PCSK9 levels are increased after the menopause (Dallas Heart Study)

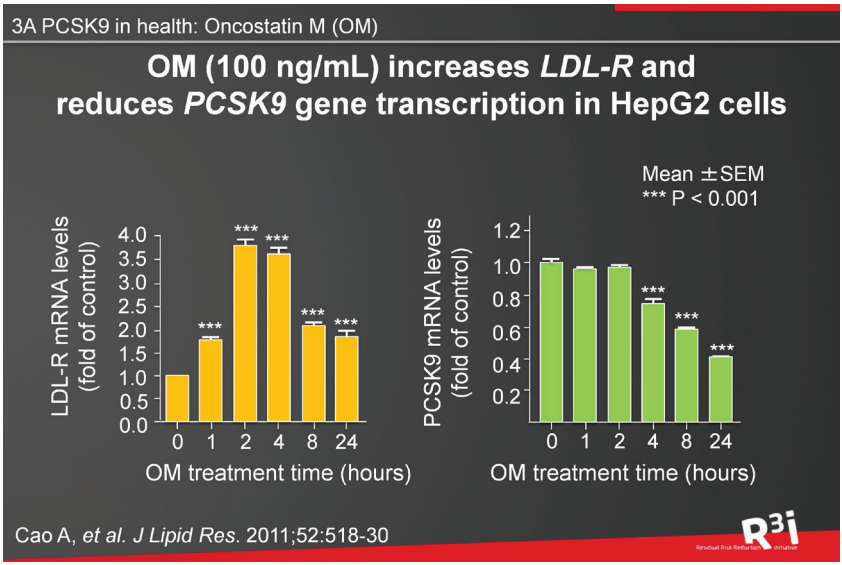
- Data from the multi-ethnic Dallas Heart Study (N = 3138) showed that premenopausal women had significantly higher plasma levels of PCSK9 than postmenopausal women
- Estrogen treatment did not significantly affect fasting PCSK9 levels in postmenopausal women
- In comparison, there was no difference in plasma PCSK9 levels in men over vs under the age of 50 years
- Median levels of PCSK9 are denoted by a horizontal line; boxes denote the 75th and 25th percentiles; whiskers represent the 95th and 5th percentiles

Naturally-occurring small molecule inhibitors

DEFINING TOMORROW'S VASCULAR STRATEGIES

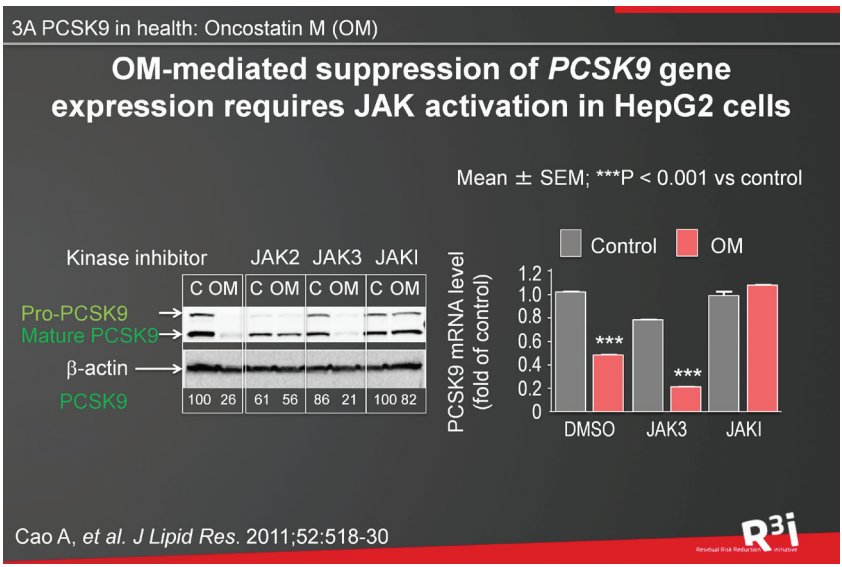
Naturally-occurring small molecule inhibitors: Oncostatin M (OM)

DEFINING TOMORROW'S VASCULAR STRATEGIES



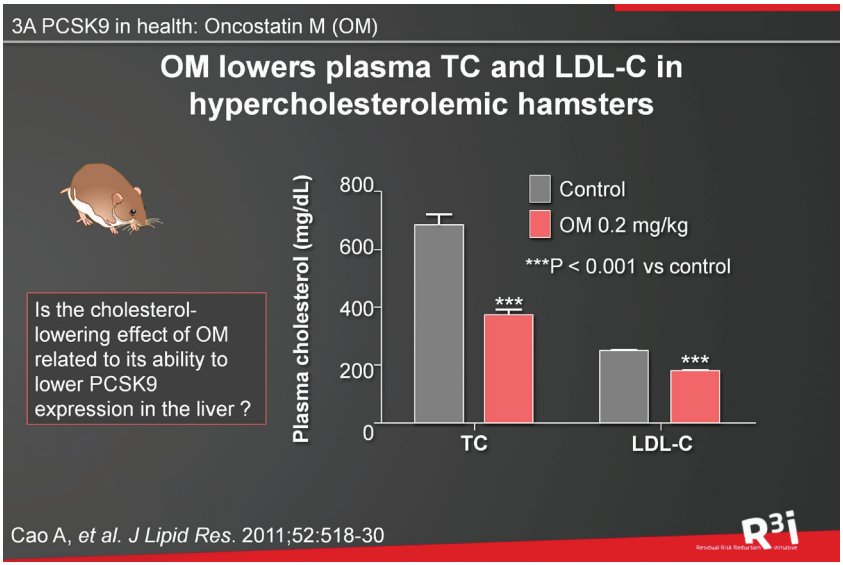
OM (100 ng/mL) increases *LDL-R* and reduces *PCSK9* gene transcription in HepG2 cells

- OM is an inflammatory cytokine (IL-6 family) that activates LDL-R transcription by binding TFs (EGF-1 and c/EBPβ) to sterol-independent regulatory elements of the LDL-R promoter
- OM (100 ng/mL) induced a rapid increase in LDL-R mRNA levels (measured by RT PCR) in HepG2 cells
- In contrast, the level of PCSK9 mRNA steadily decreased in OM-treated cells



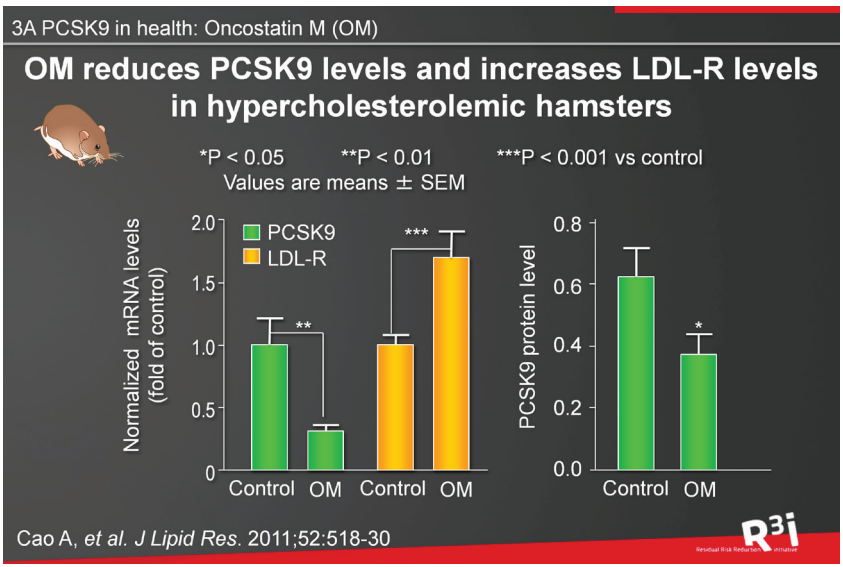
OM-mediated suppression of *PCSK9* gene expression requires JAK activation in HepG2 cells

- HepG2 cells were pre-incubated for 2 h with JAK inhibitors (JAK2 (50μM), JAK3 (100μM), and JAKI (10μM)) followed by OM treatment for 24h
- Western blotting showed that JAKI (known to block JAK1, JAK2, JAK3, and Tyk2 activity) and JAK2, but not JAK3, prevented OM-mediated suppression of PCSK9 expression
- rtPCR showed that the OM-mediated decrease in *PCSK9* mRNA was observed in DMSO-treated control cells and in JAK3 inhibitor-treated HepG2 cells but not in cells treated with JAKI
- Results suggest that JAK1 and JAK2 are essential for the OM-mediated inhibition of *PCSK9* gene expression



OM lowers plasma TC and LDL-C in hypercholesterolemic hamsters

- Hamsters were fed a cholesterol-enriched diet for 2 weeks and then treated with PBS or OM (0.2 mg/kg of body weight for 8 days)
- OM treatment lowered serum TC levels and LDL-C to 53.7% ± 10.4% and 71.2% ± 8%, respectively (P < 0.001 vs untreated control cells for both)
- Is the cholesterol-lowering effect of OM related to its ability to lower PCSK9 expression in the liver ?



OM reduces PCSK9 levels and increases LDL-R levels in hypercholesterolemic hamsters

- Total hepatic RNA was isolated from hamsters with dietary-induced hypercholesterolemia untreated or treated with 0.2 mg/kg OM for 8 days
- RT-PCR showed a 1.7-fold increase in LDL-R mRNA and a 31% reduction in *PCSK9* mRNA with vs without OM treatment
- Western blot analysis showed a 41% decrease in PCSK9 protein in OM-treated livers vs control

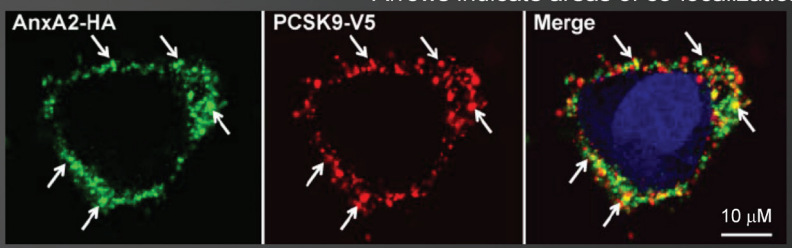
Naturally-occurring small molecule inhibitors: Annexin A2 (AnxA2)

DEFINING TOMORROW'S VASCULAR STRATEGIES

3A PCSK9 in health: Annexin A2 (AnxA2)

PCSK9 and AnxA2 co-localize at the cell surface in CHO-K1 cells

Arrows indicate areas of co-localization

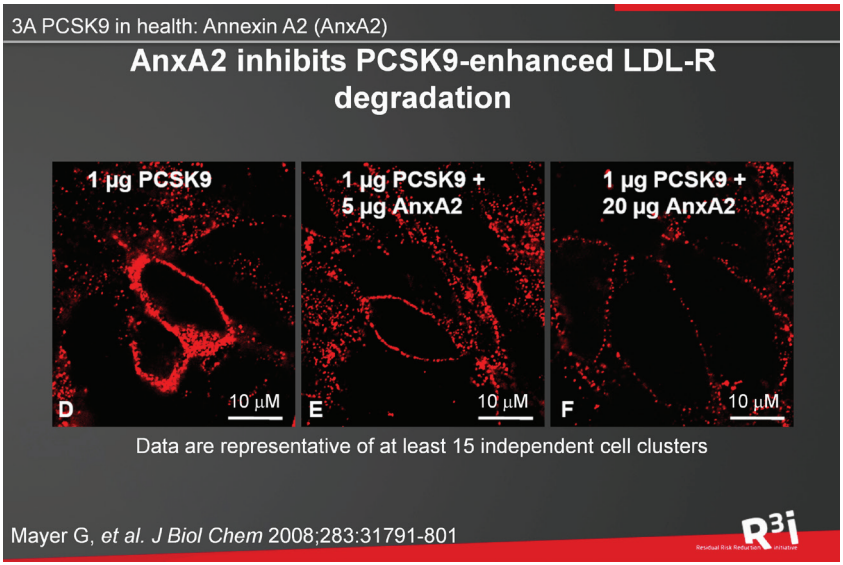


Data are representative of at least 20 independent cell clusters

Mayer G, *et al. J Biol Chem* 2008;283:31791-801

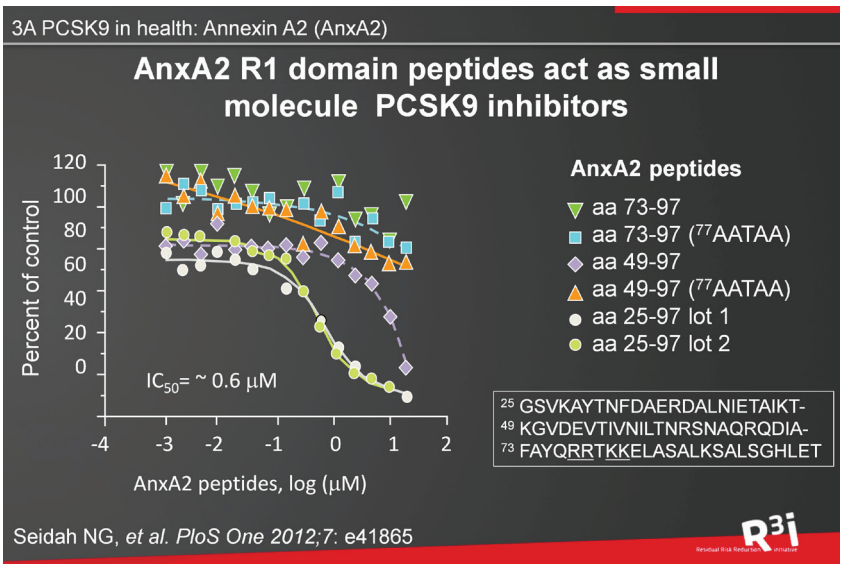
PCSK9 and AnxA2 co-localize at the cell surface in CHO-K1 cells

- AnxA2 is a heterotetrameric cytosolic and membrane-associated calcium-dependent phospholipid binding protein, strongly expressed in lungs, aorta, heart, adrenals and small intestine
- It is a co-receptor for plasminogen and TPA and promotes vascular fibrinolysis via plasmin generation
- PCSK9-V5 and AnxA2-HA transfected CHO-K1 cells were fixed under nonpermeabilizing conditions and labeled with the anti-V5 (red) and anti-HA tag (green) antibodies. The nuclei of transfected cells are marked by EGFP fluorescence (blue)
- Immunofluorescence staining demonstrates a partial co-localization of PCSK9 with AnxA2 at the plasma membrane of CHO-K1 cells



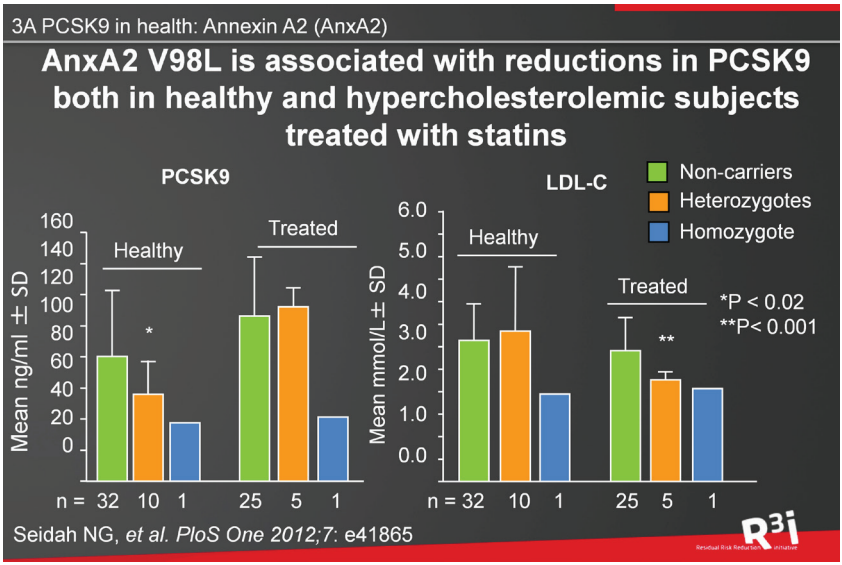
AnxA2 inhibits PCSK9-enhanced LDL-R degradation

- CHO-K1 cells over-expressing *LDL-R* were incubated at 4°C for 1 h (preventing internalization) with 1 mg of purified PCSK9-His6 alone (*D*) or with 5 mg (*E*) or 20 mg (*F*) of purified AnxA2-His6
- Cells were fixed under nonpermeabilizing conditions and cell surface PCSK9 was visualized by immunofluorescence using the anti-PCSK9 antibody
- The cell-surface localization of PCSK9 was reduced by the addition of purified AnxA2 suggesting that AnxA2 is a natural small molecule inhibitor of PCSK9



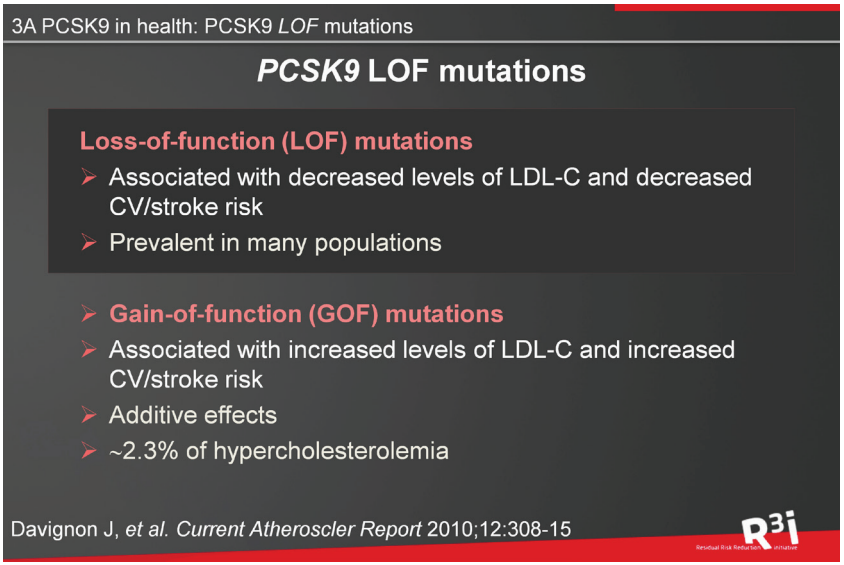
AnxA2 R1 domain peptides act as small molecule PCSK9 inhibitors

- His-tagged LDL-R ectodomain (15nM) and biotinylated PCSK9 (15nM) were incubated together with increasing concentrations of AnxA2 peptides for 60 min at 20°C
- Luminescence monitoring revealed a dose-dependent inhibition of the PCSK9/LDL-R interaction with AnxA2
- The longest AnxA2 peptide (aa 25–97) inhibited binding with an IC_{50} of 0.6 µM



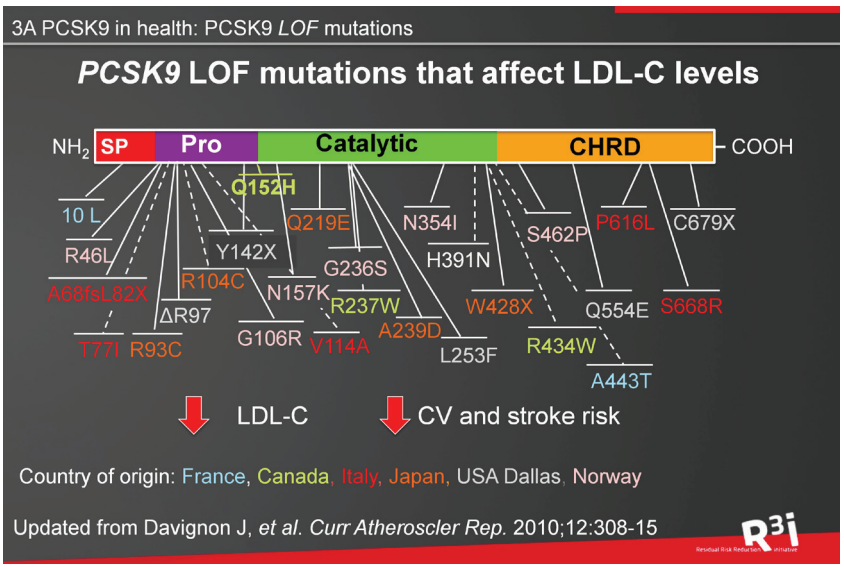
AnxA2 V98L is associated with reductions in PCSK9 both in healthy and hypercholesterolemic subjects treated with statins

- In untreated healthy subjects (n = 43), carriers of the AnxA2 V98L variant had a significant 30% reduction in circulating PCSK9 levels and no change in LDL-C vs non-carriers
- In hypercholesterolemic subjects treated with statins (n = 31), there was no change in PCSK9 levels in AnxA2 V98L carriers vs non-carriers due to the dominant effect of statins on PCSK9 levels masking the effect of the heterozygous mutation; as expected, LDL-C levels were significantly reduced
- In V98L homozygotes, reductions in PCSK9 and LDL-C levels were similar (~50% and ~30%, respectively) both in healthy and in statin-treated subjects



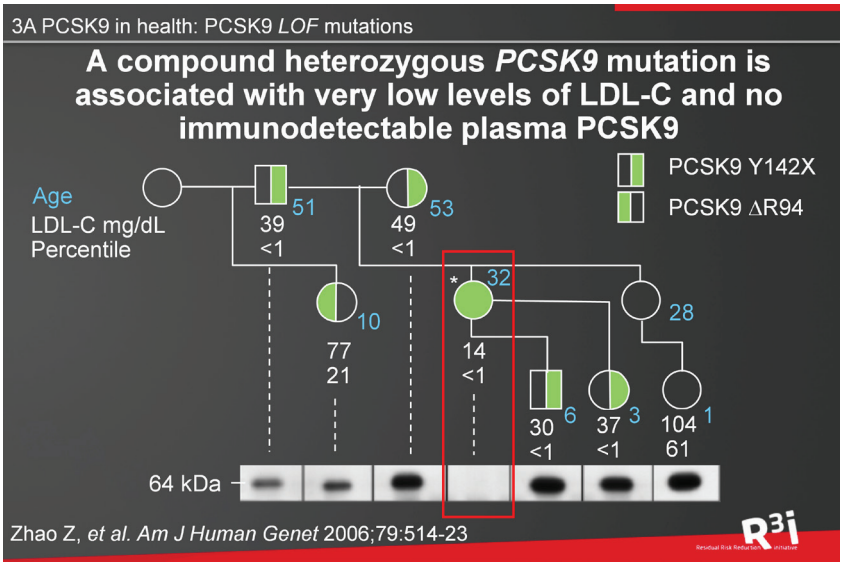
***PCSK9* LOF mutations**

- LOF mutations are associated with decreased levels of LDL-C and decreased CV/stroke risk. They are prevalent in many populations
- In contrast, GOF mutations are associated with increased levels of LDL-C and increased CV/stroke risk. They account for ~2.3% of hypercholesterolemia cases



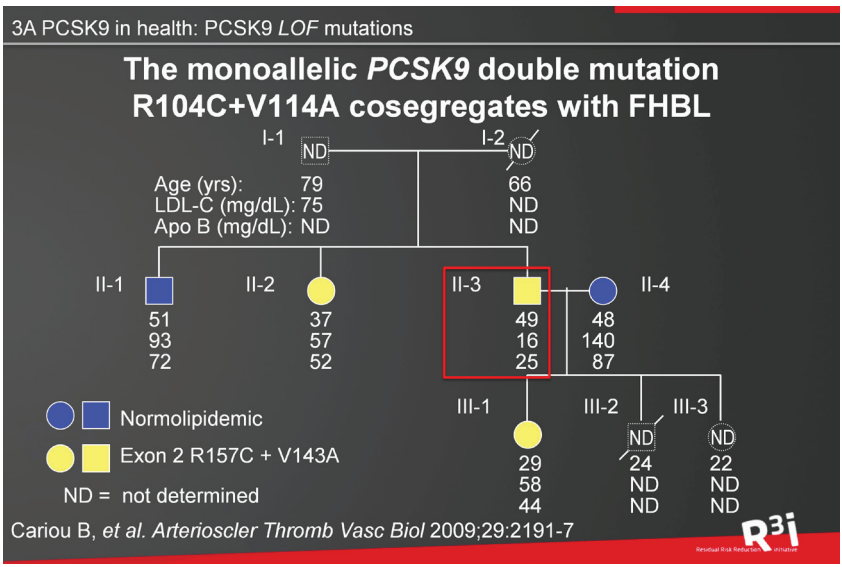
***PCSK9* LOF mutations that affect LDL-C levels**

- The LOF mutations associated with low LDL-C levels and low levels of CV and stroke risk are shown below the protein
- The color code indicates the country of origin for each mutation



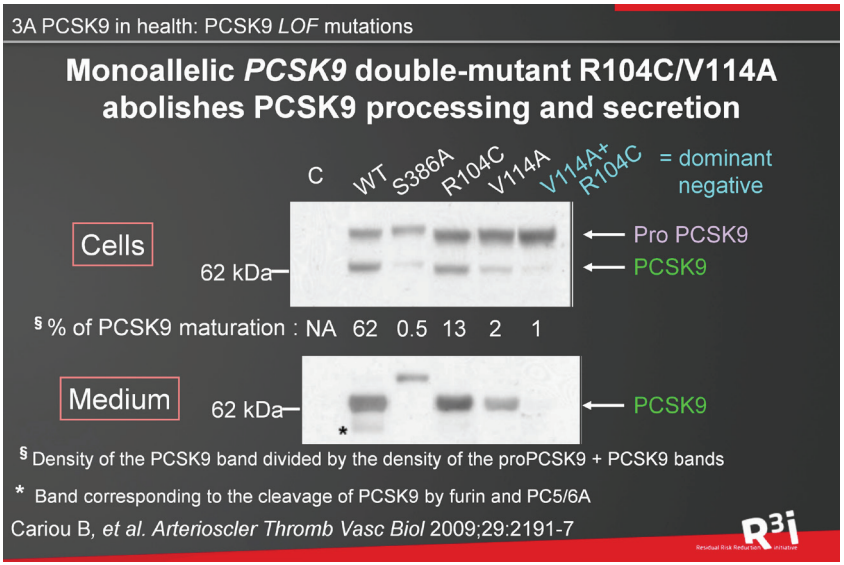
A compound heterozygous PCSK9 mutation is associated with very low levels of LDL-C and no immunodetectable plasma PCSK9

- An African-American family was identified in which 4 members were heterozygous for the nonsense Y142X allele; LDL-C levels were ~49 mg/dL (1st percentile vs age- and sex-matched controls)
- One family member (a 32 year-old female college-educated aerobics instructor) was heterozygous for an in-frame 3-bp deletion that deletes an arginine at codon 97; LDL-C levels were 14 mg/dL and no plasma PCSK9 protein was detected
- This patient was healthy, fertile, normotensive and had normal liver and renal function



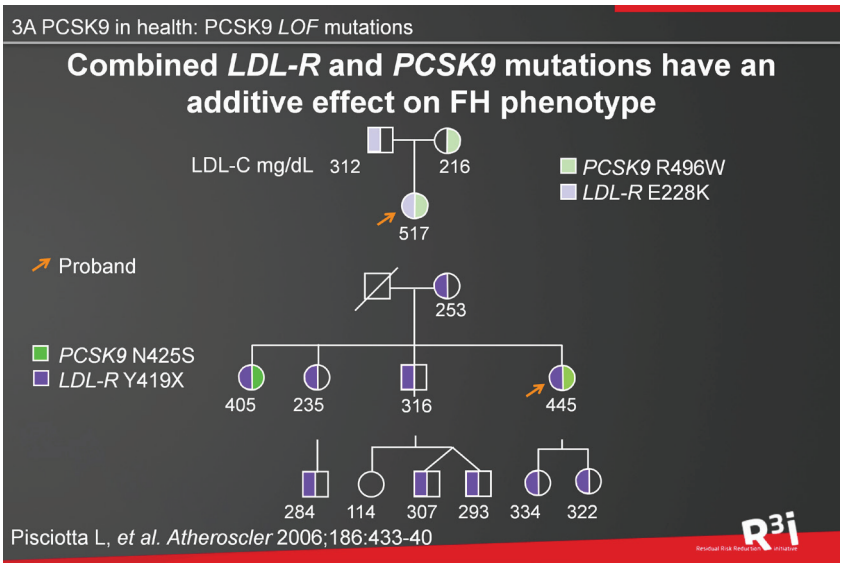
The monoallelic *PCSK9* double mutation R104C+V114A cosegregates with FHBL

- A white 49-year-old diabetic man with a monoallelic PCSK9 double mutation (R104C+V114A) had profound familial hypobetalipoproteinemia (FHBL) (LDL-C: 16 mg/dL); although his daughter and sister had the same mutations, they displayed a milder phenotype (LDL-C 44 mg/dL and 57 mg/dL, respectively)
- All family members were otherwise healthy with a normal liver function



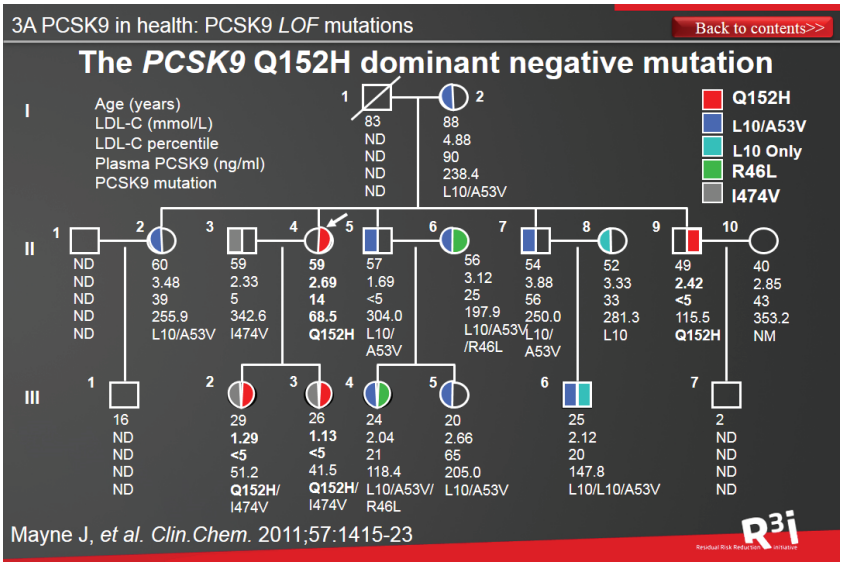
Monoallelic *PCSK9* double-mutant R104C/V114A abolishes PCSK9 processing and secretion

- IHH were transiently transfected with empty plasmid (C) or plasmid encoding either WT PCSK9, LOF PCSK9 variants S386A, R104C, V114A, or the double mutant R104C/V114A
- Western blot analysis of proteins extracted from lysates or media of cells transfected with WT PCSK9 showed bands of 73 and 64 kDA, corresponding to the pro-PCSK9 and the mature cleaved PCSK9 protein, respectively
- Cells expressing the S386A mutant appeared to secrete the uncleaved form of PCSK9 into the medium
- Mutants R104C and V114A had decreased autocatalytic activity but were secreted into the medium
- A combination of the R104C and V114A mutations abolished PCSK9 processing and secretion



Combined *LDL-R* and *PCSK9* mutations have an additive effect on FH phenotype

- *Apo B* and *PCSK9* genes were sequenced in the families of 2 patients with homozygous FH who were heterozygous for *LDL-R* gene mutations
- LDL-C levels in double heterozygotes were 56 and 44% higher than those found in simple heterozygotes for the two *LDL-R* mutations, respectively
- These observations indicate that rare missense mutations in *PCSK9* may worsen the clinical phenotype of patients carrying *LDL-R* mutations



The *PCSK9* Q152H dominant negative mutation

- White French-Canadian family members carrying the PCSK9 Q152H variant had mean decreases in circulating PCSK9 and LDL-C concentrations of 79% and 48%, respectively, compared with unrelated noncarriers (n=210)
- In cell culture, the proPCSK9-Q152H variant did not undergo efficient autocatalytic cleavage and was not secreted
- Cells transiently transfected with PCSK9-Q152H cDNA had significantly higher LDL-R concentrations vs WT

PCSK9 in disease

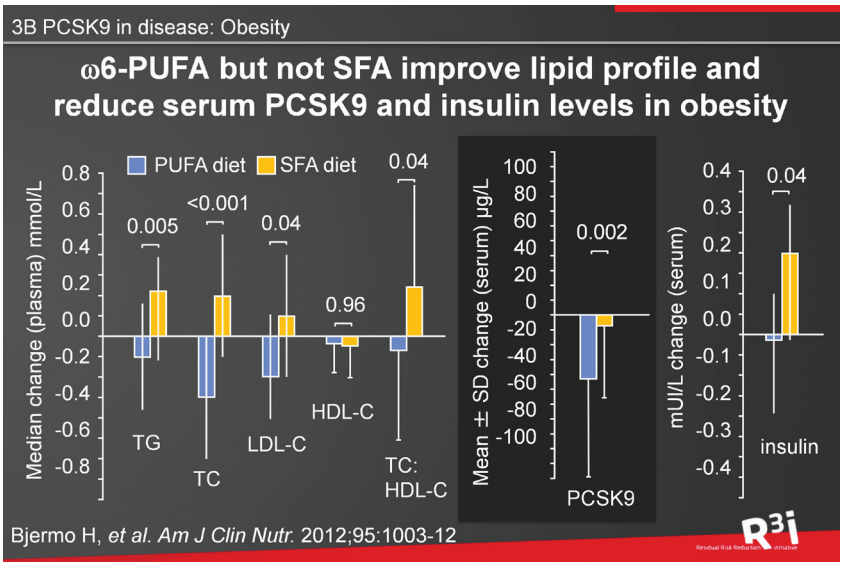
SECTION

3B

Residual Risk Reduction initiative

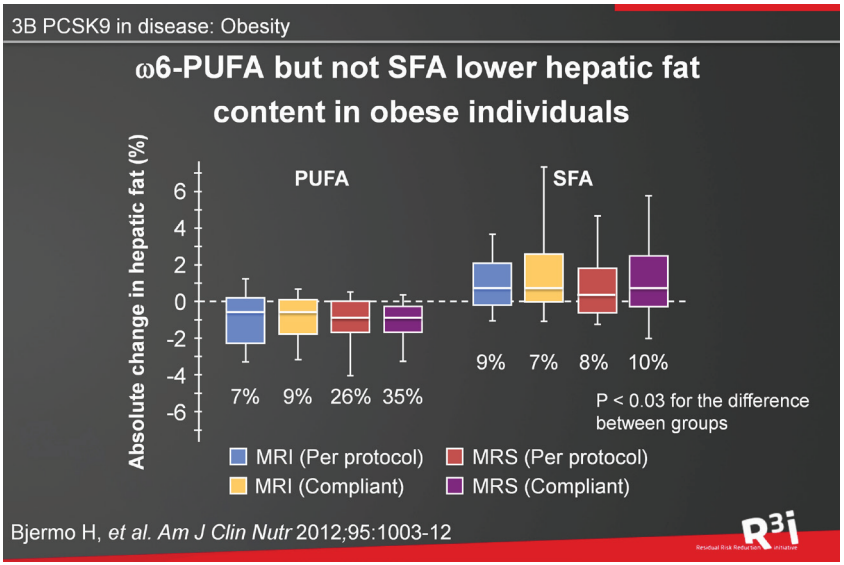
Obesity

DEFINING TOMORROW'S VASCULAR STRATEGIES



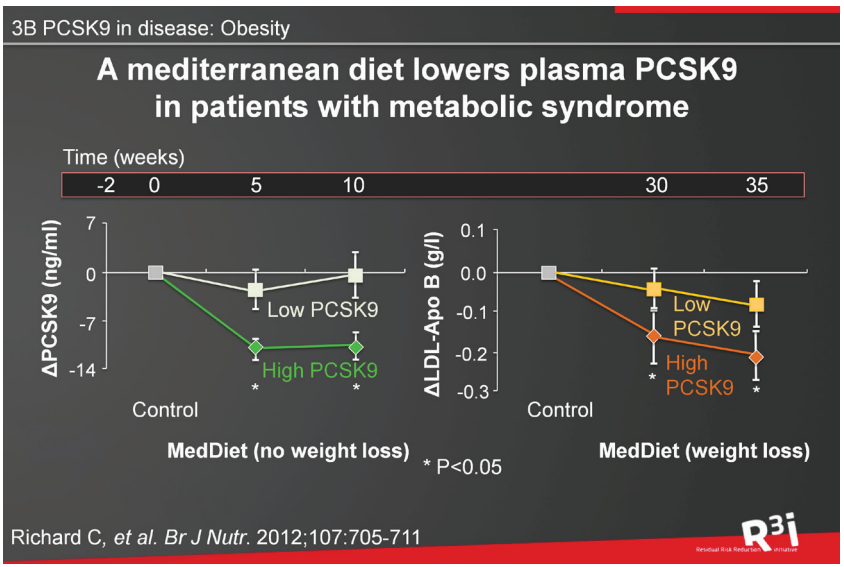
w6-PUFA but not SFA improve lipid profile and reduce serum PCSK9 and insulin levels in obesity

