PROJECT FINAL REPORT

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Name of the scientific representative of the project's co-ordinator[1], Title and Organisation:

Professor Bert Groen

University Medical Center Groningen

Tel: +31 50 3632669

E-mail: a.k.groen@umcg.nl

Project website Erreur ! Signet non défini. **address:** http://www.resolve-diabetes.org/

[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the Grant Agreement.

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1. Final publishable summary report

1.1. *Executive summary (Coordinator)*

The metabolic syndrome (MetS) is defined as a cluster of interrelated common clinical disorders, including abdominal obesity, elevated blood pressure, loss of glycemic control, high triglycerides (TG), and low high-density lipoprotein cholesterol (HDL-C). MetS is widely used as simple clinical definition of overweight individuals at increased risk of a large number of comorbidities such as type 2 diabetes (T2DM), cardiovascular disease (CVD) and non-alcoholic fatty liver disease (NAFLD). It is expected that by 2030 more than 30% of the total population (200 million individuals) in the 27 countries of the EU will be obese. Many of them will have one or more of the above co-morbidities. Without successful interventions, besides the toll on lives of EU citizens, costs of treating the comorbidities will increase to more than 200 billion Euros per year beyond 2030. Clearly there is an urgent clinical as well as economic need to conquer the sequelae and comorbidities of MetS. The central aim of RESOLVE was to develop a comprehensive computer model allowing targeting the underlying mechanism of low HDL-C, high triglyceride and loss of glycemic control in MetS patients. Previous computational modelling efforts had not fully captured the gradual progression and complexity of the disease, probably because the study of disease development is complicated by the multilevel aspects of biological systems. To be able to predict the reaction of body metabolism to the challenge of for instance a high fat diet the RESOLVE partner at Eindhoven University of Technology (TU/e) developed Analysis of Dynamic Adaptations in Parameter Trajectories (ADAPT). This method offers a novel and powerful approach for data-driven dynamic modelling in systems biology. RESOLVE applied this method in murine and human studies. ADAPT was applied in combination with a common, underlying 'core' computational model called MINGLeD (Model INtegrating Glucose and Lipid Dynamics). The core model describes the relevant biological processes in glucose and lipid metabolism, using coupled, nonlinear differential equations. Different versions of the model were implemented by changing model parameters based on experimental data. Through this concept observations in mice could be translated to humans. Experiments in a mouse model were carried out by UMCG and UKE to calibrate and validate MINGLeD. Importantly, it should be noted that studies such as organ-specific lipid uptake and potentially harmful interventions cannot be performed in humans and therefore need to be performed in preclinical models such as the mouse. To this purpose, the RESOLVE partners used a mouse model expressing the dysfunctional apolipoprotein E3 Leiden as well as the human cholesterylester transfer protein (CETP). Under conditions of a high-fat diet, these APOE3L*CETP transgenic mice (E3L*C) exhibit a lipoprotein profile with increased levels of triglyceride-rich lipoproteins (TRL) and decreased levels of HDL, a dyslipidemic phenotype characteristic of humans with MetS. Based on intervention studies with selective activation of peripheral energy expenditure in E3L*C mice, MINGLeD predicted beneficial changes in cholesterol and triglyceride metabolism that were validated in independent experiments (UKE, TU/e). High fat diet feeding showed a remarkable heterogeneity in metabolic response of E3L*C mice reminiscent of the situation in humans, including animals that did not develop MetS (non-responding animals). ADAPT was also applied to analyse this heterogeneity and several predictions were validated. For example, a prediction that fat was less absorbed in non-responding animals particularly during the first three months of the experiment was validated in subsequent series of experiments. The fecal excretion of fatty acids correlated closely with bile acid excretion in particular suggesting a direct role of bile acid metabolism in explaining the differences between responders and non-responders. The effect of the long-term high fat diet on metabolic response in E3L*C mice was reproduced by several other partners in RESOLVE. To validate the role of bile acids, the UMCG carried out a series of experiments with an FXR agonist that decreases bile acid synthesis while increasing the hydrophilicity of the bile acid pool. The

major impact of bile acid metabolism in MetS development was impressively validated. UZH also studied the effect of bariatric surgery on the high fat diet fed E3L*C mice and demonstrated interesting metabolic differences between caloric intake matched mice and mice that had undergone the surgery. Roux-en-Y gastric bypass (RYGB) improved glucose metabolism already 2 weeks after surgery. Also, clinically established biomarkers of dyslipidemia markedly improved. In addition, plasma levels of ceramides and sphingomyelin derived lipids were decreased in both RYGB and matched weight loss group, with a more pronounced drop in the RYGB group. This highlights the importance of the surgical procedure more than the body weight loss per se. A striking absence of connection between lipid and glucose metabolism was observed in all studies performed with mice. Also in the various human intervention studies performed in RESOLVE remarkably little connection was observed between lipid and glucose metabolism. Under physiological conditions only insulin itself seems to be very important. Liver samples derived from this study were analysed on effects on gene expression by FORTH. FORTH showed that the liver-specific genetic ablation of the transcription factor FOXO1 was associated with improved insulin sensitivity which could be explained, at least in part, by the transcriptomics data. Transcriptomics data from the obesity cohort of UZA was completed by IPL and the gene dermapontin was found to be involved in liver fibrosis and expression decreased after bariatric surgery. Analysis of the high fructose intervention study in humans indicated that fructose feeding induced metabolic perturbations such as increased de novo lipogenesis but overall the effects were rather small. UGOT/UH also performed a two-week intervention with a carbohydrate-restricted isocaloric diet in middle-aged obese non-diabetic subjects with NAFLD. Within just three days, a striking rapid, significant reduction in liver fat was observed with a concomitant strikingly decreased hepatic DNL, increased hepatic β-oxidation, and reduced plasma triglycerides. These results indicate that the macronutrient composition of the diet *per se* causes these striking, short-term metabolic benefits, without requiring caloric restriction or weight loss providing a therapeutic modality for treatment of NAFLD. The bariatric surgery intervention by UZH confirmed considerable weight loss after one year, and all MetS comorbidities ameliorated. Analysis of GLP-1 fluxes showed that peak performance of the GLP-1 response can only be caused by the very rapid delivery of glucose to the intestine. The generated model enables accurate predictions of the influence of modifications of the RYGB procedure on postprandial insulin and GLP-1 excursions. Model simulations indicate that the total length of the RY limbs has a major influence on post RYGB GLP-1 secretion and hence glucose homeostasis. Surgeons could use the model to estimate optimal distribution of the length of the intestinal limbs that are constructed. In another RYGB study, UZH identified several functions, proteins and lipids of HDL which significantly changed upon bariatric surgery, several of them in significant correlation with changes in glycemic control and body weight. Interestingly no major gender differences were observed in these bariatric surgery studies.

In conclusion, the RESOLVE consortium has developed a dynamic system biology approach that enables predicting the consequences of metabolic derailment of the MetS and its comorbidities in a personalized way. The methodology has been developed through a series of required experimental studies in humanized mouse model of the metabolic syndrome and has proven to successfully predict which metabolic pathway may be targeted to resolve the MetS. In human, ADAPT has also be successfully used its full integration is underway. When accomplished, it will generate detailed insight in the metabolic network dysregulation in MetS patients and will result in better, biology-based prediction of chronic disease risk, personalized prevention, and novel therapeutic strategies to rebalance the metabolic networks.

1.2. *Summary description of project context and objectives (Coordinator)*

1.2.1 Context (State of Art at the project start, Key challenges)

Epidemiological studies have unequivocally shown that high TG and low HDL-C represent strong independent risk factors for future CVD (Emerging Risk Factor Collaboration, JAMA 2009). For risk factors such as elevated LDL-C and high blood pressure this type of epidemiological evidence has been successfully translated into drugs that lower CVD risk. To date, however, it has proven difficult to successfully reduce CVD risk with TG-lowering and/or HDL increasing/modulating drugs such as fibrates (Keech et al. Lancet. 2005;366:1849), niacin (AIM-HIGH investigators, N Engl J Med. 2011;365:2255-67), CETP inhibitors (Barter et al. N Engl J Med. 2007;357:2109) and infusions of reconstituted HDL (Tardif JC et al, JAMA. 2007;297:1675-82). Following these studies, investigators have started questioning the potential of similar clinical interventions to combat CVD over the last few years (see e.g. Rosenson RS. Curr Atheroscler Rep. 2006; Vergeer M et al. J Lipid Res. 2010; 51:2058-73).

It appears that normalization of TG and HDL-C levels is not sufficient to offer atheroprotection. One aspect that has certainly not received adequate attention in MetS and CVD research is **the fact that TG and HDL are strong interrelated biological parameters which are also linked to glucose homeostasis**. An interesting example of an interaction between lipid, lipoprotein and glucose metabolism is the recently identified effect of LDL-C lowering on T2DM. Patients receiving statins showed a significant increase in incidence of T2DM (Sattar N et al., Lancet. 2010;375:735-42). The diabetogenic effect of statins is dose-dependent (Preiss D et al., JAMA. 2011;305:2556-64) and most strongly exerted in pre-diabetic patients with components of MetS (Waters DD et al. JACC., J Am Coll Cardiol. 2011;57:1535-45). Despite these findings, opinion leaders continue supporting current guidelines to lower LDL-C as much as possible in patients with MetS or T2DM (EHJ 2011 European Guidelines, Reiner Z. et al. European Heart Journal 2011 32, 1769–1818) primarily because the benefit of CVD reduction outweighs the risk of T2DM development, but also because of **lacking therapeutic alternatives**. Interestingly, prospective epidemiological studies have provided consistent evidence that high TG and low HDL-C, but not elevated LDL-C, precede the manifestation of T2DM and are independent risk factors for developing T2DM (von Eckardstein et al. JCEM 2000J Clin Endocrinol Metab 85:3101-8) as well as its microvascular complications (Fioretto P. et al. Nat. Rev. Endocrinol. 2010;6:19-25). In a post -hoc analysis of data from diabetic participants in the ILLUMINATE trial, the addition of the CETP inhibitor torcetrapib to atorvastatin was found to increase HDL-C and to improve glycemic control (Barter, PJ et al. Circulation. 2011;124:555-62). Infusion of reconstituted HDL into diabetic patients was found to acutely improve glucose tolerance (Drew BG et al. Circulation 2009 119:2103-11). Also in agreement with the anti-diabetic effect of HDL, several *in vitro* and animal experiments provided evidence that HDL improves the function and survival of pancreatic beta cells as well as the sensitivity of muscle, liver and adipose tissue towards insulin (von Eckardstein and Sibler, Curr. Opin. Lipidol. 2011 22(1):26-32). Surprisingly, HDL, TG and glucose metabolism have been studied primarily independent of each other. This despite the notion that in many inherited human disorders and genetically engineered animals, primary disturbances in TG or HDL metabolism cause simultaneous changes in plasma levels of HDL-C and TG respectively, and do often disturb carbohydrate metabolism as well. Over the past decade, hundreds of genes have been identified and implied to play a role in the regulation of TG, HDL and glucose metabolism. Extensive metabolic and transcriptional networks have been constructed on the basis of genomic information. Inspection of these networks reveals a multitude of regulatory nodes which suggest strong interconnections between carbohydrate, TG and HDL metabolism. The staggering complexity of the connections between the nodes in the networks impedes insight into the mechanisms controlling the fluxes through the different network branches**.**

This is probably caused by the fact that despite the realization of the metabolic complexity, most investigators still use the classical reductionist approach and focus on TG, HDL or glucose metabolism separately. Clearly this does not allow insight into the subtle interplay of all factors under general (patho)physiological conditions in patients with MetS and brings the risk that proposed drug targets will cure one aspect of MetS and deteriorate another. Complex network interactions preclude intuitive understanding. For example, several transcription factors and miRNAs are known to regulate pivotal genes of TG, HDL and carbohydrate metabolism antagonistically.

