FCAB6

PROTOCOL: Effects of PCSK9 inhibition by *Evolocumab* on postprandial lipid metabolism in type 2 diabetes

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INVESTIGATORS AND STUDY STAFF:

HELSINKI, Clinical Research institute Huch, Ltd. and Research Programs Unit (RPU), University of Helsinki:

- ***Marja-Riitta Taskinen***, MD, PhD, Professor, emerita, Principal Investigator
- Juhani Kahri, MD, PhD, docent, Principal clinical investigator
- Sanni Söderlund, MD, PhD, clinical investigator
- Niina Matikainen, MD, PhD, docent, clinical investigator
- Helinä Perttunen-Nio, Medical Laboratory Technologist, Laboratory coordinator
- Elisa Koivisto, Medical Laboratory Technologist, Study Nurse
- Virve Naatti, Medical Laboratory Technologist, Study Nurse
- Hannele Hilden, Medical Laboratory Technologist, Laboratory technician
- Laura Impivaara, MSSc, Research administrator

HELSINKI, Helsinki Medical Imaging Center, University of Helsinki, Helsinki, Finland

- Nina Lundbom, MD, PhD, docent
- Antti Hakkarainen, B.Sc

GOTHENBURG, Department of Molecular and Clinical Medicine, Institute of Medicine, University of Gothenburg and Sahlgrenska University Hospital, Gothenburg; and Royal Institute of Technology, Stockholm, Sweden:

- ***Jan Borén***, MD, PhD, Professor, chairman, Co-principal Investigator
- Stefano Romeo, MD, PhD, Senior Lecturer, clinical investigator
- Elias Björnson, BS, systems biology/statistics, multicompartmental modelling
- Martin Adiels, PhD, Senior Lecturer, statistics, multicompartmental modelling
- Adil Mardinoglu, PhD, Assoc Professor, bioinformatics, systems biology/statistics
- Linda Andersson, PhD, Staff Scientist
- Marcus Ståhlman, PhD, Staff Scientist
- Angelika Svensson, Laboratory technician
- Elin Stenfeldt, Molecular biologist, Senior laboratory technician
- Eva Hedman Sabler, Medical Laboratory Technologist, Senior laboratory technician

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1. OBJECTIVES

The overall aim is to clarify the postprandial dynamics of PCSK9 on the pathophysiology of postprandial hypertriglyceridemia in people with type 2 diabetes. The effect of 12 weeks treatment with *Evolocumab* 140 mg s.c. Q2W on postprandial lipid and lipoprotein metabolism will be assessed in patients with type 2 diabetes (n=12) in an one-arm unblinded clinical trial.

1.1 Primary efficacy assessment

a. ApoB48 production rate

1.2 Secondary efficacy assessments:

a. Chylomicron (Sf 400) TG IAUC and apoB48 IAUC during the standardized fat-rich mixed meal test
b. $VLDL_1$, $VLDL_2$ and LDL production and fractional catabolic rates
c. Visceral fat volume and liver fat content (%) determined by MRI

2. BACKGROUND AND RATIONALE

2.1 Disease

Insulin resistance and type 2 diabetes (T2D) incidence is constantly increasing in the Western countries with a forecast of up to 550 million people affected by 2030.[1] People with T2D remain at high risk of cardiovascular events despite being on current standard of care pharmacological therapy and the majority of individuals with T2D die from accelerated atherosclerotic cardiovascular disease (CVD). Part of this residual risk is due to a characteristic dyslipidemia consisting of high levels of both fasting and postprandial triglyceride rich lipoproteins (TRLs), accumulation of small dense LDL particles and low levels of high density lipoproteins (HDL).[2]

TRLs in the plasma consist of chylomicrons (CM) carrying triglycerides (TG) from the diet, liver-derived VLDL particles and their respective remnant particles. Since remnant particles not only contain TGs but also approximately 40 times higher levels of cholesteryl esters per particle compared with LDL,[3] elevated levels of remnants may lead to accelerated atherosclerosis and CVD. Recent epidemiological studies have identified non-fasting (postprandial) triglycerides and the remnant particles as clinically significant risk factors for CVD.[4-8] Although the genetic basis of hypertriglyceridemia is complex; very recent genetic data have established that TRLs and their remnants are causally related to coronary artery disease (CAD).[9, 10] Thus, there is strong evidence that postprandial dyslipidemia is atherogenic, making TRLs plasma levels lowering a crucial therapeutic goal in the management of diabetic dyslipidemia and in patients with high residual risk. Statins fail to correct the characteristic dyslipoproteinemia observed in diabetic and insulin resistant patients,[11] and clinical trials with fibrates, niacin and omega-3 fatty acids, showed efficient reduction of plasma triglycerides but failed to show significant effects on the hard end points considered when added to statins.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secreted protease that plays an important role in lipoproteins metabolism by mediating the degradation of the LDL receptor. PSCK9 loss-of-function (LOF) mutations in humans lead to lifelong low LDL-cholesterol and reduced risk of CVD, whereas gain-of-function mutations lead to autosomal dominant hypercholesterolemia (ADH-3).[12, 13] These findings paved the way for PCSK9 inhibitors as new tools to effectively lower LDL cholesterol and to treat hypercholesterolemia. Alirocumab (formerly SAR236553/REGN727) and Evolocumab (formerly AMG145) are humanized monoclonal antibodies against PCSK9 that have been shown to significantly reduce LDL-cholesterol as greater than 50% as monotherapies and when combined with other lipid-lowering drugs in phase 2 and 3 studies.[14, 15] Robust lipid lowering effects of Evolocumab have been reported also in patients on statins and ezetimibe.[16, 17] Long-term studies have reported sustainable robust reduction of LDL-cholesterol (approximately 55 to 75%) and reduced incidence of

cardiovascular events with both users of Evolocumab[18] and Alirocumab[19] as compared to placebo. Three large clinical trials (Fourier, Odyssey Outcomes and SPIRE I/II) aimed at addressing the effects of PCSK9 antibodies on CVD outcomes are currently ongoing.[20] Available data indicate that these agents are well tolerated and the rate of adverse events is comparable to control groups.[21, 22]

