Metabolic risk and cardiovascular disease: insights from large biobanks with genetic epidemiological approaches

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CHAPTER 1

General introduction, study populations, and thesis outline

CHAPTER 1

General Introduction

Cardiovascular disease

Cardiovascular diseases (CVDs) refer to a group of disorders of the heart and blood vessels, such as coronary artery disease (CAD) and ischemic stroke. A major underlying cause of various CVDs is atherosclerosis, which is characterized by the accumulation of fat, cholesterol, inflammatory cells and other substances in the arterial wall. This accumulation may ultimately cause (severely) reduced blood flow, and the plaque may also burst resulting in the formation of a blood clot and occlusion of major vessels (1). Various modifiable risk factors have been extensively described in scientific literature as causes for atherosclerosis and subsequent CVD, including but not limited to, smoking, hypertension, obesity, hyperglycaemia and dyslipidaemia (2). According to the World Health Organization (WHO), emphasis on CVD prevention and management of these risk factors has led to a 27% reduction in the age-standardized global mortality related to CVD between 2000 and 2019 (3). However, CVD still remains a leading cause of death, and contributes substantially to morbidity and healthcare costs (4-6).

To further reduce the disease burden of CVD, a more comprehensive and personalized understanding of disease mechanisms is essential. This requires optimal disease classification and prediction and effective treatment. Leveraging information from different biological layers, ranging from genetic polymorphisms to metabolic alterations in large datasets, is particularly valuable to identify biologically relevant pathways (7). Especially, recent advances in affordable high-throughput assessment of whole genomes, transcriptomes, proteomes, and metabolomes in large study samples have enabled a deeper understanding of underlying pathophysiology, and enabled the identification of clinical biomarkers and novel potential therapeutic targets for CVD (7-9).

Metabolomics

Metabolomics, one of the ‘omics’ sciences, utilises a variety of analytical tools for the quantitative and qualitative assessment of metabolites such as amino acids, organic acids, fatty acids, lipids, and many other small molecules (10). Metabolites are substrates, intermediates and end products of metabolism, which are characteristic for the expressed phenotype (11), and are thought to reflect the combined effects of genetic and environmental exposures (10, 12). Single biomarkers may not fully characterize complex biological phenomena, and metabolomics approaches now offer the opportunity to measure many

metabolites simultaneously, providing insight in a more comprehensive spectrum of dynamic metabolic processes (13).

The two most commonly applied analytical technologies in metabolomics are based on nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). This thesis mainly used the ^{1}H-NMR based metabolomic measures from the Nightingale platform (Nightingale Health Plc., Helsinki, Finland), which has been widely applied in large-scale epidemiological studies to identify underlying biological mechanisms and to improve disease risk prediction (14, 15). NMR spectroscopy relies on the fact that the magnetic properties of ^{1}H in a metabolite depend on their surrounding chemical environment. When exposed to a range of radio frequencies, protons absorb energy at specific frequencies and release it, which is then measured. One of the largest categories of the ^{1}H-NMR metabolomic measures from the Nightingale platform are lipoproteins. A detailed lipoprotein profile is provided consisting of characteristics and composition of the different sizes of high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) particles.

Total and LDL cholesterol are major risk factors for CVD (16). However, lipoprotein metabolism is a highly dynamic system through which lipids and specific apolipoproteins are passively and actively exchanged between the different lipoprotein classes in the course of their transport and metabolism within the circulation. Considering lipoprotein classes such as LDL in isolation could therefore disregard the intricate interdependence of plasma lipoproteins. For this reason, the availability of the ^{1}H-NMR metabolomic measures provides an opportunity to gain more insights into the pathophysiology of CVD by simultaneously considering all correlated metabolomic measures and inferring the metabolic processes underlying CVD.