- 67 abdominally obese subjects (15% with T2D) were randomized to a 10-wk isocaloric diet high in n-6 polyunsaturated fatty acids (PUFA, mostly vegetables) or saturated fatty acid (SFA, mostly butter), without altering the macronutrient intake. Liver fat was assessed by magnetic resonance imaging (MRI) and magnetic resonance proton (1H) spectroscopy (MRS)
- Compared to the SFA diet, the PUFA diet was associated with an improved plasma lipid profile and reduced levels of PCSK9 and insulin



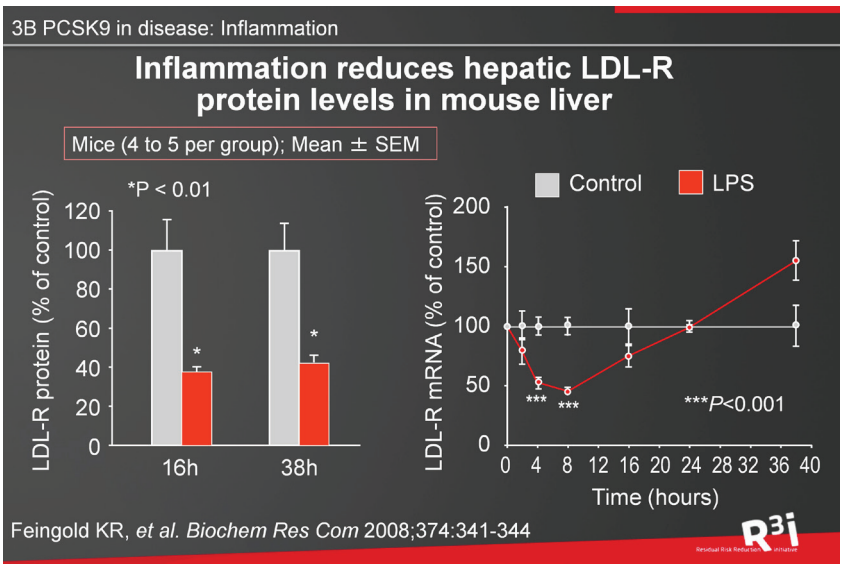
ω6-PUFA but not SFA lower hepatic fat content in obese individuals

- Boxes represent the 25th, 50th, and 75th percentiles ; the lower lines indicate the 10th percentile and the upper lines the 90th percentile
- Hepatic fat content was lower both in compliant subjects and in per protocol subjects during the PUFA diet vs the SFA diet
- A high n-6 PUFA intake was not associated with weight loss and did not cause any signs of inflammation or oxidative stress
- Down-regulation of *PCSK9* could be a novel mechanism behind the cholesterol-lowering effects of PUFAs



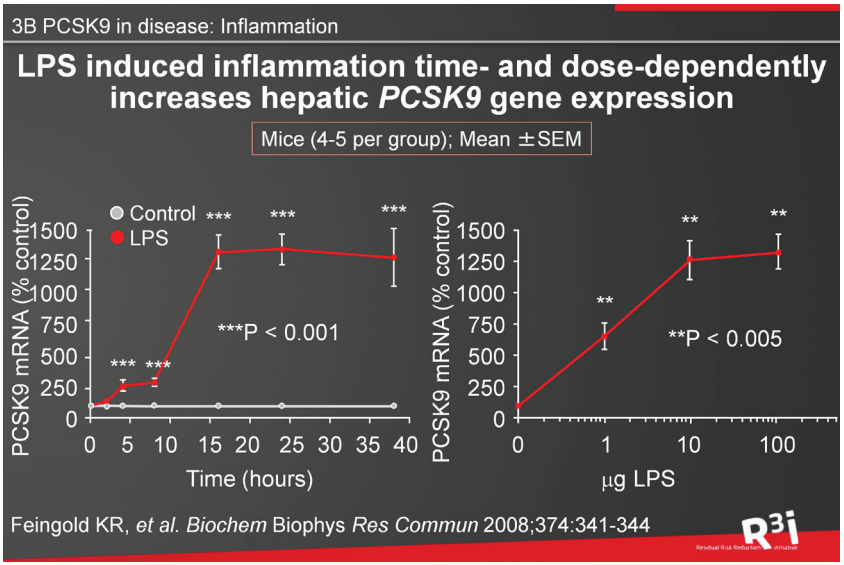
A mediterranean diet lowers plasma PCSK9 in patients with metabolic syndrome

- Subjects stratified according to baseline PCSK9 levels followed a North American control diet for 5 weeks, a mediterranean diet (MedDiet) for 5 weeks (both under isoenergetic, weight-maintaining conditions), a 20-week weight-loss period and a second MedDiet for an additional 5 weeks
- Baseline plasma PCSK9 concentrations on the control diet modulated both the PCSK9 and the LDL–Apo B response to the MedDiet with and without weight loss



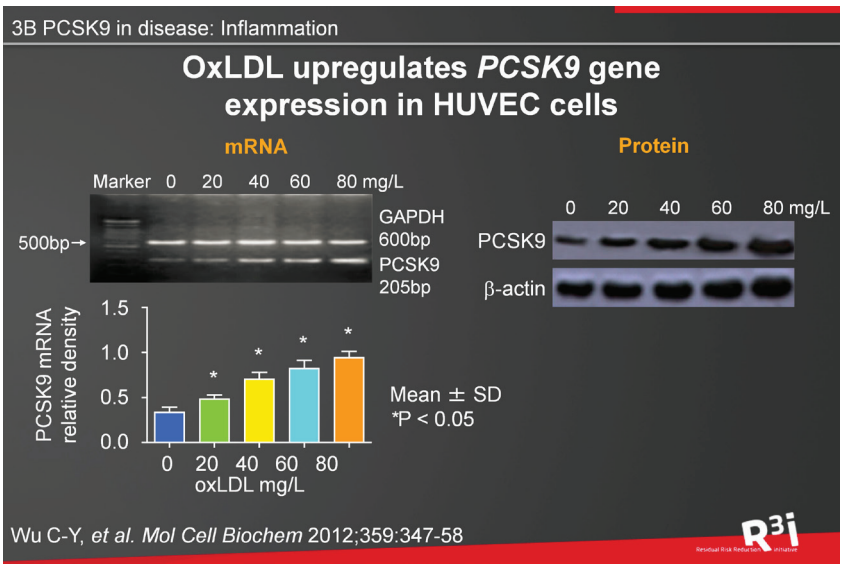
Inflammation reduces hepatic LDL-R protein levels in mouse liver

- Mice injected with i.p. LPS (5 mg/kg body weight) were euthanized at the indicated times after LPS administration
- Western blot analysis showed a significant reduction in hepatic LDL-R protein levels at 16 and 38 h after LPS injection. These reductions did not correlate with changes in LDL-R mRNA levels



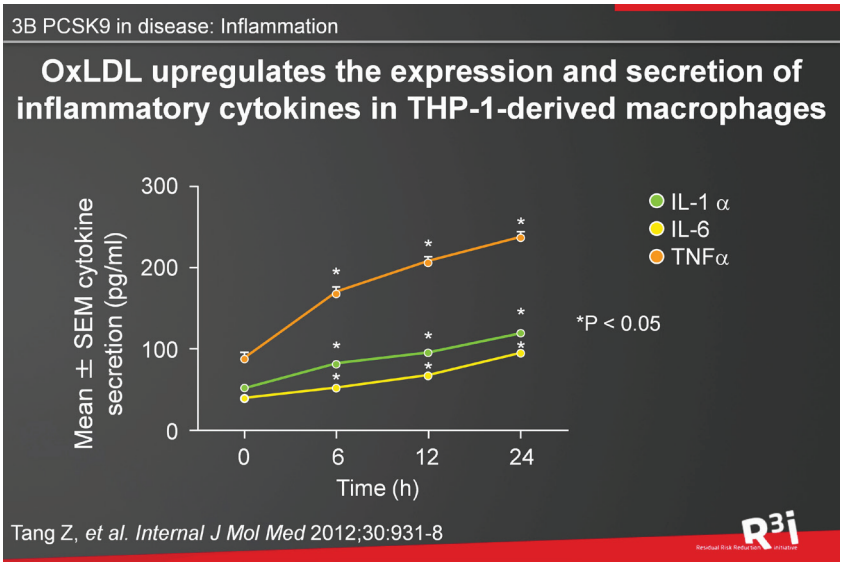
LPS induced inflammation time- and dose-dependently increases hepatic *PCSK9* gene expression

- Mice injected with i.p. LPS (5 mg/kg body weight) were euthanized at the indicated times after LPS administration
- A significant increase in PCSK9 mRNA levels in LPS-injected vs control mice was observed at all time points; this effect was dose-dependent



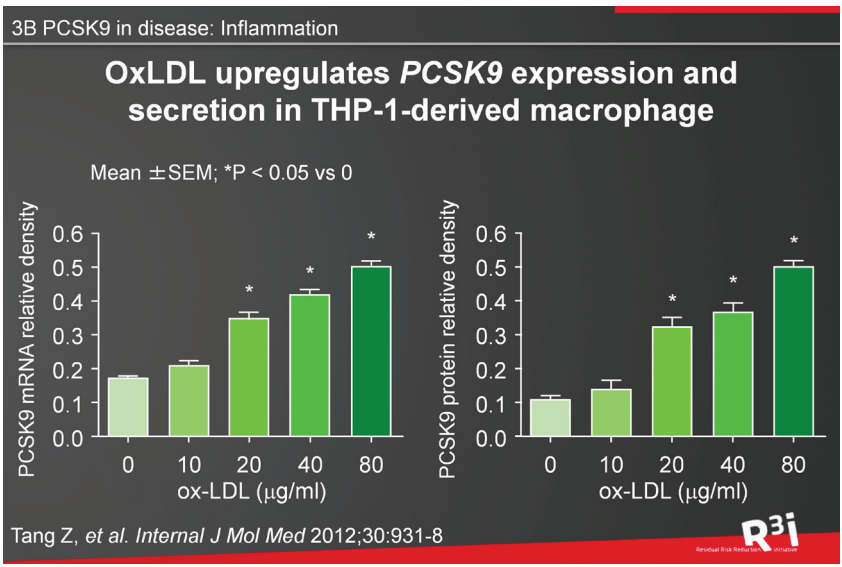
OxLDL upregulates *PCSK9* gene expression in HUVEC cells

- HUVECs were incubated with oxLDL for 24 h; PCSK9 mRNA and protein was detected by RT-PCR and Western blot, respectively
- OxLDL upregulated PCSK9 mRNA and protein levels in HUVECs in a concentration-dependent manner



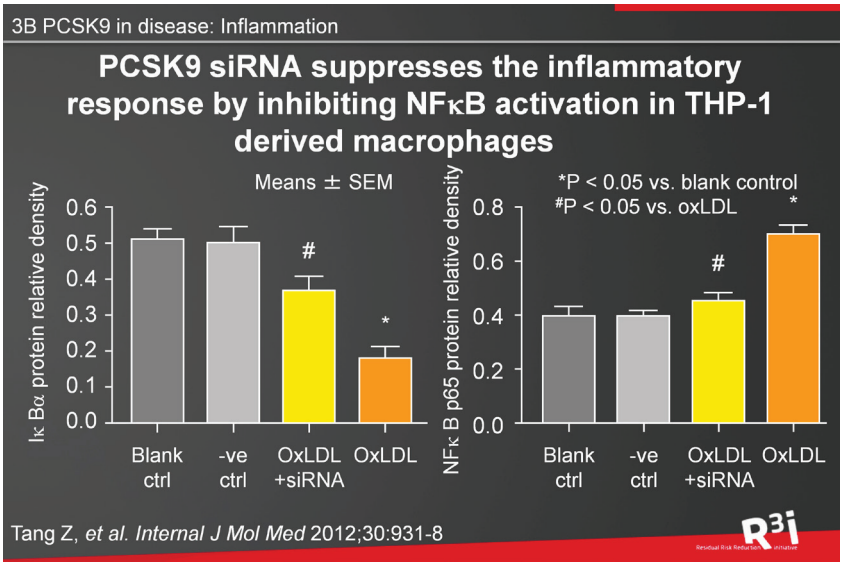
OxLDL upregulates the expression and secretion of inflammatory cytokines in THP-1 derived macrophages

- Levels of IL-lα, IL-6 and TNF-α secretion from THP-1 derived macrophages pre-incubated with 80 μg/ml oxLDL for 24 h were measured using ELISA
- OxLDL increased the expression of inflammatory cytokines in a time- and dose-dependent manner



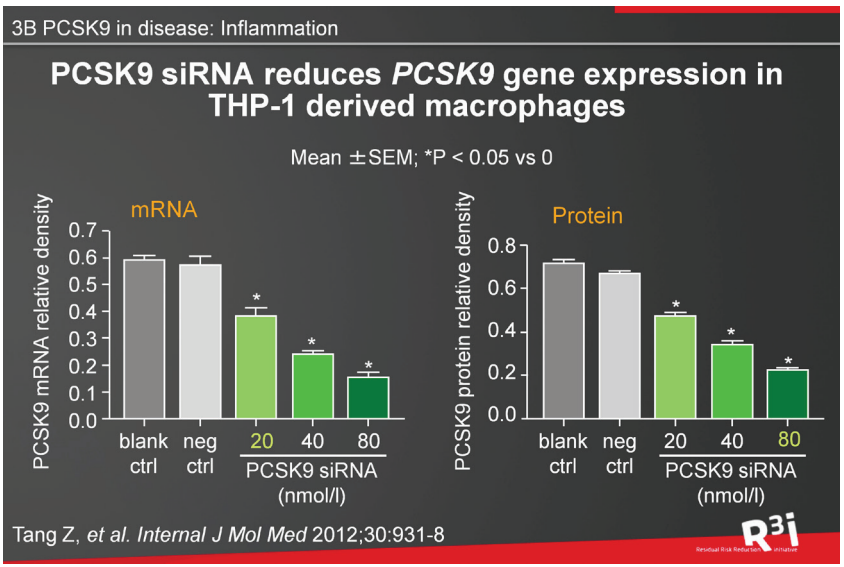
OxLDL upregulates *PCSK9* expression and secretion in THP-1-derived macrophage

- THP-1 derived macrophages were incubated with 0, 10, 20, 40 and 80 μg/ml oxLDL for 24 h
- Total RNA was extracted and PCSK9 mRNA levels were measured using rtPCR. PCSK9 protein was detected by Western blot analysis
- OxLDL dose-dependently upregulated *PCSK9* expression



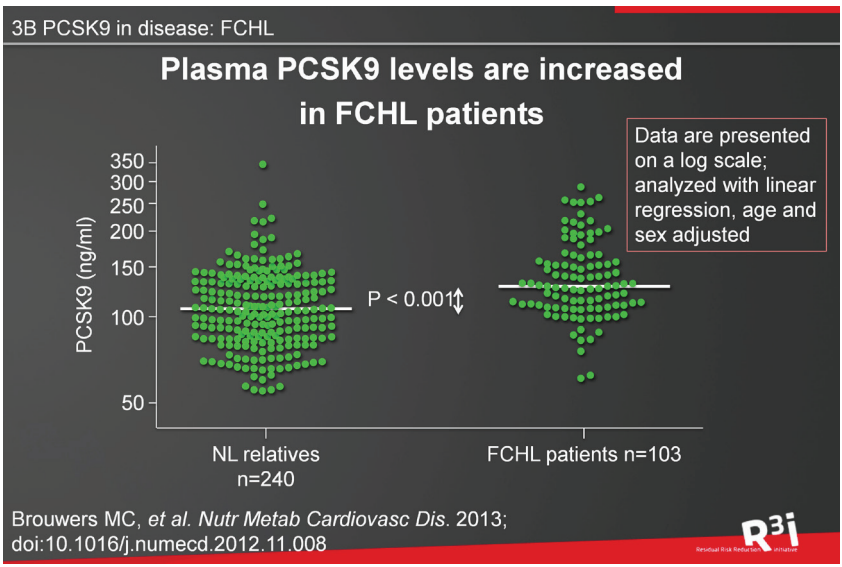
PCSK9 siRNA suppresses the inflammatory response by inhibiting NFkB activation in THP-1 derived macrophages

- THP-1-derived macrophages were transfected with or without 80 nmol PCSK9 siRNA and incubated with 80 μg/ml oxLDL for 24 h. Levels of IκBα and nuclear NFκB protein were detected by Western blotting
- OxLDL markedly increased IκBα protein degradation and NFκB protein expression in the nucleus; these effects were suppressed by PCSK9 siRNA



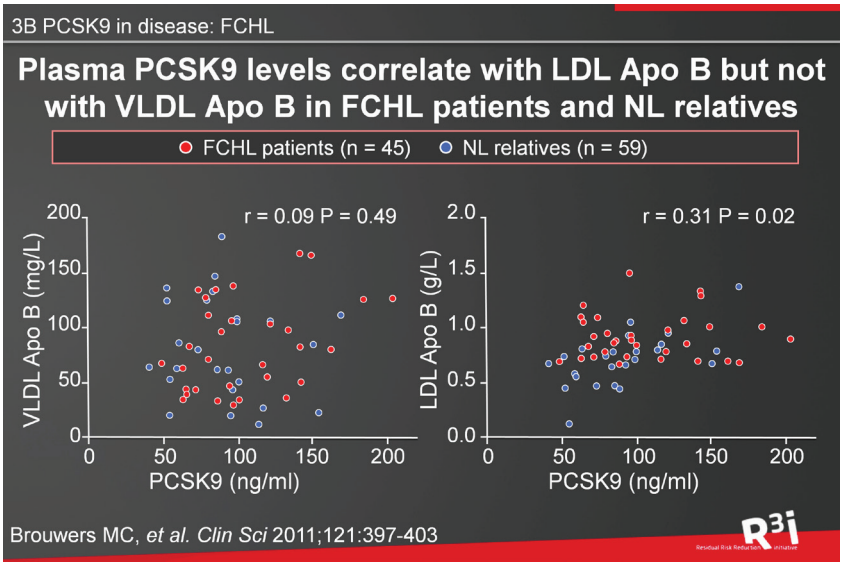
PCSK9 siRNA reduces *PCSK9* gene expression in THP-1 derived macrophages

- THP-1-derived macrophages were transfected with 20, 40 and 80 nM siRNA; PCSK9 mRNA and protein levels were detected 24 h after transfection using rtPCR and Western blotting, respectively
- PCSK9 siRNA dose-dependently reduced *PCSK9* gene expression
- Overall, this study suggests that PCSK9 expression is induced by oxLDL, and that PCSK9 siRNA protects against inflammation via the inhibition of NFκB activation in oxLDL-stimulated THP-1-derived macrophages



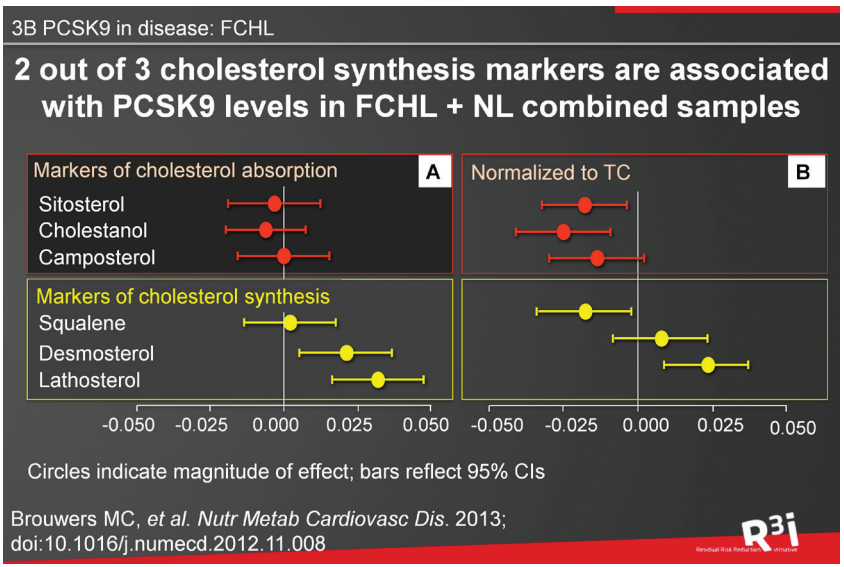
Plasma PCSK9 levels are increased in FCHL patients

- Median age- and sex-adjusted plasma PCSK9 levels were higher (127.9 ng/mL; inter-quartile range 107.7-161.6) in FCHL patients (n = 103) than in their normolipidemic (NL) relatives (106.0 ng/mL;84.6-133.6) (n = 240)



Plasma PCSK9 levels correlate with LDL Apo B but not with VLDL Apo B in FCHL patients and NL relatives

- Apo B concentrations in VLDL and LDL subfractions were measured by ultracentrifugation in FCHL patients (red circles) and NL relatives (blue circles) of a similar age and sex distribution
- Plasma PCSK9 levels were not significantly related to VLDL Apo B levels, whereas a significant relationship was observed for LDL Apo B levels
- The correlation of PCSK9 with TG levels is not accounted for by VLDL Apo B but by LDL Apo B



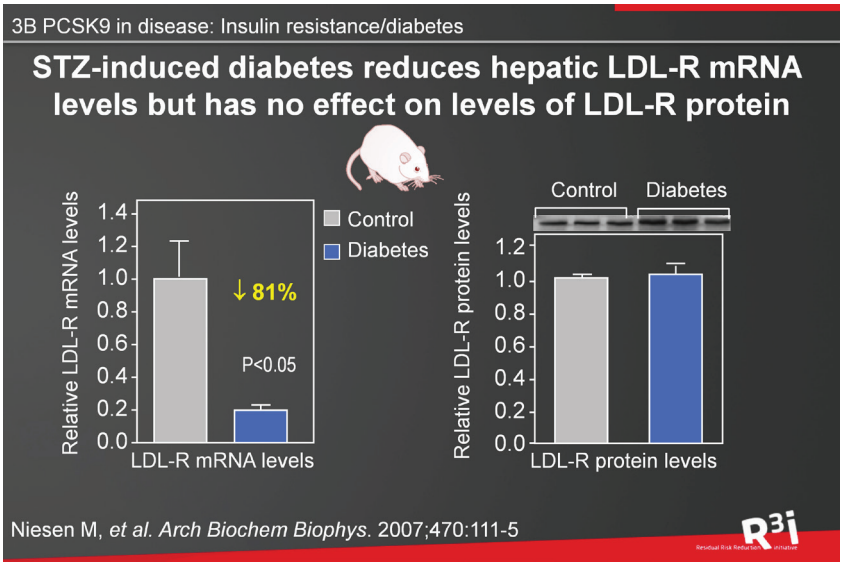
2 out of 3 cholesterol synthesis markers are associated with PCSK9 levels in FCHL + NL combined samples

(A) Two out of 3 cholesterol synthesis markers (lathosterol and desmosterol) were associated with plasma PCSK9 levels in the overall FCHL population (FCHL patients and their NL relatives combined). This was not observed for any of the cholesterol absorption markers

(B) Normalizing to TC did not affect results for lathosterol, although the strength of association was reduced. The relationship between PCSK9 and desmosterol/TC was no longer significant. Of note, all absorption markers and squalene/TC were inversely related with PCSK9 levels

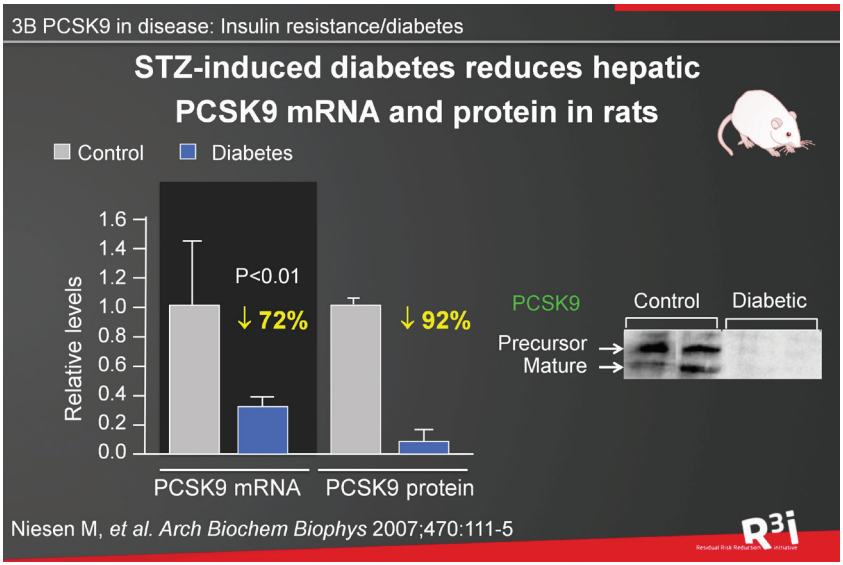
Insulin resistance/diabetes

DEFINING TOMORROW'S VASCULAR STRATEGIES



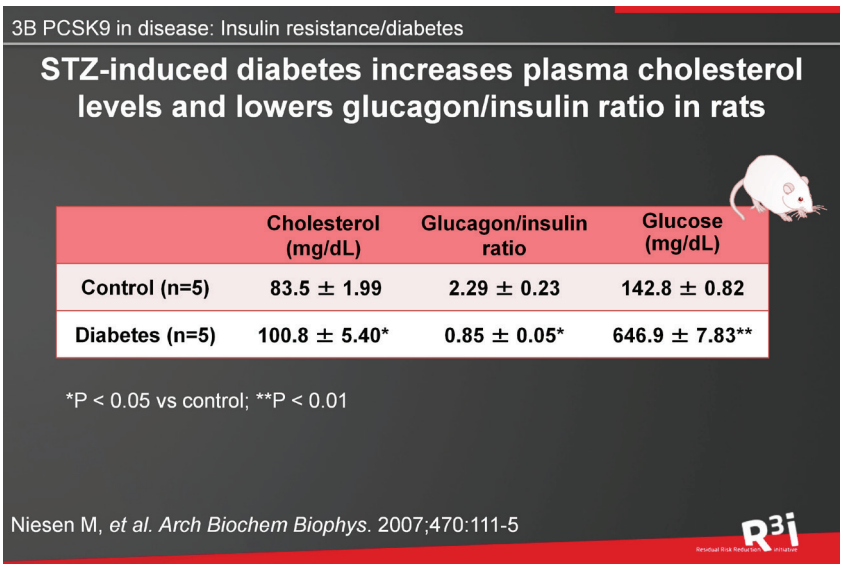
STZ-induced diabetes reduces hepatic LDL-R mRNA levels but has no effect on levels of LDL-R protein

- Rats were made diabetic by injecting with streptozotocin (STZ) and euthanized 10 days later
- qRT-PCR and Western blot analyses showed that diabetes resulted in an 81% reduction in hepatic LDL-R mRNA but no change in LDL-R protein levels
- This suggests LDL-R protein is degraded in the diabetic state



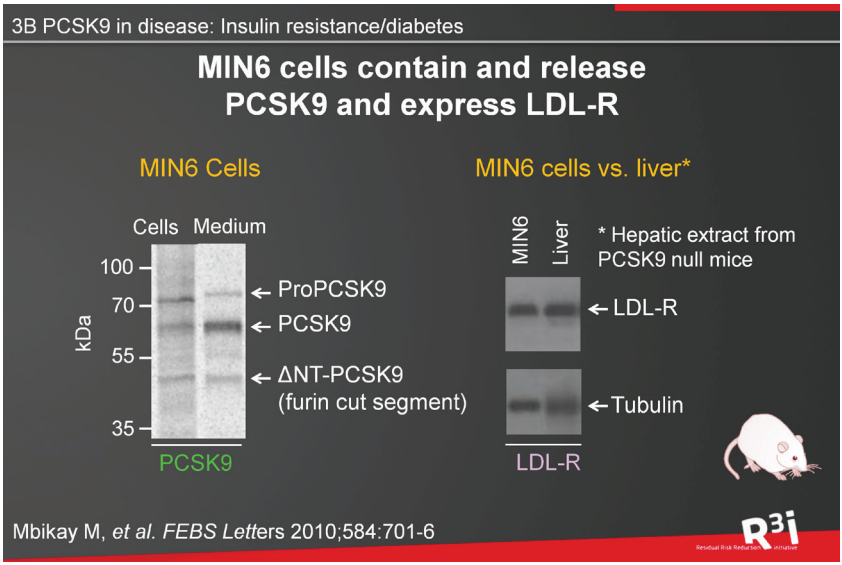
STZ-induced diabetes reduces hepatic PCSK9 mRNA and protein in rats

- PCSK9 protein levels were significantly decreased during diabetes to almost undetectable levels
- This reduction correlated with a 72% reduction in PCSK9 mRNA levels
- A reduction in PCSK9 levels could lead to reduced degradation of the LDL-R protein. This might explain why LDL-R protein levels were unaffected in diabetic rats even though LDL-R mRNA levels were reduced by 81%



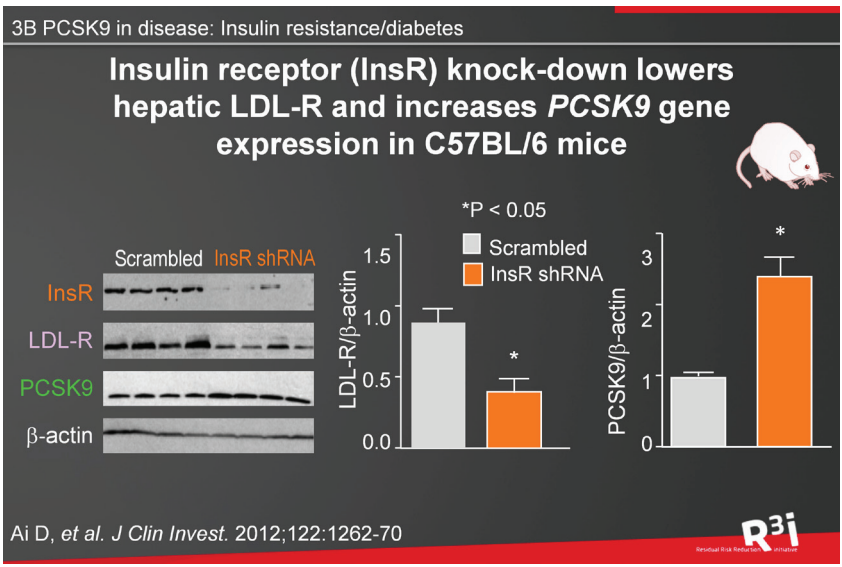
STZ-induced diabetes increases plasma cholesterol levels and lowers glucagon/insulin ratio in rats

- Serum glucose levels were 4.5 times higher in animals with vs without diabetes, whereas the glucagon/insulin ratio was significantly lower
- Serum cholesterol levels were significantly increased in animals with vs without diabetes; this increase is consistent with the observed reduction in LDL-R mRNA and the increase in PCSK9 mRNA and protein levels



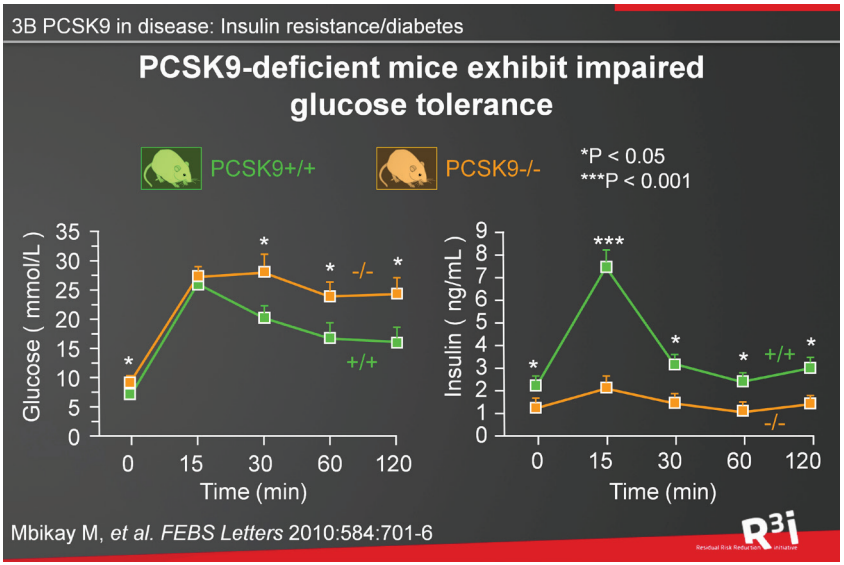
MIN6 cells contain and release PCSK9 and express LDL-R

- MIN6 cells were grown in medium containing ^{35}S-Met/Cys to metabolically label *de novo* biosynthesized proteins
- Immunoprecipitation using an anti-mouse PCSK9 antibody showed that cell lysates contained immunoreactive proteins corresponding to the mass expected for proPCSK9, PCSK9, and the furin-cleaved DNT218-PCSK9, whereas spent media contained predominantly the 62-kDa form of PCSK9
- LDL-R was detected in MIN6 cells at levels comparable to those found in normal liver



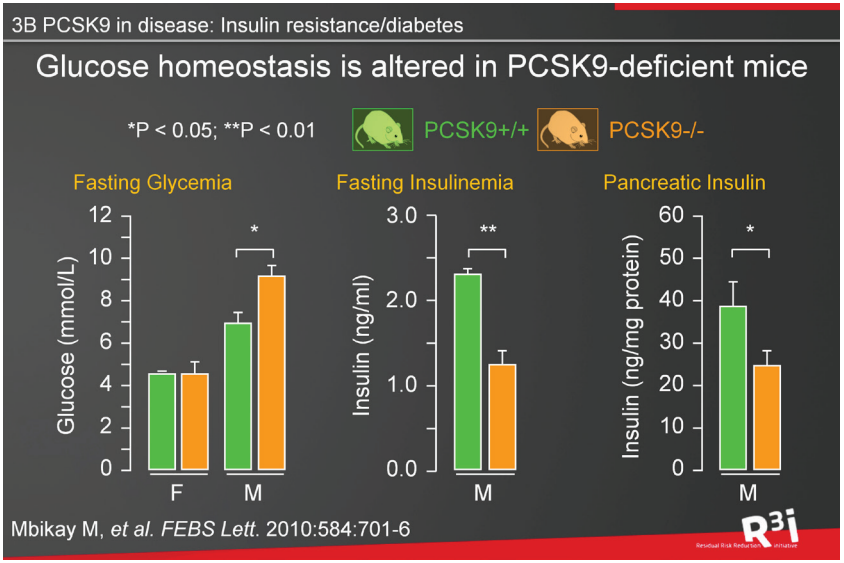
Insulin receptor (InsR) knock-down lowers hepatic LDL-R and increases *PCSK9* gene expression in C57BL/6 mice