PCSK9 is highly expressed in intestinal cells[23] and genetically modified mice lacking PCSK9 are protected against postprandial triglyceridemia.[24] Mice studies and *in vitro* studies using Caco-2 cells have confirmed a role of PCSK9 in the regulation of intestinal lipid metabolism.[23-26] Rashid *et al.* recently proposed that PCSK9 has a central role as a driver of chylomicron synthesis in intestinal cells *in vitro* by altering transcriptional and posttranscriptional processes.[23, 26] PCSK9 downregulates LDL-receptors in enterocytes. Furthermore, it associates with increased stabilization of apoB that would favor chylomicron assembly. PCSK9 also seems to affect cholesterol transport via action on CD36 and NPC1L1, SRE gene activation and activity of MTP.[27] Notably NPC1L1 is the target for ezetimibe that suppresses the activity resulting in the reduction of cholesterol absorption.[28] Thus, PSCK9 inhibition may reduce cholesterol absorption in parallel to the action of ezetimibe.[29] Overall the data suggested that PCSK9 deficiency reduce TRLs and their remnants and may thus protect against CVD. This has raised the interest to clarify and explore further the potential effect of PCSK9 inhibition on chylomicron assembly and secretion and on metabolism of TRLs and their remnant.[30, 31]

So far the data on the potential effects of PCSK9 inhibitors on plasma triglycerides and TRL metabolism is limited and mostly observational. The data from clinical trials is variable but Evolocumab has been reported to reduce triglyceride levels in a pooled analysis of 1359 patients by about 12–23 %.[30, 31] Recently, Cariou *et al.* reported that a short term high fructose diet increased serum PCSK9 levels associated with increase of postprandial triglycerides.[32] This is in agreement with recent results by Chan *et al.* who showed that both circulating PCSK9 and apoCIII associated positively with postprandial triglyceride and apoB48 responses but inversely to the fractional catabolic rate (FCR) of apoB48 to oral fat load in obese subjects. In stepwise regression analyses plasma PCSK9 was the best predictor of both plasma apoB48 responses as well as the FCR of apoB48.[33] However, PCSK9 plasma levels were not associated with VLDL-TG metabolism in another recent kinetic assessment study.[34] Notably the measurement of PCSK9 concentrations by ELISAs is challenging due to the presence of different forms of circulating PCSK9.[35]

Postprandial lipemia is a consistent component of the atherogenic dyslipidemia in T2D patients. Sterol regulatory element-binding protein 2 (SREBP-2) is the most important nuclear receptor transactivator of PCSK9[36] but also SREBP-1c regulates PSCK9.[37] In this context insulin has been shown to positively modulate PCSK9 transcription via stimulation of SREBP-1c.[37, 38] Recently, insulin was reported to induce directly PCSK9 expression *in vitro* and *in vivo* in mice.[39] Furthermore, insulin seems to enhance the degradation of LDL-receptors in a PCSK9 dependent manner. Blocking insulin signaling in different models consistently reduced PCSK9 mRNA and plasma levels by about 55−75 %. The data also suggest that insulin resistance may modify the functional relationship between insulin and PCSK9 by unmasking the potential effects of other factors like glucagon.

Notably, stimulation of SREBP-1c by insulin and carbohydrate-responsive element-binding protein (ChREPB) by glucose activates the pathway of *de novo* lipogenesis (DNL) that contributes to the overproduction of VLDL by the liver in T2D.[40] In addition, in insulin resistance state the metabolic milieu of hyperglycaemia and hyperinsulinemia may theoretically lead to enhanced circulating PCSK9 levels. Recently, PCSK9 was reported to induce the degradation of CD36 whereas the absence of PCSK9 seems to upregulate CD36 protein the major protein regulating fluxes of fatty acids and consequently triglyceride storage in both adipose tissue as well as in the

liver.[41] Interestingly, the authors demonstrated that CD36 levels were increased about 3-fold in livers of PCSK9 deficient mice reflected in increased lipid droplets in the livers of these mice. In face of these complex relationships it is not surprising that the limited available data on PCSK9 levels in subjects with T2D are inconsistent and both elevated and normal levels have been reported in small cohort studies.[33, 36, 42]

It is clear that our understanding of endogenous regulation of PCSK9 in people with diabetes is insufficient to optimize the use of PCSK9 inhibitors in people with diabetes. Current findings suggest PCSK9 inhibition as a possible effective approach to reduce the lipid burden in the postprandial state and manage the typical T2D dyslipidemia, thus contributing to CVD risk reduction in these high risk patients. So far, no studies exist on the effects of PCSK9 on postprandial lipid metabolism or on circulating TRL fluxes and the underlying molecular mechanisms. Therefore, the evaluation of the impact of PCSK9 inhibition on the postprandial responses and hepatic lipid metabolism is of particular interest in T2D subjects.[43]

2.2 Rationale

The overall aim of this research program is to clarify the postprandial dynamics of PCSK9 on the pathophysiology of postprandial hypertriglyceridemia in people with type 2 diabetes. Our team has the expertise, state-of-the-art equipment, and logistics to perform these studies. This is proven by our longstanding track of successful kinetic assessments of lipoprotein metabolism and of our track in lipid and lipoprotein associated inflammatory pathways.

2.3 Hypotheses

In this study we will answer the following clinically highly important questions:

a. Is the postprandial reduction of triglyceride levels mediated by Evolocumab associated with reduced secretion and/or improved clearance of TRLs?
b. Is decreased secretion of chylomicrons by Evolocumab associated with inhibition of apoB48 synthesis in intestinal cells and/or by reduced lipid incorporation into chylomicrons
c. Is the reduced production of intestinal TRLs (after treatment with Evolocumab) associated with improved metabolism of liver-derived VLDL?
d. Does Evolocumab reduce intestinal cholesterol absorption?
e. Are the changes of TRL fluxes induced by Evolocumab reflected in altered LDL kinetics?