Genomics

Genome-wide Association/Interaction studies

Genomics refers to the study of deoxyribonucleic acid (DNA) sequences across the entire genome of an organism, and is one of the earliest and most mature types of contemporary omics (17). Unlike rare monogenic disorders caused by pathogenic variants in a single gene, multifactorial diseases such as CVD are affected by a large number of common genetic variants with low-to-modest effect sizes, which might interact with each other as well as with lifestyle and the environment (18). With the increasing availability of genotyped and high-quality phenotypic data in large study samples, genome-wide association studies (GWAS) that aim to investigate associations between genome-wide genetic variants and phenotypes

have become increasingly popular. Evidence from GWASs have provided insight into the underlying biological mechanisms of CVD and its risk factors (19, 20). For example, the Global Lipids Genetics Consortium has identified many (ancestry-specific) loci for dyslipidaemia, resulting in improved insight in the underlying biology and fine-mapping of functional variants (21, 22).

Notably, it has been found that genetic and environmental factors, including sex and age, can contribute to a disease or phenotype in a non-additive manner, interacting to either disproportionally increase or decrease the effect size (23). Ageing is an inevitable consequence of living and a strong non-modifiable risk factor for CVD (24, 25). Other CVD risk factors, including obesity, dyslipidaemia and diabetes, and progressive defects in heart function are also closely associated with increased age (24, 26). Therefore, the prevalence, incidence and mortality of CVD is high in older adults (27). However, the role of age in the genetic architecture of CVD or its risk factors has not been widely explored yet (24), which may limit the application of genetic evidence in the elderly. Given that the number of people reaching advanced ages is increasing, it is critically important to investigate the interactions between genetic variants and age in disease risk.

Mendelian randomization

In recent years, facilitated by the availability of large-scale GWAS and high-quality data from mega biobanks, Mendelian randomization (MR) has emerged as a powerful research design for inferring causal associations in the field of observational studies. MR uses genetic variants associated with the exposure of interest, typically single nucleotide polymorphisms (SNPs) identified in GWAS, as instrumental variables (IV) to estimate the potential causal effects between exposures and outcomes (28). Based on the Mendel's First and Second Laws of Inheritance, the assortment of genetic variants from parents to offspring that occurs during gamete formation and conception is random. As a result, people are naturally randomized at conception into carrier and non-carrier groups based on their genetic carrier status, which could lead to higher or lower levels of the exposure of interest. Consequently, analogous to a randomized clinical trial (RCT) design, the MR design is considered a ‘natural experiment’, in which individuals are randomized to carry a genetic variant associated with the exposure of interest, and could be used to approximate a causal relationship between the exposure of interest and the outcome. Whereas RCTs typically investigate the effects of a specific intervention for a limited time in affected individuals, MR evaluates the causal impact of lifelong exposure to a particular factor on an outcome.

The statistical methodology for MR analysis is generally based on IV analysis to make causal effect estimates in the presence of unobserved confounding between the exposure and the

outcome (29, 30). Consistent with IV analysis, a valid MR analysis needs to satisfy three core assumptions (Figure 1): 1) genetic variants are associated with the exposure; 2) genetic variants are not associated with confounders that are related to the exposure and the outcome; 3) genetic variants exclusively affect the outcome through the exposure (30, 31).

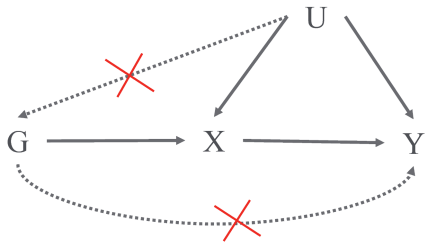

Figure 1. A directed acyclic graph for mendelian randomization analyses. G, genetic variants; X, the exposure of interest; Y, the outcome of interest; U, confounders. The solid line from G to X indicates the assumption 1). The dotted lines with crosses indicate the assumptions 2) and 3).

