Systems-based metabolomics of type 2 diabetes mellitus subtypes

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Chapter 1

General introduction and scope

Type 2 Diabetes Mellitus: the need for better diagnosis and treatment

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by hyperglycemia with disturbances of carbohydrate, lipid and protein metabolism, involving decreased production of insulin and/or reduced sensitivity of the body tissues to insulin [1-4]. According to World Health Organization (WHO), T2DM is resulted from a defect in both insulin secretion and in insulin sensitivity, as well as β-cell dysfunction, β-cell loss and its progression [5]. T2DM is now found in almost every population and epidemiological evidence suggests that without effective prevention and intervention programmes, its prevalence will continue to increase globally [6]. Because of a lack of symptoms early on in the disease, a large proportion of these individuals remain undiagnosed; this proportion is estimated to be higher than 50% [7]. Moreover, it can lead to a number of serious medical complications (e.g. retinopathy, neuropathy, myocardial infarction, stroke), which are a major cause of morbidity, hospitalization and mortality in diabetic patients; and resulting in a financial burden for public health internationally [1, 5, 8]. Indeed, diabetes care already accounts for about 2–7% of the total national health care budgets of western European countries [9]. Thus, identification of early biomarkers for prediction and monitoring is needed for adequate screening diagnostics of T2DM [7].

T2DM and cardiovascular disease (CVD) are strongly associated with various common metabolic disturbances under the name of metabolic syndrome (MetS), including abdominal obesity, insulin resistance, dyslipidaemias, hypertension and a systemic proinflammatory state [10-12]. Several modifiable risk factors for T2DM have been identified such as obesity, physical inactivity, excessive calorie intake and above mentioned MetS related disorders [10, 11]. Evidence shows that both lifestyle regulation and early pharmacotherapy during pre-diabetes are effective in slowing down the onset and progression of T2DM [2, 4, 13]. However, due to its multi-factorial causes (Figure 1) resulting from the interaction among a genetic predisposition, psychological, behavioral and environmental risk factors, the control of T2DM represents a considerable therapeutic challenge [2, 3, 5, 14] . Although life-style interventions for T2DM including appropriate diet, weight regulation and physical activity have demonstrated their efficacy to reduce cardio-metabolic risks, these interventions are often disappointing on the longer term, resulting from the poor adherence and a lack of intensive health professional support.

As a result, it is likely that the effect of lifestyle changes will be less in a real-life setting than in the published trials [5, 6]. Pharmacological intervention for the prevention of diabetes is therefore often recommended as a secondary

intervention to follow or to be used in combination with lifestyle intervention. However, the discovery of safe and effective drugs for the long-term treatment T2DM remains the challenge in diabetes research, predominantly owing to the continuing lack of understanding of the complex molecular pathogenesis and genetic basis of this disease [14]. The current treatments mainly focusing on restoring normoglycaemia generally require the use of anti-diabetic drugs; and none of these are able to correct all the anomalies involved in the complex pathogenesis of T2DM. They often fail after a few years of evolution of the disease and even insulin rarely achieve durable glycaemic control and can expose the patient to side effects, particularly hypoglycaemia and weight gain [5, 15]. It seems that either the current approach to treat hyperglycaemia in patients with T2DM does not affect the cardiovascular risk (at least not over the time course of the studies) or that current therapies exert off-target effects that neutralize any potential benefit of lowering glucose and/or do some harm [3, 15].

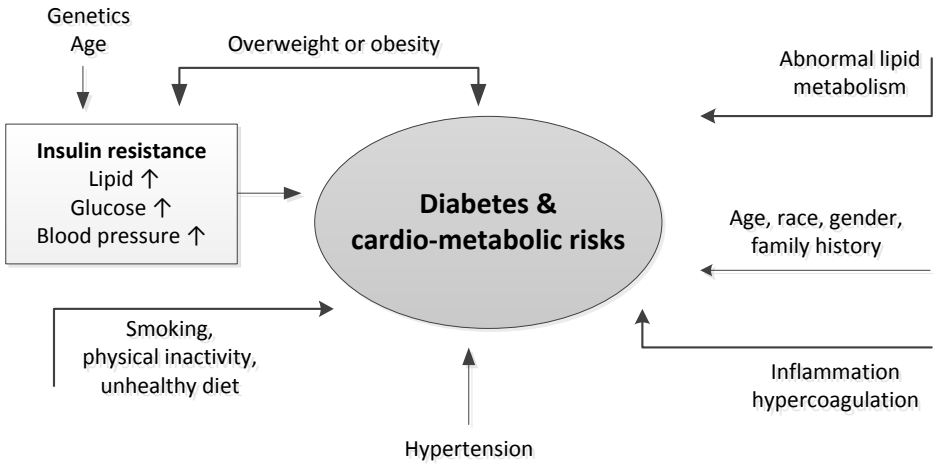

Figure 1. *A summary of multiple risk factors to cause T2DM*

Taken into account that the cardio-metabolic disorders in T2DM have diverse subtypes that involve changes in multiple molecular pathways, organs, tissue types and the central nervous system (CNS) [5, 14, 16], the strategy for new drug development for T2DM should pursue as many promising leads as possible to establish a broad range, namely a system based and personalized way, to control the factors beyond glycaemia management (e.g. hypertension, dyslipidaemia, insulin resistance, obesity) with different mechanisms of action and potential opportunities for effective combination therapies [14, 17, 18].

The discovery of novel biomarkers to dissect T2DM subtypes will help to improve its diagnosis as well will help to find new leads for actual disease modifying treatments. The attention should be drawn on the system biology based research into patient stratification with biomarkers which are disease-associated molecular changes in body tissues and fluids [19, 20]. If the correlation between diseases and changes in biomarkers could be established, the ability of health practitioners to diagnose T2DM and tailor treatments to individuals, so-called personalized healthcare solutions, would be radically improved [20].

System level based searching for diagnostic biomarkers

Systems biology and personalized medicine

A biological entity, such as the whole organism of a human being, consists of myriads of cells which in turn contain many genes, transcripts, proteins, and metabolites. These molecules participate in specific networks and systems of interactions, and it is the aberrations in network behaviour that causes disease [21]. The discipline that seeks to reconstruct how molecules interact with each other in networks is systems biology, which can be defined as 'studying biology as an integrated system of genetic, protein, metabolite, cellular, and pathway events that are in flux and interdependent' [21, 22]. Systems biology studies are typified by a shift from the more traditional reductionist approach towards more holistic approaches, with experimental strategies aimed at understanding interactions across multiple molecular entities [23].

Actually the emergence of systems-based thinking across different scientific domains has occurred in the last century, yet systems biology often stayed unnoticed by the mainstream[24]. Only recently it has gained considerable momentum in the life science and pharmaceutical research, particularly due to the fact that the target-based drug discovery strategy became unproductive and thus more interest grew for the need to better understand biology in a different perspective [21, 24, 25]. More and more scientists and healthcare practitioners realized that the classical reductionism based interventions on 'single compound focusing on a single target that links to a specific symptom' have limitations for the multifactorial abnormalities such as T2DM, obesity, metabolic syndrome, cancer and cardiovascular diseases [24-27]. Evidence [6, 28-32] has shown that because of the lack of better diagnostics and therapies, these abnormalities become prevalent globally and put great medical and economic burden for society. Personalized medicine, which is defined as customized medical care for each patient's unique condition, is regarded to make significant strides forward when a

systems approach is implemented to achieve the ultimate disease phenotyping and to develop novel therapeutics that address system-wide molecular perturbations caused by disease processes [33]. Personalized medicine, encompassing lifestyle, nutrition, psychology, environmental issues and medicine, is considered as the ultimate opportunity to improve healthcare; and it should depend on an understanding of systems biology and on systems-based developed interventions [33].

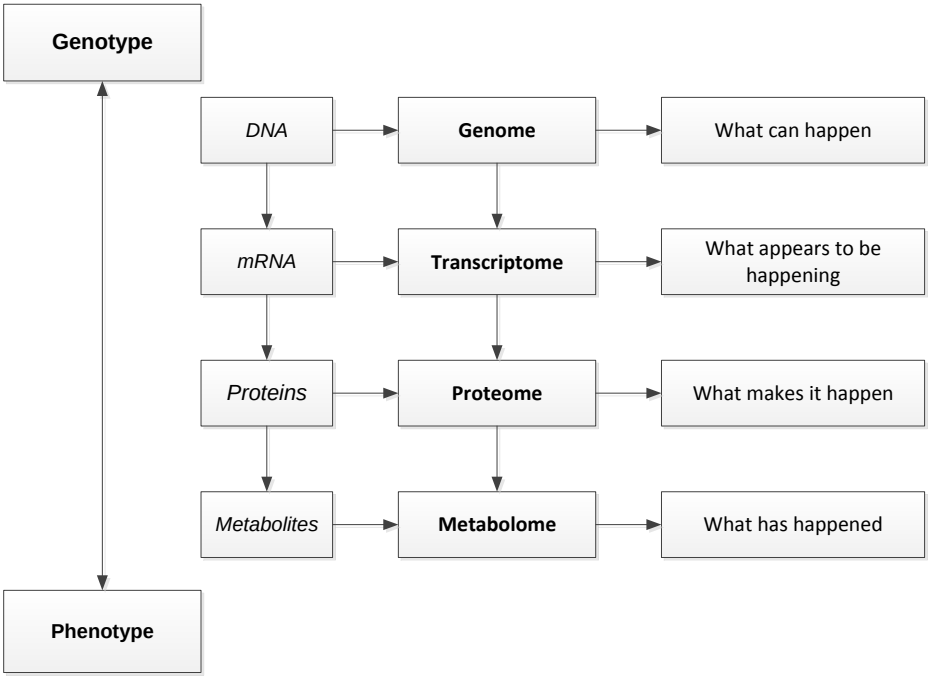

Figure 2. *The 'Omics' cascade and related research focuses.*

Novel diagnostic biomarkers as health indicators are urgently needed to move personalized medicine forward [33]. Moreover, stratifying patients on molecular biomarker profiles is a key step towards treatment response and non-response differentiation [33]. However, the current lack of knowledge of the complex underlying biochemistry in the system-based level often makes it difficult to develop or assess the value of potential biomarkers; as understanding biology requires knowledge of connectivity in systems and their self-organization and adaptation [24]. Several technologies developed in Life Sciences, particularly 'omics' techniques, may offer a possibility to improve the identification of such biomarkers. It will improve our understanding of disease pathology and will

advance translational medicine, combination therapies, integrative medicine, and personalized medicine [33].

-Omics, metabolomics and lipidomics

Driven by recent technological advances in biology, analytical chemistry and bioinformatics, measuring and analyzing dozens and even more compounds simultaneously has become feasible. This development culminated in the emergence of the 'omics' science, with the most notable variants (downstream of genomics) being transcriptomics, proteomics, and metabolomics, which comprehensively study the expression of many gene transcripts, proteins, and metabolites respectively (Figure 2) [21].

The 'omics' disciplines, widely applied in systems biology, allow researchers to achieve a 'holistic' view of the processes involved in the disease, and provide a complementary approach to the classical reductionist approach [21]. While genome approaches are powerful with regard to finding disease-associated patterns in the DNA, transcriptomics focuses on global analysis of gene expression and proteomics on protein synthesis and cell signaling by measuring the complete proteome [34, 35]. The metabolome refers to the complete set of metabolites present in cells, body fluids and tissues. It is the endpoint of the "omics cascade" and thus the closest to phenotype [34, 36].

Metabolomics is defined as 'the comprehensive quantitative and qualitative analysis of all small molecules in a system (metabolome)'. It incorporates advanced analytical technologies to measure a molecular phenotype system readout and provides the ideal technology platform for the discovery of biomarker patterns associated with healthy and diseased states, for use in personalized health monitoring programs, and for the design of individualized interventions [33].

Metabolomics dovetails with the philosophy of systems biology, because it provides a 'top-down', integrated view of biochemistry in complex organisms, as opposed to the traditional 'bottom-up' approach that investigates the network of interactions between genes, proteins and metabolites in individual cell types. A problem with systems biology is that each level of biological organization and control— genomics, gene expression, protein expression and metabolism — operates on a markedly different timescale from the others, making it difficult to find causal linkages [37]. Moreover, environmental and lifestyle influences on gene expression also make it hard to interpret genomic data, for example to predict an individual's susceptibility to diseases. Metabolomics cuts through these problems by monitoring the global outcome of all the influencing factors, without

making assumptions about the effect of any single contribution to that outcome and thus the individual contributions can be teased out [37]. For this reason, metabolomics represents a rather exhaustive metabolic phenotyping technology, which will help in understanding metabolic diseases.

A metabolomics study workflow is shown in Figure 3. For a good and valid metabolomics study, all steps, starting from the definition of the biological question until the experimental design up to the biostatistics, should be optimized; and finally the metabolites relevant to a specific phenotypic characteristic can be identified [34].

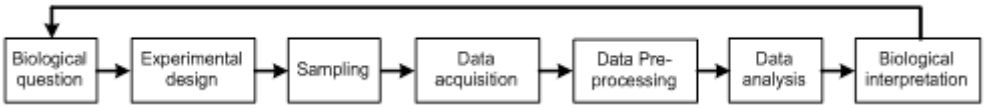

Figure 3. *A metabolomics study workflow.*

Metabolomics is mainly conducted on biofluids such as urine or plasma, which are easily obtainable in mammalian studies. Because biofluids fulfill diverse biological purposes, their metabolic composition varies with their role and the functional integrity of the organ systems that are communicating with them, and ultimately with the physiological status of the whole organism [7]. Metabolomics studies of biofluids such as urine and plasma obtained from clinical trials have been applied successfully to investigate numerous diseases and metabolic processes [7, 38-49]. Furthermore, the approach of metabolomics has proven sensitive enough for the understanding of gene function in model organisms, including yeast, plants, and mice/rats [4, 7, 50-52]. This has led to the development of a number of screening assays in recent years to be used in drug development related to human diseases such as MetS, T2DM and cardiovascular disease, as well as its potential use in toxicology as part of the drug safety assessment process [7, 52].

Lipidomics is a lipid-targeted metabolomics approach aiming at comprehensive analysis of the molecular species of lipids in biological samples. Recently, lipidomics has captured increased attention due to the well-recognized roles of lipids in numerous human diseases to which lipid-associated disorders contribute, such as diabetes, obesity, T2DM, atherosclerosis and MetS [53, 54].

Indeed, historically T2DM was considered to revolve around a glucose-insulin axis while our current understanding of the pathogenesis of T2DM has shifted to the awareness that obesity, or more accurately, the products of excess adipose tissue, precede the perturbations of glucose metabolism [55]. It is now apparent that elevation of plasma free fatty acids (FFAs) plays a pivotal role in the development of T2DM by causing insulin resistance [55]. T2DM develops because pancreatic β-

cells eventually fail to produce enough insulin to compensate for insulin resistance [55]. T2DM is tightly associated with dyslipidemia, a cluster of interrelated plasma lipid and lipoprotein abnormalities, including reduced HDL cholesterol, a predominance of small dense LDL particles, and elevated triglycerides [56]. There is evidence that each of these dyslipidemic features is associated with increased risk of cardiovascular disease, the leading cause of death in patients with T2DM and there is no single drug can successfully regulate the dyslipidemia without long-term intolerance or side-effects [56, 57]. Therefore, measuring all or subsets of lipids in order to investigating lipid biochemistry using a lipidomics approach will not only provide a thorough perspective to study intervention induced lipid changes and metabolism in the complex biological system in health and disease, but will also assist in identifying potential biomarkers for establishing new preventive or therapeutic approaches for T2DM and its related cardio-metabolic disorders, especially dyslipidemia [54].

Metabolomics and lipidomics in diabetes and metabolic syndrome research

Metabolomics studies on both animal models and human with T2DM or MetS have been reported and yielded the “fingerprints” of the biochemical changes in plasma (blood) and urine that accompany diseased states.

Several animal models have been developed and used extensively in studies of the pathophysiology of T2DM and its related metabolic abnormalities. The Zucker obese rat and Goto-kajizaki (GK) rat represent two pathogenic process of T2DM respectively, insulin resistance and hyperglycemia [4]. The urine metabolomics of both models rats demonstrates metabolic similarities between the two stages of T2DM, including reduced tricarboxylic acid (TCA) cycle and increased ketone bodies production. In addition, compared with Zucker obese rats, the GK rats have enhanced concentration of energy metabolites, which indicates energy metabolic changes produced in hyperglycemic stage more than in insulin resistant stage [4]. The db/db mouse model has autosomal recessive defects in the leptin receptor gene and produces clinical signs of leptin resistance, hyperphagia, obesity, and subsequent insulin resistance [58]. Profound changes in nucleotide metabolism including that of N - methylnicotinamide and N-methyl-2-pyridone-5-carboxamide, branched chain amino acids (BCAAs), nicotinamide metabolites and pantothenic acids were observed in such mouse model as compared with the controls [52, 58]. Kleemann et al. [59]used the ApoE*3 Leiden mouse model that expresses a mutation of the human ApoE*3 gene resulting in a slightly attenuated clearance of apoB containing particles via the LDL pathway, to elucidate the dynamics and

tissue-specificity of metabolic and inflammatory processes, the analysis of gene expression and metabolite levels in liver, white adipose tissue and muscle was performed. The results show that high fat diet induced insulin resistance is a time- and tissue-dependent process that starts in liver and proceeds in white adipose tissue. Insulin resistance development is paralleled by tissue-specific gene expression changes, metabolic adjustments, changes in lipid composition, and inflammatory responses in liver and WAT involving p65-NFkB and SOCS3. The biochemical pathways affected are numerous and different for the various tissues, indicating that the design of effective treatment regimens should be at an integral level rather than directed at a single target [59].

More and more metabolomics studies on human with T2DM, pre-diabetes and MetS have been published. Mass spectrometry techniques have been widely applied to obtain metabolite profiles of diabetic patients. The study designs can be generally summarized into three categories: 1) metabolomics differences between pre-/diseased group versus healthy controls; 2) the time-dependent metabolic profile before and after challenge test (e.g. oral glucose tolerance, lipid challenge) or drug intervention; and 3) Epidemiological –omics study. The between-group metabolomics differences are summarized in table 1. Highlighted metabolism biomarkers and pathways showed the related concentration differences between pre-diabetes/T2DM and the healthy controls [47, 49, 60-63]. The most applied challenge tests and interventions to study human system response related to metabolic profiling include oral glucose tolerance test, lipid challenge, dietary and drug interventions [42, 44, 48, 64-68]. The time dependent metabolite responses during the challenge or intervention period can be obviously detected, manifesting in multiple metabolic intermediates changes in different pathways. To name a few, the data reported the changes in fatty acids, animo acids, acylcarnitine levels, glutathione synthesis pathway, bile acids, urea cycle intermediates, and purine degradation products, etc. Finally, the European Union-funded projects, such as Molecular Phenotyping to Accelerate Genomic Epidemiology (MolPAGE) involving 17 partners from universities, pharmaceutical companies, and biotechnology companies. The MoLPAGE, formed in 2004, aims to tackle diabetes and vascular disease, at the level of genes, proteins, metabolites, and other biomarkers [7]. The ultimate goal of the MolPAGE project is to identify early diagnostic biomarkers that are able to highlight individuals likely to suffer from diabetes and vascular disease before they show any of the symptoms, biochemical abnormalities, or other features typically used in the diagnosis of these conditions, thus allowing more effective prevention programs and better treatment of the disease [7]. The results and data generated from this project are expected in the near future.

Table 1. A small summary of reported biomarkers/pathways of metabolomics studies of T2DM versus healthy controls

Published year	Platforms, biological samples	Comparison Groups	Identified Biomarkers/ pathways/major disturbed metabolite groups
Wang et al. 2005	LC-MS, plasma phospholipid	T2DM vs. Healthy controls	PE_C16:0/C22: 6, PE_C18: 0/C20: 4, Lyso-PC_C16:0, Lyso-PC_C18:0
Yi et al. 2006	GC-MS, plasma fatty acids	T2DM vs. Healthy controls	palmitic acid, stearic acid, oleic acid
Yuan et al. 2007	GC-MS, urine organic acids	T2DM vs. Healthy controls	Meleic acid,dimethyl ester, Oxy acetic acid, 4-Aminobenzoic acid, 2,5-bisoxy-benzeneacetic acid
Zhang et al. 2009	NMR spectroscopy, serum metabolites	Pre-/T2DM vs. Healthy controls	Disturbance of metabolites in choline, glucose, lipid and amino acid metabolisms and TCA cycle
Suhre et al. 2010	Multiple MS platforms, plasma, serum metabolites	T2DM vs. Healthy controls	Perturbations of metabolic pathways linked to kidney dysfunction (3-indoxyl sulfate), lipid metabolism (glycerophospholipids, free fatty acids), and the gut microflora (bile acids).
Zhao et al. 2010	UPLC-QTOF-MS, plasma and urine metabolomics	Pre-diabetes vs. Healthy controls	Alterations in fatty acid-, tryptophan-, uric acid-, bile acid-, and lysophosphatidylcholine-metabolism, and the TCA cycle

Together, in a clinical context, metabolomics offers the advantage of using readily available biofluids, i.e., urine and plasma to get information of the time-dependent fluctuations of metabolites that occurs in response to disease, drug effects or other stimuli. This approach expands the number of metabolic markers available to the practitioner by an order of magnitude (otherwise not available through routine assays)[7, 37]. Metabolomics bears promise to enable characterization of early markers of disease and prognosis, as well as drug treatment efficacy and eventually personalized medicine [7].

Chinese Medicine

Although metabolomics-based systems analysis and phenotyping will be key, it still remains a challenge to stratify patients to provide a diagnosis considering the uniqueness and the interaction of person with his environment as a whole. Comparing the generated metabolite or metabolic differences between healthy and T2DM can help us know more about multiple metabolites related pathway changes, yet not enough to stratify patients. The 'challenge test' to check people's response to the stimuli is likely to provide early disease diagnosis [33], however, it often difficult to judge which challenge to apply and whether it is the right one. A complementary approach is to get diagnostic perspectives from other medicine systems which have already regarded the human being as a whole and provided personalized intervention to improve health. Indeed, system-level based diagnosis and personalized health has long been applied in Chinese Medicine (CM), in which personalized treatment have been the sole approach to medicine [33].

Introduction of Chinese Medicine

Chinese Medicine (CM) has developed its own health care concept with over 3000 years of continuous practice and refinement through observation, testing and critical thinking [38]. Literally it means an art of healing that brings harmony and balance for human health, with an essential goal to maintain healthy condition and reach longevity with optimal quality of life.

The concept of personalized health and system diagnostic principles has long been the basis of CM, in which the focus is the 'diseased person' instead of the 'person's disease' [69]; and the 'disease process' instead of the 'disease state'. CM provides emphasis on regulating the integrity of the human body and the interaction between human individuals and their environments, and it applies multiple natural therapeutic methods for disease management as well as health promotion [38]. The aim of CM is to restore the self-regulatory ability of the human system, instead of antagonizing specific pathogenetic targets [69]. Thus CM does not focus solely on the disease defined by pathological changes but the overall maladjustments of functional status called *'syndrome type'* [69]. All diagnostic and therapeutic methods in CM are based on the differentiation of CM syndrome types [38], which can be defined as a functional status which is caused by the reaction to or interaction with environmental changes and pathogenic factors and it manifests as a group of correlated symptoms and its essence is the imbalance of the human system resulting in the perturbation of the metabolic or biological network [70] . In other words, patients suffering from the same disease

can be categorized into different CM syndrome types (Figure 4) ; and the classification of CM syndrome types clusters certain symptoms together and provides an essential base for CM guided personalized treatment [38].

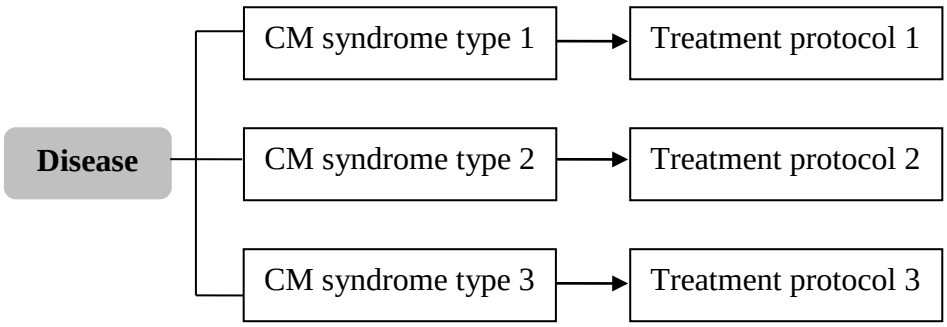

Figure 4. *Patients with same disease can be categorized into different CM syndrome types and given personalized treatment accordingly.*

Chinese Medicine diagnostic principles

Diagnosis in CM not based on objective instruments as in western medicine; instead it is achieved by using four diagnostic methods, 1) observation, 2) listening and smelling, 3) questioning and 4) pulse feeling, which are conceptual and rely entirely on clinical signs discerned by CM physicians (Figure 5) [71]. CM physicians attempt to establish the health status through qualitative symptoms collection based on one's appearance, behaviors, mental status, bio-rhythms, life style as well as his interaction and adaptation to both natural and social environment. In specific [18], 'observation' method focuses on the behavior, movement, figure, facial color, tongue, throat and finger veins of a patient; with 'listening and smelling' method, CM physicians will evaluate the speech of a patient (e.g. pitch, tone, tempo), the way a patient is coughing or breathing and smell his breath and secretion (e.g. sweat). 'Question' is one the most important diagnostic techniques and CM physician will carefully ask and analyze the complaints and symptoms of a patient. Normally the following information will be covered [71]: medical history, response to cold/heat weather or food, sweat, symptoms of head, chest and body, pain, diet and appetite, sense organs, sleep quality and life style; for females there will be questions related to menstrual cycle and pregnancy experiences. 'Pulse feeling' is an unique diagnostic method in CM, by feeling the rate, depth and tension of the pulse, CM physicians can speculate the health status of the whole system. CM physicians will then cluster the symptoms/signs together according

to CM diagnostic principles to get the CM syndrome type and provide personalized treatment.

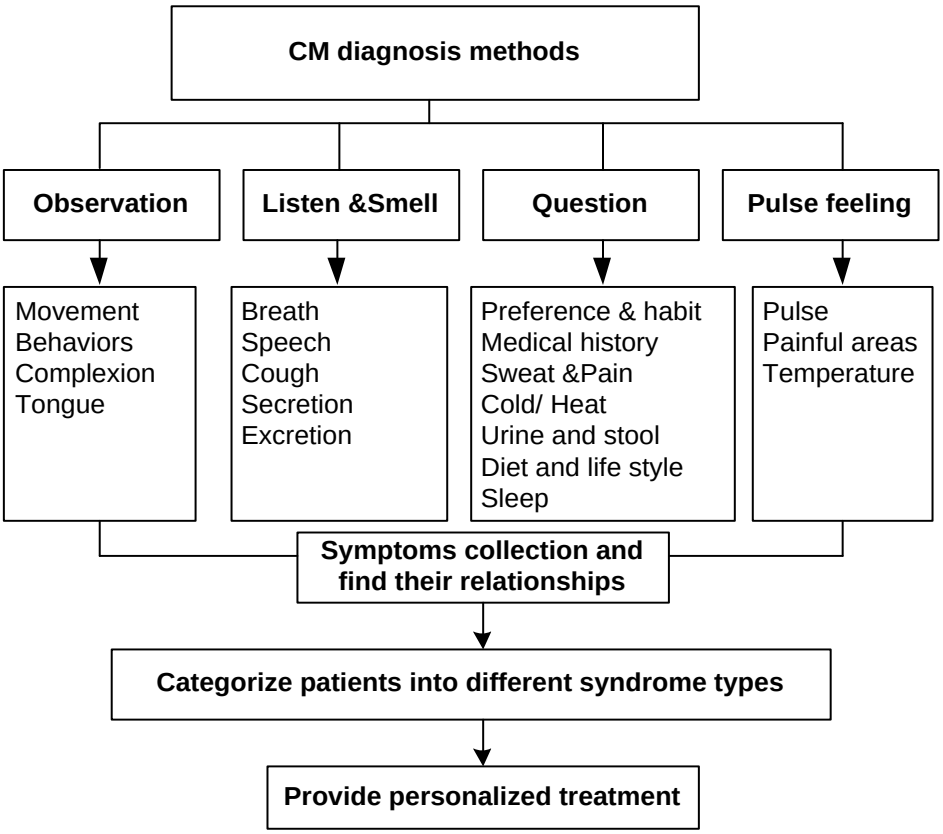

Figure5. *CM diagnostic methods*

Combining Chinese Medicine and metabolomics to subtype T2DM

CM is descriptive and phenomenological — it typically diagnoses patients using concepts based on the relationship between signs and symptoms, obtained via its four diagnostic methods [27]. It is said that CM diagnosis could be understood as a pattern recognition [71]. Patterns or syndrome types are manifestation profiles (relationships between signs and symptoms) already classified according to CM theories. It is based on pattern differentiation, together with the subject's health

status and environmental factors, that a CM physician designs and implements treatment. In contrast, Western-style, modern medicine has mainly used single biomarkers to describe disease states, for example diagnosing T2DM by measuring glucose levels. But there is a growing realization in the West that single biomarkers are not enough. A better approach is to look at patterns of biomarker responses to a challenge [27].

The concepts and practices of systems biology align very closely with CM. In CM theory, the concepts of the "whole" and the "system" rather than isolation are important, which well fits to systems biology perspectives. An emerging concept of health in the scientific literature describes an ability to adapt and self-manage in the face of social, physical and emotional challenges [27]. This perspective has long been central to the concept of health in CM, which further includes spiritual fulfilment and a sense of individual well-being. Systems biology is particularly useful when it comes to describing homeostasis — the regulation of a system's internal environment to maintain a stable condition [27]. In turn, the ability to cope with changing environments and stress is encompassed in the principle of allostasis – the physiological or behavioural changes required to stabilize the biological system [27]. The data of systems biology based challenge or intervention to health will provide insight into the resilience of allostatic mechanisms, and hence into a person's health, an approach similar as the tenets of CM.

If we can correlate the CM diagnosed syndrome types to the metabolomics quantified clusters, it may help us stratify the patients and push personalized medicine forward (Figure 6). In addition to giving WM a basis for adopting some concepts of CM, systems biology is also pushing the convergence from the other direction. Increasingly, CM uses modern biochemical measurements and tools to refine or augment diagnostic descriptions. This is starting to facilitate the translation of CM concepts into Western concepts based on biochemical, pathway or regulatory processes [27]. Several studies [39, 43, 72, 73] combining metabolomics and CM syndrome types have revealed different molecular and metabolic patterns of patients with the WM diagnosed diseases, which provided new opportunities to improve patient stratification and personalized intervention.

In China, the treatment for T2DM and/or its related metabolic disorder is often the mixture of both WM and CM strategies. Integration of CM and WM has accomplished better therapeutic effects for diabetics [74-77], manifesting as the improvement of insulin sensitivity, lipid profile and quality of life and the alleviation of diabetic foot ulcer; and the better tolerance and compliance with Western medication has been also reported. CM often provides patients with a multi-component herbal formula with tailored dosage based on the diagnosis, in order to obtain the multi-targets effects in the whole system to restore the self-

adaptation ability. The idea is quite similar to the “combination therapy”, such as ‘poly-pill’[78, 79] or ‘poly-meal’[80], proposed in western medical and scientific world. The evaluation of such multi-target effect used to be difficult, but with the systems biology based metabolomics approach, the fingerprints of whole spectrum of the active ingredients of an herbal and their concentrations can be quantified and their synergy effect in the system level [81] can be investigated. This might provide new possibilities to discover new drugs or other interventions for T2DM.

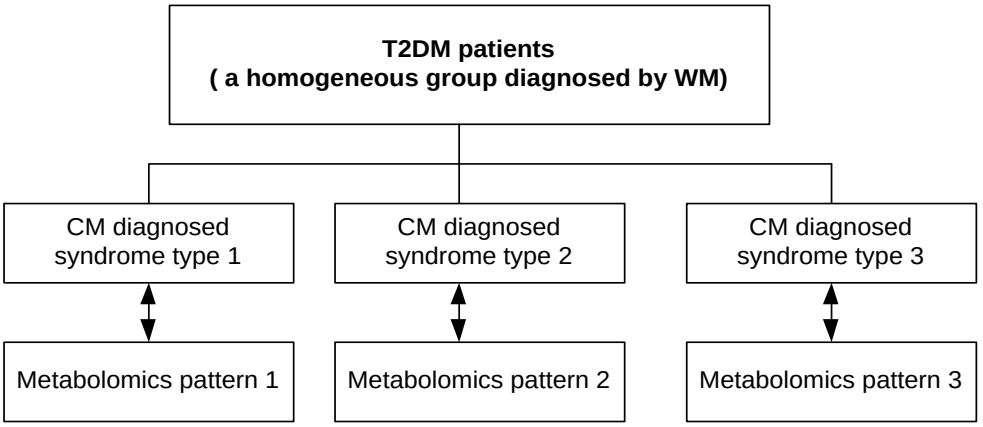

Figure 6. *Combine CM diagnosed syndrome types and metabolomics patterns after statistical pattern recognition techniques to search for the method to stratify patients*

Aim of this thesis

The aim of this thesis is to apply a system biology based metabolomics approach, lipidomics in specific, to search for novel diagnostic markers or subtypes of T2DM in its early and late stages.

The personalized diagnostic strategy of Chinese medicine will be combined with metabolomics to stratify the patients with T2DM or pre-diabetes; and explore the related metabolism changes and pathways. The multi-component preparations or drugs to treat cardio-metabolic disorders will be studied and assessed by metabolomics to understand the potential underlying multi-target or multi-pathway effects. The idea is to investigate the pre-/diabetes and its related metabolic abnormalities such as obesity and dyslipidemia from a systems-based

perspective and improve the understanding of the disease and its intervention to a personalized health direction.

Outline of this thesis

Each chapter addresses an aspect to realize the aim formulated in the thesis. In **chapter 2**, an explorative study of 50 pre-diabetic males is designed, combining GC-MS urine metabolomics with CM diagnosis to identify diagnostic biomarkers for pre-diabetic subtypes. Moreover, the inter-physician concordance of CM diagnoses is assessed. In **chapter 3 and 4**, the effects of rimonabant and a multi-component preparation, both focus on regulating weight and having beneficial effects on dyslipidemia, were assessed by lipidomics on mildly over-weighted ApoE*3Leiden.CETP Mice. In **chapter 5**, systems biology based metabolomics approaches are used to evaluate the therapeutic effects of ginseng roots grown for 3–6 years on the regulation of hyperglycemia and dyslipidemia in a Goto-Kakizaki (GK) rat model with T2DM. **Chapter 6** reports the development of a fast plasma lipid analysis platform by nanospray chip-based mass spectrometry. In **chapter 7**, conclusions and perspectives are presented.

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Chapter 2

Urine metabolomics combined with the personalized diagnosis guided by Chinese Medicine reveals subtypes of pre-diabetes

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Abstract

The prevalence of type 2 diabetes continuously increases globally. A personalized strategy applied in the pre-diabetic stage is vital for diabetic prevention and management. The personalized diagnosis of Chinese Medicine (CM) may help to stratify the diabetics. Metabolomics is regarded as a potential platform to provide biomarkers for disease-subtypes. We designed an explorative study of 50 pre-diabetic males, combining GC-MS urine metabolomics with CM diagnosis in order to identify diagnostic biomarkers for pre-diabetic subtypes. Three CM physicians reached 85% diagnosis consistency resulting in the classification of 3 pre-diabetic groups. The urine metabolic patterns of group 1'Qi-Yin deficiency' and 2 'Qi-Yin deficiency with dampness' (subtype A) and group 3 'Qi-Yin deficiency with stagnation' (subtype B) were clearly discriminated. The majority of metabolites (51%), mainly sugars and amino acids, showed higher urine levels in subtype B compared with subtype A. This indicated more disturbances of carbohydrate metabolism and renal function in subtype B compared subtype A. No differences were found for hematological and biochemical parameters except for levels of glucose and γ-glutamyltransferase that were significantly higher in subtype B compared with subtype A. This study proved that combining metabolomics with CM diagnosis can reveal metabolic signatures for pre-diabetic subtypes. The identified urinary metabolites may be of special clinical relevance for non-invasive screening for subtypes of pre-diabetes, which could lead to an improvement of personalized interventions for diabetics.

Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by hyperglycemia with disturbances of carbohydrates, fat and protein metabolism and its prevalence continuously increases globally[1-3]. Evidence shows that both lifestyle regulation and early pharmacotherapy during pre-diabetes are effective in slowing down the onset and progression of T2DM [1, 3, 4]. However, due to its multi-factorial causes resulting from the interaction between a genetic predisposition and behavioral and environmental risk factors, the control of T2DM represents a considerable therapeutic challenge[1, 2] and to diagnose and treat T2DM only based on the glucose level seems insufficient. Glycaemic control as evidenced by the reduction of glycated hemoglobin(HbA1c) with existing agents was found to have a weak and non-significant effect on the incidence of cardiovascular complications [1]. A more personalized and system based strategy to control the factors beyond glycaemia management (e.g. hypertension, dyslipidaemia, insulin resistance, obesity) is vital in reducing the diabetic morbidity and complications. The challenge towards personalized treatment is how to stratify patients to provide a diagnosis considering the uniqueness and the interaction of person with his environment as a whole [5].

The concept of personalized health and system diagnostic principles is not new, as it has long been the basis of Chinese Medicine (CM), in which the focus is the 'diseased person' instead of the 'person's disease' [6]. CM does not focus solely on the disease defined by pathological changes but the overall maladjustments of functional status called 'syndrome type' [6]. The syndrome type is a functional status which is caused by the reaction to or interaction with environmental changes and pathogenic factors [7]. It is a manifestation profile of a group of signs and symptoms and its essence is the imbalance of the human system resulting in the perturbation of the metabolic or biological network [7]. CM aims to restore the self-regulatory ability of the human system, instead of antagonizing specific pathogenetic targets [6]. Metabolomics, defined as the "comprehensive quantitative and qualitative analysis of all small molecules in a system", has been increasingly applied to study complex disease mechanisms to discover health-disease associated mechanistic biomarkers and it is regarded as a unique bridge between different healthcare perspectives on personalized medicine [8]. Urine metabolomics is of special interest because the urine collection is non-invasive and it amplifies the circulating levels of metabolites by renal concentration, which consequently ensures urine a distinct representation of metabolic response [9].

Several studies [10-13] combining metabolomics and CM syndrome types have revealed different molecular and metabolic patterns of patients with the Western Medicine (WM) diagnosed diseases, which provided new opportunities to

improve patient stratification and personalized intervention. In China, integration of CM and WM has accomplished better therapeutic effects for diabetics [14-17], manifesting as the improvement of insulin sensitivity, lipid profile and quality of life and the alleviation of diabetic foot ulcer. However, to implement CM diagnosis under WM system is not easy; as the diagnosis is not based on objective instruments. By using four diagnostic methods: observation, listening and smelling, question and pulse feeling (Figure 1), CM physicians attempt to establish the health status through qualitative symptoms collection based on one's appearance, behaviors, mental status, bio-rhythms, life style as well as his interaction and adaptation to both natural and social environment. Specifically, 'observation' method focuses on the behavior, movement, figure, facial color, tongue, throat and finger veins of a patient; with 'listening and smelling' method, CM physicians will evaluate the speech of a patient (e.g. pitch, tone, tempo), the way a patient is coughing or breathing and smell his breath and secretion (e.g. sweat). 'Question' is one the most important diagnostic techniques and CM physician will carefully ask and analyze the complaints and symptoms of a patient. Normally the following information will be covered [18]: medical history, response to cold/heat weather or food, sweat, symptoms of head, chest and body, pain, diet and appetite, sense organs, sleep quality and life style; for females there will be questions related to menstrual cycle and pregnancy experiences. 'Pulse feeling' is an unique diagnostic method in CM, by feeling the rate, depth and tension of the pulse, CM physicians can speculate the health status of the whole system. CM physicians will then cluster the symptoms/signs together according to CM diagnostic principles to get the CM syndrome type and provide personalized treatment. Due to the fact that CM diagnosis is conceptual and relies entirely on clinical signs discerned by CM physicians, the inter-physician consistency on CM diagnosis is of importance to both scientific research and clinical practice [19].

In this explorative study, we diagnosed 50 pre-diabetic males with CM syndrome types and applied urine metabolomics to search for biomarkers of pre-diabetic subtypes, with the following aims and hypotheses: (1) to assess the inter-physician concordance of CM diagnoses; (2) to find the relationships between classifications of pre-diabetics according to CM diagnosis and metabolomics. We hypothesize that combining CM diagnosis with metabolomics could help us identify pre-diabetes subtypes with related urinary metabolic patterns. The latter can provide quantitative biological evidence for CM diagnosis.