In addition, the long-held believe that the high TG, low HDL-C phenotype in MetS is caused by insulin resistance has been questioned by findings of insulin resistance or beta cell failure in genetic animal models and human inborn errors of TG and HDL metabolism. In other words, it is not known whether high TG/low HDL-C causes insulin resistance or *vice versa*, or even both. This precludes the identification of the networks that are causally related to MetS.

As a consequence, in a complex polygenic and multifactorial setting like MetS, the pathogenic sequence in the development of insulin resistance and the high TG/low HDL-C phenotype as well as their relationships to associated comorbidities like T2DM and NAFLD cannot be delineated with conventional research methodology and the application of Systems Biology approaches is clearly required. So far, however, the application of Systems Biology to improve our understanding of the pathogenesis of MetS has been limited to either kinetic modelling of very limited parts of the networks involved or approaches which identified genome-scale metabolic networks lacking mechanistic detail. Neither of these allow specific predictions on underlying mechanism causing human pathology (Bordbar and Palsson; J Int. Med., 2012; 271:131-41). Recently, two studies delineated complex genetics of HDL and obesity derived through cross breeding of different mouse strains (Langfelder et al; BBA epub 2011, Wang et al. Front Gen, 2012; 2; 41). This kind of approach yields interesting novel information about potentially involved networks, but no physiological data are presented or used to validate the identified networks.

1.2.2 Objectives of the project and the outcome

- **Project objective I:** To build a computational model for analyzing the kinetics of plasma lipids, lipoproteins and their interactions with glucose metabolism.
  During P5 both short term and long term computational models to describe lipid and glucose metabolism were finalized and shown to work successfully. Since during the course of RESOLVE an important role of bile acids in regulation of metabolic pathways important in MetS became apparent a module was developed that described the enterohepatic circulation of bile acids.

- **Project objective II**: To apply the iterative systems biology cycle for calibrating, validating and improving the computational model in dedicated studies **in mice**.
  Iterative testing of the model in experimental studies in mice was completed in P5. The success of the approach was published in PLOS computational biology. A second paper describing application of the model on a long term mouse experiment has been submitted.
- **Project objective III**: To build, calibrate and validate the computational model for use **in humans**. The completion of translating the mouse model to humans including the novel extension for bile acid metabolism was already reported in P4. In P5 the functionality of ADAPT was extended enabling application to kinetic models of metabolism and signaling pathways. The models were made publicly available and a Matlab platform was developed.

- **Project objective IV**: To analyze – based on model and experimental data – which processes in the **murine** metabolic network regulate the physiological response to perturbations in lipid, lipoprotein and glucose metabolism and how these interact. The short and long term studies in mice produced important results. High fat diet feeding showed a remarkable heterogeneity in response reminiscent of the situation in humans. ADAPT analysis revealed an important role of fatty acid absorption closely associated with a response in bile acid metabolism. A striking absence of connection between lipid and glucose metabolism was observed in all studies performed with mice.
- **Project objective V**: To analyze – based on model and experimental data – which processes in the **human** metabolic network regulate the physiological response to perturbations in lipid, lipoprotein and glucose metabolism and how these interact. In line with the mouse data also in the various human intervention studies performed in RESOLVE remarkably little connection was observed between lipid and glucose metabolism. Under physiological conditions only insulin itself seems to be very important. Network analysis did not reveal other direct connections. Important results were however obtained regarding influences on the separate networks; highlights are the relative minor influence of dietary fructose on lipid handling, the novel role of apolipoprotein F in phospholipid metabolism, the role of dermapontin in liver fibrosis, the correlation of changes in bile acid homeostasis and insulin resistance, the prognostic and potentially pathogenic role of atypical sphingolipids for diabetes and diabetic neuropathy, and the confirmation of the putative causal effect of GLP-1 in amelioration of the type 2 diabetes as well as the improvement of HDL functionality in patients who had undergone bariatric surgery.

- **Project objective VI**. To use the human model to identify network-based drug targets aimed at restoring the metabolic dyslipidemia and glycemic control in patients with MetS and associated comorbidities.
  It is generally assumed that loss of glycemic control in MetS patients also induces dyslipidemia**.** The various experimental and computational approaches applied in RESOLVE have clearly falsified this hypothesis. Perturbation of glucose metabolism did not alter lipid metabolism in mice and humans showing that under 'normal" *in vivo* conditions these metabolic networks are not intimately connected. A significant proportion of the patients undergoing bariatric surgery in the intervention study in WP6 had no dyslipidemia despite suffering from major insulin resistance. Clearly this calls for therapeutical approaches with combinations of drugs targeting the pathways independently. RESOLVE identified bile acid metabolism as an important novel target to treat MetS as well as more traditional targets such as lipid absorption.

1.3. *Description of the main S&T results/foregrounds*

1.3.1 Computational modelling of the pathophysiological processes underlying MetS (WP2)

Metabolic syndrome (MetS) is a collection of several factors: obesity, insulin resistance, elevated lipid levels in the blood, and high blood pressure. A person with metabolic syndrome faces increased risk of cardiovascular disease, type 2 diabetes, and non-alcoholic fatty liver disease. Computational modelling of MetS can provide new insights into its development, but previous modelling efforts had not fully captured the gradual progression and complexity of the disease.
The study of progressive adaptations during disease or intervention is complicated by the multilevel aspects of the biological system. One of the aims of systems medicine is to improve understanding of these adaptations by integrating data from the different molecular levels with mathematical models. ADAPT (Analysis of Dynamic Adaptations in Parameter Trajectories) has been developed, which offers a novel and powerful approach for data-driven dynamic modelling in systems biology. ADAPT provides a methodology to deal with structural and parametric uncertainties. ADAPT translates the problem of structural uncertainty in dynamic systems models into a problem of parameter uncertainty that is solved by (iterative) learning of model parameters from experimental data using machine learning and Artificial Intelligence (AI) algorithms. ADAPT has been successfully applied to simulate metabolic adaptations and therapeutic effects using longitudinal data from human studies and in studies with mouse models of MetS. In addition to modelling longitudinal data of disease progression and treatment, ADAPT can also be applied to kinetic models of metabolism and signalling pathways. A Matlab package with Graphical User Interface was developed for the ADAPT method. The software is open source and is publicly available for download from GitHub.

Different computational models have been developed in RESOLVE. These differ primarily with respect to species (human and mouse) and time-scale of their dynamics (short-term, long-term). Metabolic challenges (e.g., Oral Glucose Tolerance Test / OGTT) are applied in humanized mice for extensive metabolic phenotyping (WP3). Similar metabolic challenge tests are performed at different longitudinal time points in the intervention studies, both in mice (WP4) and human subjects (WP5, WP6). Important in the RESOLVE modelling concept is that the different models are all based on a common, underlying ‘core’ model MINGLeD (Model INtegrating Glucose and Lipid Dynamics). The core model describes the relevant biological processes, using coupled, nonlinear differential equations. MINGLeD describes pools and fluxes of glucose and various fatty acid, cholesterol and bile acids species in the circulation, the liver, intestine and periphery. Different versions of the model are implemented by changing model parameters based on experimental data. Through this concept observations from mice can be translated to humans (Figure 1). Moreover, with this approach we integrate short-term metabolic challenge models with the long-term disease progression models in a single framework for simulation and analysis.

Simulation of MINGLeD with ADAPT and using data from experiments in which mice were fed diets that resulted in development of MetS, allowed for accurate prediction of gradual, long-term development of the disease. We found that our modelling approach correctly predicted progression of MetS in the mice, as well as development of comorbidities, such as fatty liver disease. The model also uncovered the unexpected existence of two disease subtypes in the mice: those with elevated

lipid levels and those without. It correctly predicted underlying metabolic differences that could explain the two subtypes, which were confirmed with experimental data. The model is an important step in understanding the development of MetS, offering new opportunities to identify strategies to prevent the disease and its comorbidities.

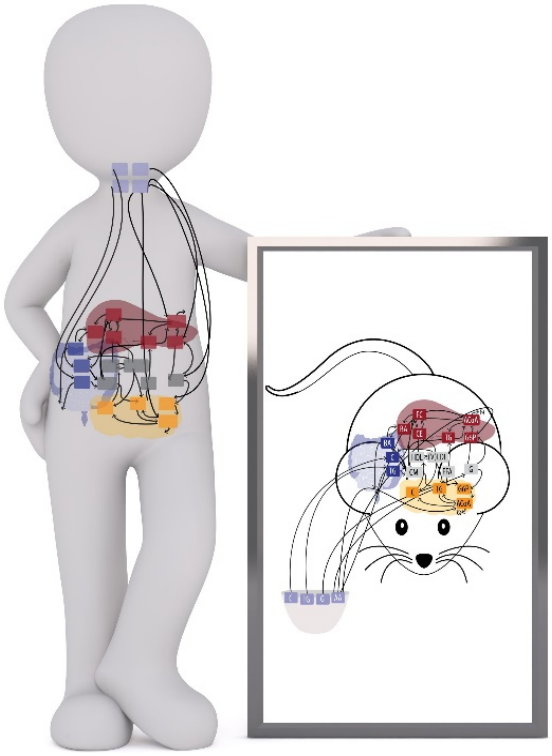

Figure 1 RESOLVE has made use of a genetically modified animal model of which its metabolic system resembles that of the human with MetS. A computational model (called MINGLeD) has been created that describes the major pathways in carbohydrate, fat and cholesterol metabolism. This model is also valid for the human metabolic system. The human version is obtained by changing model parameters based on experimental data and clinical studies. Through this concept observations from mice can be translated to humans, and vice versa.
The computational model is graphically depicted in the block diagrams. The arrows indicate transfer and exchange of different metabolites (denoted by the abbreviations in the boxes) between different tissues.

1.3.2 Short-term mouse experiments (WP3)

The role of WP3 was to generate glucose and lipid flux data in mice with Metabolic Syndrome (MetS). Several aspects of this research such as organ-specific lipid uptake and potentially harmful interventions cannot be performed in humans and therefore need to be performed in preclinical models such as the mouse. For this purpose, WP3 partners used a mouse model expressing the dysfunctional apolipoprotein E3 Leiden as well as the human cholesterylester transfer protein (CETP). Under conditions of a high-fat diet, these E3L*C mice exhibit an altered lipoprotein profile with increased levels of triglyceride-rich lipoproteins (TRL) and decreased levels of HDL, a dyslipidemic phenotype characteristic of humans with MetS. The experiments were primarily conducted to generate lipoprotein flux data after short-term perturbation of glucose homeostasis and triglyceride metabolism, which were subsequently used for computational modelling of lipid and lipoprotein metabolism in the MetS. Finally, metabolic parameters that were predicted by the computational model were validated in independent mouse experiments.

Short-term perturbation of glucose metabolism

In order to define the optimal dietary regimens, various conditions to induce the major hallmarks of the MetS in E3L*C mice were investigated. Based on the results obtained in the laboratories from the collaborating partners, the RESOLVE consortium agreed on a 0.25% cholesterol-containing

high fat diet. Significantly increased body weights were observed in the MetS group already 2 weeks after starting feeding, which became more pronounced after 4 weeks and even more after 8 weeks. In order to generate data during the initiatory phase of the disease, we decided to conduct lipoprotein flux experiments under conditions of short-term glucose perturbations four weeks after feeding. Obesity is central for the development of impaired glucose tolerance, which is another major hallmark of the MetS in humans. Increased plasma glucose levels after an oral glucose tolerance test were observed in E3L*C mice only after feeding the cholesterol-containing high-fat MetS diet (Figure 1), indicating the suitability of this mouse model to study initiation and progression of MetS.