In the field of CVD, MR analyses have been widely used and have provided convincing causal evidence (32), especially for lipids and lipoproteins such as LDL cholesterol (33), apolipoprotein B (ApoB) (34), and triglycerides (35). However, lipids and lipoproteins are biologically interconnected, sharing metabolic pathways and regulatory mechanisms. This interrelated nature often results in horizontal pleiotropy, where a genetic variant influences multiple traits, leading to biased causal estimates. For example, HDL and triglycerides are metabolically linked through key enzymes like lipoprotein lipase (LPL) and cholesteryl ester transfer protein (CETP) (36, 37). LPL hydrolyses triglycerides in VLDL, releasing components that increase HDL-C, while CETP exchanges triglycerides from VLDL for cholesterol esters in HDL, reducing HDL-C. Consequently, genetic variants in *LPL* and *CETP* genes could affect both triglycerides and HDL cholesterol (38), introducing pleiotropy. This confounds the results from MR studies, as it obscures whether CVD risk associations are driven by TG, HDL-C, or their combined effects, complicating causal inference (39).

DNA Repeat Sequences

Given that approximately 50% of the human genome is comprised of DNA repeat sequences and that GWAS does not usually include genetic variation in DNA repeats, some variant-disease associations may be missed (40, 41). Polyglutamine diseases are one of the so-called expanded repeat disorders, and are characterised by the expansion of tandem cytosine-adenine-guanine (CAG) repeats in the translated regions that are beyond the normal range (42). For example, Huntington disease, the most common polyglutamine disease, is caused by a CAG repeat expansion in the Huntingtin gene (*HTT*) (43). Interestingly, studies of

patients with polyglutamine diseases have shown that they also suffer from symptoms of disrupted sleep (44-46), like sleep apnea, daytime sleepiness and disturbances in their circadian rhythm.

Sleep health was added to the Life's Essentials by the American Heart Association in 2022 (47), and sleep disturbances are increasingly recognized as important modifiable risk factors for various metabolic diseases including CVD and type 2 diabetes (48, 49). This recognition highlights the importance of sleep health for CVD prevention. Notably, recent studies have revealed that a CAG repeat size below the pathogenic threshold of polyglutamine diseases is associated with other metabolic parameters, such as plasma lipids and body mass index (BMI) (50, 51). In addition, CAG repeat sizes within the non-pathogenic threshold were found to account for 0.75% of the total variation in BMI (51). Therefore, investigating the effects of CAG repeat sizes on sleep traits, which are still unclear, may provide novel insight in sleep health and its associated health outcomes, including CVD.

Cardiovascular Risk Assessment

The application of omics techniques has provided ample insight into the underlying mechanisms of CVD development (7-9). However, a main challenge in CVD prevention remains the role of interindividual variations among individuals and populations (e.g., age, sex, family history, ethnicity), which could result in heterogenous associations of risk factors with disease development and treatment response (52).

Metabolic syndrome, a cluster of abnormalities consisting of waist circumference, plasma triglycerides, HDL cholesterol, blood pressure, and fasting plasma glucose, is defined when at least three out of the five components are beyond population- and sex-specific cutoffs (53). It has become a major and still escalating public health and clinical challenge worldwide, influenced by factors such as urbanization, sedentary lifestyles, dietary changes, and ageing (54). Previous evidence has shown that metabolic syndrome is strongly associated with (incident) cardiometabolic diseases (55, 56). However, given the intrinsic heterogeneity of the metabolic syndrome definition ($\geq$ 3 out of 5 symptoms), the dichotomous nature of the metabolic syndrome for the purpose of clinical utility has been frequently criticized (57). Detailed insight into the heterogeneous presentation of the metabolic syndrome in populations and their potential differential risks for cardiometabolic diseases are limited. This not only restricts the exploration of the pathophysiology of CVD driven by the metabolic syndrome, but also impedes the precise management of CVD risk in individuals with diverse clinical manifestations.