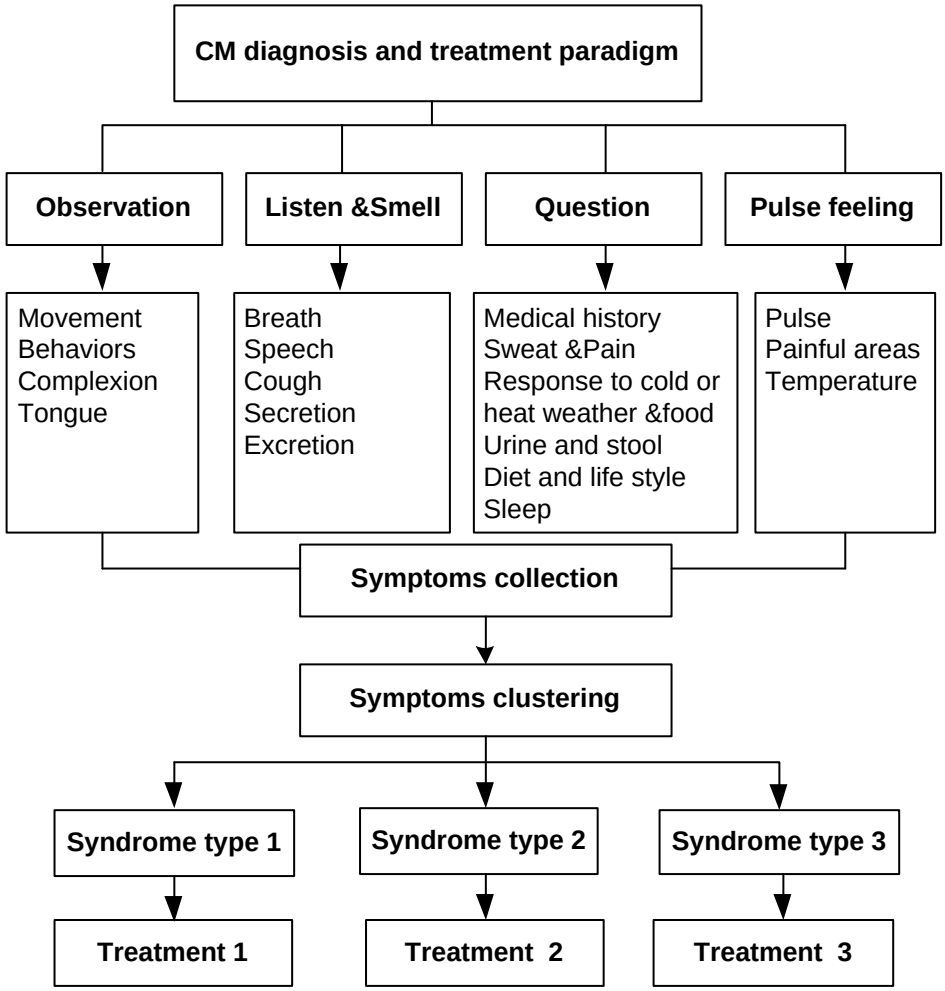

Figure 1. *CM diagnosis and treatment paradigm.*

Experimental section

Subjects and study design

The study was conducted at TNO (Zeist, the Netherlands) and 69 overweight pre-diabetic males were recruited from TNO's candidate database. A pre-study screening comprised of a physical examination, clinical laboratory tests and the

evaluation of anamnesis and life style was performed in order to confirm that the recruited subjects have no other clinical abnormalities than pre-diabetes. Subjects were declared eligible when meeting the inclusion and exclusion criteria. Inclusion criteria: 1) males aged 30 –70 years; 2) Body Mass Index (BMI) 26 – 35 kg/m^2; 3) pre-diabetes as determined by fasting glucose level of 6.1 – 6.9 mmol/L; 4) normal Dutch eating habits. Exclusion criteria: 1) smokers; 2) alcohol consumption ≥28 units/week; 3) unexplained weight fluctuation of > 2kg in the month before the pre-study screening; 4) currently on CM therapies. Finally 50 overweight pre-diabetic males, all of Dutch ancestry, were included in the study. All study subjects signed the informed consent and their baseline characteristics are summarized in Table 1. The study was approved by the Medical Ethics Committee of Tilburg (METOPP; Dutch acronym for medical ethical research in patients and volunteers).

Table 1. Baseline characteristics of 50 pre-diabetic males

Male (n = 50)	**Mean ± SD**	**Range**
Age /years	57 ± 8	40 – 69
Body weight /kg	94.2 ± 10.1	75.4 – 117.6
BMI /kg m^{-2}	28.8 ± 2.4	25.6 – 35.5
BP systolic /mm Hg^{-1}	137.7 ± 17.8	93 – 177
BP diastolic /mm Hg^{-1}	83.0 ± 10.1	56 – 104
Waist circumference /cm	105.0 ± 8.1	92 – 128
Blood glucose /mmol L^{-1}	6.3 ± 0.3	5.9 – 6.9

The study was designed as an explorative study without intervention (Figure 2). Fifty pre-diabetic males were diagnosed by three different CM physicians separately and in a randomized order. The CM physicians had to have least ≥ 5 year CM training with a valid certificate and ≥ 10 year clinical experience in the Netherlands. Each CM examination took 15- 20 minutes and the physicians were not allowed to exchange ideas or know the diagnosis from each other. They were asked to apply CM diagnostic theories of organ and Qi-Blood-Fluid to give the personalized descriptions with percentage scaling to each subject. CM physicians first questioned subjects with symptoms and signs, then summarized and clustered these signs into CM diagnostic terms/syndromes (Figure 1). Finally CM physicians wrote down all the diagnosed syndromes, often 3-4 syndromes for each subject, and then based on severity and frequency of these syndromes ranked with percentage scaling for each diagnosed syndrome under 100% in total.

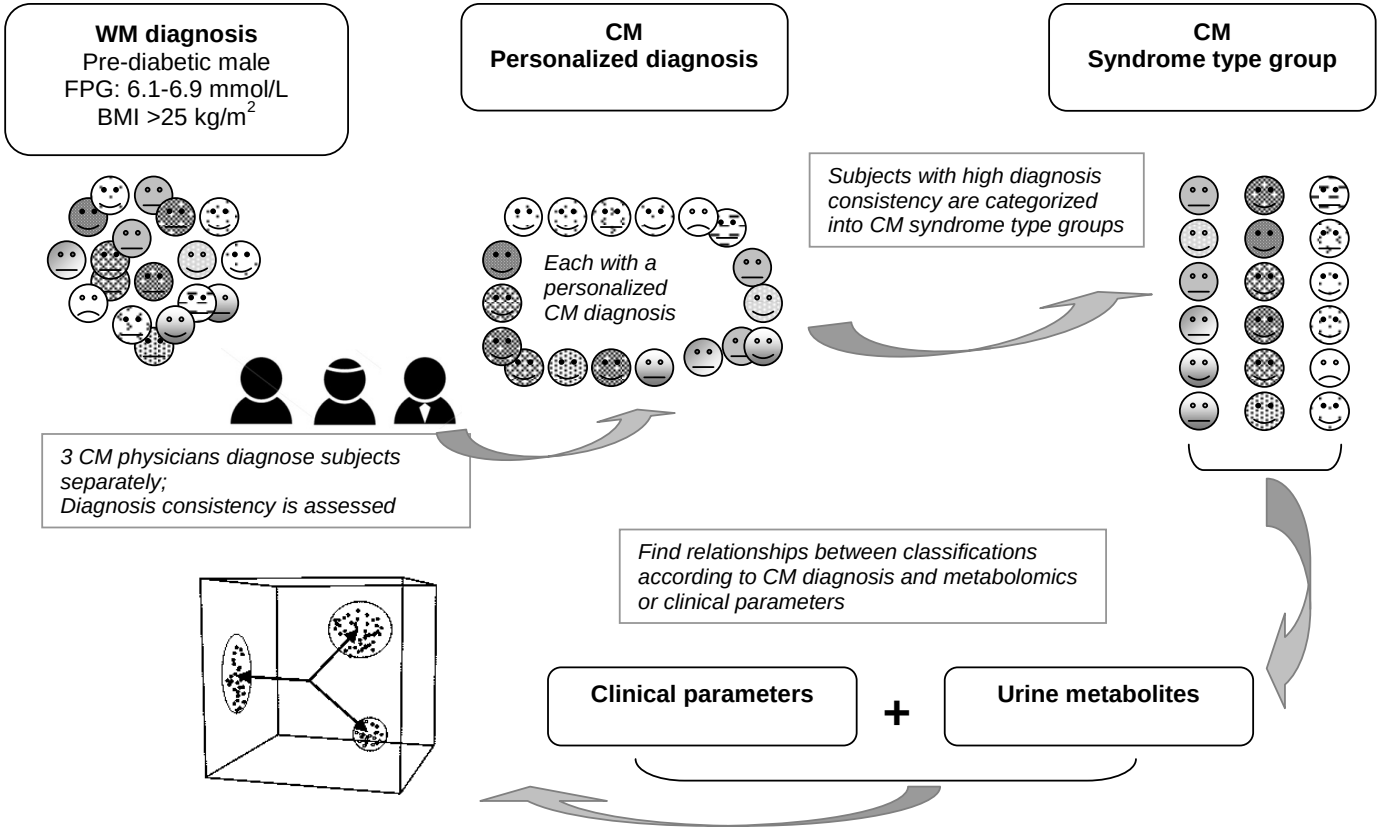

Figure 2. *Study design and objectives*

For example, the diagnosis was written down per subject by each physician in the form of "55% liver yang ascending, 35% yin deficiency and 10% liver qi stagnation". CM diagnoses were considered consistent when ≥ 80% inter-physician agreement was reached based on generalized procrustes analysis (GPA) results [20]. GPA is a multivariate exploratory technique that involves transformations (i.e., translation, rotation, reflection, isotropic rescaling) of individual data matrices to provide optimal comparability [20]. Subjects who were diagnosed differently by three CM physicians were excluded from further data analysis. The blood and the first void urine samples of study subjects were collected after an overnight fast (≥ 12 h). The blood samples were used for clinical laboratory measurements and urine samples for GC-MS metabolomics. The relationships between classifications of pre-diabetics according to CM diagnosis and metabolomics or clinical parameters were studied.

Clinical parameter analysis

Blood samples were obtained from antecubital vein of forearms and collected in tubes containing clot activator for serum and in anticoagulant tubes (Vacutainer Systems, Becton Dickinson, UK) of either K_3EDTA or Li-heparin for plasma. The K_3EDTA tubes were used for Hb1Ac determination while the Li-heparin tubes were used for the determination of glucose and lipids. Blood samples were centrifuged for 15 min at 2000 × g at 4 ºC within 30 min after collection. Samples were aliquoted and stored at -20°C till analysis. All biochemical determinations in blood were analyzed with enzymatic techniques on the Olympus analytical equipment (Olympus-Diagnostica, Germany), including the haematology profile and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, bilirubin, urea, creatinin, total- and high density lipoprotein-cholesterol (HDL-C) and the ratio of total cholesterol and HDL-C, triacylglycerols, glucose.

GC-MS of urinary metabolomics

Urine sample preparation

After collection, the first void spot urine samples were aliquoted and then stored in -20°C till gas chromatography–mass spectrometry (GC-MS) analysis. Urine samples were first thawed to room temperature, then homogenized by vortexing, and afterward centrifuged for 20min at 3500 RPM. One hundred µL urine sample of each study subject was added to 10 µL internal standard (IS) mixture (nr.1) consisting of leucine-D3, glutamic acid-D3, phenylalanine-D5, cholic acid-D4 with a

final IS concentration of 10ng/µL in a clean PTFE vial. Afterwards the urine samples with IS mixture (nr.1) were frozen at -80 ºC and lyophilized or freeze dried. Subsequently, 10µL IS mixture (nr.2) consisting of alanine-D4 and glucose-D7 with final IS concentration of 10 ng/µL were firstly added to the dried extracts. The dried extracts were then oximized with 20 µL of 16 mg/mL pyridine hydrochloride solution and 20 µL of 56mg/mL ethoxyamine hydrochloride solution in pyridine for 90 min at 40 °C, followed by adding 10 µL IS mixture (nr.3) consisting of DCHP, trifluoroacetyl- anthracene (TFAA) and DFBP with corresponding final concentrations of 18ng/ µL, 19 ng/µL and 20 ng/µL in IS nr.3. Finally, the extracts were silylated for 50 min at 40 ºC with 200 µL of MSTFA and centrifuged for 20 min at 3500 RPM.

Instrument settings

The derivatized (oximation and silylation) urine extracts were analyzed with an Agilent 6890 gas chromatograph coupled with an Agilent 5973 mass selective detector. The GC-MS method applied in the study can analyze a broad range of metabolites and was reported by Koek el al. [21]. The 1-µL aliquots of the extracts were injected into a HP5-MS capillary column (30 m × 250 µm i.d., 0.25-µm film thickness; J&W Scientific, Folson, CA) using PTV injection (Gerstel CIS4 injector) in the splitless mode. The temperature of the PTV was 70 ˚C during injection, and 0.6 min after injection, the temperature was raised to 300 ˚C at a rate of 2 ˚C /s and held at 300 ˚C for 51 min. The initial GC oven temperature was 70 ˚C; 5 min after injection the GC oven temperature was increased with 6 ˚C /min to 325 ˚C and held for 3 min at 325 ˚C. Helium was used as a carrier gas and pressure programmed such that the helium flow was kept constant at a flow rate of 1.7 mL/min. Detection was achieved with MS using electron impact ionization and full scan data acquisition (m/z 15-800).

The performance of the applied GC-MS platform was assessed through the repeated analysis of the quality control (QC) samples. The QC samples, used to monitor the GC-MS response in time, were prepared by pooling aliquots of 50 urine samples to represent the full biochemical diversity of the study samples and allow the calculation of the analytical precision for all metabolites measured. The QC sample data is also used to correct systematic errors such as batch to batch response differences by a single point calibration model [22, 23]. Twenty QC samples were processed exactly in the same way as the study samples and analyzed after every 2-3 study samples with double injections. Furthermore, method performance was carefully monitored using multiple internal standards and duplicate analysis of samples.

Metabolomics data preprocessing

The GC-MS raw data was processed by Agilent ChemStation software. Only metabolites with 80% of the samples having a value were kept in the dataset. Data for each sample was corrected for the recovery of the IS for injection. Batch to batch differences in data were removed by synchronizing medians of QC-samples per batch. Duplicate measurements were combined into a single measurement. When both analytical duplicates had a zero value or when both had a non-zero value, measurements were averaged. The single value was taken when only one of the duplicates was above zero. Metabolites with high imprecision (i.e. relative standard deviation ≥50%) were removed from the dataset. Finally, the GC-MS dataset contained 128 metabolites and 46 of which were identified.

Data analysis

In order to assess the inter-physician diagnostic consistency, generalized procrustes analysis (GPA) was performed [20]. GPA is a method for comparing shapes of objects, in which the shape of an object is defined in a mathematical context as all the remained geometrical information after location, scale and rotational effects being filtered out. Three objects (CM physicians) were defined in the study, namely a matrix with concept-scores for each of three CM physicians. Each matrix size was 50 (number of pre-diabetic males) × 26 (number of CM diagnostic terms with percentage scaling). To explore urinary metabolic patterns and its relation to CM diagnosed groups, an unsupervised Principal component analysis (PCA) [24] was carried out in MATLAB (version 7.7.0471, the Mathworks) with the PLS toolbox (version 5.0.3, Eigenvector Research, InC.). Partial least squares discriminant analysis (PLS-DA) [25] was further applied to identify the specific metabolites which contribute most to discriminate between the subtypes; and PLS-DA models were validated using double cross validation (DCV) [26]. To avoid the possibility that a few high-intensity variables dominate the final results [27], metabolomics dataset was standardized per variable, meaning that first the means of each variable were subtracted, and then all variables were divided by their standard deviation (SD). Statistical differences of blood clinical parameters and urine metabolite regulation between subtypes of pre-diabetics were assessed by univariate data analyses by SPSS 17.0 and the results are presented as means ± SD unless indicated otherwise, with p value < 0.05 as statistical significance. To correct for false positives, the multiple test correction (MTC) of Benjamini and Hochberg false discovery rate (FDR) analysis[28] was applied to adjust p values derived from the univariate results of the metabolomics data.

Results

The diagnostic consistency of CM physicians and the CM syndrome types

Twenty six CM diagnostic terms (data not shown) were used to provide the personalized descriptions for each subject. The GPA analysis revealed a diagnostic consensus proportion of 85% among three CM physicians. The residuals from GPA can provide information of the inter-physician diagnosis agreement for a subject. The higher the residual, the lower agreement the CM physicians have upon this subject. Six subjects with relatively high residuals (≥ 0.02) were excluded from further analysis. An example of subjects with high and low inter-physician diagnostic consistency is illustrated in Figure 3. The GPA residual value was low for subject 35 and high for subject 11. Obviously, the similar diagnoses of three CM physicians were given for subject 35 (Figure 3A) while different diagnoses were given for subject 11 (Figure 3B).

The chief CM physician categorized the remaining 44 study subjects into three CM syndrome type groups: group 1 “Qi-Yin deficiency” (n= 15), group 2 “Qi-Yin deficiency with dampness” (n=20) and group 3 “Qi-Yin deficiency with stagnation” (n=9). The description and main symptoms under each syndrome type were summarized in Table 2. All subjects actually had symptoms of CM syndrome type “Qi-Yin deficiency”, mainly manifesting as fatigue, spontaneous or night sweat and afternoon heat. Only subjects in group 1 were diagnosed solely as “Qi-Yin deficiency” while subjects in group 2 and 3 had basic symptoms of “Qi-Yin deficiency” with additional CM pathological factors, either “dampness” or “stagnation”.

Metabolomics patterns and the relevant clinical characteristics

Metabolomics patterns

The GC-MS urine metabolomics data matrix of 44 subjects × 128 metabolites was used for PCA. The first two principal components (PCs) were selected, which described 45.4% of the total variance of the urine metabolome for 3 CM groups (Figure 4). A clear separation was observed (gray line in Figure 4) of group 1 (Qi-Yin deficiency) with group 2 (dampness) versus group 3 (stagnation). Thirteen out of 15 subjects (87%) of group 1 were clustered with subjects of group 2; while only three subjects numbered 22, 29 and 31 of group 1 were closer with subjects of group 3. Eight of 9 subjects of group 3 appeared at the right side of PCA score plot, except for subject 4 who was clustered with subjects diagnosed with ‘dampness’ and ‘Qi-Yin deficiency’ (Figure 4). This separation in PCA indicated

underlying concentration differences of urine metabolites between two potential pre-diabetic subtypes: 'group 1 plus 2' (subtype A) versus 'group 3' (subtype B). PLS-DA with DCV was used to further identify urine metabolites that differed in concentrations between subtypes A and B. The DCV error rate was 11%, indicating that 5 out of 44 subjects were misclassified. Among top 10 metabolites that contributed most to discriminate between subtype A and B, only three were identified. Gluconic acid and tryptophan showed significantly higher concentration in subtype B subjects (Figure 5B) while D-xylose appeared no significant differences between the two subtypes (data not shown).

Subsequently, quantitative differences in urine metabolites between subtypes A and B were tested by an independent t-test with MTC-FDR to correct for false positives. This resulted in 65 out of 128 urine metabolites (51%) with significant higher concentrations (p = 0.00 − 0.02) and 38% metabolites with higher concentration trend in subtype B compared with subtype A (Figure 5A). Twenty four out of the 65 significantly changed metabolites could be identified, mainly sugars, amino acids and organic acids, and were ranked based on the coefficients of PLS-DA regression vector (Figure 5B). The urine levels of these 24 metabolites were found to be 60 – 460% higher in subtype B compared to subtype A.

Table 2. Descriptions and main symptoms of three CM syndrome types

Group 1 **Qi-Yin deficiency** **(n= 15)**	**Group 2** Qi-Yin deficiency with **Dampness** **(n= 20)**	**Group 3** Qi-Yin deficiency with **Stagnation** **(n=9)**
Fatigue	*The similar symptoms as "Qi-Yin deficiency" plus symptoms below:*	*The similar symptoms as "Qi-Yin deficiency" plus symptoms below:*
Short of breath	Uncomfortable fullness of belly	Chest oppression;
Palpitation	Inactive, lack of appetite	Tingling in feet or limbs
Dizziness	Heaviness of the body	Dry skin
Spontaneous or night sweat	Obesity	Cold finger tips or toes
Dry mouth and throat	Edema	Unexplained pain
Thirsty	Feels sick and vomiting;	Light purple lips
Insomnia and irritation	Bad breath	Dilated veins under the tongue
Hot flush		
Afternoon heat		
Concentration problems		

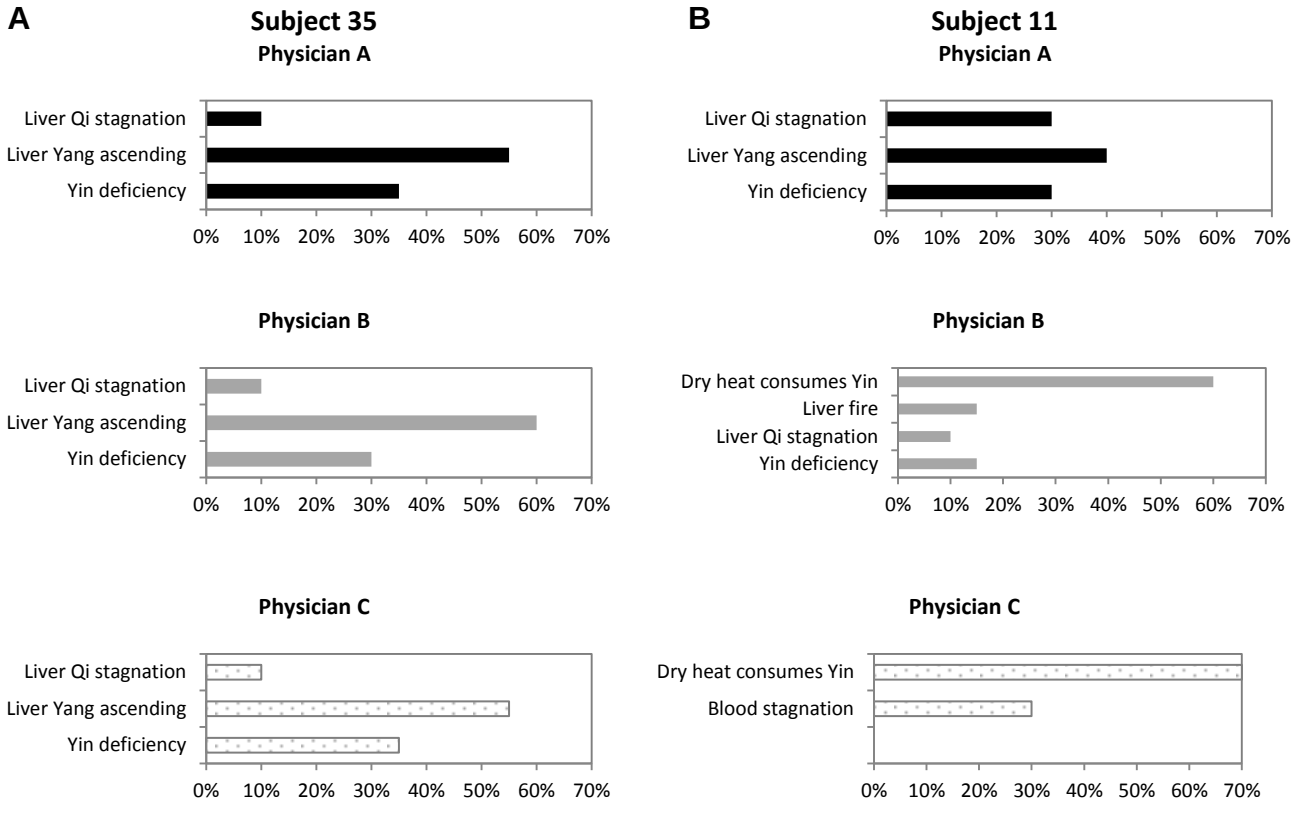

Figure 3. *Illustration of inter-physician diagnostic consistency. (A) Subject 35 was with a low GPA residual meaning consistent inter-physician diagnoses; (B) subject 11 was with a high GPA residual meaning discrepant inter-physician diagnoses.*

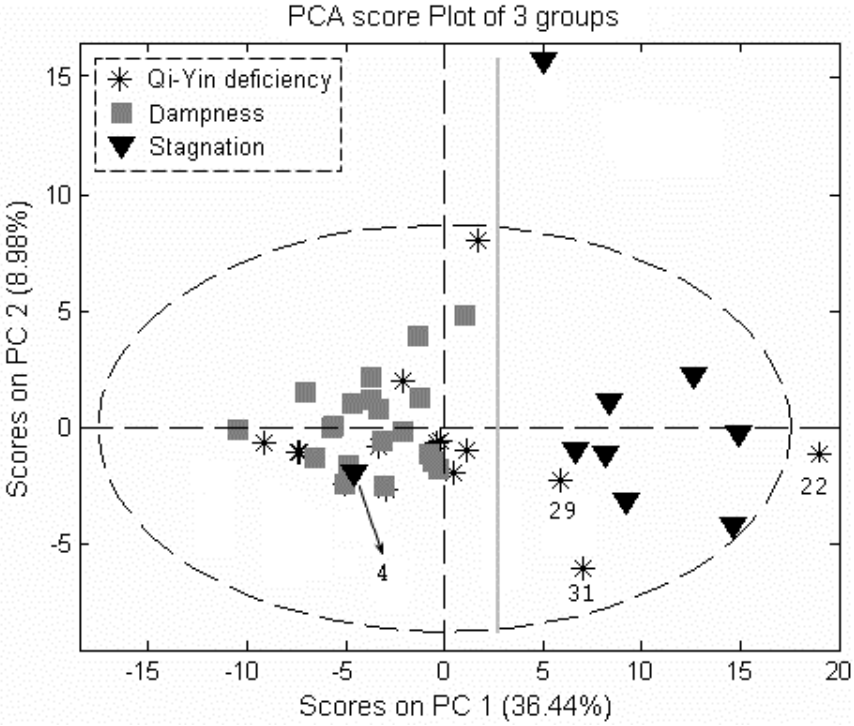

Figure 4. *PCA score plot of urine metabolomics data reflecting pre-diabetic subtypes based on CM diagnosis. Three CM syndrome groups (n=44): group 1 'Qi-Yin deficiency', group 2 'Qi-Yin deficiency with dampness', group 3 'Qi-Yin deficiency with stagnation'. Group 3 was clearly separated from group 1 and 2. The gray line highlighted the separation between "group 1 plus 2" and "group 3".*

Clinical characteristics

Clinical characteristics of two pre-diabetic subtypes were analyzed by independent t-test. No significant differences were observed for parameters of haematology (data not shown) or physical examination including age, BMI, waist circumference and blood pressures (BP) (Table 3). Subtype B showed significant higher levels of fasting plasma glucose (p =0.04) and γ-glutamyltransferase (γ-GT) (p =0.03) as compared to subtype A (Table 3). No other clinical parameters showed significant differences between the two subtypes.

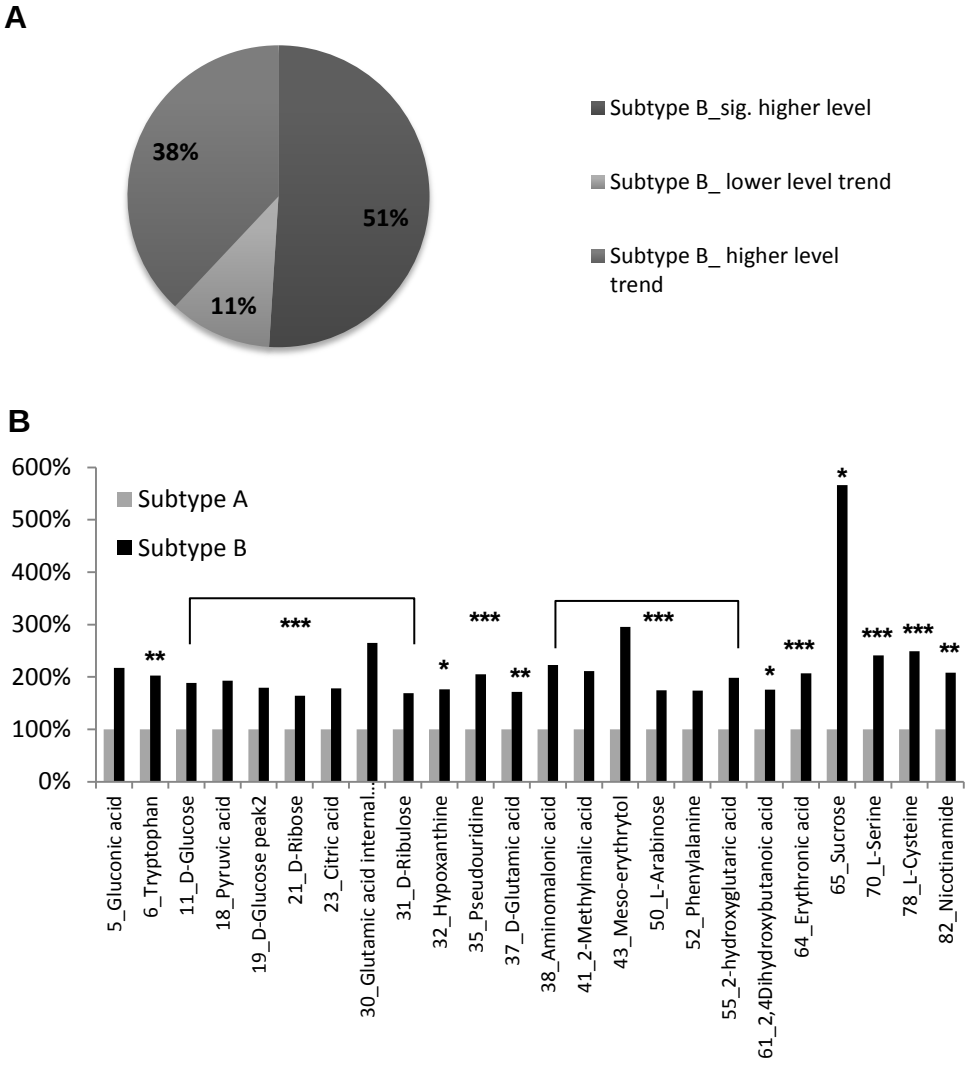

Figure 5. *Urinary metabolite level differences between subtypes A and B. (A) The distribution of urinary metabolite changes in subtype B (vs. subtype A); (B) Twenty four identified metabolites showed significantly higher urine levels in subtype B. The urinary metabolite level of subtype A was set to be 100% and relative changes of subtype B were illustrated in % compared with it (*p <0.05; ** p < 0.01; *** p <0.001 after MTC-FDR). The number before each metabolite refers to the ranking of metabolite based on coefficients of PLD-DA regression vector.*

Table 3. Clinical characteristics of two PCA separated CM syndrome type groups

	Subtype A Qi-Yin deficiency & Qi-Yin deficiency with **Dampness** (n= 35)	**Subtype B** Qi-Yin deficiency with **Stagnation** (n= 9)	***p***
Age /years	56±8	58±7	NS
BMI (kg/m^2)	28.8±2.1	29.1 ±3.4	NS
Waist circumference / cm	105±7	105±12	NS
Systolic BP (mm/Hg)	137±17	135 ± 22	NS
Diastolic BP(mm/Hg)	83±9	81 ± 13	NS
Glucose (mmol/L)	6.1 ±0.4	6.4±0.6	0.04
Hb1Ac (%)	5.8±0.3	5.9 ±0.3	NS
TC (mmol/L)	5.7±0.9	5.8 ± 1.0	NS
HDL-C (mmol/L)	1.3±0.3	1.2 ±0.2	NS
LDL-C (mmol/L)	3.7±0.8	3.7 ±1.0	NS
Ratio TC/HDL-C	4.7±1.1	4.8 ± 1.0	NS
TG (mmol/L)	1.7 ±0.8	1.9 ± 0.9	NS
ALP (U/L)	79±20	72 ± 13	NS
ALT (U/L)	29±11	29 ± 10	NS
AST(U/L)	23±5	22 ± 4	NS
γ -GT(U/L)	36±15	50±20	0.03
Urea (mmol/L)	6.1±1.5	6.1 ± 1.0	NS
Creatinine (mmol/L)	93±13	87 ± 14	NS

NS: No significance, $p > 0.05$

Discussion

This is an explorative study combining urine metabolomics with CM syndrome type diagnosis to search for different metabolic patterns in pre-diabetic subtypes. First of all, this study demonstrated that three CM physicians have reached a high (85%) diagnostic consistency. Taken into account the many different CM diagnostic principles, the three CM physicians involved in the current study were

asked to apply CM theories of Organ and Qi-Blood-Fluid and to quantify the diagnoses in percentage scaling. In this way, the CM diagnostic theory was clarified and standardized in advance, which may increase the inter-physician diagnostic consistency. Zhang et al. [19] also reported that consensus on CM diagnostic criteria results in the improved agreement of diagnosis. Thus the standardization of CM diagnostic procedures including diagnostic principle selection and symptoms scaling can lead to a more quantitative and reproducible CM diagnosis.

In real CM practice, there are many CM syndrome/pattern diagnostic principles and CM physicians are free to choose one or more of them to provide a syndrome diagnosis, in order to provide an optimal personalized treatment. The diagnostic terms under these principles can be translated into one another in certain extent. As shown in figure 6, most frequently used CM diagnostic terms of T2DM were summarized in different diagnostic principle levels. In the organ level, CM terms under 'Organ' diagnostic principle were listed and they can be translated to the relevant higher 'functional level'. For example, the diagnostic terms in 'Organ level' such as 'Lung stomach heat' can be categorized under diagnostic term 'Yin deficiency with excessive heat' under 'functional level'. According to the 'Standard for diagnosis and therapeutic effect evaluation of diabetes mellitus' established by Chinese integrative medicine academy diabetes committee in the year 2005 [29], the T2DM and related disorders including metabolic syndrome and obesity were categorized into CM syndromes under CM 'functional level', which is based on the combination of 'CM theories of Organ and Qi-Blood-Fluid'. As for research reasons, the first step to understand the subtypes in 'functional level' is more practical. Followed this concept, three CM physicians in current study were asked to freely write down the CM diagnostic terms ranked with percentage scaling based on the severity and frequency of the syndrome, which is a purely personalized diagnosis for each patient (Figure 2). Then 44 subjects with high CM diagnosis agreement were set to the CM syndrome types in CM 'functional level' based on the relations of CM syndromes shown in figure 6. Therefore, the CM diagnostic strategy applied in the current study is semi-unsupervised. The fully unsupervised CM diagnosis need much more study subjects and will be investigated in the future.

Furthermore, this study demonstrated that three different pre-diabetic syndrome types could be diagnosed based on CM principles: "Qi-Yin deficiency" (group 1), "Qi-Yin deficiency with dampness" (group 2) and "Qi-Yin deficiency with stagnation". "Qi-Yin deficiency" can be described as abnormalities induced by hypermetabolism. Its symptoms showed certain similarities with "chronic fatigue syndrome" and/or "people with mild inflammatory status". Its main complaints (Table 2) included fatigue, short of breath, dizziness, afternoon heat,

concentration problems and sleep disorders. “Qi-Yin deficiency” is regarded as the main CM syndrome type of T2DM in many Chinese national protocols and guidelines for diabetes management [29, 30]; while “stagnation” and “dampness” are two CM pathological factors closely associated with T2DM and diabetic complications [29, 30]. In CM theory, “dampness” refers to the imbalanced metabolism due to dysfunction of liquid and humor, usually by an over intake of

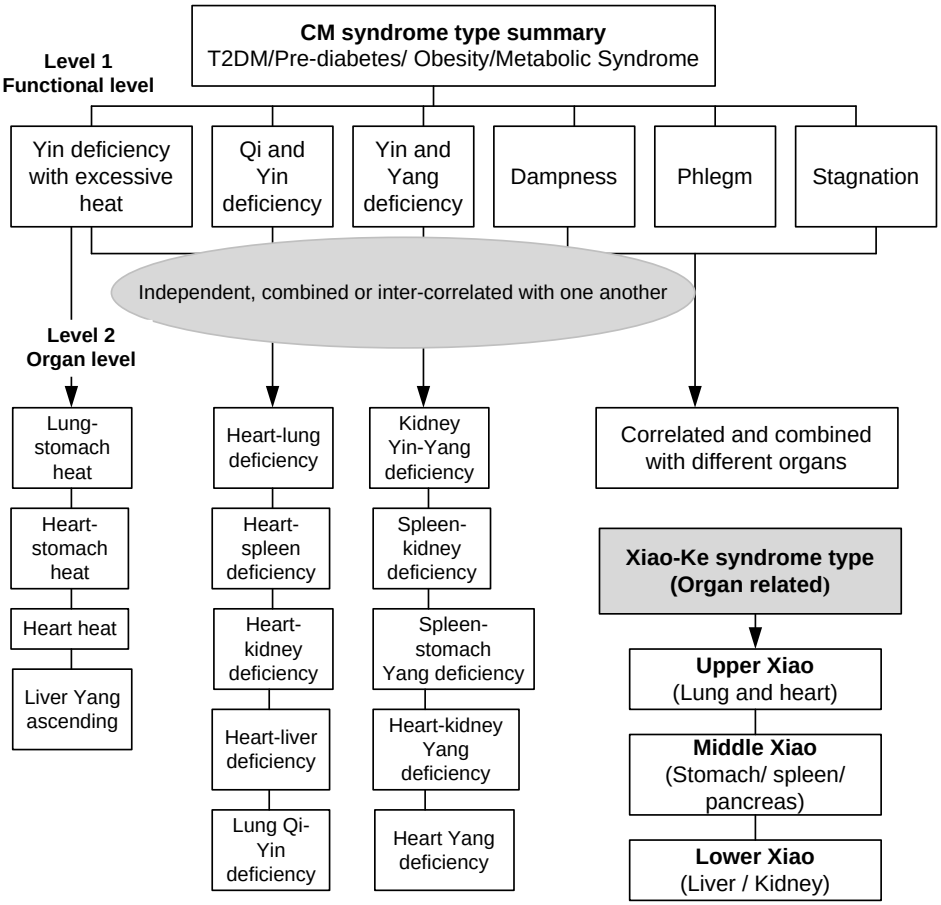

Figure 6. *Chinese Medicine syndrome type summary for T2DM and its related disorders including pre-diabetes, obesity and metabolic syndrome.*

raw, cold, greasy, or sweetened food [31]; while “stagnation” covers a wide range of abnormalities including blood circulation disturbance (especially microcirculation), metabolic and immune disorders, connective tissue pathological changes and some mental problems [32]. Chinese animal models

related to these CM syndrome types might provide us with an idea of the underlying biological mechanisms. The animals model of “dampness” was built by feeding animal with high carbohydrate and fat diets in the warm and humid environment to induce the symptoms analogous to humans such as fatigue, appetite loss and reduction of Na+-K+-ATPase activity [31]. Animal models of “stagnation” were made by using repetitive external stimuli (e.g. electronic needle, noise, light) to induce pain, stress, increased blood noradrenalin levels, a sympathetic hyperfunction and microcirculatory disturbances [33]. In Chinese population, “dampness” was more prevalent in patients with fatty liver [34], hypertension [35] and diabetic angiopathic complications [36]. “Stagnation” or its related CM syndromes (e.g. Yang deficiency) refers to T2DM patients with higher occurrence of proliferative diabetic retinopathy [11] or cerebral infarction [37]. Although CM syndrome type based animal models have many more varieties[31-33] and shall differ from real human situations, we still could infer that “dampness” is more associated with unhealthy life-style (i.e. intake of high energy diets) induced abnormalities; while “stagnation” may represent either catecholamines induced autonomic nervous system derangement or blood circulation problems.

The most importantly, this study demonstrated that we were able to distinguish two out of three defined pre-diabetic subtypes based on their urinary metabolic patterns: subtype A: “Qi-Yin deficiency” (group 1) and “Qi-Yin deficiency with dampness” (group 2), subtype B: “Qi-Yin deficiency with stagnation”. Although the “Qi-Yin deficiency” group could not be clearly distinguished from the other two groups based on the urinary metabolome, its biological background could be reflected by understanding the other two groups as they shared the same basic complaints. Subtype A was highlighted by “dampness” symptoms such as heaviness of the body, obesity and oedema while subtype B by “stagnation” symptoms such as cold and tingling limbs and pain. The two pre-diabetic subtypes showed a clear separation by PCA and PLS-DA and 51% of the measured urinary metabolites were significantly different between these pre-diabetic subtypes. Subtype B showed higher urinary levels of metabolites compared to subtype A.

Firstly, some metabolites (i.e. D-glucose, pyruvic acid, D-ribose, citric acid, D-ribulose, L-cysteine, Meso-erythritol, nicotinamide) are related to carbohydrate and energy metabolism. The higher urinary concentrations of glucose and other sugars in subtype B may corroborate with a higher hyperglycemic status of these study subjects. The fasting plasma glucose level of subtype B also showed a significantly higher concentration (p =0.04) than that of subtype A (Table 3). There might be enhanced endogenous glucose production from gluconeogenesis and the urinary loss of tricarboxylic acid (TCA) cycle intermediates such as citric acid and pyruvic acid in subjects of subtype B may indicate increased hepatic gluconeogenesis to provide extra pyruvate as a substrate for glucose [38]. Meso-

erythritol and nicotinamide can provide beneficial effects for diabetics as food supplements [39-42].