- Knockdown of hepatic InsR by shRNA in chow-fed, 5-hour fasted C57BL/6 mice resulted in an 85% reduction in InsR and a 57% reduction in LDL-R protein levels compared with controls
- In contrast, PCSK9 protein levels were increased 2.4-fold
- This suggests that PCSK9 increases might be responsible for the reduction in LDL-R that results from decreased insulin signalling



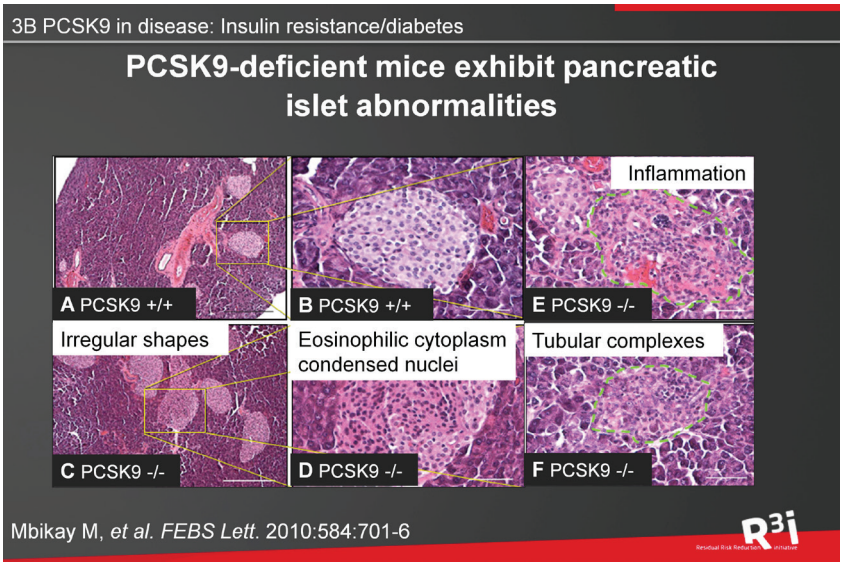
PCSK9-deficient mice exhibit impaired glucose tolerance

- *PCSK9*+/+ and *PCSK9*-/- male mice aged 4 months or older (n = 9/genotype) were subjected to an oral glucose tolerance test
- Plasma glucose was not different between the 2 genotypes at 15 min, but was significantly higher in mutant mice at later time points
- Insulin levels barely changed in *PCSK9*-/- mice but dramatically increased in *PCSK9*+/+ mice after 15 minutes; levels returned to baseline at subsequent time points



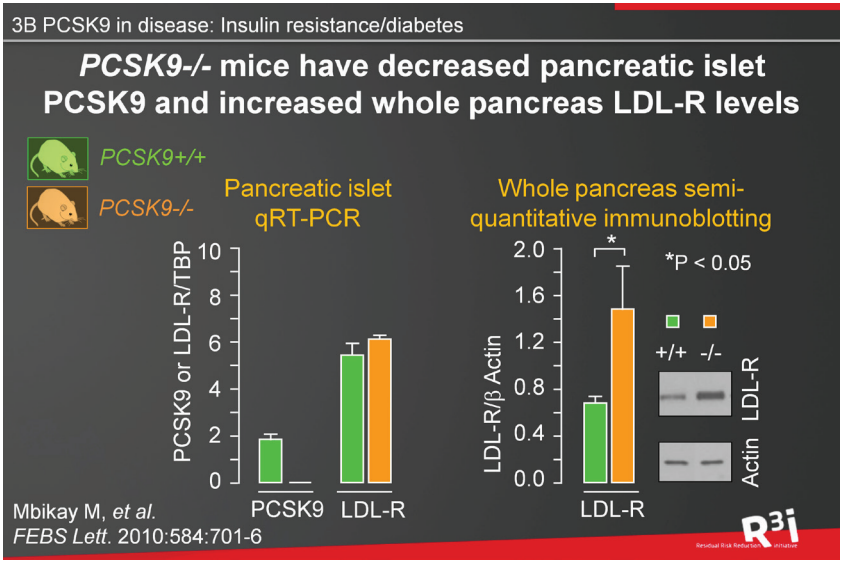
Glucose homeostasis is altered in PCSK9-deficient mice

- Female (F) and male (M), *PCSK9*+/+ and *PCSK9*-/- 4-month-old mice (n = 8/gender/genotype) were phenotyped for glucose homeostasis parameters
- Fasting glycemia was similar in *PCSK9*+/+ and -/- females but was increased in male *PCSK9*-/- mice; insulinemia and pancreatic insulin content were significantly reduced in male *PCSK9*-/- mice, suggesting insulin insufficiency
- The reduced susceptibility of female mice to this process is attributed to the beneficial effects of female sex hormones on β-cell function



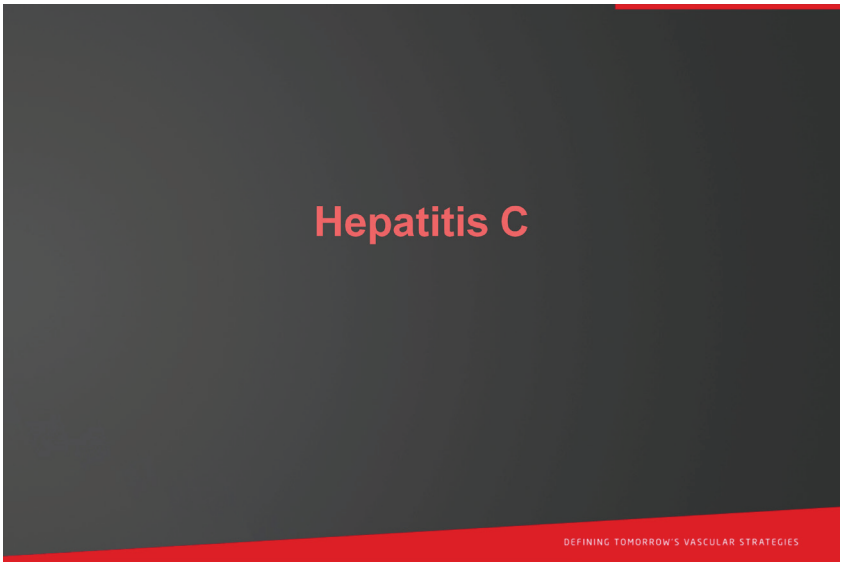
PCSK9-deficient mice exhibit pancreatic islet abnormalities

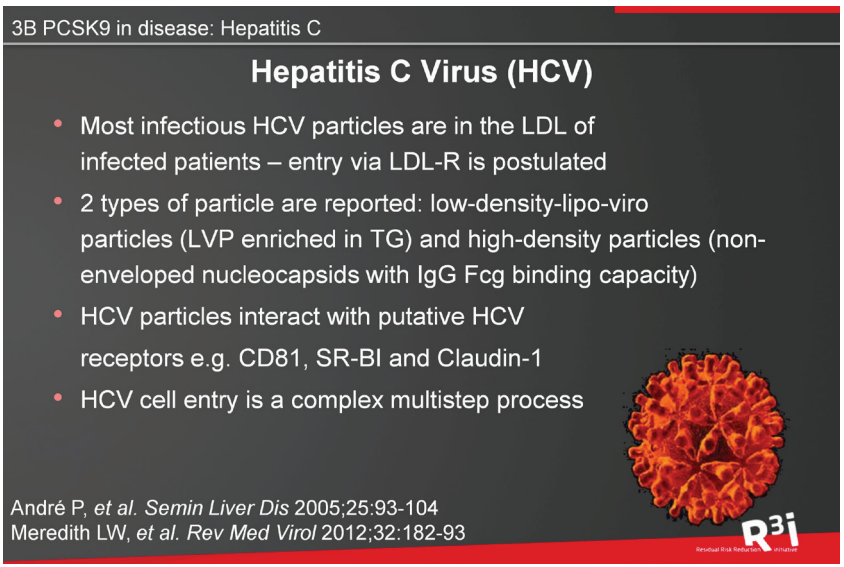
- Pancreatic cells were stained with Gill's hematoxylin solution (nuclear staining) and 0.5% eosin (cytoplasmic staining)
- Whereas pancreatic islets from *PCSK9+/+* mice appeared generally normal (panels A and B), those from *PCSK9-/-* often exhibited irregular shapes (panel C), with most β-cells displaying eosinophilic cytoplasm and condensed nuclei (panel D), suggesting early apoptosis
- Also observed in the pancreas of these mutant mice were sites of inflammation (outlined in panel E) and occasional tubular complexes (outlined in panel F)



***PCSK9-/-* mice have decreased pancreatic islet PCSK9 and increased whole pancreas LDL-R levels**

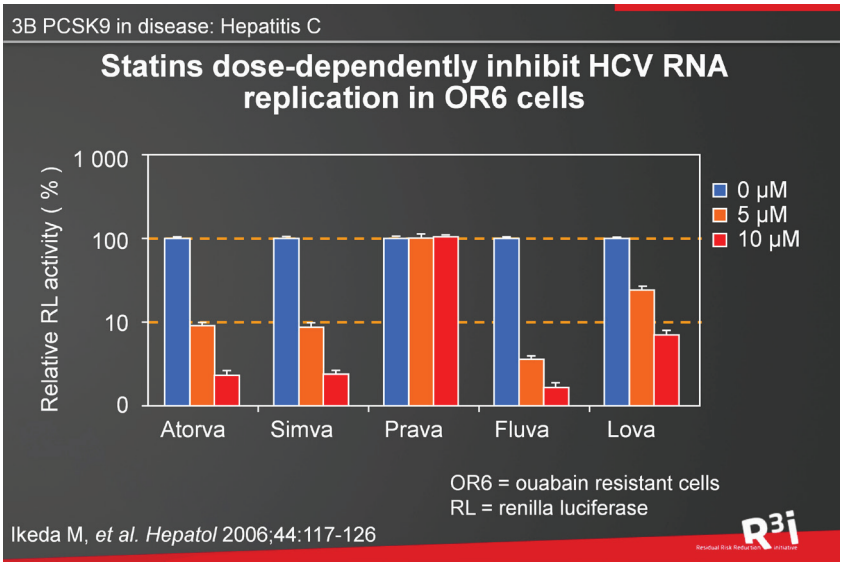
- qRT-PCR using TBP mRNA level for normalization showed that LDL-R transcripts were present at similar levels in islets from *PCSK9+/+* and *PCSK9-/-* mice, whereas *PCSK9* transcripts were present in islets from *PCSK9+/+* mice only
- sqIB analysis of proteins extracted from whole pancreas showed that *PCSK9-/-* mice contained 2-fold more LDL-R in this organ than *PCSK9+/+* mice
- Together, these changes might explain the pancreatic islet abnormalities observed in PCSK9-deficient mice





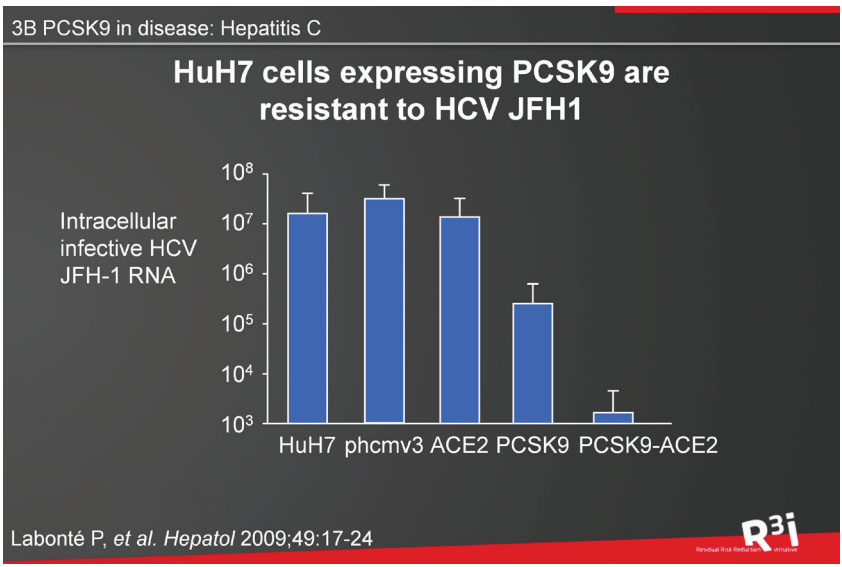
Hepatitis C Virus (HCV)

- Most infectious HCV particles are in the LDL of infected patients – entry via LDL-R is postulated
- 2 types of particle are reported: low-density-lipo-viro particles (LVP enriched in TG) and high-density particles (non-enveloped nucleocapsids with IgG Fcg binding capacity)
- HCV particles interact with a number of putative HCV receptors, including CD81, scavenger receptor class B type I (SR-BI) and Claudin-1
- HCV cell entry is a complex multistep process



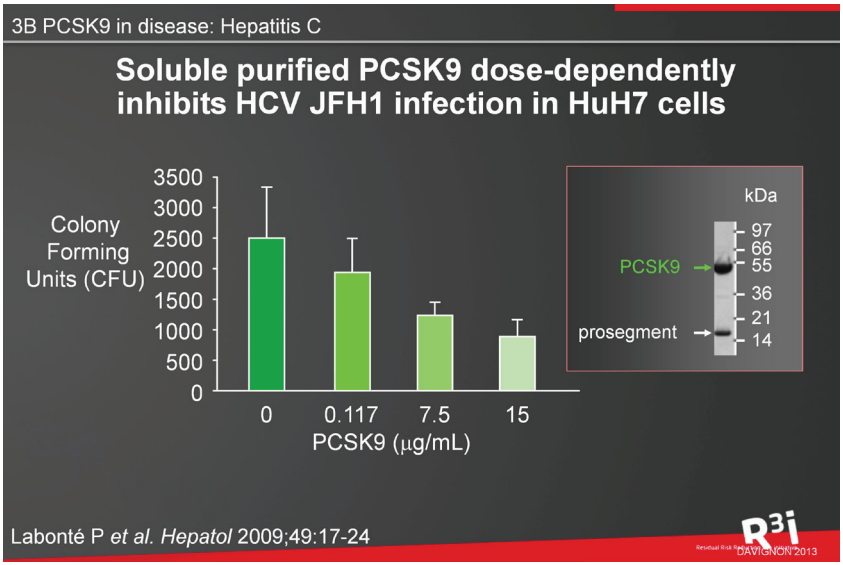
Statins dose-dependently inhibit HCV RNA replication in OR6 cells

- OR6 cells were incubated with atorvastatin, simvastatin, pravastatin, fluvastatin or lovastatin (0, 5, and 10 µmol/L) for 72 h
- Using a genome-length HCV RNA (strain O of genotype 1b) replication system with renilla luciferase (RL) as a reporter, atorvastatin, simvastatin and fluvastatin were shown to exhibit stronger anti-HCV activity than lovastatin; pravastatin had no inhibitory effect on HCV RNA replication
- With the exception of pravastatin, inhibitory effects were dose-dependent



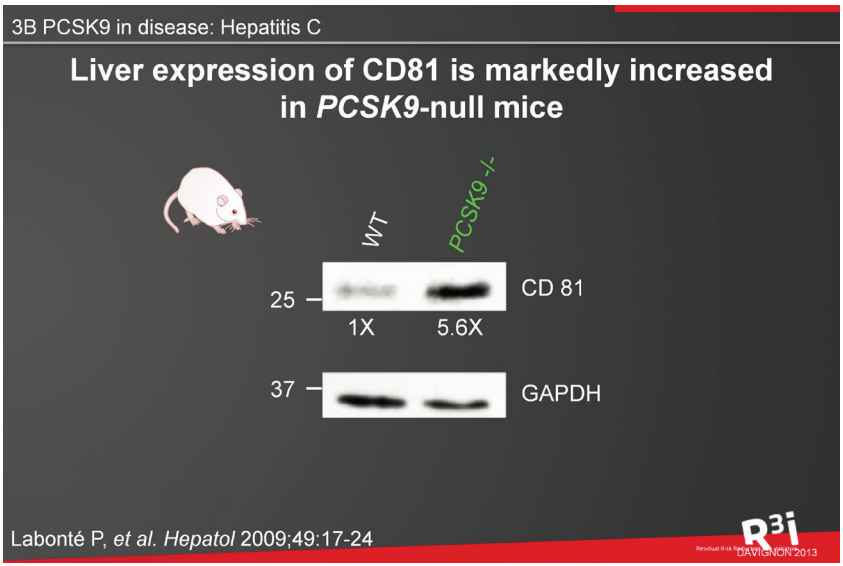
HuH7 cells expressing PCSK9 are resistant to HCV JFH1

- HuH7 cells were stably transfected with empty vector (phcmv3) or a vector coding for angiotensin converting enzyme 2 (ACE2), PCSK9 or PCSK9-ACE2 (a more active membrane-bound form of PCSK9)
- Levels of intracellular HCV genotype 2a (JFH-1) RNA 15 days post infection were calculated using qPCR and input RNAs were normalized to RNA 18S
- WT PCSK9 and PCSK9-ACE2 protein reduced the quantity of HCV genome by 90% and 99.9%, respectively; the full-length ACE2 protein had little or no effect



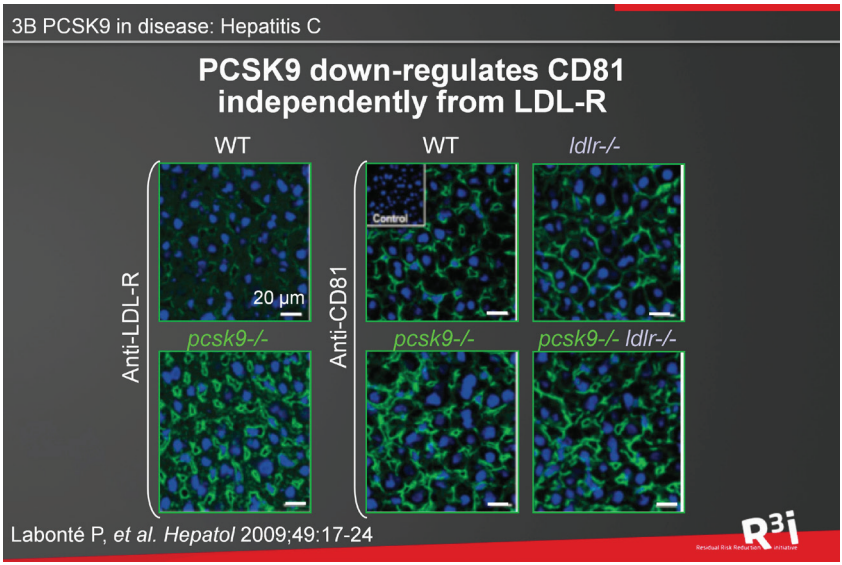
Soluble purified PCSK9 dose-dependently inhibits HCV JFH1 infection in HuH7 cells

- HuH7 cells were preincubated with 0 to 15 μg/mL of purified soluble PCSK9 for 4 h prior to infection with J6/JFH1
- After 3 days, HCV NS5A protein was stained with a rabbit polyclonal antibody and the activity of soluble PCSK9 was calculated from the colony-forming units (CFU) observed
- PCSK9 can inhibit HCV infection up to 2.5-fold in a dose-dependent manner



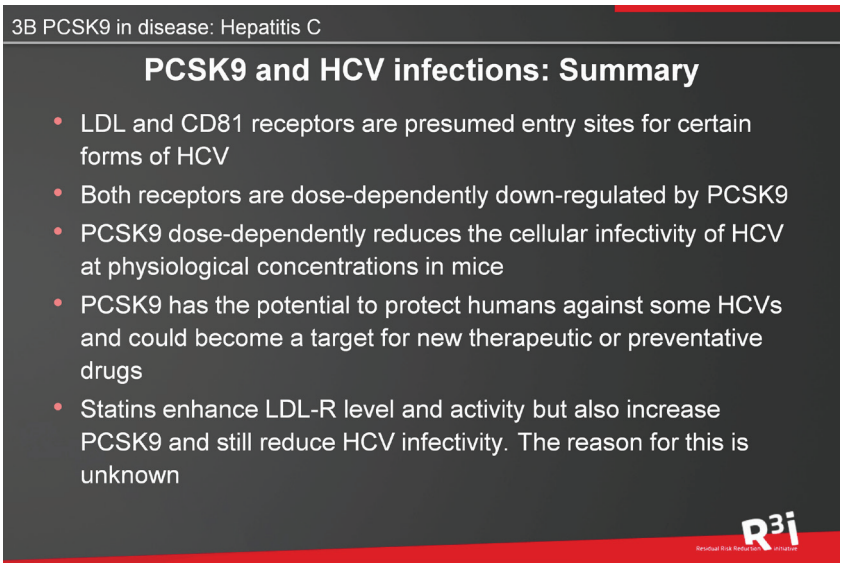
Liver expression of CD81 is markedly increased in *PCSK9*-null mice

- CD81 expression in liver extracts from WT or *PCSK9*-/- mice was normalized using GAPDH as a control
- CD81 expression was substantially higher in mice that did not express PCSK9



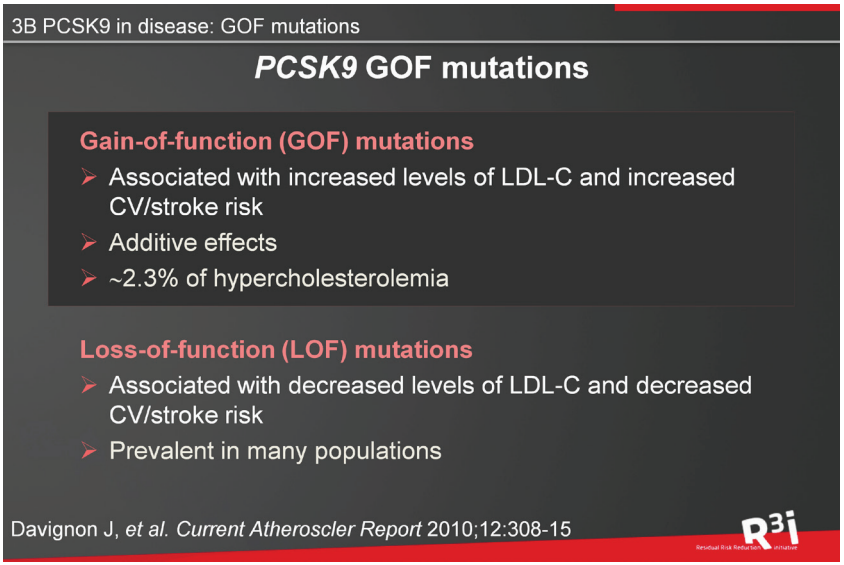
PCSK9 down-regulates CD81 independently from LDL-R

- LDL-R and CD81 expression were analyzed by immunohistochemistry (green) in liver cryosections of WT, *PCSK9-/-* and double knockout *PCSK9-/- LDL-R-/-* mice (n = 2 for each genotype). Nuclei were stained with TOPRO- 3 (blue)
- LDL-R was highly expressed in the livers of *PCSK9-/-* mice (left-hand panel)
- Compared to WT mice, labeling of CD81 appeared noticeably higher over the basolateral membrane of hepatocytes, facing sinusoids, in the *PCSK9-/-* mice (middle panel)
- Compared to *LDL-R-/-* mice, the level of CD81 was distinctly higher in the livers of the double mutant (right panel), suggesting that PCSK9 down-regulates CD81 independently from LDL-R



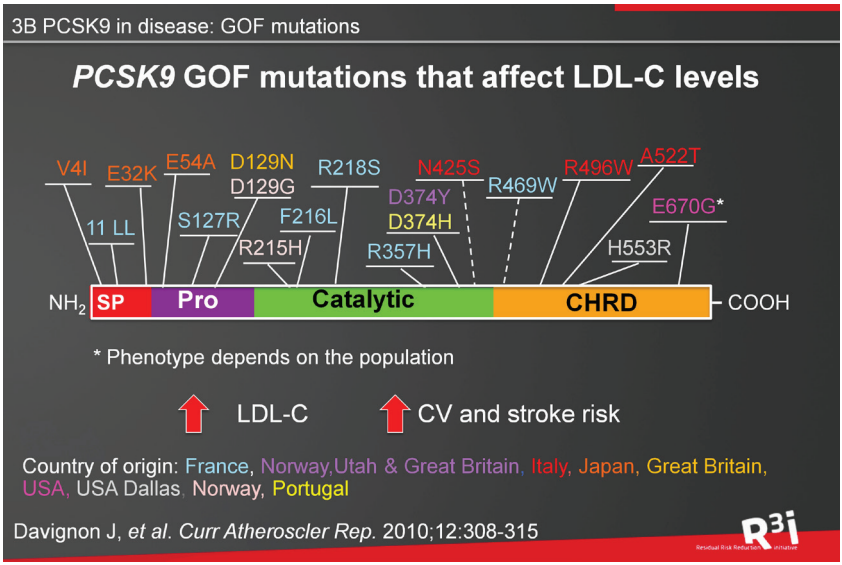
PCSK9 and HCV infections: Summary

- LDL and CD81 receptors (presumed entry sites for certain forms of HCV) are dose-dependently down-regulated by PCSK9
- PCSK9 dose-dependently reduces the cellular infectivity of HCV in mice
- PCSK9 has the potential to protect humans against some HCVs and could become a target for new therapeutic or preventative drugs
- Statins enhance LDL-R level and activity but also increase PCSK9 and still reduce HCV infectivity. The reason for this is unknown



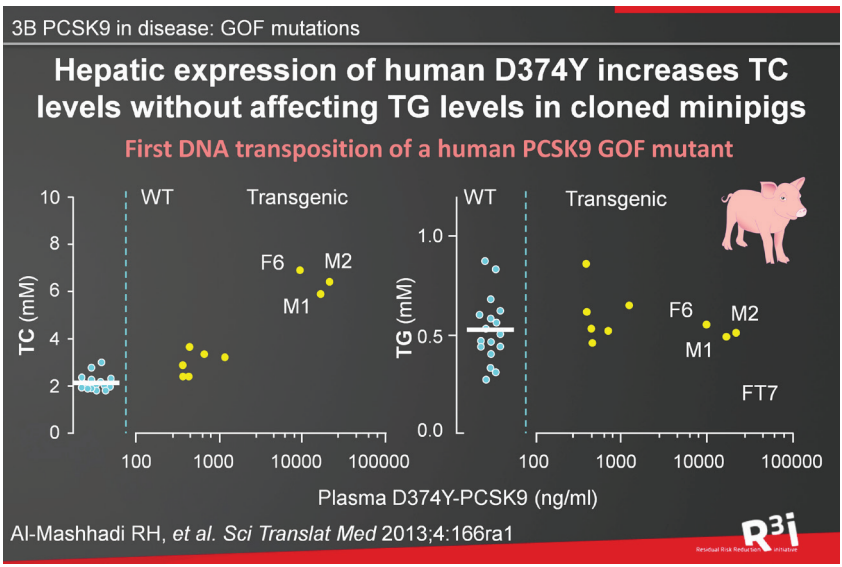
***PCSK9* GOF mutations**

- GOF mutations are associated with increased levels of LDL-C and increased CV/stroke risk. They account for ~2.3% of hypercholesterolemia cases
- In contrast, LOF mutations are associated with decreased levels of LDL-C and decreased CV/stroke risk. They are prevalent in many populations



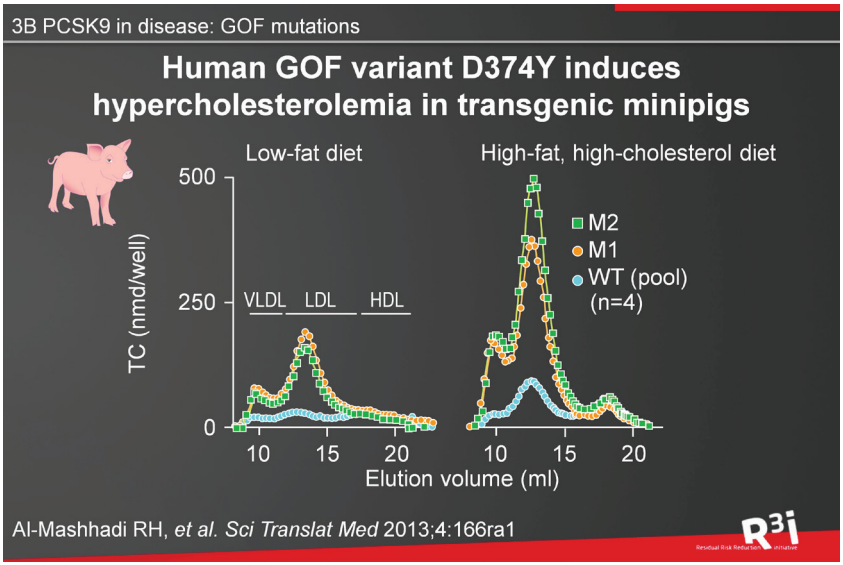
***PCSK9* GOF mutations that affect LDL-C levels**

- The mutations associated with high LDL-C levels and increased CV/stroke risk are shown below the protein
- The color code indicates the country of origin for each mutation



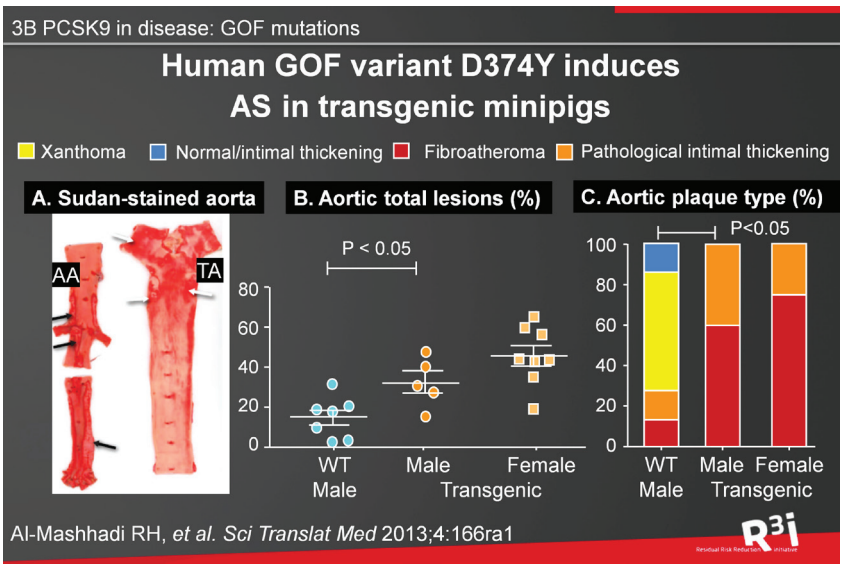
Hepatic expression of human D374Y increases TC levels without affecting TG levels in cloned minipigs

- Yucatan minipigs with liver-specific expression of the human GOF PCSK9 mutation, D374Y, were created using Sleeping Beauty DNA transposition and cloning by somatic cell nuclear transfer
- In high-expressing founders (M1, M2 and F6) on a standard low-fat diet, plasma TC levels were about 3-fold higher than in WT pigs
- Fasting plasma TGs were not increased by D374Y-*PCSK9* expression



Human GOF variant D374Y induces hypercholesterolemia in transgenic minipigs

- FPLC analysis showed that 14-week old high-expressing M1 and M2 founders fed a low-fat diet had increased levels of Apo B-containing VLDL and LDL compared to WT
- The contrast between transgenic and WT animals was more pronounced following a high-fat high-cholesterol diet at 22 weeks of age

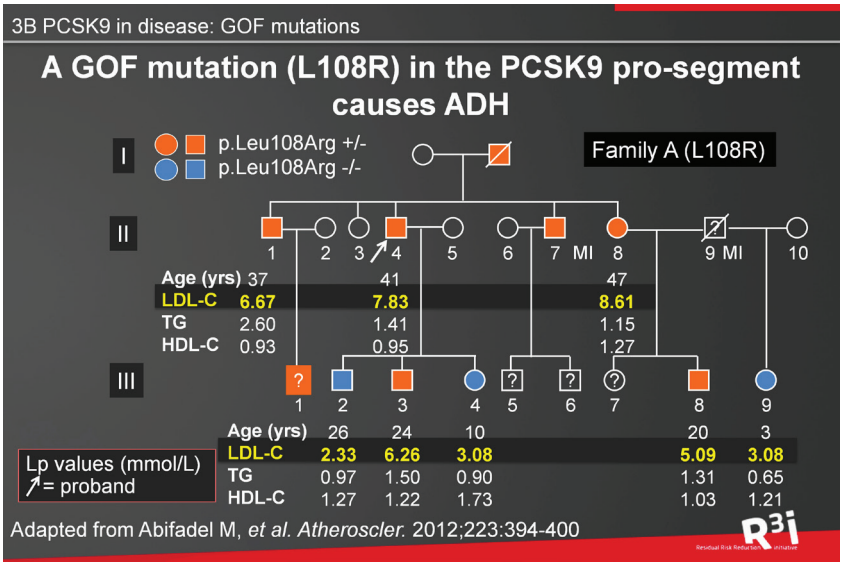


Human GOF variant D374Y induces AS in transgenic minipigs

(A) Sudan IV–stained aorta (AA, abdominal aorta; TA, thoracic aorta) from a male D374Y-PCSK9 transgenic pig showed raised lesions (black arrows) in the abdominal aorta and non-raised lesions (white arrows) in the short ascending aorta and aortic arch

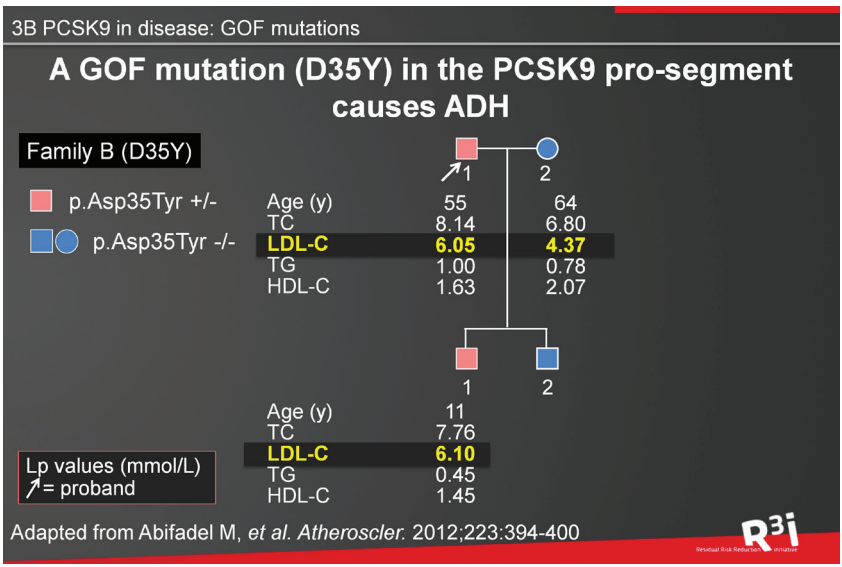
(B) Mean aortic surface area covered by atherosclerotic lesions was increased 2.1-fold and raised lesions 2.8-fold in transgenic compared with WT males; lesion area was greatest in transgenic female animals

(C) Microscopic analysis of the largest raised lesion in the AA showed progressive atherosclerotic lesions (pathological intimal thickening or fibroatheroma) in 100% of transgenic males vs 28% of WT males