3. EXPERIMENTAL PLAN

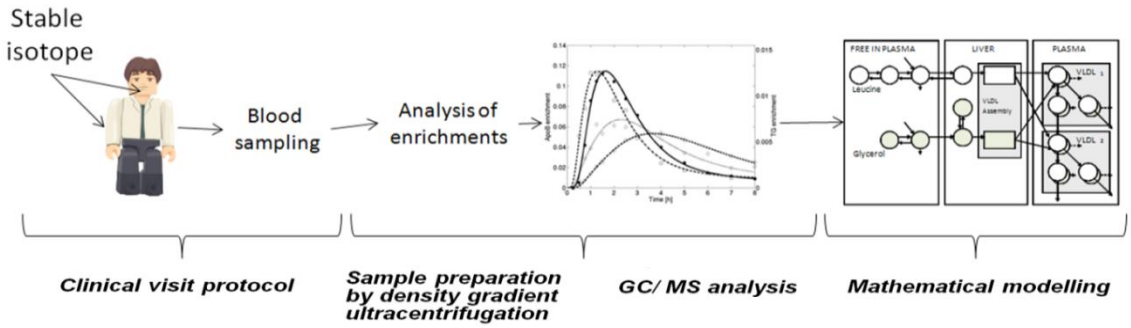
3.1 Study Design

The study is designed to investigate of the impact of Evolocumab on the postprandial kinetics of TRLs metabolism and LDL fluxes. Lipoprotein metabolism is a complex system in which abnormal concentrations of various pro- and anti-atherogenic particles can result from alterations in their rates of production, conversion and/or catabolism. Traditional methods that measure plasma lipoprotein concentrations only provide static estimates of lipoprotein metabolism and hence limited mechanistic information. By contrast, we use tracers labelled with stable isotopes (**Figure 1 and Table 1**), which provide us with a powerful and cutting-edge tool for probing lipid and lipoprotein kinetics *in vivo*, thus improving the understanding of the pathogenesis of dyslipoproteinaemia.

The study specifically clarifies the postprandial response of TRLs and focuses on the synthesis and turnover of chylomicrons and hepatically derived large triglyceride-rich $VLDL_1$ and smaller $VLDL_2$, and the interaction between these lipoproteins. This will also allow us also to follow the effects of changes in VLDL metabolism on LDL kinetics. The subjects will be extensively phenotyped including imaging of liver fat and measurements of hepatic *de novo* lipogenesis (DNL), biomarkers of cholesterol absorption and synthesis. We will also perform PCSK9

genotyping in the study subjects.

Figure 1. *Outline of a kinetic procedure that includes three major steps:* ***(i)*** *clinical visit protocol,*

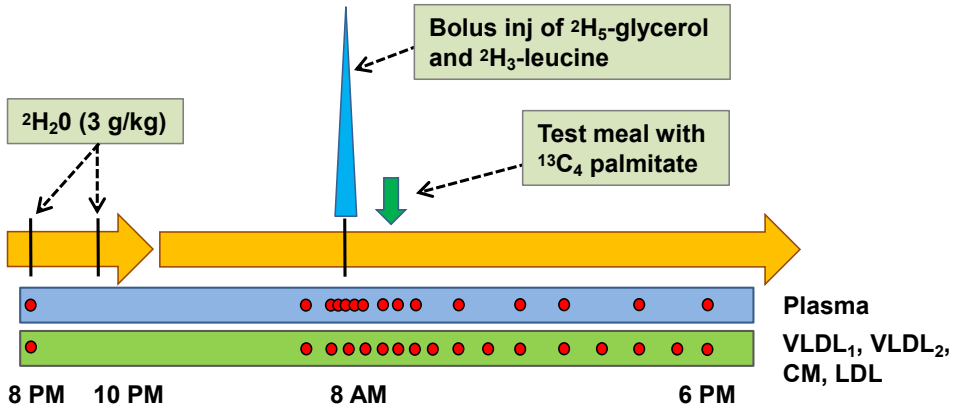


*(**ii**) sample preparation/processing and enrichment analysis by GC/MS, and (**iii**) mathematical modeling.*

Table 1. **Stable isotopes**	
$^{2}H_5$**-Glycerol**	*Injected tracer that is esterified to triglycerides in the liver but not in the intestine as it lacks glycerol kinase activity. It is thus only incorporated into VLDL–triglycerides.*
$^{2}H_3$**-Leucine**	*Injected tracer that is incorporated into apoB100 and apoB48. Chylomicrons secreted from the enterocytes contain apoB48, while VLDL secreted from the liver contain apoB100*
$^{13}C_4$**-Palmitate**	*The dietary tracer [1,2,3,4-^{13}C]-palmitate will be administered in the high fat meal to track the contribution of dietary lipids on VLDL–triglyceride secretion*
$^{2}H_2O$ **(heavy water)**	*Given orally. Incorporated into all fatty acids. Used to quantify the amount of fatty acids derived from de novo lipogenesis in the liver in $VLDL_1$ and $VLDL_2$*

These studies will clarify the dynamics of PCSK9 after an oral fat load, and will correlate the kinetics of PCSK9 with the postprandial kinetics of chylomicrons and liver derived VLDL and LDL in extensively phenotyped obese subjects.

Clinical protocol: Each subject enrolled will participate in two kinetic procedures to compare kinetic data *before* and *after* 12 weeks treatment with Evolocumab. On the evening of the first visit, $^{2}H_2O$ (3 g/kg) will be served between 8 PM and 10 PM to assess the contribution of *de novo* lipogenesis to fatty acids in chylomicrons and VLDL **(Figure 2)**.



At 8 AM the following morning, the subjects will receive a bolus injection of $^{2}H_5$-glycerol and $^{2}H_3$-leucine to assess VLDL and LDL-apoB100 and chylomicron–apoB48 kinetics; two hours later they will consume a defined mixed high fat meal (57 g fat, 63 g carbohydrates and 40 g protein, total 934 kcal) also containing $^{13}C_4$-palmitate tracer. These procedures will be repeated identically after the Evolocumab treatment.

The study design does not contain a control group. Instead, each subject will function as their own control. This study design is common in kinetic studies since it minimizes variation, and thus the number of study subjects needed. This is critical since kinetic studies are extremely labor intensive and time demanding.

3.2 Number of Centers

The study is a single-center one-arm unblinded clinical trial. The clinical part of the study will be conducted in the research facilities of Research Programs' Unit, Diabetes & Obesity, University of Helsinki; Clinical Research Institute, Huch ltd. Biomedicum Helsinki, Finland. Laboratory work and analysis will be made in the Helsinki and Gothenburg centers. All enrichment studies of stable isotopes, proteomics and lipidomics analyses, as well as statistical and modelling work will be performed in Gothenburg, Sweden.

3.3 Number of Subjects

Total 12 subjects (*i.e.,* 24 kinetic procedures).

3.4 Estimated Study Duration

The duration of the run-in period is at least 4 weeks. The study procedure comprises about 10 days followed by treatment over 12 weeks followed by another 10 days period for study procedures.