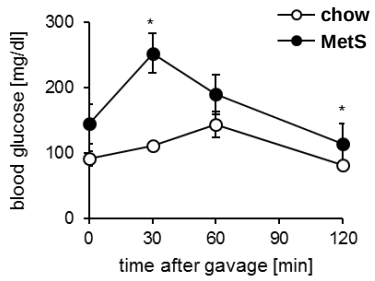

Fig. 1. Glucose tolerance test in ApoE3L*C mice. Male E3L*C mice were fed either a cholesterol-containing high fat diet (MetS) or a control diet (chow) for 4 weeks. Animals were fasted overnight and 1g glucose/kg body weight were i.p. injected. Blood glucose levels were measured at indicated time points using a glucometer. *p<0.05.

In order to establish a reproducible method that is suitable to analyse lipid and lipoprotein fluxes under conditions of an altered glucose homeostasis, several intervention strategies disturbing adequate insulin signaling were established, such as administration of the beta cell toxin alloxan. As it has been shown in humans and rodent models that the activation status of brown adipose tissue is an important determinant of glucose homeostasis, the blockade of insulin signaling was tested by determining glucose and lipid disposal rates under conditions of inactive and active brown adipose tissue. Under these conditions, the disposal of radiolabelled glucose, lipids and lipoproteins was followed in E3L*C mice with MetS pre-treated with alloxan (to blunt insulin signaling) in combination with or without an oral glucose gavage (to increase plasma glucose levels). A robust and significant increase of plasma glucose levels was detected after alloxan treatment, whereas glucose gavage induced only a slight increase in plasma glucose levels (Figure 2A). TRL disposal rates into metabolically active organs such as brown and white adipose tissue was impaired after treatment with alloxan leading to the accumulation of pro-atherogenic TRL in the plasma compartment (Figure 2B). These data provide evidence that insulin signaling rather than increased glucose levels is the main determinant of TRL disposal.

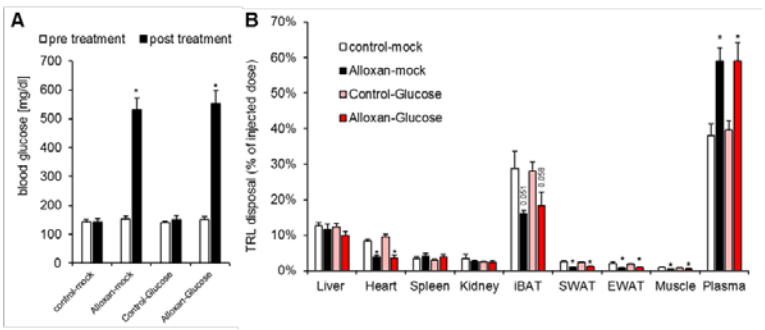

Fig. 2. Glucose perturbation by alloxan and glucose gavage effects TRL disposal in E3L*C mice with MetS. E3L*C mice were fed for four weeks with a diet inducing MetS. Mice were treated with saline (control) or alloxan three days prior to the metabolic lipoprotein turnover study. An oral gavage with 1g/kg glucose was performed and blood glucose (A) as well as the organ specific uptake of TRL (B) were determined. *P< 0.05.

Short-term perturbation of triglyceride metabolism

A central objective of this part was to determine the effect of interventions modulating the activity of lipoprotein lipase (LPL), and thus intravascular lipolysis of TRL, on lipoprotein metabolism and fluxes. TRL lipolysis in BAT can be increased by transferring mice to a housing temperature of 6°C. This triggers sympathetic flow to BAT and activation of brown adipocytes via beta-adrenergic signaling, leading to increased uncoupled mitochondrial oxidation of fatty acids and consequently the generation of heat for maintaining core body temperature. Fatty acids are provided by both LPL-dependent intravascular lipolysis of TRL and intracellular lipolysis at lipid droplets. The physiological relevance of fatty acids released by intravascular lipolysis is demonstrated by the observation that genetic deficiency of the fatty acid transporter CD36 blunts the uptake of TRL fatty acids in cold-activated BAT and makes the mice cold-sensitive. The opposite intervention, reduction of intravascular lipolysis, can be performed by acute inhibition using the lipase inhibitor tetrahydrolipstatin (THL). To investigate the effect of LPL inhibition during the course of an oral fat load in dependence of BAT activation, plasma lipid levels were determined in E3L*C mice with and without MetS (Figure 3).

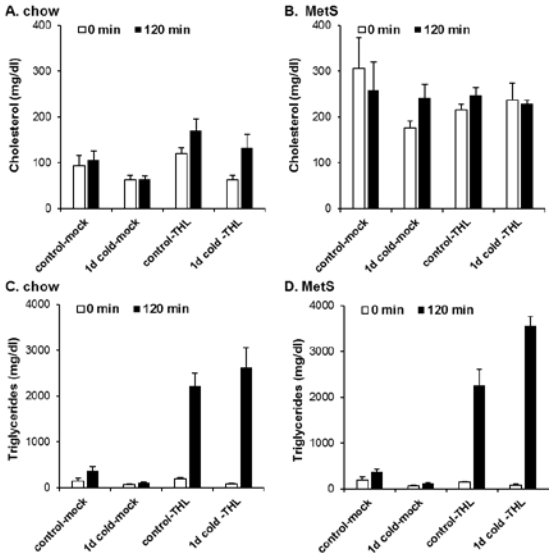

Fig 3. BAT-dependent lowering of plasma lipids in E3L*C mice depends on LPL and MetS. E3L*C mice kept on either a chow diet (A,C) or the MetS diet for 8 weeks (B,D). Mice were housed at thermoneutral temperature (control) or exposed to 6°C for 1 day (cold). Blood was collected from fasted mice (0 min), subsequently olive oil (200µl per mouse) was administered by oral gavage and the mice received an intravenous injection of saline (mock) or THL. Blood was collected again at the end of the experiment before necropsy (120 min). Total cholesterol (A,B) and triglycerides (C,D) levels were determined in plasma.

In comparison to chow controls, cholesterol plasma levels were increased in E3L*C with MetS. Furthermore, the experiments indicated that BAT activation by cold exposure lowers both cholesterol and triglyceride levels. As expected, an oral fat load leads to increased triglyceride but not cholesterol. Notably, THL injection results in a profound rise in plasma triglyceride levels and blunts the triglyceride lowering effect of BAT activation. Dynamic flux data were generated in E3L*C under brown adipose tissue activation (Figure 4).

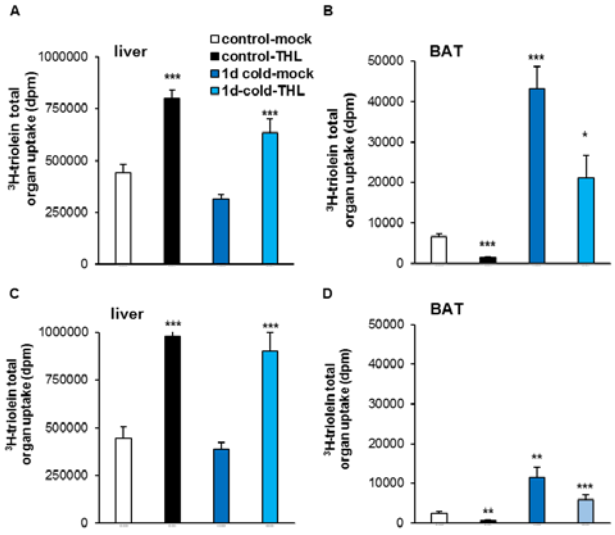

Fig. 4. Liver and BAT disposal of TRL in E3L*C mice with and without MetS. TRL labeled with ^{3}H-triolein were injected intravenously into E3L*C mice without (A,B) and with MetS (C,D). 20 min after injection of labelled TRL, mice were perfused and organs were harvested to determine the specific uptake of TRL into liver (A,C) and BAT (B,D). Statistically significant differences between Mock and THL-treated mice are indicated (*p<0.05; **p<0.01; ***p<0.001).

The organ-specific uptake of TRL indicates that LPL inhibition leads to increased hepatic TRL disposal whereas TRL uptake into BAT of E3L*C mice without and with MetS is decreased. Activation by cold-exposure resulted in a profound increased uptake into BAT, which is associated with a diminished clearance by the liver in chow-fed animals. These data indicate that LPL-mediated peripheral processing determines plasma clearance rates and shifts TRL fluxes in an organ-specific manner. In comparison to insulin-sensitive mice, metabolic studies in E3L*C mice with MetS indicate that TRL uptake into BAT is less pronounced after cold-exposure and less dependent on LPL inhibition. The diminished uptake into BAT is associated with higher uptake into liver. Altogether, TRL flux data in E3L*C mice with and without MetS showed that perturbations of triglyceride hydrolysis by BAT activation and LPL inhibition significantly alter TRL catabolism.

Validation of computational models

The results of perturbation experiments encompassing steady state metabolite concentrations in different compartments as well as flux data generated in E3L*C mice show the relevance of energy combustion by brown adipose tissue for energy balance and thus the development of obesity and MetS. To refine the basal computational model (MINGLeD), an additional module mimicking increased energy expenditure by peripheral tissues was implemented. Peripheral energy expenditure was increased within the model by an activation factor that represents the respiration of acetyl-CoA. An incremental increase of this activation factor predicted major changes in peripheral, hepatic and intestinal metabolites including triglycerides and cholesterol. To validate the model, metabolites and metabolic fluxes were determined in mice that were subjected to cold exposure as a stimulus to increase peripheral energy expenditure. In line with the assumption that cold-activated BAT uses high amounts of triglycerides to generate heat and with the model predictions, decreased triglyceride stores were detected in BAT isolated from E3L*C mice with and without MetS. Interestingly, the model predicted also lower plasma total and HDL cholesterol as well as a concomitant higher cholesterol uptake into BAT and the liver, which was accompanied by both accelerated lipoprotein processing and increased cholesterol transport from peripheral tissues toward the liver. These predictions were confirmed by metabolic tracer studies using radiolabelled

HDL and by determining the reverse cholesterol flux to the liver. These data suggested increased metabolisation of cholesterol to bile acids in the liver, which could be confirmed by investigating bile acid metabolism (Figure 5).

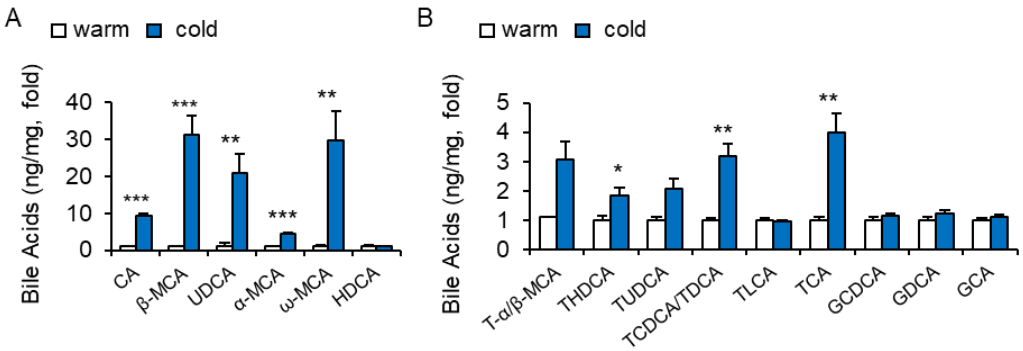

Fig. 5. Hepatic bile acids in warm and cold-exposed mice. Relative levels of (A) unconjugated bile acid and (B) conjugated bile acid species in the livers of C57BL6/J wild type mice that were housed at warm conditions or at 6°C (cold). Significant changes are indicated by asterisks (*, $p<0.05$; **, $p<0.01$, ***, $p<0.001$, determined by Students T-test; n=5-9 per group).