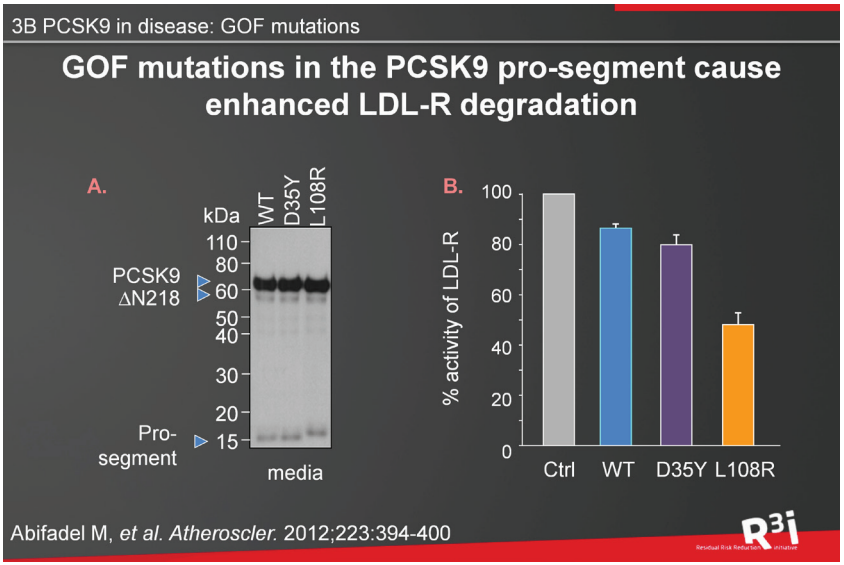
A GOF mutation (L108R) in the PCSK9 pro-segment causes ADH

- Pedigree and genetic analysis of Family A showing birth age at given lipid measurements and TC, LDL-C, HDL-C and TG levels (mmol/L) (with untreated values for affected individuals). The proband is indicated by an arrow
- Sequence analysis showed the proband and affected members are heterozygous with respect to the p.Leu108Arg mutation in the *PCSK9* gene



A GOF mutation (D35Y) in the PCSK9 pro-segment causes ADH

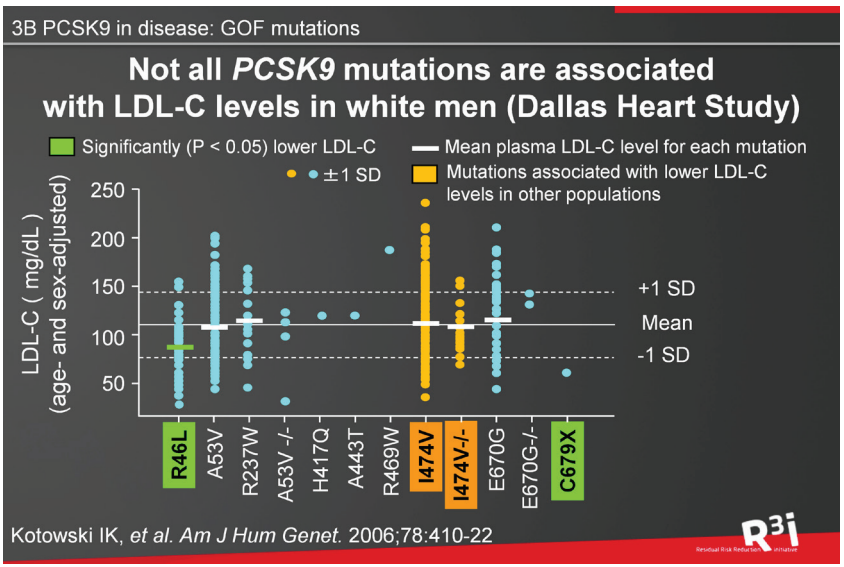
- Pedigree and genetic analysis of Family B showing age at given lipid measurements, TC, LDL-C, HDL-C and TG levels (mmol/L) (with untreated values for affected individuals). The probands are indicated by an arrow
- Sequence analyses showed that the proband and his son are heterozygous with respect to the p.Asp35Tyr substitution in exon 1 of *PCSK9*



GOF mutations in the PCSK9 pro-segment cause enhanced LDL-R degradation

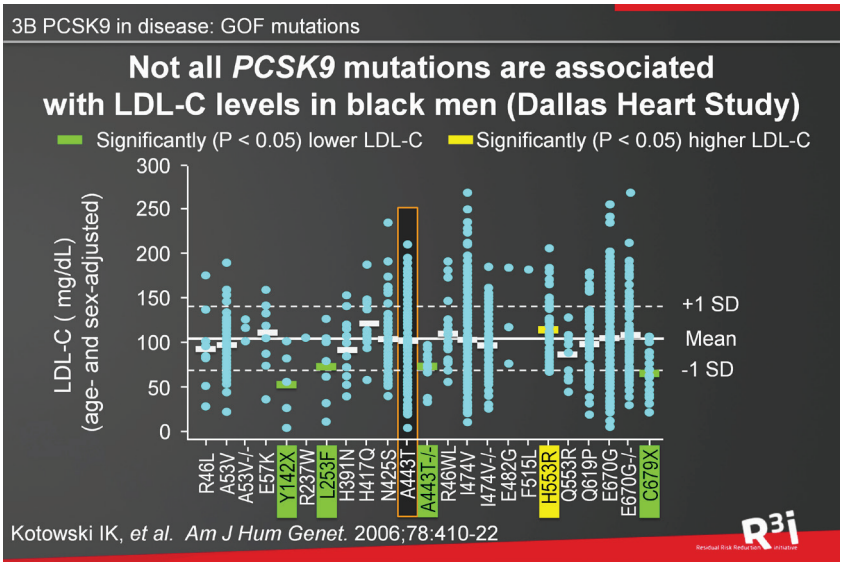
(A) Western blotting of proteins from HepG2 cells overexpressing WT, D35Y or L108R PCSK9 showed similar levels of PCSK9 protein. All forms were autocatalytically cleaved and were similarly susceptible to furin cleavage resulting in comparative levels of PCSK9-ΔN_{218} in the cell media

(B) HuH7 cells were incubated for 4 h with equal amounts of WT, D35Y and L108R protein (730ng/ml); cell surface LDL-R was measured by fluorescence-activated cell sorting (FACS). The reduction in LDL-R activity associated with D35Y was slightly higher than with WT. L108R led to a ~2-fold decrease in LDL-R activity vs WT



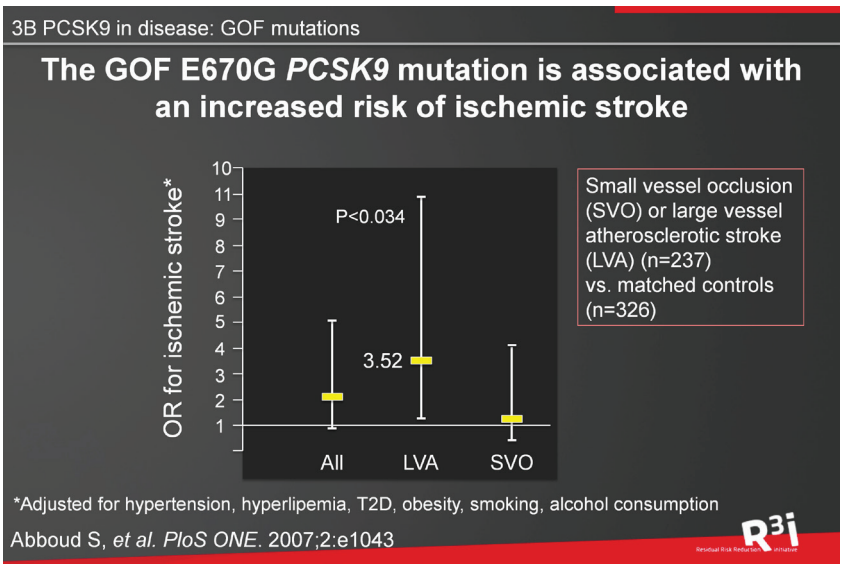
Not all PCSK9 mutations are associated with LDL-C levels in white men

- DNA sequencing of Dallas Heart Study participants (n = 1822 blacks, 1045 whites, 601 hispanics) revealed 19 non-synonymous sequence variations in *PCSK9*, including 17 missense mutations and 2 nonsense mutations. All 19 mutations were present in blacks, whereas only 9 were found in whites
- Of the 9 non-synonymous mutations found in whites, only one (R46L) was significantly associated with plasma LDL-C levels
- Large inter-individual LDL-C variations occurred for each given PCSK9 mutation



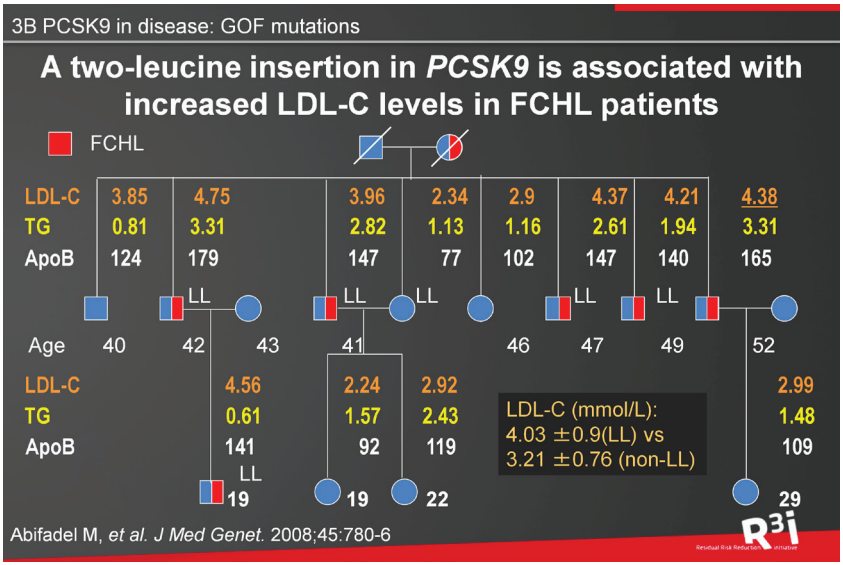
Not all *PCSK9* mutations are associated with LDL-C levels in black men

- Among blacks, 3 missense mutations (L253F, A443T, and H553R) were associated with plasma LDL-C levels
- The L253F and A443T mutations were associated with low LDL-C levels: heterozygotes for the F253 allele had a 30% reduction in mean plasma LDL-C levels (L/L 104.6 mg/dl; L/F 73.2 mg/dl), whereas homozygotes for the T443 variant had a 29% reduction in plasma levels of LDL-C
- The H553R mutation was associated with significantly increased plasma levels of LDL-C



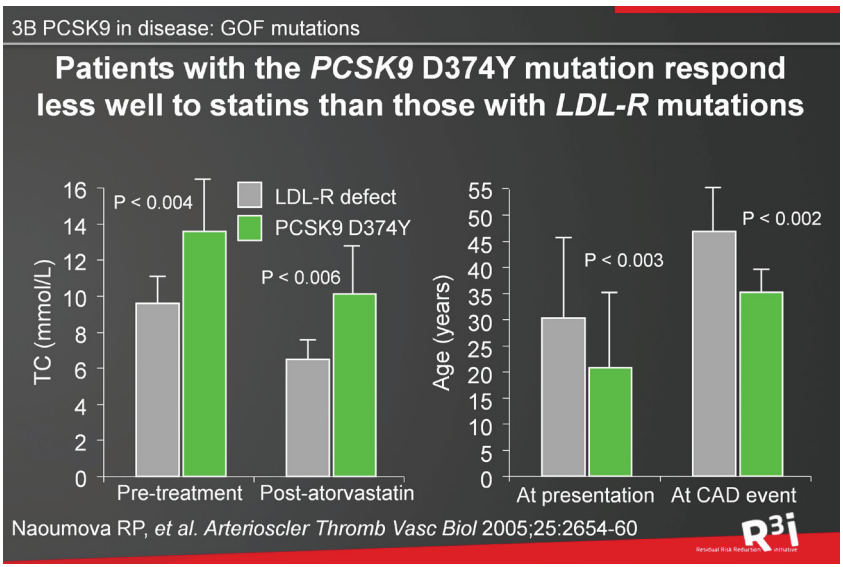
The GOF E670G *PCSK9* mutation is associated with an increased risk of ischemic stroke

- In a multivariate analysis, the G allele tended to be more common among ischemic stroke cases than controls (8.1% vs. 4.3%; P = 0.095 OR 2.10, 95% CI 0.87-5.05)
- In particular, the G allele was significantly more common among large vessel atherosclerotic (LVA) stroke patients than in control subjects (10.8% vs. 4.3%; P =0.017 OR 3.52, 95% CI 1.25–9.85)
- The E670G variation was not related to the risk of small vessel occlusion (SVO)



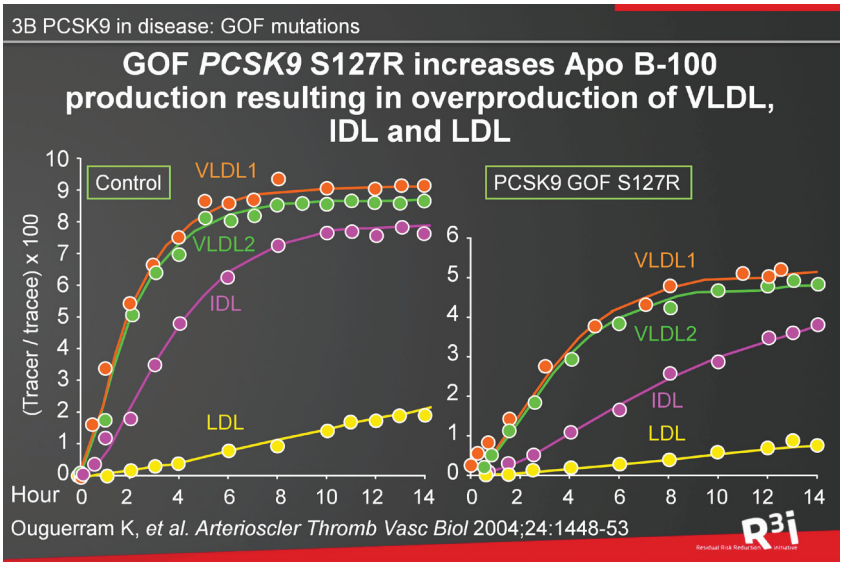
A two-leucine insertion in *PCSK9* is associated with increased LDL-C levels in FCHL patients

- Insertion of two leucines (p.L21tri also designated p.L15_L16ins2L) in the leucine stretch of the PCSK9 signal peptide is associated with FCHL
- This mutant was associated with high TC and LDL-C values



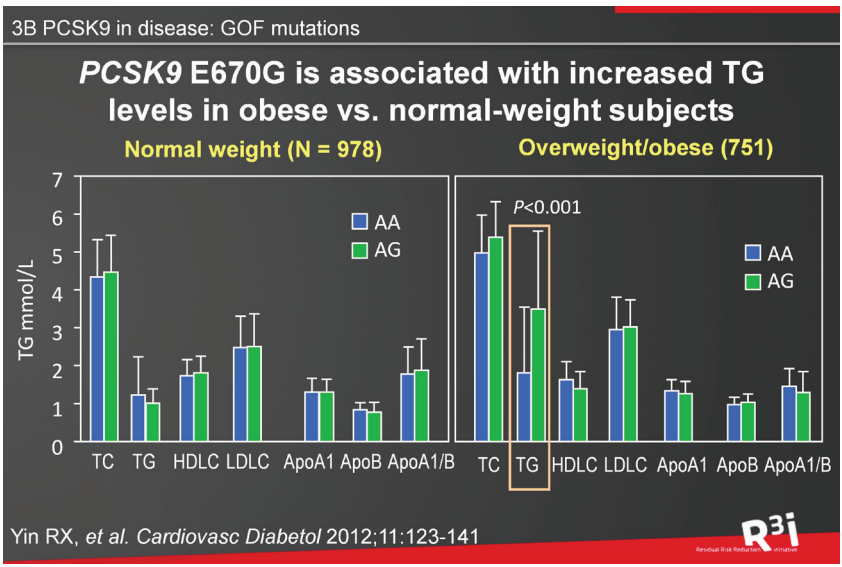
Patients with the *PCSK9* D374Y mutation respond less well to statins than those with *LDL-R* mutations

- 4 unrelated white British families comprising 13 individuals with severe ADFH attributable to the D374Y variant of the *PCSK9* gene were compared with typical HeFH patients with known mutations in *LDL-R*
- Mean serum TC concentrations remained significantly higher in the *PCSK9* vs *LDL-R* defective patients both before and during treatment with atorvastatin
- *PCSK9* D374Y patients developed premature CHD 10 years earlier than HeFH patients



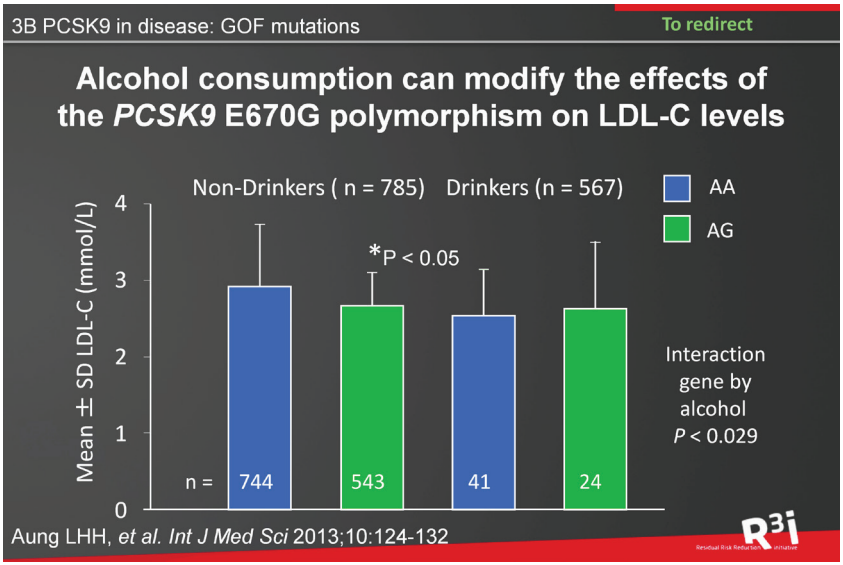
GOF PCSK9 S127R increases Apo B-100 production resulting in overproduction of VLDL, IDL and LDL

- Fasted subjects (n = 3) with and without *PCSK9* S127R received an i.v. infusion of [2H3]-leucine followed by a 14-h tracer to label endogenous Apo B-100
- S127R dramatically increased the production rate of Apo B-100 (3-fold) vs control, an effect that corresponded with the direct overproduction of VLDL (3-fold), IDL (3-fold), and LDL (5-fold)
- S127R caused a decrease in VLDL and IDL conversion (10% to 30% of the controls) and the rate of LDL fractional catabolism (by 30%)



***PCSK9* E670G is associated with increased TG levels in obese vs. normal-weight subjects**

- 978 normal weight and 751 overweight/obese subjects from the Chinese Bai Ku Yao population were randomly selected from previously stratified randomized cluster samples
- Overweight/obese subjects had higher *PCSK9* 670A allele frequencies than normal weight subjects
- The level of TG varied between PCSK9 genotypes ($P < 0.001$) in overweight/obese but not normal-weight subjects
- An interaction exists between PCSK9 SNPs and overweight/obesity



Alcohol consumption can modify the effects of the *PCSK9* E670G polymorphism on LDL-C levels

- The genotypic and allelic frequencies of *PCSK9* E670G were not different between non-drinkers and drinkers *(P > 0.05 for each)*
- The interactions between *PCSK9* E670G genotypes and alcohol consumption on serum lipid parameters were detected using a factorial design covariance analysis after controlling for potential confounders
- In non-drinkers (but not drinkers), subjects with the AA genotype had significantly (P < 0.05) higher LDL-C levels than those with the AG genotype
- In terms of LDL-C levels, subjects with the AA genotype benefited more from alcohol consumption than those with the AG genotype

PCSK9: Drug effects

SECTION

4

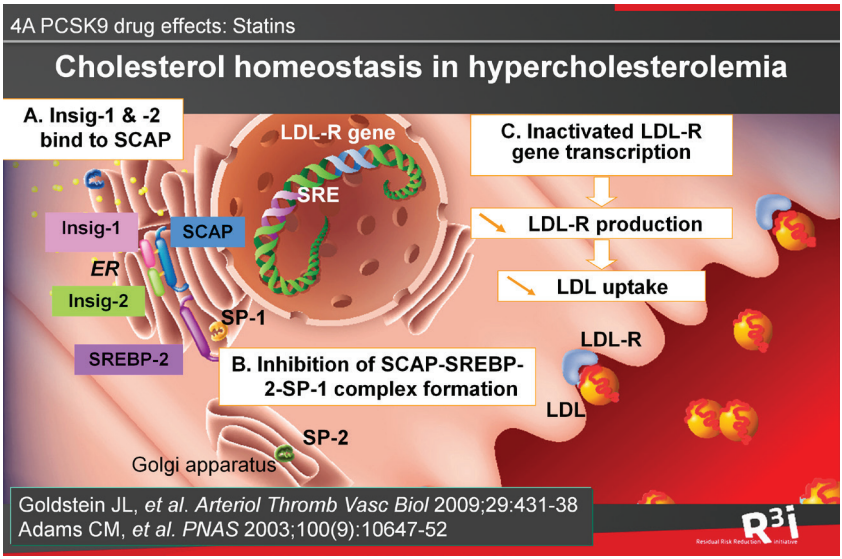
Residual Risk Reduction initiative

Statins

SECTION

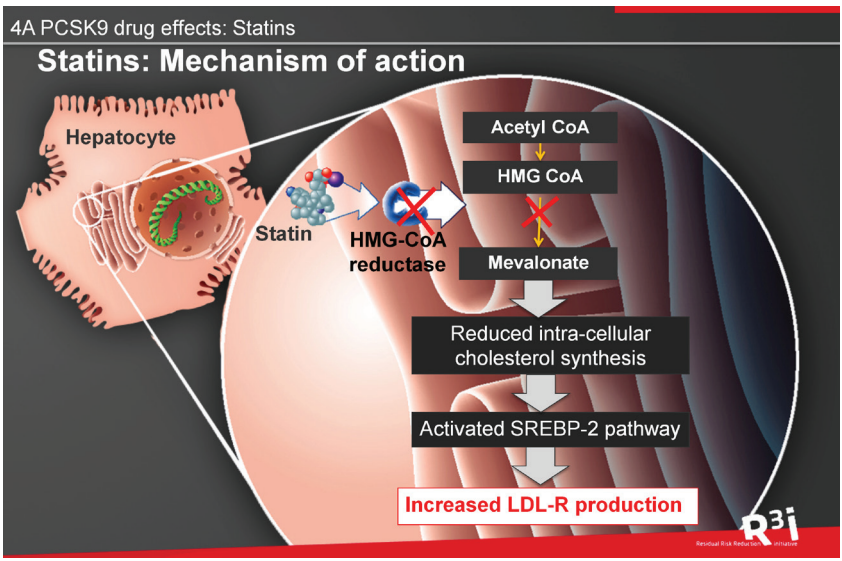
4A

Residual Risk Reduction initiative



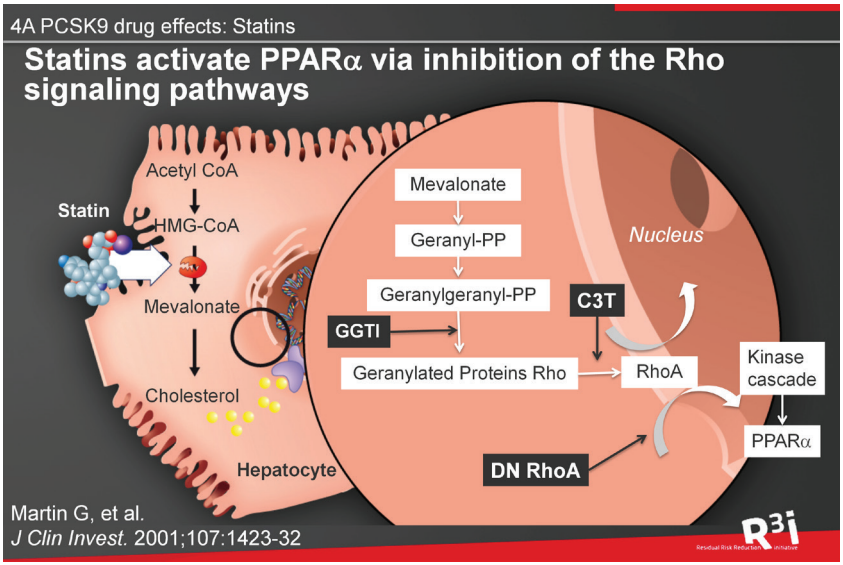
Cholesterol homeostasis in hypercholesterolemic patients

- Sterol-regulatory element binding proteins (SREBPs) are transcription factors that contribute to cholesterol homeostasis by regulating the expression of the LDL-R
- When intracellular cholesterol levels are high, membrane-bound Insulin-induced gene-1 and -2 (Insig-1 and Insig-2) proteins bind to SREBP cleavage activated protein (SCAP), preventing it from binding to, and activating SREBP-2
- Inactive SREBP-2 is retained in the ER and is therefore unable to activate LDL-R expression
- Low levels of LDL-R result in a slower rate of LDL-C removal from the circulation and increased plasma levels of LDL-C



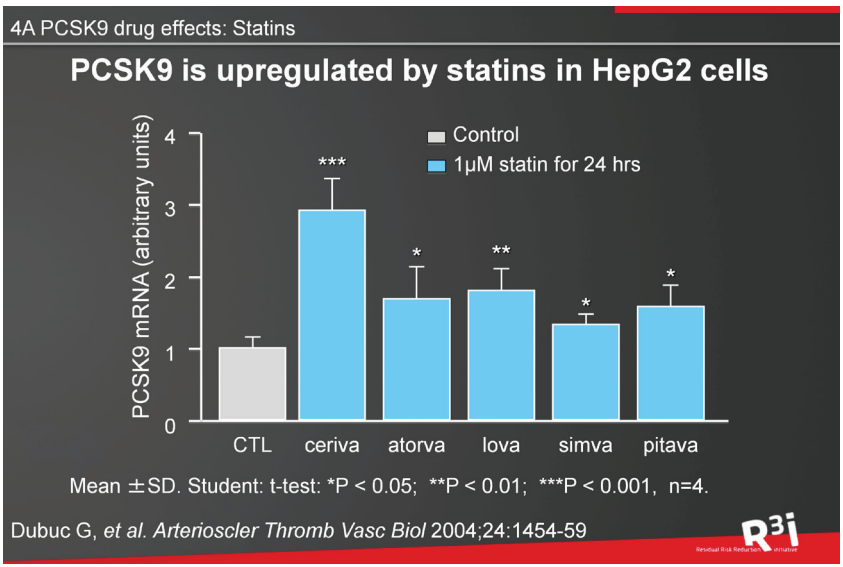
Statins: Mechanism of action

- Statins (3-hydroxy-3-methylglutaryl coenzyme A [HMG-CoA] reductase inhibitors) reduce intra-cellular cholesterol levels by inhibiting the rate-limiting step of cholesterol synthesis in the liver (the conversion of HMG CoA into mevalonate)
- Activated SREBP-2 is then transported to the nucleus, where it upregulates genes encoding LDL-R and HMG CoA synthase. This results in:
  - Increased levels of cell-surface LDL-Rs leading to faster clearance of LDL particles from the plasma, increased cellular cholesterol levels and reduced plasma levels of LDL-C
  - Increased levels of HMG CoA synthase leading to increased cellular cholesterol levels



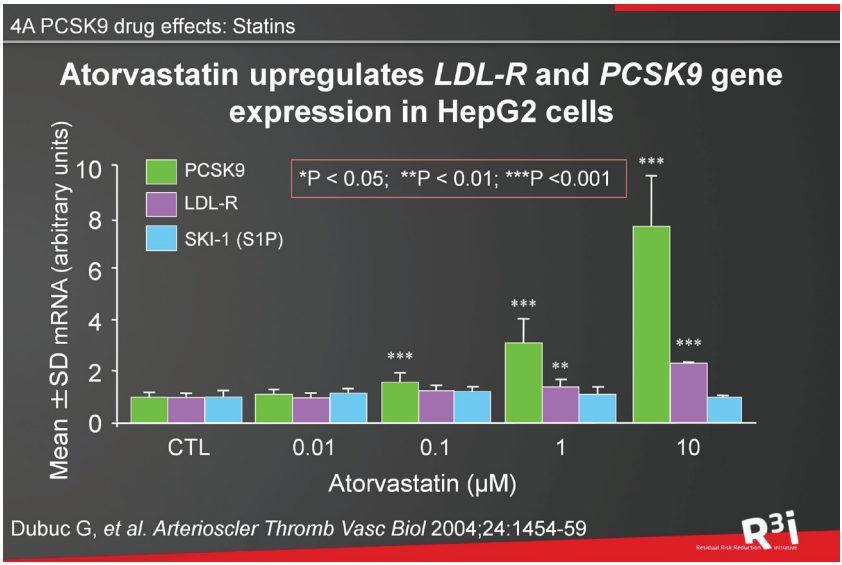
Statins activate PPARα via inhibition of the Rho signaling pathways

- Statins inhibit the formation of mevalonate, thereby suppressing the formation of isoprenoids, such as geranyl pyrophosphate (geranyl-PP) and geranylgeranyl pyrophosphate (geranylgeranyl-PP)
- In ECs, the effects of statins is mimicked by C3 exoenzyme, an inhibitor of Rho activity. As such statins inhibit the prenylation of proteins, such as Ras and Rho that activate the mitogen-activated protein (MAP) kinase cascade or the NF-κB pathway, leading to PPARα activation



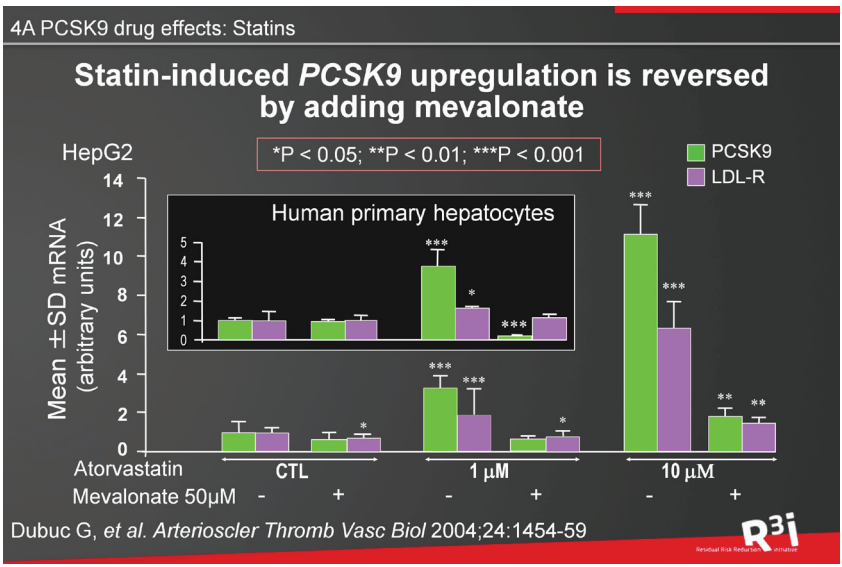
PCSK9 is upregulated by statins in HepG2 cells

- HepG2 cells were treated for 24 with 1 μmol/L of cerivastatin, atorvastatin, lovastatin, simvastatin, or pitavastatin and gene expression levels were quantified by qPCR
- Statin treatment significantly increased *PCSK9* expression by a factor of 3 for cerivastatin and by 1.5 for the other statins



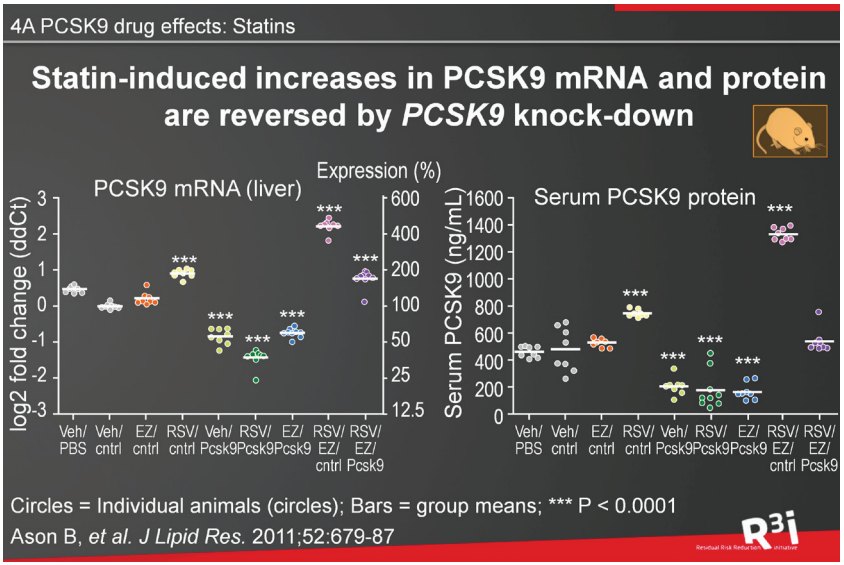
Atorvastatin upregulates *LDL-R* and *PCSK9* gene expression in HepG2 cells

- HepG2 cells were treated for 48 h with increasing concentrations of atorvastatin; gene expression levels were quantified by qPCR
- *PCSK9* gene expression was dose-dependently upregulated up to 7.5-fold
- Under the same conditions, *LDL-R* expression increased by only 2.5-fold
- SKI-1 (that cleaves SREBPs on cholesterol depletion) was not regulated at the transcriptional level by statin treatment



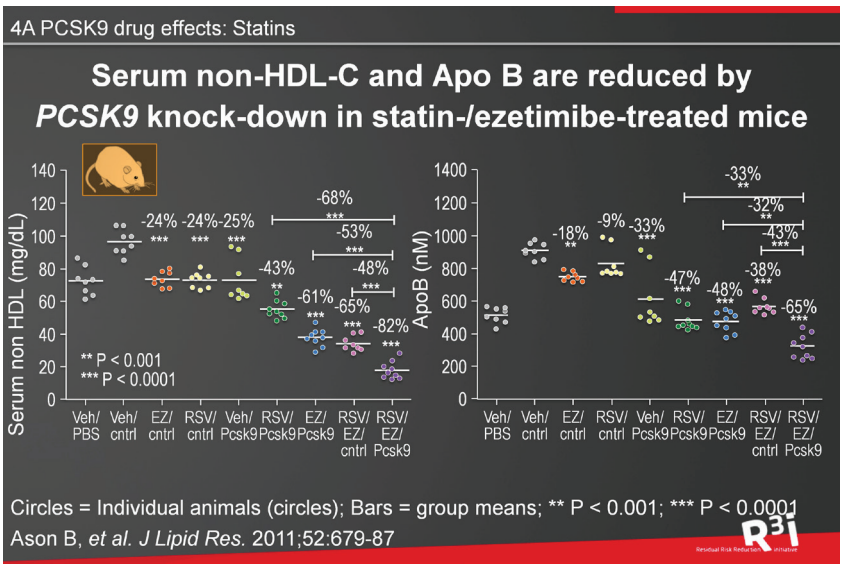
Statin-induced *PCSK9* upregulation is reversed by adding mevalonate

- HepG2 cells or human primary hepatocytes were pre-incubated for 24 h with 10% LPDS followed by 48 h without (CTL) or with atorvastatin 1 or 10 mmol/L and with or without 50 mmol/L mevalonate; gene-specific expression was measured by qPCR
- Statin-induced PCSK9 upregulation was 2-fold greater than that of LDL-R
- Upregulation of both PCSK9 and LDL-R was reversed with mevalonate; the effect on *PCSK9* was greater than for LDL-R
- In cells incubated with 1 mmol/L of statin, mevalonate lowers PCSK9 (but not LDL-R) to below the level of control, suggesting that PCSK9 is regulated more tightly by cholesterol than the *LDL-R* gene