Various risk factors for CVD could be modified by adjusting environmental and behavioural factors, such as stress, nutrition and physical activity. Additional risk factors may be controlled by appropriate medication, such as statins to reduce LDL-C and decrease CVD risk. However, some risk factors may not be amenable to changes in behaviour and lifestyle and cannot be controlled by available medication. Lipoprotein(a) [Lp(a)], is one such risk factor. Lp(a) was first identified in 1963 (58) and is an LDL-like particle covalently bound to an apolipoprotein(a) molecule. Lp(a) blood levels are predominantly determined by genetic factors (59), and thus far no easy preventive strategies (e.g., weight loss, nutrition) have been identified. According to current guidelines, Lp(a) levels higher than 50 mg/dL are regarded as a cardiovascular risk-increasing factor (60-63). The proportion of individuals with Lp(a) ≥ 50 mg/dL ranges from 31% for Mexican individuals to 63% for non-Hispanic-Black individuals (64). Except for lipoprotein apheresis for individuals with elevated Lp(a) (65), there are currently no approved drug therapies for lowering Lp(a). It is thus essential to know whether Lp(a) has differential effects in subgroups with different risk levels defined by the concomitant presence of other common cardiovascular risk factors. These questions have not been fully investigated in previous studies.

In today's ageing society, it is particularly important to better understand the effects of ageing, especially the effects of ageing on subclinical symptoms that may be unnoticed and might develop into serious diseases over time. Thyroid hormones, having vital roles in development, growth and metabolism, have also been implicated in ageing, longevity and the development of age-related diseases, including cardiovascular disease (66). Subclinical hypothyroidism, biochemically characterised by elevated thyroid-stimulating hormone (TSH) concentrations in conjunction with thyroid hormone (T4) levels within the population reference range, is a common condition among older adults. The prevalence of subclinical hypothyroidism ranges from 4% to 20% in the adult population, with a higher prevalence in women and older people (67). While severe subclinical hypothyroidism (TSH > 10 mIU/L) has been associated with increased risk for cardiovascular events and mortality (68), the cardioprotective effects of levothyroxine therapy in older adults with subclinical hypothyroidism remain controversial (68, 69), and might vary according to pretreatment TSH concentrations. Moreover, older people may be at enhanced risk of levothyroxine overtreatment. Studies utilising biomarkers for CVD risk are needed to reveal potential earlier effects of levothyroxine therapy, depending on the amount of TSH elevation in older adults, which could be taken into account for better CVD prevention and risk management in older adults.

Main Study Populations

UK Biobank

UK Biobank (UKB) is a prospective cohort study, which recruited approximately 500,000 individuals aged 40-70 years across the entire United Kingdom during the baseline survey between 2006 and 2010. Invitation letters were sent to eligible adults registered to the National Health Services (NHS) and living within a 25 miles distance from one of the assessment centres. Extensive phenotypic and genotypic details of the participants have been collected since the baseline assessment, including sociodemographic data, lifestyle, physical measures, biological samples (blood, urine and saliva), genome-wide genotyping, and prospective follow-up on a wide range of health-related outcomes, etc. The UKB cohort study was approved by the North-West Multicentre Research Ethics Committee (MREC). All participants provided electronic written informed consent for the study. A detailed description of the UKB cohort study has been presented elsewhere (70). In Chapters 2-4 and Chapters 6-7, we made use of individual UKB data with large sample sizes to perform solid and robust statistical analysis.

Netherlands Cohorts

The Netherlands Epidemiology of Obesity (NEO) study

The NEO study is a population-based prospective cohort study, which includes 6,671 individuals aged 45-65 years with an oversampling of overweight individuals (71). Men and women aged between 45 and 65 years with a self-reported BMI of 27 kg/m^2 or higher, living in the greater area of Leiden (in the west of the Netherlands) were eligible to participate in the NEO study. In addition, all inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited irrespective of their BMI, allowing for a reference distribution of BMI. The collection of baseline data started in September 2008 and was completed at the end of September 2012. Participants were invited to come to the NEO study center of the Leiden University Medical Center (LUMC) for a baseline study visit after an overnight fast. During this study visit, participants would undergo an extensive physical examination, including blood and urine sampling. Prior to this study visit, participants completed a general questionnaire at home in terms of their demographic, lifestyle, and clinical data in addition to specific questionnaires on diet and physical activity. The NEO

study was approved by the medical ethical committee of LUMC, and all participants gave written informed consent. Individual data from NEO study is used in Chapter 5.