The levels of most of the unconjugated and conjugated bile acid species were significantly higher in the cold-housed mice than in the warm-housed control mice. Bile acids are exclusively synthesized in the liver by a number of enzymatic reactions using two different routes. The classical bile acid synthesis pathway starts with the rate-limiting enzyme cholesterol 7-alpha-hydroxylase and it prevails under normal conditions. Bile acids can also be formed by the alternative bile acid synthesis pathway which is initiated by action of sterol 27-hydroxylase followed by 25-hydroxycholesterol 7-alpha-hydroxylase. In further experiments validating the effect of increased peripheral energy expenditure, it was demonstrated that the alternative pathway is responsible for maintaining cholesterol homeostasis under conditions of BAT activation.
In conclusion, BAT activation is accompanied by a marked increase in intravascular LPL activity, driving fatty acid uptake into thermogenic brown adipocytes. LPL-dependent TRL remodeling results in the generation of cholesterol-enriched lipoprotein remnants and HDL, accelerating cholesterol flux to the liver under conditions of BAT activation. Together with a strongly induced hepatic bile acid synthesis capacity, this leads to increased biliary excretion of bile acids. These results are in line with the predictions by the MINGLeD model that in silico BAT activation lowers plasma and hepatic cholesterol pools. Thus, the predicted effects of increased peripheral energy expenditure on metabolic parameters including peripheral, plasma and hepatic lipid values as well as lipid fluxes were experimentally verified.

1.3.3 Long-term mouse experiments (WP4)

1.3.3.1 Comparisons of responder and non-responder ApoE*3Leiden.CETP mice

The E3L*C mouse model is an important animal model to study the metabolic syndrome (MetS), since these mice have a humanised lipoprotein metabolism and a heterogeneous response to the MetS, similarly to humans. The reported heterogeneity among these mice and their common classification refers to responder (R) and non-responder mice (NR mice); only R mice show

increased body weight, and cholesterol and triglycerides levels. To better define the differences between R and NR mice, we focused on feeding behavior, body weight gain, glucose tolerance, and lipid parameters, and liver histology analysis. Our data confirmed that only R mice resemble the pathological features of human MetS: obesity, dyslipidaemia, glucose intolerance. NR mice do not develop the full dysmetabolic phenotype because of a severe inflammatory hepatic condition, which may heavily affect liver function. We conclude that R mice and NR are metabolically different and that NR mice have indications of severely impaired liver function. Hence, it is critical to properly identify and separate the respective mice to decrease heterogeneity in the data. Data of biochemical and histological analyses should be used to confirm retrospectively the classification of the animals.

1.3.3.2 RYGB surgery in ApoE*3Leiden.CETP responder mice

Roux-en-Y gastric bypass (RYGB) is a bariatric surgery technique that allows to achieve sustained and long-term weight loss and to improve comorbidities in the MetS. We elucidated the improvements in lipid and glucose metabolism after RYGB surgery. Male E3L*C R mice fed Western type diet for 6 weeks underwent RYGB or Sham surgery. Sham groups were either fed *ad libitum* or were body weight-matched to the RYGB mice, to discriminate and identify surgical effects from body weight loss associated effects. 20 days after surgery, mice were sacrified, liver was collected to assess metabolic, histological and global gene expression changes after surgery. RYGB induced a greater reduction in body weight, achieved by high food restriction in the body weight matched (BWm) mice, and total fat mass compared to Sham *ad libitum* mice (Sham AL). Plasma levels of total cholesterol, non HDL-C and ceramides were strongly reduced upon RYGB compared to BWm mice. Glucose tolerance and insulin sensitivity improved 2 weeks after surgery in RYGB and BWm mice. Liver histology confirmed lipid reduction in RYGB and BWm mice while the transcriptomics data proved modification in lipid metabolism, especially altered hepatic gene expression and upregulation in genes related to cholesterol metabolism in RYGB compared to BWm and Sham AL. RYGB surgery improves glucose metabolism and greatly ameliorates lipid metabolism in part in a body weight-dependent manner.

In conclusion, our initial comparison between R and NR mice allowed us to better understand the patho-physiology of glucose and lipid metabolism in these mice and to provide baseline data for the second study about the effect of RYGB surgery in R mice. We showed that RYGB leads to a rapid improvement in glucose and lipid metabolism in our mouse model, but it was surprising to see that many beneficial RYGB-induced effects were also seen in the BWm controls. Hence, the rapid decrease in body weight which is linked to a marked reduction in caloric intake in the BWm mice during this period had a similarly beneficial effect on overall metabolism as RYGB.

1.3.3.3 Impact of dietary composition

In comparison to glycerolipids, sphingolipids (SL) show vast structural diversity, which arises by the combinatorial composition of different sphingoid base backbones in conjunction with different N-acyl chains and headgroups. 1-deoxy-sphinglipids (1-deoxySL) are a particularly interesting subclass of SL species which are formed in a side reaction during SL de-novo synthesis when the key regulatory enzyme serine-palmitoyltransferase uses alanine instead of its canonical substrate serine. As 1-deoxySL are toxic to beta cells and their plasma levels associated with non-alcoholic fatty liver disease (NAFLD), we investigated whether they are involved in impaired glucose homeostasis and hypertriglyceridemia. This hypothesis was tested in male and female ApoE3L.hCEPT transgenic mice by perturbing sphingolipid metabolism via a dietary

supplementation with L-alanine and L-serine. The mice were kept for 6 months on a high fat caloric diet (HFD) either as such or supplemented with L-serine or L-alanine (5% w/w, each: HFD+ala and HFD+ser, respectively). The average weight gain over time was not different between the different feeding groups but we observed a considerable heterogeneity between individual mice even within the same treatment groups. These metabolic changes were associated with increased liver fat and hepatic steatosis. Responder animals exhibited the classical macro- and microvesicular centrilobular steatosis with little inflammatory changes. Low-responder mice, however, accumulated less lipid droplets in the liver while on HFD.

L-serine supplementation efficiently suppressed 1-deoxySLs formation whereas L-alanine supplementation was associated with an early increase in 1-deoxySL but not with any elevation in total 1-deoxySL levels compared to mice on HFD only. However, besides elevated plasma and liver TGs, the mice presented neither impaired glucose homeostasis nor any pathological changes in beta cell morphology.

Despite the differences in the sphingoid base profile, we could not detect any significant difference in the metabolic profile between the HFD+ala and HFD+ser group. As the mice neither developed impaired glucose homeostasis nor showed obvious changes in beta cell histology, the expected primary endpoint for comparison was not met by the model. However, we observed a significant lowering of plasma and liver TGs in the amino acid fed animals compared to the HFD only group. This lipid lowering effect appeared to be independent of SL or 1-deoxySL formation as SL were not different between the groups and 1-deoxySL formation only suppressed in the HFD+ser group. The observed effect might, however, be related to recently published data that showed that supplementation with serine but also with glycine positively affects the C1 carbon, S-adenosyl methionine and homocysteine metabolism. In a model for alcohol induced steatosis, serine supplementation reduced liver TGs and supplementation with glycine and serine suppressed methionine-induced hyper-homocysteinemia in rats. The underlying mechanism how dietary alanine or serine lowers TGs will be followed up and investigated in more detail as it could promote a novel therapeutic approach to reduce steatosis in NAFLD.

1.3.3.4 Impact of genetic interventions

The critical position of FOXO1 at a node in the insulin signaling pathway linking glucose and lipid homeostasis prompted RESOLVE partner FORTH to investigate its role in the pathogenesis of MetS via a conditional gene silencing approach. It was hypothesized that silencing of FOXO1 specifically in the liver of apoE3L*C mice on a 12-weeks high fat diet (a condition that causes insulin resistance) would ameliorate glucose production via gluconeogenesis as well as VLDL production thus reducing the clinical symptoms of MetS in these mice. For this purpose, adeno-associated viruses (AAVs) expressing short hairpin RNAs (shRNAs) for FOXO1 or control (scrambled shRNAs) were designed and produced in collaboration with partner UMCG. The protocol of AAV-mediated genetic ablation of the transcription factor FOXO1 in apoE3L*C mice was established and the efficiency of FOXO1 gene silencing was demonstrated using Real Time quantitative PCR and Western blotting. Using these AAVs, FORTH conducted a large scale genetic intervention experiment in apoE3L*C mice that received a HFD for 12 weeks. These mice were fully characterized for various ‘clinical’ and histopathological parameters and a full transcriptomic analysis was performed using next generation RNA sequencing (Illumina). It was found that silencing of FOXO1 in E3L*C mice did not affect body weight or plasma lipoprotein levels but it

was associated with reduced glucose levels and statistically significant improvements of glucose and insulin tolerance tests suggesting that genetic ablation of FOXO1 had a positive impact on glucose homeostasis. These findings could be explained, at least in part, by the liver transcriptomics data that were obtained from the same mice. The clinical and the transcriptomics data would be valuable for a better understanding of the molecular mechanisms that are implicated in the pathogenesis of the metabolic syndrome and for the design of novel diagnostic tools and therapeutic strategies.

1.3.4 Humanization of the computational model through the direct use of clinical data (WP5)

The main objective of **WP5** was to humanize and calibrate the computational mouse models (**WP2**) using existing data sets derived from healthy, overweight and morbidly obese human cohorts with/without metabolic syndrome before and after interventions (bariatric surgery, hypercaloric diets, omega-3 enriched diets, administration of intravenous lipid emulsions).
An aim of **WP5** was to humanize, test the performance and extend the computational model by using pre-existing data sets from **AMC** patient cohorts (Xu X et *al*., *J Cell Metabol*, 2013 ; de Weijer BA et *al*., *Obesity (Silver Spring)*, 2013 ; Brands M et *al*., *J Lipid Res*, 2013 ; Brands M et *al*., *Clin Endocrinol (Oxf)*, 2013 ; Bonet D et *al*., *Obes Rev*, 2014) and patient cohorts from literature (**TU/e/UMCG/AMC**). The computational model should describe the dynamics of the metabolic processes, their regulation and adaptation by combining glucose and fatty acid kinetics and the regulation by insulin. Moreover, at this time, it has been shown that only measured metabolic fluxes can be used to develop/test performance/extend the model. To overcome this limitation, it was proposed to introduce Genome-Scale Metabolic Modeling (GSMM) in RESOLVE, a technology that permits analysis of metabolic datasets with only a single time point (**TU/e**). Then, concretely, 4 independent **AMC** cohorts were used to provide data on 1/ oral glucose tolerance (obese and healthy patients), 2/ glucose flux data during iv lipid emulsion (healthy patients), 3/ glucose flux and lipolysis, transcriptomics and targeted metabolomics data (liver, adipose tissue) (morbidly obese patients before/after bariatric surgery or before/after a high-dose ω-3 fatty acid supplementation intervention) (**AMC/TU/e**). Moreover, a module of the gut was incorporated in the MINGLeD model to describe postprandial metabolism of glucose, lipids/lipoproteins and bile acids with the aim to predict the metabolic effects of bariatric surgery (**AMC/TU/e**). In parallel, AMC showed that basal endogenous glucose production, glucose and insulin are reduced in obese women within two weeks after RYGB, whereas no beneficial effects on hepatic or peripheral insulin sensitivity were observed (**AMC** ; *Data not published*).
Another part of **WP5** aimed to generate a new overweight and obese patient cohort to identify genes/pathways differentially expressed according to the metabolic status. **UZA** has collected metabolic data and biopsies from overweight and obese patients followed at the Antwerp University Hospital. This collection consists of metabolic data, including biochemical parameters and NASH scoring, liver tissue biopsies and in a sub-cohort adipose tissue biopsies. This collection groups the data and biopsies for 474 overweight and obese patients at baseline (before weight loss intervention) and 108 after intervention (12 months after a weight loss program (hypocaloric diet or bariatric surgery); see **WP6**). The patient cohort can be used, even after the end of the RESOLVE consortium, for specific scientific questions and further analyses. Transcriptome analyses have been performed by **IPL** on RNA prepared from liver biopsies of overweight and obese patients from **UZA** cohort with different metabolic status and before and after a weight loss program (see **WP6**).