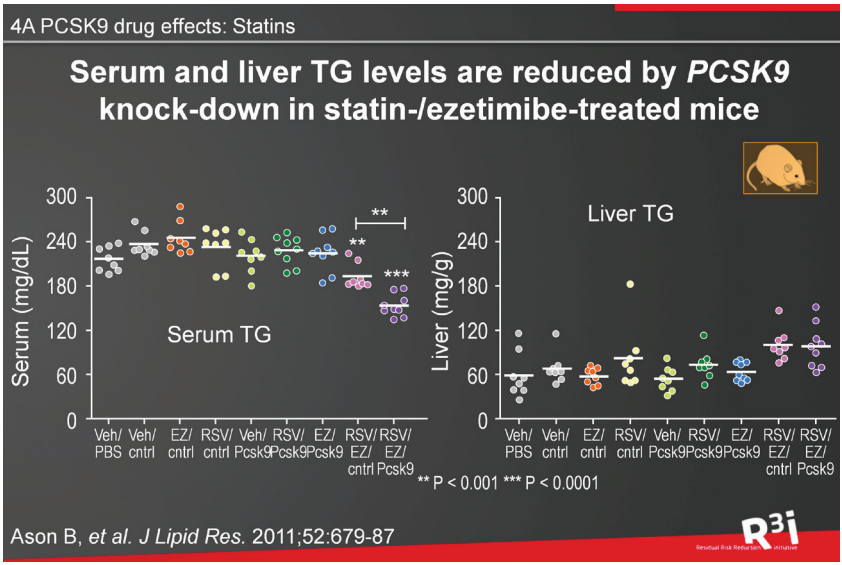
Statin-induced increases in PCSK9 mRNA and protein are reversed by *PCSK9* knock-down

- C57Bl/6 mice hemizygous for *LDL-R* and the overexpression of *hCetp* were treated with ezetimibe (EZ; 10 mg/kg/day), rosuvastatin (RSV; 20 mg/kg/day), or the combination for 14 days; on day 11 they received a 6 mg/kg dose of PCSK9 siRNA
- Liver PCSK9 mRNA and serum PCSK9 protein levels were slightly increased with ezetimibe and were significantly increased 2-fold with rosuvastatin and 4-fold with ezetimibe/rosuvastatin
- Administration of PCSK9 siRNA with either ezetimibe or rosuvastatin reduced PCSK9 levels below those observed for the vehicle/control, whereas the ezetimibe/rosuvastatin combination reduced PCSK9 to vehicle/control levels



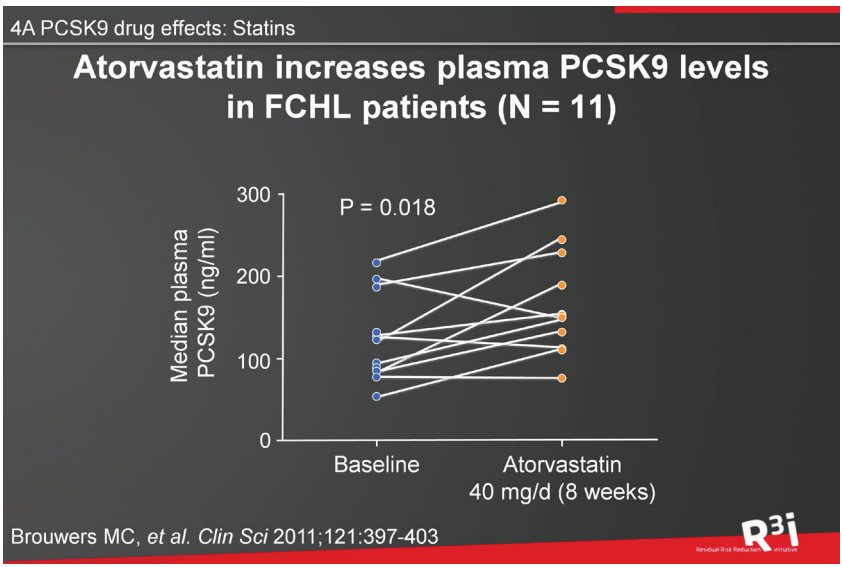
Serum non-HDL-C and Apo B are reduced by *PCSK9* knock-down in statin-/ezetimibe-treated mice

- Ezetimibe, rosuvastatin, and PCSK9 siRNA significantly reduced serum non-HDL levels vs vehicle/control siRNA treatment in C57Bl/6 mice hemizygous for *LDL-R* and the overexpression of *hCetp*
- Both ezetimibe and PCSK9 siRNA were associated with a significant reduction in serum Apo B
- PCSK9 siRNA plus either ezetimibe or rosuvastatin, led to even greater reductions in both serum non-HDL and Apo B vs the individual treatments



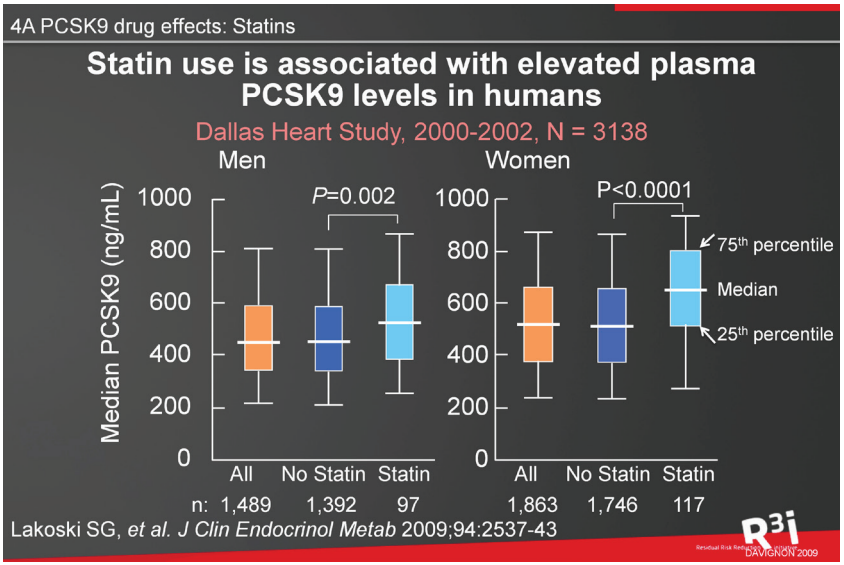
Serum and liver TG levels are reduced by *PCSK9* knock-down in statin-/ezetimibe-treated mice

- Significant reductions in serum TG levels were observed in mice treated with ezetimibe/rosuvastatin/PCSK9 siRNA vs the negative control group (Veh/cntrl)
- A trend toward increased hepatic TG levels was observed for the ezetimibe/rosuvastatin/PCSK9 siRNA treatment
- Attenuating the induction of *PCSK9* improves the efficacy of ezetimibe and rosuvastatin



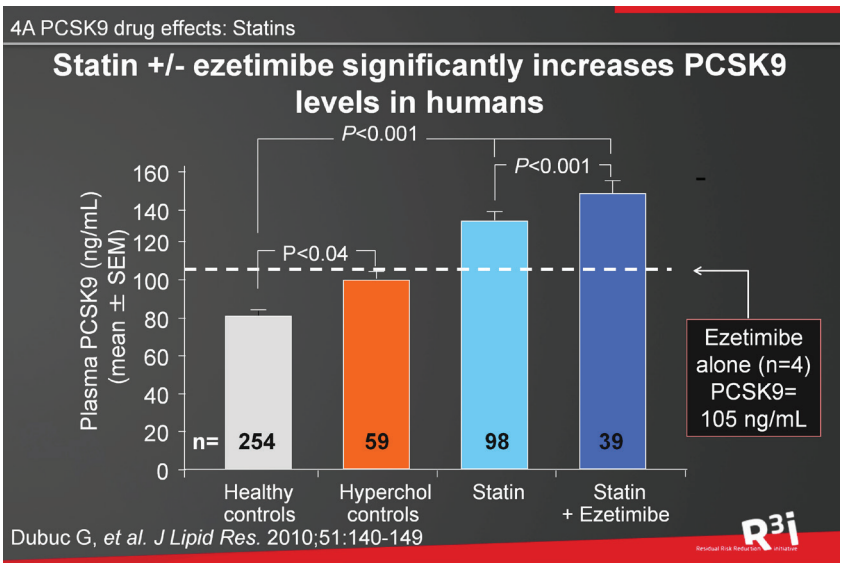
Atorvastatin increases plasma PCSK9 levels in FCHL patients (N = 11)

- PCSK9 protein levels were measured by dual monoclonal antibody sandwich ELISA in 11 patients with FCHL
- Median plasma PCSK9 protein levels increased significantly from 122.0 (81.9–188.0) ng/ml to 150.0 (113.0–228.0) ng/ml after 8 weeks of treatment



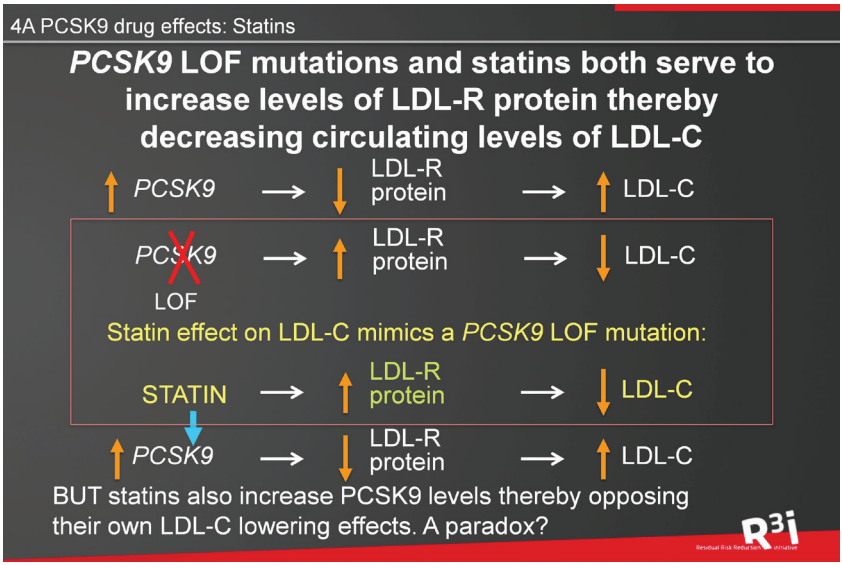
Statin use is associated with elevated plasma PCSK9 levels in humans

- Statin use was associated with a significant increase in circulating levels of PCSK9 among both men and women in the multi-ethnic Dallas Heart Study (N = 3138)



Statin +/- ezetimibe significantly increases PCSK9 levels in humans

- Compared to healthy controls (n = 254), significantly higher plasma PCSK9 levels were observed amongst untreated patients with hypercholesterolemia (n = 59) and amongst hypercholesterolemic patients treated with statins with (n = 39) or without (n = 98) ezetimibe
- Significantly higher PCSK9 levels were observed in people receiving ezetimibe-statin combination therapy vs statin monotherapy
- In the 4 subjects receiving ezetimibe alone, PCSK9 levels were near the mean of the untreated hypercholesterolemic control subjects



PCSK9 LOF mutations and statins both serve to increase levels of LDL-R protein thereby decreasing circulating levels of LDL-C

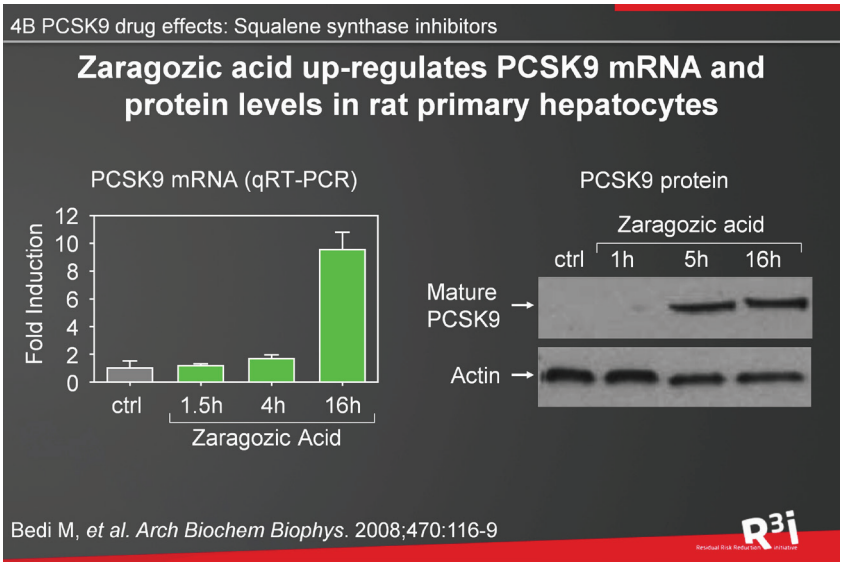
- PCSK9 reduces levels of LDL-R protein and increases circulating levels of LDL-C
- _PCSK9_ LOF mutations and statins both serve to increase levels of LDL-R protein thereby decreasing circulating levels of LDL-C
- Paradoxically, statins also increase levels of PCSK9

Squalene synthase inhibitors

SECTION

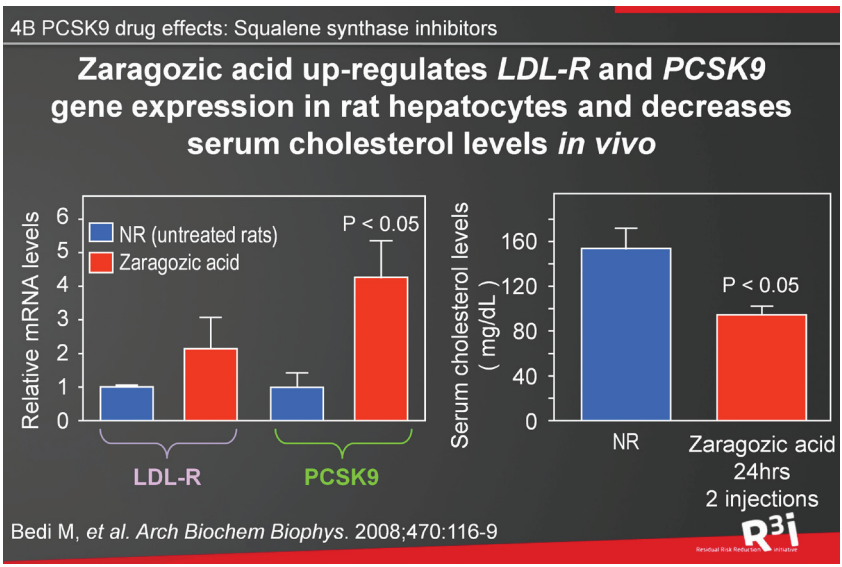
4B

Residual Risk Reduction initiative



Zaragozic acid up-regulates PCSK9 mRNA and protein levels in rat primary hepatocytes

- Rats received a 2mg/kg i.p. dose of zaragozic acid (a squalene synthase inhibitor) and were killed at the indicated times (n = 10 per condition)
- qRT-PCR showed that zaragozic acid treatment increased hepatic PCSK9 mRNA levels as a function of time
- Western blotting showed that the increase in mRNA levels was accompanied by an increase in PCSK9 protein levels



Zaragozic acid up-regulates *LDL-R* and *PCSK9* gene expression in rat hepatocytes and decreases serum cholesterol levels *in vivo*

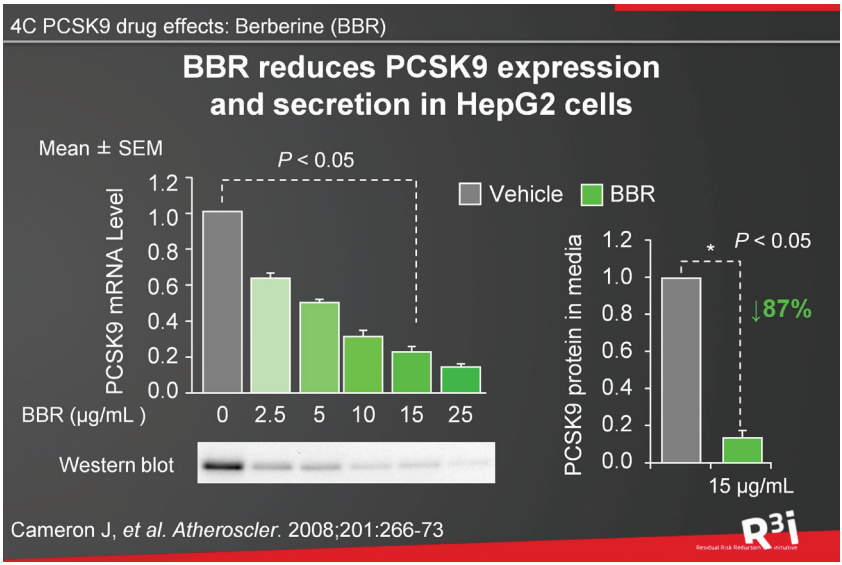
- Rats (n = 7 per condition) were injected with 2 mg/kg of zaragozic acid 24h and 2h before euthanization
- Zaragozic acid increased both LDL-R and PCSK9 mRNA levels and led to a significant reduction in serum cholesterol levels

Berberine (BBR)

SECTION

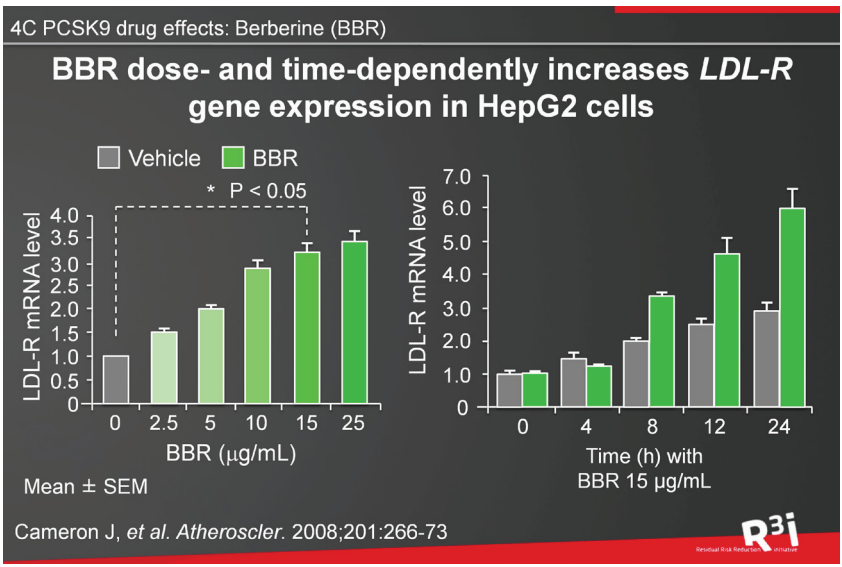
4C

Residual Risk Reduction initiative



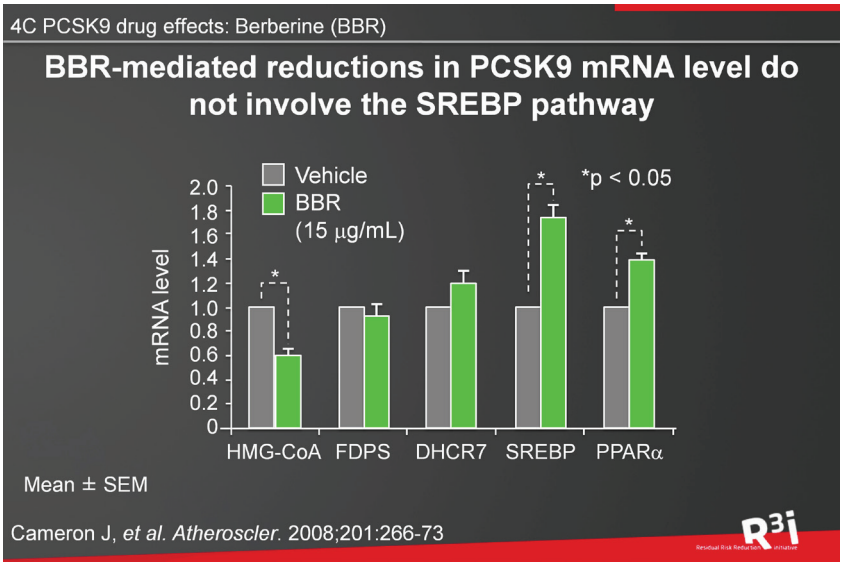
BBR reduces PCSK9 expression and secretion in HepG2 cells

- BBR is an isoquinoline plant alkaloid that, although not licensed as a drug, is commonly used in herbal medicine to treat a range of conditions, including T2D, hypercholesterolemia, glaucoma, eye infections, bacterial diarrhea, parasitic infestations and skin disorders
- BBR reduces LDL-C levels by stabilizing LDL-R mRNA
- rtPCR showed that BBR reduced the amount of PCSK9 mRNA in HepG2 cells a dose-dependent manner (87% at 15μg/ml)
- Western blot analysis showed that BBR dose-dependently reduced the amount of secreted PCSK9



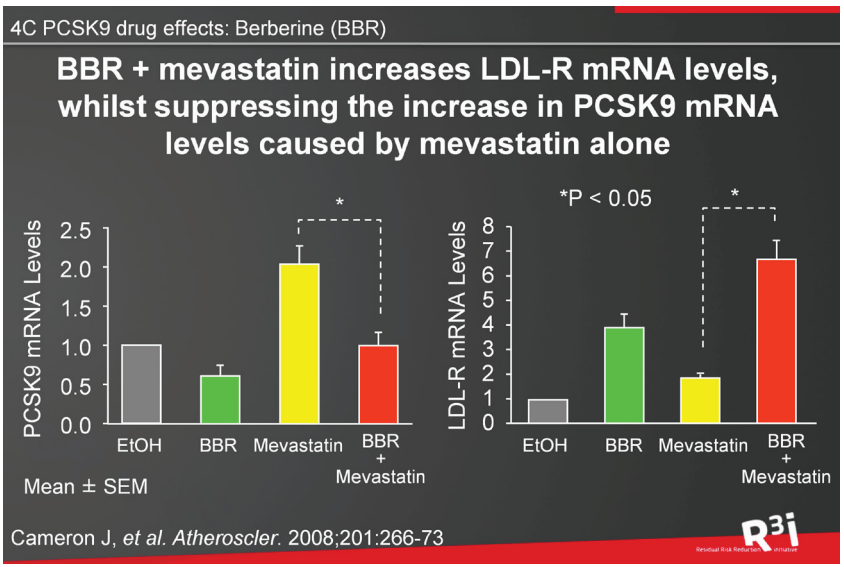
BBR dose- and time-dependently increases *LDL-R* gene expression in HepG2 cells

- A dose-dependent increase in the amount of LDL-R mRNA was observed when BBR was added to HepG2 cells (3-fold at 15μg/ml)
- Time-course experiments also revealed that BBR increased the amount of LDL-R mRNA in a time-dependent manner; after 12h and 24h the amount of LDL-R mRNA was increased by 1.9-fold and 2.1-fold, respectively



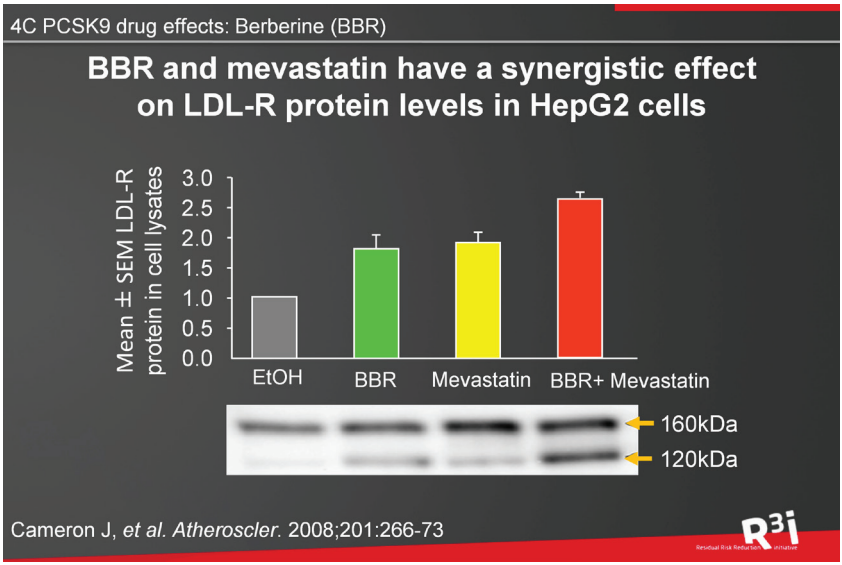
BBR-mediated reductions in PCSK9 mRNA level do not involve the SREBP pathway

- 15μg/ml BBR reduced the level of SREBP-regulated HMG-CoA reductase gene expression 39% in HepG2 cells but had no significant effect on the amounts of farnesyl diphosphate synthase (FDPS) or 7-dehydrocholesterol reductase (DHCR7) mRNA, both of which are also regulated by SREBP
- In contrast, BBR 15μg/ml increased the amounts of 2 non-SRE containing genes involved in lipid metabolism (PPARα mRNA increased by 39% and SREBP-2 mRNA increased by 74%)
- The inconsistent effects of BBR on the amounts of mRNA from genes with or without a SRE, suggests that the BBR-mediated reduction in PCSK9 mRNA level does not involve the SREBP pathway



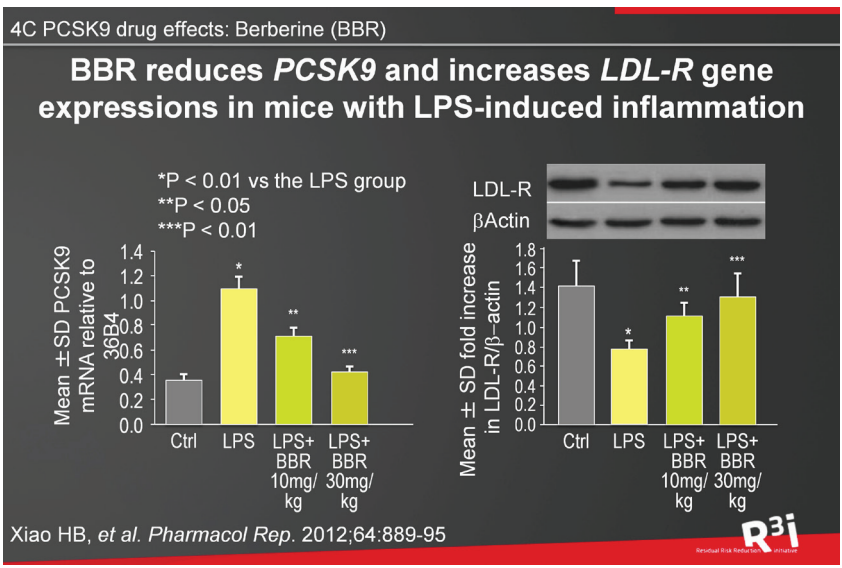
BBR + mevastatin increases LDL-R mRNA levels, whilst suppressing the increase in PCSK9 mRNA levels caused by mevastatin alone

- Although a combination of BBR and mevastatin increased the amount of LDL-R mRNA in HepG2 cells 6-fold, BBR suppressed the increase in PCSK9 mRNA induced by mevastatin by 56%



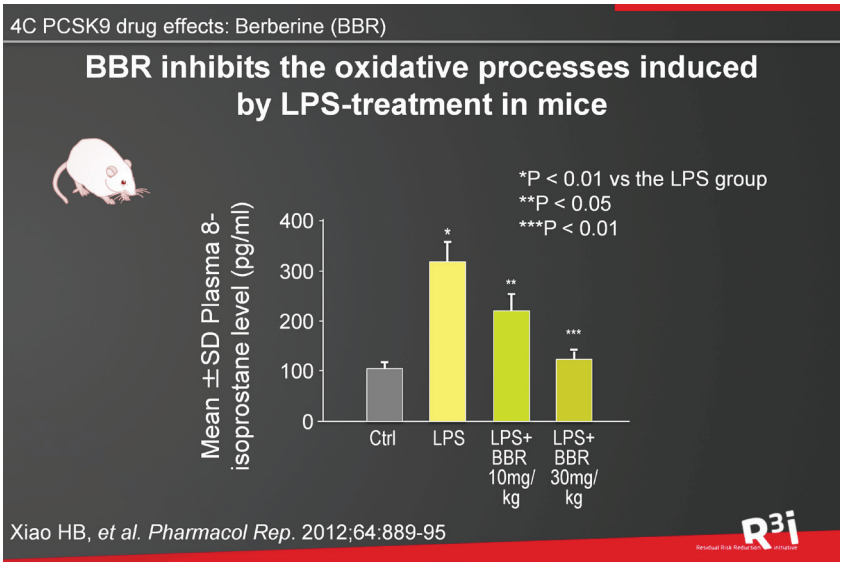
BBR and mevastatin have a synergistic effect on LDL-R protein levels in HepG2 cells

- Western blot analysis of HepG2 cell lysates revealed that cells treated with a combination of BBR and mevastatin had a 2.6-fold increase in LDL-R protein levels compared to a 1.4-fold increase in cells treated with mevastatin alone



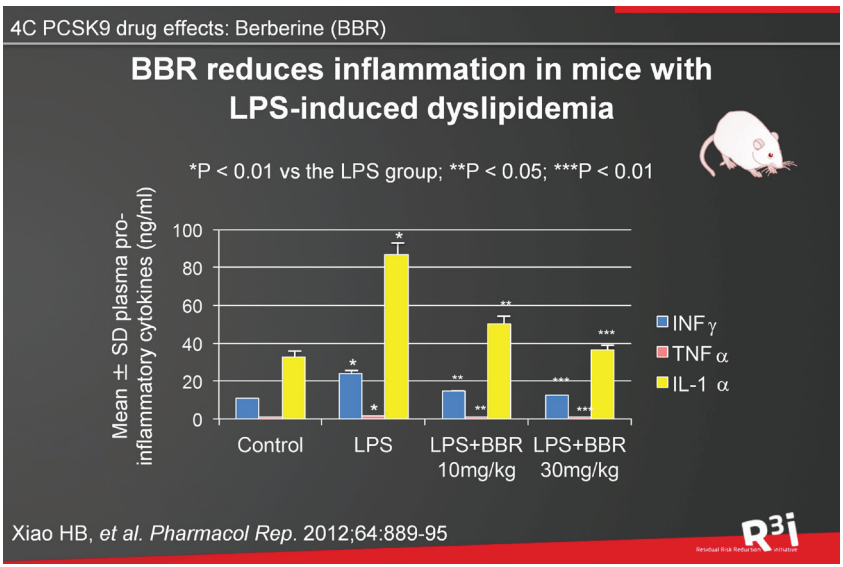
BBR reduces *PCSK9* and increases *LDL-R* gene expressions in mice with LPS-induced dyslipidemia

- LPS (lipopolysaccharide) administration decreases HDL levels whilst increasing VLDL and LDL levels in rodents
- Hepatic *PCSK9* expression significantly increased in LPS-injected mice, whereas *LDL-R* expression significantly decreased; in both cases, effects were dose-dependently suppressed when mice were pre-treated with BBR (10 or 30 mg/kg)



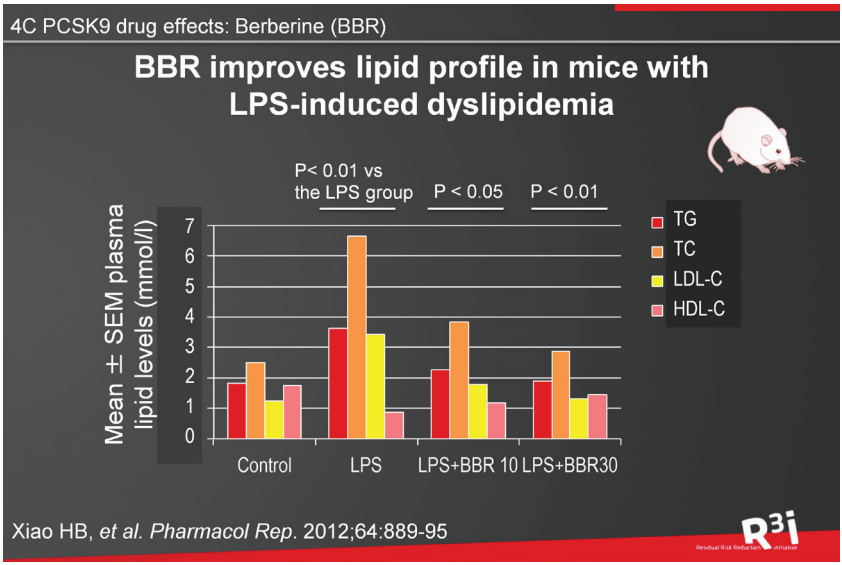
BBR inhibits the oxidative processes induced by LPS-treatment in mice

- Plasma 8-isoprostane generation was higher in the LPS-injected mice than in control mice; this effect was dose-dependently suppressed when mice were pre-treated with BBR (10 or 30mg/kg)



BBR reduces inflammation in mice with LPS-induced dyslipidemia

- Plasma IFNγ, TNFα and IL-1α levels were significantly increased in LPS-injected mice; this effect was markedly decreased in mice pre-treated with BBR 10 or 30mg/kg



BBR improves lipid profile in mice with LPS-induced dyslipidemia

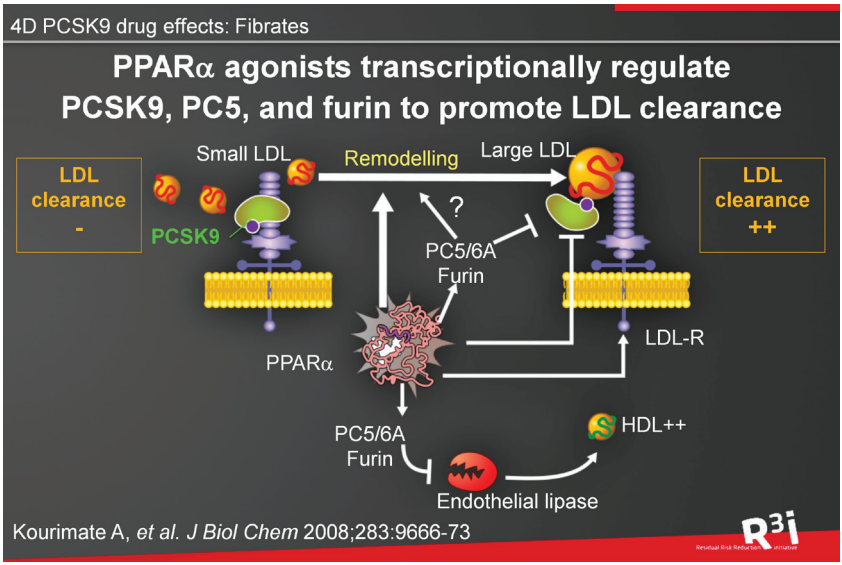
- LPS-injected mice had significantly reduced levels of plasma HDL-C vs control, and significantly elevated levels of plasma LDL-C, TG and TC; these effects were significantly decreased in mice pre-treated with BBR 10 or 30mg/kg
- Based on its antioxidant properties, it is probable that BBR regulates *PCSK9* and *LDL-R* gene expressions by inhibiting lipid peroxidation, which in turn, improves lipid profiles in mice with LPS-induced inflammation