4. SUBJECT ENROLLMENT AND ELIGIBILITY

Subjects will be recruited from previous studies and by using an online recruitment service that is provided by Clariness GmbH, Germany.

4.1 Inclusion Criteria

- Male or female (non-fertile or using a medically approved birth control method) overweight/obese subjects with T2D treated with lifestyle counselling and a stable metformin dose for at least three months
- age 18–77 yrs.
- BMI 25–40 kg/m^2
- triglycerides between 1.5–4.5 mmol/L and LDL cholesterol >1.8 but ≤4.0 mmol/L (on Atorvastatin 20 mg/day)
- HbA1c: ≤9%.
- Each patient will attend a pre-screening visit (at week −5) where eligibility criteria will be evaluated. If the patient uses another statin than atorvastatin (20 mg) at screening visit the used statin is stopped and atorvastatin 20 mg will be initiated. If the patient is not using any statin, atorvastatin 20 mg will be initiated and the lipid values will be checked after 4 weeks when all inclusion/exclusion criteria will be assessed.

4.2 Exclusion Criteria

- Type 1 diabetes
- apoE2/2 phenotype
- ALT/AST > 3×ULN
- CK>3×ULN

- GFR <60 ml/min
- clinically significant TSH outside the normal range
- BMI >40 kg/m^2
- HbA1C > 9.0 %
- fasting TG > 4.5 mmol/l
- total chol > 7.0 mmol/l
- positive urine or serum pregnancy test
- untreated or inadequately treated hypertension defined as blood pressure >160 mmHg systolic and/or >105 mmHg diastolic, use of thiazide diuretics at a dose of ≥25 mg/day
- subject not on a stable dose of atorvastatin (20 mg/ day before randomization)
- lipid-lowering drugs other than statins within 3 months
- any other diabetes medication except diet + metformin
- history/diagnosis of diabetes nephropathy / retinopathy
- current smoking
- weekly alcohol use over 24 doses for men and 16 for women
- history of MI, ACS or coronary revascularization (PCI or CABG) within the last 6 mos.
- planned revascularization (eg CABG, PCI, carotid or peripheral revascularization procedures) within 3 months of screening
- NYHA class III/IV congestive heart failure persisting despite treatment
- history of hemorrhagic stroke
- hypersensitivity to (evolocumab or*)* any of the excipients found in the drug product
- use of estrogen therapy
- current use of antithrombotic or anticoagulant therapy
- known bleeding tendency that would be an contraindication to heparin test
- history of cancer within the past 5 years (except for adequately treated basal cell skin cancer, squamous cell skin cancer or in situ cervical cancer)
- women of childbearing potential not protected by effective birth control method and/or not willing to be tested for pregnancy
- patient considered by the investigator or any sub-investigator as inappropriate for this study for any reason

Restrictions during the study:

- No aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) within 1 week before heparin tests
- No alcohol or strenuous exercise within 72 hours of each special test

5. TREATMENT PROCEDURES

The protocol comprises two periods: 1. Run-in period and 2. Treatment period

5.1 The run-in period

The run-in period starts by the screening visit when the eligibility to enter the treatment period will be evaluated. If the subjects is not using statin at all or is using another statin, then atorvastatin is initiated at a dose of 20 mg/ day. The qualification labs are obtained a minimum of 4 weeks after the change in statin or statin dose.

5.2 Treatment period

Eligible subjects will be assigned to Evolocumab monotherapy 140 mg subcutaneously every 2 weeks (Q2W) as add on to the statin therapy. Intervention with Evolocumab will start at the last day of the first kinetic visit and continue over a period of 12 weeks. The patients are invited to visit the clinic monthly for safety blood samples. The 2nd kinetic procedure will be scheduled to

be started on day 5 (± 2) after Evolocumab injection at week 12.

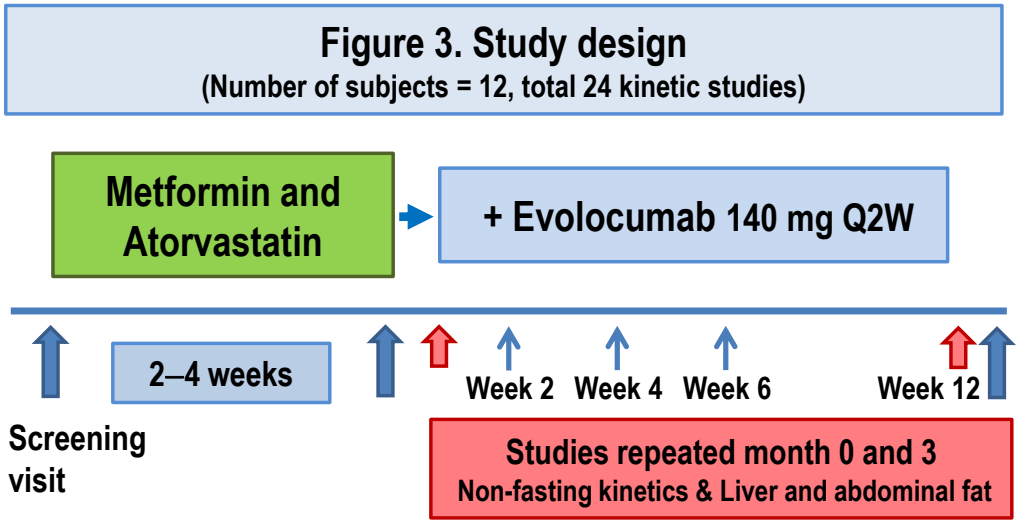


Figure 3. *Design of the clinical study with study visits (blue arrows) and two kinetic procedures to compare kinetic data before and after 12 weeks treatment with Evolocumab (red arrows). An additional visit is needed for measurements of liver fat content in the fasting state by magnetic resonance imaging (MRI visit). This visit should be performed within 7 days before the start of the kinetic* procedure*. Safety visits at 2, 4 and 8 weeks.*

Table 2. Summary of clinical visits in the study (Appendix 1)		
CLINICAL VISITS	*REASON FOR VISIT(S)*	*NUMBER OF VISITS*
Screening visit (1) Screening visit (2)	Screening Heparin test	50 subjects × 1 visit = 50 12 subjects × 1 = 12
Kinetic visits	Injections of stable isotopes and blood sampling	12 subjects × 7 visits × 2 (two kinetic procedures) = 168
MRI visits	Measurement of hepatic and visceral fat	12 subjects × 1 × 2 (one visit for each kinetic procedure) = 24
Safety visits	Safety monitoring	12 subjects × 4 visits = 48
Final visit	Heparin test, evaluation	12 subjects × 1 = 12

Thus, the study protocol includes 264 clinical visits (*i.e.,* 22 visits for each study subjects) in addition to 50 screening visits.