TU/e has developed patient-derived hepatic metabolic network models derived from 150 patients in the **UZA** cohort on which transcriptomic analysis was performed by **IPL**. Different GSMM's were thus used (**UZA/TU/e** , *Data not published*). In parallel, data from arrays were analyzed using different softwares with the aim to identify genes and/pathways differentially expressed according to the metabolic status of the patients (**IPL**). Thus, it was found that the expression of the nuclear receptor PPARalpha gene, a major player in hepatic and plasma lipid metabolism, is inversely correlated with the severity of NASH. Analysis of liver biopsies has shown that gene expression of PPARalpha and its targets correlates positively with liver status improvement in patients with NASH (**IPL/UZA/UZH**; Francque S et al., *J Hepatol.*, 2015). Moreover, bile acid alterations were shown to be related to the metabolic phenotype associated with NASH, especially insulin resistance, but not liver necroinflammation, in obese patients (**IPL/UZA** ; Legry V et *al.*, *J Clin Endocrinol Metab*., 2017). This study prompted to extend the human computational model MINGLeD by integrating bile acid metabolism (**IPL/UZA/TU/e**). Other genes/pathways have been identified and are under investigation (**IPL/UZA/UMCG**; Mogilenko DA et *al*., Submitted & *Data not published*). Thus, the identification of one of these genes allowed to extend the human computational model MINGLeD concerning lipid metabolism, especially phospholipid metabolism (**IPL/UZA/TU/e**; *Data not published*).
Thanks to pre-existing datasets from published and **AMC** patient cohorts, the computational model has been humanized and calibrated and allows now to predict metabolic impacts on liver, adipose tissue and gut after a glucose/insulin/lipid modulation, as for example, after bariatric surgery (**AMC/TU/e/UMCG/UZA**). Moreover, a new overweight and obese patient cohort (**UZA** cohort) is now available for specific scientific questions and further analyses. Transcriptomic analyses have been performed on this cohort and allowed to identify genes modulated in these patients. Others are still under investigation. These genes allowed the extension of the humanized computational model and constitute new markers of metabolic and/or liver status.

1.3.5 Interventions in humans using validated computational models (WP6)

The objectives of WP6 were to undertake interventions in humans that perturb lipid, lipoprotein and glucose metabolism using validated computational model analysis.

Studies in overweight individuals: Studies in overweight individuals were performed to (1) clarify the dynamics of postprandial lipid metabolism of triglyceride-rich lipoproteins, (2) to quantitate the contribution of different sources of FAs in the liver to production of triglyceride-rich lipoproteins, and (3) to define the mechanisms for impaired lipid clearance. The main achievements were to develop an integrated model for postprandial metabolism of apoB100 and apoB48-containing lipoproteins, and establishment of novel methods for enrichment analysis of low abundant apoB48 from chylomicrons and chylomicron subfractions. These studies have demonstrated (1) the importance of hepatic *de novo* lipogenesis for hepatic biosynthesis of triglyceride-rich lipoproteins, (2) clarified the interplay between intestinal and hepatic lipoprotein secretion, and (3) clarified the mechanisms for impaired metabolism of triglyceride-rich lipoproteins in patients with ectopic fat accumulation in the liver.

To assess the effects of dietary fructose on liver fat content and other cardiometabolic risk factors, abdominally obese men (n=71) were served 75 g fructose/day with their habitual diet over 12 weeks. We analyzed changes in body composition, dietary intake, an extensive panel of cardiometabolic risk markers, hepatic de novo lipogenesis (DNL), liver fat content, and postprandial lipid responses after a

standardized oral fat tolerance test (OFTT). Results showed that fructose consumption had modest adverse effects on cardiometabolic risk factors. However, fructose consumption significantly increased liver fat content and hepatic DNL and decreased β-hydroxybutyrate (a measure of β-oxidation). Our data demonstrated adverse effects of moderate fructose consumption for 12 weeks on multiple cardiometabolic risk factors in particular on liver fat content despite only relative low increases in weight and waist circumference. Our study also indicates that there are remarkable individual differences in susceptibility to visceral adiposity/liver fat after real world daily consumption of fructose sweetened beverages over 12 weeks. In line, results from a short-term intervention with an isocaloric low-carbohydrate diet in obese subjects with NAFLD demonstrated rapid and dramatic reductions of liver fat and other cardiometabolic risk factors paralleled by marked decreases in hepatic DNL, and large increases in serum β-hydroxybutyrate concentrations, reflecting increased mitochondrial β-oxidation.

Studies in bariatric surgery patients: To improve our understanding of the molecular pathways involved in the development of insulin resistance and dyslipidemia we assessed the glucose and lipid metabolism using state of the art techniques in 41 morbidly obese subjects (18 men and 23 women). Detailed metabolic fluxes and tissue transcriptomics in obese humans were performed before and after bariatric surgery-induced weight loss. Results showed that one year after bariatric surgery, insulin sensitivity in multiple tissues was restored in the majority of patients despite a mean remaining BMI within the obese range. The latter suggests additional weight loss-independent metabolic effects of bariatric surgery such as an increase in GLP-1 secretion. Liver fat was dramatically reduced after weight loss and might account for some of the beneficial metabolic effects. The metabolic improvements were not gender-specific except for a higher insulin sensitizing effect of weight loss on adipose tissue insulin sensitivity in women. The first analysis of the RNA sequencing data suggested major effects of weight loss on inflammatory pathways in skeletal muscle and adipose tissue, paving the way for interventions that specifically target the low inflammatory state of obesity with the aim to restore insulin sensitivity. Overall, this unique and extensively phenotyped human cohort increases our understanding of the molecular networks of the metabolic syndrome and opens up new avenues in the development of diabetes and dyslipidemia treatment.

Lifestyle interventions: In **WP2** a computational version of the bariatric surgery model was developed. As part of **WP6** this model has been applied in an '*in silico* systems surgery' study to show how differences during surgery might impact glycaemic control one-year post-surgery. For this analysis we extended the bariatric surgery model with the previously developed model of glucose homeostasis included in MINGLeD and data of the bariatric surgery patients. The metabolic response to a mixed meal before and after surgery was used as input for the modelling. MINGLeD was extended to include GLP-1 signalling. To our knowledge, this model is the first model capable of simulating the characteristic postprandial GLP-1 peak observed after RYGB. The model confirms that GLP-1 contributes to the enhanced insulin secretion after bariatric surgery. Analysis of the GLP-1 fluxes show that this peak is practically solely caused by an increased delivery of glucose to the ileum. This supports the hindgut theory that the accelerated delivery of nutrients to the distal intestine augments an insulinotropic signal.

To identify key components during the progression toward NASH and fibrosis, we investigated the liver transcriptome in a human cohort of NASH patients. The transition from histologically proven fatty liver to NASH and fibrosis was characterized by gene expression patterns that successively reflected altered functions in metabolism, inflammation, and epithelial-mesenchymal transition. A meta-analysis combining human transcriptomic datasets with murine models of NASH and fibrosis

defined a molecular signature characterizing NASH and fibrosis and evidencing abnormal inflammation and extracellular matrix (ECM) homeostasis. Dermatopontin expression was found increased in fibrosis, and reversal of fibrosis after gastric bypass correlated with decreased dermatopontin expression. Functional studies in mice identified an active role for dermatopontin in collagen deposition and fibrosis.

Studies have revealed the role of ApoF in the control of lipid metabolism illustrated by the finding that ApoF knockdown reduces fatty acid uptake in human hepatocyte IHH cells. Lipidomic flux measurements were performed in IHH cells after transfection of a specific ApoF gene siRNA and in the plasma of a subgroup of patients for whom liver microarray data were available. The results indicate that ApoF may be involved in hepatic PC synthesis or secretion.

1.3.6 Large-scale data generation, data management and data validation (WP7)

1.3.6.1 Data mining guidance and Generation of a database for project-specific datasets and integration

For storage, management and analysis of large-scale data generated in the project as well as publicly available data related to the project a systems biology software platform, the geneXplain platform (https://platfrom.genexplain.com), was used and adopted to deal with the metabolic data. The geneXplain platform has been installed on the secure servers accessible for all partners, which provides an online toolbox and workflow management system for a broad range of bioinformatics and systems biology applications. The platform provides an integrated view on several databases and analysis tools, public domain as well as commercial ones that are necessary for creation the database for the project-specific data. Extension of the geneXplain platform has been done with import methods for a number of biological file formats ranging from various table formats with measurements of concentrations of various biological molecules, microarray formats, NGS data formats as well as formats for complex network models. Additional software architecture on the servers has been created helping to upload, maintain, and utilize project-specific and publicly available data using a combination of the SEEK / FAIRDOM database management system and geneXplain platform database management system. Novel analysis workflows have been created in the geneXplain platform for analysis of metabolism-related data including their mapping onto metabolic pathways and networks and performing complex queries of the respective enzymes and their regulatory networks (using databases such as Recon2, Human Metabolic Atlas, Reactome, HumanCyc, TRANSFAC and TRANSPATH). Finally, the new workflow in the geneXplain platform combines analysis of metabolic pathways with the analysis of transcription regulation of genes encoding key metabolic enzymes followed by investigation of signal transduction networks in order to identify novel biomarkers that potentially can be used for the (differential) diagnosis, prognosis and monitoring of metabolic abnormalities and for identification of novel targets for potential treatment of metabolic syndrome.

1.3.6.2 Targeted proteomics of human and murine lipoproteins

UMCG has established quantitative targeted proteomics for key players in lipid and lipoprotein metabolism.