Secondly, many amino acids (e.g. L-cysteine, L-serine, phenylalanine, tryptophan) were increased in the urine of subtype B. Pre-/diabetes is characterized by insulin resistance and catabolic/anabolic hormonal imbalance, which will affect the metabolism of the whole body. Insulin resistance can cause protein degradation and amino acid release [43, 44]. In addition to being used as substrate for gluconeogenesis, these amino acids are cleared from the circulation by the kidneys and thus appear in higher concentrations in the urine [43, 44]. We speculate that subtype B might have more insulin resistance or impaired insulin action compared with subtype A. Moreover, L-serine, a non-essential amino acid, was reported to be excreted significantly more in the diabetics than in healthy controls [45]. Phenylalanine and tryptophan are essential amino acids which are either incorporated into proteins or broken down for energy and metabolic intermediates [46]. Tryptophan is the precursor of brain neurotransmitter serotonin and phenylalanine is the precursor of catecholamines which are adrenalin-like substances such as tyramine, dopamine, epinephrine and norepinephrine in the body [42]. Increased phenylalanine and tryptophan in urine might reflect the catecholamines related autonomic nervous system derangement [47-50]. Interestingly, “stagnation” animal modeling was also developed with the increased blood noradrenalin level.

Finally, the higher urinary excretion of the majority of measured metabolites in subtype B subjects could indicate that they might have more potential disturbance of renal function; therefore they lost metabolites that are necessary for carbohydrate and energy metabolism. Specifically, hypoxanthine’s relation with renal problems has been well demonstrated. On one hand, when xanthine oxidase converts hypoxanthine to xanthine in the presence of molecular oxygen, superoxide radical is generated. Reactive oxygen species, which leads to a lipid peroxidation, protein denaturation and DNA oxidation, contributes to the microvascular dysfunction and exert direct renal tissue damage [51]. On the other hand, uric acid is the end product of hypoxanthine catabolism and could deposit in renal tissue and form kidney stones [51]. Increased urinary citric acids were observed in diabetics and created more risks of kidney stones [52-54].

Clinical parameters did not show significant differences among the two pre-diabetic subtypes, except for higher blood glucose and γ -GT in subtype B. Elevated γ -GT level is associated with diseases of the liver and pancreas as well as cardiovascular mortality [55, 56]. Though both are still within the clinical reference range, the potential diabetic related problems might be indicated, which need further investigation. This study is an early phase investigation on subtype of the pre-diabetics based on a small number of study subjects and CM

physicians. As such whilst this data is very promising, there needs to be larger investigations using more subjects and CM physicians to demonstrate the further reliability and external validity/ generalizability of this novel approach to diagnosing pre-diabetic subtypes.

Conclusion

This is the first study combining non-invasive GC-MS urine metabolomics with CM personalized diagnosis to find metabolic subtypes in pre-diabetics. This study demonstrated that 85% inter-physician consistency of CM diagnosis was reached when the diagnostic principles were standardized and symptom descriptions were clarified in advance. Based on 3 different CM syndrome groups, two pre-diabetic subtypes can be identified by urine metabolomics and had different urinary metabolic patterns. The subtype B excreted higher levels of sugars and amino acids compared to the subtype A, indicating more disturbances of carbohydrate metabolism and renal function. The identified urinary metabolites may be of special clinical relevance for easy and non-invasive screening for subtypes of pre/diabetes and uncovering the diabetic development and prognosis in some extent, which could lead to a better understanding and improvement of personalized interventions for diabetics. This study proved that the understanding and improvement of diagnosis plays a key role in bridging between CM and WM on personalized healthcare. Future studies are needed to validate the subtypes yielded in the current study and to assess the intervention response of these subtypes to metabolic or hyperglycemic drugs.

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Chapter 3

Plasma and liver lipidomics response to an intervention of rimonabant in ApoE*3Leiden.CETP transgenic mice

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Abstract

Lipids are known to play crucial roles in the development of life-style related risk factors such as obesity, dyslipoproteinemia, hypertension and diabetes. The first selective cannabinoid-1 receptor blocker rimonabant, an anorectic anti-obesity drug, was frequently used in conjunction with diet and exercise for patients with a body mass index greater than 30 kg/m² with associated risk factors such as type II diabetes and dyslipidaemia in the past. Less is known about the impact of this drug on the regulation of lipid metabolism in plasma and liver in the early stage of obesity.

We designed a four-week parallel controlled intervention on apolipoprotein E3 Leiden cholesteryl ester transfer protein (ApoE*3Leiden.CETP) transgenic mice with mild overweight and hypercholesterolemia. A liquid chromatography-linear ion trap-Fourier transform ion cyclotron resonance-mass spectrometric approach was employed to investigate plasma and liver lipid responses to the rimonabant intervention. Rimonabant was found to induce a significant body weight-loss (9.4%, $p < 0.05$) and a significant plasma total cholesterol reduction (24%, $p < 0.05$). Six plasma and three liver lipids in ApoE*3Leiden.CETP transgenic mice were detected to most significantly respond to rimonabant treatment. Distinct lipid patterns between the mice were observed for both plasma and liver samples in rimonabant treatment vs. non-treated controls. This study successfully applied, for the first time, systems biology based lipidomics approaches to evaluate treatment effects of rimonabant in the early stage of obesity.

The effects of rimonabant on lipid metabolism and body weight reduction in early stage obesity were shown to be moderate in ApoE*3Leiden.CETP mice on high-fat diet.

Introduction

Obesity, a major risk factor for serious diet-related chronic diseases such as diabetes and cardiovascular disease, is commonly stated as critically important compositions of the metabolic syndrome [1, 2]. In recent decades, obesity has reached epidemic proportions globally due to the rapid economic growth, modernization and urbanization. The major causes of its rising epidemic are excessive consumption of energy-dense food high in saturated fats and sugars and reduced physical activity [3, 4]. Obesity is known to be associated with dyslipoproteinemia characterized by increased levels of plasma triacylglycerides (TG) and low density lipoprotein cholesterol (LDL-C) and decreased level of high density lipoprotein cholesterol (HDL-C) [5]. Chronic liver disease associated with obesity has been identified in adults since 1970s and soon after this condition was also reported in childhood and adolescence [6, 7]. Most commonly, non-alcoholic fatty liver is observed in obese subjects with liver disease. This disease is frequently caused by complex hepatocellular metabolic dysfunctions due to the impaired insulin action, leading to disordered metabolism of fat and free fatty acids and subsequent oxidant mediated damage to the hepatocytes [6].

Traditionally, prevention and treatment of obesity focus on individual behavior interventions through increased regular exercise and a low-fat, low refined carbohydrate diet [4]. It has proven inadequate probably because the sociological factors are not taken into account. Medical treatment approaches for obesity have largely been developed in modern societies and appear to be effective on the short term [8, 9]. However, data reporting on long-term health outcome based on successful treatment strategies are very limited [10].

Previously, it has been demonstrated that early obesity is associated with endothelial dysfunction in high-fat fed pigs [11]. The observed abnormalities such as mild dyslipidemia, vascular oxidative stress and hypertension indicated that the early phases of obesity play a key role in the progression of coronary atherosclerosis and cardiovascular events and can be considered as the center point of the metabolic syndrome [6, 11-13]. Collectively, effective strategies for prevention and recognition of overweight and obesity in an early stage are critical.

Since lipids are involved in obesity-associated pathology, novel tools that enable a large-scale study of individual lipids in biological systems are highly demanded for understanding the potential pathogenic mechanisms. Lipidomics technology can provide an integrated view of lipid metabolites present in cells, tissues and biological fluids [14-16]. This tool can not only provide insights into the specific roles of lipids in monitoring health status, but will also assist in identifying potential preventive or biomarkers [17, 18]. The availability of novel analytical and advanced instrumental as well as powerful informatics technologies has facilitated

the characterization of global changes of lipids in metabolic conditions such as insulin resistance [19], obesity [20], atherosclerosis [21], diabetes [22], and hepatic steatosis [23] and has facilitated data integration in order to understand the biological system.

Rimonabant, as the first selective cannabinoid-1 (CB_1) receptor blocker, was proved to lead to reduced food intake, long-term maintained weight loss, and improved cardiovascular risk factors, manifesting as elevated plasma HDL-C, reduced plasma TG and inhibited insulin resistance in obese subjects [24-26]. In 2008 the European Medicines Agency withdrew the drug from the market in countries where it was commercially approved and marketed because of the psychiatric side-effects (e.g. depression and even suicide attempt) [27]. The aim of the current study was to unravel the underlying effects of rimonabant on plasma and hepatic lipid metabolism in stages of early obesity.

For this we used a double transgenic mouse model, i.e. apolipoprotein E3 Leiden cholesteryl ester transfer protein (ApoE*3Leiden.CETP) transgenic mice, that matches with human lipid metabolism as closely as possible. The presence of ApoE*3Leiden hampers the uptake of very low density lipoprotein (VLDL)-remnants by the liver thus leading to increased VLDL/LDL-C levels in the plasma. CETP is a plasma glycoprotein that is responsible for the transportation of cholesterol ester (ChoE) from HDL to apoB-containing lipoproteins (e.g. VLDL and LDL) in exchange of TG, leading to decreased HDL-C levels [28, 29]. This gene is not present in wild type mice. Since in wild type mice the plasma cholesterol (Cho) is almost completely confined to the HDL fraction while VLDL and LDL are virtually absent due to the lack of CETP, wild type mice hardly develop dyslipidemia and, as a consequence, atherosclerosis [30]. The ApoE*3Leiden.CETP mice, however, have a higher VLDL/LDL-C level and relatively low HDL-C level. Taken together, ApoE*3Leiden.CETP mice have a human-like atherogenic lipoprotein profile. They not only respond in a human-like manner to pharmaceutical interventions with respect to lipid lowering efficacy [31, 32] but also respond to HDL modulating therapy. Many studies proved that the ApoE*3Leiden.CETP transgenic mouse is a valuable model to investigate the pathogenesis of vascular atherosclerotic lesion development and the effect of combination therapies on dyslipidemia and atherosclerosis [33-38]. In this paper we reported the results of the study of large-scale lipids in plasma and liver tissues of 16 female ApoE*3Leiden.CETP mice, 8 of which were subjected to a period of 4 weeks of rimonabant intervention and 8 untreated animals.

Based on our study, we proposed that the rimonabant treatment intervention on early obesity of ApoE*3Leiden.CETP mice would affect plasma and hepatic lipid metabolism relative to the non-treated controls, leading to increased HDL-C concentrations and decreased VLDL/LDL-C levels.

Methods

Ethics statement

All animals received humane care conforming to the rules and regulations set forward by the Netherlands Law on Animal Experiments. All animal experiments were approved by an independent institutional ethical committee on animal care and experimentation (Dierexperimenten Commissie DEC of Netherlands Organization for Applied Scientific Research, Zeist, the Netherlands) with a permit No. of DEC2489.

Animals

ApoE*3Leiden.CETP transgenic mice, expressing a human CETP gene [34], were bred at TNO (Leiden). In this study, sixteen female ApoE*3Leiden.CETP mice were used. All mice were housed under standard conditions in conventional cages (4 mice per cage) with free access to water and food. At the age of 6-10 weeks, mice were fed a semi-synthetic modified Western-type diet (Hope Farms, Woerden, Netherlands) containing 15% (w/w) saturated fat, 0.2% (w/w) Cho and 40% (w/w) sucrose as described by Nishina et al [39] as a 4 weeks run-in diet in order to get mildly elevated lipid levels (plasma Cho levels of about 14-18 mmol/L) and a moderate increase in body weight. Thereafter (t = week 0), mice were matched on body weight and plasma Cho and TG levels (after 4 h fasting) and set into 2 groups. Subsequently, mice received a Western-type diet (Hope Farms, Woerden, Netherlands) without or with rimonabant (Sanofi-Aventis Netherlands B.V., Gouda, The Netherlands) at a concentration of 10 mg/kg body weight/day for a period of 4 weeks. Table 1 presents the study design and time points at which both biochemical parameter and lipidomics profiling measurements were done.

Sacrifice and Sample Collection

Animals were sacrificed with rapid asphyxiation with CO_2 and opened longitudinally after 4-week intervention experiment. Blood was collected before start of the intervention (t = week 0) and just before sacrifice (t = week 4) via tail vein bleeding into CB 300 LH microvettes (Sarstedt, Nümbrecht, Germany), containing lithium heparin and were placed on ice immediately after blood collection.

Table 1. Study design and time points at which both biological parameters and lipidomic profiling were done

Time points of experiment (week)	-4	-3	-2	-1	0	1	2	3	4
	Run-in period					Intervention period			
Group 1, control	×	→			×	→			×
Group 2, rimonabant treatment	×	→			×	→			×
Randomization					×				
Body weight and food intake					×	×	×	×	×
Plasma cholesterol and triacylglyceride					×				×
Lipoprotein profile					×				×
HDL-C measurement					×				×
CETP level & activity					×				×
Sacrifice with plasma & liver collection for lipidomics									×

Plasma samples were obtained after centrifugation of the blood samples for 10 min at 6000 rpm at 4 °C. Liver tissues were dissected on ice and immediately weighted before being snap-frozen in liquid nitrogen. Both the plasma and the tissue samples were stored at -80 °C until use.

Plasma biochemical analyses and lipoprotein profile analysis

Plasma samples collected at t = week 0 and t = week 4 were assayed for total cholesterol (TC), total triacylglycerides (TG), HDL-C and lipoprotein profile. Plasma TC and TG were quantified using the commercially available enzymatic kits 236691 and 11488872 (Roche Molecular Biochemicals, Indianapolic, IN, USA), respectively. Plasma HDL-C was quantified after precipitation of apoB-containing lipoproteins. Pooled lipoprotein profiles were measured by fast performance liquid chromatography (FPLC) using an AKTA apparatus (Amersham Biosciences). Cho, TG and Phospholipid (PL) levels were measured in the fractions of freshly obtained samples. PLs were determined in the FPLC fractions using kit “phospholipids B” (Instruchemie Co., The Netherlands).

Measurement of cholesteryl ester transfer activity in plasma

CETP level was measured in each animal using the commercially available enzymatic kit “RB-CETP” (Roar Biomedical, Inc.). The transfer of newly synthesized ChoE in plasma was assayed by a radioisotope method as described before [40]. Briefly, [^{3}H] Cho mixed with bovine serine albumin was equilibrated with plasma free Cho for 24 h at 4 °C followed by incubation for 3 h at 37 °C. Subsequently, apoB-containing lipoproteins were precipitated by addition of heparin/$MnCl_2$. Lipids were extracted from the precipitation and the labeled cholesteryl esters were separated from labeled unesterified Cho on silica columns and assayed by liquid scintillation counting.

Lipidomics analyses

Lipid extraction for plasma samples

Briefly, 30 μL of internal standard (IS) mixture containing LPC (17:0) at 1.5 μg/mL, PE (34:0) at 5 μg/mL, PC (34:0) at 5 μg/mL and TG (51:0) at 5 μg/mL in 2:1 of CH_2Cl_2/MeOH and 30 μL of IS mixture containing LPC (19:0) at 30 μg/mL, PE (30:0) at 30 μg/mL, PC (38:0) at 150 μg/mL and TG (45:0) at 60 μg/mL in 2:1 of CH_2Cl_2

/MeOH were added to 30 μL of plasma which was placed in a new 2 mL eppendorf vial (Eppendorf, Hamburg, Germany). Following this, 180 μL MeOH and 360 μL CH_2Cl_2 were successively added. Thorough vortex was performed both before and after CH_2Cl_2 addition. Subsequently, 120 μL water was added to form a two-phase system in which lipids were located in the bottom organic phase. After 10 min centrifugation at a rotation speed of 6000*g* at 10 °C, 300 μL of lipid extracts from the bottom layer were transferred into a new brown auto-sampler vial. The extracts were diluted 20 times with ACN/IPA/water (65:30:5, v/v/v) before LC-MS running.

Lipid extraction for liver samples

Sixty microliters of IS mixture containing LPC (17:0) at 1.5 μg/mL, PE (34:0) at 7.5 μg/mL, PC (34:0) at 12.5 μg/mL and TG (51:0) at 45 μg/mL in 2:1 of CH_2Cl_2/MeOH and 60 μL of IS mixture containing LPC (19:0) at 18 μg/mL, PE (30:0) at 90 μg/mL, PC (38:0) at 150 μg/mL and TG (45:0) at 480 μg/mL in 2:1 of CH_2Cl_2/MeOH were added to 10 mg of dry liver powder followed by addition of 160 μL of MeOH containing 0.02% antioxidant butylated hydroxytoluene (BHT), and then 320 μL of CH_2Cl_2 was added. The mixture was thoroughly vortexed for 1 min both before and after CH_2Cl_2 addition. After that, the resulted suspension was placed for 5 min in an ultrasonic bath at -4 °C and then placed in a shaker followed by 45 min incessantly shaking at -4 °C. A 10 min centrifugation at a rotation speed of 6000*g* at 10 °C was needed before 500 μL of the supernatant was transferred into a new 2 ml eppendorf vial. Subsequently, 100 μL of 0.9 % NaCl was added to the supernatant to give rise to a two-phase system: the nonlipid compounds were located in the upper aqueous phase, while most of the lipids were in the lower organic phase. After being centrifuged at 2000*g* for 10 min at 10 °C, a total of 300 μL of lipid extract was collected from the bottom organic phase. Diluted the lipid extracts 40× with ACN/IPA/water (65:30:5, v/v/v); 10 μL was loaded for LC-MS lipidomics analysis.

LC-MS lipid profiling

Diluted lipid extracts from both plasma and liver tissue samples were measured on a liquid chromatography-linear ion trap-Fourier transform ion cyclotron resonance-mass spectrometric (LC-FTMS) system equipped with a Surveyor HPLC MS pump, an autosampler (Thermo Fischer, San Jose, CA) and an Ascentis Express C_8 2.1 × 150 mm (2.7 μm particle size) column (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). The binary solvent consisted of water/ACN (2:3, 10 mM ammonium formate) and ACN/IPA (1:9, 10 mM ammonium formate). The LC

separation conditions were identical to those described previously [41]. The lipidomics profiling was carried out in the full ESI positive ion mode with a mass range of m/z 430-1500. The heated capillary was set at 300 °C. The voltages of the sampling cone and capillary were 3.8 kV and 48 V, respectively. The tube lens was optimized to be 140 V. Nitrogen was used as sheath gas (60 units), auxiliary gas (5 units) and sweep gas (3 units). The LC-MS data were acquired by Xcalibur (Thermo Fisher) with 200 ms maximum injection time. The number of μscans was 2. Both the ion trap and FT scan events were recorded during data acquisition.
Specifically, samples of interest (i.e. plasma or liver samples) were randomly analyzed and the quality control (QC) samples, prepared by pooling of all plasma or liver samples, were regularly placed in the measurement sequence. Of note, plasma and liver samples were analyzed separately.

Preprocessing of lipidomics data

Lipid peaks including spiked IS such as LPC (17:0), LPC (19:0), PE (34:0), PE (30:0), PC (34:0), PC (38:0), TG (51:0) and TG (45:0) were extracted based on their expected retention time and accurate masses according to an in-house lipid database using LCquan v2.5 (Thermo Fisher). The peak area of each extracted lipid ion was calibrated by an appropriate IS. Duplicate measurements were combined into a single measurement after IS calibration. Data quality was assessed by calculating the relative standard deviation (RSD) of all calibrated lipid peaks in the QC samples. Peaks with a RSD larger than 20% were excluded leaving 131 lipids in the plasma lipidomics data set and 133 lipids in the liver lipidomics data set for subsequent data analyses. General information about the lipidomics protocol was provided in the Supplementary Text, Tables and Figures as Supporting Information.

Statistical analysis

Statistical significance of biochemical parameters was analyzed by independent student t-test. Lipidomics data were first analyzed by independent student t-test and later extended with Benjamini and Hochberg multiple testing corrections. Data were expressed as mean $\pm$ SD unless otherwise stated. A value of $p < 0.05$ was considered statistically significant.
In order to visualize possible relations between the samples from treated and non-treated groups, principal component analysis (PCA) was carried out for the mean centered plus unit variance scaled plasma lipidomics data and liver lipidomics data, respectively using Matlab software (version 6.5.1, release 13, The Mathworks, 2003).

One control mouse (marked as 3733) was excluded from statistical data analyses throughout the article, because it did not respond to Western-type diet during run-in period and failed to reach hypercholesterolemia criteria essential for our experiment. We observed that the relative levels of most hepatic lipids were much lower in this mouse as compared to the other control mice. In this animal, the biochemical markers such as plasma TC, TG and liver weight were lowest among all control mice (data not shown).

Results

Food intake and body weight

The variation in food intake and body weight during the 4 weeks of intervention is shown in Figure 1 A and B, respectively. The body weight was significantly reduced in mice on rimonabant compared to control throughout the whole intervention period. In total, the weight loss was 9.4% (p = 0.03) at the end of the experiment. This decline in body weight might be explained by reduced food intake in the initial states of the experiment, although statistical significance was not reached.

Plasma cholesterol, triacylglycerides, HDL-C and lipoprotein profiles

After a period of 4 weeks of intervention, plasma TC was significantly reduced by 24% (p = 0.04) (Figure 2A) and plasma TG reached a reduction trend (e.g. 1.34 $\pm$ 0.96 vs. 2.35 $\pm$ 1.34 mM, p = 0.11) in the rimonabant group as compared to the control mice (Figure 2B). As compared to the control, we could not see a significant increase in plasma HDL-C upon rimonabant intervention (Figure 2C).
The 4-week rimonabant intervention led to decreased levels of Cho, TG and PLs in the VLDL for 1.5, 2.5 and 2 fold respectively (Figure 2 D-F) and to a slightly increased level of Cho in HDL particles (magnified part in Figure 2D). Concentrations for Cho and TG as well as PLs were unaffected in LDL particles, whereas TG and PL concentrations were unaffected in HDL particles.

Rimonabant does not significantly affect plasma CETP activity

The CETP level was constant during the intervention (Figure 3A). The four-week rimonabant intervention resulted in a non-significant change of plasma CETP activity (e.g. 90.8 $\pm$ 27.0 vs. 70.6 $\pm$ 33.3 nmol/mL/h, p = 0.22) as compared to the control (Figure 3B).

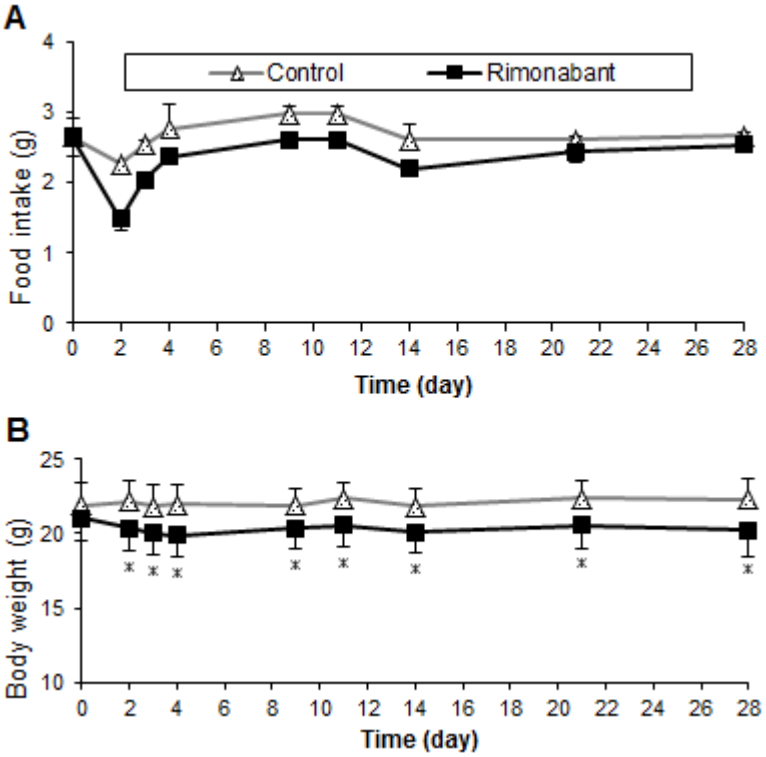

Figure 1. *Food intake and body weight. The 4-week intervention effect of rimonabant on food intake (A) and body weight (B). * P < 0.05 vs. the control. Body weight per mouse and food intake per cage were measured at day 0, 2, 3, 4, 9, 11, 14, 21 and 28 respectively. Values are expressed as means ± SD. P values correspond to the mean difference between the rimonabant group and the control group.*

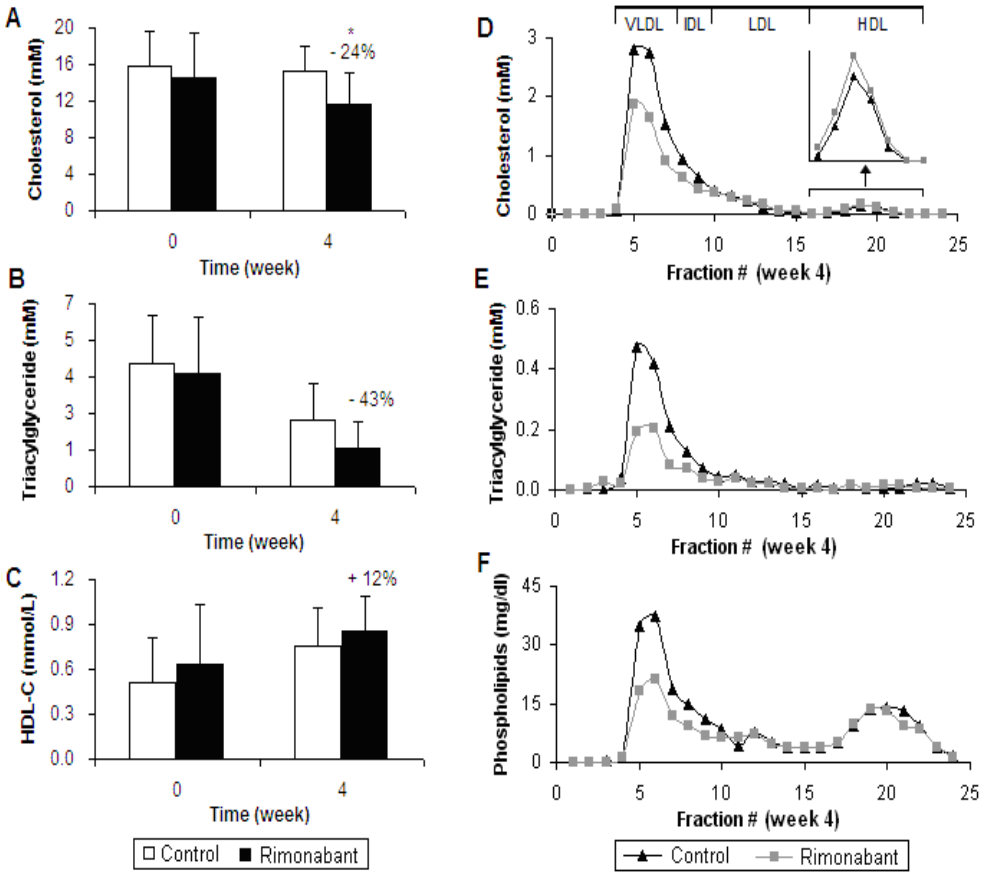

Figure 2. *Four-week intervention effect of rimonabant on plasma TC (A) and TG (B) as well as HDL-C (C) levels. Plasma TC, TG and HDL-C were measured at week 0 and 4. Values are shown as means ± SD. *P < 0.05 vs. the control. P values correspond to the mean difference between the rimonabant and the control group; (D-F) alterations of Cho and TG as well as PLs in the pooled lipoprotein profiles on the rimonabant treatment as compared to the controls. Fractions 4-7 represent VLDL; fractions 8-9 represent intermediate-density lipoprotein (IDL); fractions 10-15 represent LDL; fractions 16-23 represent HDL.*

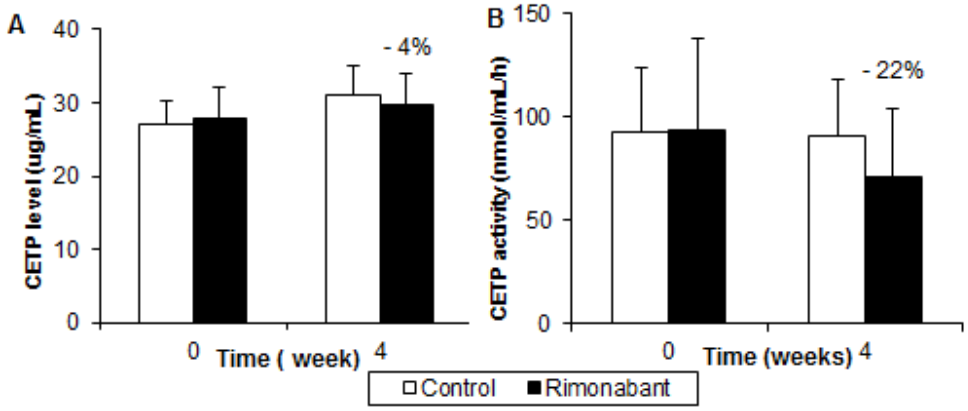

Figure 3. *Rimonabant does not significantly affect plasma CETP activity. Effect of rimonabant on plasma CETP level (A) and CETP activity (B) in ApoE*3Leiden.CETP mice at time points of t = week 0 and 4; (white bars: the control group; black bars: the rimonabant group). Values are means ± SD. There were no statistically significant changes found in CETP level and CETP activity during the intervention treatment.*

Lipidomics reveals differences between nontreatment and rimonabant treatment mice for both plasma and liver samples

To get an overview of existing patterns in the lipidomics data such as clusters of mice of nontreated controls and mice undergoing rimonabant treatment and which lipids contributed most to these clusters, we performed PCA for the plasma and liver lipidomics datasets, respectively. Figure 4 displays the PCA biplots (A, plasma samples; B, liver samples). In both plasma and liver the rimonabant treated group was separated well from the control group. Two rimonabant treated mice (marked as 3736 and 3758) deviated from the others within the group causing some overlap with the mice from the control liver group. The deviations of these two mice from other group members were further checked with data from biological parameters and lipidomics. The biological parameters revealed that among all rimonabant treated mice the liver weights of these two mice were the heaviest and the total plasma TG levels were the lowest (data not shown). The liver lipidomics data showed that the TG levels were more abundant in these two mice than other rimonabant treated mice. In addition, the loadings in the biplot (lipid species represented by colored symbols in Figure 4A and B) indicated that TG lipid species dominated the differentiation between non-treated controls and the rimonabant-treated mice for both plasma and liver lipidomics data sets.

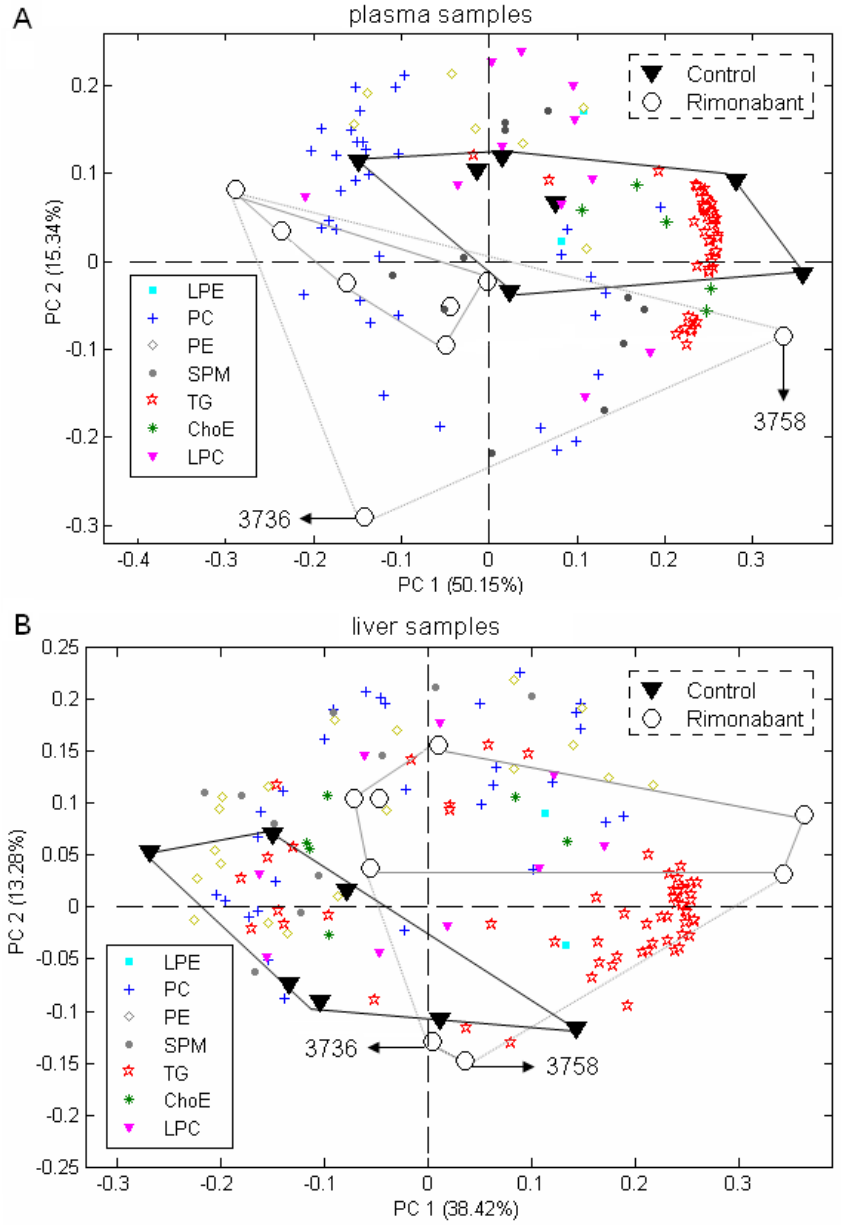

Figure 4. *PCA biplots for the first two principal components of lipid profiling of plasma samples (A) and liver samples (B) in control animals (n = 7) and animals treated with rimonabant (n = 8). Both PCA models used mean centred plus unit variance scaled data.*

Rimonabant significantly affects individual lipids in both plasma and liver of treated mice

To investigate quantitative changes of lipids in the rimonabant intervention group as compared to the control, statistical significance for the mean difference of all identified lipids between the rimonabant group and the control group was tested using independent student t-test. It was found that 33 plasma and 25 liver lipid species out of 131 and 133 lipids respectively were significantly changed after the 4-week rimonabant intervention as compared to the controls. Four lipids changed significantly upon rimonabant treatment in both plasma and liver, i.e. PE (36:3), TG (50:1), TG (52:2) and TG (56:7). Within these perturbed lipids, one interesting observation was that 31 out of 33 lipid species were significantly *decreased* in plasma samples whereas 22 out of 25 lipid species were significantly *increased* in liver samples in the rimonabant group versus the controls (Table 2 and 3).
Remarkably, after multiple testing correction only 6 plasma lipids (LPC-18:1, LPC-18:2, LPE-20:4, PC-38:2, PE-38:2 and SPM-16:0) and 3 liver lipids (PC-40:4, ChoE-16:1 and TG-56:6) remained significant out of 33 and 25 plasma and liver lipids respectively (lipids with *p* values marked in bold in Table 2 and 3).

Rimonabant significantly decreases the overall responses of plasma lipid classes

The summation of the individually measured lipids into different lipid classes showed for plasma concentrationsa significant reduction of PE with 20% ($p = 0.02$), ChoE with 22% ($p = 0.02$) and TG with 46% ($p = 0.04$) in rimonabant treated mice vs. the control (white bar graph in Figure 5); the summation of individually measured lipids into lipid classes of LPC, PC and SPM in plasma of mice receiving rimonabant treatment were comparable to those in untreated controls under the current LC-MS conditions. In total, the level of phospholipids in plasma samples was comparable in rimonabant treated mice vs. the control (data not shown). In liver tissues, none of the summation of the individually measured lipids within each of lipid classes was significantly changed after the rimonabant intervention in relation to the control (black bar graph in Figure 5).

Table 2. Significantly influenced lipid molecular species in plasma upon rimonabant treatment as compared to non-treated controls

Table 2.1 LPC, PC, PE, SPM and ChoE

Lipid species	**control (mean ± SD)**	**rimonabant (mean ± SD)**	**rimonabant vs. control change (%)**	***p* value**	**(↑) (↓)**
LPC (18:1)	0.2896 ± 0.0146	0.2414 ± 0.032	83	**0.0030****	↓
LPC (18:2)	0.1402 ± 0.0105	0.1069 ± 0.0138	76	**0.0002*****	↓
LPC (20:3)	0.0264 ± 0.0023	0.0219 ± 0.0034	83	0.0109*	↓
LPE (20:4)	0.0022 ± 0.0001	0.0017 ± 0.0002	76	**0.0001*****	↓
PC (36:2)	0.0848 ± 0.0088	0.0738 ± 0.0059	87	0.0133*	↓
PC (38:2)	0.5067 ± 0.0589	0.3773 ± 0.0624	74	**0.0012****	↓
PE (36:3)	0.7573 ± 0.1050	0.6087 ± 0.1137	80	0.0214*	↓
PE (38:2)	0.2678 ± 0.0457	0.2042 ± 0.0205	76	**0.0035****	↓
PE (38:4)	0.1453 ± 0.0243	0.1126 ± 0.0146	77	0.0068**	↓
SPM (16:0)	0.0046 ± 0.0003	0.0054 ± 0.0004	118	**0.0006*****	↑
SPM (18:0)	0.0008 ± 0.0001	0.0010 ± 0.0001	122	0.0102*	↑
ChoE (18:1)	0.1869 ± 0.0171	0.9168 ± 0.2101	77	0.0180*	↓

*$P < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the contrl.

P values correspond to the mean difference between the rimonabant group and the control group.

Note: lipids with *p* values marked in bold mean those remain significant after multiple testing correction.

Table 2.2 TG's

Lipid species	**control (mean ± SD)**	**rimonabant (mean ± SD)**	**rimonabant vs. control change (%)**	***p* value**	**(↑) (↓)**
TG (44:0)	0.0169 ± 0.0009	0.0151 ± 0.002	89	0.0437*	↓
TG (46:0)	0.0328 ± 0.0037	0.0273 ± 0.0047	83	0.0259*	↓
TG (46:1)	0.0127 ± 0.0038	0.0078 ± 0.0033	62	0.0198*	↓
TG (48:0)	0.0167 ± 0.0057	0.009 ± 0.0056	54	0.0202*	↓
TG (48:1)	0.0084 ± 0.0028	0.0048 ± 0.0032	57	0.0387*	↓
TG (48:2)	0.1037 ± 0.0302	0.0558 ± 0.0411	54	0.0247*	↓
TG (50:0)	0.0493 ± 0.0196	0.0251 ± 0.0197	51	0.0329*	↓
TG (50:1)	0.1568 ± 0.0685	0.0805 ± 0.0631	51	0.0429*	↓
TG (52:2)	1.0304 ± 0.4185	0.5248 ± 0.4136	51	0.0353*	↓
TG (52:6)	0.0025 ± 0.0008	0.0013 ± 0.001	52	0.0298*	↓
TG (54:2)	0.3189 ± 0.1698	0.1508 ± 0.1247	47	0.0459*	↓
TG (54:3)	0.9160 ± 0.3800	0.4568 ± 0.3700	50	0.0341*	↓
TG (54:4)	0.1780 ± 0.0750	0.0921 ± 0.0693	52	0.0382*	↓
TG (56:3)	0.1001 ± 0.0481	0.0453 ± 0.0314	45	0.0200*	↓
TG (56:4)	0.0765 ± 0.0328	0.0396 ± 0.0253	52	0.0289*	↓
TG (56:7)	0.3749 ± 0.1634	0.2055 ± 0.134	55	0.0459*	↓
TG (58:3)	0.0057 ± 0.0028	0.0029 ± 0.0017	52	0.0367*	↓
TG (58:4)	0.0051 ± 0.0022	0.0028 ± 0.0014	55	0.0290*	↓
TG (58:5)	0.0155 ± 0.0069	0.0084 ± 0.005	54	0.0384*	↓
TG (58:6)	0.0162 ± 0.0055	0.0098 ± 0.0044	60	0.0253*	↓
TG (58:7)	0.0264 ± 0.0091	0.016 ± 0.0078	60	0.0322*	↓

*$P < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the contrl.

P values correspond to the mean difference between the rimonabant group and the control group. *Note*: lipids with *p* values marked in bold mean those remain significant after multiple testing correction.