The Netherlands Study of Depression and Anxiety (NESDA)

NESDA is an ongoing longitudinal cohort study, consisting of 2,981 participants aged 18-65 years. Participants were recruited from the general population, general practices, and secondary mental health centres, of whom with depressive/anxiety disorders and healthy controls (72). Blood samples were collected after an overnight fast at the baseline visit (2004-2007). The Ethical Committees of all participating universities approved the NESDA project, and all participants provided written informed consent. Individual data from NESDA is used in Chapter 5.

Randomized Clinical Trials: TRUST and IEMO80+

TRUST (Thyroid hormone Replacement for Untreated older adults with Subclinical hypothyroidism - a randomised placebo-controlled Trial) and IEMO80+ thyroid trial (the Institute for Evidence-Based Medicine in Old Age 80-plus thyroid trial) are two clinical trials that aimed to investigate the effects of levothyroxine therapy in older population. Detailed description and protocols for the two RCT have been published previously (73, 74). In brief, both trials recruited community-dwelling participants with subclinical hypothyroidism, defined as elevated thyrotropin levels (4.6 to 19.9 mIU/L) measured on at least two occasions between 3 months and 3 years apart, and free T4 levels within the reference range. TRUST recruited participants aged 65 years and older in the Netherlands, Switzerland, Ireland, and the United Kingdom between April 2013 and May 2015, and IEMO80+ recruited participants aged 80 years and older in the Netherlands and Switzerland between May 2014 and May 2017. Both trials shared a near-identical design and recruitment strategy. Trial protocols were approved by the relevant ethics committees and regulatory authorities in all countries involved in the trials. The trials were conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent was obtained from all participants (trials registrations: ClinicalTrials.gov NCT01660126 [TRUST and IEMO], Netherlands Trial Register NTR3851 [IEMO]).

Thesis Outline

This thesis aims to generate deeper insights into the metabolic risk assessment for a more precise prevention of cardiovascular disease, through the integration of multiple omics data with large-scale biobanks. **Chapter 1** describes the general introduction, study populations, and thesis outline, respectively.

The research **chapters (2-8)** of this thesis are structured in three parts:

Part I: Lipoprotein metabolism and CVD risk

Part I aims to investigate the role of ^{1}H-NMR metabolomic measures in CVD risk and to provide additional insight into possible underlying mechanisms of atherosclerotic CVD. In **Chapter 2**, we investigated the associations between independent metabolomic profiles, derived from the ^{1}H-NMR metabolomic measures, and CVD through several different epidemiological approaches. This aligns with the principles of triangulation (75), aiming to synthesize evidence from diverse methodologies that are subject to unrelated sources of bias. Subsequently, in **Chapter 3,** building upon our own findings from **Chapter 2** and evidence from previous studies, we examined the role of phospholipid transfer protein (PLTP) activity, determined by genetic predisposition, in lipoprotein metabolism and in the risk of developing CVD.

Part II: Genetic variation and CVD risk

Part II aims to provide more insights into the risk factors associated with CVD from a genetic perspective. In **Chapter 4**, we performed genome-wide interaction studies on different cardiovascular risk factors to identify age-specific genetic risks in a large UKB sample. Subsequently, replications were performed in two other independent samples, i.e. the Copenhagen General Population Study and the Estonian Biobank. In **Chapter 5**, embedded in two Dutch cohorts, we explored the associations between CAG repeat sizes and sleep health, and investigated the extent to which genetic variations could explain the variation of sleep traits.

Part III: CVD risk assessment

Part III aims to investigate the risk associated with CVD in different subpopulations with specific risk profiles. In **Chapter 6**, we identified the heterogeneous presentation of the metabolic syndrome components and investigated their age- and sex-specific associations with CVD. In **Chapter 7**, we examined whether Lp(a) has differential effects in groups with different risk levels defined by the concomitant presence of other common modifiable cardiovascular risk factors. In **Chapter 8**, based on two RCTs in older adults with subclinical hypothyroidism, we assessed the levothyroxine treatment response of cardiometabolic biomarkers in populations with different pretreatment TSH levels. These preclinical biomarkers may be indicative of future cardiovascular risk.

Chapter 9 summarizes and discusses the main findings of this thesis, and the future outlook.

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