5.2.1 Evolocumab

PCSK9 is a protein secreted by liver. Evolocumab is a fully human monoclonal IgG2 antibody against PCSK9. Inactivation of PCSK9 prevents PSCK9/LDL receptor interaction and results in the decreased LDL-receptor degradation and lowers LDL-C by enhancing LDL receptor-mediated clearance. Evolocumab (dose 140 mg) is injected subcutaneously once every 2 weeks. This dosing maintains a consistent reduction of LDL cholesterol levels reported to be up to 55–70% in the drug development program. The efficacy, tolerability and safety of PCSK9 inhibition has been confirmed in intervention trials including >8000 subjects. Regulatory approval for Evolocumab (Repatha) occurred in Europe and USA in 2015.

5.2.2 Dosage, Administration, and Schedule

Intervention with Evolocumab will start at the last day of the first kinetic visit and continue over a

period of 12 weeks. The patients are invited to visit the clinic at weeks 2, 4, and 8 for safety blood samples. The 2nd kinetic procedure will be scheduled to start on day 5 (± 2) after Evolocumab injection at week 12. The dose of Evolocumab will be 140 mg s.c. Q2W without any dose adjustments or escalation.

5.3 Metformin and Atorvastatin

5.3.1 Dosage, Administration, and Schedule

The subjects will use metformin at a stable dose (500−3000 mg per day) throughout the study. If the patient uses another statin than atorvastatin (20 mg) at screening visit the used statin is stopped and atorvastatin 20 mg is initiated. If the patient is not using any statin, atorvastatin 20 mg will be initiated and the lipid values will be checked after 4 weeks. If eligible (LDL>1.8 mmol/L), the patient can be recruited. The subjects will continue using atorvastatin 20 mg throughout the study. The subjects will use the metformin as a prescription medication. Atorvastatin will be administered by the study centre to the subjects.

6. STUDY PROCEDURES

6.1 General Study Procedures

Blood samples will be taken at the time points indicated by the red dots in **Figure 2 and at days 1, 2, 3, 4, 5 and 7** and lipoprotein fractions (chylomicrons, $VLDL_1$ and $VLDL_2$) will be separated by density gradient ultracentrifugation. LDL will be isolated separately by ultracentrifugation using a fixed angle rotor. Concentrations of lipids and apolipoproteins (apoB100, apoB48) will be performed in whole plasma and in all fractions at each time point and enrichments of isotopes in lipids (TG, FAs) and proteins (apoB100, apoB48, PCSK9, apoCIII) by mass spectrometry analysis at each time point. Hepatic DNL will be calculated from enrichment of deuterated water ingested during the kinetic procedure at specified time points (0, 4 and 8 hrs). The following biomarkers will be measured:

- PCSK9, ApoCIII, apoA5, FGF21 and resistin in samples at fasting, 4 and 8 hrs.
- Biomarkers of cholesterol absorption (cholestanol, campesterol, sitosterol, avenasterol) and synthesis (cholestenol, desmosterol, lathosterol) in samples at fasting, 4 and 8 hrs.
- Markers of energy balance (FFA, β-OHB), glucose and insulin during the mixed meal
- Fasting samples: apoB100, apoAI, apoAII, Lp(a), adiponectin, and ghrelin, leptin, and LDL- and HDL size
- Lipolytic enzymes: we will measure lipoprotein lipase and hepatic lipase activities as well as lipoprotein lipase mass after injection of 75 IE/kg heparin. Blood samples will be taken before and after 10 min heparin injection. See **Appendix 1**.

Determination of liver, subcutaneous and intra-abdominal fat: Proton magnetic resonance spectroscopy will be performed to determine liver fat content, and magnetic resonance imaging to determine subcutaneous abdominal and intra-abdominal fat (a separate visit).

Modeling of the data: We will use multicompartmental models to describe the kinetics and dynamics of material. Enrichment of deuterated water to measure DNL is measured in fasting and at 4-h and 8-h samples.

6.2. Sample Storage and Destruction

Samples will be stored at Sahlgrenska University Hospital, Gothenburg, Sweden in -80°C freezers in locked freezers that have alarm, in locked rooms.

7. REMOVAL AND REPLACEMENT OF SUBJECTS

We estimate that 10 successfully finalized subjects are needed. Therefore, we enroll 12 subjects in the study.

Withdrawal from the study:

- Withdrawal of consent
- Clinical information/condition/adverse event that potentially endangers study subject/ study results judged by the PI of the study
- any information or clinical event described in Evolocumab SPC that is a contraindication for the use of Evolocumab

All study withdrawals will be recorded in the CRFs and in the patient's medical records when considered as confirmed.

8. SAFETY DATA COLLECTION, RECORDING, AND REPORTING

Safety visits will occur at weeks 2, 4 and 8 during the intervention and 3 months after the termination of the study drug. Safety visits include inspection of the injection sites, evaluation and recording of possible side effects, clinical examination (blood pressure, pulse, weight, waist circumference), and blood sampling (blood cell count, ALT, AST, cholesterol values, blood glucose, and creatinine kinase (CK)).

8.1 Adverse Events

In addition to adverse events defined below, safety data collection includes also collection and recording of adverse device effects and other safety events such as overdose, misuse/abuse, lack of effect.

8.1.1 Definition of Adverse Events

An adverse event (AE) is any untoward medical occurrence in a patient or clinical investigation.

8.1.2 Reporting Procedures for Adverse Events

All AEs, regardless of seriousness, or relationship to IMP, are to be recorded to CRF and reported in compliance with all applicable regulations. All adverse events will be collected from the first study-related activity (from the signing of the informed consent) and in all following contacts with the study-subject through-out the project. This includes events from the first trial related activity after the subject has signed the informed consent, and until the post treatment follow-up period. Laboratory or vital signs are to be recorded as AEs only if they are symptomatic, require corrective treatment or consultation, lead to IMP discontinuation, ***or*** are considered as clinically relevant.