Fibrates

SECTION

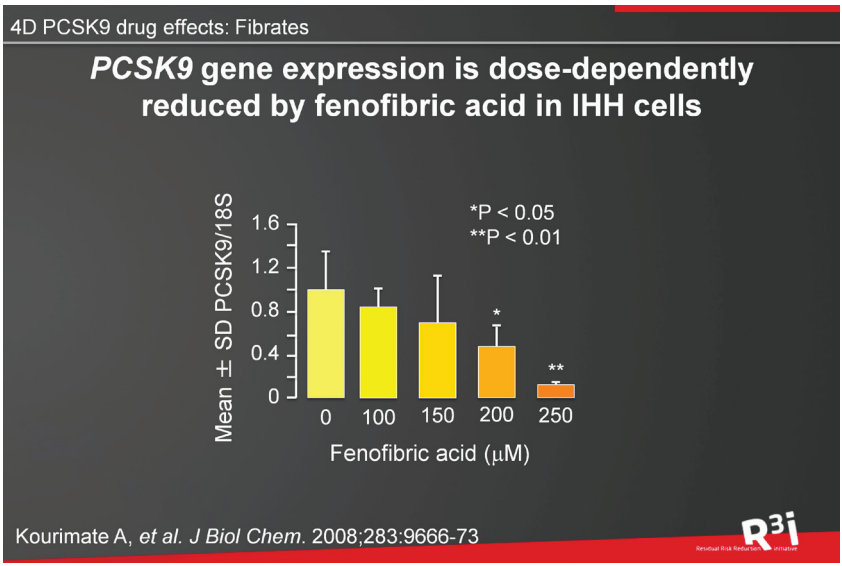
4D

Residual Risk Reduction initiative



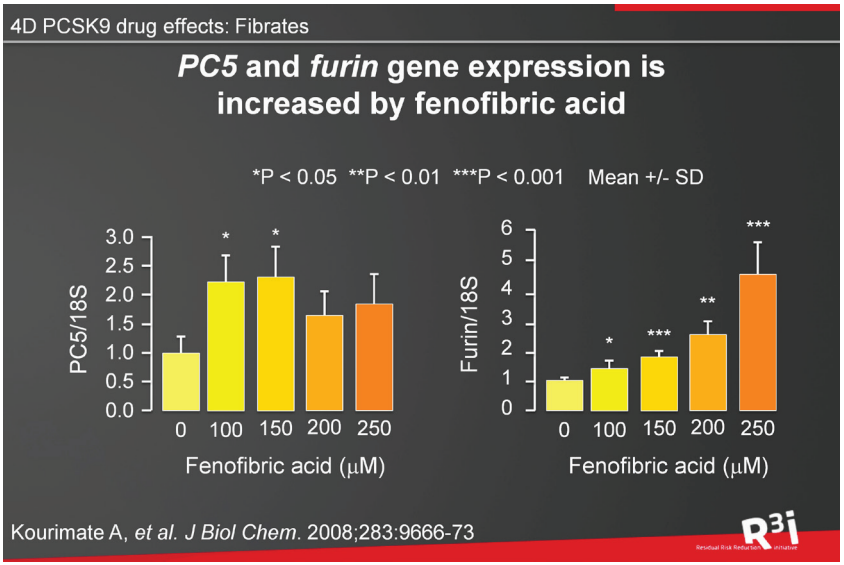
PPARα agonists transcriptionally regulate PCSK9, PC5, and furin to promote LDL clearance

- PPARα agonists (e.g. fibrates) promote LDL clearance by:
  - Directly promoting LDL remodelling leading to fewer dense pro-atherogenic particles and more large LDLs with an optimal binding capacity for LDL-R
  - Increasing LDL-R levels
  - Increasing the production of PC5/6A and furin, which:
    - Degrades endothelial lipase, thereby inhibiting the hydrolysis of HDL-phospholipids and the conversion of large LDL into smaller LDL; the net effect is increased levels of plasma HDL-C and lower levels of plasma LDL-C
    - Cleaves the active form of PCSK9 to form less active/inactive PCSK9



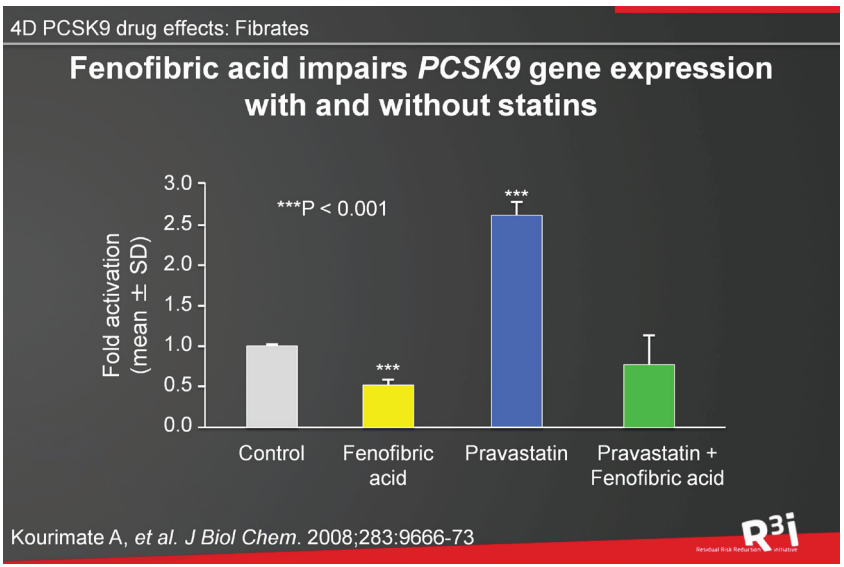
***PCSK9* gene expression is dose-dependently reduced by fenofibric acid in IHH cells**

- Fenofibric acid dose-dependently decreased PCSK9 mRNA levels in immortalized human hepatocytes (IHH), with an EC_{50} of 200μM



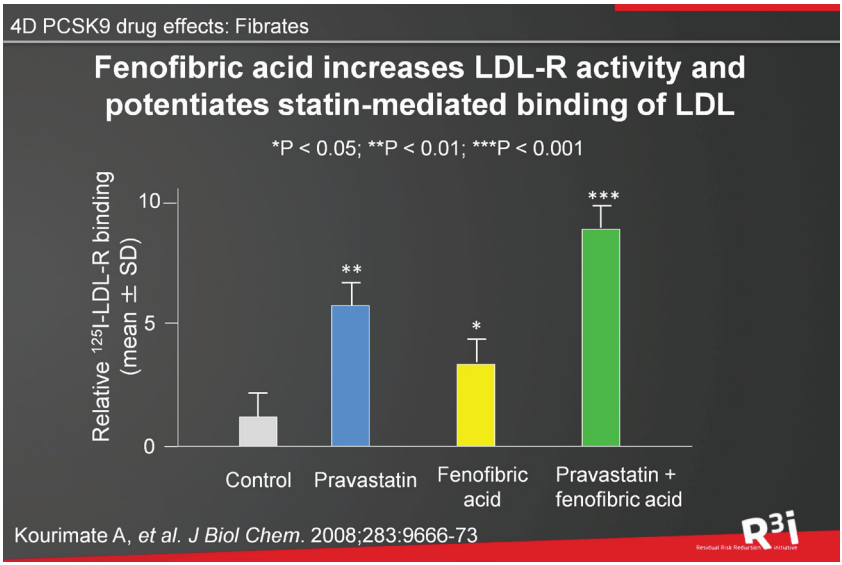
PC5 and furin gene expression is increased by fenofibric acid

- Fenofibric acid increased levels of PC5 and furin mRNA in IHH cells; the effect on furin was significant and dose-dependent



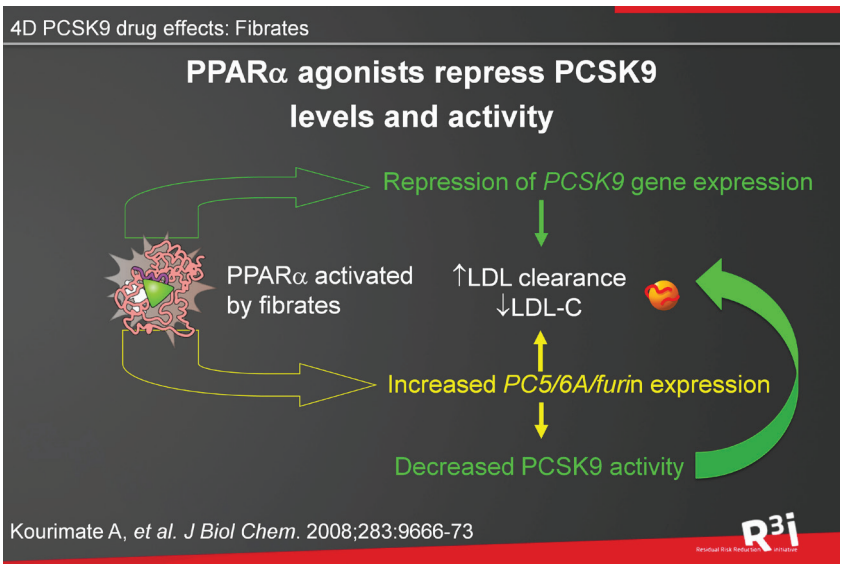
Fenofibric acid impairs *PCSK9* gene expression with and without statins

- IHH cells were exposed for 24h to fenofibric acid (250μM), and/or pravastatin (10μM) in the presence of 5% LPDS
- RT-PCR analysis showed that *PCSK9* gene expression was significantly increased by statins and significantly reduced with fenofibric acid, both alone and in combination with the statin



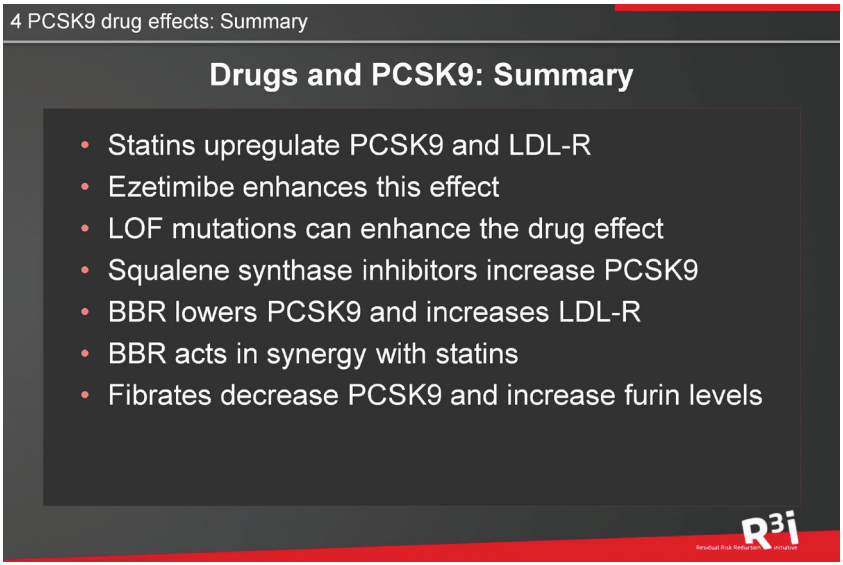
Fenofibric acid increases LDL-R activity and potentiates statin-mediated binding of LDL

- PCSK9-depleted IHH were incubated with or without fenofibric acid (250μM) for 24h followed by a 24h-treatment with pravastatin 5μM
- Separately, pravastatin and fenofibric acid significantly increased the amount of specifically bound ^{125}I-LDL by 500% and 170%, respectively
- Both drugs together provided an additional 30% increase in LDL-R activity vs pravastatin alone



PPARα agonists repress PCSK9 levels and activity

- PPARα-activation by fibrates inhibits *PCSK9* gene expression and increases *PC5/6A* and *furin* gene expression
- Increased levels of PC5/6A and furin decrease PCSK9 activity
- The combined effect is an increase in LDL clearance rates and decreased levels of plasma LDL-C



Drugs and PCSK9: Summary

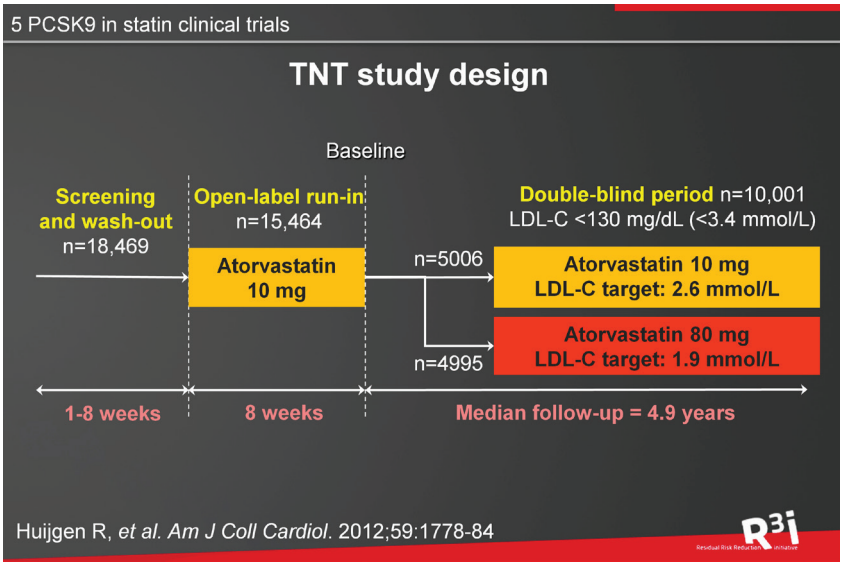
- Statins upregulate PCSK9 and LDL-R
- Ezetimibe enhances this effect
- LOF mutations can enhance the drug effect
- Squalene synthase inhibitors increase PCSK9
- BBR lowers PCSK9 and increases LDL-R
- BBR acts in synergy with statins
- Fibrates decrease PCSK9 and increase furin levels

PCSK9 in statin clinical trials

SECTION

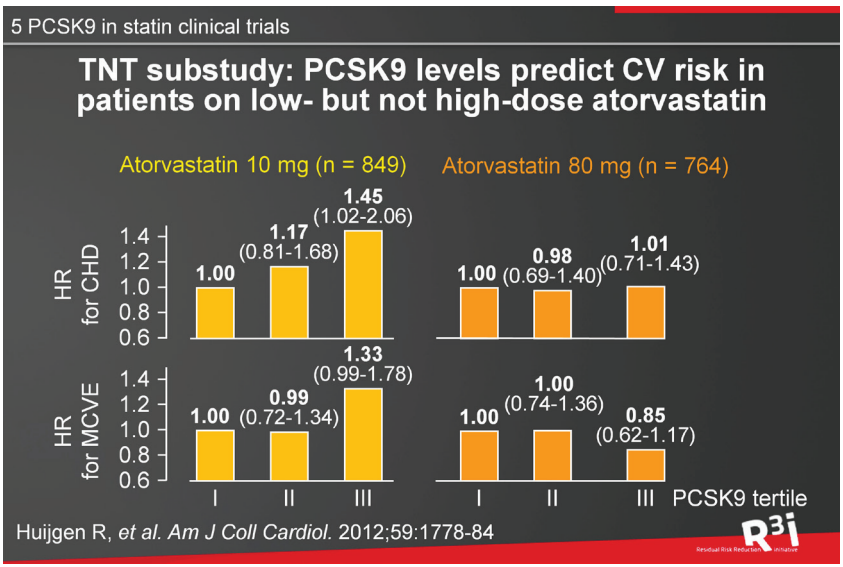
5

Residual Risk Reduction initiative



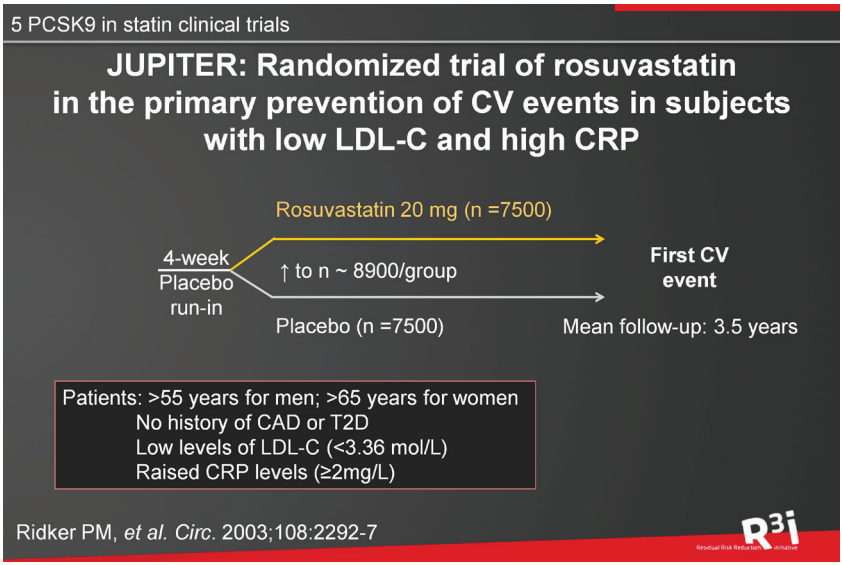
TNT study design

- The Treating to New Targets (TNT) study compared the efficacy of high- versus low-dose atorvastatin for the reduction of major CV events (CHD death, non-fatal/non-procedure-related MI, resuscitated cardiac arrest, fatal or non-fatal stroke) in patients with CHD and dyslipidemia
- After a run-in period with atorvastatin 10mg/day, patients were randomized to either continue with 10mg or be up-titrated to 80mg, and followed for ~5 years



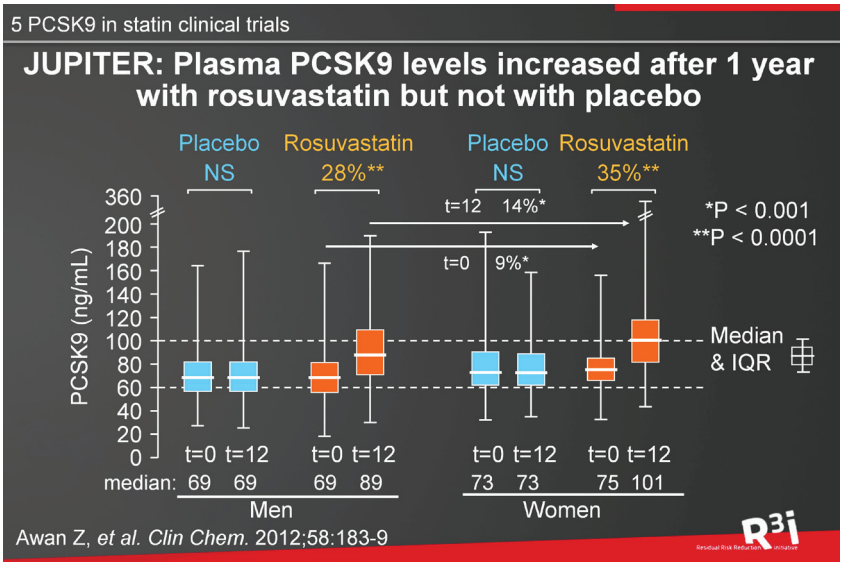
TNT substudy: PCSK9 levels predict CV risk in patients on low- but not high-dose atorvastatin

- A nested case-control TNT substudy (n=1613) showed that baseline circulating PCSK9 levels were predictive of CHD and major CV events (MCVE) in subjects randomized to remain on atorvastatin 10mg (P = 0.039), but not in those receiving atorvastatin 80mg (P = 0.24)
- Please note: Due to errors in the anonymization process, this paper has now been retracted. A new anonymized clinical and biomarker database has been created by restoring the original set of anonymized identifiers and data are currently being re-analyzed according to the original study plans



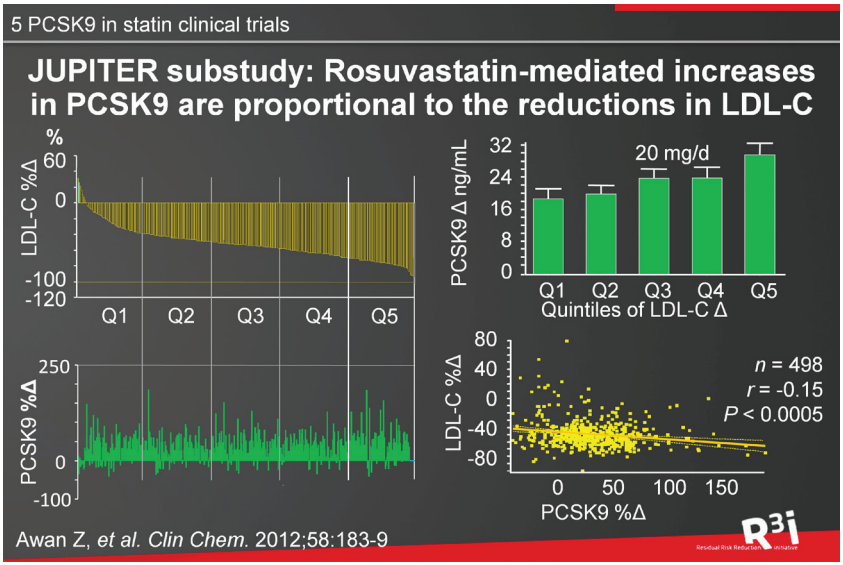
JUPITER: Randomized trial of rosuvastatin in the primary prevention of CV events in subjects with low LDL-C and high CRP

- The Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) trial was a randomized, double-blind, placebo-controlled study, designed to investigate whether treatment with rosuvastatin 20mg/day vs placebo decreased the rate of first CV events (MI, stroke, unstable angina, CV death, CABG/PTCA) in older patients (>55 years for men; >65 years for women) with no history of CAD or T2D and moderate- to low- levels of LDL-C (<3.36 mol/L) and raised CRP levels (≥2mg/L)



JUPITER: Plasma PCSK9 levels increased after 1 year with rosuvastatin but not with placebo

- A JUPITER substudy (n = 500 men and 500 women) showed that patients randomized to placebo had no significant change after 1 year in any measured variable, including PCSK9, suggesting considerable stability of PCSK9 concentrations over time
- In contrast, participants randomized to rosuvastatin had significant reductions in TC, LDL-C, TGs, and hsCRP as well a modest increase in HDL-C (not shown). These effects were accompanied by a 35% increase in median concentrations of PCSK9 in women (from 75ng/mL to 101ng/mL) and a 28% increase in men (from 69ng/mL to 89 ng/mL)



JUPITER substudy: Rosuvastatin-mediated increases in PCSK9 are proportional to the reductions in LDL-C

(A) Although individual variation among those allocated to rosuvastatin was wide, a significant relationship was observed between the magnitude of LDL-C reduction and the increase in PCSK9 concentrations

(B) This relationship was seen in both an analysis of quintiles of LDL-C reduction and when the LDL-C change was treated as a continuous variable

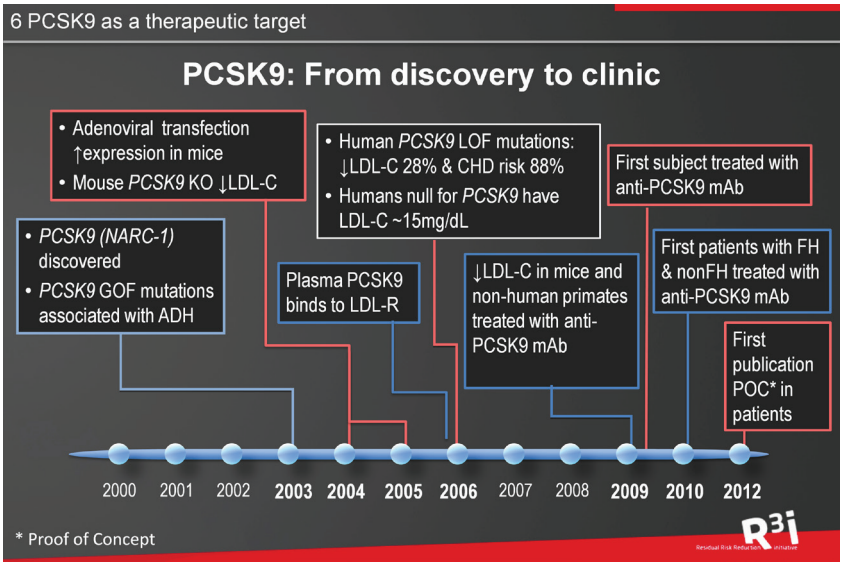
- A similar response was observed for Apo B and non–HDL-C changes and PCSK9 changes (data not shown)

PCSK9 as a therapeutic target

SECTION

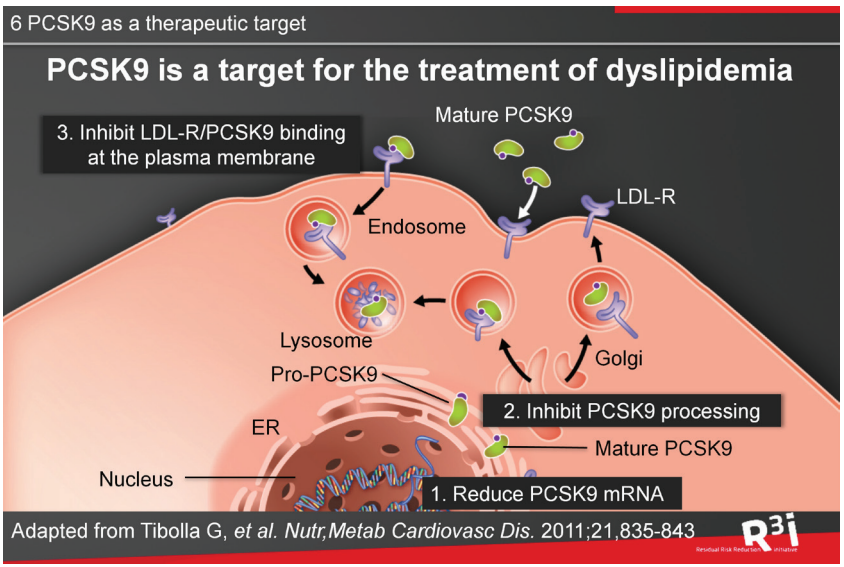
6

Residual Risk Reduction initiative



PCSK9: From discovery to clinic

- The pace of research, from PCSK9 discovery through to clinical trials, has been rapid



PCSK9 is a target for the treatment of dyslipidemia

Several strategies have been investigated for reducing PCSK9 activity:

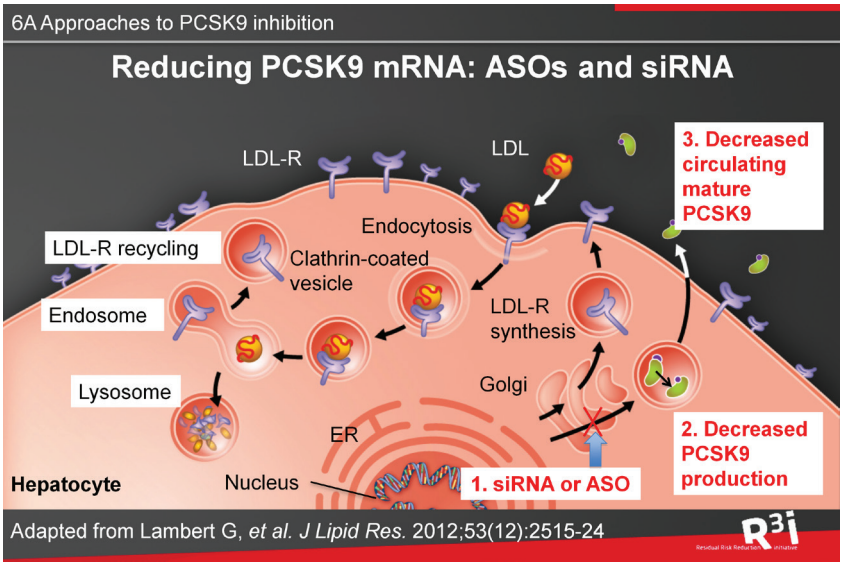
1. Modulation of PCSK9 mRNA expression using antisense oligonucleotides (ASO) or small interfering RNA (siRNA)
2. Reduction of PCSK9 processing and secretion using small molecules to inhibit autocatalytic processing
3. Inhibition of PCSK9 binding to LDL-R using mimetic small peptides, adnectin or monoclonal antibodies (mAb) that target extracellular PCSK9

Approaches to PCSK9 inhibition

SECTION

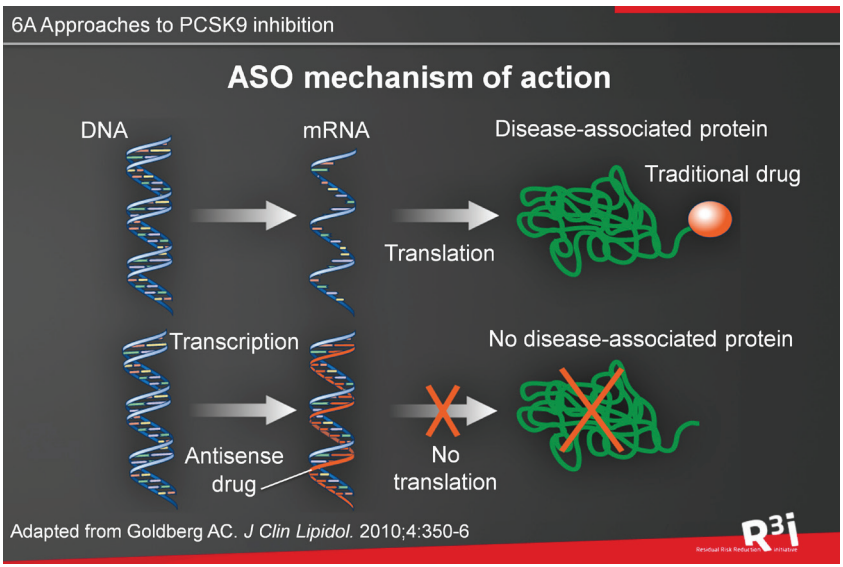
6A

Residual Risk Reduction initiative



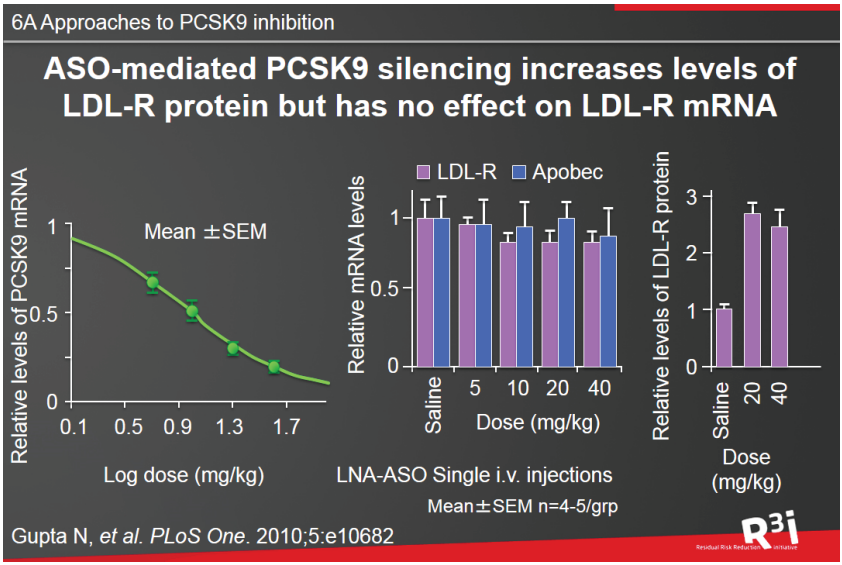
Reducing PCSK9 mRNA: ASOs and siRNA

- ASOs and siRNAs directed against PCSK9 mRNA have the potential to reduce PCSK9 mRNA levels in the liver



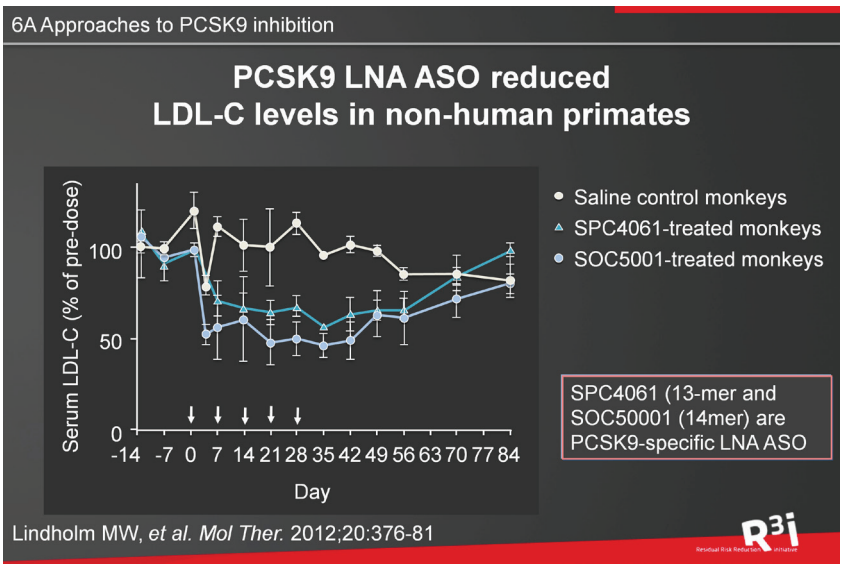
ASO mechanism of action

- ASOs are short synthetic analogues of natural nucleic acids that hybridize to specific mRNA sequences. This induces selective RNase H-mediated mRNA degradation, thereby preventing translation of the selected mRNA into protein



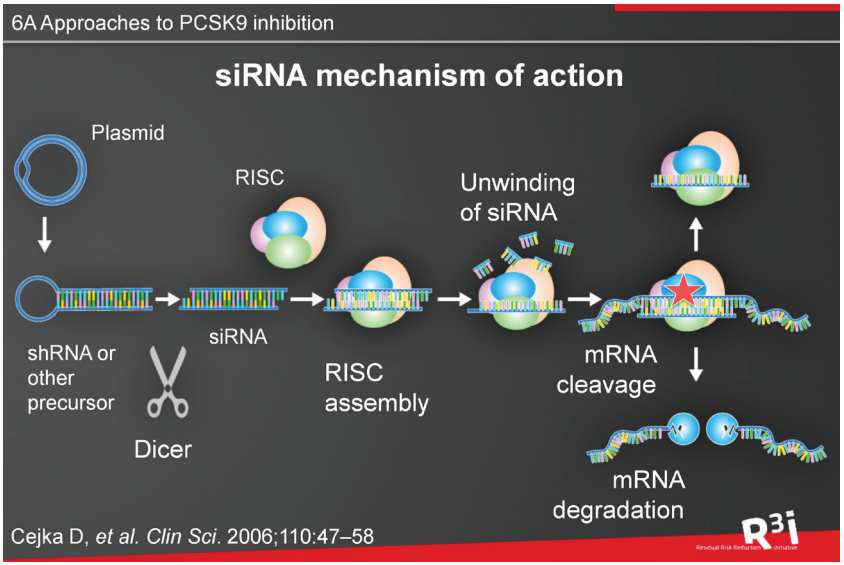
ASO-mediated PCSK9 silencing increases levels of LDL-R protein but has no effect on LDL-R mRNA

- A single i.v. injection of a locked nucleic acid (LNA) ASO (5-40mg/kg) targeting PCSK9 efficiently and dose-dependently reduced PCSK9 mRNA levels in female mice (ED_{50} ~9 mg/kg). The maximum effect of the ASO on PCSK9 mRNA levels was 95%
- This was accompanied by a 2- to 3-fold increase in LDL-R protein in animals treated with the highest dose. LDL-R mRNA levels were unaffected



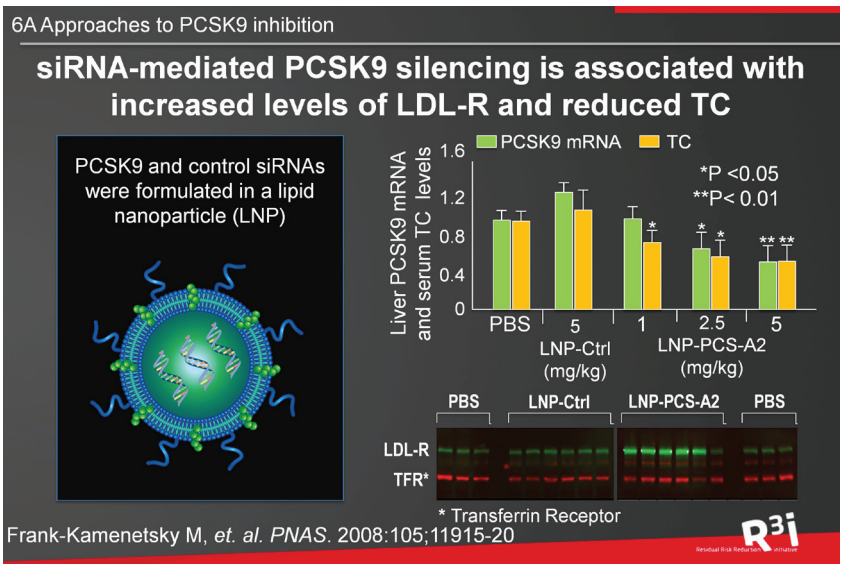
PCSK9 LNA ASO reduced LDL-C levels in non-human primates