Table 3. Significantly influenced lipid molecular species in liver tissue upon rimonabant treatment as compared to non-treated controls

Lipid species	**control (mean ± SD)**	**rimonabant (mean ± SD)**	**rimonabant vs. control change (%)**	**p value**	**(↑) (↓)**
PC (38:4)	2.160 ± 0.299	2.563 ± 0.319	119	0.0262*	↑
PC (40:4)	0.157 ± 0.012	0.196 ± 0.024	125	**0.0018****	↑
PC (40:5)	0.834 ± 0.069	1.052 ± 0.184	126	0.0113*	↑
PC (40:6)	0.546 ± 0.048	0.668 ± 0.086	122	0.0054**	↑
PC (40:8)	0.272 ± 0.040	0.225 ± 0.036	83	0.0318*	↓
PE (34:1)	0.254 ± 0.038	0.308 ± 0.055	121	0.0484*	↑
PE (36:3)	0.529 ± 0.126	0.418 ± 0.066	79	0.0493*	↓
PE (38:6)	1.474 ± 0.079	1.785 ± 0.341	121	0.0355*	↑
PE (40:6)	0.563 ± 0.057	0.711 ± 0.125	126	0.0133*	↑
ChoE (16:1)	0.628 ± 0.232	1.078 ± 0.160	172	**0.0007*****	↑
ChoE (22:6)	0.043 ± 0.008	0.054 ± 0.005	127	0.0054**	↑
TG (50:1)	1.357 ± 0.363	1.773 ± 0.362	131	0.0450*	↑
TG (50:2)	1.779 ± 0.570	2.497 ± 0.636	140	0.0396*	↑
TG (50:3)	0.347 ± 0.132	0.581 ± 0.211	167	0.0251*	↑
TG (52:2)	7.234 ± 1.170	8.572 ± 1.160	118	0.0448*	↑
TG (52:4)	0.462 ± 0.116	0.679 ± 0.229	147	0.0411*	↑
TG (52:5)	0.526 ± 0.165	0.840 ± 0.346	160	0.0478*	↑
TG (54:5)	0.354 ± 0.078	0.504 ± 0.136	143	0.0235*	↑
TG (54:6)	0.616 ± 0.181	1.021 ± 0.305	166	0.0091**	↑
TG (54:7)	0.078 ± 0.025	0.141 ± 0.058	180	0.0195*	↑
TG (56:5)	0.517 ± 0.100	0.687 ± 0.151	133	0.0250*	↑
TG (56:6)	0.135 ± 0.027	0.209 ± 0.033	155	**0.0005*****	↑
TG (56:7)	1.047 ± 0.281	1.562 ± 0.558	149	0.0461*	↑
TG (56:8)	0.168 ± 0.049	0.274 ± 0.108	163	0.0326*	↑
TG (60:1)	0.015 ± 0.002	0.012 ± 0.002	83	0.0352*	↓

*$P < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the contrl.

P values correspond to the mean difference between the rimonabant group and the control group.

Note: lipids with *p* values marked in bold mean those remain significant after multiple testing correction.

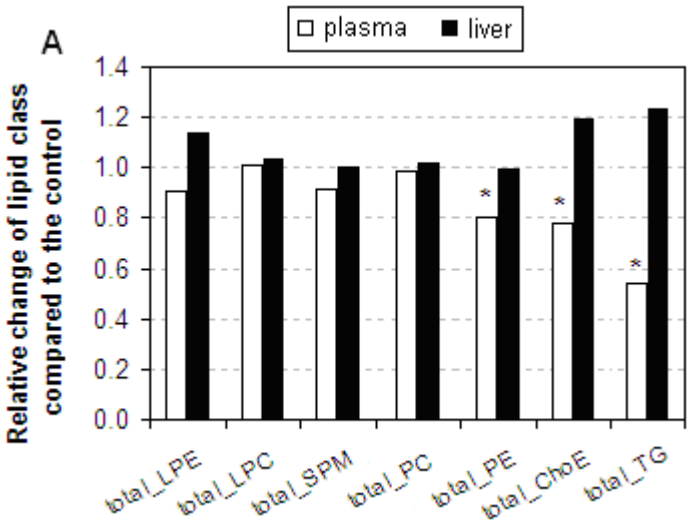

Figure 5. *Relative change of the summation of the individually measured lipids in different lipid classes based on the present LC-MS lipidomics data acquired from plasma and liver samples in the rimonabant group vs. the control. The summation of the individually measured lipids in each lipid class in the control is set to be 1. * P < 0.05 vs. the control. P values correspond to the mean difference between the rimonabant group and the control group.*

Discussion

Rimonabant, a selective CB_1 receptor antagonist, is known for reducing body weight and improving cardiovascular risk factors in obese subjects[24, 26, 42-44]. However, studies of the obesity regulation effects of rimonabant mainly focus on the plasma/serum biochemical and lipid profile [45-47]. Less is known about the regulation of individual plasma and hepatic lipid species upon rimonabant treatment in obese subjects.

The present study describes the rearrangement and relocalisation of lipids in an early obese ApoE*3Leiden.CETP mouse model with humanized lipoprotein metabolism exposed to a 4-week rimonabant intervention. Lipoprotein profiling and lipidomics approaches were used for this purpose.

Throughout the experiment, food intake upon rimonabant dropped sharply during the first two days but quickly recovered to some extent during the first 3 weeks; since day 21, food intake has returned to nearly the levels in identical high-fat fed

mice without rimonabant treatment. Although the rapid return to high energy intake within 3 weeks, the rimonabant group exhibited a significant and maintained weight reduction throughout the intervention period as compared with the non-treated controls. Collectively, this body weight reduction may be attributed to 1) the transient reduction in energy intake; 2) a reduction in energy efficiency (i.e. wastage of energy) caused by interruption of cannabinoid signalling upon rimonabant treatment [48]. Notably, the 9.4% body weight reduction induced by rimonabant treatment in our study seems not to be comparable with the at least 20% body weight-loss induced by rimonabant reported in literature for obese mice and humans [43, 44]. However, it should not be neglected that the mouse model used was in the early stage of obesity with only mildly increased body weight and plasma Cho.

Rimonabant intervention studies both in rodents and humans [24, 26, 45, 47, 49] have shown significant reduction of plasma TC and TG and a significant increase in HDL-C concentrations. Our 4-week rimonabant treatment of ApoE*3Leiden.CETP transgenic mice in early stage of obesity showed similar reduction of plasma TC and a reduction trend of plasma TG (Figure 2A and B). However, the treatment did not significantly increase levels of HDL-C (Figure 2C), which was probably due to our short intervention period combined with an early stage of obesity in our animal model. In line with these observations, plasma Cho and TG in VLDL were reduced, whereas Cho in HDL was slightly increased after the rimonabant intervention (Figure 2D and E). These effects may be mediated via adiponectin, a major adipocyte cytokine involved in the regulation of hyperglycaemia, hyperinsulinaemia, and fatty acid oxidation at the peripheral adipocyte level [42, 50, 51]. Adiponectin release from adipocytes is known to be regulated by inhibition of CB_1 [43, 52]. Furthermore, no significant reduction effect of rimonabant on CETP activity and levels was found in the ApoE*3Leiden mouse model.

The LC–MS-based plasma lipidomics analysis revealed a significant decrease of a large number of plasma lipids (Table 2) and a significant reduction in plasma PE, ChoE and TG lipid classes (Figure 5) after rimonabant intervention, indicating the beneficial effect of rimonabant on plasma lipid metabolism in early stage of obesity. Specifically, as ChoE, TG and phospholipids (e.g. PE) are the main components of VLDL particles, their reduction in plasma indicated a lower circulated VLDL induced by rimonabant, which is in line with the result of plasma lipoprotein profiles, i.e., the VLDL fractions of Cho, TG and PLs were all reduced in rimonabant group (Figure 2D-F). Evidence from animal studies and clinical trials indicated that the beneficial metabolic effects of rimonabant on plasma/serum lipid profiles are caused by the absence of basal endocannabinoid signaling, leading to reduced energy efficiency of food [24, 48, 53]. In addition, after

multiple testing corrections six plasma lipid species showed a *most* significant reduction in response to rimonabant except for SPM (16:0) which increased. Although it is unknown if these lipid compounds would be useful for understanding the pharmacological manipulation of rimonabant on improving obesity-related metabolic abnormalities under current experimental conditions, it may give hints point to an important role of individual lipid molecular species in rimonabant's management of obesity.

It is recognized that high-fat diet-induced obesity is highly associated with fatty liver due to the expression of hepatic CB_1 receptor [49, 54]. Liver-specific deletion of CB_1 or blockage of CB_1 receptor was frequently used to protect against obesity-associated hepatic steatosis [45, 49, 53]. The results from available studies suggest that rimonabant, as a CB_1 receptor antagonist, potentially has clinical applications in the treatment of high-fat diet-induced liver diseases [45, 53]. In the present study, down-regulation of lipids was not observed in liver tissues as in plasma after rimonabant treatment. It could be owing to the early stage of obesity in our animal model, which displays a mild increase in body weight and moderately elevated plasma Cho levels (14-18mmol/L). It also needs to be noted that our animal model is very different from those used in literature to investigate rimonabant's management in high-fat diet-induced liver disease. Our ApoE*3Leiden.CETP mice express a natural mutation of the human APOE3 gene in addition to the human APOC1 gene. Introduction of these genes induces an attenuated clearance of apoB-containing lipoproteins via the LDL receptor pathway [34, 55]. Mice with such genetic background show mildly increased Cho and TG levels on a chow diet and a human-like lipoprotein profiles on high fat diet [56]. Taken collectively, we proposed that 4-week rimonabant treatment has moderate effect on liver lipid metabolism in the early stage of obesity of ApoE*3Leiden.CETP mice under current experimental conditions.

This study shows that LC-MS lipidomics approaches hold promises for searching potential lipid biomarkers in relation to disease prevention and health promotion. Moreover, it was demonstrated that a 4-week rimonabant intervention improves body weight and cardiovascular risk factors during the early stage of obesity in ApoE*3Leiden.CETP mice. Finding of only limited amount of significant lipid changes may be attributed to the early stage of obesity in the animal model used. Taken together, it indicates that the effects of rimonabant on body weight and cardiovascular risk factors are moderate in the case of early stage obesity.

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Supporting Information

Materials

Synthetic lipid standards including lyso-phosphocholine (LPC-17:0, LPC-19:0), phosphatidylethanolamine (PE-30:0, PE-34:0), phosphatidylcholine (PC-34:0, PC-38:0) and triglyceride (TG-45:0, TG-51:0) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). ULC-MS grade of acetonitrile (ACN), methanol (MeOH), isopropanol (IPA), and water as well as LC-MS grade of dichloromethane (CH_2Cl_2) were obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium formate (AmFm, 99.995%) was of LC-MS grade and purchased from Sigma-Aldrich Chemie (St. Louis, MO, USA). Rimonabant was purchased from Sanofi-Aventis Netherlands B.V. (Gouda, The Netherlands).

Lipid standards for plasma lipidomics profile and liver lipidomics profile

In brief, stock solutions of 8 lipid standards from 4 different lipid classes were separately prepared in a CH_2Cl_2/MeOH mixture (2:1, v/v) for all standards except TG by weighing an exact amount of each lipid standard in new glass autosampler vials and stored at -20°C until further use. The TG standard was dissolved in CH_2Cl_2 instead of a CH_2Cl_2/MeOH mixture due to its weak polarity.
For LC-MS lipidomics analysis of study samples, the stock solutions were separated into two sets. Set 1 consist of LPC (19:0), PC (38:0), PE (30:0), and TG (45:0), which was used for quantification of less intermediate $\rightarrow$ high abundant lipids in the samples; set 2 consist of LPC (17:0), PC (34:0), PE (34:0) and TG (51:0), which was used for quantification of very low $\rightarrow$ low abundant lipids in the samples. The final working solution for each set was prepared by pipetting a certain volume of corresponding stock solutions, "thawed" to room temperature followed by thorough vortex, into a new glass autosampler vial and diluting it to appropriate concentrations with a CH_2Cl_2 /MeOH mixture (2:1, v/v). Detailed information for these standards is provided in Supplementary Tables S1.
For method validation of LC-MS liver lipidomics profile, two sets of standard mixture were also used. Set 1 was used as internal standard (I.S.) mixture, consisting of LPC (17:0) at 30 μg/mL, PC (34:0) at 180 μg/mL, PE (34:0) at 120 μg/mL, and TG (51:0) at 90 μg/mL in a CH_2Cl_2 /MeOH mixture (2:1, v/v); and set 2 was used as "validation standard mixture" containing LPC (19:0), PC (38:0), PE (30:0), and TG (45:0) at the following C_0-C_8 concentration levels: LPC (19:0) at 0, 0.1, 0.5, 1, 2.5, 10, 30, 90, 180 μg/mL; PC (38:0) at 0, 0.5, 2.5, 5, 12.5, 50, 150, 450, 900 μg/mL; PE (30:0) and TG (45:0) at 0, 0.3, 1.5, 3, 7.5, 30, 90, 270, 540 μg/mL.

From C_1 to C_8, the concentration was gradually increased, i.e., working solution of C_8 was prepared first and then gradually diluted towards C_7–C_1. Three different concentrations (e.g. C_4, C_6 and C_8, corresponding to low, medium and high concentration levels, respectively), were selected for evaluation of repeatability and calculation of recovery.

Table S1.Information of 8 lipid internal standards used in lipidomics analyses.

Lipid Standard	monoisotopic mass (m/z)	Ion adduct	plasma lipidomics		liver lipidomics	
			stock (mg/ml)	working (μg/ml)	stock (mg/ml)	working (μg/ml)
LPC (17:0)	510.3557	$[M + H]^+$	0.75	6	0.75	6
PE (34:0)	720.5583	$[M + H]^+$	1.05	20	1.80	30
PC (34:0)	762.6025	$[M + H]^+$	1.20	20	1.50	50
TG (51:0)	866.8209	$[M + NH_4]^+$	1.25	20	2.25	180
LPC (19:0)	538.3880	$[M + H]^+$	0.90	120	0.75	72
PE (30:0)	664.4927	$[M + H]^+$	2.50	120	2.45	360
PC (38:0)	818.6658	$[M + H]^+$	3.50	600	3.75	600
TG (45:0)	782.7264	$[M + NH_4]^+$	2.50	240	4.20	1920

Liver samples for method validation of LC-MS liver lipidomics profile

Liver tissues from five healthy male mice at age of 8-12 weeks with C57BL/6 background were used for method validation of LC-MS liver lipidomics profile. These samples were kindly provided by Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University (The Netherlands).

Liver sample pre-processing

The frozen liver samples stored at -80°C were immediately put into a lyophilizer after taken out from the freezer and lyophilized for 48 hours for the purpose of full dryness. After lyophilization, the liver was placed on clean aluminum foil followed by folding of the aluminum foil in order to cover the liver within it. A hammer was then used to triturate the crisp dry liver, wrapped inside the aluminum foil. Ten milligrams of liver powder was weighed in a clean 1.5 mL eppendorf vial for the subsequent lipid extraction. Notably, the triturated liver tissues from 5 mice used for method validation were mixed homogeneously before use.

Lipid extraction for method validation of liver lipid profiling

i) Spiking before sample preparation. Sixty microliters of I.S. mixture and 60 μL of the validation standard mixture were added to 10 mg of dry liver powder followed by addition of 160 μL of MeOH containing 0.02% antioxidant butylated hydroxytoluene (BHT), and then 320 μL of CH_2Cl_2 was added. The mixture was vortexed for 1 min both before and after CH_2Cl_2 addition. After that, the resulted suspension was placed for 5 min in an ultrasonic bath at -4°C and then placed in a shaker followed by 45 min incessantly shaking at -4°C. Then 10 min centrifugation at a rotation speed of 6000*g* at 10°C was needed before 500 μL of the supernatant was transferred into a new 1.5 ml eppendorf vial. 100 μL of 0.9 % NaCl was subsequently added to the supernatant to give rise to a two-phase system: the nonlipid compounds were located in the upper aqueous phase, while most of the lipids were in the lower organic phase. After being centrifuged at 2000*g* at 10 °C for 10 min, a total of 300 μL of lipid extract was collected from the bottom organic phase followed by addition of 60 μL of a CH_2Cl_2/MeOH mixture (2:1, v/v). Diluted the lipid extracts 40× with ACN/IPA/water (65:30:5, v/v/v); 10 μL was loaded for LC-MS lipidomics analysis. ii) Spiking after sample preparation. The same procedures as described for spiking before sample preparation were conducted except that 60 μL of 2:1 CH_2Cl_2/MeOH instead of 60 μL of validation standard mixture was added to 10 mg dry liver powder before sample preparation, while 60 μL of the validation standard mixture instead of 60 μL 2:1 CH_2Cl_2/MeOH was added into the collected 300 μL of liver lipid extract.

QC samples

For the purpose of monitoring the LC-MS response in time and assessing data quality, quality control (QC) samples, were obtained by pooling all plasma or liver samples. Sample preparation for QC samples was conducted as the same manner as study samples of interest. The number of QC samples (number of aliquots of pooled plasma or pooled liver samples) needed depends on the size of the study. In the present study, two injections of one QC sample are performed after every 6^{th} study sample injections.

LC-MS chromatography

Lipid extracts were performed on a liquid chromatography-linear ion trap-Fourier transform ion cyclotron resonance-mass spectrometric (LC-FTMS) system equipped with a Surveyor HPLC MS pump, an autosampler (Thermo Fischer, San Jose, CA) and an Ascentis Express C8 2.1 × 150 mm (2.7 μm particle size) column (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). The binary gradient

system consisted of water/ACN (2:3, 10 mM ammonium formate) and ACN/IPA (1:9, 10 mM ammonium formate). The performance of the elution for the gradient was identical to that reported before [41]. The flow rate was 0.26 mL/min. The column oven was maintained at 55 °C and the temperature of the autosampler tray was 12 °C. The lipidomics profiling was carried out on Thermo Fischer linear ion trap-Fourier transform ion cyclotron resonance-mass spectrometry in the full ESI positive scan mode with a mass range of m/z 430-1500. IS of PC (34:0) was used to tune the mass spectrometry for optimizing the MS parameters. During tuning, 1 μg/mL of PC (34:0) at a flow rate of 10 μL/min from a Hamilton syringe pump and 30% A/70% B of elution at a flow rate of 250 μL/min from the Surveyor MS pump were continuously flowing into the LC-MS system. Finally, the optimized MS parameters are as following: the heated capillary was at 300°C. The voltages of the sampling cone and capillary were 3.8 kV and 48 V, respectively. The tube lens was 140 V. Nitrogen was used as sheath gas (60 units), auxiliary gas (5 units) and sweep gas (3 units). The LC-MS data were acquired by Xcalibur (Thermo Fisher) with 200 ms maximum injection time. The number of μ scans was 2. Both the ion trap and FT scan events were recorded during data acquisition.

Data processing

Lipid peaks including spiked internal standards (IS) were extracted based on their expected retention time and accurate masses according to an in-house lipid database using LCquan v2.5 (Thermo Fisher). The extracted lipid peaks were initially integrated by LCquan and manually corrected if needed. The parameters for the integration were peak detection algorithm, ICIS; smoothing points, 7; window, 60 s; view width, 3 min; baseline, 100; area noise factor, 5; peak noise factor, 10. The LCquan pre-processed data were exported as an Excel report. The standards, used for quantification, were picked out from the pre-processed Excel data sheet and the relative standard deviation (RSD) of their absolute peak areas from all injected samples was subsequently calculated to check the stability of the LC-MS system during the measurement. Lipids detected in study samples were quantified as the peak area ratios of lipids in study samples to lipid standards. Supplementary Table S2 showed the spiked concentration of 8 standards, the quantification strategies and RSD of absolute peak area of spiked standard from all injected samples. After quantification, duplicate measurements were combined into a single measurement. And then data quality was assessed by calculating the RSD of the peak area ratios of lipids in study samples to appropriate lipid standards in all QC samples. The summary of the calculation results is presented in Supplementary Table S3. Peaks with a %RSD more than 20% were excluded leaving 131 lipids in plasma lipidomics data set and 133 lipids in liver lipidomics data set

for further data analysis.
The lipidomics datasets were first analyzed by independent student t-test and late extended with Benjamini and Hochberg multiple testing corrections. $P < 0.05$ was considered to be statistically significant. In order to visualize possible relations between the samples from treated and non-treated groups, principal component analysis (PCA) was carried out for the mean centered plus unit variance scaled plasma lipidomics data set and liver lipidomics data set, respectively using Matlab software (version 6.5.1, release 13, The Mathworks, 2003).

Table S2. The spiked concentrations of 8 standards used in lipidomics analyses and quantification strategies used for LC-MS lipidomics data analyses.

Internal standards	Spiked conc. (μg/mL)		Quantified lipids	Quantified lipid abundance	% RSD	
	plasma	liver			plasma	liver
LPC (17:0)	1.5	1.5	LPC, LPE	very low → low	13.6	14.7
PE (34:0)	5	7.5	PE		23.9	26.3
PC (34:0)	5	12.5	PC, SM		9.0	14.8
TG (51:0)	5	45	ChoE, TG		12.2	13.0
LPC (19:0)	30	18	LPC	less intermediate → high	8.3	14.4
PE (30:0)	30	90	PE		9.3	16.0
PC (38:0)	150	150	PC, SM		10.8	13.6
TG (45:0)	60	480	TG		11.0	11.4

Table S3. The RSD of the peak area ratios of lipids in study samples to corresponding lipid standards calculated in all QC samples

Plasma lipidomics dataset		Liver lipidomics dataset	
% RSD	Number of peaks	% RSD	Number of peaks
0 - 5	45	0 - 5	71
5 - 10	56	5 - 10	47
10 - 15	19	10 - 15	8
15 - 20	11	15 - 20	7
> 20	1	> 20	3

Chapter 4

Lipidomics reveals multiple pathway effects of a multi-components preparation on lipid biochemistry in ApoE*3Leiden.CETP mice

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Abstract

Causes and consequences of the complex changes in lipids occurring in the metabolic syndrome are only partly understood. Several interconnected processes are deteriorating, which implies that multi-target approaches might be more successful than strategies based on a limited number of surrogate markers. Preparations from Chinese Medicine (CM) systems have been handed down with documented clinical features similar as metabolic syndrome, which might help developing new intervention for metabolic syndrome. The progress in systems biology and specific animal models created possibilities to assess the effects of such preparations. Here we report the plasma and liver lipidomics results of the intervention effects of a preparation SUB885C in apolipoprotein E3 Leiden cholesteryl ester transfer protein (ApoE*3Leiden.CETP) mice. SUB885C was developed according to the principles of CM for treatment of metabolic syndrome. The cannabinoid receptor type 1 blocker rimonabant was included as a general control for the evaluation of weight and metabolic responses.

ApoE*3Leiden.CETP mice with mild hypercholesterolemia were divided into SUB885C-, rimonabant- and non-treated control groups. SUB885C caused no weight loss, but significantly reduced plasma cholesterol (−49%, p <0.001), CETP levels (−31%, p <0.001), CETP activity (−74%, p <0.001) and increased HDL-C (39%, p <0.05). It influenced lipidomics classes of cholesterol esters and triglycerides the most. Rimonabant induced a weight loss (−9%, p <0.05), but only a moderate improvement of lipid profiles. *In vitro,* SUB885C extract caused adipolysis stimulation and adipogenesis inhibition in 3T3-L1 cells.

SUB885C, a multi-components preparation, is able to produce anti-atherogenic changes in lipids of the ApoE*3Leiden.CETP mice, which are comparable to those obtained with compounds belonging to known drugs (e.g. rimonabant, atorvastatin, niacin). This study successfully illustrated the power of lipidomics in unraveling intervention effects and to help finding new targets or ingredients for lifestyle-related metabolic abnormality.

Introduction

The incidence of lifestyle-related cardiovascular and metabolic health complications, often collectively named metabolic syndrome, continues to increase world-wide [1, 2]. Although the major risk factors, including a sedentary lifestyle, overweight, unfavorable dietary habits and smoking are essentially modifiable, lifestyle measures often prove difficult and disappointing on the longer term. Because of the complex and multi-factorial manifestations of the metabolic syndrome, pharmacological strategies for primary prevention are increasingly focusing on the use of low-dose drug combinations. An example is the development of "polypill" concepts with a statin, one or more anti-hypertensive compounds and acetylsalicylic acid to reduce risks for cardiovascular disease in middle-aged individuals [3, 4]. At the same time it has been shown that dietary measures may be of comparable efficacy. Such a "polymeal" could provide a "more natural, safer and probably tastier alternative" than a polypill [5]. New insights and leads for dietary prevention or intervention can also be acquired from other healthcare systems like Chinese Medicine (CM). In CM [6], the gap between food and drugs has always been small, and nutrition is seen as a normal part of prevention and healthcare. A remarkable high number of preparations have been handed down over the centuries with documented activity related to clinical features of what is now described as metabolic syndrome. The possibilities to analyze the subtle and multiple-pathway effects of such preparations have increased by the developments in systems biology-based metabolomics and specific animal models [7]. Here we report the plasma and liver lipidomic analysis of the effects of a CM preparation, SUB885C, in apolipoprotein E3 Leiden cholesteryl ester transfer protein (ApoE*3Leiden.CETP) transgenic mice. SUB885C is a multi-components preparation developed according to the principles of CM containing eight natural ingredients. The core formula is used in China for treatment of metabolic syndrome and early stage type 2 diabetes with obesity. A SUB885C intervention study [7] in prediabetic ApoE*3 Leiden mice has shown that SUB885C significantly improved insulin sensitivity as compared with non-treated controls. Meanwhile, several other anti-inflammatory and metabolic effects of the active ingredients in SUB885C have been reported [8-12]. Therefore, we hypothesized that SUB885C exerts a multi-target activity on lipid metabolism and insulin sensitivity. To investigate this, a parallel controlled intervention study was designed with female ApoE*3Leiden.CETP transgenic mice [13-16] showing mild hypercholesterolemia and overweight. The ApoE*3Leiden.CETP mouse model is obtained by cross-breeding ApoE*3Leiden mice with mice expressing human CETP. It has been shown to respond in a human-like manner to both lipid-lowering and high density lipoprotein cholesterol (HDL-C) raising interventions [13-19].

Outcome parameters included body weight, food intake, plasma lipids and lipoproteins, and lipidomics of plasma and liver. Lipidomics measures all or subsets of lipids and provides a thorough perspective to study intervention induced lipid changes and metabolism in the complex biological system [7, 20].The cannabinoid receptor type 1 (CB1) blocker rimonabant was included as a general control for the evaluation of weight and metabolic responses in the study. To further explore our findings, cell-based assays in 3T3-L1 adipocytes focusing on adipolytic and adipogenic activities of SUB885C were performed.

Materials and Methods

Ethics statement

The experiments were performed according to the rules set by the Netherlands Law on Animal Experiments and approved by the Institutional Ethical Committee on Animal Care and Experimentation (Dierexperimenten Commissie DEC of Netherlands Organization for Applied Scientific Research, Zeist, the Netherlands) with a permit number of DEC2489.

Materials and Chemicals

Materials used for intervention studies

SUB885C was provided by SU Biomedicine, The Netherlands. SUB885C consists of eight natural ingredients: *Fructus Crataegi* (Shan Zha), *Folium Nelumbinis* (He Ye), *Folium Apocyni* (Luo Bu Ma Ye), *Flos Rosae rugosae* (Mei Gui Hua), *Radix et Rhizoma Rhei* (Da Huang), *Depuratum mirabilitum* (Mang Xiao, also known as mirabilite or Glauber's salt), *Thallus Sargassi* (Hai Zao*)*, and honey fried *Radix Glycyrrhizae* (Gan Cao). Above dry and sliced compounds were used for a water based decoction. After decoction and water solvent was dried into solid phase, it was used as the preparation for the intervention study. For the quality control of the preparation, the quantities of defined chemical markers were used for assessment according to pharmacopeia guideline.

Chemicals and lipid internal standards

Synthetic lipid standards including 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC-17:0), 1-nonadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC-19:0), 1,2-dipentadecanoyl-*sn*-glycero-3-phosphatidylethanolamine (PE-30:0), 1,2-diheptadecanoyl-*sn*-glycero-3-

phosphoethanolamine (PE-34:0), 1,2-diheptadecanoyl-*sn*-glycero-3-phospholcholine (PC-34:0) and 1,2-dinonadecanoyl-*sn*-glycero-3-phospholcholine (PC-38:0) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). 1,2,3-tripentadecanoylglycerol (TG-45:0) and 1,2,3-triheptadecanoylglycerol (TG-51:0) were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Ultra liquid chromatography-mass spectrometry (Ultra LC-MS) grade of acetonitrile (ACN), methanol (MeOH), isopropanol (IPA) and water as well as LC–MS grade of dichloromethane (CH_2Cl_2) were purchased from Biosolve (Valkenswaard, The Netherlands). Phosphate Buffered Saline (PBS), and Hanks Balanced Salt Solution (HBBS) were supplied by Gibco. Isoproterenol, 10% sterile bovine serum albumin (BSA) solution, methanol and dimethyl sulfoxide (DMSO) were supplied by Sigma Aldrich. Plastic ware for tissue culture was supplied by Greiner Bio-One. The adipolysis assay kit was purchased fom Chemicon Int. (Temecula, CA).

Intervention studies on mice

Twenty-four female ApoE*3Leiden.CETP transgenic mice (age 6–10 weeks) were obtained from specific pathogen free (SPF) breading stock (TNO, Leiden, The Netherlands). The animals were fed a semi-synthetic modified Western-type diet (Hope Farms, The Netherlands), containing 0.2% cholesterol (Cho), 15% saturated fat and 40% sucrose as described by Nishina et al. [21], for a run-in period of 4 weeks to get a mild hypercholesterolemia (plasma Cho levels of 14–18 mmol/L) and body weight increase. Following this run-in period, mice were matched on body weight, plasma Cho and triglycerides (TG) levels (after 4 hours fasting) and divided in three groups of eight animals each (non-treated control, rimonabant- and SUB885C- treated). Preparations for intervention were given orally as admix to a Western-type diet for 4 weeks. Briefly, mice received the Western-type diet, without or with either SUB885C (SU Biomedicine, The Netherlands) at a concentration of 2% or rimonabant (Sanofi-Aventis, The Netherlands) at 10 mg/kg body weight/day. Flavoured sugar lumps were added to the diet of all groups to mask possible tastes of intervention preparations. Body weight per mouse and food intake per cage was measured at intervention day 0, 2, 3, 4, 9, 11, 14, 21 and 28. Blood was collected at the start of the intervention (week 0) and just before sacrifice of the mice (week 4) via tail vein bleeding using CB 300 LH microvettes (Sarstedt, Nümbrecht, Germany). Plasma samples were collected by centrifugation of the blood samples for 10 min at 6000 rpm at 4°C. At the end of the intervention (week 4), animals were sacrificed with rapid asphyxiation using CO_2. Livers were dissected on ice and samples were weighted and snap-frozen in liquid nitrogen. Both plasma and liver samples were stored at −80°C until use.

Adipocyte studies

The 3T3-L1 preadipocyte cell line (ATCC) was used to study possible direct lipolysis or adipogenetic activity by SUB885C. The 3T3-L1 cells were cultured and differentiated from pre-adipocytes into full grown adipocytes as described by Niwano et al. [22]. Three hundred micrograms of SUB885C was extracted with 4 ml methanol. After evaporation of the methanol, the extract was dissolved in 100 µl DMSO. To study adipolysis the growth medium was removed from the cells and cells were washed twice with 1 ml HBSS per well. Then, to each well 250 µl HBSS was added containing 2% BSA and 2.5 µl of the diluted SUB885C extracts in DMSO. As a positive control, 2.5 µl of 1 mM isoproterenol in DMSO was added (final concentration 10 µM isoproterenol). For the negative controls, either 2.5 µl DMSO (DMSO control) or nothing (blank) was added. After 3 h incubation glycerol release was measured with a commercial adipolysis assay kit (Chemicon Int., Temecula, CA). For the adipose conversion assay the addition of SUB885C started at the initiation of the cell differentiation. Together with the differentiation medium, SUB885C extract was added at different dilutions. At every medium replacement, fresh extract was added as well. After nine days of differentiation, the adipose conversion of the cells was analyzed by measuring the amount of fat produced. This was done by staining the fat with Oil Red O as described by Ramírez-Zacarías et al. [23].

Plasma biochemical analyses and lipoprotein profile analysis

Plasma Cho, TG, HDL-C, lipoprotein profiles, CETP levels and activities and alanine aminotransferase (ALT) were measured at week 0 and week 4. Plasma total cholesterol (TC) and TG concentrations were determined in each animal using enzymatic kits (Roche Molecular Biochemicals, Indianapolis, Ind, USA). Pooled lipoprotein profiles were measured by fast performance liquid chromatography (FPLC) using an AKTA apparatus (Amersham Biosciences). TC, TG and phospholipid levels were measured in the fractions of freshly obtained samples. Phospholipids were determined in the FPLC fractions using a phospholipids B kit (Instruchemie Co., The Netherlands). Plasma HDL was determined by quantification of HDL-C in plasma after precipitation of apoB-containing lipoproteins. Thus, 10 µl of heparin (LEO Pharma, The Netherlands) and 10 µl of 0.2 mol/L $MnCl_2$ were added to 20 µl plasma, and mixtures were incubated for 20 min at room temperature and centrifuged for 15 minutes at 13000*g* at 4°C. Plasma ALT was measured in pooled samples using a Boehringer Reflotron system. CETP levels were measured per mouse using RB-CETP kits from Roar Biomedical, Inc. Endogenous CETP activity was measured as described before [24]. Briefly, ^{3}H cholesterol was equilibrated

for 24 h with plasma cholesterol at 4°C followed by incubation at 37°C for 3 h. Subsequently, apoB-containing lipoproteins were precipitated by addition of heparin/$MnCl_2$. Lipids were extracted from the precipitate and labeled cholesteryl esters were separated from labeled unesterified cholesterol on silica columns and assayed by liquid scintillation counting.

Lipidomics analyses

Lipid extraction for plasma samples

Plasma samples were thawed to room temperature and extracted with 2:1 of CH_2Cl_2/MeOH as described previously [25]. Briefly, 30 µl of heparin plasma was placed in a 2 ml vial (Eppendorf, Hamburg, Germany). Thirty microliters of the internal standard (IS) mixture consisting of LPC-19:0, PE-30:0, PC-38:0 and TG-45:0 with corresponding concentrations of 30, 30, 150 and 60 µg/ml was first added, followed by 190 µl of MeOH and then 380 µl of CH_2Cl_2. The mixture was thoroughly vortexed both before and after CH_2Cl_2 addition. Afterwards, 120 µl of water was added and thoroughly vortexed. After centrifuging for 10 min at 6000g at 10ºC, 300 µl of the lower organic phase was transferred into a new autosampler vial and stored at −20 ºC until analysis. For LC-MS analysis, 25 µl of the lipid extract was diluted with 475 µl ACN/IPA/water 65/30/5 (v/v/v) and 10 µl was injected.

Lipid extraction for liver samples

The frozen liver samples stored at −80°C were lyophilized and ground into powder. Ten milligrams of liver powder was weighed in a clean 1.5 ml eppendorf vial for the subsequent lipid extraction. Liver lipid extraction was achieved by liquid-liquid extraction (LLE) with a CH_2Cl_2/MeOH mixture (2:1, v/v) based on the method of Bligh and Dyer [26] with some modifications. Briefly, 60 µl of IS were added to 10 mg of dry liver powder followed by addition of 180 µl of MeOH containing 0.02% antioxidant butylated hydroxytoluene (BHT), and then 360 µl of CH_2Cl_2 was added. The mixture was vortexed for 1 min both before and after CH_2Cl_2 addition. Afterwards, the resulting suspension was placed for 5 min in an ultrasonic bath at 4°C and then put in a shaker followed by 45 min incessantly shaking at 4°C. Thereafter 10 min centrifugation at a rotation speed of 6000*g* at 10°C was applied before 500 µl of the supernatant was transferred into a new 1.5 ml eppendorf vial. One hundred microliters of 0.9% NaCl was subsequently added to the supernatant to get a two-phase system where most of the lipids were in the lower organic phase. After being centrifuged at 2000*g* at 10°C for 10 min, a total of 300 µl of

lipid extract was collected from the bottom organic phase. The final extract was diluted 40 times by injection solvent as described previously [25] and then 10 µl was injected for LC-MS analysis.

LC-MS lipid profiling

LC-MS analysis was performed on a hybrid liquid chromatography-linear ion trap-Fourier transform ion cyclotron resonance-mass spectrometric system (LC-FTMS) consisting of a Surveyor HPLC MS pump and an autosampler (Thermo Fisher) equipped with an Ascentis Express C8 2.1 × 150 mm (2.7 µm particle size) column (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). The LC-MS method used has been described previously by Hu et al. [25]. With this method, seven different lipid classes including both polar lipids such as lyso-phosphatidylcholine (LPC), lyso-phosphoethanolamine (LPE), phosphatidylcholine (PC), phosphoethanolamine (PE), sphingomyelin (SPM) and non-polar lipids such as cholesterol esters (ChEs) and TG were eluted from the column ionized with electrospray ionization in the positive ion mode. The MS detection was performed in the full scan mode with a range of mass to charge ratio (m/z) 400-1500. The identification of the detected lipid peaks was performed as described previously [25] and the current accurate mass data acquired by FT and linear-ion-trap MS/MS fragmentation. For those peaks without MS/MS fragmentations, identification was based on the observed accurate m/z and the relative retention times of specific m/z peaks. Forty-eight plasma samples (week 0 and week 4) and 24 liver samples (week 4) from ApoE*3.CETP transgenic mice of three groups were prepared in duplicate and injected once according to the procedures described above. The performance of the applied lipid profiling platform was assessed through the repeated analysis of the quality control (QC) samples [27]. The QC samples, used to monitor the LC-MS response in time, were prepared by pooling aliquots of 48 plasma samples for plasma lipidomics and 24 liver samples for liver lipidomics respectively to represent the full biochemical diversity of the study samples and allow the calculation of the analytical precision for all lipids measured. The QC sample data is also used to correct systematic errors such as batch to batch response differences by a single point calibration model [28, 29]. Ten QC plasma samples and 5 QC liver samples were processed exactly in the same way as the study samples. In total 106 plasma samples including 96 study samples and 10 QC samples and 53 liver samples including 48 study samples and 5 QC samples were injected into the LC-MS system. The study samples were randomly analyzed and the QC samples were placed at regular intervals in the analysis sequence (one QC after every 10 samples). Furthermore, method

performance was carefully monitored using multiple IS and duplicate analysis of QC samples. Of note, plasma and liver samples were analyzed separately.

Lipidomics data processing

In total, 140 plasma lipid peaks and 137 hepatic lipid peaks were identified and selected as target lipids based on retention time and m/z of peaks processed by LC-Quan 2.5 (Thermo Fisher). The peak detection algorithm was used for peak integration using the following parameters: ICIS; smoothing points, 7; window, 30 s; view width, 3 min; baseline, 40; area noise factor, 5; peak noise factor, 1045. Data for each mouse was normalized for the recovery of the IS for injection. Batch to batch differences in the data were removed by synchronizing the medians values of the QC samples per batch. Data was used only if the duplicates corresponded after visual inspection and the duplicates were averaged.

Statistical data analysis

One mouse (number 3733) in the control group was excluded from data analyses, because it did not respond to the diet during the run-in period and failed the inclusion criteria for hypercholesterolemia. Univariate data analyses were done by SPSS 17.0 and the results are presented as means ± standard deviation (SD) unless indicated otherwise. Statistical differences of biochemical parameters and lipidomics regulation at the end of the intervention of the three groups were analyzed parametrically by one-way analysis of variance (ANOVA). Data were log transformed if homogeneity of variance assumption was rejected. The Dunnett post hoc method was used to identify which treatment was significantly different from the control. One-tailed and two-tailed tests were used for biochemistry parameters and the lipidomics data respectively, with p values < 0.05 as statistical significance. To correct for false positives, the multiple test correction (MTC) of Benjamini and Hochberg false discovery rate (FDR) analysis was applied to adjust p values derived from the univariate results of the lipidomics data. To avoid the possibility that a few high-intensity variables dominate the final results [30], plasma and liver lipidomics datasets were autoscaled per variable, meaning that first the means of each variable were subtracted, and then all variables were divided by their SD. To find treatment dependent lipidomics grouping effects, both lipidomics datasets were analyzed by Principal Component Analysis (PCA) [31] in MATLAB (version 7.7.0471, the Mathworks) with the PLS toolbox (version 5.0.3, Eigenvector Research, InC.). PCA was used to find patterns in the data such as clusters of mice (scores) of non-treated controls and undergoing the treatments; and to identify which lipids contributed most to these clusters (loadings). Partial

least squares discriminant analysis (PLS-DA) [32] was further applied to identify the specific metabolites which contribute most to discriminate between the subtypes; and PLS-DA models were validated using double cross validation (DCV) [33]. Week 4 lipidomics were used for data analysis because (1) the study interest is in the effect of SUB885C in the end of the intervention and all baseline parameters are homogeneous among the three groups; (2) liver lipidomics results are only available at week 4. Finally data matrices of 15 mice (7 controls, 8 SUB885C) ×140 lipids for plasma lipidomics and 15 mice × 137 lipids for liver lipidomics were used for multivariate data analysis.

Results

SUB885C does not influence food intake or body weight in ApoE*3Leiden.CETP mice

Compared to the controls, SUB885C did not have an effect on food intake in ApoE*3Leiden.CETP mice until day 20, and did not affect mean body weight (Figure 1). After day 21, an increase in food intake was observed with SUB885C. According to published results in the same experiment [34], mice treated with rimonabant showed a reduced food intake with a pronounced dip at day 2. A significant reduction of mean body weight of approximately 9% remained visible until the end of the intervention [34].

SUB885C decreases plasma cholesterol, triglycerides and increases HDL-C

SUB885C treatment of ApoE*3Leiden.CETP mice caused a significant decrease in plasma Cho by 49% (8±1 mM versus 15±2 mM, $p<0.001$) after 4 weeks as compared to the controls (Figure 2A). During the 4 week intervention period plasma TG levels in both the SUB885C treated group and in control mice were significantly reduced by 67% (1.4 ± 0.5 mM versus 4 ± 2 mM, $p<0.01$) and 46% (2 ± 1 mM versus 4 ± 2 mM, $p< 0.01$), respectively (data not shown). At week 4, TG levels in the SUB885C group tended to be lower compared to the control mice (Figure 2A), but this difference did not reach significance (1.4±0.5 mM versus 2±1 mM, $p = 0.06$). Plasma HDL-C in the SUB885C group was significantly increased by 39% (1.1±0.3 mmol/L versus 0.8±0.3 mmol/L, $p<0.05$) as compared to that of the controls in week 4. Lipoprotein fraction analyses showed that SUB885C treatment caused a 3-fold decrease of very low density lipoprotein-cholestol (VLDL-C) and 2.5 fold of VLDL-TGs and VLDL-phospholipids, as compared to those of the control group (Figure 2 B–D).