8.1.3 Reporting Procedures for Product Complaints

Any concerns or irregularities about the packaging, appearance or usage of the prefilled AI/Pen are to be reported to Amgen within 24 hours of discovery or notification of the concern or irregularity. Should any such concerns or irregularities occur the IP will not be used until Amgen confirms that it is permissible to use the product.

8.2 Serious Adverse Events

8.2.1 Definition of Serious Adverse Events

A serious adverse event (SAE) is any untoward medical occurrence that at any dose: results in death, or is life-threatening, or requires inpatient hospitalization or prolongation of existing hospitalization, or results in persistent or significant disability/incapacity, or is a congenital anomaly / birth defect, or is a medically important event.

8.2.2 Reporting Procedures for Serious Adverse Events

All SAEs will be reported in compliance with all applicable regulations. An SAE is to be reported within 24 hours. The investigators will copy Amgen Finland Safety department ***(EU-Nordic Baltic Drug Safety nordic.baltic.drugsafety@amgen.com*** when expediting to FIMEA any serious adverse reactions (SAR) or suspected unexpected serious adverse reactions (SUSAR) which occurred during the use of Evolocumab in the study **(Appendix 2)**.

9. STATISTICAL CONSIDERATIONS

The primary and secondary efficacy assessment will be compared before and after Evolocumab using paired t-test and ANCOVA. Efficacy assessments 1.1.a and 1.2.b are outputs from the mathematical model (the production and clearance rates), 1.2.a is calculated from time series data using the trapetzoid method (AUC for chylomicron triglycerides and apoB48) and 1.2.c is calculated from within the software in the MRI/MRS scanner (Visceral fat volume and liver fat content).

Using 10 subjects we estimate that we can detect a difference of at least 20% for TG kinetics and 15% for apoB kinetics (both under 1.2.b) at an alpha of 0.05 and a power of 80% (Reproducibility of glucose, fatty acid and VLDL kinetics and multi-organ insulin sensitivity in obese subjects with non-alcoholic fatty liver disease. Magkos et al Int J Obes 2011).

Based on our paper *Improved Estimation of Human Lipoprotein Kinetics with Mixed Effects Models* by Berglund M et al. PLoS One. 2015 Sep 30;10(9):e0138538, we hope to further increase study power. It is hard to perform this type of estimates in kinetic assessments since the effect of the intervention is unclear. Therefore, we enroll 12 subjects in the study.

Subjects completing the protocol are included in the statistical analyses. Paired t-test and ancova will be used to compare estimates of kinetic parameters in all subjects, from kinetic studies before and after Evolocumab intervention**.**

10. PROTOCOL-SPECIFIED PRODUCT(S)

Evolocumab (AMG 145) is supplied as a sterile, single-use, preservative free solution for subcutaneous injection in a disposable, spring-based prefilled autoinjector (AI)/pen. The AI/pen contains a 1.0 mL deliverable volume of 140 mg/mL Evolocumab in 220 mM proline, 20 mM acetate, 0.01% (w/v) polysorbate 80, pH 5.0. Evolocumab will be administered 140 mg s.c. Q2W without any dose adjustments or escalation.

11. REGULATORY OBLIGATIONS

11.1 Informed Consent

Only subjects who sign an informed consent are included to the study. No study specific interventions will take place before the patient signs informed consent.

11.2 Independent Ethics Committee/Institutional Review Board

The study protocol will be subjected to the consideration of the Medical Ethics committee of the Helsinki University Central Hospital and permission from the Finnish Medicines Agency (Fimea) will be obtained. The study will be conducted according to GCP and GLP standards.

11.3 Subject Confidentiality

All information disclosed or produced during this clinical study (the trial protocol, CRFs, the results) are confidential prior to the publication of results and treated in compliance with the applicable laws and regulations.

12. ADMINISTRATIVE AND LEGAL OBLIGATIONS

12.1 General considerations

The clinical part of the study will be conducted in the research facilities of Research Programs' Unit, Diabetes & Obesity, University of Helsinki; Clinical Research Institute, Huch ltd. Biomedicum Helsinki, Finland. Laboratory work and analysis will be made in Helsinki and Gothenburg centers. The study protocol will be subjected to the consideration of the Medical Ethics committee of the Helsinki University Central Hospital and permission from the Finnish Medicines Agency (Fimea) will be obtained. The study will be conducted according to GCP and GLP standards. The subjects taking part to the study are insured by a patient insurance taken out by the Helsinki University Central Hospital.

12.2 Protocol Amendments and Study Termination

In case of any protocol amendment, the amendment will be submitted to the Ethics committee and requires written approval by the Ethics committee. The written amendment should be signed by the investigator and filed with the clinical protocol. Amgen will be informed of protocol amendments and will provide a review.

12.3 Data Collection, study Documentation and Archive

All study documentation and data collected during the study will be documented and archived in compliance with all applicable regulations.

12.4 Publication Policy

The Principal Investigator agrees to provide Amgen with a copy of any manuscript, at least forty-five (45) days prior to submission. In accordance with scientific custom, the contribution of Amgen will be expressly noted in all written or public disclosures, by acknowledgment, as appropriate.

12.5 Compensation

Amgen will provide funding in support of the Study. The funding constitutes an Investigator-Initiated Research Grant for this Study.

13. REFERENCES

1. Whiting DR, Guariguata L, Weil C, Shaw J. Idf diabetes atlas: Global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes Res Clin Pract*. 2011;94:311-321
2. Boren J, Matikainen N, Adiels M, Taskinen MR. Postprandial hypertriglyceridemia as a coronary risk factor. *Clin Chim Acta*. 2014;431:131-142
3. Proctor SD, Vine DF, Mamo JC. Arterial retention of apolipoprotein b(48)- and b(100)-containing lipoproteins in atherogenesis. *Curr Opin Lipidol*. 2002;13:461-470
4. Nordestgaard BG, Benn M, Schnohr P, Tybjaerg-Hansen A. Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. *JAMA*. 2007;298:299-308
5. Bansal S, Buring JE, Rifai N, Mora S, Sacks FM, Ridker PM. Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. *JAMA*. 2007;298:309-316
6. Freiberg JJ, Tybjaerg-Hansen A, Jensen JS, Nordestgaard BG. Nonfasting triglycerides and risk of ischemic stroke in the general population. *JAMA*. 2008;300:2142-2152
7. Varbo A, Freiberg JJ, Nordestgaard BG. Extreme nonfasting remnant cholesterol vs extreme ldl cholesterol as contributors to cardiovascular disease and all-cause mortality in 90000 individuals from the general population. *Clin Chem*. 2015;61:533-543
8. Nordestgaard BG, Varbo A. Triglycerides and cardiovascular disease. *Lancet*. 2014;384:626-635
9. Do R et al. Kathiresan S. Common variants associated with plasma triglycerides and risk for coronary artery disease. *Nat Genet*. 2013;45:1345-1352