1.3.6.3 Targeted metabolomics

UZH developed and validated a novel MS based lipidomics method with a special focus on sphingolipid profiling. The method allows the analyses of free sphingoid bases, ceramides,

sphingomyelines, glycosphingolipids and sphingosine-1-phosphates by high resolution, accurate mass spectrometry (HRAM-MS). Beyond previously established methods, UZH put a special focus on the analysis of atypical and isomeric sphingolipid species. Deoxysphingolipids (deoxySL) and deoxymethlyssphingolipids are formed when serine-palmitoyltransferanse (SPT), the rate limiting enzyme of sphingolipid synthesis, uses alanine or glycine, respectively, instead of serine. Because of the missing hydroxyl-group these atypical sphingolipids are neither processed to complex sphingolipids nor metabolized to sphingosine-1-phosphate for degradation. They are neurotoxic and the molecular pathogen causing hereditary sensory neuropathy which clinically resembles to diabetic neuropathy. SPT also forms other atypical sphingolipids by using acyl-CoAs which are shorter or longer than the typically used palmitoyl-CoA. Additionally, UZH established and verified an analytical workflow based on the commercial software package "Lipid Search" (Thermo Scientific) which allows the identification and quantification of unknown lipid species based on a proprietary database and detection algorithm. The new method as well as a prior method that allows the differentiation and quantification of typical and atypical backbones were applied to several studies in humans and genetic or interventional mouse models as well as to the elucidation of the metabolism of atypical sphingolipids:

- ✓ UZH resolved the chemical structure of 1-Deoxysphingosine (1-deoxySO). This downstream metabolite of 1-Deoxysphinganine was believed to bear a Δ4 trans double bond as expected for a canonical sphingosine base. By using two independent tandem mass spectrometry approaches and tailored synthesis of Δ14 unsaturated 1-deoxySO UZH showed that 1-deoxySLs undergo a different metabolic conversion than canonical sphingoid bases. This information was crucial to unravel the metabolism of the downstream metabolites formed from deoxysphingolipids and identify the involvement of CYP4F enzymes in these metabolic pathways *(J. Lipid Res. 2016, 57: 1194-203; J. Lipid Res. 2017;58:60-71)*. Furthermore UZH resolved the metabolism of methylsphingolipids that are formed by the use of glycine. Altogether these findings are likely important for the prevention and treatment of diabetes mellitus type 2 and its complications, notably diabetic sensory neuropathy (DSN) and non-alcoholic fatty liver disease (NAFLD).
- ✓ In prospective studies, UZH showed that plasma levels of atypical sphingolipids are associated with increased risks for either diabetes or cardiovascular events (*BMJ Open Diabetes Res Care. 2015, 3:e000073; Atherosclerosis 2015, 240:216-21; PLoS One. 2017, 12:e0175776*). In agreement with a causal prodiabetic role, deoxysphingolipids were found to interfere with beta cell survival and function (*Diabetes. 2014, 63:1326-39*).
- ✓ In cross-sectional studies 1-Deoxysphingolipid plasma levels were found increased in patients with diabetic neuopathy (*Eur J Neurol. 2015, 22:806-14; PLoS One. 2017,12:e0170583*). In addition, UZH provided evidence that deoxysphingolipids contribute to the pathogenesis of diabetic neuropathy and that serine treatment lowers deoxysphingolipids and ameliorates the neuropathy (*Diabetes. 2015, 64:1035-45*).
- ✓ UZH analyzed the sphingoid base profile in plasma samples from the HEPADIP Cohort provided by partner UZA. The study included 350 individuals at different stages of NAFLD. We observed a close association of canonical, serine based SL's with classical CVD markers like LDL- and total cholesterol whereas 1-deoxySL showed a correlation with T2DM related risk factors. Plasma deoxySL levels correlated to visceral but not to total abdominal or subcutaneous fat depots. We also observed a significant increase in plasma 1-deoxySL levels at early steatosis (5%-33%). SAdiene, a polyunsaturated downstream product of sphingosine, correlated negatively with the number of inflammatory foci.

- ✓ In a cross-over study UZH showed that fenofibrate but not nicotinic acid treatment lowers plasma concentration of deoxysphingolipids and other atypical sphingolipids (J Clin Lipidol. 2015, 9:568-75).
- ✓ UZH characterized the sphingoid base profile in a series of mouse models for MetS. We observed significant changes in the plasma sphingolipid profile of ApoEL.hCETP mice either after RYGB surgery (1.3.3.2) or dietary intervention (1.3.3.3). In liver specific HNF4 α KO mice we observed significant changes in the liver and plasma lipid profile (T4.6, FORTH). In KO mice, canonical and 1-Deoxysphingolipid species were significantly decreased in plasma, whereas the same classes were increased in liver tissue. ORMDL proteins are believed to be negative regulators of serine palmitoyltransferase (SPT). We used Ormdl3-knockout (Ormdl3-/-) and transgenic (Ormdl3Tg/wt) mice to study the effect of ORMDL3 on total SL levels in plasma and tissues. Ormdl3-knockout (Ormdl3-/-) and transgenic (Ormdl3Tg/wt) mice were generated to study the effect of ORMDL3 on total SL levels in plasma and tissues. Sphingoid bases with atypical chain lengths (C16, C17) were decreased in plasma and liver of ORMDL3-KO mice. (FASEB J. 2016 Dec;30(12):4289-4300)
- ✓
- ✓ UZH established a method to measure trimethylaminoxide (TMAO). This metabolite is formed from dietary choline and carnitine by gut microbiota and liver enzymes. It very recently attracted a lot of scientific, clinical and public attention as a causal biomarker of cardiovascular disease with dietary origin. UZH found significant correlations with MetS, diabetes, glycemic control and kidney function but no independent association with history, presence or incidence of coronary heart disease (CHD) (*Atherosclerosis 2015, 243:638-644*).

1.3.6.4 Effects of bariatric surgery on the molecular composition and functionality of HDL

A low plasma level of HDL-cholesterol is a component of the metabolic syndrome and an independent risk factor for coronary heart disease (CHD) and diabetes mellitus type 2 (T2DM). Nevertheless and despite their many vasoprotective and anti-diabetic effects, HDL is exploited for neither prevention nor treatment of these diseases. An important reason for this shortfall is the structural and functional complexity of HDL particles. Therapeutic interventions including bariatric surgery may change HDL composition and hence function, however, with as yet unknown clinical consequences. UZH therefore investigated in 26 patients the effect of Roux-en Y gastric bypass surgery (RYGB) on the functionality and molecular composition of HDL. Compared to the pre-operative baseline visit, the follow-up visits 3 months and 12 months after RYGB revealed expected significant changes in body weight, glucose, HbA1c, nonHDL-cholesterol, triglycerides, and CRP. HDL-cholesterol and apoA1 increased significantly after 12 months (+57% and +35%, respectively) but not after 3 months. Both 3 months and 12 months after RYGB, HDL showed increased activities to elicit cholesterol efflux from macrophage, to inhibit apoptosis of human aortic endothelial cells (HAECs), and to increase mitochondrial potential of C2C12 myotubes. Only 12 months after RYGB, HDL showed increased activity to inhibit apoptosis of INS1e beta cells. Out of 88 lipid species with a focus on sphingolipids and 176 proteins, only the decrease of two proteins and the increase of three proteins as well as six lipid species observed after 3 months was sustained until twelve months after surgery. The lipids include ether phospholipids and sphingoine-1-phosphate that in a case-control study were found by UZH to be associated with CHD and correlated with anti-apoptotic functionality of HDL (*Atherosclerosis 2015, 241:539-546; Atherosclerosis 2016 246:130-40*). The changes in the content of HDL in ether phospholipids and

sphingosine-1-phosphate but also an atypical sphingolipid as well as the anti-apoptotic activities of HDL towards HAECs and INS1e cells correlated with the improvement in glycemic control. In conclusion, RYGB improves several potentially vasoprotective and anti-diabetic functions of HDL as well as protein and lipid composition before changes in HDL-cholesterol and apoA1 plasma levels become significant. These quantitative and qualitative changes may contribute to the improved glycemic control after RYGB. It will be interesting to test the identified structural correlates of improved HDL functionality for clinical exploitation as diagnostic biomarkers or therapeutic targets.

1.3.6.5 Large scale transcriptomics data

Using transcriptomics for de novo gene and pathway investigation has contributed significantly to the understanding of metabolic health. However, information related to the gene signatures that are characteristic of MetS is still missing and it is urgently needed because it may lead to new insights regarding the diagnosis and the therapies of MetS. One of the aims of RESOLVE was to generate transcriptomics data from a mouse model of diet-induced MetS (E3L*C mouse). The key findings were:

- ✓ High fat diet administration to E3L*C mice affected the transcriptomic profiles in the liver and the adipose tissue;
- ✓ Key genes of metabolic diseases were altered which could explain the metabolic abnormalities that are present in these mice;
- ✓ Bioinformatic analysis identified liver and adipose gene clusters with similar trends of expression over time which could be useful as “gene signatures” of MetS;
- ✓ Comparison of two diet intervention studies revealed a limited number of common genes. However the expression of the common genes was affected in a similar way;
- ✓ RYGB and weight reduction interventions also affected significantly the gene expression profiles in the liver. Both interventions restored the gene expression changes caused by the HFD;
- ✓ Genetic ablation of the transcription factor FOXO1 in the livers of E3L*C mice had a strong impact on the expression of genes belonging to different metabolic pathways.

This transcriptomics data could explain at least in part, the clinical phenotype of these mice which included improved glucose and insulin tolerance and augmented steatosis. The data give the RESOLVE consortium the opportunity to make important correlations between the expression levels of specific genes and the predisposition or the protection from the development of MetS. These gene signatures could also prove to have clinical applications for the prognosis or the treatment of MetS.

1.3.6.6 Data on miRNA profiles in plasmas of humans and mice

One of the goals of RESOLVE was to monitor changes in the levels of circulating miRNAs at the various stages of the pathogenesis of the MetS in the E3L*C mouse strain that was used as a model of MetS as well as in human patients with MetS and to correlate these changes with the incidence of diabetes. It was found that certain microRNAs were increased in the serum of apoE3L*C mice at the late but not the early or intermediate stages of MetS or at the very late stage. The data from the apoE3L*C mice were validated in a mouse model of diabetes (Akt2-/- mouse). Two of these circulating miRNAs (Mir192 and Mir194) were also found to be associated with the incidence of diabetes type 2 in a cohort of patients followed for a period of 6 years. These findings reveal for the first time characteristic circulating miRNA signatures during the pathogenesis of MetS which could be exploited further for diagnostic or therapeutic purposes.

1.3.7 Dissemination, Exploitation and IPR (WP8)

This WP was dedicated to set up internal and external communication tools for the partners of RESOLVE as well as the outside community.

In the first period of RESOLVE, a project website has been developed for external communication to the general public (www.resolve-diabetes.org). The website was updated and improved on a regular basis. A secure portal was also implemented on the website, only accessible for members of the RESOLVE team, useful for the internal communication within RESOLVE. This collaborative website (http://cbio.bmt.tue.nl/resolve) provided a secure and private repository to share confidential documents and promote internal communication between partners which was of special importance in view of the fact the computational models of Partner 2 (TU/e) 'needed to be fed' with data from all RESOLVE partners.

The RESOLVE strategy was to extend excellence and disseminate the knowledge throughout both the scientific community and the general public at large. The RESOLVE consortium is strongly motivated and highly committed to present the outcomes of the project in high quality conferences and in publishing their work related to the project in top tier journals. The RESOLVE communication strategy also aimed at providing an interface for the various public domains to be kept informed about the aims, development and impacts of RESOLVE research activities.
The dissemination plan targeted the scientific community, industry, the general public, policy makers and contributes to develop citizen awareness.
To disseminate the results, the RESOLVE consortium organised training courses and workshops and participated to conferences. Disseminations activities are listed in the 'use and dissemination of foreground' section.

Following, the outcomes of the project have been largely disseminated through the world-wide web:

- RESOLVE website (www.resolve-diabetes.org)
- RESOLVE collaborative portal (http://cbio.bmt.tue.nl/resolve/)
- Facebook page of RESOLVE (https://www.facebook.com/resolvediabetes)
- YouTube animation explaining ADAPT (http://www.youtube.com/watch?v=x54ysJDS7i8)
- YouTube animation explaining modeling of the MetS (https://www.youtube.com/watch?v=aC9F8qqVMO4)
- Publication of Leaflet on website
- The RESOLVE consortium has published >75 peer reviewed papers including many in prestigious journals such as Nature Medicine, Cell Metabolism, Nature Communications.
- The partners have disseminated the progress of RESOLVE in numerous high profile scientific conferences including Keystone meetings, Gordon Conferences and European Atherosclerotic Society meetings
- The recent Resolve publication in PLos Computational Biology, (https://www.ncbi.nlm.nih.gov/pubmed/29879115), was welcomed by a Press Release which can be

found on EurekAlert (https://www.eurekalert.org/pub_releases/2018-06/p-nct053118.php). This press release also led to an invitation by Health Europe to write an article in their quarterly journal (https://www.healtheuropa.eu/category/publications/health-europa-quarterly/) on MetS.

