Metabolite Alterations and Cardiometabolic Disease: A Nutritional Perspective

Smith, Einar

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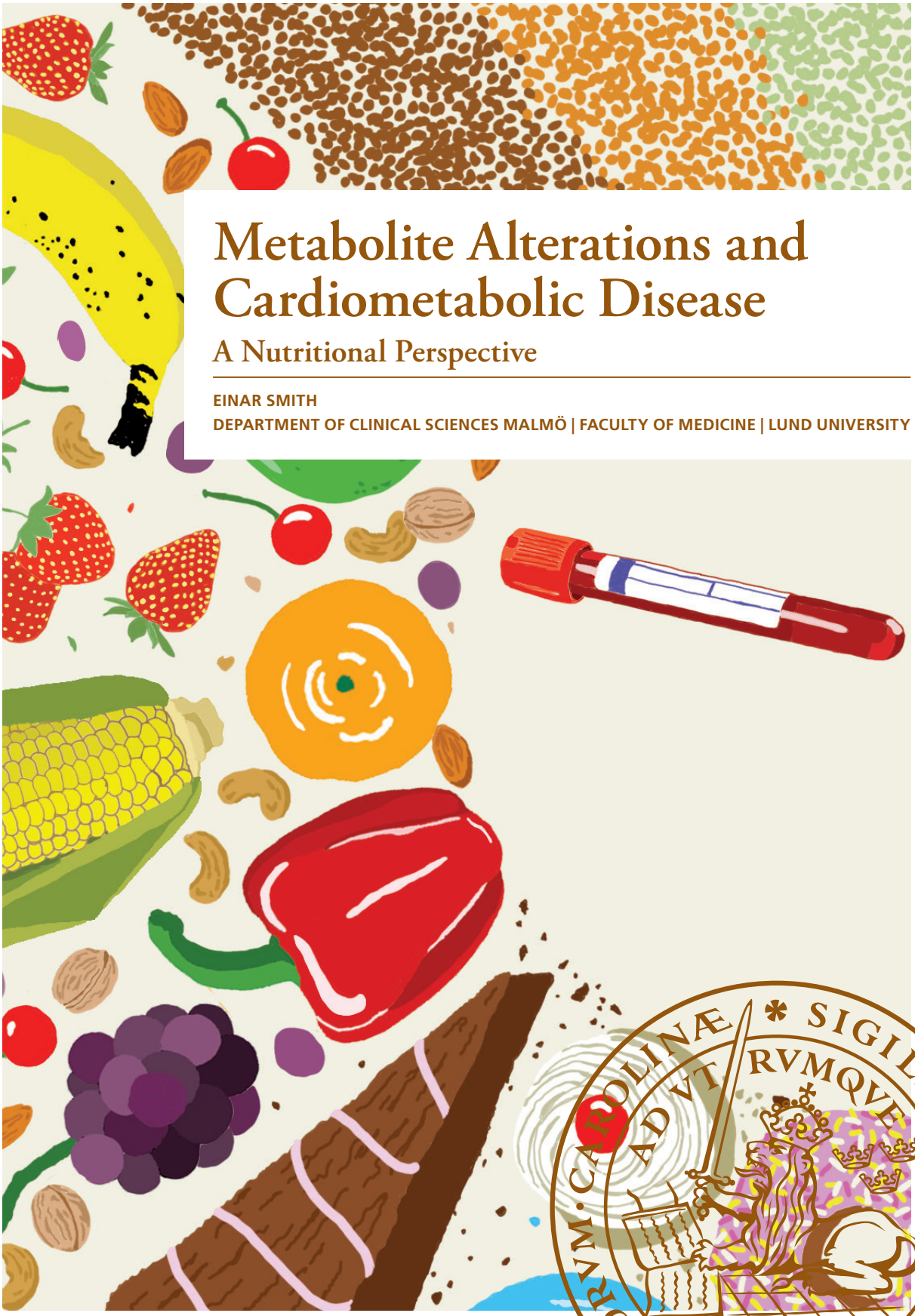

Metabolite Alterations and Cardiometabolic Disease

A Nutritional Perspective

EINAR SMITH
DEPARTMENT OF CLINICAL SCIENCES MALMÖ | FACULTY OF MEDICINE | LUND UNIVERSITY

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Einar Smith

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Rikard Landberg PhD
Professor and Head of Division in Food and Nutrition Science
Chalmers University of Technology

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Abstract

Background

Cardiovascular disease (CVD), type 2 diabetes (T2DM), and atrial fibrillation (AF) collectively impact millions globally, necessitating a comprehensive understanding of preceding metabolic alterations for early intervention. This thesis aims to explore metabolic shifts across populations-based cohorts and evaluate the metabolic impact of a dietary intervention.