- Monkeys injected with a 20mg/kg s.c. dose of LNA ASO (SPC 4061/5001) or saline followed by a 5mg/kg s.c. dose at regular intervals thereafter had a significant reduction in serum LDL-C levels that occurred 24h after the first injection
- From Day 4 onwards, SPC5001 treatment resulted in a mean 50% reduction in LDL-C vs pre-dose levels, with a 70% reduction in the highest responder
- SPC4061 was less potent that SPC5001, resulting in a mean 35% (maximum 50%) reduction



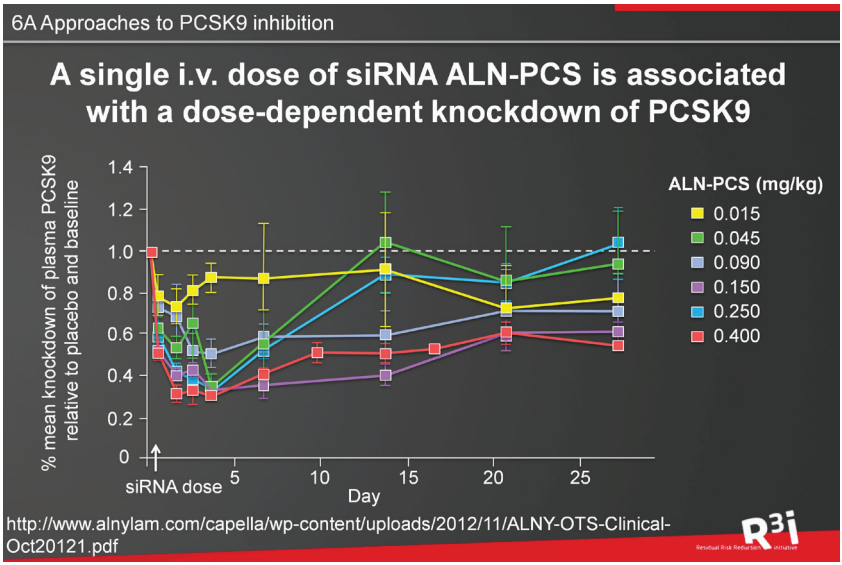
siRNA mechanism of action

- siRNAs are short stretches of paired nucleotides with a nucleotide overhang at both 3'-ends that associates with RISC (RNA-induced silencing complex)
- Within RISC, the siRNA is unwound and the sense strand removed for degradation by cellular nucleases
- The antisense strand of the siRNA directs RISC to the complimentary target mRNA sequence, ready for degradation by RISC endonuclease



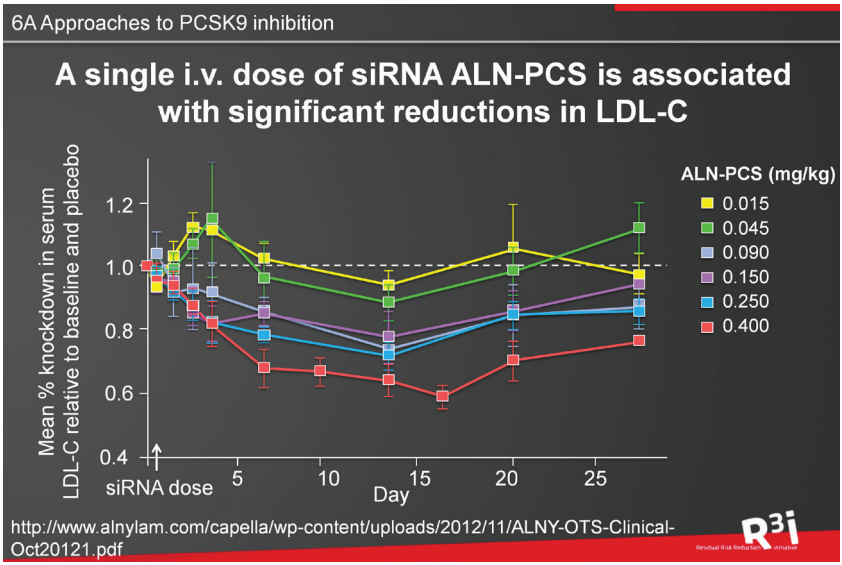
siRNA-mediated PCSK9 silencing is associated with increased levels of LDL-R and reduced TC

- Water-soluble siRNAs targeting PCSK9 (PCS-A2) were packaged into a lipid nanoparticle (LNP)
- In statin-resistant rats, LNP-PCS-A2 (1–5 mg/kg) resulted in dose-dependent reductions in PCSK9 mRNA levels up to 50-60%. This effect was associated with an acute 50–60% decrease in serum TC lasting 10 days, returning to pre-dose levels by 3 weeks
- Immunoblot of liver extracts showed that LNP-PCS-A2-treated animals had a significant 3- to 5-fold induction of LDL-R vs PBS- or LNP-Crtl-treated animals
- This suggests that lowering PCSK9 mRNA levels with anti-PCSK9 siRNAs in the liver results in an acute and durable decrease in serum cholesterol as a result of increased hepatic LDL-R expression



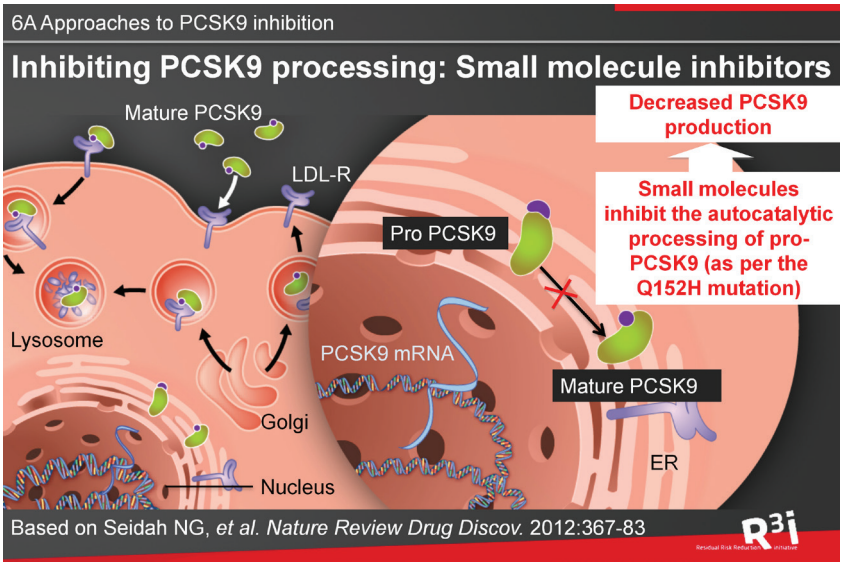
A single i.v. dose of siRNA ALN-PCS is associated with a dose-dependent knockdown of PCSK9

- A randomized, single-blind, placebo-controlled Phase I study in which 32 healthy, untreated volunteers with elevated LDL-C (>116 mg/dL) were randomized 3:1 to a single 60-minute I.V. infusion of an antisense siRNA targeting PCSK9 (ALN-PCS 0.015-0.400 mg/kg) or placebo
- Active treatment was associated with a rapid, dose-dependent, and durable knockdown of plasma PCSK9 of up to 84% (mean lowering 68%) in the highest-dose group ($P < 0.0001$ vs placebo)



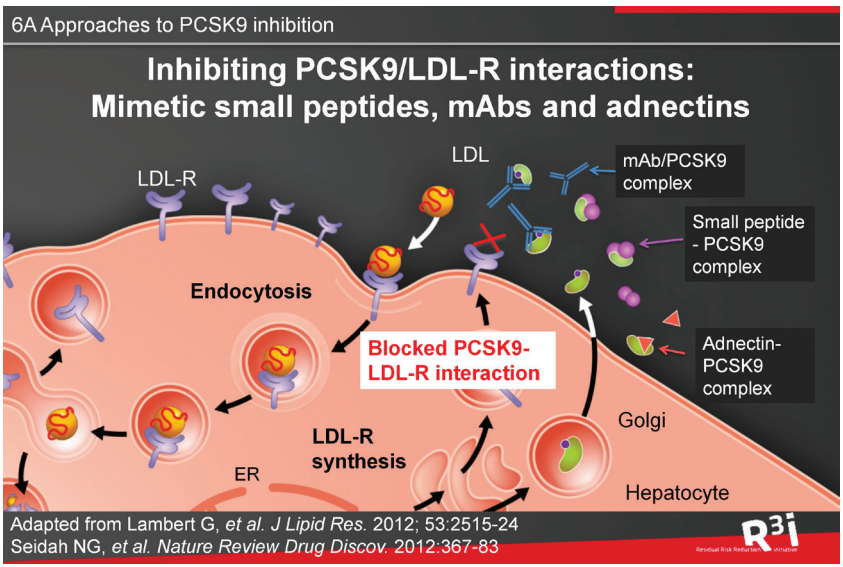
A single i.v. dose of siRNA ALN-PCS is associated with significant reductions in LDL-C

- Active treatment was associated with major dose-dependent reductions in LDL-C of up to 50% (mean 41%) in the highest-dose (0.4 mg/kg) group ($P < 0.01$ vs placebo)



Inhibiting PCSK9 processing: Small molecule inhibitors

- A dominant negative *PCSK9* LOF Q152H mutation prevents the zymogen pro-PCSK9 from autocatalytically cleaving itself in the ER, thereby preventing PCSK9 secretion
- *In silico* docking studies have been used to design compounds that can block the catalytic site of PCSK9
- The safety and efficacy of these compounds in animal models and non-human primates must be confirmed before clinical trials can begin



Inhibiting PCSK9/LDL-R interactions: Mimetic small peptides, mAbs and adnectins

- The extracellular PCSK9/LDL-R interaction can be blocked using:
  - EGF-A mimetic peptides that bind to secreted PCSK9 molecules preventing the PCSK9–LDL-R interaction
  - mAbs (~150 kDa) that target PCSK9
  - Inhibitory adnectins (~12 kDa) that are specific for PCSK9. (Adnectins are a new family of therapeutic proteins based on the 10th fibronectin type III domain and designed to bind with high affinity and specificity to therapeutically relevant targets)

6A Approaches to PCSK9 inhibition

PCSK9-targeted agents in development (1)

Treatment type	Company	Development stage	LDL-lowering effect in non-human primates
Adnectin	BMS/Adnexus	Pre-clinical	
LNP siRNA	Alnylam/Novartis	Phase I	41-50% decrease in LDL-C
LNA ASO	Santaris/Novartis	Phase I	50% decrease in LDL-C Program discontinued
Mimetic small peptide	Serometrix	Pre-clinical	
Small molecule inhibitor	Shifa Biomedical Corporation, Cadila Healthcare	Pre-clinical	

Seidah NG, *et al. Nature Review Drug Discov.* 2012:367-83

PCSK9-targeted agents in development (1)

- A number of therapeutic approaches for reducing PCSK9 levels are being developed. These include: Adnectins, siRNAs/ASOs, mimetic small peptides, small molecule inhibitors and anti-PCSK9 mAbs

6A Approaches to PCSK9 inhibition

PCSK9-targeted agents in development (2)

Treatment	Company	Development stage	LDL-lowering effect in humans
Fully human mAb (SAR236553/REGN727)	Sanofi/Regeneron	Phase II	30-65% decrease in LDL-C 73% decrease in LDL-C with mAB + atorvastatin 80mg vs vs 17% with atorvastation alone
Fully human mAb (AMG 145)	Amgen	Phase II	63-75 % decrease in LDL-C
Fully human mAb (RN316)	Pfizer/Rinat Neuroscience	Phase II	
Fully human mAb (LGT209)	Novartis	Phase II	
Fully human mAb (1B20)	Merck	In development	

Seidah NG, *et al. Nature Review Drug Discov.* 2012:367-83

PCSK9-targeted agents in development (2)

- A number of therapeutic approaches for reducing PCSK9 levels are being developed. These include: Adnectins, siRNAs/ASOs, mimetic small peptides, small molecule inhibitors and anti-PCSK9 mAbs

Anti-PCSK9 mAbs

SECTION

6B

Residual Risk Reduction initiative

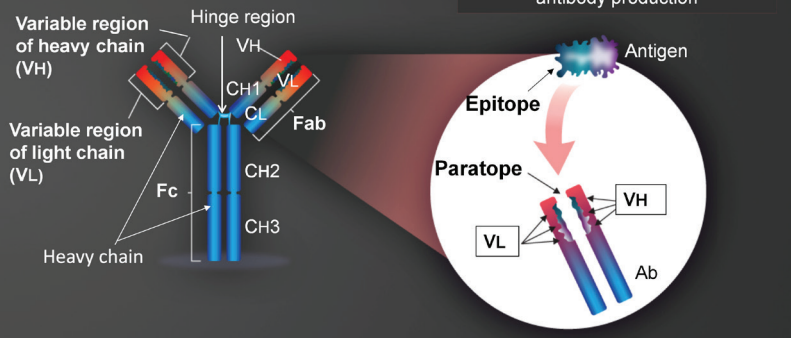
Therapeutic mAbs: Biology

DEFINING TOMORROW'S VASCULAR STRATEGIES

Antibodies: Structure and terminology

Antibody
Immunoglobulin secreted by B cells

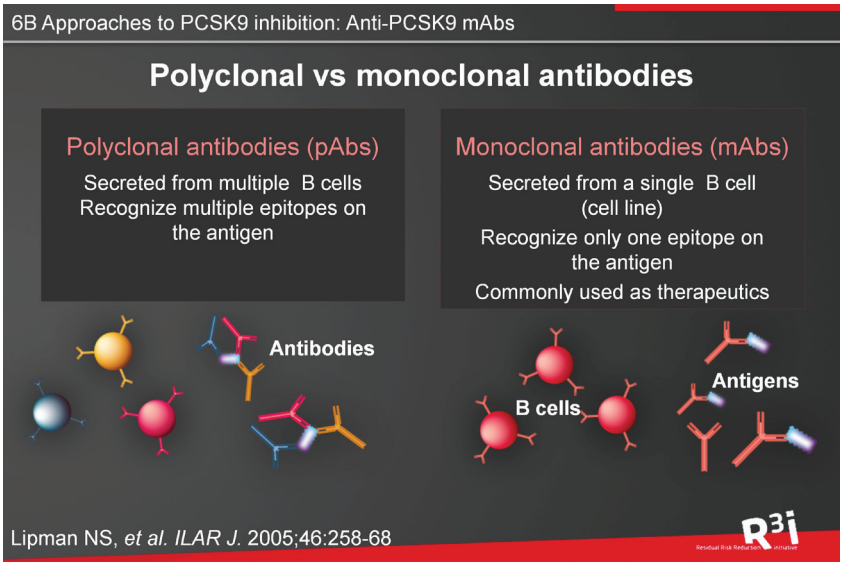
Antigen
Foreign substance that stimulates antibody production



Foltz IN, *et al. Circulation* 2013;127:2222-30

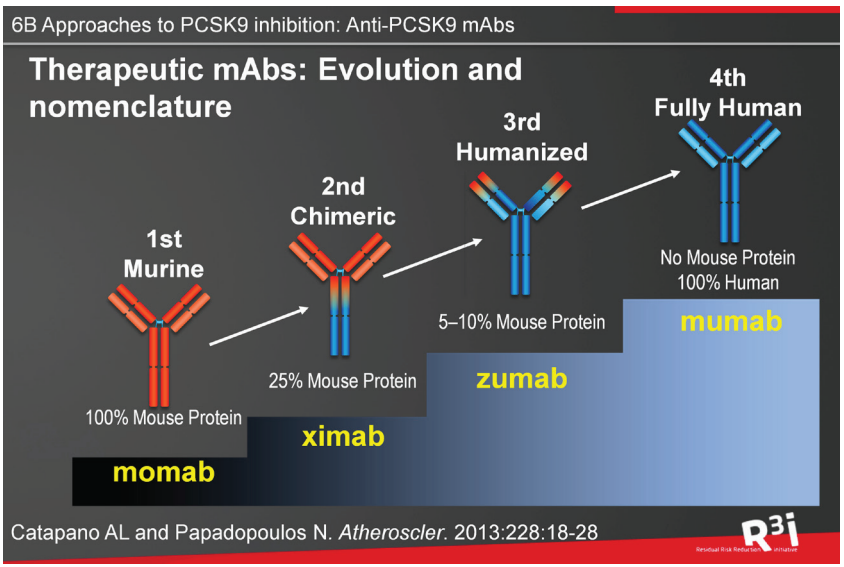
Antibodies: Structure and terminology

- Antibodies are immunoglobulins (Igs) that are produced and secreted by specialized B lymphocytes in response to an antigen (foreign substance)
- The Y-shaped molecules (~150 kD) comprise 2 identical heavy chains (H; ~55 kD) and 2 light chains (L; ~25 kD), each of which contains multiple constant (C) and one variable (V) regions linked by disulfide bonds
- The antigen-binding domains (Fab) reside in the arms; their effector domain (Fc) resides in the tail
- In mammals, there are 5 classes of Ig (IgG, IgM, IgA, IgD, and IgE). The Ig class determines both the type and the temporal nature of the immune response



Polyclonal vs monoclonal antibodies

- Polyclonal antibodies (pAbs) are secreted from multiple B cells and recognize multiple epitopes on the antigen
- Monoclonal antibodies (mAbs) are secreted from a single B cell (cell line) and recognize only one epitope on the antigen. They are commonly used as therapeutics



Therapeutic mAbs: Evolution and nomenclature

- Patients treated with the first therapeutic mAbs (mouse mAbs eg muromomab) often developed side effects due to the formation of human anti-mouse antibodies
- To reduce the risk of these events, chimeric mAbs (eg abciximab, infliximab) and humanized mAbs (eg daclizumab, omalizumab) were produced by fusing the antigen-binding variable domain (Fab) from the species used for immunization (typically mouse) onto variable amounts of human constant domains
- More recently, fully human mAbs (eg adalimumab) have been produced which, compared with chimeric and humanized mAbs, generally have reduced immunogenicity

Therapeutic mAbs are distinct from small molecule drugs

	Small molecule	mAb
Size	0.5 kDa	150 kDa
Structure	Chemical entity	Immunoglobulin
Target	Intracellular, CNS	Extracellular
Target specificity	Low(er)	High
Metabolism	Hepatic/renal	Target-mediated disposition
Administration	Oral	s.c. or i.v.
Dosing frequency	Daily	Q1W-Q4W
Crosses the blood-brain barrier	Potentially yes	No

Tabrizi MA, *et al. Drug Discov Today.* 2007;12:540-7; Gabathuler R. *Neurobiol Dis.* 2010;37:48-57; Gerber DE. *Am Fam Physician.* 2008;77:311-9

Therapeutic mAbs are distinct from small molecule drugs

- Compared to small molecule drugs, therapeutic mAbs are larger, have a higher target specificity, require less-frequent dosing and cannot cross the blood-brain barrier
- They are typically administered via s.c. or i.v. infusion/injection and do not undergo hepatic or renal metabolism (i.e. they have a reduced potential for drug-drug interactions)

Therapeutic mAbs: Clinical safety

- The majority of AEs are target-related
  - Infections, cancer
  - Autoimmune disease
  - Organ-specific adverse events such as cardiotoxicity
- Non specific (off-target) toxicities
  - Acute anaphylaxis
  - Serum sickness
  - Infusion reactions
  - Immunogenicity

Hansel TT, *et al. Nature Rev. Drug Discov* 2010;9:325-38

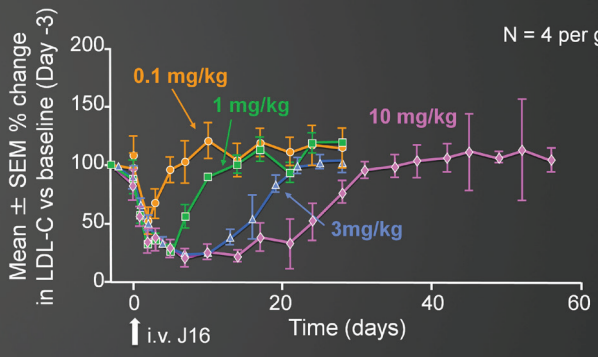
Therapeutic mAbs: Clinical safety

- In general, mAbs are well tolerated
- The majority of AEs are target- (antigen-) related and are specific to their target and therapeutic area of use
- Non specific (off-target) toxicities are consistent with those commonly observed with protein therapeutics and are related to immune reactions (e.g. hypersensitivity reactions, infusion-related reactions and immunogenicity)
- The risk of these AEs is considerably lower with chimeric and humanized mAbs than with human anti-mouse Abs

Anti-PCSK9 mAbs: Pre-clinical trials

DEFINING TOMORROW'S VASCULAR STRATEGIES

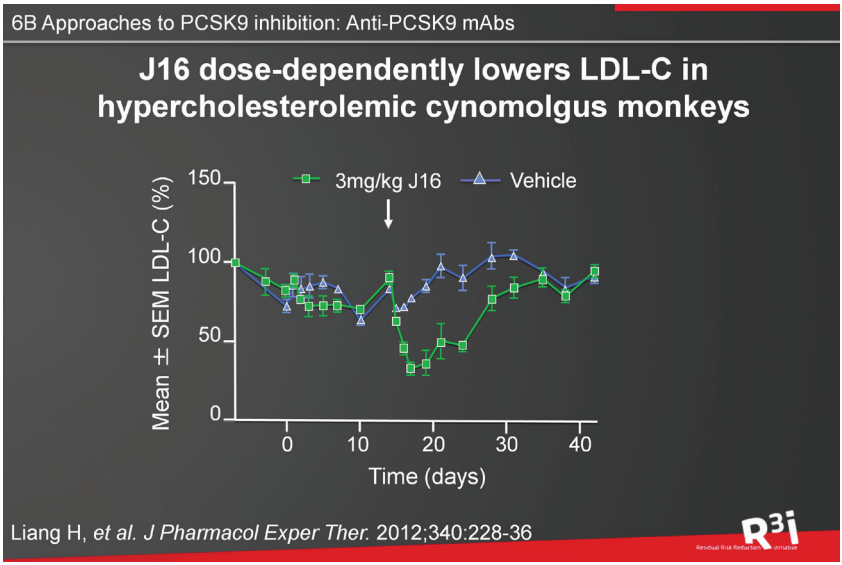
Humanized anti-PCSK9 mAb, J16, dose-dependently lowers LDL-C in healthy cynomolgus monkeys



Liang H, *et al. J Pharmacol Exper Ther* 2012;340:228-36

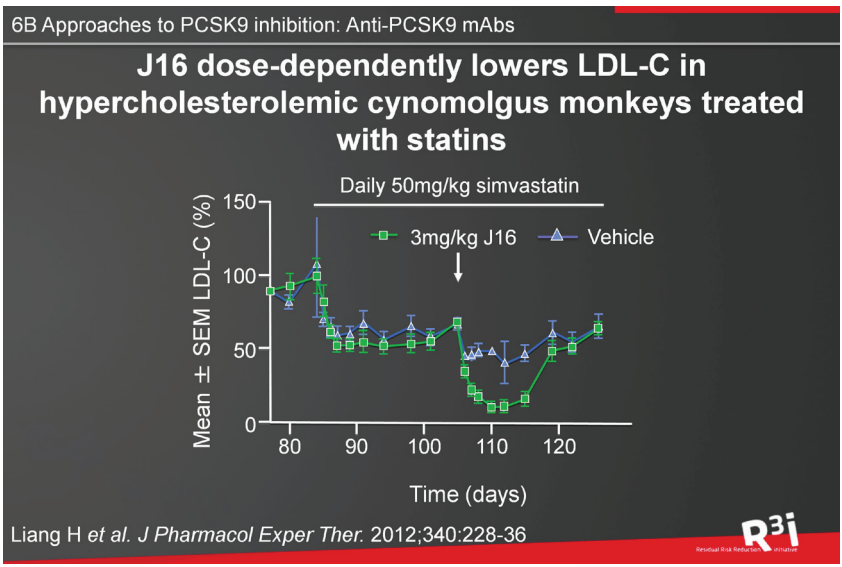
Humanized anti-PCSK9 mAb, J16, dose-dependently lowers LDL-C in healthy cynomolgus monkeys

- Cynomolgus monkeys fed a normal diet received a single i.v. injection of J16 at doses of 0.1, 1, 3, and 10 mg/kg (n = 4/group)
- J16 significantly and rapidly reduced LDL-C at all doses vs baseline (Day -3); levels recovered by 2 to 4 weeks after dosing
- The magnitude, duration, and cumulative percentage reduction over time calculated by area under the curve of the LDL-C lowering was dose-dependent



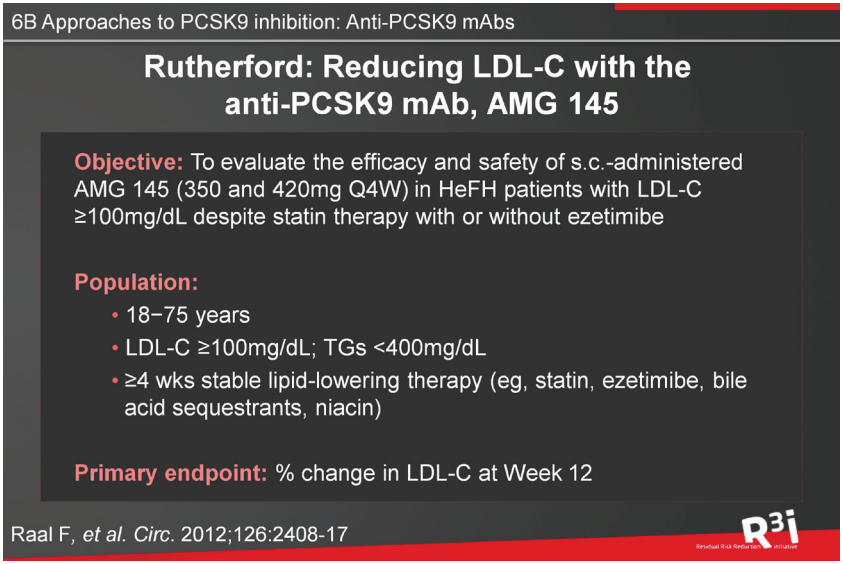
J16 dose-dependently lowers LDL-C in healthy cynomolgus monkeys

- Hypercholesterolemic cynomolgus monkeys fed a diet comprising 62% kcal fat and 0.1 mg/kcal cholesterol for >18 months (mean LDL-C 120 mg/dL) received a single i.v. injection of 3 mg/kg J16 or vehicle on day 14
- J16 effectively lowered serum LDL-C levels by 64% by day 3; levels gradually returned to predosing levels by 2.5 to 3 weeks post-treatment
- The percentage reduction and the duration of the effect were similar to that observed in monkeys fed normal chow



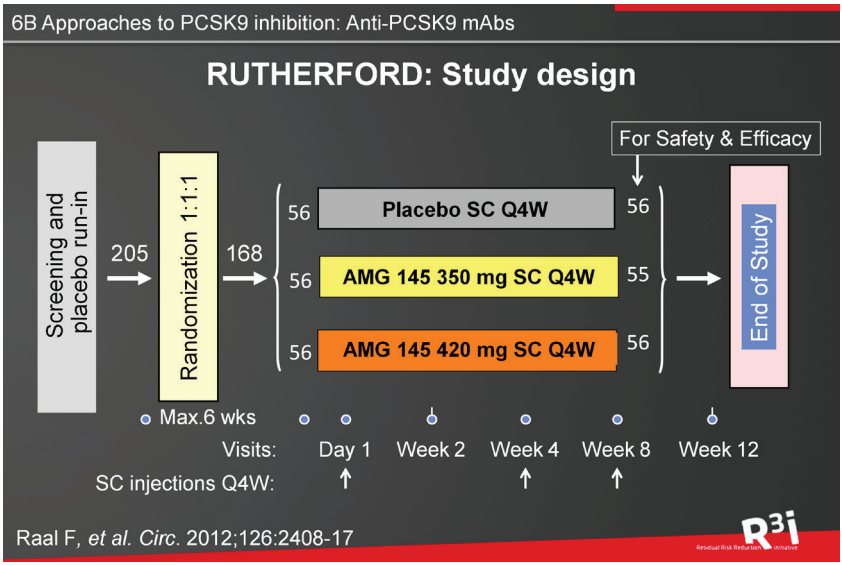
J16 dose-dependently lowers LDL-C in hypercholesterolemic cynomolgus monkeys treated with statins

- Hypercholesterolemic cynomolgus monkeys fed a diet comprising 62% kcal fat and 0.1 mg/kcal cholesterol for >18 months (mean LDL-C 120 mg/dL) received simvastatin 50 mg/kg from days 84 to 126 together with a single i.v. injection of 3 mg/kg J16 or vehicle on day 105
- LDL-C levels were reduced by up to 43% with simvastatin treatment alone (day 89) and stabilized thereafter
- J16 dosing caused an additional 65% reduction in LDL-C (achieved 5 days after J16 dosing) and returned to predosing levels within 2 weeks



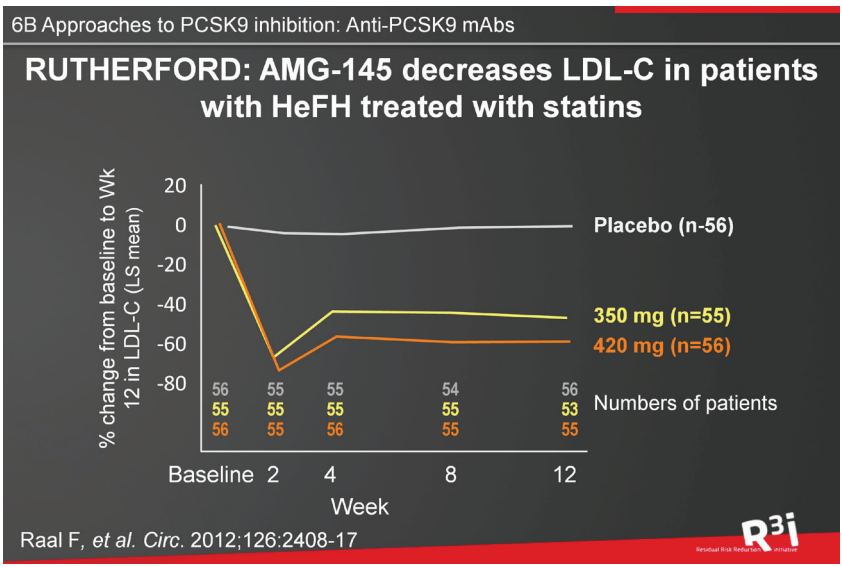
Rutherford: Reducing LDL-C with the anti-PCSK9 mAb, AMG 145

- AMG 450 is a fully human anti-PCSK9 mAb
- The Reduction of LDL-C With PCSK9 Inhibition in Heterozygous Familial Hypercholesterolemia Disorder (RUTHERFORD) trial was designed to evaluate the efficacy and safety of AMG 145 in HeFH patients aged 18-75 years, with TGs <400mg/dL and LDL-C ≥100mg/dL despite stable lipid-lowering therapy (statin +/- ezetimibe, bile acid sequestrants, niacin) for at least 4 weeks prior to treatment
- The primary endpoint was the % change in LDL-C at Week 12



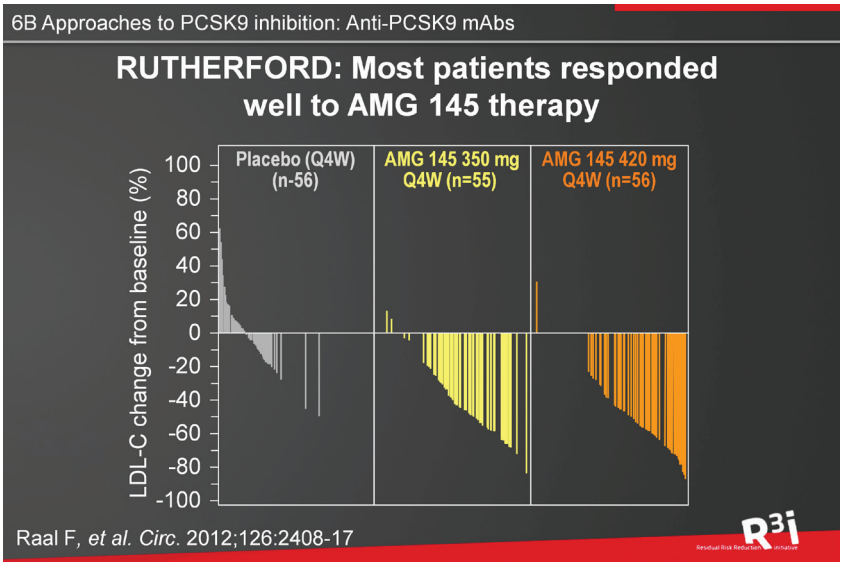
RUTHERFORD: Study design

- Rutherford was a Phase 2 double-blind, placebo-controlled trial in which 168 patients with HeFH were randomized to receive a s.c. dose of placebo or AMG 145 (either 350mg or 420mg) every 4 weeks



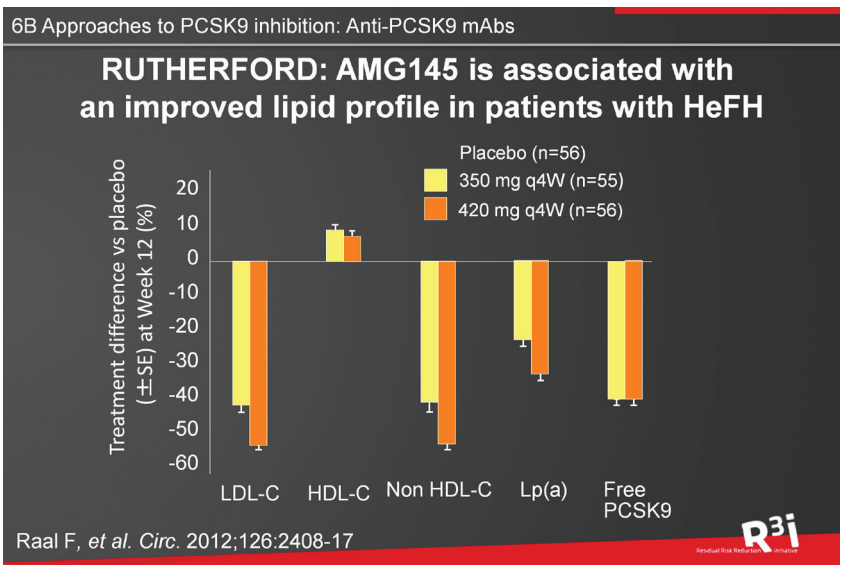
RUTHERFORD: AMG-145 decreases LDL-C in patients with HeFH treated with statins

- AMG 145 resulted in a rapid and sustained dose-dependent reduction in LDL-C (measured by ultracentrifugation and calculated using the Friedewald formula)
- At week 12, the LS mean reduction in LDL-C was 43% and 55% with AMG 145 350mg and 420 mg, respectively, compared with a 1% increase with placebo ($P < 0.001$ for both dose groups)