10. Rosenson RS, Davidson MH, Hirsh BJ, Kathiresan S, Gaudet D. Genetics and causality of triglyceride-rich lipoproteins in atherosclerotic cardiovascular disease. *J Am Coll Cardiol*. 2014;64:2525-2540
11. Chapman MJ, Ginsberg HN, Amarenco P, Andreotti F, Boren J, Catapano AL, Descamps OS, Fisher E, Kovanen PT, Kuivenhoven JA, Lesnik P, Masana L, Nordestgaard BG, Ray KK, Reiner Z, Taskinen MR, Tokgozoglu L, Tybjaerg-Hansen A, Watts GF, European Atherosclerosis Society Consensus P. Triglyceride-rich lipoproteins and high-density lipoprotein cholesterol in patients at high risk of cardiovascular disease: Evidence and guidance for management. *Eur Heart J*. 2011;32:1345-1361
12. Stein EA, Raal F. Reduction of low-density lipoprotein cholesterol by monoclonal antibody inhibition of pcsk9. *Annu Rev Med*. 2014;65:417-431
13. Abifadel M, Elbitar S, El Khoury P, Ghaleb Y, Chemaly M, Moussalli ML, Rabes JP, Varret M, Boileau C. Living the pcsk9 adventure: From the identification of a new gene in familial hypercholesterolemia towards a potential new class of anticholesterol drugs. *Curr Atheroscler Rep*. 2014;16:439
14. Dadu RT, Ballantyne CM. Lipid lowering with pcsk9 inhibitors. *Nat Rev Cardiol*. 2014;11:563-575
15. Marais AD, Kim JB, Wasserman SM, Lambert G. Pcsk9 inhibition in ldl cholesterol reduction: Genetics and therapeutic implications of very low plasma lipoprotein levels. *Pharmacol Ther*. 2015;145C:58-66
16. Blom DJ, Hala T, Bolognese M, Lillestol MJ, Toth PD, Burgess L, Ceska R, Roth E, Koren MJ, Ballantyne CM, Monsalvo ML, Tsirtsonis K, Kim JB, Scott R, Wasserman SM, Stein EA, Investigators D. A 52-week placebo-controlled trial of evolocumab in hyperlipidemia. *N Engl J Med*. 2014;370:1809-1819
17. Stroes E, Colquhoun D, Sullivan D, Civeira F, Rosenson RS, Watts GF, Bruckert E, Cho L, Dent R, Knusel B, Xue A, Scott R, Wasserman SM, Rocco M, Investigators G-. Anti-pcsk9 antibody effectively lowers cholesterol in patients with statin intolerance: The gauss-2 randomized, placebo-controlled phase 3 clinical trial of evolocumab. *J Am Coll Cardiol*. 2014;63:2541-2548
18. Sabatine MS, Giugliano RP, Wiviott SD, Raal FJ, Blom DJ, Robinson J, Ballantyne CM, Somaratne R, Legg J, Wasserman SM, Scott R, Koren MJ, Stein EA, Open-Label Study of Long-Term Evaluation against LDLCI. Efficacy and safety of evolocumab in reducing lipids and cardiovascular events. *N Engl J Med*. 2015;372:1500-1509
19. Robinson JG, Farnier M, Krempf M, Bergeron J, Luc G, Averna M, Stroes ES, Langslet G, Raal FJ, El Shahawy M, Koren MJ, Lepor NE, Lorenzato C, Pordy R, Chaudhari U, Kastelein JJ, Investigators OLT. Efficacy and safety of alirocumab in reducing lipids and cardiovascular events. *N Engl J Med*. 2015;372:1489-1499
20. Giugliano RP, Sabatine MS. Are pcsk9 inhibitors the next breakthrough in the cardiovascular field? *J Am Coll Cardiol*. 2015;65:2638-2651
21. Norata GD, Tibolla G, Catapano AL. Pcsk9 inhibition for the treatment of hypercholesterolemia: Promises and emerging challenges. *Vascul Pharmacol*. 2014;62:103-111
22. Joseph L, Robinson JG. Proprotein convertase subtilisin/kexin type 9 (pcsk9) inhibition and the future of lipid lowering therapy. *Prog Cardiovasc Dis*. 2015;58:19-31
23. Rashid S, Curtis DE, Garuti R, Anderson NN, Bashmakov Y, Ho YK, Hammer RE, Moon YA, Horton JD. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking pcsk9. *Proc Natl Acad Sci U S A*. 2005;102:5374-5379
24. Le May C, Kourimate S, Langhi C, Chetiveaux M, Jarry A, Comera C, Collet X, Kuipers F, Krempf M, Cariou B, Costet P. Proprotein convertase subtilisin kexin type 9 null mice are protected from postprandial triglyceridemia. *Arterioscler Thromb Vasc Biol*. 2009;29:684-690
25. Levy E, Ben Djoudi Ouadda A, Spahis S, Sane AT, Garofalo C, Grenier E, Emonnot L, Yara S, Couture P, Beaulieu JF, Menard D, Seidah NG, Elchebly M. Pcsk9 plays a significant role in cholesterol homeostasis and lipid transport in intestinal epithelial cells. *Atherosclerosis*. 2013;227:297-306
26. Rashid S, Tavori H, Brown PE, Linton MF, He J, Giunzioni I, Fazio S. Proprotein convertase subtilisin kexin type 9 promotes intestinal overproduction of triglyceride-rich apolipoprotein b lipoproteins through both low-density lipoprotein receptor-dependent and -independent mechanisms. *Circulation*. 2014;130:431-441