Method

Utilizing liquid-chromatography mass spectrometry, we quantified approximately 110 metabolites in over 6000 subjects from the Malmö Preventive Project (MPP), Malmö Diet and Cancer (MDC), Malmö Offspring Study (MOS), and the Cilento dietary intervention study (CDI). Paper I investigates associations between metabolites and future atrial fibrillation in MDC. Paper II examines associations between metabolites and a healthy dietary pattern in MDC, and their associations with future CVD, T2DM, and mortality. Paper III presents a metabolite-based model for healthy dietary intake assessed in MOS, testing its association with future T2DM and CVD in MDC and MPP. Paper IV assesses the metabolic effects of a 6-day Mediterranean diet intervention among Swedish participants in the CDI.

Results

Paper I identifies 15 metabolites with significant associations with AF, particularly acylcarnitines (1). Paper II associates six metabolites with healthy dietary intake, with ergothioneine especially inversely related to CVD and overall mortality (2). Paper III's metabolic signature for healthy dietary intake associates with lower T2DM and CVD incidence in both MPP and MDC (3). Paper IV reports significant post-intervention metabolite changes, especially in the dietary related metabolome.

Discussion

This thesis provides a comprehensive analysis of metabolite alterations associated with CVD, T2DM, and AF, elucidating the relationships between metabolic and dietary pattern biomarkers and disease risk. The findings emphasize the utility of plasma metabolites as potential predictors and intermediaries in the pathways leading to these major diseases. Paper 3 and 4 combined acts as a proof of concept that plasma metabolites can be used to identify subgroups with higher risk for CVD and T2DM that might be caused by poor dietary intake Similar methods could be used to develop validated metabolic analyses as biomarkers for healthy dietary intake, with potential application in personalized preventive medicine.

Key words: Metabolomics, nutrition, nutritional metabolomics, biomarkers, cardiometabolic disease, cardiovascular disease, type 2 diabetes, prospective cohort studies. Dietary intervention.

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Table of Contents

Abbreviations and Acronyms

95% CI	95 % Confidence Interval
AHEI	Alternative Healthy Eating Index
AF	Atrial Fibrillation
ARIC	Atherosclerosis Risk in Communities
BMI	Body Mass Index
CAD	Coronary Artery Disease
CDI	Cilento Dietary Intervention
CMD	Cardiometabolic Disease
CV	Coefficient of Variation
CVD	Cardiovascular Disease
DASH	Dietary Approaches to Stop Hypertension
FDR	False Discovery Rate
FFQ	Food Frequency Questionnaire
HCFP	Health-Conscious Food Pattern
HDL	High Density Lipoprotein
HDMS	Healthy Dietary Metabolic Signature
HEI	Healthy Eating Index
HILIC	Hydrophilic Interaction Liquid Chromatography Column
ICC	Interclass Correlations
ICD	International Classification of Diseases
LDL	Low Density Lipoprotein
LC	Liquid Chromatography
LC-MS	Liquid Chromatography – Mass Spectrometry
LOESS	Locally Estimated Scatterplot Smoothing
MD	Mediterranean Diet
MDS	Mediterranean Diet Score
MDC	Malmö Diet and Cancer Study
MDC-CC	Malmö Diet and Cancer Study – Cardiovascular Cohort

MOS	Malmö Offspring Study
MPP	Malmö Preventive Project
MS	Mass Spectrometry
m/z	Mass-over-charge ratio
MURDOCK	Measurement to Understand Reclassification of Disease of Cabarrus/Kannapolis
NMR	Nuclear Magnetic Resonance
NT-proBNP	N-Terminal prohormone of Brain Natriuretic Peptide
PEth	Phosphatidylethanol
PCA	Principal Component Analysis
PLS	Partial Least Squares (also known as Projection to Latent Structures)
RCT	Randomized Controlled Trial
SCAAR	Swedish Coronary Angiography and Angioplasty Register
SD	Standard Deviation
QC	Quality Control
QTOF	Quadrupole Time-of-Flight
T2DM	Type 2 Diabetes Mellitus

List of papers

Paper I

Smith E, Fernandez C, Melander O, Ottosson F. Altered Acylcarnitine Metabolism Is Associated With an Increased Risk of Atrial Fibrillation. Journal of the American Heart Association. 2020;9(21):e016737.

Paper II

Smith E, Ottosson F, Hellstrand S, Ericson U, Orho-Melander M, Fernandez C, Melander O. Ergothioneine is associated with reduced mortality and decreased risk of cardiovascular disease. Heart. 2020;106(9):691-7.

Paper III

Smith E, Ericson U, Hellstrand S, Orho-Melander M, Nilsson PM, Fernandez C, Melander O, Ottosson F, A healthy dietary metabolic signature is associated with a lower risk for type 2 diabetes and coronary artery disease. BMC Med. 2022;20(1):122.