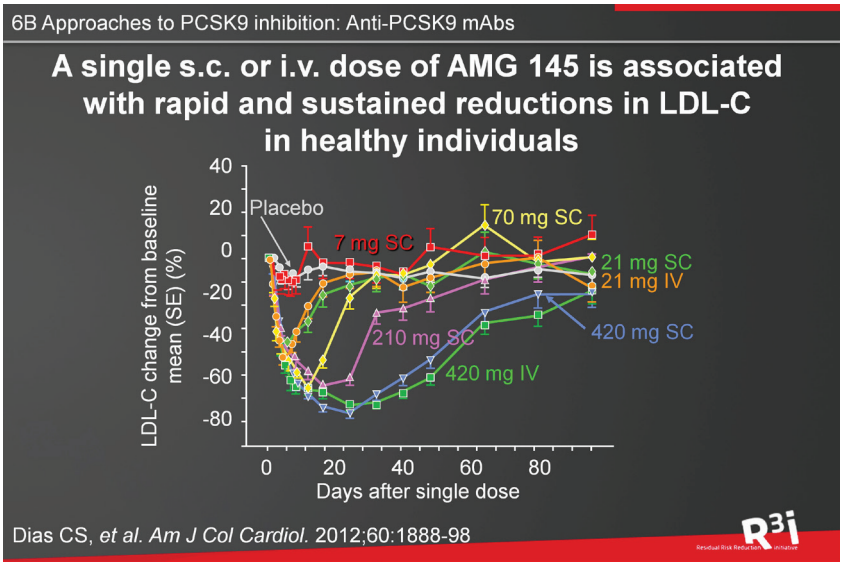
RUTHERFORD: Most patients responded well to AMG 145 therapy

- 95% of patients on AMG 145 experienced reductions in LDL-C of at least 15%; 52% of patients had reductions ≥50%
- 4 patients in the AMG 145 350mg group and 1 in the 420mg group were considered to be poor responders (i.e. had <15% reduction in LDL-C at Week 12)
- 2 patients (1 on AMG 145 350mg and 1 on 420mg) discontinued therapy after just 1 dose; both continued the study visits but did not receive the study drug for 12 weeks



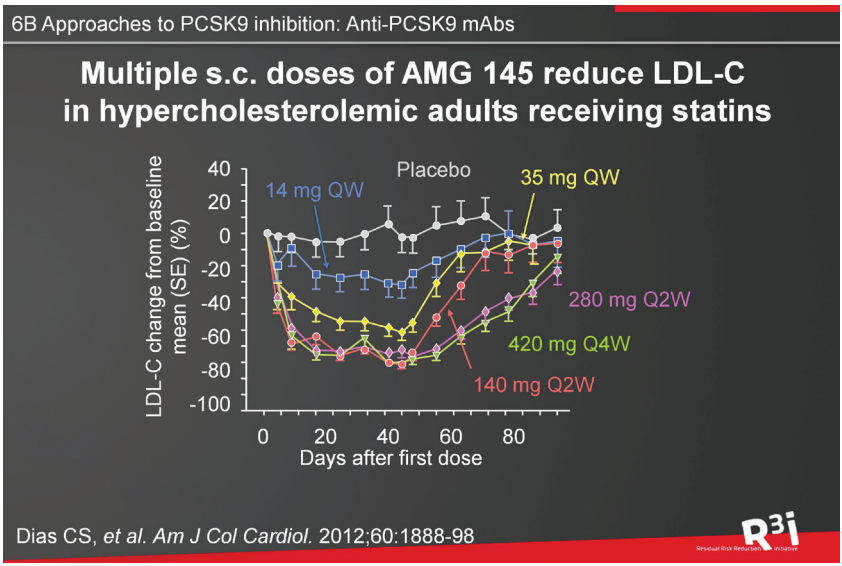
RUTHERFORD: AMG145 is associated with an improved lipid profile in patients with HeFH

- Reductions in TC, non–HDL-C, and Apo B were consistent with those seen in LDL-C
- Compared with placebo, there were modest, but statistically significant, dose-dependent reductions of 15% and 20% in TGs and a modest, statistically significant increase of approximately 7% in HDL-C
- There was a significant dose-dependent reduction in Lp(a) with AMG 145 (23% and 32% vs placebo)
- AMG 145 significantly reduced free PCSK9 levels by 41% vs placebo with both doses; lower LDL-C levels were associated with lower free PCSK9 levels at all doses and at all visits



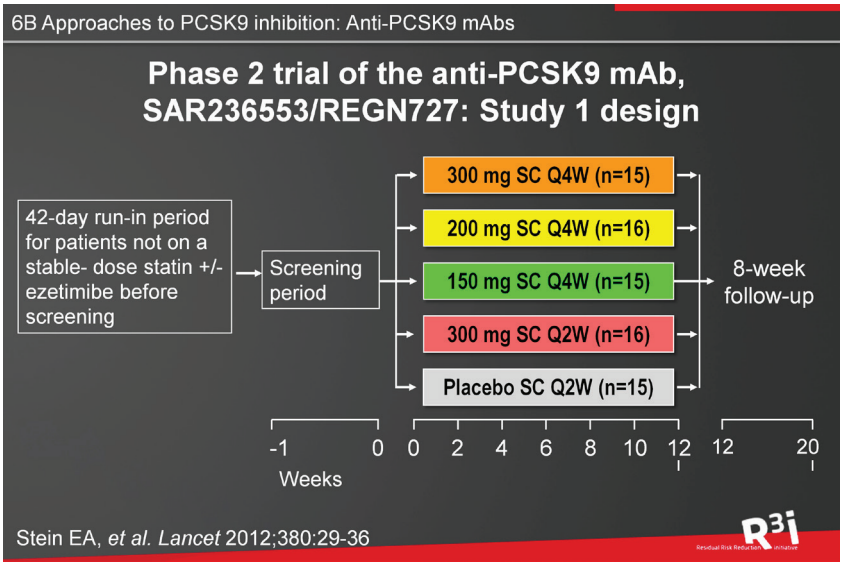
A single s.c. or i.v. dose of AMG 145 is associated with rapid and sustained reductions in LDL-C in healthy individuals

- Healthy adults were randomized to receive either a single s.c. dose of AMG 145 (7, 21, 70, 210, or 420mg), an i.v. dose (21 or 420mg) or matching placebo
- AMG 145 dose-dependently reduced LDL-C by up to 64% ($P < 0.0001$) vs placebo after 1 dose ≥21 mg



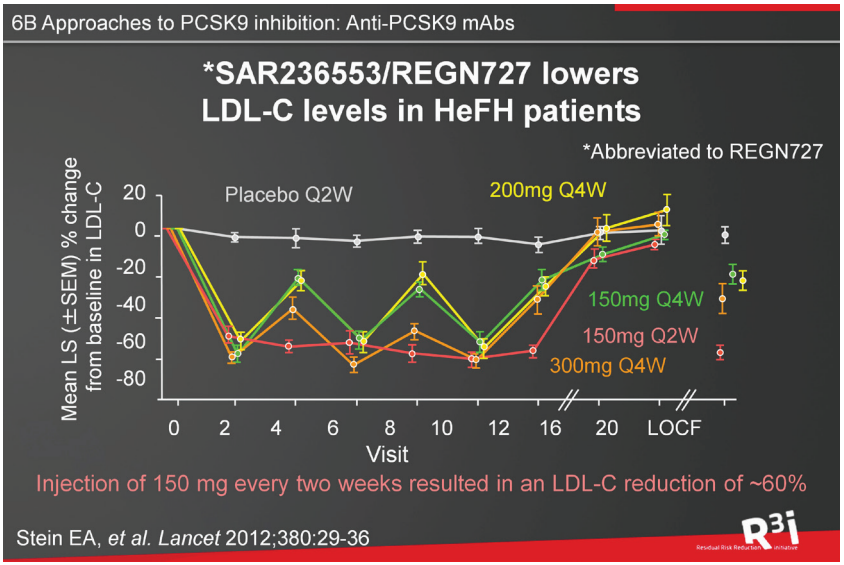
Multiple s.c. doses of AMG 145 reduce LDL-C in hypercholesterolemic adults receiving statins

- Hypercholesterolemic adults receiving low- to moderate-dose statins were randomized to receive s.c. AMG 145 at doses of either 14 or 35mg QW x6; 140 or 280mg Q2W x3; 420mg Q4W x2; or matching placebo
- AMG 145 dose-dependently reduced LDL-C by up to 81% ($P < 0.001$) with repeated doses ≥35mg QW



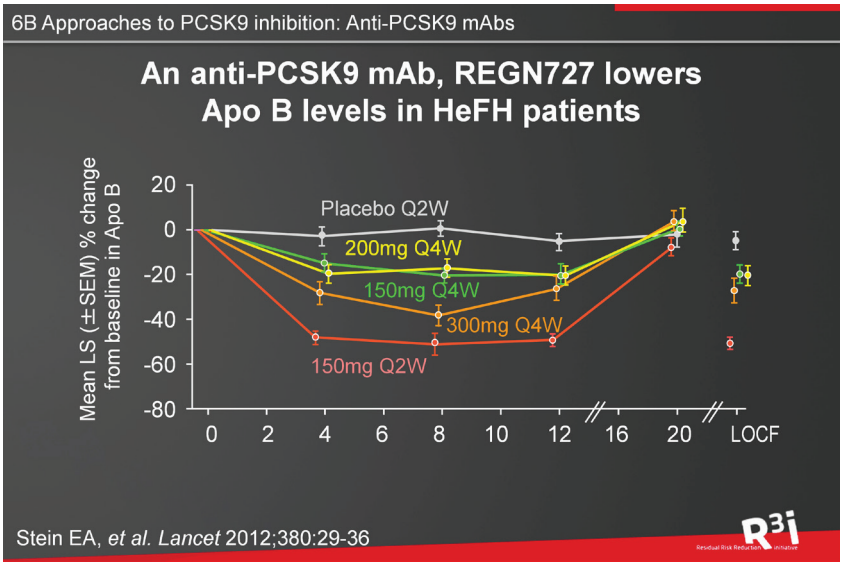
Phase 2 trial of the anti-PCSK9 mAb, SAR236553/REG727: Study design

- A randomized, placebo-controlled, multicentre, Phase 2 trial in adults with HeFH and LDL-C concentrations ≥2·6 mmol/L on a stable diet and statin dose, with or without ezetimibe
- Patients were randomized to receive s.c. injections of SAR236553/REG727 (REGN727) 150mg, 200mg, or 300mg Q4W; REGN727 150mg Q2W; or placebo Q2W (ratio 1:1:1:1:1). Randomization was stratified by concomitant use of ezetimibe at baseline
- The primary endpoint was the mean percent reduction in LDL-C from baseline at Week 12



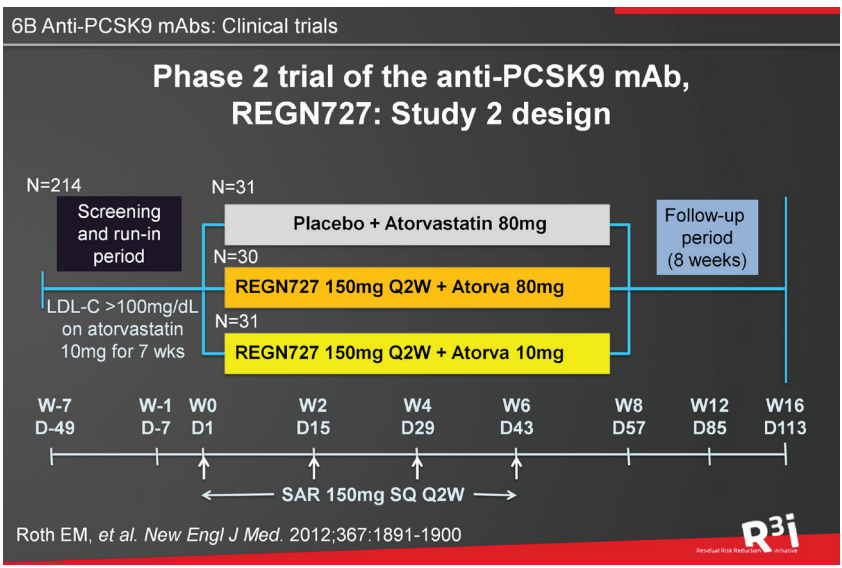
SAR236553/REGN727 lowers LDL-C levels in HeFH patients

- The least squares (LS) mean reduction in LDL-C from baseline to Week 12 was 28.9–67.9% with REGN727 vs 10.65% with placebo
- The percentage changes in LDL-C at Week 12 were statistically significant vs placebo for all REGN727 doses
- LDL-C reductions were rapid, reaching a maximum at 2 weeks for every dose other than 150mg Q2W, which continued to decrease with subsequent dosing and stabilized after the third dose



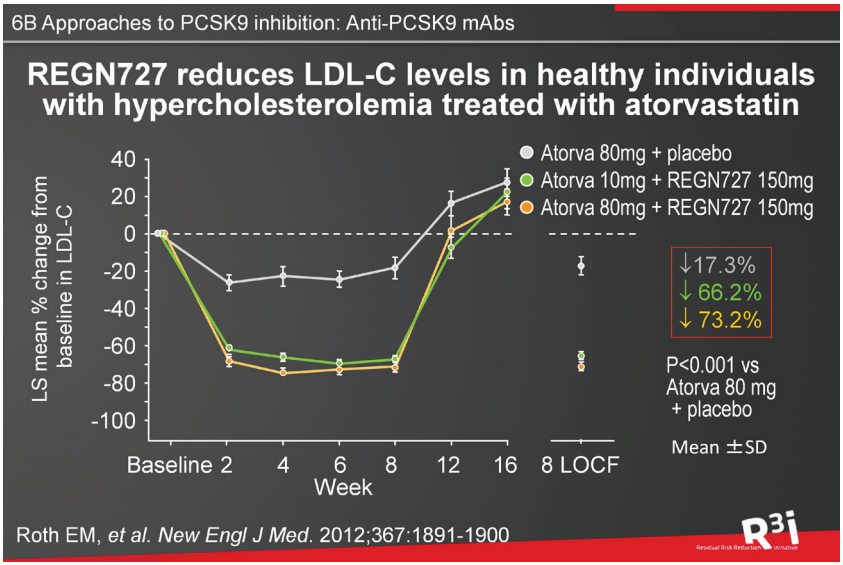
An anti-PCSK9 mAb, REGN727, lowers Apo B levels in HeFH patients

- The LS mean reduction in Apo B from baseline to Week 12 was 20.91% to 50.19% with REGN727 vs 6.39% with placebo
- Reductions were consistent with those observed for LDL-C



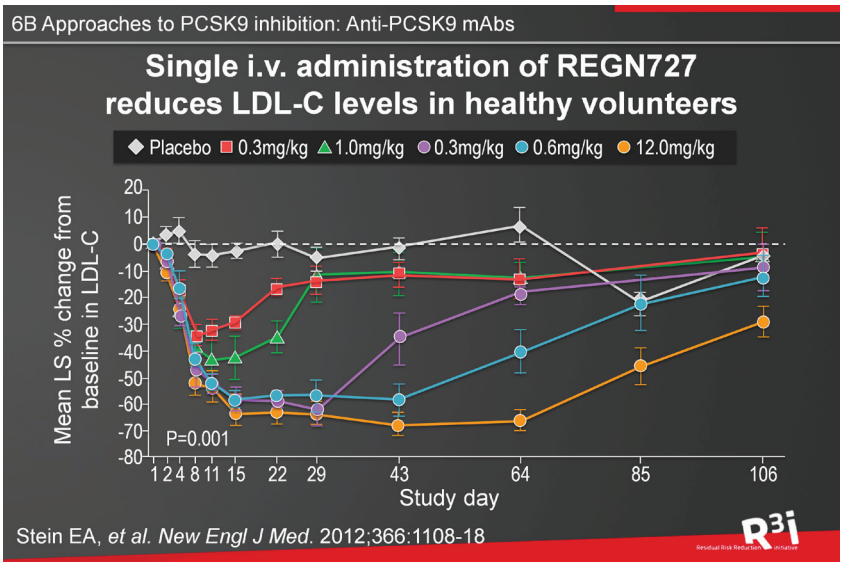
Phase 2 trial of the anti-PCSK9 mAb, REGN727: Study 2 design

- A Phase 2, multicenter, double-blind, placebo-controlled trial involving 92 patients with LDL-C levels ≥100 mg/dL (2.6 mmol/L) after treatment with 10mg of atorvastatin for at least 7 weeks
- Patients were randomly assigned to receive 8 week's treatment with atorvastatin 80mg/day plus REGN727 Q2W, atorvastatin 10mg/day plus REGN727 Q2W, or atorvastatin 80mg/day plus placebo Q2W and were followed for an additional 8 weeks after treatment
- The primary endpoint was the percent reduction from baseline at Week 16



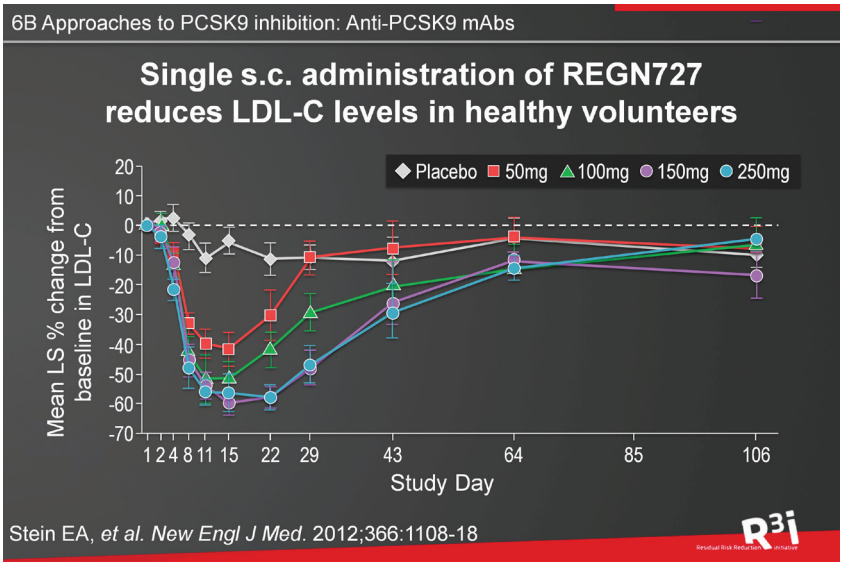
REGN727 reduces LDL-C levels in healthy individuals with hypercholesterolemia treated with atorvastatin

- After 8 weeks of treatment, the LS mean percent reduction in LDL-C from baseline was 73.2 with atorvastatin 80mg plus REGN727 vs 17.3 with atorvastatin 80mg plus placebo (LS mean difference of –55.9%; P<0.001) The corresponding reduction with atorvastatin 10mg plus REGN727 150mg was 66.2



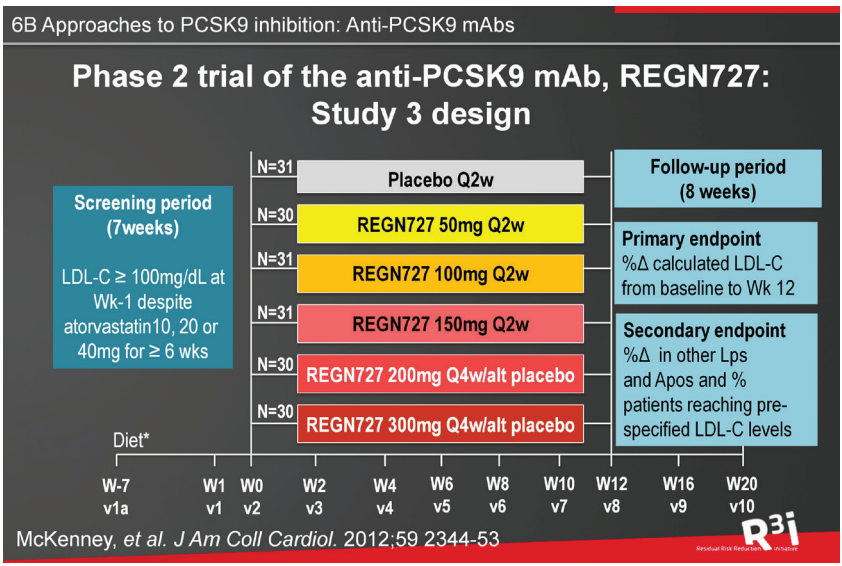
Single i.v. administration of REGN727 reduces LDL-C levels in healthy volunteers

- Single i.v. administration of REGN727 in healthy volunteers with hypercholesterolemia (LDL-C >2.59 mmol/L) resulted in a LS mean difference in the change from baseline in LDL-C of up to 65% vs placebo
- The degree and duration of LDL-C lowering were dose-dependent, with higher doses producing prolonged reductions that were sustained up to Day 64



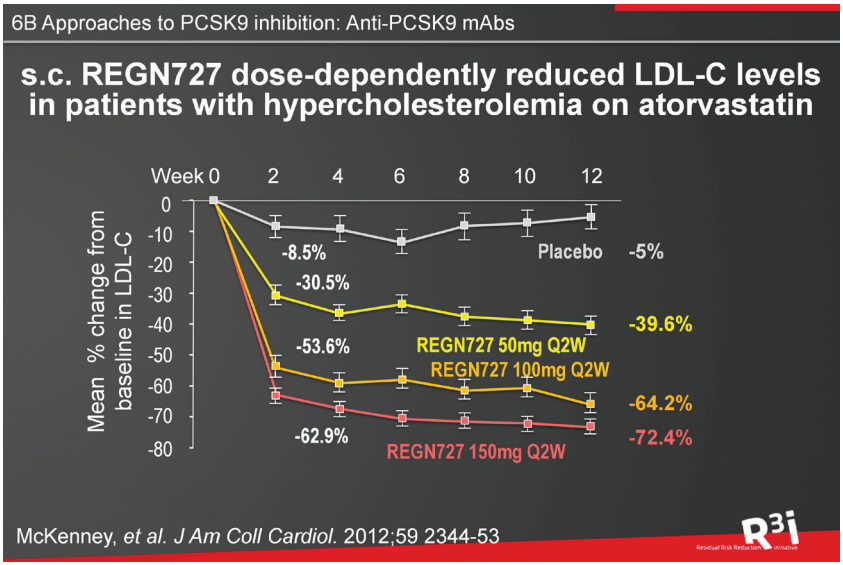
Single s.c. administration of REGN727 reduces LDL-C levels in healthy volunteers

- Single s.c. administration of REGN727 in healthy volunteers with hypercholesterolemia (LDL-C >2.59mmol/L) resulted in a LS mean difference in the change from baseline in LDL-C of up to 46% vs placebo
- The degree and duration of LDL-C lowering were dose-dependent, with higher doses producing prolonged reductions that were sustained up to Day 22



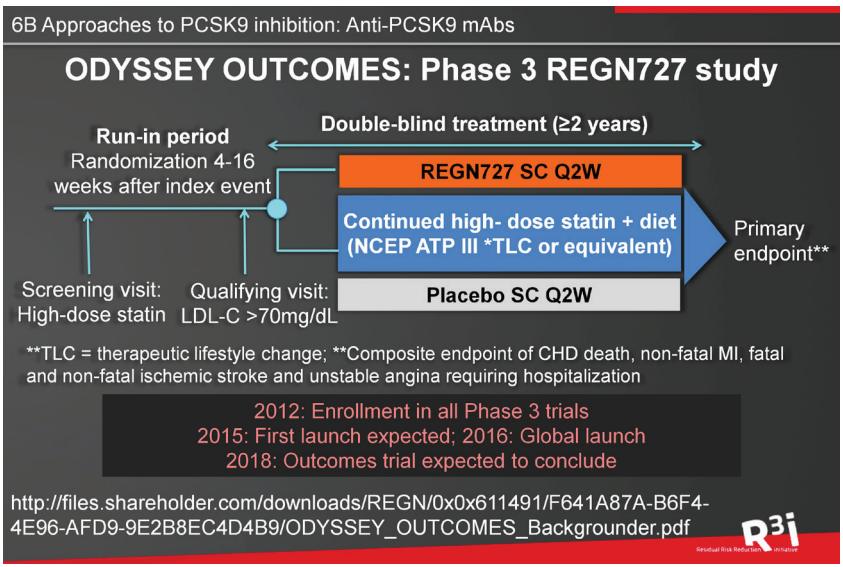
Phase 2 trial of the anti-PCSK9 mAb, REGN727: Study 3 design

- Double-blind, parallel-group, placebo-controlled Phase 2 trial in which 183 patients with LDL-C ≥100 mg/dl (2.59 mmol/l) on stable-dose atorvastatin 10, 20, or 40 mg for ≥6 weeks were randomized to s.c. placebo Q2W; s.c. REGN727 50, 100, or 150mg Q2W; or s.c. REGN727 200 or 300mg Q4W, alternating with placebo for a total treatment period of 12 weeks
- Primary endpoint: % change in LDL-C from baseline to Week 12
- Secondary endpoints included % changes in other Lps and Apos and the % of patients achieving their target LDL-C level



s.c. REGN727 dose-dependently reduced LDL-C levels in patients with hypercholesterolemia on atorvastatin

- s.c. REGN727 Q2W demonstrated clear dose-dependent reductions in LDL-C
- The mean percent changes in LDL-C from baseline to Week 12 were 40%, 64%, and 72% for 50, 100, and 150 mg Q2W, respectively, compared to 5% for placebo



ODYSSEY OUTCOMES: Phase 3 REGN727 study

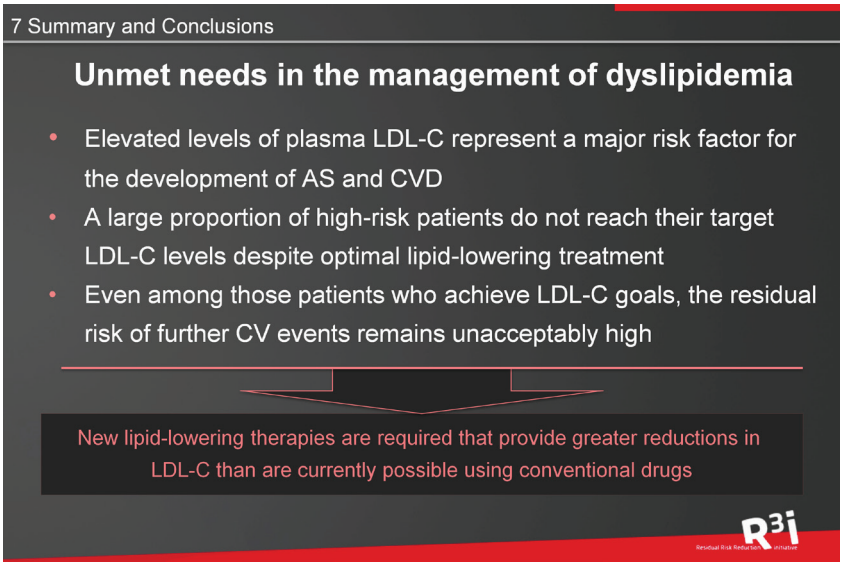
- The ODYSSEY OUTCOMES trial is a randomized, double-blind, placebo-controlled study designed to evaluate the safety and efficacy of REGN727 on the incidence of CV events in patients who have experienced an ACS and are not at LDL-C goal
- ~18,000 patients >40 years of age are being recruited from 49 countries across 6 continents
- Patients receiving optimal lipid-lowering therapy (high-dose statin plus NCEP ATP III-recommended therapeutic lifestyle change (TLC) or equivalent) will receive either a s.c. 75 mg injection of REGN727 or placebo every two weeks; patients not at LDL-C goal with the initial dose will be up-titrated to 150mg

Summary and conclusions

SECTION

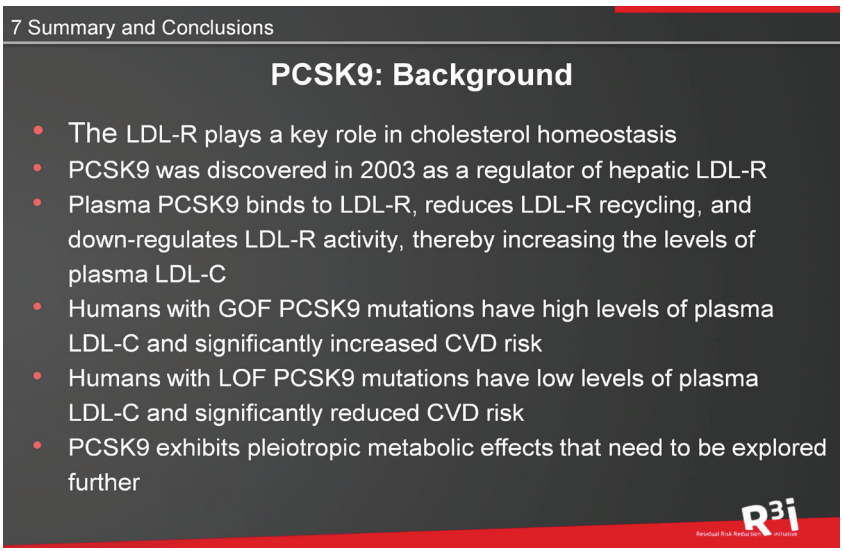
7

Residual Risk Reduction initiative



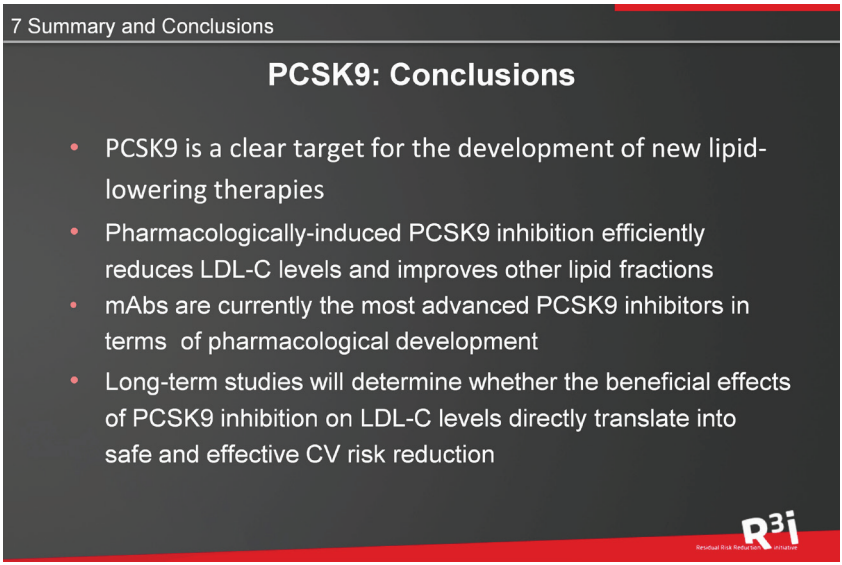
Unmet needs in the management of dyslipidemia

- Elevated levels of plasma LDL-C represent a major risk factor for the development of AS and CVD
- A large proportion of high-risk patients do not reach their target LDL-C levels despite optimal lipid-lowering treatment
- Residual CV risk remains unacceptably high even among those patients who achieve their LDL-C goals
- New lipid-lowering therapies are needed that provide greater reductions in LDL-C than are currently possible using conventional drugs



PCSK9: Background

- The LDL-R plays a key role in cholesterol homeostasis
- PCSK9 was discovered as hepatic LDL-R regulator in 2003
- Plasma PCSK9 binds to LDL-R, reduces LDL-R recycling, and down-regulates LDL-R activity resulting in increased plasma LDL-C
- Humans with GOF *PCSK9* mutations have higher levels of plasma LDL-C and increased CVD risk
- Humans with LOF *PCSK9* mutations have lower levels of plasma LDL-C and reduced CVD risk
- PCSK9 exhibits pleiotropic metabolic effects that need to be explored further



PCSK9: Conclusions

- PCSK9 is a clear target for the development of new lipid-lowering therapies
- Pharmacologically-induced PCSK9 inhibition efficiently reduces LDL-C levels and improves other lipid fractions
- mAbs are currently the most advanced PCSK9 inhibitors in terms of pharmacological development
- Long-term studies will determine whether the beneficial effects of PCSK9 inhibition on LDL-C levels directly translate into safe and effective CV risk reduction

Abbreviations

AA	Abdominal aorta
ACE2	Angiotensin converting enzyme 2
ACS	Acute coronary syndrome
Ad	Adenovirus
ADFH	Autosomal dominant familial hypercholesterolemia
ADH	Autosomal dominant hypercholesterolemia
AE	Adverse event
AnxA2	Annexin A2
Apo	Apolipoprotein
ARH	Autosomal recessive hypercholesterolemia
AS	Atherosclerosis
ASO	Antisense oligonucleotide
BBR	Berberine
BMI	Body mass index
CAD	Coronary artery disease
CE	Cholesteryl ester
CHD	Coronary heart disease
CHO	Chinese hamster ovary (cells)
CI	Confidence interval
CM	Chylomicron
CMr	Chylomicron remnant
CRP	C-reactive protein
CTTC	Cholesterol Treatment Trialists Collaboration
CV	Cardiovascular
CVD	Cardiovascular disease
DHCR7	Dehydrocholesterol reductase
DMSO	Dimethyl-sulfoxide
EC	Endothelial cell
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbant assay
ER	Endoplasmic reticulum
FCHL	Familial combined hyperlipidemia
FDB	Familial defective Apo B
FDPS	Farnesyl-diphosphate synthetase
FFA	Free fatty acid
FH	Familial hypercholesterolemia
FHBL	Familial hypobetalipoproteinemia
FPLC	Fast protein liquid chromatography
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GOF	Gain-of-function
HBL	Hypobetalipoproteinemia
HCV	Hepatitis C Virus
HDL	High-density lipoprotein
HeFH	Heterozygous familial hypercholesterolemia
HepG2	Liver hepatocellular carcinoma cells
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HUVEC	Human umbilical vein endothelial cells
ICU	Intensive care unit
IDL	Intermediate-density lipoprotein
Ig	Immunoglobulin

IHH	Immortalized human hepatocytes
IL	Interleukin
Insig	Insulin-induced gene protein
InsR	Insulin receptor
i.p.	Intraperitoneal
i.v.	Intravascular
JAK	Janus kinase
KO	Knock-out
LCAT	Lecithin cholesterol acyl transferase
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein-cholesterol
LDL-R	LDL receptor
LNA	Locked nucleic acid
LNP	Lipid nanoparticle
LOCF	Last observation carried forward
LOF	Loss-of-function
Lp	Lipoprotein
LPDS	Lipoprotein-deficient serum
LPS	Lipopolysaccharide
LS mean	Least squares mean
LVA	Large vessel atherosclerosis
mAb	Monoclonal antibody
MAP	Mitogen-activated protein
MCVE	Major coronary/vascular events
MI	Myocardial infarction
MIN	Mouse insulinoma cells
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MRS	Magnetic resonance proton (1H) spectroscopy
mRNA	Messenger ribonucleic acid
NARC-1	Neural apoptosis-regulated convertase 1
NFkB	Nuclear factor kappa B
NL	Normolipidemic
OM	Oncostatin M
OR6	Ouabain resistant cells
OxLDL	Oxidized low-density lipoprotein
pAb	Polyclonal antibody
PACE	Paired basic amino acid cleaving enzyme
PAI-1	Plasminogen activator inhibitor-type 1
PAR-1	Proteinase-activated receptor 1
PBS	Phosphate-buffered saline
PC	Proprotein convertase
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin kexin type 9
PPAR	Peroxisome proliferator activated receptor
Pro	Pro-domain
Veh	Vehicle control
Ez	Ezetimibe
RSV	Rosuvastatin
PUFA	Polyunsaturated fatty acid
qRT-PCR	Quantitative reverse transcriptase PCR
ROS	Reactive oxygen species
rtPCR	Real-time PCR
s.c.	Subcutaneous
SBP	Systolic blood pressure
SCAP	SREBP cleavage activating protein
SFA	Saturated fatty acid
shRNA	Short hairpin RNA

siRNA	Small interfering RNA
SKI-1	Subtilisin kexin isozyme 1
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
SP	Signal peptide
SP-1/-2	Site 1 or 2 protease
Sq-IB	Semi-quantitative immunoblotting
SR-BI	Scavenger receptor class B type I
SREBP	Sterol regulatory element binding protein
STZ	Streptozotocin
SVO	Small vessel occlusion
T2D	Type 2 diabetes
TA	Thoracic aorta
TAT	Total abdominal adipose tissue
TC	Total cholesterol
TF	Tissue factor
TIMP	Tissue inhibitor of metalloproteinase
TG	Triglyceride
TNF	Tumour necrosis factor
tPA	Tissue plasminogen activator
VLDL	Very-low density lipoprotein
WT	Wild-type