27. Tavori H, Rashid S, Fazio S. On the function and homeostasis of pcsk9: Reciprocal interaction with ldlr and additional lipid effects. *Atherosclerosis*. 2015;238:264-270
28. Myocardial Infarction Genetics Consortium I, Stitziel NO, Won HH, Morrison AC, Peloso GM, Do R, Lange LA, Fontanillas P, Gupta N, Duga S, Goel A, Farrall M, Saleheen D, Ferrario P, Konig I, Asselta R, Merlini PA, Marziliano N, Notarangelo MF, Schick U, Auer P, Assimes TL, Reilly M, Wilensky R, Rader DJ, Hovingh GK, Meitinger T, Kessler T, Kastrati A, Laugwitz KL, Siscovick D, Rotter JI, Hazen SL, Tracy R, Cresci S, Spertus J, Jackson R, Schwartz SM, Natarajan P, Crosby J, Muzny D, Ballantyne C, Rich SS, O'Donnell CJ, Abecasis G, Sunyaev S, Nickerson DA, Buring JE, Ridker PM, Chasman DI, Austin E, Ye Z, Kullo IJ, Weeke PE, Shaffer CM, Bastarache LA, Denny JC, Roden DM, Palmer C, Deloukas P, Lin DY, Tang ZZ, Erdmann J, Schunkert H, Danesh J, Marrugat J, Elosua R, Ardissino D, McPherson R, Watkins H, Reiner AP, Wilson JG, Altshuler D, Gibbs RA, Lander ES, Boerwinkle E, Gabriel S, Kathiresan S. Inactivating mutations in npc1l1 and protection from coronary heart disease. *N Engl J Med*. 2014;371:2072-2082
29. Fazio S. The role of pcsk9 in intestinal lipoprotein metabolism: Synergism of statin and ezetimibe. *Atheroscler Suppl*. 2015;17:23-26
30. Tavori H, Giunzioni I, Fazio S. Pcsk9 inhibition to reduce cardiovascular disease risk: Recent findings from the biology of pcsk9. *Curr Opin Endocrinol Diabetes Obes*. 2015;22:126-132
31. Druce I, Abujrad H, Ooi TC. Pcsk9 and triglyceride-rich lipoprotein metabolism. *J Biomed Res*. 2015;29
32. Cariou B, Langhi C, Le Bras M, Bortolotti M, Le KA, Theytaz F, Le May C, Guyomarc'h-Delasalle B, Zair Y, Kreis R, Boesch C, Krempf M, Tappy L, Costet P. Plasma pcsk9 concentrations during an oral fat load and after short term high-fat, high-fat high-protein and high-fructose diets. *Nutr Metab (Lond)*. 2013;10:4
33. Chan DC, Wong AT, Pang J, Barrett PH, Watts GF. Inter-relationships between proprotein convertase subtilisin/kexin type 9, apolipoprotein c-iii and plasma apolipoprotein b-48 transport in obese subjects: A stable isotope study in the postprandial state. *Clin Sci (Lond)*. 2015;128:379-385
34. Sullivan S, Fabbrini E, Horton JD, Korenblat K, Patterson BW, Klein S. Lack of a relationship between plasma pcsk9 concentrations and hepatic lipoprotein kinetics in obese people. *Transl Res*. 2011;158:302-306
35. Awan Z, Baass A, Genest J. Proprotein convertase subtilisin/kexin type 9 (pcsk9): Lessons learned from patients with hypercholesterolemia. *Clin Chem*. 2014;60:1380-1389
36. Cui CJ, Li S, Li JJ. Pcsk9 and its modulation. *Clin Chim Acta*. 2015;440:79-86
37. Costet P, Cariou B, Lambert G, Lalanne F, Lardeux B, Jarnoux AL, Grefhorst A, Staels B, Krempf M. Hepatic pcsk9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c. *J Biol Chem*. 2006;281:6211-6218
38. Xiao X, Song BL. Srebp: A novel therapeutic target. *Acta Biochim Biophys Sin (Shanghai)*. 2013;45:2-10
39. Miao J, Manthena PV, Haas ME, Ling AV, Shin DJ, Graham MJ, Crooke RM, Liu J, Biddinger SB. Role of insulin in the regulation of proprotein convertase subtilisin/kexin type 9. *Arterioscler Thromb Vasc Biol*. 2015;35:1589-1596
40. Taskinen MR, Boren J. New insights into the pathophysiology of dyslipidemia in type 2 diabetes. *Atherosclerosis*. 2015;239:483-495
41. Demers A, Samami S, Lauzier B, Des Rosiers C, Sock ET, Ong H, Mayer G. Pcsk9 induces cd36 degradation and affects long-chain fatty acid uptake and triglyceride metabolism in adipocytes and in mouse liver. *Arterioscler Thromb Vasc Biol*. 2015;35:2517-2525
42. Verges B, Duvillard L, Brindisi MC, Gautier E, Krempf M, Costet P, Cariou B. Lack of association between plasma pcsk9 and ldl-apob100 catabolism in patients with uncontrolled type 2 diabetes. *Atherosclerosis*. 2011;219:342-348
43. Cohen JC, Boerwinkle E, Mosley TH, Jr., Hobbs HH. Sequence variations in pcsk9, low ldl, and protection against coronary heart disease. *N Engl J Med*. 2006;354:1264-1272
44. Bateman RJ, Munsell LY, Morris JC, Swarm R, Yarasheski KE, Holtzman DM. Human amyloid-beta synthesis and clearance rates as measured in cerebrospinal fluid in vivo. *Nat Med*. 2006;12:856-861
45. Lee AY, Yates NA, Ichetovkin M, Deyanova E, Southwick K, Fisher TS, Wang W, Loderstedt J, Walker N, Zhou H, Zhao X, Sparrow CP, Hubbard BK, Rader DJ, Sitlani A, Millar JS, Hendrickson

RC. Measurement of fractional synthetic rates of multiple protein analytes by triple quadrupole mass spectrometry. *Clin Chem*. 2012;58:619-627

46. Lassman ME, McAvoy T, Lee AY, Chappell D, Wong O, Zhou H, Reyes-Soffer G, Ginsberg HN, Millar JS, Rader DJ, Gutstein DE, Laterza O. Practical immunoaffinity-enrichment lc-ms for measuring protein kinetics of low-abundance proteins. *Clin Chem*. 2014;60:1217-1224

14. APPENDICES

LIST OF APPENDICES

Appendix 1. Clinical data files
Appendix 2. Adverse Event reporting procedure