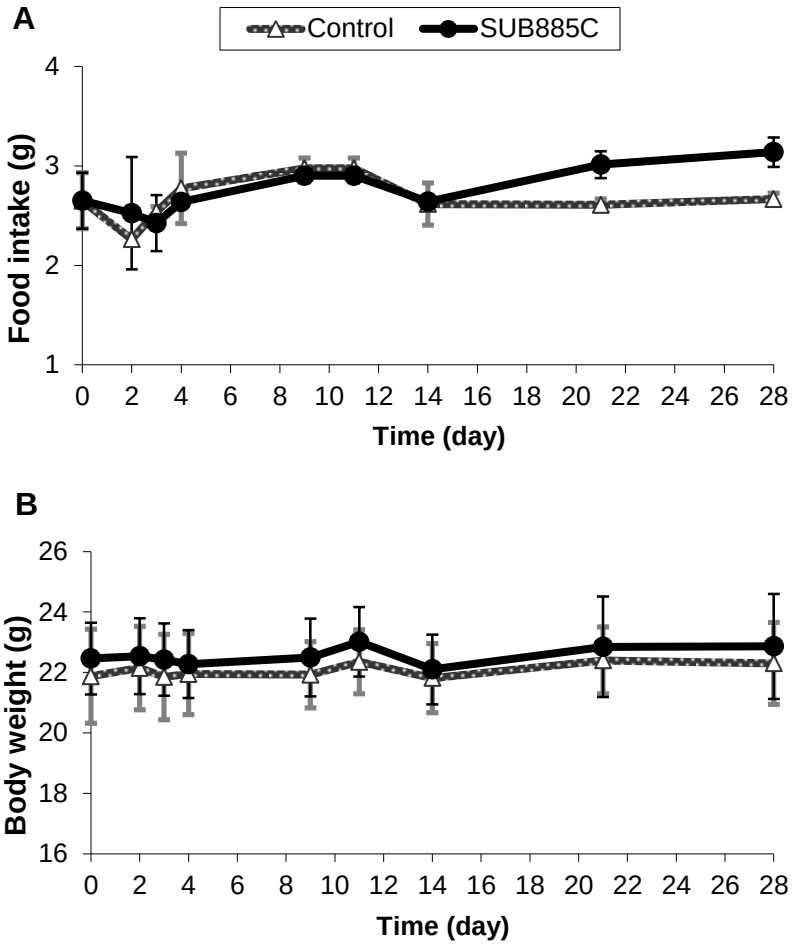

Figure 1. *Food intake and body weight. (A) Food intake (per cage) and (B) body weight (per mouse) were recorded and measured at day 0, 2, 3, 4, 9, 11, 14, 21 and 28 for SUB885C-treated mice and control (*p <0.05 vs. control).*

In ApoE*3Leiden.CETP mice, the reduction of plasma TC was caused by the decrease of (V)LDL-C; while the increase of plasma HDL-C was in line with the obvious increase in HDL-C fraction.

Based on previous published results [34], rimonabant treatment of ApoE*3Leiden.CETP mice induced a significant decrease of plasma Cho with 24% (p <0.05) when compared with that of the controls. However, there were no significant changes of plasma TG and HDL-C at the end of the intervention. No

adverse signs were observed during the study and ALT levels of all three groups were in the reference range.

SUB885C decreases plasma CETP level and activity

Four weeks of SUB885C intervention (Figure 2A) of ApoE*3Leiden.CETP mice caused a significant decrease in CETP level by 31% (22 ± 2 µg/mL versus 31±4 µg/mL, $p<0.001$) and CETP activity by 74% (24 ± 11 µg/mL versus 91±27 µg/mL, $p<0.001$) as compared to the controls. However, rimonabant did not significantly affect the CETP level or the CETP activity in the same experimental setting [34].

Lipidomics reveals detailed lipid changes caused by SUB885C

Two Principal Components (PCs) were selected for plasma and liver lipidomics. These PCs described 67% and 57% of the total variance of the plasma- and liver-lipidomics, respectively (Figure 3A and B). In both PCA biplots, a separation was observed between control- and SUB885C treated-mice, indicating major changes of measured plasma and liver lipids between two groups. The loadings in the biplots (lipid species represented by colored symbols in Figure 3A and B) indicated that the lipids separating the SUB885C treated-mice from the control mice were TG, ChE and SPM. PLS-DA with DCV was used to further investigate the main discriminating lipids between SUB885C and control groups. The DCV error rates in both plasma and liver were 20%, indicating that 3 out of 15 mice were misclassified. Among 30 main discriminating lipids between the control and SUB885C treated mice, ChEs, SPMs, PCs, TGs contributed most for the separation in plasma while SPMs, PCs, TGs contributed most in liver (Table S1 Supporting information).

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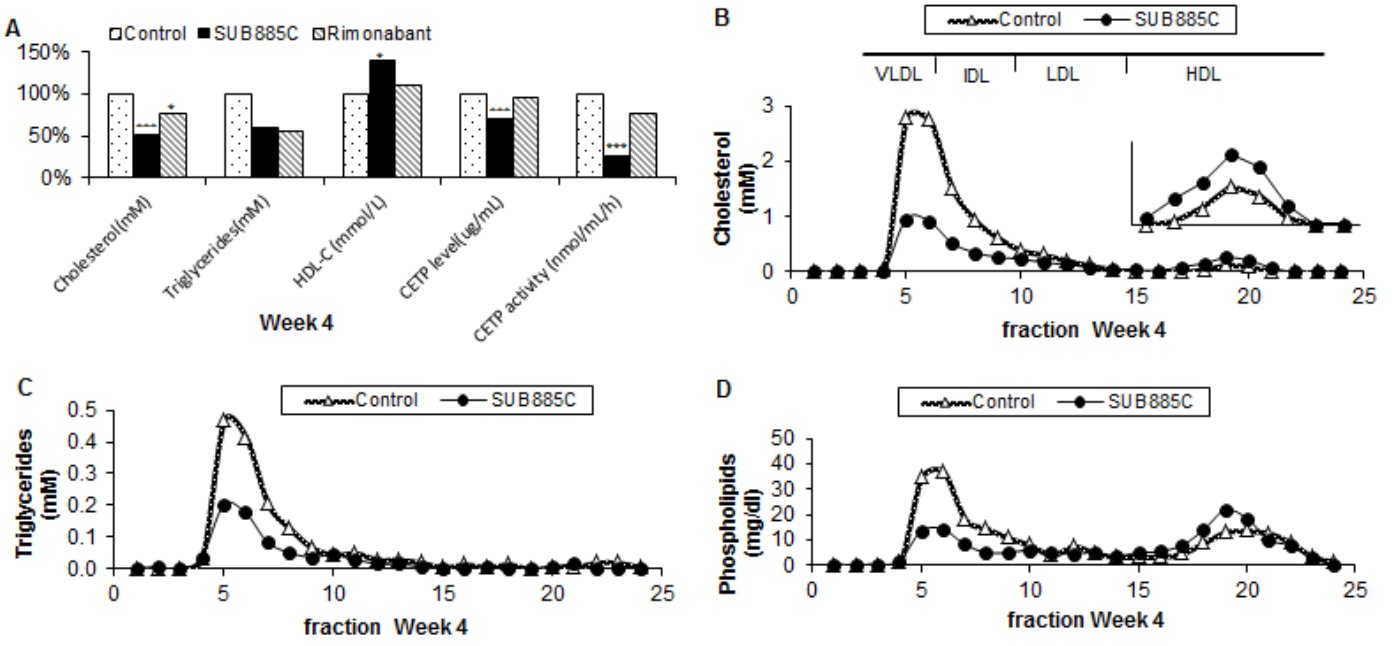

Figure 2. *Plasma lipid, CETP and lipoprotein. Plasma concentrations are shown for TC, TG, HDL-C, CETP level and activity (A) of the SUB885C-, rimonabant- and non-treated mice at week 4 (concentrations in the control group are set to be 100% and relative changes of treated groups were illustrated in % compared with the control, *p < 0.05, ** p < 0.01, *** p < 0.001). Alterations of Cho (B), TG (C) and phospholipids (D) in the pooled lipoprotein profiles of the SUB885C- and non-treated mice at week 4. Fractions 4–7 as VLDL; 8–9 as IDL; 10–15 as LDL and 16–23 as HDL.*

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Quantitative differences in liver and plasma lipids between the SUB885C treatment group and untreated controls were statistically further analyzed by ANOVA with a two-tailed Dunnett post hoc method. SUB885C caused a significant reduction of plasma lipid classes of SPM, ChE, and TG by −28% (p<0.001), −46% (p<0.001), and −60% (p<0.01) compared to the controls, respectively. It caused a 12% reduction (p<0.05) of total liver SPM, without significant influence on total liver TG or ChE lipids. It was found that 86 (61%) plasma and 22 (16%) liver lipids out of 140 and 137 lipids respectively were significantly changed after the 4-week SUB885C intervention as compared to the controls. Fifteen lipids changed significantly under SUB885C intervention in both plasma and liver, including LPC (18:2), PC (36:5), PE (34:2), SPM (22:1), SPM (24:1), ChE (18:2), ChE (20:4), TG (50:1), TG (54:0), ether TG (TG-O) (50:0), TG-O (50:1), TG-O (50:2), TG-O (52:1),TG-O (52:2) and TG-O (58:2) (Supporting information Table S2 and S3). The correlation analysis, data with normal distribution by Pearson's and without by Spearman's, was performed to evaluate whether there is a relation between the concentrations of these lipids in plasma and liver. Only TG-O (50:0) showed a positive correlation between its concentration of plasma and liver; the others showed no correlation (data not shown).

After MTC analysis, 70 out of 86 plasma lipids and 3 out of 22 hepatic lipids remained significant (p = 0.00−0.02, p values marked in bold) by SUB885C intervention (Supporting information Table S2 and S3). All three significantly changed hepatic lipids and 68 out of 70 (97 %) significantly changed plasma lipids were down regulated by SUB885C treatment. In plasma, SUB885C caused a significant reduction of 80% neutral lipids including ChE and TG, 50% SPM, 17% LPC and 14% PE. Three out of 5 significantly changed plasma PC (14%) by SUB885C was down-regulated (Table 1). In liver, SUB885C caused a significantly reduction of 17% ChE and 3% TG, but no obvious changes in phospholipids. Thus SUB885C It affected the lipid classes of TG, ChE and SPM the most, which was in line with PCA results.

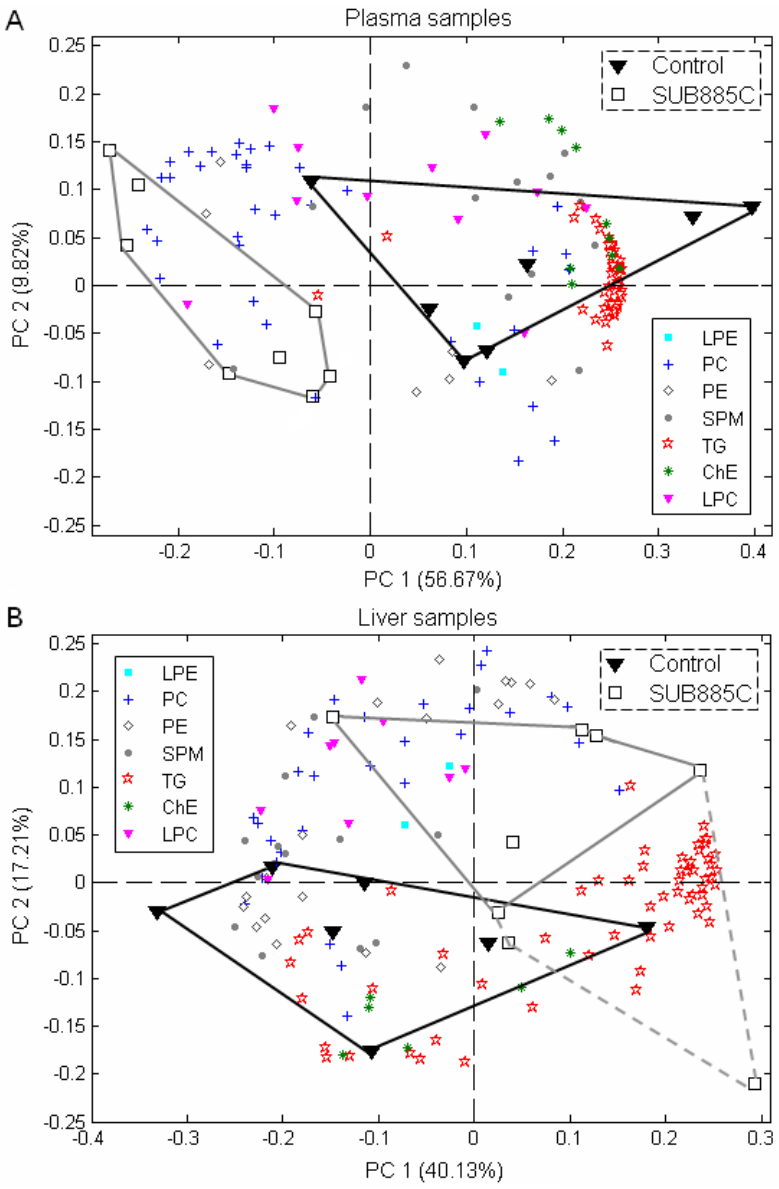

Figure 3. *Lipidomics of plasma and liver reveals differences between non-treated and SUB885C-treated mice. Principle component analyses (PCA) of plasma and liver lipidomics datasets were applied to differentiate the non-treated controls (n = 7) and the SUB885C treated mice (n = 8). PCA biplots for (A) plasma samples and (B) liver samples.*

Table 1. Significantly changed lipids in each lipid class

Plasma Lipid class	All measured lipids (n)	Sig. changed lipids (n, % in each lipid class)	Sig. reduced lipids (n, % in each lipid class)
LPC	12	3 (17%)	3 (17%)
LPE	2	0	0
PC	36	5 (14%)	3 (8%)
PE	7	1 (14%)	1 (14%)
SPM	14	7 (50%)	7 (50%)
ChE	10	8 (80%)	8 (80%)
TG	59	47 (80%)	47 (80%)
Total	**140**	**70 (50%)**	
Liver lipid class	**All measured lipids (n)**	**Sig. changed lipids (n, % in each lipid class)**	**Sig. reduced lipids (n, % in each lipid class)**
LPC	9	0	0
LPE	2	0	0
PC	29	0	0
PE	19	0	0
SPM	13	0	0
ChE	6	1 (17%)	1 (17%)
TG	59	2 (3%)	2 (3%)
Total	**137**	**3 (2%)**	

Compared with the results of rimonabant induced lipid changes from the previous publication [34], twenty four plasma lipids including LPC (18:2), PC (38:2), SPM (16:0), ChE (18:1), TG (46:0), TG (46:1), TG (48:0), TG (48:1), TG (48:2), TG (50:0), TG (50:1), TG (52:2), TG (52:6), TG (54:2), TG (54:3), TG (54:4) TG (56:3), TG (56:4), TG (56:7), TG (58:3), TG (58:4), TG (58:5), TG (58:6), TG (58:7) were significantly changed by both SUB885C and rimonabant, all of which were down regulated except for SPM (16:0) that SUB885C induced a down-regulation while rimonabant a up-regulation. After MTC, only LPC (18:2) and PC (38:2) remained significant in both SUB885C and rimonabant groups; while all lipids except for SPM (16:0), TG (56:3) and TG (58:5) still remained significant under SUB885C intervention. Two liver lipids, PE (36:3) and TG (50:1), were significantly changed by SUB885C and rimonabant. The former was down-regulated by both treatments while the latter was down-regulated by SUB885C but up-regulated by rimonabant. Both lipids did not hold significance after MTC in SUB885C and rimonabant treatment.

SUB885C is able to stimulate adipolysis and inhibit adipogenesis *in vitro*

To investigate SUB885C's effect on adipolysis, the release of glycerol in the medium was measured after incubation of 3T3-L1 adipocytes with SUB885C. The positive control isoproterenol, a non-selective agonist of the β-adrenergic receptors, is known to increase the hydrolysis of TG [22, 35] and to stimulate adipolysis in 3T3-L1 adipocytes [22] (Figure 4A). When at a 500 × dilution, SUB885C extract caused a 22% higher glycerol release as compared to isoproterenol at a concentration of 20 μM. When SUB885C extract was 2500 × diluted, glycerol release was 20% lower than 20 μM isoproterenol, yet still higher than DMSO and Blank.

The effect of SUB885C on lipid accumulation in 3T3-L1 adipocytes was investigated during their differentiation process. As positive control the cytokine tumor necrosis factor-α (TNF-α) was used, which interferes with adipocyte differentiation [22]. SUB885C was found to dose-dependently inhibit *in vitro* adipogenesis (Figure 4B). At 5000 × dilution there was 30% lipid inhibition in the cells as compared to the control (0.1% DMSO). At 1000 × dilution there was 45% lipid inhibition and at 500 × dilution there was 100% lipid inhibition in the cells. This was similar for TNF-α at a concentration of 2 ng/ml.

The appearance of 3T3-L1 cells cultured for 9 days in the presence of SUB885C extract was shown in Figure 4C. The number of nuclei did not differ much among the 0.1% DMSO control, 500 × and 1000 × diluted SUB885C extracts. With 500 × dilution (top row) there was a decrease in the amount of nuclei, indicating toxicity induced cell loss, which was in line with the phase contrast picture of microscope with the 500 × dilution (bottom row). The other dilutions did not show a clear effect on the cell morphology. TNF-α, as a control, did not show any effect on nuclei numbers and a densely packed cell layer. Inhibition of lipid accumulation in 3T3-L1 cells after exposure to SUB885C extract could be clearly seen at 1000 × and 500 × dilutions.

Discussion

Causes and consequences of the complex changes in lipid patterns occurring during development of the metabolic syndrome are still only partly understood. Several interconnected processes are deteriorating which implies that in the end multi-target approaches might be more successful than prevention or intervention strategies based on a limited number of surrogate markers.

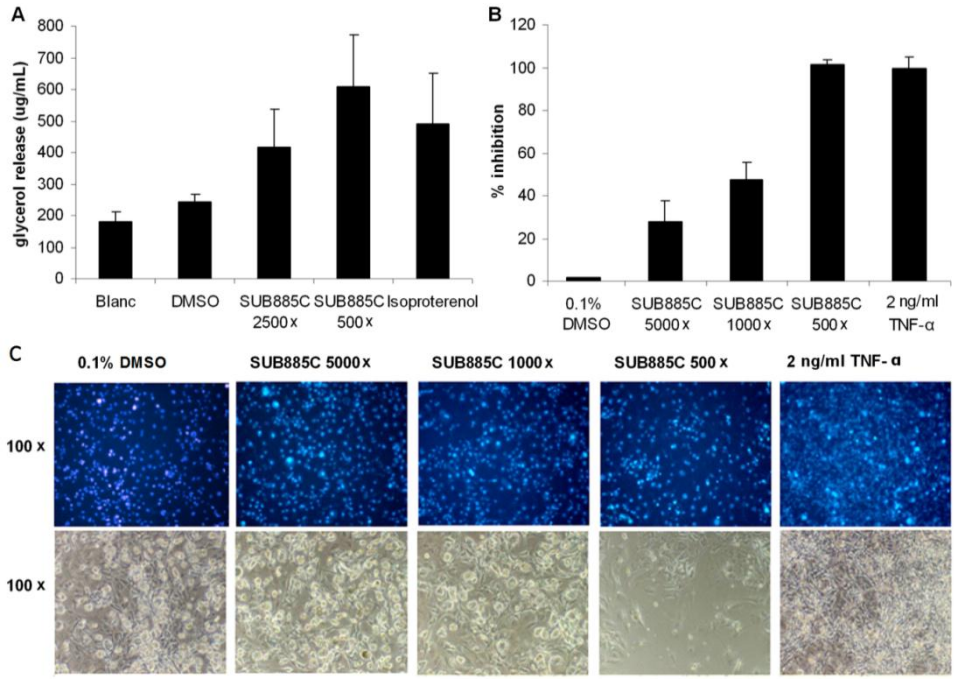

Figure 4. *Adipolytic and adipogenic activities of SUB885C in 3T3-L1 adipocytes. (A) Glycerol release as the result of adipolysis in 3T3-L1 adipocytes after incubation with SUB885C (500× and 2500× diluted) and 20 µM isoproterenol dissolved in DMSO with 1% as final concentration. (B) Inhibition of adipose conversion in differentiating 3T3-L1 adipocytes by SUB885C. (C) Morphological analysis of the differentiated 3T3-L1 adipocytes. The cells were treated with 20 µg/ml DAPI (top row), which stained the nuclei of the cells and gave an indication of cell numbers. The phase contrast of the microscope showed the outlines of the cells (bottom row) and gave an overview of cell density and morphology. The magnification is on the left.*

Results of the present study illustrated that a metabolomics-based analysis of the effects of a multi-component preparation can be used to study potential target processes or novel ingredients. Preparations having their origin in other healthcare systems such as CM have been shown to provide interesting starting points [36-42]. In a previous study it was found that SUB885C improved insulin sensitivity compared to control in pre-diabetic ApoE*3Leiden mice fed with a high fat diet for 10 weeks [7]. The ApoE*3Leiden.CETP mouse model used in the present study is a cross-bred of the ApoE*3Leiden mouse with mice expressing human CETP [16]. This further shifts the distribution of Cho from HDL to VLDL/LDL, reduces plasma-mediated scavenger receptor class B type I (SR-BI)-dependent

Cho efflux, and produces a strong pro-atherogenic condition [43]. The model has shown its value for the evaluation of interventions that reduce plasma lipids or increase HDL-C [14, 15, 17, 34, 44].

In the present study, SUB885C showed multiple effects to improve metabolic parameters and lipid patterns, which were summarized in figure 5. Specifically, SUB885C treatment induced a increase of plasma HDL-C, which was accompanied with a decrease of (V)LDL levels. Both effects are commonly regarded as anti-atherogenic [14, 17]. Levels and activity of CETP, an important regulator of HDL metabolism, were found to be decreased, which may be related to the reduced levels of VLDL-TG, a substrate for CETP [44]. Taken together, the effects of SUB885C on HDL-C may be due to: 1) a decreased CETP activity and CETP level; 2) a reduced level of (V)LDL, an acceptor for HDL-ChE, which would reduce CETP transfer activity by decreasing the transfer of ChE from HDLs to TG–enriched lipoproteins, and more ChE-enriched HDL particles remain; 3) improved insulin sensitivity. SUB885C significantly reduced plasma TC, which likely reflected the reduction of (V)LDL-C concentrations in the SUB885C treated mice.

During the intervention period total plasma TG levels when measured enzymatically decreased significantly in both the control and the SUB885C groups. At the end of intervention TG level tended to be lower in the SUB885 group compared to that of the control mice, but mainly due to a relatively large within group variance this did not reach significance. With the lipidomics, however, we found a significant reduction of 60% of total plasma TG (p<0.01) as compared to that of the controls. This supports our observation that SUB885C treatment induced a TG-lowering effect. SUB885C particularly modulated a wide range of lipids such as TGs, ChEs and SPMs in plasma while in the liver only a few of these lipids were influenced (Table 1). After the correlation analysis of significantly changed lipids both in plasma and liver, only TG-O (50:0) showed a positive correlation and others not. Instead of a redistribution of lipids between plasma and liver, the effects of SUB885C might relate more to plasma lipoprotein metabolism. Based on the fact that the reduced lipids (i.e. ChE, SPM and TG) in plasma are core lipids of the lipoprotein particle, it seems conceivable that the circulating lipoprotein particles in plasma were reduced by SUB885C due to its influence on lipoprotein regulators such as CETP whose activities are mainly in plasma. This hypothesis is confirmed by the fact that SUB885C (not rimonabant) significantly reduced the level and activity of CETP (Figure 2A) in ApoE*3Leiden.CETP mice.

In Table 2, some of the overall effects of SUB885C were compared with those of the drugs (i.e. rimonabant, atorvastatin, torcetrapib, niacin and tesaglitazer) that were used for modulating plasma lipid profiles obtained in the ApoE*3Leiden.CETP mouse model.

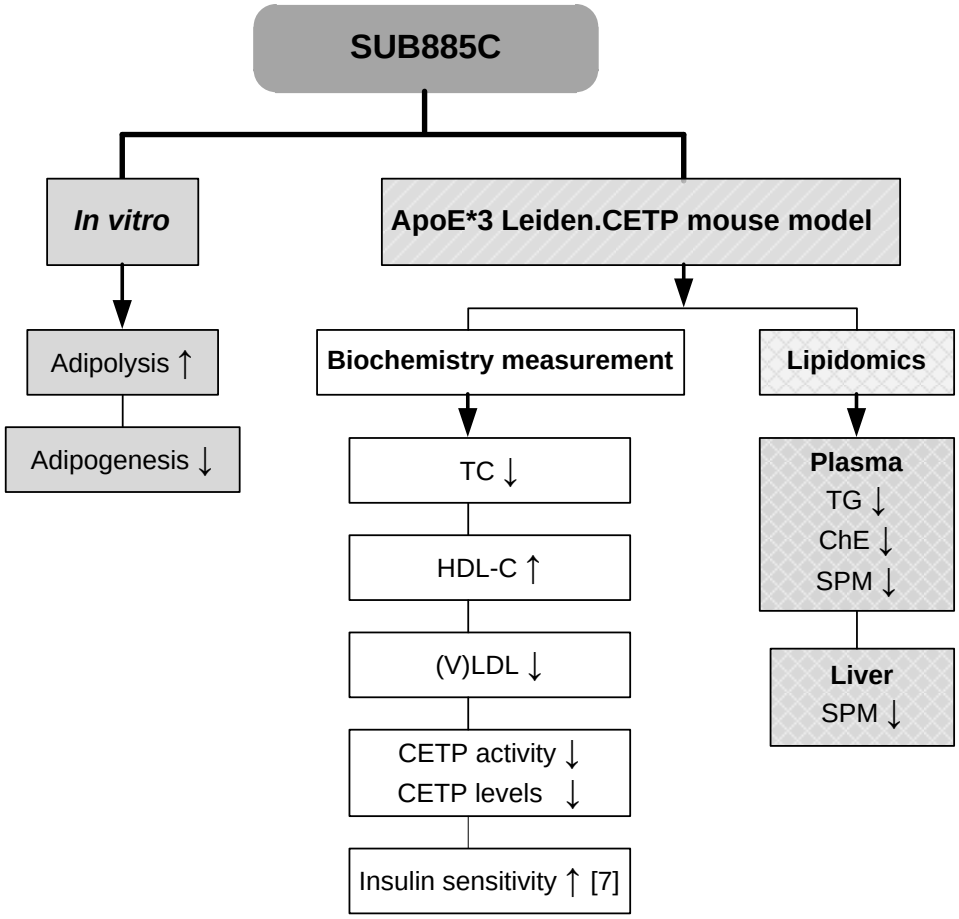

Figure 5. *The summary of SUB885C effects. The effects of SUB885C were illustrated both in vitro and in ApoE*3Leiden.CETP mice with biochemical and lipidomics measurements. The improvement for insulin sensitivity was result from Wang et al. [7].*

Although detailed comparisons cannot be made due to different study designs, effects of SUB885C on these variables appear to be comparable to those of the drugs investigated. Compared with the side-effects induced by these drugs, including psychiatric abnormalities (rimonabant) [45, 46], severe flushing (niacin) [44], headache (atorvastatin), increased risks of cardiovascular morbidity and mortality (torcetrapib) [14, 47], the elevated serum creatinine and associated decreases in glomerular filtration rate (tesaglitazer) [48]; the reported side-effects of the active compounds in SUB885C are scarce. Only herbs containing anthraquinones (i.e. emodin and chrysophanol) were reported to cause diarrhea

[12]. Thus, SUB885C might have a higher benefit-risk ratio than other therapeutic interventions.

In contrast to rimonabant, SUB885C did not reduce body weight or food intake during the 4-week intervention period. Rimonabant is a CB1 inverse agonist, which has been developed and briefly marketed for weight management and improvement of symptoms of the metabolic syndrome. Shortly after its introduction in Europe it was withdrawn because of its central side-effects such as depression and other psychiatric abnormalities [45, 46]. It is now commonly assumed that its action on the central CB1 receptors produces a relatively rapid but transient decrease of appetite [49]. In our study this was observed as a sharp dip in food intake around day 2 (Figure 1). In addition, rimonabant acts on peripheral CB1 receptors leading to a more sustained reduction of body weight and beneficial effects on a number of symptoms of the metabolic syndrome [50-52]. Binding studies using a cell membrane preparation expressing the human cannabinoid CB1 receptor showed that a SUB885C total extract was able to displace bound radioactive ligand 3H-CP55940 (data not shown). This could indicate that one or more compounds present in the mixture have affinity for CB1 receptors. However, more data are needed to confirm this. The lack of the effect of SUB885C on body weight and food intake at least suggests that there is no dominating overall effect on the central or peripheral regulation of food-intake or energy regulation.

The *in vitro* results showed that SUB885C is able to stimulate lipolysis and inhibit adipogenesis in 3T3-L1 cells. At this stage it is difficult to speculate on a possible mechanism and its relevance for the overall *in vivo* effects of the preparation since there are several compounds classes that produce such effects, including, but not limited to CB1 blockers [22, 42, 53].

Further studies are needed to reveal the pathways and processes modulated by SUB885C in more detail. Effects of the individual active components in SUB885C have been studied. For example, extracts of Fructus crataegi (Hawthorn berries) are used in CM for treatment of several cardiovascular problems and have been reported to possess anti-inflammatory properties and to modulate mitochondrial functioning [8, 11]. Flos Rosae rugosae (rose flower) extract has been reported to increase the activities of antioxidant enzymes and to reduce lipid peroxidation [10]. Radix et Rhizoma Rhei (rhubarb root) and Radix Glycyrrhizae (licorice root) have been reported to produce inhibition of fatty acid synthase, thus contributing to weight reduction [12]. Radix et Rhizoma Rhei contains emodin which is known as an inhibitor of 11 β-hydroxysteroid dehydrogenase type 1 and has been shown to ameliorate metabolic disorders in diet-induced obese mice [9].

Table 2. Effects on lipid parameters of SUB885C and existing drugs in the ApoE*3Leiden.CETP female mice model

	SUB885C	**Rimonabant** [34]	**Atorvastatin** [15]	**Torcetrapib** [14]	**Niacin** [44]	**Tesaglitazer** [17]
Mice age	6-10 wk	6-10 wk			12 wk	18 wk
Run-in diet	4 wk	4 wk	2 wk	4 wk	3 wk	11wk:0.3% w/w
(cholesterol)	0.2% w/w	0.2% w/w	0.1% w/w	0.25% w/w	0.1% w/w	4wk:0.1%w/w
Study diet	same as	same as	same as	same as	same as	0.1% w/w
(cholesterol)	run-in diet	run-in diet	run-in diet	run-in diet	run-in diet	
Study period	4 wk	4 wk	6wk	14 wk	3 wk	8 wk
TC	-49%	-24%	-33%	-20%	-44 - 68% *	-55%
TG	-41% (NS)	-43% (NS)	NS		-57 - 77% *	-71%
HDL-C	+ 39%	+12% (NS)	+52%	+30%	+77- 87% *	+38%
(V)LDL-C	- 67%	-33%	-88%	-26%	-52 - 79% *	- 80%
CETP level	-31%	-4% (NS)	-29% (NS)	+ 33%	-24 - 45% *	-42%
CETP activity	-74%	-22% (NS)	-36%	-73%	-24 - 52% *	-56%

Basic diet for ApoE*3Leiden.CETP female mice during run-in and study period: Western type diet with 15% w/w fat plus different content of cholesterol

NS: no significance. *dependent on different doses of Niacin

This enzyme is now receiving considerable interest as a pharmacological target in metabolic syndrome [49]. Folium apocyni is obtained from leaves of Apocynum venetum L. (Venetian dogbane) and used in CM to prepare herbal teas against various cardiovascular and other problems.
In conclusion, our study has shown that a CM principle-based multi-components preparation is able to produce anti-atherogenic changes in lipid spectra of the ApoE*3Leiden.CETP mouse model, which are comparable to those obtained with compounds belonging to known drug classes. Our data also illustrate the power of lipidomics in unraveling effects in detail and to help finding new targets or ingredients. These findings can be used to develop new preparations at the nutrition-pharma interface that can be used to prevent metabolic syndrome or ameliorate its first symptoms. Forthcoming studies should include dose-titrations and studies on lipid fluxes in human volunteers.

Acknowledgement

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Supporting Information

Table S1. Thirty lipids contributed most to discriminate between control and SUB885C treated mice in plasma and liver

Ranking *	Plasma lipids	Liver lipids
1	ChE (18:1)	PC (34:3)
2	ChE (18:1)-Dimeric	LPC (16:0)
3	ChE (18:2)	SPM (20:0)
4	PC (38:6)	TG (52:6)
5	ChE (18:3)-Dimeric	TG (52:3)
6	ChE (18:3)	SPM (15:0)
7	PC (36:6)	PC (40:5)
8	PC (38:4)	PC (36:5)
9	PC (40:5)	TG (52:2)
10	PE-O (38:5)	TG (50:2)
11	PC-O (42:6)	ChE (22:6)
12	SPM (24:2)	TG (50:1)
13	ChE (18:2)-Dimeric	PE (38:6)
14	PE (38:2)	SPM (22:1)
15	PE (38:4)	PC (40:8)
16	SPM (14:0)	TG (50:3)
17	SPM (22:1)	PC (36:1)
18	SPM (18:1)	TG (50:0)
19	SPM (18:0)	PC (38:6)
20	SPM (24:1)	LPE (20:4)
21	PC (40:4)	PC (38:3)
22	SPM (15:0)	PC-O (38:4)
23	SPM (22:0)	TG (52:1)
24	PC (40:7)	PC (40:7)
25	PE (36:3)	PE (34:1)
26	SPM (23:0)	PC-O (34:1)
27	TG (54:5)	TG (52:5)
28	TG (46:1)	SPM (22:0)
29	TG (50:3)	PE (38:3)
30	SPM (20:0)	PC-O (36:4)

* The ranking is calculated based on the coefficients of PLSDA regression vector.

Eight gray highlighted lipids are similar lipids present in both plasma and liver for the discrimination between control and treated mice.

Table S2. Lipid molecular species are significantly influenced in plasma upon SUB885C treatment as compared to non-treated controls

Table S2.1 LPC, PC and SPM

Lipid species	control (mean ± SD)	SUB885C (mean ± SD)	SUB885C vs. control change (%)	*p* value	(↑) (↓)
LPC (14:0)	0.0008 ± 0.0001	0.0007 ± 0.0001	15	**<0.05**	↓
LPC (18:2)	0.14 ± 0.01	0.12 ± 0.01	16	**<0.01**	↓
LPC -O(18:1)	0.0033 ± 0.0008	0.0024 ± 0.0003	28	<0.05	↓
PC (32:0)	0.0034 ± 0.0004	0.0029 ± 0.0003	16	**<0.01**	↓
PC (36:5)	0.0006 ± 0.0001	0.0007 ± 0.0001	34	<0.05	↓
PC (38:2)	0.51 ± 0.06	0.36 ± 0.04	29	**<0.001**	↓
PC (38:4)	0.020 ± 0.003	0.029 ± 0.007	43	**<0.05**	↑
PC (38:6)	0.010 ± 0.002	0.014 ± 0.003	31	<0.05	↑
PC (40:7)	0.0025 ± 0.0003	0.0030± 0.0004	22	**<0.05**	↑
PC-O (34:3)	0.00006 ± 0.00001	0.00005 ± 0.00001	17	<0.05	↓
PC-O (38:7)	0.00008 ± 0.00003	0.00004 ± 0.00002	44	**<0.01**	↓
PE (34:2)	0.014 ± 0.003	0.008 ± 0.003	42	**<0.01**	↓
SPM (14:0)	0.00037 ± 0.00004	0.00026 ± 0.00002	31	**<0.001**	↓
SPM (16:0)	0.0046 ± 0.0003	0.0042 ± 0.0004	11	<0.05	↓
SPM (16:1)	0.15 ± 0.02	0.10 ± 0.02	31	**<0.001**	↓
SPM (22:0)	0.27 ± 0.03	0.19 ± 0.05	31	**<0.001**	↓
SPM (22:1)	0.0009 ± 0.0001	0.0010 ± 0.0001	17	<0.05	↑
SPM (23:0)	0.13 ± 0.01	0.09 ± 0.01	28	**<0.001**	↓
SPM (23:1)	0.00031 ± 0.00004	0.00027 ± 0.00002	13	<0.05	↓
SPM (24:0)	0.0095 ± 0.0008	0.0081 ± 0.0007	15	**<0.001**	↓
SPM (24:1)	0.0044 ± 0.0004	0.0034 ± 0.0003	22	**<0.001**	↓
SPM (24:2)	0.0018 ± 0.0001	0.0015 ± 0.0002	17	**<0.01**	↓

p values correspond to the mean difference between the SUB885C group and the control group.
Note: lipids with *p* values marked in bold mean those remain significant after MTC.
LPC-O: ether LPC; PC-O: ether PC;
* Homogeneity of variance assumption is deviated of this lipid. Data was log transformed and the mean ± SD was shown as the normal data value for a reference.

Table S2.2 ChE, TG's

Lipid species	control (mean ± SD)	SUB885C (mean ± SD)	SUB885C vs. control change (%)	*p* value	(↑) (↓)
ChE (18:1)	0.30 ± 0.04	0.18 ± 0.02	40	**<0.001**	↓
ChE (18:2)	2.9 ± 0.6	1.2 ± 0.3	57	**<0.001**	↓
ChE (18:3)	0.0011 ± 0.0003	0.0004 ± 0.0001	65	**<0.001**	↓
ChE (20:4)	0.14 ± 0.03	0.10 ± 0.01	29	<0.05	↓
ChE (22:6)	0.015 ± 0.004	0.009 ± 0.002	38	**<0.001**	↓
ChE (18:1)-Dimeric	12 ± 2	7± 1	43	**<0.01**	↓
ChE (18:2)-Dimeric*	1.6 ± 0.5	0.7 ± 0.2	55	**<0.001**	↓
ChE (18:3)-Dimeric	0.0045 ± 0.0015	0.0011 ± 0.0005	75	**<0.001**	↓
ChE (22:6)-Dimeric	0.02 ± 0.01	0.007 ± 0.003	65	**<0.01**	↓
TG (46:0)	0.033 ± 0.004	0.027 ± 0.004	19	**<0.05**	↓
TG (46:1)	0.013 ± 0.004	0.007 ± 0.002	47	**<0.01**	↓
TG (48:0)	0.17 ± 0.06	0.06 ± 0.03	63	**<0.01**	↓
TG (48:1)	0.008 ± 0.003	0.003 ± 0.002	61	**<0.01**	↓
TG (48:2)	0.10 ± 0.03	0.04 ± 0.02	62	**<0.01**	↓
TG (50:0)	0.05 ± 0.02	0.015 ± 0.010	70	**<0.01**	↓
TG (50:1)	0.16 ± 0.07	0.06 ± 0.03	65	**<0.01**	↓
TG (50:2)	0.11 ± 0.04	0.05 ± 0.02	59	**<0.01**	↓
TG (50:3)	0.020 ± 0.006	0.009 ± 0.004	54	**<0.01**	↓
TG (50:4)	0.017 ± 0.004	0.011 ± 0.003	38	<0.05	↓
TG (52:0)	0.06 ± 0.03	0.02 ± 0.01	68	**<0.01**	↓
TG (52:1)	0.22 ± 0.11	0.07 ± 0.04	70	**<0.01**	↓
TG (52:2)	1.0 ± 0.4	0.4 ± 0.2	59	**<0.01**	↓
TG (52:3)	0.31 ± 0.11	0.14 ± 0.07	54	**<0.05**	↓
TG (52:4)	0.7 ± 0.3	0.3 ± 0.1	64	**<0.01**	↓
TG (52:5)	0.004 ± 0.001	0.0021 ± 0.0006	51	**<0.01**	↓
TG (52:6)	0.0025 ± 0.0008	0.0011 ± 0.0006	57	**<0.01**	↓

p values correspond to the mean difference between the SUB885C group and the control group.
Note: lipids with *p* values marked in bold mean those remain significant after MTC.
* Homogeneity of variance assumption is deviated of this lipid. Data was log transformed and the mean ± SD was shown as the normal data value for a reference.