The rights of intellectual property generated in the project were regulated by the Consortium Agreement (CA). Equally knowledge generated in the project was handled according to the CA. Industrial property monitoring and patent survey is an indispensable prerequisite to ensure that the research is driven in the right direction. An Intellectual Property Committee (IPC) was implemented at the beginning of the project.

1.4 Potential impact, main dissemination activities and exploitation of results

1.4.1 Potential impact

Metabolic syndrome (MetS) and its related cardiometabolic conditions are an epidemic threat to public health. Worldwide, more than one in three adults is now overweight or obese, and the prevalence of overweight and obesity continues to rise in adults and children of both developed and developing countries. Metabolic syndrome has a large impact on health and well-being. Its medical complications include, but are not limited to, hypertension, dyslipidemia, type 2 diabetes mellitus, atherosclerosis, non-alcoholic fatty liver disease (NAFLD), obstructive sleep apnea, osteoarthritis, and some forms of cancer. In addition, obese people commonly face social stigma and experience psychological stress. Clearly a better understanding of underlying mechanism to cause development of MetS related comorbidities can have a major impact on health and wellbeing of humans.

Impact of model development

A scientific and clinical challenge to adequately fight MetS is the identification of keys to the metabolic networks controlling its pathogenesis. Mathematical models describing parts of the pathways exist but a comprehensive model integrating lipid, lipoprotein and glucose metabolism is lacking. Construction of detailed kinetic models requires knowledge of kinetic parameters of the enzymes and transporters that constitute the metabolic networks and their multilevel regulation. **Measuring all the relevant kinetic parameters (under *in vivo* conditions) is a task that cannot (for sure) be achieved in the coming decades**. **To take this hurdle, RESOLVE has therefore used a novel approach.** First an unique 'core' model to describe the main lipid and carbohydrate fluxes has been developed. This model called MINGLeD (Model INtegrating Glucose and Lipid Dynamics) describes the relevant biological processes, using coupled, nonlinear differential equations. MINGLeD describes pools and fluxes of glucose and various fatty acid, cholesterol and bile acids species in the circulation, the liver, intestine and periphery. To compensate for the lack of sufficient kinetic parameter values for all the individual steps in the computational model sophisticated parameter estimation procedures were developed. In short, mathematical modeling is first used to quantitatively integrate sets of time-dependent metabolite and flux data that reflect different physiological conditions. Multiple sets of different parameter values are identified that all can describe the data. In a next step, the trajectories of parameter adaptations required to accurately describe the difference between the experimental conditions are calculated. To accomplish this, a

large-scale parameter estimation protocol is applied to capture multiple parameter sets which accurately describe the different experimental data. A number of steady-state simulations is performed with randomly sampled parameter sets to identify parameters and therefore parts of the network that are important to describe the experimental data. Then a collection of the most likely parameter sets is optimized by applying a weighted non-linear least squares algorithm. Finally, a limited number of acceptable parameter sets is obtained that describe the experimental data. When this is accomplished, these parameter sets are scrutinized for predicting physiologically acceptable concentrations/fluxes for non-measured quantities. This approach was called ADAPT (Analysis of Dynamic Adaptations in Parameter Trajectories) and offers a novel and powerful approach for data-driven dynamic modelling in systems biology. In addition to modelling longitudinal data of disease progression and treatment, ADAPT can also be applied to kinetic models of metabolism and signalling pathways. **A Matlab package with Graphical User Interface was developed for the ADAPT method. The software is open source and is publicly available for download from GitHub.**

Application and impact in experimental models

Mice

To calibrate and validate MINGLeD, experiments in a mouse model had to be carried out. It should be noted that organ-specific lipid uptake and potentially harmful interventions cannot be performed in humans and therefore need to be performed in preclinical models such as the mouse. It may also be noted that some flux data sets can only be obtained after sacrifice. Thus, the RESOLVE partners used a mouse model expressing the dysfunctional apolipoprotein E3 Leiden (E3L*C) as well as the human cholesterylester transfer protein (CETP). Under conditions of a high-fat diet, these E3L*C mice exhibit an altered lipoprotein profile with increased levels of triglyceride-rich lipoproteins (TRL) and decreased levels of high-density lipoprotein cholesterol (HDL-C), a dyslipidemic phenotype characteristic of humans with MetS. In order to define the optimal dietary regimens, various conditions to induce the major hallmarks of the MetS in E3L*C mice were investigated. Based on the results obtained in the laboratories from the collaborating partners, the RESOLVE consortium agreed on using a 0.25% cholesterol-containing high fat diet. Significantly increased body weights were observed in the MetS group already 2 weeks after starting this diet, which became more pronounced after 4 weeks and even more after 8 weeks. Obesity is central for the development of impaired glucose tolerance, which is another major hallmark of the MetS in humans. Increased plasma glucose levels after an oral glucose tolerance test were observed in E3L*C mice only after feeding the cholesterol-containing high-fat MetS diet, **indicating the suitability of this mouse model to study initiation and progression of MetS**. Simulation of MINGLeD with ADAPT and using data from experiments in which mice were fed the 0.25% cholesterol-containing high fat diet, allowed for accurate prediction of gradual, long-term development of the disease. We found that our modelling approach correctly predicted progression of MetS in the mice, as well as development of comorbidities, such as fatty liver disease. Unexpectedly, the model also mimicked the heterogeneity in disease development characteristic for humans. ADAPT analysis correctly predicted underlying metabolic differences that could explain this heterogeneity. Importantly, the predictions were validated in additional experiments. The eventually mathematical model is an important step in understanding the development of MetS, offering new opportunities to identify strategies to prevent the disease and its comorbidities.

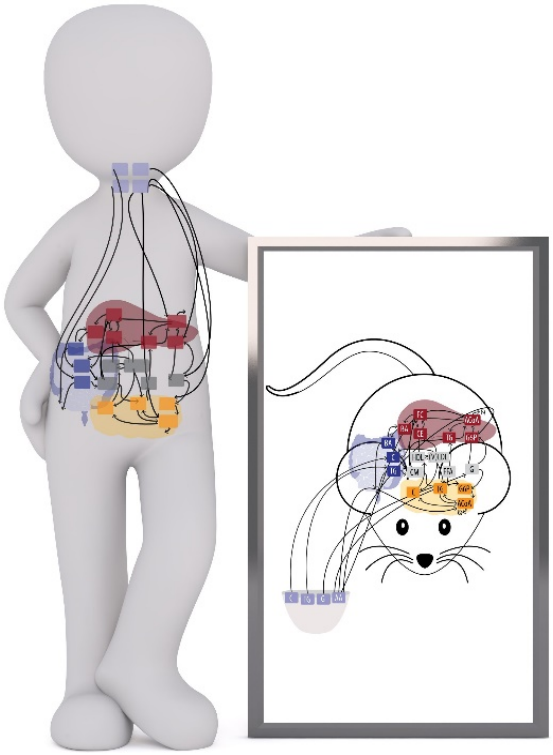

Figure 1 RESOLVE has made use of a genetically modified animal model of which its metabolic system resembles that of the human with MetS. A computational model (called MINGLeD) has been created that describes the major pathways in carbohydrate, fat and cholesterol metabolism. This model is also valid for the human metabolic system. The human version is obtained by changing model parameters based on clinical studies. Through this concept observations from mice can be translated to humans, and vice versa. The computational model is graphically depicted in the block diagrams. Arrows indicate transfer and exchange of different metabolites (denoted by the abbreviations in the boxes) between different tissues and organs.

In order to establish a reproducible method that is suitable to analyse lipid and lipoprotein fluxes under conditions of an altered glucose homeostasis, several intervention strategies disturbing adequate insulin signaling were established, such as administration of the beta cell toxin alloxan. As it has been shown in humans and rodent models that the activation status of brown adipose tissue is an important determinant of glucose homeostasis, the blockade of insulin signaling was tested by determining glucose and lipid disposal rates under conditions of inactive and active brown adipose tissue. Under these conditions, the disposal of radiolabelled glucose, lipids and lipoproteins was followed in E3L*C mice with MetS pre-treated with alloxan (to blunt insulin signaling) in combination with or without an oral glucose gavage (to increase plasma glucose levels). A robust and significant increase of plasma glucose levels was detected after alloxan treatment, whereas glucose gavage induced only a slight increase in plasma glucose levels. Triglyceride rich lipoprotein (TRL) disposal rates into metabolically active organs such as brown and white adipose tissue was impaired after treatment with alloxan leading to the accumulation of pro-atherogenic TRL in the plasma compartment. **These data provided evidence that insulin signaling rather than increased glucose levels is the main determinant of TRL disposal.**

Humans

Lipids

Studies in overweight individuals were performed to (1) clarify the dynamics of postprandial lipid metabolism of TRL (2) to quantitate the contribution of different sources of FAs in the liver to production of TRL, and (3) to define the mechanisms for impaired lipid clearance. The main achievements were to develop an integrated model for postprandial metabolism of apoB100 and apoB48-containing lipoproteins, and establishment of novel methods for enrichment analysis of low abundant apoB48 from chylomicrons and chylomicron subfractions. **These studies have demonstrated (1) the importance of hepatic *de novo* lipogenesis for hepatic biosynthesis of triglyceride-rich lipoproteins, (2) clarified the interplay between intestinal and hepatic lipoprotein secretion, and (3) clarified the mechanisms for impaired metabolism of triglyceride-rich lipoproteins in patients with ectopic fat accumulation in the liver.**

Bile acids

Since literature reports from mouse studies and bariatric surgery patients suggest an important role of bile acids (BA) in glycemic and lipid control as well we decided to extend MINGLeD, with a bile acids module. This module describes the dynamics of different BA species (primary and secondary, conjugated and unconjugated) as function of time and location in the intestine (duodenum, jejunum, ileum and colon). Since the mathematical framework of MINGLeD is based on ordinary differential equations (ODE's) also the gut module was modelled with ODE's. The BA response dynamics in different compartments are described and predicted, including liver, gallbladder (GB), ileum, proximal colon and plasma. Through the modelling we obtained a fascinating novel insight into BA kinetics. The initial rise (within 30-60 minutes) in plasma BA's after a meal is caused by propulsion of BA's residing in the gastrointestinal (GI) tract from prior meals, which precedes gallbladder emptying induced by the new meal. In healthy subjects BA's released from the gallbladder are responsible for the peak in plasma BA's observed around 90 minutes. The shoulder in the response results from BA recycling through the enterohepatic circulation. Data analysis from patients in which the gallbladder has been removed confirmed these results. It should be noted that BA's in plasma, which can also be measured, are only a minor fraction of the total BA's in other compartments.

Impact of dietary composition

Mice

In comparison to glycerolipids, sphingolipids (SL) show vast structural diversity, which arises by the combinatorial composition of different sphingoid base backbones in conjunction with different N-acyl chains and headgroups. 1-deoxy-sphinglipids (1-deoxySL) are a particularly interesting subclass of SL species which are formed in a side reaction during SL de-novo synthesis when the key regulatory enzyme serine-palmitoyltransferase uses alanine instead of its canonical substrate serine. As 1-deoxySL are toxic to beta cells and their plasma levels associated with non-alcoholic fatty liver disease (NAFLD), we investigated whether they are involved in impaired glucose homeostasis and hypertriglyceridemia. This hypothesis was tested in male and female ApoE3L.hCEPT transgenic mice by perturbing sphingolipid metabolism via a dietary supplementation with L-alanine and L-serine. L-serine supplementation efficiently suppressed 1-deoxySLs formation whereas L-alanine supplementation was associated with an early increase in 1-

deoxySL but not with any elevation in total 1-deoxySL levels compared to mice on HFD only. However, besides elevated plasma and liver TGs, the mice presented neither impaired glucose homeostasis nor any pathological changes in beta cell morphology.