Table S2.3. TG's

Lipid species	**control (mean ± SD)**	**SUB885C (mean ± SD)**	**SUB885C vs. control change (%)**	***p* value**	**(↑) (↓)**
TG (54:0)	0.025 ± 0.012	0.008 ± 0.006	67	**<0.05**	↓
TG (54:1)	0.12 ± 0.07	0.04 ± 0.03	68	**<0.05**	↓
TG (54:2)	0.32 ± 0.17	0.11 ± 0.06	66	**<0.01**	↓
TG (54:3)	0.9 ± 0.4	0.4 ± 0.2	58	**<0.01**	↓
TG (54:4)	0.18 ± 0.08	0.08 ± 0.04	57	**<0.01**	↓
TG (54:5)	0.05 ± 0.02	0.021 ± 0.009	55	**<0.05**	↓
TG (54:6)	0.012 ± 0.004	0.007 ± 0.002	47	**<0.05**	↓
TG (54:7)	0.0012 ± 0.0004	0.0007 ± 0.0002	40	<0.05	↓
TG (56:0)	0.0028 ± 0.0013	0.0011 ± 0.0007	61	**<0.05**	↓
TG (56:1)	0.012 ± 0.006	0.004 ± 0.003	64	**<0.05**	↓
TG (56:2)	0.021 ± 0.010	0.008 ± 0.004	61	**<0.01**	↓
TG (56:3)	0.10 ± 0.05	0.04 ± 0.02	55	**<0.05**	↓
TG (56:4)	0.08 ± 0.03	0.03 ± 0.02	55	**<0.01**	↓
TG (56:5)	0.12 ± 0.04	0.06 ± 0.03	48	<0.05	↓
TG (56:6)	0.05 ± 0.02	0.03 ± 0.01	49	**<0.05**	↓
TG (56:7)	0.37 ± 0.16	0.15 ± 0.06	60	**<0.01**	↓
TG (56:8)	0.0043 ± 0.0017	0.0022 ± 0.0006	48	**<0.05**	↓

p values correspond to the mean difference between the SUB885C group and the control group.
Note: lipids with *p* values marked in bold mean those remain significant after MTC.
* Homogeneity of variance assumption is deviated of this lipid. Data was log transformed and the mean ± SD was shown as the normal data value for a reference.

Table S2.4. TG's

Lipid species	control (mean ± SD)	SUB885C (mean ± SD)	SUB885C vs. control change (%)	*p* value	(↑) (↓)
TG (58:1)	0.004 ± 0.002	0.002 ± 0.001	64	**<0.05**	↓
TG (58:2)	0.004 ± 0.002	0.0013 ± 0.0009	65	**<0.05**	↓
TG (58:3)	0.006 ± 0.003	0.002 ± 0.001	59	**<0.01**	↓
TG (58:4)	0.005± 0.002	0.003 ± 0.001	51	**<0.01**	↓
TG (58:5)	0.016 ± 0.007	0.008± 0.005	49	<0.05	↓
TG (58:6)	0.016 ± 0.006	0.008 ± 0.004	51	**<0.01**	↓
TG (58:7)	0.026 ± 0.009	0.013 ± 0.007	50	**<0.01**	↓
TG (58:8)	0.015 ± 0.005	0.007 ± 0.002	54	**<0.01**	↓
TG (58:9)	0.09 ± 0.04	0.04 ± 0.01	55	**<0.05**	↓
TG (60:1)	0.002 ± 0.001	0.0007 ± 0.0005	63	<0.05	↓
TG (60:2)	0.002 ± 0.001	0.0007 ± 0.0005	67	**<0.05**	↓
TG (60:3)	0.0011 ± 0.0006	0.0004 ± 0.0002	67	**<0.01**	↓
TG-O (50:0)	0.011 ± 0.006	0.003 ± 0.002	70	**<0.05**	↓
TG-O (50:1)	0.0035 ± 0.0016	0.0011 ± 0.0007	70	**<0.05**	↓
TG-O (50:2)	0.0008 ± 0.0003	0.0003 ± 0.0002	67	**<0.05**	↓
TG-O (52:0)	0.016 ± 0.008	0.005 ± 0.004	71	**<0.05**	↓
TG-O (52:1)	0.0037 ± 0.0017	0.0011 ± 0.0008	70	**<0.05**	↓
TG-O (56:2)	0.008 ± 0.004	0.003 ± 0.002	70	<0.05	↓
TG-O (56:3)	0.002 ± 0.001	0.0007± 0.0005	69	**<0.05**	↓
TG-O (58:2)	0.006 ± 0.004	0.002 ± 0.001	69	<0.05	↓
TG-O (58:3)	0.0023 ± 0.0012	0.0008 ± 0.0006	67	<0.05	↓

p values correspond to the mean difference between the SUB885C group and the control group.
Note: lipids with *p* values marked in bold mean those remain significant after MTC.
TG-O: ether TG
* Homogeneity of variance assumption is deviated of this lipid. Data was log transformed and the mean ± SD was shown as the normal data value for a reference.

Table S3. Lipid molecular species are significantly influenced in liver tissue upon SUB885C treatment as compared to non-treated controls

Lipid species	control (mean ± SD)	SUB885C (mean ± SD)	SUB885C vs. control change (%)	*p* value	(↑) (↓)
LPC (18:1)	0.34 ± 0.04	0.29± 0.05	12	<0.05	↓
LPC (18:2)	0.11 ± 0.02	0.08 ± 0.02	28	<0.01	↓
PC (32:0)	0.29 ± 0.02	0.26 ± 0.02	10	<0.05	↓
PC (36:5)	0.55 ± 0.06	0.64 ± 0.08	16	<0.05	↑
PC-O (38:5)*	0.33 ± 0.04	0.25 ± 0.02	21	<0.05	↓
PC-O (40:6)	0.025 ± 0.002	0.020 ± 0.004	33	<0.05	↓
PE (34:2)	0.25 ± 0.05	0.20 ± 0.03	20	<0.05	↓
PE (36:2)	0.76 ± 0.11	0.63 ± 0.06	17	<0.05	↓
PE (36:3)	0.53 ± 0.13	0.39 ± 0.06	26	<0.05	↓
SPM (22:1)	0.63 ± 0.05	0.51 ± 0.05	19	<0.01	↓
SPM (24:1)*	0.86 ± 0.11	0.68 ± 0.07	21	<0.05	↓
ChE (18:2)	0.29 ± 0.06	0.16 ± 0.05	44	**<0.01**	↓
ChE (20:4)	0.14 ± 0.06	0.07 ± 0.01	50	<0.01	↓
TG (50:1)	1.4 ± 0.4	1.8 ± 0.3	30	<0.05	↑
TG (54:0)	0.04 ± 0.01	0.024 ± 0.004	50	<0.01	↓
TG-O (50:0)	0.038 ± 0.007	0.026 ± 0.005	30	**<0.01**	↓
TG-O (50:1)	0.065 ± 0.008	0.053 ± 0.004	29	<0.01	↓
TG-O (50:2)	0.016 ± 0.002	0.013 ± 0.002	50	<0.05	↓
TG-O (52:1)	0.11 ± 0.02	0.077 ± 0.008	28	**<0.001**	↓
TG-O (52:2)	0.12 ± 0.02	0.10 ± 0.01	17	<0.01	↓
TG-O (58:1)	0.015 ± 0.003	0.009 ± 0.002	40	<0.01	↓
TG-O (58:2)*	0.034 ± 0.006	0.024 ± 0.005	33	<0.05	↓

p values correspond to the mean difference between the SUB885C group and the control group.
Note: lipids with *p* values marked in bold mean those remain significant after MTC.
PC-O: ether PC; TG-O: ether TG
* Homogeneity of variance assumption is deviated of this lipid.
Data was log transformed and the mean ± SD was shown as the normal data value for a reference.

Chapter 5

Linking biological activity with herbal constituents by systems biology-based approaches: effects of Panax ginseng in type 2 diabetic Goto-Kakizaki rats

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Abstract

Although a number of animal experiments and clinical trials have investigated the effects of ginseng roots on diabetes, the relationship between its therapeutic effects on diabetes and the quality and the growth age of this herb have not yet been reported. This study systematically investigated the effects of 3- to 6-y-old ginseng roots on glycemic and plasma lipid control in a rat model of type 2 diabetes. Six groups of male Goto-Kakizaki (GK) rats received either metformin, 3- to 6-y-old ginseng root, or no treatment. The treatments were administered twice daily for 9 weeks. A combined approach was used that involved applying liquid chromatography–mass spectrometry-based lipidomics, measuring biochemical parameters and profiling the components of ginseng roots of different ages. Compared to the untreated controls, treatment with 4- and 6-y-old ginseng roots significantly improved glucose disposal, and 5-y-old ginseng treatment significantly increased high density lipoprotein cholesterol. Treatment with 6-y-old ginseng significantly decreased total plasma triacylglyceride (TG) and very-low-density lipoprotein cholesterol and improved plasma glycated hemoglobin (HbA1c). In addition, treatment with 4- to 6-y-old ginseng influenced plasma lipidomics in diabetic GK rats by reducing TG lipid species. Metformin significantly reduced fasting blood glucose by 41% and reduced HbA1c by 11%, but showed no effects on the plasma lipid parameters. The present study demonstrates that ginseng roots show growth age-dependent therapeutic effects on hyperlipidemia and hyperglycemia in diabetic GK rats. These age-dependent effects may be linked with the variation in both the ratios and concentrations of specific bioactive ginsenosides in ginseng roots of different growth ages. This study introduced novel systems biology-based approaches for linking biological activities with potential active components in herbal mixtures.

Introduction

The incidence and prevalence of diabetes, particularly type 2 diabetes mellitus (T2DM), is increasing rapidly worldwide [1]. T2DM is a chronic metabolic disorder characterized by increased insulin resistance, impaired insulin secretion, and progressive β-cell dysfunction leading to hyperglycemia [2, 3]. The majority (50–70%) of T2DM patients have an atherogenic profile, manifested as an elevation in plasma triacylglyceride (TG) and apolipoprotein B levels, a decrease in high-density lipoprotein cholesterol (HDL-C) levels, and a preponderance of small, dense, low-density and very-low-density lipoprotein (LDL and VLDL) particles [2-4]. Individuals with T2DM have a substantial risk of morbidity and mortality from major coronary events, such as cardiovascular, cerebrovascular, and peripheral vascular diseases [5-7]. To this end, the prevention and treatment of diabetes is crucial to reduce the risk factors for diabetic complications.

Clinical trials have provided adequate evidence that hyperglycemia and hyperlipidemia are fundamental risk factors for the vascular complications associated with diabetes [8, 9]. To reduce these two risk factors, proper diet and regular exercise have been emphasized as a high priority. Lipid-lowering and glycemia-lowering medications are currently used to treat diabetes and its associated complications in mainstream medical approaches [10]. However, the current drug treatments for diabetes have three major limitations: 1) the high frequencies of side effects, 2) the high rates of secondary failure, and 3) the rapidly increasing costs of new diabetic drug development. Due to these concerns, researchers have been searching for more effective and multi-targets therapies. This strategy differs from the ‘one drug fits all’ treatment concept and may reduce side effects.

Natural products, such as herbs, have a much longer history of use in the treatment of diabetes, than modern pharmaceuticals. For example, in China, as early as the period from 1368 to 1644, the root of *Panax ginseng* C. A. Mey (often simply referred to as ginseng in this text) was reported to treat diabetic symptoms in an ancient medicinal textbook titled *Compendium of Materia Medica* (*Ben Cao Gang Mu*) by Shizhen Li (1518–1593). Since then, the popularity of treating illnesses like diabetes with ginseng has continued to grow worldwide. Reports obtained from *in vivo* animal experiments and clinical trials have demonstrated that radix ginseng, the dried root of *Panax ginseng* C.A. Meyer, has a wide range of therapeutic effects on the central nervous system, the cardiovascular system, and the immune system [11-13]. Pharmacological studies have indicated that radix ginseng can ameliorate diabetes by improving glucose homeostasis and insulin sensitivity and alleviate diabetes-induced oxidative stress by inhibiting lipid peroxidation [14-16]. However, the lack of standardization in quality control and

quality assurance in producing ginseng and ginseng products often leads to inconclusive results when they are used to treat diabetes [17]. In part, this lack of standardization is due to the complex chemical composition of ginseng roots, which exhibit regional variation and age-dependent variation in growth that influence the compositional ratios of certain ginsenosides [18]. Sengupta et al. [17] observed that reconstituting a ginseng extract by adding two ginsenosides, Rg1 and Rb1, in a defined ratio could alter the angiogenic outcome: the dominance of Rg1 led to angiogenesis, whereas the dominance of Rb1 exerted an opposing effect. These observations strongly suggest that quality control and quality assurance must be addressed when making ginseng products.

Ginseng is a perennial herb that can grow for dozens of years. The major components in ginseng are ginsenosides, which are responsible for most of ginseng's biological and pharmacological activities. Ginseng cultivators have long observed that the ginseng growth period is directly related to its therapeutic quality. Ginseng grown for $\geq$ 4 y is regarded to be for the appropriate quality for medicinal use [19]. Although ginseng has been demonstrated to have beneficial effects on diabetes management, no research has yet been performed to determine how the ginseng's age and its related quality affect its therapeutic efficacy.

With these considerations, we used systems biology based metabolomics approaches to evaluate the therapeutic effects of ginseng roots grown for 3–6 y on the regulation of hyperglycemia and dyslipidemia in a Goto-Kakizaki (GK) rat model with spontaneous T2DM. The GK rat model displays fasting hyperglycemia and has a non-obese phenotype [20]. Metformin, a widely used oral medicine in T2DM treatment to reduce hepatic gluconeogenesis and improve glucose uptake [21, 22], was administered as a positive control. We aimed to evaluate possible ginseng-induced effects on lowering glucose and improving glucose tolerance. We hypothesized that ginseng roots show growth age-dependent effects on improving glycemia and lipid metabolism in diabetic GK rats. We hypothesized that these age-dependent effects may be due to the fact that the ratios and concentrations of specific ginsenosides in the ginseng roots change during growth.

Experimental Section

Chemicals and Reagents

Liquid chromatography grade dichloromethane (CH_2Cl_2), methanol (MeOH), isopropanol (IPA), and acetonitrile (ACN) were purchased from Tedia (Fairfield, USA). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Analytical grade ammonium formate (AmFm) was obtained from

Sigma-Aldrich (St. Louis, USA). Analytical grade glucose was from Chengdu Kelong Chemical Reagent Plant (Chengdu, China). Chemical grade sodium carboxymethylcellulose was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Ginseng samples, harvested after 3–6 y of growth, were purchased as dried roots from Fusong Chengan Ecology Ginseng Co., Ltd. (Jilin, China). Nine ginsenoside standards, including Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, and Rg2, were purchased from Chengdu Cogon Bio-tech Co., Ltd. (Chengdu, China). Metformin was purchased from Shandong Linuo Kefeng Pharmaceutical Company Co., Ltd. (Shandong, China). Synthetic lipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA) and Sigma-Aldrich (Munich, Germany). Digoxin and leucine-enkephalin was obtained from Sigma-Aldrich (Munich, Germany).

Ginseng Plant Cultivation

Radix Panax ginseng C.A. Mey was used in this study. All of the ginseng plants were cultivated at one farm located GAP station in a village (Fusong Chengan Ecology Ginseng Co., Ltd) in Jilin Province (Northern China). Ginseng plants of different ages were cultivated at the same location and harvested at the same time (i.e., in autumn). After harvesting the plants, the ginseng roots were air dried in the sun. According to Chinese Pharmacopeia, the water content is not more than 12.0% and total ash is not more than 5.0% and acid-insoluble ash is not more than 1.0%. The main ginseng roots containing secondary roots without root hair were used in the present study.

Animal Protocols and Ginseng or Metformin Administration

All animal experiments were approved by an institutional ethical committee on animal care and experimentation under authorization number 113 (West China School of Pharmacy, Sichuan University, Chengdu, China). Four-week-old, male GK rats, weighing 260–320 g, were purchased from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China). All animals were housed in a temperature-controlled room with a 12-h light/dark cycle. Food and water was freely available, except during fasting periods before some experiments.

After a one-week adaptive feeding period, 37 GK rats were randomly divided into six groups for a 9-week treatment intervention: group 1, non-treated control (n = 5); group 2, positive control (n = 8) receiving intraperitoneal administration of metformin at a dosage of 75 mg/kg body weight/day, groups 3, 4, 5, and 6 (n = 4, 5, 5, and 5, respectively), receiving intraperitoneal administration of 3-, 4-, 5-, or

6-y-old ginseng roots, respectively, at a dosage of 1.875 g/kg body weight/day, twice daily. Both ginseng and metformin were ground into powder and mixed with 0.5% sodium carboxymethylcellulose before administration. The non-treated animals were injected with 0.5% sodium carboxymethylcellulose at 2× 1 mL/100 g body weight/day without any herbal medicine or drug. No adverse effects were observed in animals after treatments with ginseng roots, metformin, or the vehicle.

Notably, plasma biochemical parameters including total cholesterol (TC), TG, HDL-C, low-density lipoprotein cholesterol (LDL-C), very-low-density lipoprotein cholesterol (VLDL-C), and plasma glycated hemoglobin (HbA1c) were measured at the end of the experiment. Fasting blood glucose (FBG) was measured at week 0 and week 9. The intraperitoneal glucose tolerance test (IPGTT) was also performed at week 9. In addition, body weight and food intake were measured weekly throughout the experiment.

Sacrifice and Sample Collection

Animals were sacrificed with rapid asphyxiation with CO_2 and opened longitudinally after the 9-week experiment (endpoint). Blood was collected via saphenous vein puncture before the start of the treatment (i.e., t = week 0) and via heart puncture after sacrifice (i.e., t = week 9) in CB 300 LH microvettes (Sarstedt, Nümbrecht, Germany), containing lithium heparin, and the samples were placed on ice immediately after collection. Plasma samples were obtained after centrifugation at 3000*g* for 5 min at 4°C. Aliquots of plasma were frozen and stored at -80°C until use.

Plasma Lipid Biochemical Parameters

Plasma TC and TG were measured by the fully automatic enzymatic method using enzymatic kits obtained from Shanghai Fosun Long March Medical Science Co., Ltd. (Shanghai, China). Plasma HDL-C, LDL-C, and VLDL-C were measured using enzymatic kits purchased from Wenzhou Dongou Jinma Bio-tech Co., Ltd. (Wenzhou, China). All measurements were carried out on plasma samples taken after animals were fasted for 4 h.

Plasma Glycemic Metabolic Parameters

HbA1c was measured using an immune-based assay. After the animals were fasted for 4 h, FBG concentrations were measured before and after treatment. Blood glucose levels were determined in blood samples taken from the tail vein with a glucose assay kit using the GOD-PAP method (Sichuan Maker Bio-tech Co., Ltd., Chengdu, China). The IPGTT was performed on the final day of treatment on the 9th week using an intraperitoneal administration of glucose (1.25 g/kg body weight) after the animals were fasted for 4 h. Blood glucose levels were also determined in blood samples from the tail vein at 0 min (prior to glucose administration), and 30, 60, 120, 180 and 240 min after the glucose administration.

Liquid Chromatography–Mass Spectrometry (LC-MS) Lipid Profiling

Lipid Extraction

Lipid extracts were obtained via a modified Bligh/Dyer extraction procedure, as described previously [23]. Briefly, 30 μL of internal standard (IS) mixture were added to 30 μL of plasma followed by the addition of 540 μL of 2:1 CH_2Cl_2/MeOH. The mixture was thoroughly vortexed, and then 120 μL of water was added to form a two-phase system. The lipids were dissolved in the bottom organic phase. After being centrifuged at 6000*g* for 10 min at 10°C, 100 μL of the lipid extracts from the bottom layer were transferred and diluted 5 times with ACN/IPA/water (65:30:5, v/v/v). Then 10 μL of the lipid extracts were loaded for LC-MS lipid profiling analysis. Notably, each sample was prepared in duplicate and each prepared sample was injected once.

LC-MS Analysis

LC-MS lipidomics analysis was performed on a hybrid ion-trap time-of-flight mass spectrometer (IT-TOF-MS; Shimadzu, Kyoto, Japan). The mass spectrometer was coupled with an ultra fast liquid chromatography (LC) system (Shimadzu, Kyoto, Japan). An Ascentis® Express C_8 column (2.7 μm particle size, 90 Å, 2.1 × 150 mm; Sigma-Aldrich, Munich, Germany) was used for the LC separation. The LC separation conditions used were identical to the previously published methods [23]. The plates with diluted lipid extracts were maintained at 12°C.

Plasma lipid profiling was carried out on the Shimadzu IT-TOF-MS equipped with an electrospray ion source. MS survey scans were acquired in the positive ion mode. The voltages of the interface and the detector of the TOF analyzer were set to 4.5 kV and 1.6 kV, respectively. The temperatures of the curved desorption line and heat block were both set to 200°C. The flow rate of the nebulizing gas was 1.5

L/min. The dry gas pressure was 0.2 MPa. The flight tube temperature was stable at 40°C, and the ion trap pressure was maintained at 1.6×10^{-2} Pa. Ultra-high purity argon was used for collision and ion cooling. The data were collected at a mass range of m/z 400–1500 with an ion scan duration of 20 ms using LCMS solution software (Shimadzu, Kyoto, Japan).
All study samples were randomly analyzed. Quality control (QC) samples, prepared by pooling all of the plasma samples, were regularly measured in the sequence to monitor the response of the LC-MS system and assess the lipid profiling platform.

LC-MS Ginsenoside Profiling

Ginseng Extraction

Thirty milligrams of powdered ginseng root were weighed in a new 2 mL Eppendorf vial followed by the addition of 1 mL MeOH/water (4:1, v/v) containing 0.32 μg/mL digoxin, which was used as the IS for ginsenoside analysis. After thoroughly vortexing the mixture for 3 min, the resulted suspension was placed in a shaker and incessantly shaken for 1 h at room temperature. The suspension was then placed in an ultrasonic bath at 4°C for 1 h. Next, the suspension was centrifuged at 10000*g* for 10 min at 20°C. Subsequently, 600 μL of supernatant was transferred and filtered using a Whatman polypropylene syringe filter with a 0.2 μm pore size and a 25 mm diameter (Whatman, Netherlands B.V.). The filtered ginseng extracts were diluted 5 times with MeOH/water (1:1, v/v) before being analyzed by LC-MS.

Ginsenoside Analysis

The diluted ginseng root extracts were analyzed on an Acquity™ ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS, Synapt™ G1 HDMS system, Water Corp., Milford, USA) equipped with an MS pump, an autosampler (Waters Corp., Milford, USA), and a Waters UPLC T_3 column (2.1 mm × 100 mm, i.d., 1.8 μm, Waters Corp., Milford, USA). The separation was carried out with a binary solvent consisting of water (15 mM AmFm) and ACN. The binary gradient started with 20% B until 0.5 min, increased to 21% B from 0.5 to 7 min, to 30% B from 7 to 10 min, to 40% B from 10 to 18 min, to 80% B from 18 to 20 min, and was maintained at 80% B from 20–23 min. Then from 23–23.1 min, solvent B was decreased to 20%, and it was then maintained for 4.9 min for column re-equilibration. The flow rate was 0.50 mL/min. The column oven temperature was set to 35°C, and the

temperature of the autosampler tray was maintained at 10°C. The following parameters were used for mass spectrometry: capillary voltage, 2.5 kV; reference cone voltage, 50 V; sampling cone voltage, 35 V; extraction cone voltage, 4.0 V; source temperature, 80°C; desolvation temperature, 400°C; desolvation gas flow, 700 L/h; cone gas flow, 20 L/h; reference scan frequency, 10 s; scan time, 1.0 s; interscan time, 0.02 s; and lock mass, 554.2615 (leucine-enkephalin). The ginsenoside profiling was acquired under negative ionization mode. The ginsenoside profiling data were recorded by using MassLynx™ (MassLynx V4.1 SCN 639).

Before carrying out the ginsenoside profiling of the ginseng root samples, a compact validation of the method was performed, and the analytical characteristics were evaluated in terms of linearity, repeatability, recovery, and limit of detection (LOD) for nine, representative ginsenoside standards. Digoxin was used as the IS The results of the compact method validation were satisfactory for the profiling analysis of complex herb mixtures [24].

Statistical Analysis

All measured biological parameters are presented as means ± SD. Univariate statistics were performed with SPSS (Statistical Product and Service Solutions) using one-way analysis of variance (ANOVA) with the two-sided Dunnett post hoc test for multiple comparisons to investigate the differences between the control and treatment groups. Differences among groups were considered statistically significant if $p < 0.05$.

During the analysis of the lipid metabolism parameters, the appropriate lipid IS was used to correct the signal intensities of the lipids in each ionization mode (see Supplementary Table S1 for details). Thereafter, peak areas were calculated for each lipid species relative to the standards to account for the variability in MS signals among the different groups of samples. Principle component analysis (PCA) was chosen to visualize the clustering pattern related to the samples and the conditions.

Results

Treatment Effects on Biochemical Parameters

Throughout the 9-week treatment period, neither food intake nor body weight was significantly changed in any of the treated rats when compared to the untreated controls. Within each group, food intake was comparable, and the body

weight of the animals showed a continuous increase from week 1 to week 9 (data not shown).

The plasma biochemical parameters (i.e., TC, TG, HDL-C, LDL-C, and VLDL-C) were measured after 4 h fasting in all the GK rats at week 9. These results indicated that there was a significant decrease in the levels of TG (1.27 ± 0.33 vs. 0.85 ± 0.28 mmol/L, $p < 0.05$) and VLDL-C (0.58 ± 0.15 vs. 0.39 ± 0.13 mmol/L, $p < 0.05$) in the GK rats that received the 6-y-old ginseng root treatment compared to the untreated control. There was a significant increase in HDL-C (1.27 ± 0.13 vs. 1.48 ± 0.09 mmol/L, $p < 0.05$) in the GK rats that received the 5-y-old ginseng root treatment compared to the untreated controls. Additionally, there was a trend of improvement (i.e., $0.05 < p < 0.1$) in TG (1.27 ± 0.33 vs. 0.90 ± 0.27 mmol/L), HDL-C (1.27 ± 0.13 vs. 1.39 ± 0.15 mmol/L), and VLDL-C (0.58 ± 0.15 vs. 0.41 ± 0.12 mmol/L) levels in GK rats that received the 4-y-old ginseng root treatment vs. the untreated control. Interestingly, the metformin-treated animals did not show the above trends in the lipid biochemical parameters. The mean baseline (i.e., t = week 0) FBG in all of the treatment groups was comparable to that in the untreated control group (data not shown). At the end of the experiment, a significant reduction in FBG (9.36 ± 4.27 vs. 5.52 ± 1.26 mmol/L, $p = 0.03$) was observed only in the metformin treated group vs. untreated controls, but no reduction in FBG was observed in the ginseng treated groups.

No reduction in HbA1c was observed in animals following the treatments with 3- to 5-y-old ginseng roots at the end of the study; however, animals receiving treatment with either 6-y-old ginseng root or metformin displayed a decrease tendency of –11% ($p = 0.094$) and –11% ($p = 0.067$), respectively (vs. untreated controls) (Figure 1).

In addition, glucose tolerance was evaluated by IPGTT at week 9. Compared to the nontreated control group, significant glucose disposal was observed in GK rats responding to 4- and 6-y-old ginseng root treatments at 180 min (i.e., 19.91 $\pm$ 4.73 vs. 9.83 $\pm$ 3.83 and 19.91 $\pm$ 4.73 vs. 11.28 $\pm$ 3.18, respectively; both $p < 0.05$) and at 240 min (i.e., 17.75 $\pm$ 3.96 vs. 6.88 $\pm$ 2.18 and 17.75 $\pm$ 3.96 vs. 8.63 $\pm$ 2.01, respectively; both $p < 0.01$) (Figure 2). In order to further evaluate the overall glucose exposure, the area under the curve (AUC) of glucose disposal was calculated. The glucose AUC corresponding to the 4- and 6-y-old ginseng treatments decreased by 15% and 8%, respectively, in comparison to the untreated rats. Meanwhile, the glucose AUC of the metformin-treated animals was comparable with the 3- and 5-y-old ginseng treatment groups, and there was no significant decrease when compared to that of the untreated rats. Collectively, the ginseng treatments displayed a similar glucose response curve to the metformin treatment. The treatments with 4- and 6-y-old ginseng induced better glucose tolerance than metformin.

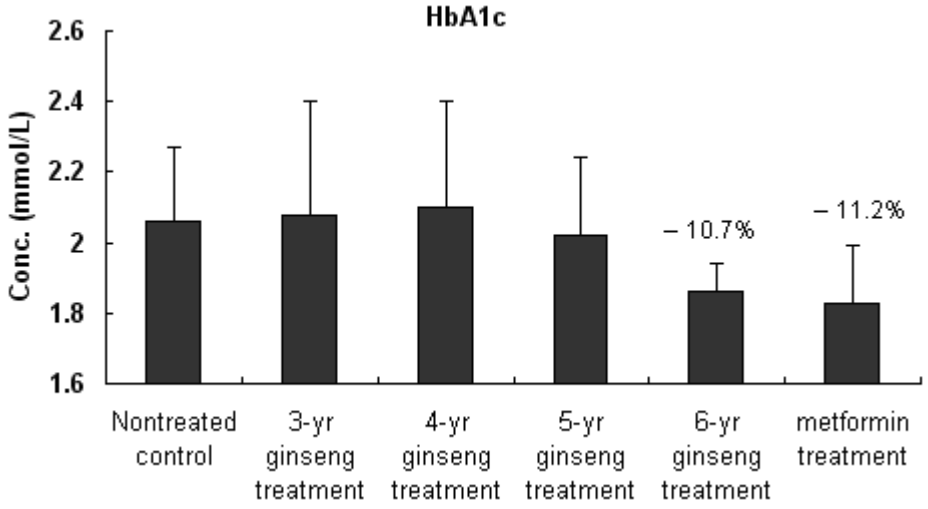

Figure 1. *HbA1c levels in GK rats that received treatment with 3- to 6-y-old ginseng root or metformin, or nontreatment. The HbA1c levels exhibited a tendency to decrease ($p < 0.1$, for both) in the 6-y-old ginseng root and metformin treatment groups vs. untreated controls, respectively.*

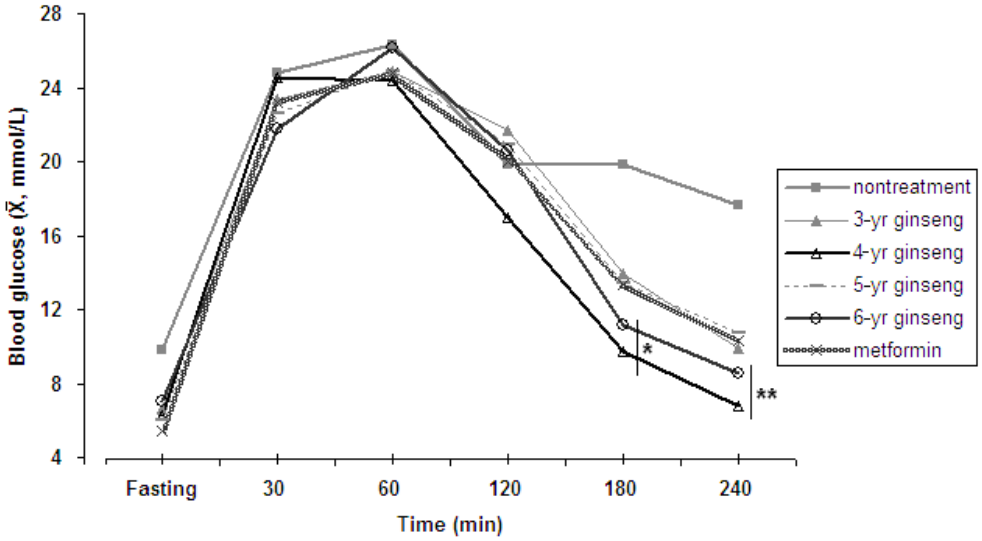

Figure 2. *Intraperitoneal glucose tolerance test results. The intraperitoneal glucose tolerance test was performed at the end of the experiment in untreated GK rats and in animals treated with 3- to 6-y-old ginseng roots or metformin. Significantly higher rates of glucose disposal at 180 min and 240 min were observed in GK rats after treatment with 4- and 6-y-old ginseng roots. *$P < 0.05$, **$p < 0.01$ compared to untreated rats.*

Relationship between efficacy and the growth ages of the ginseng roots

A total of 96 individual lipids including lyso-phosphatidylcholine (LPC), phosphatidylcholine (PC), phosphoethanolamine (PE), sphingomyelin (SPM), diacylglyceride (DG), TG and cholesterol ester (ChE) were identified and quantified in the lipidomics study. In order to identify general clusters in the data from the nontreated controls and the rats undergoing the different treatments and to examine which lipids contributed most to the clusters, we carried out PCA on the plasma lipidomics data from all of the study samples, including the 3- to 6-y-old ginseng treatment, the metformin treatment, and the untreated control groups. Figure 3 displays the PCA score plot based on a PC2 vs. PC1 model. The score plot (consisting of the symbols '▲,' '★,' '+,' and '○') showed a clear separation between the 6-y-old ginseng treated group (symbols connected with bold dashed lines) and the nontreated control animals (symbols connected with bold solid lines). The first two principal components accounted for 55.1% of the variance, i.e., more than half of the total variance in the model. In addition, there was a clear trend of separation between the 4-y-old ginseng treated group (symbols connected with gray bold solid lines in Figure 3) and the nontreated controls. No separation was found between the untreated controls and 3- or 5-y-old ginseng root treatment groups. Furthermore, the PCA score plot showed that metformin-treated animals (symbols connected with gray dot dashed lines in Figure 3) largely overlapped with that of the nontreated animals. The PCA biplot indicated that the TG lipids dominated the clustering patterns of the 6-y-old ginseng treatment group vs. the nontreated controls and of the 4-y-old ginseng treatment vs. nontreated controls (Supplementary Figure S1 in the Supporting Information). Interestingly, TG lipids were found to be even more abundant in metformin-treated rats than in the untreated rats according to the biplot, suggesting that metformin has very limited effects on plasma lipid metabolism in diabetic GK rats. In summary, this initial PCA suggested striking differences between the patterns depending on the growth age of the ginseng roots, in spite of the fact that the ginseng had the same genetic background, were planted in the same region, and were under identical harvesting and processing conditions.

To further identify any potential underlying patterns causing the observed differences in the PCA model, we analyzed the content of individual lipid molecular species in the groups treated with 3- to 6-y-old ginseng vs. the untreated controls using one-way ANOVA with the 2-sided Dunnett post hoc test. In total, only TG (58:6) and TG (58:7) showed significant reductions (p = 0.021 and 0.022, respectively), and TG (56:6) and TG (60:9) showed a decreasing trend (p = 0.085 and 0.097, respectively) in the 6-y-old ginseng treatment group vs. the nontreated controls. Although rarely statistical significance was observed, most of

the TG lipids showed a decreasing trend in groups treated with 4- to 6-y-old ginseng roots (data not shown) compared to the nontreated controls, which suggests that the effects of ginseng root treatment on plasma lipid metabolism in diabetic GK rats are growth age-dependent.

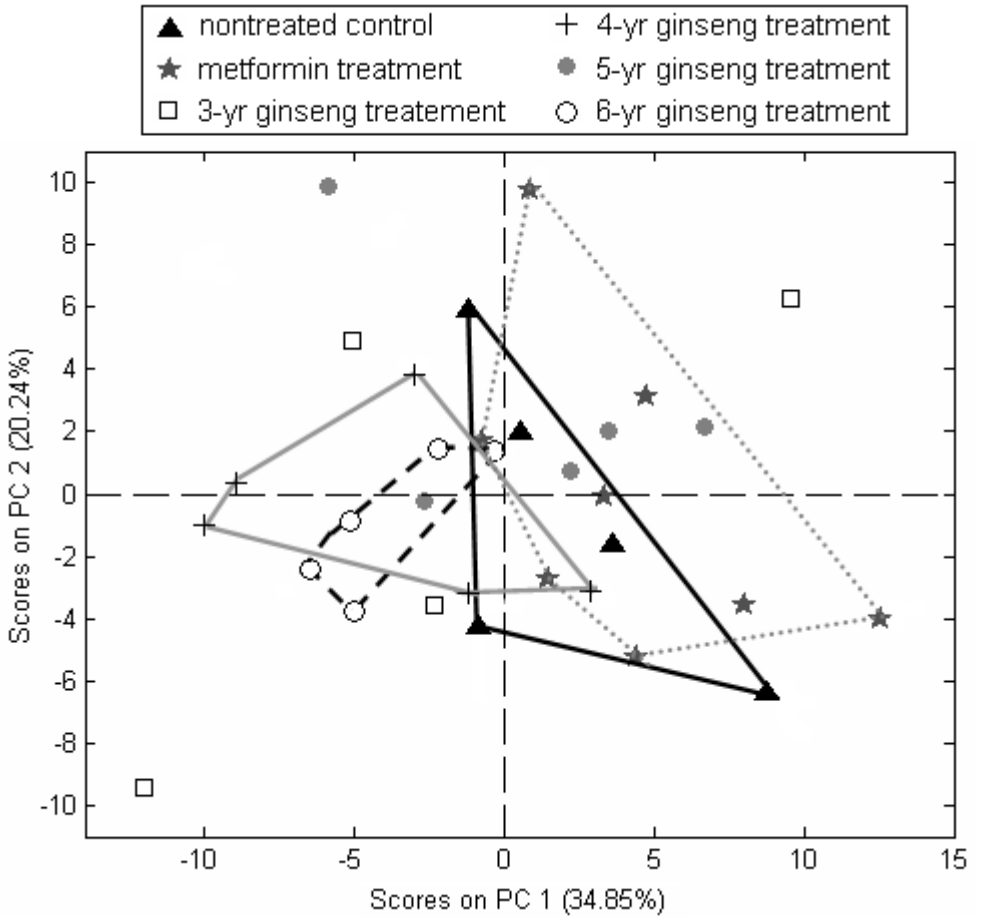

Figure 3. *PCA score plot of lipidomics data. PCA score plot of plasma lipidomics data from all of the study samples revealed general clusters in the nontreatment and treatment groups. '▲' connected with bold solid lines represents the nontreated control group, '★' connected with gray dot dashed lines represents the metformin-treated group, '+' connected with gray bold solid lines represents the 4-y-old ginseng root treatment group, and '○' connected with bold dashed lines represents the 6-y-old ginseng root treatment group.*

Relationships between the Biologically Active Components in Ginseng Roots and the Biological Responses

In order to find correlations between the biologically active components in ginseng roots and biological responses, we first investigated whether there were growth age-dependent differences in the ginsenosides of ginseng roots. PCA was performed on the mean-centered plus unit variance-scaled LC–MS data from all the ginseng root samples with 3–6 y of growth. Figure 4A presents the PCA score plot (consisting of the symbols '▼,' '□,' '×,' and '+') on the basis of the PC3 vs. PC1. The variation in PC3 may reflect the metabolites responsible for the age-dependent differences. The 3- to 6-y-old ginseng roots are discriminated in PC3, and the 6-y-old ginseng samples are located close to the 4-y-old ginseng samples. Meanwhile, the 5- and 3-y-old ginseng samples, which partially overlap with each other, are located relatively far away from the 6-y-old samples, but close to the 4-y-old ginseng samples. The age-dependent differences were also related to the sizes of ginseng roots, as shown in Figure 4B.

In the next step, we analyzed the variation in the concentrations of the individual components in ginseng roots of different growth ages on a univariate basis. Figure 5 displayed the concentration variation (in %) of 8 specific ginsenosides in the 3- to 6-y-old ginseng roots. In the 4-y-old ginseng roots, the most abundant four compounds were Rb2, notoginsenoside R1 (Noto-R1), malonly-Rb2 (mal-Rb2), and malonly-Rb3 (mal-Rb3). In the 5-y-old ginseng roots, the most abundant two compounds were Rb3 and Rd. In the 6-y-old ginseng roots, two compounds, namely Re and gypenoside XVII, were most abundant. Interestingly, Rb2, Noto-R1, mal-Rb2, and mal-Rb3 continued decreasing in abundance with age in the 4- to 6-y-old ginseng roots, and Rb3 and Rd continued increasing with age in the 3- to 5-y-old ginseng roots. The variation in the concentrations of these eight compounds with growth age may be account for the different therapeutic effects of 4- to 6-y-old ginseng roots. The concentrations and ratios of these compounds might serve as valuable markers for therapeutic quality control.

Discussion

Panax ginseng C.A. Mey root and its products have long been used as herbal treatments and dietary supplements with documented health benefits, including antioxidation, anti-hyperglycemia, anti-atherosclerosis, and anti-cancer effects [25-29]. For diabetes management, ginseng root extracts have been demonstrated to lower blood glucose [30], increase insulin sensitivity [31], and regulate lipid metabolism [32, 33].

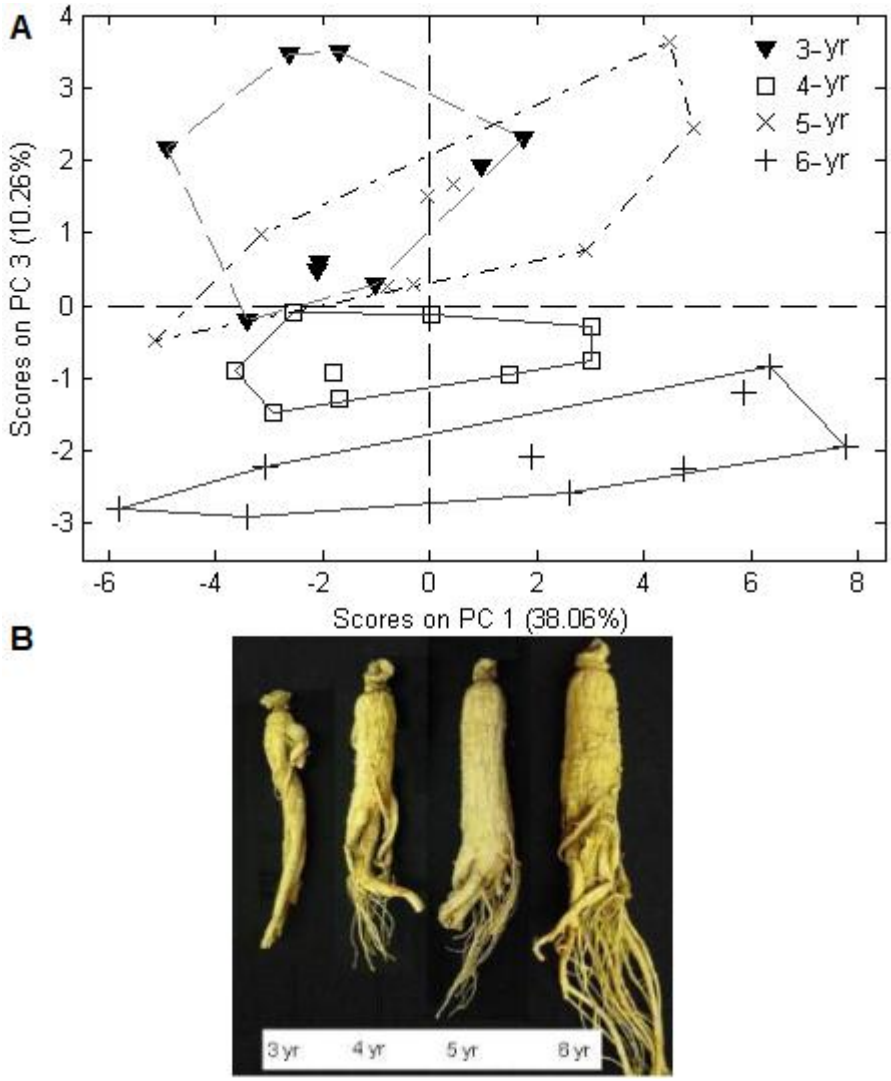

Figure 4. *PC3 vs. PC1 score plot of extracted Panax ginseng roots and dry morphology of ginseng roots. (A) PC3 vs. PC1 score plot of all MeOH/H_2O extracted Panax ginseng roots '▼,' '□,' '×,' and '+' represent 3-, 4-, 5- and 6-y-old ginseng roots, respectively. The PCA score plot reveals that 3- to 6-y-old ginseng roots are discriminated in PC3. The 6-y-old ginseng samples are located close to the 4-y-old ginseng samples, while the 5- and 3-y-old ginseng samples partially overlap with each other, but are located relatively far away from the 6-y-old ginseng samples and close to the 4-y-old ginseng samples. (B) Dry morphology of 3- to 6-y-old ginseng roots.*

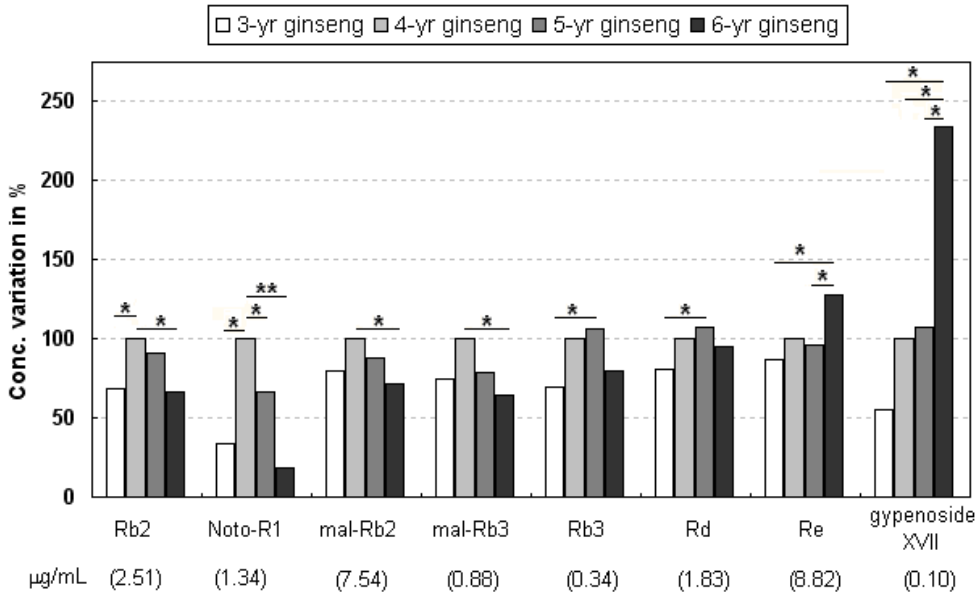

Figure 5. *Concentration variation (in %) of 8 specific ginsenosides in the 3- to 6-y-old ginseng roots. The concentrations of the ginsenosides in the 4-y-old ginseng roots were set equal to 1. The concentrations values (μg/mL) of the ginsenosides in the 4-y-old ginseng are given along the X-axis below the name of 8 ginsenosides. The ginsenosides Rb2, noto-R1, mal-Rb2 and mal-Rb3 were most abundant in 4-y-old ginseng, ginsenosides Rb3 and Rd were most abundant in the 5-y-old ginseng, and ginsenosides Re and gypenoside XVII were most abundant in the 6-y-old ginseng. These 8 ginsenosides are responsible for the age-dependent differentiation of the ginsenoside profiles (*: $p < 0.05$, **: $p < 0.01$).*

Although the research investigating the effects of ginseng roots in diabetes is rapidly growing, researchers have not yet examined how the therapeutic effects of this ginseng are related to its quality and the growth age. The aims of this study were: (1) to evaluate the effects of ginseng roots grown for 3–6 y on regulating hyperglycemia and hyperlipidemia in a T2DM GK rat model, and (2) to link the biologically active components in ginseng roots to their biological effects. To this end, we first tested lipid- and glycemic-related biological parameters, and then incorporated two advanced technologies as part of a systems biology approach. These two technologies involved: (a) the application of lipidomics to a study of 37 GK rats with T2DM that received 9-week, parallel interventions, which included a nontreatment control group, and (b) the application of profiling ginsenosides to identify the differences among ginseng roots of different growth ages. Our study presents three major findings.

Firstly, 4- to 6-y-old ginseng roots were observed to improve hyperlipidemia and hyperglycemia in diabetic GK rats. Six-year-old ginseng had the most beneficial effect on hyperlipidemia, followed by 5-year-old and then 4-y-old ginseng, based on the observation that 6-y-old ginseng significantly improved plasma TG and VLDL-C and 5-y-old ginseng significantly improved HDL-C in comparison to the untreated controls. Treatment with 4-y-old ginseng led to a trend of improvement in TG, HDL-C, and VLDL-C, although these improvements were not statistically significant. In contrast to the ginseng treatments, metformin showed very limited effects on these lipid-related biochemical parameters. However, in terms of glycemic control, metformin significantly improved FBG (reduced by 41%, p = 0.030 vs. control) and produced a substantial improvement tendency in HbA1c (reduced by 11.2% vs. control). Our results are consistent with those reported in a previous review, which concluded that metformin has beneficial effect on glucose-lowering but rather limited effect on plasma lipid profile in T2DM [34]. Meanwhile, 4- to 6-y-old ginseng roots produced positive effects on glycemic control by improving glucose tolerance (4- and 6-y-old ginsengs in IPGTT) and a decreasing tendency of HbA1c (6-y-old ginseng). In our current study, the glycemic regulation effects produced by ginseng were equal to or better than those produced by metformin treatment. Accordingly, under the current experimental conditions, the treatments with 4- to 6-y-old ginseng roots, especially 6-y-old ginseng, were superior to metformin treatment in terms of producing beneficial effects on lipid regulation and glucose tolerance. Moreover, metformin is associated with adverse gastrointestinal effects, including taste disturbance, appetite loss, nausea or vomiting, abdominal pain, and diarrhea [21], which cause patients to become intolerant to metformin. Ginseng is commonly used as a dietary supplement and herbal medicine in China, and its adverse effects have been scarcely reported. Therefore, ginseng may be an effective and safe initial intervention to improve glucose- and lipid-related parameters in people with T2DM.

Secondly, we identified distinct clusters in the lipid profiles, which demonstrate differences between the levels of lipid metabolites in the intervention groups after the 9-week parallel treatments. The PCA of the plasma lipidomics data revealed a clear difference between the 6-y-old ginseng-treated group and the nontreated controls. The TG lipids contributed the most to this difference. Four TG species were identified as discriminating lipids (i.e., TG 56:6, 58:6, 58:7, and 60:9) in the total lipid pattern. In addition, a large number of TG molecules showed non-significant decreasing trends in the GK rats receiving 4- to 6-y-old ginseng treatments in comparison to the untreated controls, indicating that the TG-lowering effect of ginseng is growth-age dependent. The growth age of the

ginseng roots can account for the variation in the contents of specific biologically active components.

Thirdly, we identified general growth and age-related trends by profiling components in ginseng roots, and we precisely defined changes in individual components. Through further integration with biological parameters, we predicted linkages between bioactive components in ginseng roots and the bioactivities related to growth ages. Ginsenosides (i.e. ginseng saponins) have been well recognized as major bioactive components, which are responsible for the biological and pharmacological activities of ginseng roots and tissues. In the present study, we examined the identified 27 ginsenosides found in all ginseng root extracts. We combined our biochemical and lipidomic results in the animal model together with the variations in the concentrations of specific ginsenosides in the 3- to 6-y-old ginseng roots. This method allowed us to link the improvements in lipid and glycemic metabolism due to treatment with 4-y-old ginseng root to Noto-R1, Rb2, mal-Rb2, and mal-Rb3. Meanwhile, the improvements due to treatment with 5-y-old ginseng root were linked to Rb3 and Rd, and the beneficial effects of 6-y-old ginseng root treatment were associated with Re and gypenoside XVII. However, it is possible that the efficacy of 6-y-old ginseng root treatment could be due to a combination of many different ginsenosides in a specific concentration ratio (e.g., high concentrations of gypenoside XVII and Re, and a low concentration of Noto-R1, see Figure 5). We observed that four ginsenosides were related to the 4-y-old ginseng root treatment effects, which is partially supported by the results of a previous study on the medicinal use of pure ginseng components to treat diabetes. Yang et al. provided experimental evidence on the clinical application of six representative notoginsenosides, including notoginsenoside R1, in the diabetic, obese KK-Ay mouse model and demonstrated that notoginsenosides improved insulin and leptin sensitivity [35]. Liu et al. reported that mal-ginsenosides, including mal-Rb1, mal-Rb2, mal-Rc, and mal-Rd, significantly lowered plasma glucose without changing the hepatic glycogen and cholesterol levels in streptozotocin-induced diabetic mice [36]. Moreover, ginsenoside Rb2 is reported to be capable of lowering TG levels in 3T3-L1 adipocytes cultured under high energy conditions by stimulating the expression of SREBP and leptin mRNA [37]. Although the present study could not precisely determine the beneficial actions of these four ginsenosides on glycemic and lipid control in diabetes, it provided experimental evidence on the hyperglycemic and hyperlipidemia regulation effects of Asian ginseng root and identified potential bioactive components.

Although no relevant research on diabetes has been reported in literature for the two specific ginsenosides identified in the 5-y-old ginseng root (i.e. Rb3 and Rd), several studies indicated that these components play an important role in health

promotion and disease prevention. For example, a recent study demonstrated that ginsenoside Rb3 can significantly ameliorate myocardial injury and heart function impairment induced by isoproterenol in rats by increasing the activities of myocardial antioxidant enzymes and inhibiting myocardial lipid peroxidation in myocardial ischemia [38]. Ginsenoside Rd was considered to be a potential compound for cancer prevention due to its inhibitory action on 26S proteasome activity and its low toxicity [39].

For the two ginsenosides identified in the 6-y-old ginseng root (i.e., Re and gypenoside XVII), there has been substantial research related to their biological effects in diabetic/diseased animal models and humans [40-42]. For instance, ginsenoside Re was demonstrated not only to exhibit a dose-dependent anti-hyperglycemic effect in diabetic *ob/ob* mice [25, 43], but it also had a significant anti-hyperlipidemic efficacy in streptozotocin-induced diabetic rats [44]. Gypenoside XVII has been reported to have a protective effect against oxidative stress in phagocytes, vascular endothelial cells, and liver microsomes, which indicates that it may play an important role in the prevention and treatment of atherosclerosis, liver disease, and inflammation [45]. Based on our findings and the reported effects of the above-mentioned ginsenosides in the literature, we summarized the observed ginseng therapeutic effects and the correlated ginsenoside biological effects in Figure 6. We considered only the ginsenosides with the highest concentrations for the ginseng of each growth ages. However, it is likely that combinations of different ginsenosides in various ratios will be the key to explain the observed multi-dimensional pharmacologic effects. By using the Matrigel implant model and reconstituting the extracts using distinct ratios of Rg1 and Rb1, Sengupta et al.[17] observed that different defined ratios led to opposing biological effects, which could alter angiogenic outcomes. In the future, additional pre-clinical and clinical experiments on the medicinal use of purified individual compounds or combination of specific ginsenosides will be necessary to confirm their efficacy on T2DM. These studies may provide an opportunity to develop a new class of agents for diabetes care. Furthermore, this line of research can also lead to the development of new bioactive markets for quality control of herbal extracts.

Conclusion

In conclusion, by applying LC–MS-based lipidomics, measuring biochemical parameters, and profiling the components of ginseng roots of different ages, we demonstrate that ginseng roots show growth age-dependent therapeutic effects on hyperlipidemia and hyperglycemia in diabetic GK rats. These effects may be

linked with the age-dependent variations in concentrations of specific bioactive ginsenosides in the ginseng roots. The present study provides novel and valuable experimental evidence on the age-dependent biological actions of ginseng in the treatment of diabetes. The results demonstrate that 4- to 6-year-old (i.e. $\geq$ 4 y) ginseng roots contain bioactive ginsenosides that may prove to be valuable in the development of drugs or dietary supplements to regulate lipid levels and increase glucose tolerance. Our results suggest that future investigations should examine the biological effects of combinations of specific ginsenosides from ginseng roots.

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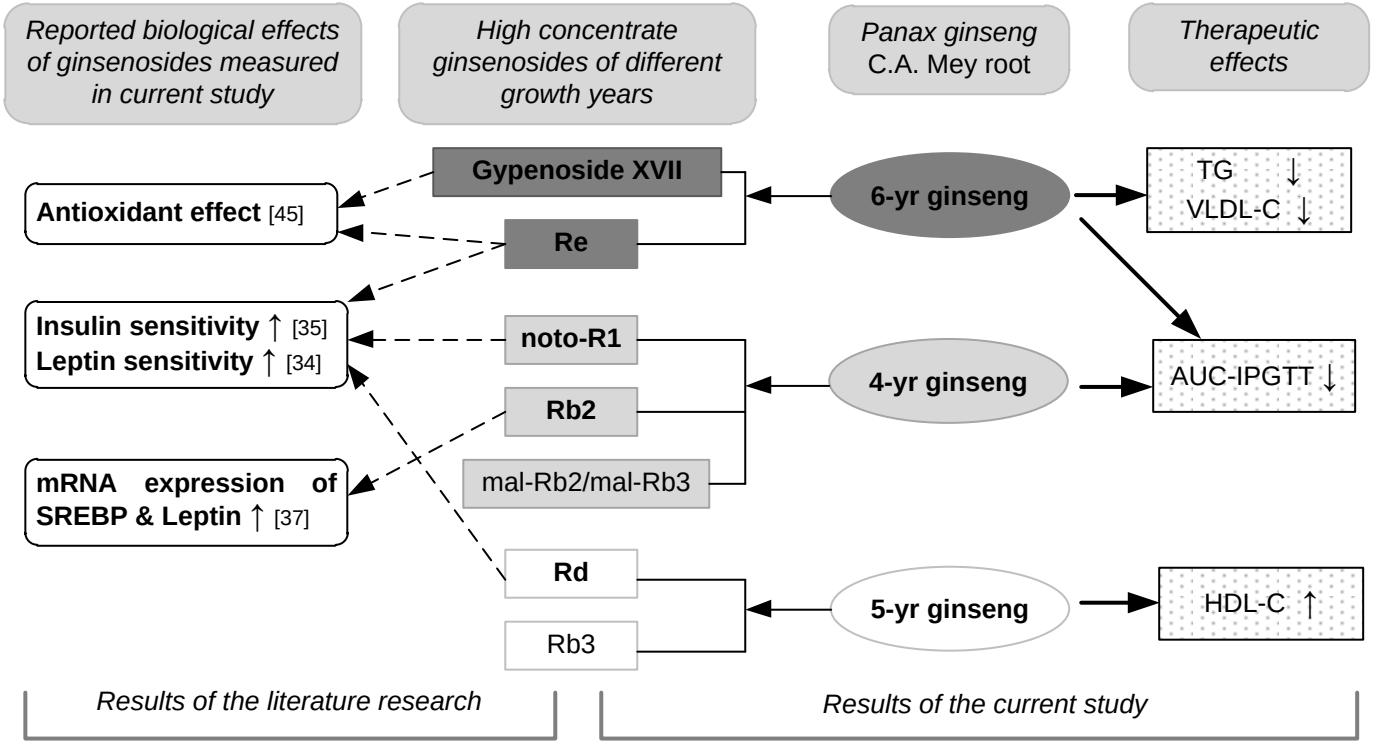

Figure 6. *Illustration of observed ginseng therapeutic effects and the correlated ginsenoside biological effects. The columns on the left summarize the literature reports of the biological effects of ginsenosides that were measured in the current study. The references are displayed with the relevant biological effects. The remaining three columns display the results found in current study. The 4- to 6-y-old ginseng roots shared some similar effects.*

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Supporting information

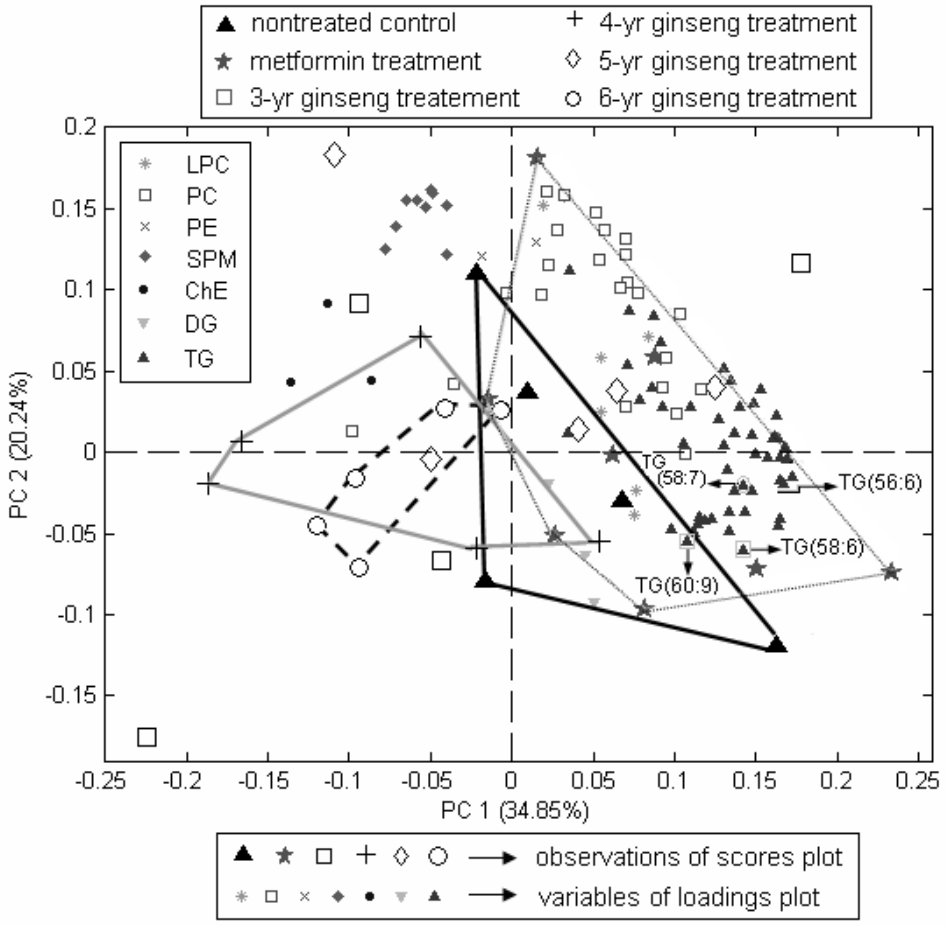

Figure S1 *Score and loading biplots of PCA of plasma lipidomics data from all study samples to reveal general clusters of rats of nontreatment and receiving different treatments (scores) and to examine which lipids contributed most to the clusters (loadings). Symbols of '▲' connected with black bold solid lines stand for nontreated control group, symbols of '★' connected with square dot lines stand for metformin treated group, symbols of '+' connected with gray bold solid lines stand for 4 yr old ginseng treated group, symbols of '○' connected with black bold dashed lines stand for 6 yr old ginseng treated group. Symbols of '✳', '□', '×', '◆', '●', '▼' and '▲' in smaller size represent lipid species of LPC, PC, PE, SPM, ChE, DG and TG, respectively.*

Chapter 6

Fast plasma lipid analysis by nanospray chip-based mass spectrometry

Abstract

Lipidomics has become a powerful tool to study phenotypes and changes in life style associated abnormalities such as obesity, diabetes type 2 and cardiovascular diseases. A quick lipid profiling and the detection of changes in concentration of lipid classes in biological samples will provide valuable information for elucidating the mechanism of above mentioned diseases.
Lipid profiling is often performed with LC/ MS or GC/MS (after fractionation). These methods are relatively time consuming. Direct nanospray infusion in combination with mass spectrometry has been established as a powerful technology for lipid analysis. The method is fast and enables high throughput of samples with low sample consumption. Following this idea, a method of direct nanospray infusion using a TriVersa chip-based infusion instrument in combination with high resolution LTQ-Orbitrap mass spectrometer has been developed as a tool for fast lipid analysis in serum and tissue after a very simple lipid extraction with isopropanol. However, this method cannot be applied to plasma due to clogging of the nanospray emitters caused by relatively high salt concentrations from the anti-coagulants (EDTA, citrate, heparin salts, etc).
The standard Folch lipid extraction method was modified to solve the problem of salt (anti-coagulant) formation in the nanospray chip. The salts were removed and we have developed a fast lipid analysis method by direct nanospray infusion (DI method) for plasma. With the sample volume as low as 5 µL of plasma, a snapshot of all different lipid class can be obtained within 5 minutes per sample. The following lipid classes are covered: triacylglycerols, cholesterol esters, phosphatidylcholines, lyso-phosphatidylcholines and sphingomyelins. When compared with the validated LC/MS method, the DI method is equivalent to it for high abundance lipids. However, it is less precise than LC/MS method. However, it still holds promise for the fast lipids analysis for plasma samples and worth further development and validation.

Introduction

Lipids, the fundamental components of biological membranes, play an important role in biological systems, energy metabolism and storage, and contribute in great deal to pathophysiological states such as diabetes mellitus, fatty liver, hepatotoxic induced insulin resistance and atheroslecrosis [1, 2]. Lipids comprise an enormous number of chemically distinct molecular species arising from the various combinations of fatty acids and a series of backbone structures [2]. Lipid profiling and measurement in biological samples, so called lipidomics research, is a lipid-targeted metabolomics approach aiming at comprehensive analysis of lipids in biological systems [2]. It is regarded as an important technology to provide valuable information for investigating the effect of lifestyle, diet and drug intervention on lipid metabolism. However, a priori the characterization of complex lipids presents a considerable challenge for lipid profiling as they are characterized by a huge diversity of chemical structure with different physicochemical properties [3]. Once extracted, the lipids must then be fractionated, usually requiring a multi-step chromatography process to allow identification and quantitation of the individual molecular species [3]. These intricate operations are time consuming and it is often not possible to cover the whole lipidome by a single analytical method.

Recently, direct infusion nano-electrospray with an LTQ/Orbitrap mass spectrometer (MS) has been established as a powerful technology for lipid analysis and identification [4-8]. The LTQ Orbitrap is a hybrid mass spectrometer consisting of two mass analyzers that can function in parallel or in series. The linear ion trap provides the MS^n capability and the Orbitrap can acquire the spectra at high resolution (100.000) and high mass accuracy [9]. The LTQ/Orbitrap is able to make mass measurements with a resolving power of up to 100K (at m/z 400) and mass accuracies of better than 2 ppm have been demonstrated [10]. Nanospray- electrospray ionization (ESI) is a development of ESI for spraying very low amounts of very low concentration samples (nmol/mL). The technique has an increased tolerance to high aqueous solvents and the spectra can be obtained from pg of material with very little clean up being required. This increased performance is the result of lowering the inner diameter of the spray needle and reducing potentials normally used in ESI. The nanospray chip system (TriVersa NanoMate) (Figure 1) was developed by Advion Biosciences as an add-on to most ESI sources.

In order to make a snapshot of a wide range of lipid classes, we have combined the TriVersa nanospray chip-based system with the LTQ/Orbitrap and developed a fast lipid analysis method by direct nanospray infusion (DI method). By a simple lipid extraction with isopropanol (IPA), the extracts of serum and tissue samples

can easily applied with the direct nanospray infusion to LTQ/Orbitrap. However, this method cannot be applied to plasma due to clogging of the nanospray emitters caused by high salt concentrations from the anti-coagulants (EDTA, citrate, heparin salts, etc). The Folch lipid extraction method was modified to remove the salts.

Figure 1. *TriVersa NanoMate device and a nanospray chip holding 400 nanospray emitters.*

Method and materials

A modified Folch lipid extraction method (Figure 2) was applied to extract the in-house reference human plasma. Firstly 10µL human plasma was mixed with 30µL isopropanol (IPA) containing the internal standards (IS) including lyso-phosphatidylcholine (LPC)_C17:0, phosphatidylcholine (PC)_C24:0, ceramide (CER)_C17:0, fatty acid (FA)_C16-D31, LCA-D4, triacylglycerol(TG)_C51:0, cholesterol ester (ChE) _C17:0. Afterwards add 400 µL methanol, 800 µL chloroform and 300 µL water (Milli Q) and vortex well in each step. After 5 min centrifuge at speed 2500RPM at 20ºC, the upper layer consists of water while the lower layer consists of methanol-chloroform containing the plasma lipids. The upper aqueous layer was removed. The organic phase was evaporated with nitrogen at 45 ºC. The residue was reconstituted with 900 µL IPA. After being vortexed and 5 min centrifuged at speed 14000 RCF at 20 ºC, the extract was diluted 5 fold with IPA. And then being vortexed and 5 min centrifuged at the speed 14000 RCF at 20 ºC, the extract was diluted 1.5 fold with 2mM ammonium acetate (NH_4Ac)/ Methanol. Finally, the extract was infused using the automated

TriVersa NanoMate apparatus (Nanomate HD, Advion Bioscience Ltd., USA) fitted on a LTQ-Orbitrap MS (Thermo Fisher Scientific, Netherlands).

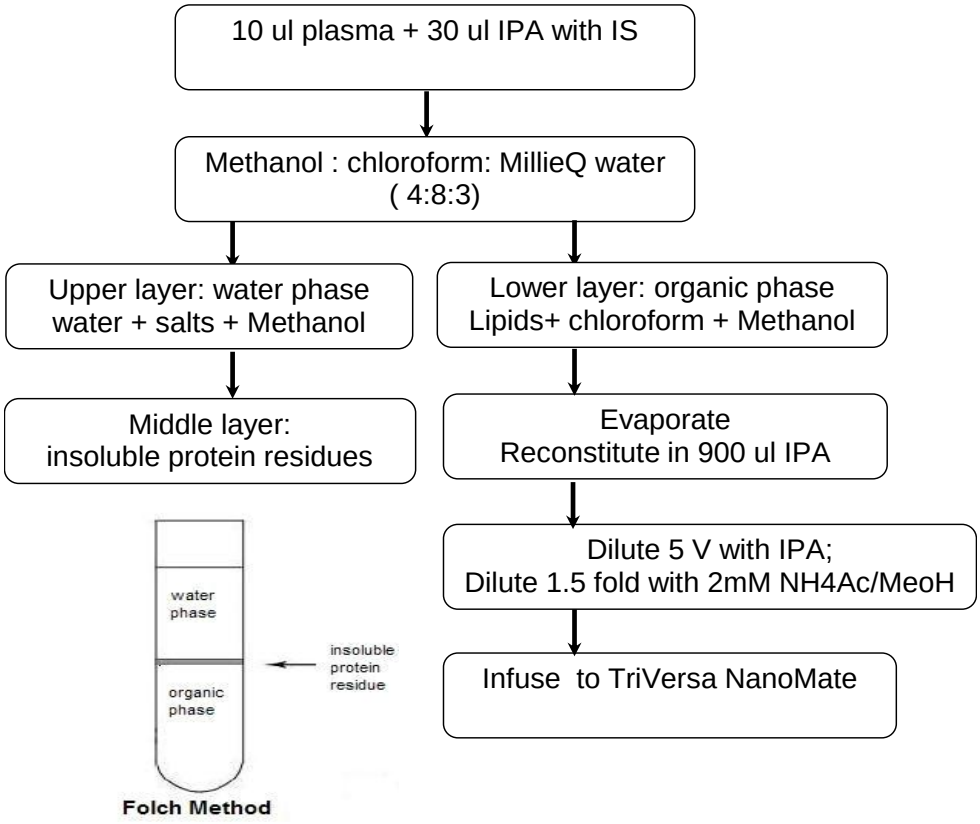

Figure 2. *Modified Folch method for lipid extraction*

The TriversaNanomate was loaded with 5 µL of the extracts. Full scan data acquisition (m/z range 300-1200 in positive mode while 200-1200 in negative mode) was performed to obtain a global snapshot of the plasma lipids followed by SIM scans (m/z subranges for improved sensitivity) in both positive and negative modes. The SIM scanranges were defined (see below) to measure all members of the various lipid classes in a single SIM scan range (e.g. cholesterol esters from m/z 600 to 700). The total analysis time is only 10 minutes per extract. The instrumental method illustrated in table 1.

Table 1. TriVersa- LTQ Orbitrap instrument method

TriVersa- LTQ Orbitrap instrument method	
Infusion volume (µL)	5
Polarity	positive /negative
Ionization	nano-ESI
Total MS run time (min)	5
Number of scan events	1
Resolution	100000
San segment 1	
duration (min)	1.5
san event info	FTMS+ full scan
m/z range	300-1200 (positive)/200-1200 (negative)
San segment 2-8	
duration (min)	0.5
scan event info	FTMS + SIM scan
m/z range - segment 2	300-530 (positive)/ 200-530 (negative)
m/z range - segment 3	450-600
m/z range - segment 4	550-700
m/z range - segment 5	650-800
m/z range - segment 6	750-900
m/z range - segment 7	850-1000
m/z range - segment 8	950-1200

Result and discussion

The spectrum of the full m/z range (1.5 minutes) covers all lipid classes. Combined with SIM scans, 0.5 minute per m/z range, the sensitivity of the method for the various lipid classes is increased. The following lipid classes are covered: TGs and ChEs (positive mode); PCs, LPCs and SPMs (positive/negative mode); FFAs (negative mode) (Figure 3A-B).

Recovery

Table 2 summarizes the extraction recovery of the general lipid classes. The recovery of the lipid extraction method was 100% for TG's and ChE's, 85% for PC's and 75% for LPC's (determined with a validated LC/MS lipid method [11]).

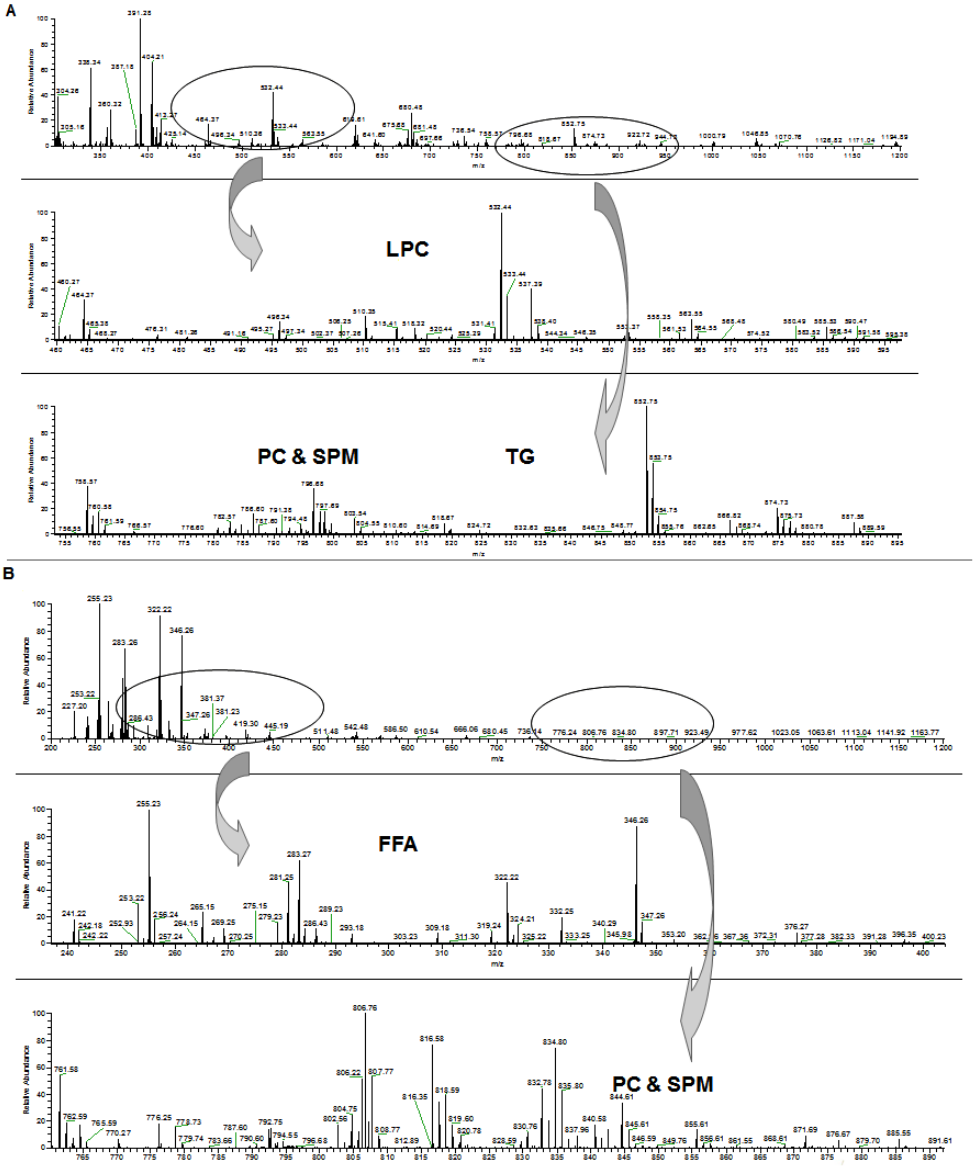

Figure 3. *Mass spectrum of lipids analyzed by DI method*

Table 2. Recovery of selected lipid classes

Lipid classes	LPC	PC	ChE	TG
Recovery (%)	75%	85%	100%	100%

Relative standard deviation (RSD)

RSD (5 replicates) for main lipid classes using DI method is 5-34%, which is lower than that by using LC/MS method (1-10%). (Table 3)

Table 3. RSD of response ratio to IS (5 replicates)

	C16:0 LPC	C18:1 LPC	C34:3 PC	C36:2 PC	C16:0 SPM	C24:1 SPM	C50:3 TG	C52:2 TG
DI method	5%	9%	16%	20%	23%	24%	34%	8%
LC/MS method	6%	1%	8%	5%	6%	6%	10%	8%

Linearity

The linearity of the method was investigated by analyzing different volumes of the same plasma sample. Triplicate samples were prepared and infused in two series: (1) 8 µL, 10 µL, 12 µL; and (2) 10 µL, 20 µL, 30 µL. The response versus plasma volume was linear for all lipid classes (R^2 > 0.98). Examples of LPC_C16:0 and ChE_C20:4 measured by the DI method and a validated LC/MS lipid method are shown in Figure 4 A-B respectively.

Standard addition linearity

Standards of high abundance plasma lipids (LPC_C18:0, PC_C36:2, TG_C54:3) and low abundance plasma lipids platelet-activating factor ((PAF) _C16:0, PC_C40:0 and TG_C42:0) were added to plasma at 1.5, 2 and 10 times the native concentrations independently (determined with LC/MS method, denoted as 1.5*, 2* and 10*).

For high abundance lipids the method failed to accurately detect a concentration increase from 1.5* to 2*. The concentration increase from 2* to 10* for C54:3-TG can be accurately determined. However, the signal increase was less than proportional for C18:0_LPC and C36:2_PC, which indicates ion suppression (most likely by very abundant TG's). For low abundance lipids, LC/MS can accurately detect concentration increases at 1.5*, 2* and 10* for PAF_C16:0 and TG_C42:0. The DI method is also linear, but the accuracy was not as good, especially for C16:0-PAF. The data quality for PC 40:0 was very poor (experimental failure) (Figure 5 A-D). The TG concentration was used to dilute the extract to prevent

saturation, and as a consequence this resulted in very low concentrations of LPC and PC, which precluded their accurate analysis (very low signal to noise ratio).

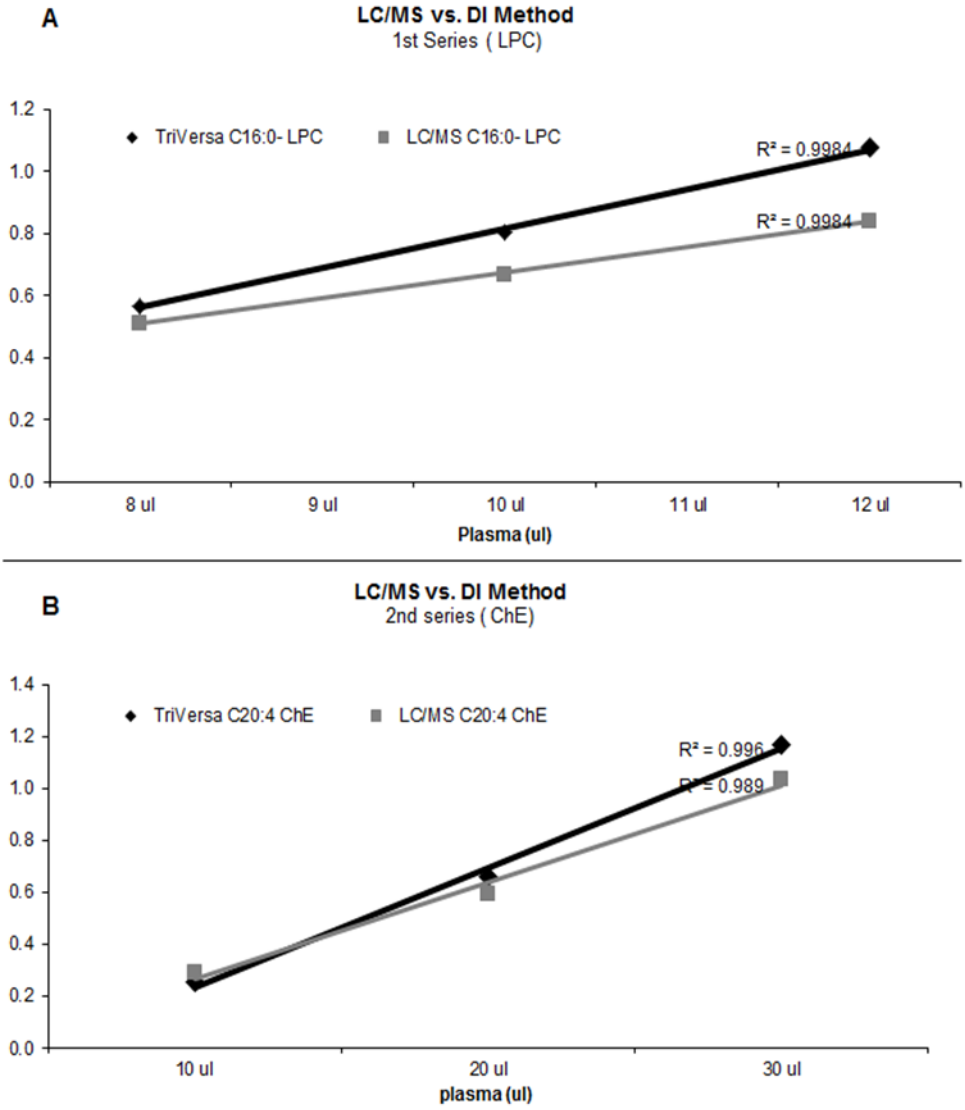

Figure 4. *Plasma volume linearity. (A) First series, LPC_C16:0 measured by DI method and a validated LC/MS lipid method; (B) Second series, ChE_C20:4 measured by DI method and a validated LC/MS lipid method.*

Conclusion

This study showed that the modified Folch method effectively lowers the salt concentration of the final extracts. Clogging of nanospray emitters did no longer occur when analyzing large numbers of plasma samples. The DI method is fast and enables high throughput of samples with low sample consumption, without sample-to-sample carry-over effect. When compared with the validated LC/MS method, the DI method is equivalent for high abundance lipids. However, it is less precise than the LC/MS method. The DI method is not applicable to samples with high triglycerides levels because extracts needs to be diluted too much (poor detectability of low abundance lipids). For samples with high triglyceride concentrations additional fractionation, e.g. liquid–liquid extraction (LLE), solid-phase extraction (SPE), or high-performance liquid chromatography (HPLC), should be a good remedy. The DI method is promising for the fast lipids analysis for plasma samples and worth further development and validation.