Despite the differences in the sphingoid base profile, we could not detect any significant difference in the metabolic profile between the HFD+ala and HFD+ser group. As the mice neither developed impaired glucose homeostasis nor showed obvious changes in beta cell histology, the expected primary endpoint for comparison was not met by the model. However, we observed a significant lowering of plasma and liver TGs in the amino acid fed animals compared to the HFD only group. This lipid lowering effect appeared to be independent of SL or 1-deoxySL formation as SL were not different between the groups and 1-deoxySL formation only suppressed in the HFD+ser group. The observed effect might, however, be related to recently published data that showed that supplementation with serine but also with glycine positively affects the C1 carbon, S-adenosyl methionine and homocysteine metabolism.

Humans

To assess the effects of dietary fructose on liver fat content and other cardiometabolic risk factors, abdominally obese men (n=71) were served 75 g fructose/day with their habitual diet over 12 weeks. We analyzed changes in body composition, dietary intake, an extensive panel of cardiometabolic risk markers, hepatic de novo lipogenesis (DNL), liver fat content, and postprandial lipid responses after a standardized oral fat tolerance test (OFTT). Results showed that fructose consumption had modest adverse effects on cardiometabolic risk factors. However, fructose consumption significantly increased liver fat content and hepatic DNL and decreased β-hydroxybutyrate (a measure of β-oxidation). Our study also indicates that there are remarkable individual differences in susceptibility to visceral adiposity/liver fat after real world daily consumption of fructose sweetened beverages over 12 weeks. **Results from another short-term intervention with an isocaloric low-carbohydrate diet in obese subjects with NAFLD demonstrated rapid and dramatic reductions of liver fat and other cardiometabolic risk factors paralleled by marked decreases in hepatic DNL, and large increases in serum β-hydroxybutyrate concentrations, reflecting increased mitochondrial β-oxidation**.

Target identification

Brown adipose tissue (BAT)

The results of experiments encompassing steady state metabolite concentrations in different compartments as well as flux data generated in E3L*C mice show the relevance of energy combustion by brown adipose tissue for energy balance and thus the development of obesity and MetS. To refine the basal computational model (MINGLeD), an additional module mimicking increased energy expenditure by peripheral tissues was implemented. Peripheral energy expenditure was increased within the model by an activation factor that represents the respiration of acetyl-CoA. An incremental increase of this activation factor predicted major changes in peripheral, hepatic and intestinal metabolites including glucose, triglycerides and cholesterol. To validate the model, metabolites and metabolic fluxes were determined in mice that were subjected to cold exposure as a stimulus to increase peripheral energy expenditure. **In line with the assumption that cold-**

activated BAT uses high amounts of triglycerides to generate heat and with the model predictions, decreased triglyceride stores were detected in BAT isolated from E3L mice with and without MetS. Interestingly, the model predicted also lower plasma total and HDL cholesterol as well as a concomitant higher cholesterol uptake into BAT and the liver, which was accompanied by both accelerated lipoprotein processing and increased cholesterol transport from peripheral tissues toward the liver. These predictions were confirmed by metabolic tracer studies using radiolabelled HDL and by determining the reverse cholesterol flux to the liver. These data suggested increased metabolization of cholesterol to bile acids in the liver, which could be confirmed by investigating bile acid metabolism.

Bile acids

In both mouse and human studies bile acids were identified as important regulators of lipid and carbohydrate metabolism. To validate the role of bile acids in the E3L*C mouse MetS model series of experiments were carried out with an FXR agonist that decreases bile acid synthesis while increasing the hydrophilicity of the bile acid pool. The major impact of bile acid metabolism in MetS development was impressively validated. Two weeks exposure to the FXR agonist decreased plasma cholesterol and triglyceride in the E3L*C mice mice on high fat diet by ~75%, the animals lost weight as well suggesting **that bile acid metabolism maybe a target to treat metabolic syndrome in humans**

FOXO1

The critical position of FOXO1 at a node in the insulin signaling pathway linking glucose and lipid homeostasis prompted to investigate its role in the pathogenesis of MetS via a conditional gene silencing approach. For this purpose, adeno-associated viruses (AAVs) expressing short hairpin RNAs (shRNAs) for FOXO1 or control (scrambled shRNAs) were designed and produced. Using these AAVs, we conducted a large scale genetic intervention experiment in E3L*C mice that received the 0.25% cholesterol HFD for 12 weeks. These mice were fully characterized for various physiological and histopathological parameters and a full transcriptomic analysis was performed using next generation RNA sequencing (Illumina). It was found that silencing of FOXO1 in E3L did not affect body weight or plasma lipoprotein levels but it was associated with reduced glucose levels and statistically significant improvements of glucose and insulin tolerance tests **suggesting that genetic ablation of FOXO1 had a positive impact on glucose homeostasis.** These findings could be explained, at least in part, by the liver transcriptomics data that were obtained from the same mice. The clinical and the transcriptomics data that were generated will be valuable for a better understanding of the molecular mechanisms that are implicated in the pathogenesis of MetS and for the design of novel diagnostic tools and therapeutic strategies.

Non-alcoholic steatohepatitis

Investigation of the liver transcriptome in a human cohort of NASH patients revealed additional interesting novel candidate targets. The transition from histologically proven fatty liver to NASH and fibrosis was characterized by gene expression patterns that successively reflected altered functions in metabolism, inflammation, and epithelial-mesenchymal transition. A meta-analysis combining human transcriptomic datasets with murine models of NASH and fibrosis defined a molecular signature characterizing NASH and fibrosis and evidencing abnormal inflammation and extracellular matrix

(ECM) homeostasis. **Dermatopontin expression was found increased in fibrosis, and reversal of fibrosis after gastric bypass correlated with decreased dermatopontin expression. Functional studies in mice identified an active role for dermatopontin in collagen deposition and fibrosis.**

Studies also revealed a role of ApoF in the control of lipid metabolism: ApoF knockdown reduces fatty acid uptake in human hepatocyte IHH cells. Lipidomic flux measurements were performed in IHH cells after transfection of a specific ApoF gene siRNA in mice and in the plasma of a subgroup of patients for whom liver microarray data were available. **The results indicate that ApoF may be involved in hepatic phosphatidylcholine synthesis or secretion.**

Bariatric surgery

Detailed metabolic fluxes and tissue transcriptomics in obese humans were performed before and after bariatric surgery-induced weight loss . Results showed that one year after bariatric surgery, insulin sensitivity in multiple tissues was restored in the majority of patients despite a mean remaining BMI within the obese range. The latter suggests additional weight loss-independent metabolic effects of bariatric surgery such as an increase in GLP-1 secretion. Liver fat was dramatically reduced after weight loss and might account for some of the beneficial metabolic effects. The metabolic improvements were not gender-specific except for a higher insulin sensitizing effect of weight loss on adipose tissue insulin sensitivity in women. In line with the mouse data also in the various human intervention studies performed in RESOLVE remarkably little connection was observed between lipid and glucose metabolism. Under physiological conditions only insulin itself seems to be very important.
The first analysis of the RNA sequencing data suggested major effects of weight loss on inflammatory pathways in skeletal muscle and adipose tissue, paving the way for interventions that specifically target the low inflammatory state of obesity with the aim to restore insulin sensitivity. **Overall, this unique and extensively phenotyped human cohort increases our understanding of the molecular networks of the metabolic syndrome and opens up new avenues in the development of diabetes and dyslipidemia treatment.**

The gut module of MINGLeD was used to analyse postprandial GLP-1 excursions to investigate the underlying mechanism of the strong GLP-1 response after bariatric surgery. Analysis of GLP-1 fluxes showed that peak performance can only be caused by the very rapid delivery of glucose to the ileum were most L-cells are located. The model enables accurate predictions of the influence of modifications of the RYGB procedure on postprandial insulin and GLP-1 excursions. To our knowledge, this model is the first model capable of simulating the characteristic postprandial GLP-1 peak observed after RYGB. The model confirms that GLP-1 contributes to the enhanced insulin secretion after bariatric surgery. Analysis of the GLP-1 fluxes show that this peak is practically solely caused by an increased delivery of glucose to the ileum. This supports the hindgut theory that the accelerated delivery of nutrients to the distal intestine augments an insulinotropic signal. Model simulations indicate that the total length of the RY limbs has a major influence on post RYGB GLP-1 secretion and hence glucose homeostasis. **Surgeons could use the model to estimate optimal distribution of the length of the intestinal limbs that are constructed.**

Potential socioeconomical impact.

Metabolic Syndrome is defined as the clustering of abdominal obesity, elevated blood pressure, elevated fasting glucose, high triglycerides (TG), and low HDL-C and is widely used as a simple clinical definition to identify, often obese, individuals at increased morbidity risk. It is expected that by 2030 close to 200 million individuals (33% of the total population) in the 27 countries of the EU will be obese. Many of them will have one or more of the following co-morbidities: cardiovascular disease, Type2 diabetes mellitus, fatty liver disease, chronic kidney disease, alzheimer, as well as various frequent cancers (notably of the breast, prostate or colon). Without successful interventions, costs will - in 2030 - increase to more than 100 billion Euros per year. Although widely used, the metabolic syndrome is an insensitive tool to identify obese individuals at the highest risk of morbidity. Also it is not suitable to identify the co-morbidity that a particular obese individual is most likely to develop, whether it is cancer or cardiovascular disease. Thus, at present we cannot predict who will remain healthy, and who will develop one or multiple co-morbidities, and if so, which ones. Treating all patients with metabolic syndrome is costly, and probably unnecessary. The challenge we are facing is to improve individual risk classification of obese individuals to improve personalized prevention and treatment. The RESOLVE consortium has addressed this issue by developing a dynamic system biology approach that enables predicting the consequences of metabolic derailment in a personalized way. The methodology has been successfully applied in a mouse model of the metabolic syndrome and has been successful in analysing different aspects of MetS in humans but the full integration is still underway. When accomplished it will generate detailed insight in the metabolic network dysregulation in Mets patients and will result in better, biology-based prediction of chronic disease risk, personalized prevention, and novel therapeutic strategies to rebalance the metabolic networks.

1.4.2 Main dissemination activities

Website. Implementation of a public website has ensured the dissemination of selected information to a wide audience. It has developed harmonised and shared communication tools (logo, electronic presentation) to ensure project promotion.

Portal. RESOLVE has implement a general collaborative portal. This collaborative portal has provided a secure and private repository to share confidential documents and data to promote internal communication between partners.

Issue of leaflets and newsletters presenting the project. RESOLVE has interviewed his researches. Summarized have been disseminated through FaceBook.

Webshops. RESOLVE has organized workshops for all participants to get familiar with computational modeling in TU/e. In addition, RESOLVE's researchers were offered training courses for data management and analysis using the geneXplain platform.

International Symposium. On May 5th 2018 all RESOLVE participants joined in a dedicated final international symposium in Lisbon preceding the yearly European Atherosclerosis Society meeting. This meeting was a great success.

Promotion of scientific results:

The RESOLVE consortium has published its results in more than 75 peer reviewed journals including prestigious journals such as Nature, Cell Metabolism, Nature Communication with accompanying editorial comments.

1.4.3 Exploitation of results

Resolve has not generated data that needed protection. The mathematical models that were generated will be made available to the scientific community for further development. The exploitation of the work performed lies in future work in this field.

1.5 Address of the project public website and relevant contact details

http://www.resolve-diabetes.org/