Abbreviations

CER, ceramide; ChE, cholesterol ester; DI: direct infusion method; FA, fatty acid; FFA, free fatty acid; GC, gas chromatography; HPLC, high-performance liquid chromatography; IPA, isopropanol; IS, internal standard; LC, liquid chromatography; LLE, liquid–liquid extraction; LPC, lyso-phosphatidylcholine; MeOH, methanol; MS, mass spectrometry; NH4Ac, Ammonium acetate; PAF, platelet-activating factor; PC, phosphatidylcholine; SPE, solid-phase extraction; SPM, sphingomyelin; TG, triacylglycerol/triglycerides; *, times

Acknowledgement

The great appreciation is dedicated to the help and support from Elwin Verheij, Invana Bolbeldijk and Raymond Ramaker for the development of this method.

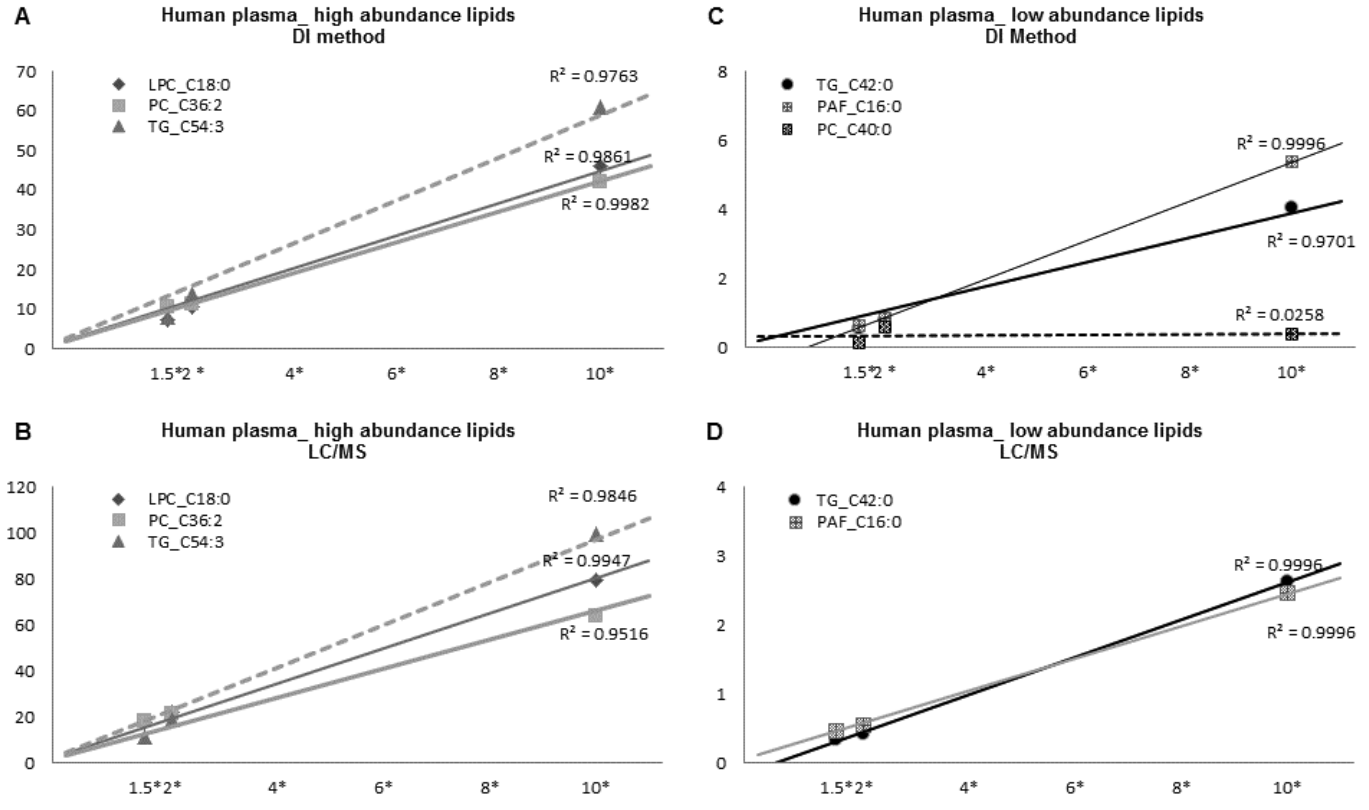

Figure 5. *Standard addition linearity. (A) High abundance lipids DI method; (B) High abundance lipids LC/MS method; (C) Low abundance lipids DI method; (D) Low abundance lipids LC/MS method (The PC_C40:0 was not added to the spike solution for LC/MS method)*

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Chapter 7

Conclusions and perspectives

Conclusions

Type 2 diabetes mellitus (T2DM) leads to a number of serious medical complications (e.g. retinopathy, neuropathy, myocardial infarction), which are a major cause of morbidity, hospitalization and mortality in diabetic patients; and resulting in a major threat and financial burden for public health internationally [1-3]. Due to its multi-factorial causes resulting from the interaction between a genetic predisposition together with behavioral and environmental risk factors, the diagnosis and treatment of T2DM remains unsatisfactory. A personalized and system based strategy applied in the pre-diabetic stage is vital for diabetic prevention and management.

Emerging technologies have made it feasible to acquire high-throughput profiles of a whole organism's metabolic status (metabolite profiling or metabolomics) [4]. These techniques, which allow assessment of large numbers of metabolites that are substrates and products in metabolic pathways, are particularly relevant for studying metabolic diseases such as T2DM [4]. Metabolomics has been increasingly applied to study complex disease mechanisms to discover health-disease associated mechanistic biomarkers and is regarded as a unique bridge between different healthcare perspectives on personalized medicine [5, 6]. The concept of personalized health with system diagnostic principles has long been the basis of CM, in which the focus is on the overall maladjustments of functional status called 'syndrome type'[7]. The essence of 'syndrome type' is the human system imbalance resulting in the perturbation of the metabolic network [8]. If we can correlate the CM diagnosed syndrome types to the metabolomics quantified patterns, it may help us stratify the patients and push personalized medicine forward.

In light of this idea, the aim of this thesis was to combine metabolomics and CM diagnostic strategy to search for the diagnostic makers to subtype T2DM. The multi-component preparations or drugs to treat cardio-metabolic disorders were studied and assessed by metabolomics to understand the potential underlying multi-pathway effects, leading to a better understanding of the personalized health of T2DM.

In **Chapter 2**, an explorative study of 50 pre-diabetic males was designed, combining urine metabolomics with CM diagnosis to identify metabolic patterns. Due to the fact that CM diagnosis is conceptual and relies entirely on clinical signs discerned by CM physicians, the inter-physician consistency on CM diagnosis is of importance to be investigated at the first place. Three CM physicians reached 85% diagnosis consistency when the diagnostic principles were standardized and symptom descriptions were clarified in advance. Based on 3 different CM syndrome groups, two pre-diabetic subtypes can be identified by urine

metabolomics and had different urinary metabolic patterns; and one of which indicated more disturbances of carbohydrate metabolism and renal function. The identified urinary metabolites may be of special clinical relevance for easy screening for subtypes of pre-/diabetes, which may lead to a better understanding and improvement of personalized interventions for diabetics. This study set an example to combine non-invasive gas chromatography–mass spectrometry (GC-MS) urine metabolomics with CM personalized diagnosis to find metabolic subtypes in pre-diabetics; and proved that the improvement of diagnosis plays a key role in bridging between CM and western medicine on personalized healthcare.

Obesity is one of the major risk factors for T2DM. Compliance with lifestyle modifications such as reduced caloric intake and increased physical activity has proved to be difficult for the general population, meaning that pharmacological intervention may be the only recourse for some people with obesity [9]. Therefore, in **Chapter 3 and 4,** the effects of rimonabant and a multi-component preparation (SUB885C), both with effects of regulating weight and the improvement on cardio-metabolic risk factors, were assessed by lipidomics on mildly over-weight ApoE*3Leiden.CETP Mice. Rimonabant is a selective cannabinoid-1 receptor antagonist for treatment of obesity and targets the endocannabinoid system. SUB885C is developed according to the principles of CM containing eight natural ingredients. The core formula is used in China for treatment of metabolic syndrome and early stage T2DM with obesity. For rimonabant and SUB885C, little is known about the impact on the regulation of lipid metabolism in the early stage of obesity. A 4-week rimonabant intervention brought a significant reduction of the body weight, yet a moderate effects on lipid profile and limited lipid metabolism changes in the ApoE*3Leiden.CETP mice. Although no weight reduction was observed, SUB885C was able to produce multiple anti-atherogenic changes in lipids of the mice to improve metabolic parameters and lipid patterns, manifesting as decreased plasma cholesterol, (V)LDL, and triglycerides, increased HDL-C and a change in wide range of neutral lipids. These effects were comparable to those obtained with compounds belonging to known drugs (e.g. atorvastatin, niacin). *In vitro,* SUB885C extract caused adipolysis stimulation and adipogenesis inhibition in 3T3-L1 cells. These two studies successfully illustrated the power of lipidomics in unraveling intervention effects and can help researchers pinpoint novel ways to treat lifestyle-related metabolic abnormality.

Panax ginseng C. A. Mey (often simply referred to as ginseng) is reported to have effects to treat diabetic symptoms and is widely used in China as the dietary or drug ingredient for daily health maintenance. So far no research has been performed to determine how the ginseng's growth year and its related quality

affect its therapeutic efficacy. In **Chapter 5**, lipidomics was applied to evaluate the effects of the root of ginseng grown for 3–6 years on the regulation of hyperglycemia and dyslipidemia in a diabetic Goto-Kakizaki (GK) rat model. By applying liquid chromatography–mass spectrometry (LC-MS) based lipidomics, measuring biochemical parameters, and profiling the components of ginseng roots of different ages, we demonstrate that ginseng roots show growth age-dependent therapeutic effects on hyperlipidemia and hyperglycemia in diabetic GK rats. These effects may be linked with the age-dependent variations in concentrations of specific bioactive ginsenosides in the ginseng roots. The $\geq$ 4 year ginseng roots contain bioactive ginsenosides that may prove to be valuable in the development of drugs or dietary supplements to regulate lipid levels and increase glucose tolerance. This study introduced novel systems biology-based approaches for linking biological activities with potential active components in herbal mixtures.

Chapter 6 reports the preliminary development of a fast plasma lipid analysis platform by direct nanospray infusion using a TriVersa chip-based instrument in combination with high resolution LTQ-Orbitrap mass spectrometer. The modified Folch method was applied for the plasma lipid extraction. With the sample volume of 5 µL plasma, a snapshot of the following lipid classes can be obtained within 5 minutes per sample: triacylglycerols, cholesterol esters, phosphatidylcholines, lyso-phosphatidylcholines, sphingomyelins and fatty acids. When compared with the validated LC-MS method, the fast method is equivalent to it for high abundance lipids; yet it is less precise than LC-MS method. However, it still holds promise for the fast lipids analysis for plasma samples and worth further development and validation.

To sum up, the early metabolomics studies carried out in this thesis set an example to combine analytical bioscience, clinical approach and other health system perspectives such as CM to provide the systems biology view on the metabolism patterns of T2DM and the intervention effects of possible drug and dietary approaches. It is promising to fusion metabolomics and CM diagnosis to search for subtypes of T2DM. Metabolomics, lipidomics in specific, is able to investigate multi-dimensional pharmacological effects and detect potential biomarkers related to these effects. These findings can be used to develop future new research strategies and products at the nutrition-pharma interface to manage T2DM and related metabolic risk factors. However, it will only be realized when analytical methods will further improve, and system biology based metabolomics approaches are more integrated in the medical research paradigm.

Perspectives

Improved diagnosis to stratify patients is important to push personalized medicine forward

The only one human study reported in this thesis (**Chapter 2**) showed the promise to fuse metabolomics and CM diagnosis to search for subtypes of T2DM. However, it is an early phase investigation on subtypes of pre-diabetes based on a small number of study subjects and CM physicians. As such whilst this data is very promising, there needs to be larger investigations using more subjects and CM physicians to demonstrate the further reliability and external validity/ generalization ability of this novel approach to diagnosing pre-diabetic subtypes. Future studies are needed to validate the subtypes yielded in the current study and to assess the time dependent response of these subtypes to challenge test or metabolic drug intervention.

This study proved that the understanding and improvement of diagnosis plays a key role in bridging between CM and Western medicine (WM) on personalized healthcare. Stratifying patients on molecular biomarker profiles is a key step towards treatment responder/nonresponder differentiation and personalized medicine. The CM method of qualitative subtyping could be of use to help decide the course of treatment for patients in modern medicine, and provide momentum for the move towards personalized medicine [13]. A diagnostic tool such as a questionnaire can be designed, combing CM symptoms and WM quality of life items, to search for patients subtypes based on different health and disease related manifestations.

Interventions for T2DM

It is obvious that multiple mechanisms are involved in T2DM development, and so far no universal cure exists for all of the metabolic abnormalities that are embodied in this disease. For example, drugs aimed at metabolic targets, such as the enhancement of fatty acid oxidation, may have desirable effects on hepatic insulin action and steatosis, but possible deleterious effects in muscle or islet β-cells [9]. Moreover, drugs that stimulate insulin secretion beyond the already elevated levels of the obese and insulin-resistant state may eventually cause β-cell stress and permanent damage [9]. It is unlikely that effective treatments will be based on single bioactive compounds. These complexities may force a focus on combined drug/nutrition therapies with individualized optimization.

To restore energy balance and improve metabolic risks, rimonabant, a cannabinoid-1 receptor antagonist which regulates central appetite control in

response to endogenous ligands, used to show promise. However, it has incompletely characterized peripheral effects, and has been associated with psychic (neurologic, mental) side-effects such as depression and even suicide attempt. Moreover, the lipidomics response to an intervention of rimonabant in transgenic mice, as reported in **Chapter 3**, only showed moderate effects in early stage obesity. Projects are underway to develop cannabinoid-1 receptor blockers that do not pass the blood-brain barrier (to prevent mental side effects) are non-brain penetrant and act only on peripheral cannabinoid-1 receptor [10]. Metabolomics can again be a good method to investigate the effects and mechanism of such agents.

Meanwhile, pharmacological strategies for primary prevention are increasingly focusing on the use of low-dose drug combinations. An example is the development of “polypill” concepts with a statin, one or more anti-hypertensive compounds and acetylsalicylic acid to reduce risks for cardiovascular disease in middle-aged individuals [11]. While at the same time it has been shown that dietary measures such as “polymeal” may be of comparable efficacy or even provide a “more natural, safer and probably tastier alternative” than a polypill [12]. Therefore, T2DM prevention through nutritional intervention is realized to be crucial for increased human quality of life. Metabolomics based nutritional profile is useful to give indications for personalized intervention that can influence on multiple targets in the human system with fewer side-effects. For example, an intervention with selected dietary products affected inflammatory processes, oxidative stress, and metabolism in humans, as shown by large-scale profiling of genes, proteins, and metabolites in plasma, urine, and adipose tissue [13]. To make it possible for health-care and nutrition practitioners to give nutrition recommendations, more trials using metabolomics and a suitable database based on a large number of measurements of accurate metabolite concentrations from healthy people might be necessary [14]. Furthermore the concept of health promotion alongside disease management will help to improve the current healthcare system [11]

Such new insights and leads for dietary prevention or intervention can also be acquired from CM where the gap between food and drugs is small. The study results of the muti-components preparation SUB885C and ginseng reported in **Chapter 4 and 5** [15, 16] may shed light on this approach. Both SUB885C and ginseng showed beneficial effects on dyslipidemia and may have influence on multiple pathways, which need further investigation. In China a remarkable high number of preparations have been handed down over the centuries with documented activity related to clinical features of what is now described as metabolic syndrome. The diabetic regulation health claims of these Chinese herbs included increasing insulin, decreasing blood glucose, increasing glucose

metabolism, stimulating pancreatic function, and alleviate diabetes-induced oxidative stress by inhibiting lipid peroxidation [17-19]. Only a few side effects of Chinese herbs used in treating diabetes have been reported, however, further comprehensive studies are warranted as evidence is still scarce with likely underreporting [19].

The use of Chinese herbal medicines, either as food ingredients or drug therapy, in diabetes seems promising but still far from proven. First of all, the lack of standardization in quality control and quality assurance in producing the herb and herbal products often leads to inconclusive intervention results. **Chapter 5** clearly illustrated the age-dependent effects of ginseng intervention and these age-dependent effects may be due to the fact that the ratios and concentrations of specific ginsenosides in the ginseng roots change during growth [15]. The possibilities to evaluate the quality control and analyze the subtle and multiple-pathway effects of Chinese herb or/and preparations have increased by the developments in systems biology-based metabolomics and specific animal models, as shown in **Chapter 4 and 5**.

In addition, many of the herbs are still untested or tested only in limited trials, thus further clinical trials are needed to evaluate their effects and mechanisms [19]. To avoid the underestimation of the Chinese herb intervention, it is suggested that study subjects in such clinical trials should at first be stratified according to CM diagnosed subtypes as the CM diagnosis is on the syndrome types instead of a disease in WM perspective. Therefore, improved diagnosis and intervention go hand in hand, and metabolomics can certainly contribute to find and understand the molecular phenotypes.

The challenge of metabolomics study and the future direction

With respect to the technology of metabolite profiling, further progress is required to determine the chemical identity of peaks that can be determined with metabolite-profiling methods. Given the fact that the phenotype of any biological system is largely determined by its metabolite composition, the future development of metabolite-profiling technologies is of crucial importance to biomedical research [20].

It seems wise to combine the untargeted metabolomics platform with targeted platforms in the same study. As for an untargeted global metabolite analysis, so many of the changes detected are possibly associated with high concentration metabolites. Many of the metabolites detected in studies by GC-MS are involved in amino acid metabolism, the Krebs cycle, glycolysis and β-oxidation, and thus it is perhaps not so surprising that the approach detects changes in these pathways [21]. To additionally apply a targeted approach where a number of predefined

metabolites are targeted, this has the advantage that this type of approach can also be readily made quantitative by nature and more biological interpretation of results can be drawn.
Lipidomics measures all or subsets of lipids and provides a thorough perspective to study intervention induced lipid changes and metabolism in the complex biological system. To study more in-depth the involvement of specific lipid species and pathways, more understanding of lipid metabolism is needed. T2DM is not only a disorder of glucose metabolism, but also associated with a cluster of interrelated plasma lipid and lipoprotein abnormalities. Combining lipidomics with biochemically measured lipid profiles (e.g. total cholesterol, triglycerides, HDL-Cl, etc) can provide us with a better understanding of lipid metabolism. **Chapter 3-5** describes the successful application of this approach, using LC-MS based lipidomics, to investigate the changes in the lipidome of animal models following interventions and illustrated possible underlying mechanism how lipid were re-distributed in the biological system after intervention.
The amount of information generated with metabolomics studies does not in itself a guarantee for the discovery of a biomarker or a metabolic pattern. It is important to carry out biomarker validation and follow-up studies to complete the cycle of hypothesis generation and hypothesis testing. Approaches can be like MolPAGE project [22], using large cohorts and extensive statistical material from clinical settings will enable the method to expand into a better understanding of the disease pathophysiology, which could lead to the biomarkers for clinical settings.
Another obstacle to progress in biomarker research resides as much in the culture and organization of academic research as in deficiencies in collaborative approaches. To become clinically useful, biomarker research must operate more like the large, collaborative networks mobilized for international genome-wide association studies and the multi-institution, multi-investigator big-science projects, involving industry and experts in molecular biology, genetics, analytical chemistry, computation, engineering, clinical-trial design, epidemiology, statistics, regulation and health-care economics [23]. To reach this, organizational and funding reforms should persuade the research community to adopt common standards and a cross disciplinary, systems-based approach to biomarker discovery and validation [23].

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Summary

The incidence of Type 2 diabetes mellitus (T2DM) and its cardio-metabolic complications continues to increase globally. This epidemiological reality heightens the urgency for gaining a deeper understanding of the complex molecular pathogenesis and genetic basis of this disease. Treatment focusing on glycaemic control is not enough to reduce the cardiovascular morbidities. A more personalized and system based strategy should be applied for diabetic prevention and management.

Systems biology based metabolomics is regarded as a promising approach to provide an overview at a system level. It has been increasingly applied to study complex disease mechanisms to discover health-disease associated biomarkers. It also creates a possibility to study and evaluate the system based diagnosis and intervention of other healthcare systems such as Chinese Medicine (CM). The concept of personalized health with system diagnostic principles is the basis of CM which focuses on the 'disease subtype'. If we can correlate CM diagnosed subtypes to quantitative metabolomics patterns, it may help us stratify the patients and find the related pattern of changes in metabolites, namely diseased related metabolic profile/fingerprint.

The aim of this thesis was to combine metabolomics and CM diagnosis to search for diagnostic makers or metabolic profiles to subtype T2DM. The multi-component preparations or drugs to treat cardio-metabolic disorders were studied and assessed by metabolomics to understand the potential underlying multi-pathway effects and move further to personalized health of T2DM.

In **Chapter 2**, an explorative study of 50 males with pre-diabetes was designed, combining urine metabolomics with CM diagnosis to identify metabolic patterns. Three CM physicians reached 85% diagnosis consistency resulting in the classification of 3 pre-diabetic groups. Based on 3 different CM diagnosed groups, two pre-diabetic subtypes (A and B) could be identified by urine metabolomics with different metabolic patterns. The majority of metabolites (51%), mainly sugars and amino acids, showed higher urine levels in subtype B compared with subtype A. This indicated more disturbances of carbohydrate metabolism, the Krebs cycle and renal function in subtype B. No differences were found for hematological and biochemical parameters except for levels of glucose and γ-glutamyltransferase that were significantly higher in subtype B. This study proved that combining metabolomics with CM diagnosis can reveal the metabolic fingerprint for pre-diabetic subtypes. The identified urinary metabolites may be of special clinical relevance for non-invasive screening for subtypes of pre-diabetes,

which could lead to an improvement of personalized interventions for diabetics. However, due to the relatively small number of subjects, future studies with more subjects are needed to validate the subtypes yielded in the study and to demonstrate the further reliability and generalization of this novel approach to diagnose pre-diabetic subtypes.

In **Chapter 3 and 4,** the effects of rimonabant and a multi-component preparation (SUB885C), both with reported effects of regulating weight and the improvement on cardio-metabolic risk factors, were assessed by lipidomics on mildly over-weight ApoE*3Leiden.CETP Mice. Mice with 4-week intervention, either rimonabant or SUB885C, were compared with the ones without. Rimonabant is a selective cannabinoid-1 receptor antagonist for treatment of obesity and targets the endocannabinoid system. SUB885C is developed according to the principles of CM containing eight natural ingredients. The core formula is used in China for treatment of metabolic syndrome and early stage T2DM with obesity. Little is known about the impact of these two therapeutics on the regulation of lipid metabolism in the early stage of obesity. A 4-week rimonabant intervention brought a significant reduction of the body weight, and moderate effects on lipid profile in mildly obese ApoE*3Leiden.CETP mice. Though no weight reduction was observed, SUB885C was able to produce multiple anti-atherogenic changes in lipids of the mice to improve metabolic parameters and lipid patterns, manifesting as decreased plasma cholesterol, triglycerides, increased HDL-C and a change in wide range of neutral lipids. These effects were comparable to those obtained with compounds belonging to known drugs (e.g. atorvastatin, niacin). In *vitro*, SUB885C extract caused adipolysis stimulation and adipogenesis inhibition in 3T3-L1 cells. These two studies successfully illustrate the power of lipidomics in unraveling intervention effects and to help finding new targets or ingredients for lifestyle-related metabolic abnormality.

Panax ginseng C. A. Mey (often simply referred to as ginseng) is reported to have effects to treat diabetic symptoms in China. However, the relationship between its therapeutic effect on diabetes and the quality and the growth age of this herb has not yet been reported. In **Chapter 5**, a combined approach involving lipidomics, biochemical parameters and herbal component profiling was used to evaluate the effects of the ginseng roots grown for 3–6 years on the regulation of hyperglycemia and dyslipidemia in a diabetic Goto-Kakizaki (GK) rat model. Six groups of GK rats received either metformin, 3- to 6-y-old ginseng root, or no treatment. The treatments were administered twice daily for 9 weeks. Compared to the untreated controls, treatment with 4- and 6-y-old ginseng roots significantly improved glucose disposal, and 5-y-old ginseng treatment significantly increased HDL-cholesterol. Treatment with 6-y-old ginseng significantly decreased total plasma triacylglyceride (TG) and VLDL-cholesterol and

improved plasma glycated hemoglobin (HbA1c). In addition, treatment with 4- to 6-y-old ginseng influenced plasma lipidomics in diabetic GK rats by reducing TG lipid species. Metformin significantly reduced fasting blood glucose by 41% and reduced HbA1c by 11%, but showed no effects on the plasma lipid parameters. This study demonstrates that ginseng roots show growth age-dependent therapeutic effects on hyperlipidemia and hyperglycemia in diabetic GK rats. These age-dependent effects may be linked with the variation in both the ratios and concentrations of specific bioactive ginsenosides in ginseng roots of different age. The $\geq$ 4 year ginseng roots contain bioactive ginsenosides that may prove to be valuable in the development of drugs or dietary supplements to regulate lipid levels and increase glucose tolerance. This study introduced novel systems biology-based approaches for linking biological activities with potential active components in herbal mixtures.

In **Chapter 6** we reported the preliminary development of a fast plasma lipid analysis platform by direct nanospray infusion using a TriVersa chip-based instrument in combination with high resolution LTQ-Orbitrap mass spectrometer. The modified Folch method was applied for the plasma lipid extraction. With a volume of 5 µL plasma, a snapshot of the following lipid classes can be obtained within 5 minutes per sample: triacylglycerols, cholesterol esters, phosphatidylcholines, lyso-phosphatidylcholines, sphingomyelins and fatty acids. When compared with the validated liquid chromatography–mass spectrometry (LC-MS) method, the fast method is equivalent for high abundance lipids; yet it is less precise than LC-MS method. However, it still holds promise for the fast lipids analysis for plasma samples and worth further development and validation.

To conclude, the early systems biology based metabolomics investigations performed in this thesis converged analytical bioscience, clinical approach and the diagnostic perspectives in other health system such as CM to provide the systems biology view on the pre-stage of T2DM. It is promising to fusion metabolomics and CM diagnosis to search for subtypes of T2DM. Metabolomics, lipidomics in specific, is able to investigate multi-dimensional pharmacological effects and detect potential biomarkers related to these effects. These findings can be used to develop new research strategies and products at the nutrition-pharma interface to manage T2DM and related metabolic abnormalities. However, it will only be realized when analytical methods will further improve, and the system biology based metabolomics approaches are more integrated in the medical research paradigm.

Summary

Samenvatting

De incidentie van type 2 diabetes mellitus (T2DM) en de daaraan verbonden cardio-metabole complicaties blijft wereldwijd toenemen. Deze epidemiologische werkelijkheid verhoogt de urgentie voor het verkrijgen van een beter begrip van de complexe moleculaire pathogenese en de genetische basis van deze ziekte. Behandeling gericht op glykemische controle is veelal niet genoeg om de cardiovasculaire morbiditeit te verminderen. Een meer persoonlijke en systeem-gebaseerde strategie heeft de voorkeur voor diabetische preventie en therapie. Systeembiologie op basis van metabolomics wordt beschouwd als een veelbelovende aanpak om van een patient een beeld op systeemniveau te geven en daarmee gepersonaliseerde geneeskunde mogelijk maakt. De systeembiologie wordt in toenemende mate toegepast om complexe ziektes en de onderliggende mechanismen te bestuderen, en voor de gezondheid-ziekte relatie biomarkers te ontdekken. Het creëert ook de mogelijkheid om het systeem te bestuderen en evalueren van op basis van diagnose en tussenkomst van andere medische systemen zoals Chinese Geneeskunde (CM). Het concept van gepersonaliseerde gezondheidszorg met diagnostische principes is de basis van CM die zich richt op het 'ziekte subtype'. Als CM gediagnosticeerde subtypes gecorreleerd kunnen worden met kwantitatieve metaboliet-patronen, kan het helpen patiënten te stratificeren en het bijbehorende patroon van veranderingen in metabolietconcentraties, het ziekte gerelateerde metabolietprofiel te identificeren. Het doel van het onderzoek beschreven in dit proefschrift was om metabolomics en CM diagnostiek te combineren om te zoeken naar de diagnostische markers of metabolietprofielen voor T2DM subtypes. De multi-component preparaten of medicijnen om cardio-metabole stoornissen te behandelen werden bestudeerd met metabolomics om de onderliggende multifactoriele effecten te begrijpen, en daarmee de persoonlijke therapie van T2DM te versterken.

Hoofdstuk 2 beschrijft een studie aan 50 mannen met pre-diabetes waarin een combinatie van urine metabolomics met CM diagnose is gedaan met als doel de metabolietpatronen te identificeren die correleren met de CM diagnose. Drie CM artsen bereikten 85% overeenkomst in de diagnose wat resulteerde in de indeling van de proefpersonen in drie pre-diabetische subgroepen. Op basis van de metabolietprofielen in urine konden twee pre-diabetische subtypes A en B geïdentificeerd woorden. De meerderheid van de metabolieten (51%), vooral suikers en aminozuren, hadden hogere urineconcentraties voor subtype B in vergelijking met subtype A. Dit duidt op grotere verstoringen van het koolhydraat metabolisme, de Krebs-cyclus en de nierfunctie bij subtype B. Er werden geen verschillen gevonden voor hematologische en biochemische parameters, behalve

voor het gehalte aan glucose en γ-glutamyltransferase die significant hoger waren in subtype B. Uit dit onderzoek blijkt dat de combinatie van metabolomics en CM diagnose resulteert in een metabole vingerafdruk voor pre-diabetische subtypen. De geïdentificeerde metabolieten in de urine zijn potentieel klinisch relevant voor niet-invasieve screening op subtypen van pre-diabetes, en kunnen leiden tot een verbetering van gepersonaliseerde interventies voor diabetici. Vanwege het relatief kleine aantal proefpersonen in studie zijn toekomstige studies met meer deelnemers noodzakelijk om de betrouwbaarheid en generalisatie van deze nieuwe benadering voor pre-diabetische subtypering te onderbouwen en te valideren.

In **hoofdstuk 3 en 4** is onderzoek gedaan naar de effecten van Rimonabant en een multi-component CM geneesmiddel (SUB885C), beiden met gerapporteerde effecten op de regulering van gewicht en de verbetering van cardio-metabolische risicofactoren, op het plasmalipidenprofiel in ApoE * 3Leiden.CETP muizen met licht overgewicht. De muizen kregen een 4-weken durende interventie, hetzij Rimonabant of SUB885C, en werden vergeleken met een controlegroep. Rimonabant is een selectieve cannabinoïde-1 receptor antagonist voor de behandeling van obesitas en richt zich op het endocannabinoïdsysteem. SUB885C is ontwikkeld volgens de principes van CM en bevat acht natuurlijke ingrediënten. De kernformulering van SUB885C wordt in China gebruikt voor de behandeling van metabool syndroom en T2DM bij obese patienten in een vroeg stadium. Er is weinig bekend over de impact van deze twee geneesmiddelen op de regulering van het vetmetabolisme in de vroege fase van obesitas. De behandeling met Rimonabant gedurende 4 weken leidde tot een aanzienlijke vermindering van het lichaamsgewicht en had een geringe invloed op het plasmalipiden profiel in licht obese ApoE * 3Leiden.CETP muizen. Hoewel er geen gewichtsverlies werd waargenomen na behandeling met SUB885C, leidde dit CM geneesmiddel tot meerdere anti-atherogene veranderingen in het plasmalipidenprofiel van de muizen. De verbetering van metabole parameters en lipidenpatronen manifesteerde zich als een verlaagd plasma cholesterol, triglyceriden, verhoogde HDL-C en een verandering in een scala van neutrale lipiden. Deze effecten waren vergelijkbaar met die verkregen met bekende geneesmiddelen (bijv. atorvastatine, niacine). In vitro, stimuleert een SUB885C extract adipolysis en remt adipogenese in 3T3-L1 cellen. Deze twee studies met illustreren de kracht van lipidomics in het ontrafelen van interventie-effecten en kan daarmee helpen bij het vinden van nieuwe targets en/ of ingrediënten voor interventies bij lifestyle-gerelateerde metabole aandoeningen.

Panax ginseng CA Mey (vaak simpelweg aangeduid als ginseng) wordt in China gebruikt als een middel om diabetische symptomen in China behandelen. Echter, de relatie tussen de therapeutische werking bij diabetes en de chemische

samenstelling (kwaliteit en leeftijd) is nog onduidelijk. In **hoofdstuk 5** is een gecombineerde aanpak van lipidomics, biochemische parameters en samenstellings analyse van Panax toegepast om de effecten van de ginseng wortels, variërend in leeftijd van 3-6 jaar, op hyperglycemie en dyslipidemie in een diabetisch Goto-Kakizaki (GK) rat model te evalueren. Zes groepen van GK ratten kregen ofwel metformine, 3 tot 6 jaar oude ginseng wortel, of geen behandeling. De behandelingen werden tweemaal daags toegediend gedurende 9 weken. Vergeleken met de onbehandelde groep gaf zowel 4 als 6 jaar oude ginsengwortel een aanzienlijk verbeterd glucosemetabolisme, en de 5 jaar oude ginseng-behandeling gaf een significant verhoogd HDL-cholesterol. De behandeling met 6 jaar oude ginseng leidde tot een significante daling van plasma-triacylglyceriden (TG) en VLDL-cholesterol en een verbetering van geglycosyleerd hemoglobine (HbA1c) in plasma. Bovendien, resulteerde de behandeling met 4, 5 en 6 jaar oude ginseng in een verlaging van plasma-triglyceriden in diabetische GK. Metformine verminderde de bloedsuikerspiegel na vasten met 41% en verlaagde de HbA1c-waarde met 11%, maar toonde geen effecten op de plasmalipiden. Deze studie toont aan dat ginseng een leeftijdsafhankelijke (groei) therapeutisch effect op hyperlipidemie en hyperglycemie in diabetische GK ratten heeft. Deze leeftijdsafhankelijke effecten kunnen worden gerelateerd aan de variatie in zowel de verhoudingen als concentraties van specifieke bio-actieve ginsenosiden in ginseng wortels. D≥4 jaar oude ginseng wortels bevatten bioactieve ginsenosiden die waardevol zijn voor de ontwikkeling van medicijnen of voedingssupplementen om dyslipidemie te reguleren en glucose tolerantie te verhogen. Deze studie heeft nieuwe systeembiologische benaderingen geïntroduceerd voor het koppelen van biologische activiteit met actieve componenten in kruidenmengsels.

In **hoofdstuk 6** is de ontwikkeling beschreven van een snelle plasmalipiden analyseplatform door middel van directe nanospray infusie met behulp van een TriVersa nanospraychip systeem in combinatie met een hoge resolutie LTQ-Orbitrap massaspectrometer. Een aangepaste Folch methode werd toegepast voor de extractie van lipiden uit plasma. Met een volume van slechts 5 µl plasma, kan binnen 5 minuten per monster data verkregen worden van de volgende lipidenklassen: triacylglycerolen, cholesterol esters, fosfatidylcholines, lyso-fosfatidylcholines, spingomyelinen en vetzuren. In vergelijking met de gevalideerde vloeistofchromatografie-massaspectrometrie (LC-MS)-methode, is deze snelle methode ondanks een mindere nauwkeurigheid nagenoeg gelijkwaardig voor plasmalipiden met een hoge concentratie. Deze methode is in principe bijzonder geschikt voor high-throughput plasmalipiden analyse maar vraagt nog verdere ontwikkeling en validatie.

Tot slot, het systeembiologie-metabolomics onderzoek verricht in dit proefschrift convergeerde analytische biowetenschappen, klinisch onderzoek en de diagnostische concepten in andere gezondheidszorgsystemen, zoals CM om een systeembiologisch beeld van de pre-fase van T2DM te verkrijgen. Het is veelbelovend om met een combinatie van metabolomics en CM diagnosemethodieken te zoeken naar subtypes van T2DM. Metabolomics, en i.h.b. lipidomics, is in staat om multi-dimensionele farmacologische effecten te onderzoeken en potentiële biomarkers die verband houden met deze effecten op te sporen. Deze bevindingen kunnen worden gebruikt om nieuw onderzoekstrategieën en/of producten op het voeding-farma grensvlak te ontwikkelen voor T2DM behandeling en de daarmee samenhangende metabole afwijkingen te beheren. Dit zal echter alleen worden gerealiseerd als analytische methoden verder verbeteren, en systeembiologie en metabolomics beter geïntegreerd zijn binnen biomedisch onderzoek.

List of abbreviations

ACN, acetonitrile
ALT, alanine aminotransferase
ANOVA, analysis of variance
ApoE*3Leiden, Apolipoprotein E3 Leiden
BHT, butylated hydroxytoluene
CB1, cannabinoid 1
CETP, cholesteryl ester transfer protein
CER, ceramide
CH_2Cl_2, dichloromethane
Cho, cholesterol
ChoE / ChE, cholesterol ester
CM, Chinese medcine
CNS, central nervous system
CVD, cardiovascular disease
DCM, dichloromethane
DG, diacyglyceride
DI: direct infusion
FA, fatty acid
FBG, fasting blood glucose
FFA, free fatty acid;
FPLC, fast performance liquid chromatography
FTMS, Fourier transform ion cyclotron resonance mass spectrometry
GC, gas chromatography
GK, Goto-kajizaki
HbA1c , glycated hemoglobin;
HDL, high density lipoprotein;
HPLC, high-performance liquid chromatography
IDL, Intermeidate-density lipoprotein
IPA, isopropanol
IPGTT, intraperitoneal glucose tolerance test
IS, internal standard
LC, liquid chromatography
LDL, low density lipoprotein
LLE, liquid–liquid extraction
LPC, lysophosphatidylcholine
MeOH, methanol
MetS, metabolic syndrome

Molecular Phenotyping to Accelerate Genomic Epidemiology (MolPAGE)
MS, mass spectrometry
MTC; multiple test correction
MVDA, multivariate data analysis
NH4Ac, Ammonium acetate
PAF, platelet-activating factor
PCA, principal component analysis
PC, phosphatidylcholine/ Principal Component
PE, phosphatidylethanolamine
PG, phosphatidylglycerol
PLs, phospholipids
QC, quality control
RSD, relative standard deviation
SD, standard deviation
SPE, solid-phase extraction
SPM, sphingomyelin
T2DM, Type 2 diabetes mellitus
TCA, tricarboxylic acid
TG, triacylglycerol/triglyerides
TNF-α, tumor necrosis factor-α
UFLC, ultra-fast liquid chromatography
(V) LDL, (very) low density lipoprotein
*/ ×, times
WM, Western medicine

List of publications

Wei H, Pasman W, Rubingh C, Wopereis S, Tienstra M, Schroen J, Wang M, Verheij E, Greef Jvd: Urine metabolomics combined with the personalized diagnosis guided by Chinese medicine reveals subtypes of pre-diabetes. *Molecular BioSystems* **2012**, 8(5):1482-1491.

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Curriculum Vitae

Heng Wei , often referred to as Koko, was born on December 30, 1979 in Chengdu, P.R China. After six year study in Chengdu foreign language school, a key middle school with extra training on English and mathematics, she started to study in Chengdu Chinese Medical University, majoring in internal medicine with the combination strategy of Western medicine and Chinese medicine. She got the Bachelor degree of Medicine in China and afterwards went to the Netherlands. She received her MSc degree of Human Nutrition in 2007 from Wageningen University and directly began a four-year PhD in TNO (Zeist, the Netherlands) in the personalized health research project focused on metabolic health using systems biology based technologies. Her PhD was to search for type 2 diabetes diagnostic biomarkers or metabolism phenotypes by metabolomics, with special focus on lipid metabolism. During her PhD, she also coordinated scientific cooperation projects between the Netherlands and China for Sino-Dutch Center of preventive and personalized medicine.

Koko currently works as a research scientist in nutrition clinical studies in Centre for Human Drug Research (CHDR), Leiden.

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Overview of completed training activities

Training institute	*Type of training*	*Year*	*Certificate*
Amsterdam University (UvA)	Biosystem data analysis (6 ECT)	2008	Yes
James Boswell Institute, Utrecht	Nederlands voor vergevorderden	2008	Yes
ThermoFisher,UK	LTQ/LXQ/LCQ Fleet operation training	2008	Yes
Montreux LC/MS symposium	Practical LC/MS course	2008	Yes
Netherlands Metabolomics Center, NuGo and TNO	Hands-on training course in metabolomics	2008	Yes
Leiden University	The course Intellectual Property	2011	Yes
Leiden University	Time management, self-management	2011	Yes
Leiden University	Writing an excellent research grant proposal	2011	Yes
Leiden University	Research Based Business Opportunity (5 ECT)	2011	Yes
Leiden University	Research Based Business Ventures (5 ECT)	2012	Yes
HVCC	ICH GCP training	2012	Yes