Paper IV

Smith E, Ottosson F, Ericson U, Hellstrand S, Rizzo M, Sukruang K, Pizza V, Orho-Melander M, Nilsson P.M, Kennbäck C, Fernandez C, Antonini P, Di Somma S, Melander O, Enhancement of Cardiometabolic Disease-Related Metabolic Signature through a Six Day Mediterranean Diet Intervention. Unpublished Manuscript

Papers not included in the thesis

1. Ottosson F, **Smith E**, Melander O, Fernandez C. Altered Asparagine and Glutamate Homeostasis Precede Coronary Artery Disease and Type 2 Diabetes. The Journal of clinical endocrinology and metabolism. 2018;103(8):3060-9.
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6. Hellstrand S, Ottosson F, **Smith E**, Brunkwall L, Ramne S, Sonestedt E, et al. Dietary Data in the Malmö Offspring Study-Reproducibility, Method Comparison and Validation against Objective Biomarkers. Nutrients. 2021;13(5).
7. Ottosson F, **Smith E**, Ericson U, Brunkwall L, Orho-Melander M, Di Somma S, et al. Metabolome-Defined Obesity and the Risk of Future Type 2 Diabetes and Mortality. Diabetes Care. 2022;45(5):1260-7.
8. Yan Y, **Smith E**, Melander O, Ottosson F. The association between plasma metabolites and future risk of all-cause mortality. J Intern Med. 2022.

Introduction and background

Introduction

Coronary artery disease (CAD), stroke, type 2 diabetes (T2DM), and atrial fibrillation (AF) are among the largest health concerns causing mortality and decreased quality of life globally (4). While the former three are commonly grouped under the umbrella of cardiometabolic diseases (CMD) (5), AF though closely associated (6), is often not classified as such. The development of these diseases, including AF, is intricate and multifactorial (7-9). There is strong evidence indicating that healthy dietary intake can delay and sometimes prevent development of CMD and AF (10-13).

In this thesis I aimed to investigate the complex interaction between risk factors and development of disease, with a focus on dietary intake and circulating metabolites. Using large cohort studies, I associated individual metabolites with development of AF (paper I), healthy dietary intake, and reduced development of CAD, stroke, and mortality (paper II). Furthermore, I discovered a metabolic signature associated with healthy dietary intake, and reduced CMD development (paper III). Lastly, I showed that the identified metabolic signature can be altered by a short dietary intervention (paper IV).

As an internal medicine resident, I encounter patients with co-existing CVD, T2DM and AF on a daily basis. These individuals are marked by years of disease with a lowered quality of life, a life that will be much shorter than their healthy counterparts. Despite substantial development in the pharmacological treatments we can offer these patients, preventive measures have not received the same focus. This thesis aims to uncover novel insights into the development of CVD, T2DM, and AF, with the ultimate goal of improving preventive measures to avoid future incidences of these conditions.

Pathogenesis

Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death globally claiming an estimate of 17.9 million lives per year (4). CVD include the diseases of the heart and the blood vessels, with CAD – which impacts the vessels supplying the heart, and ischemic stroke – affecting the vessels to the brain, being the two predominant killers. Combined, they account for approximately 85% of CVD related deaths (4). The common pathophysiological foundation of CVD is atherosclerosis, a condition characterized by accumulation of plaque within vessel walls (7). The plaques can either chronically restrict blood flow or, when they rupture, cause an acute lack of oxygen to the tissues downstream, exemplified by a heart attack, a type of CAD. Ruptured plaques may also form emboli, which can travel through the bloodstream and, if lodged in cerebral vessels, obstruct blood flow, one of the mechanisms causing ischemic stroke (14).

The development of atherosclerosis is caused by multiple factors including hypertension, high LDL-cholesterol, smoking, and unhealthy dietary intake (7, 12). While smoking has decreased and the treatment of high LDL-cholesterol and hypertension have improved, leading to fewer cases of CVD (15-17), dietary intake has only seen a slight improvement (12, 18).

Dietary impact on CVD can be distilled into three core components: the overconsumption of calories, the ingestion of harmful substances, and the omission of beneficial foods (19). Some estimate that suboptimal intake of dietary factors can explain up to 45% of deaths due to CMD in the United States (12). Given the significance of diet in CVD development there is a pressing need for further research to better understand and address this problem.

Type 2 diabetes

T2DM is a complex metabolic disorder characterized by persistently elevated blood glucose levels, primarily resulting from insulin resistance and a progressive decline of beta-cell function (20). This condition leads to an insufficient insulin response, culminating in elevated blood glucose levels. The prevalence of T2DM is escalating, with projections suggesting that over 640 million people could be affected by the year 2040 (21). This trend is closely associated with the global increase in obesity (22).

The chronically elevated blood glucose level in T2DM is a precursor to a range of complications, both microvascular and macrovascular. Among these, atherosclerosis is particular significant, often resulting in CVD, the principal cause of mortality in patients with T2DM (23). Recent advances in research have unveiled

the heterogeneous nature of T2DM, highlighting a need for sub-classification. Ahlqvist et al. discovered distinct T2DM subgroups, and reclassification could lead to more tailored and effective treatment strategies (9). This reclassification acknowledges the diverse etiological pathways and risk factors contributing to T2DM, underscoring its multifaceted nature.

Diet plays a critical role in the onset of T2DM, with certain dietary patterns being linked to an increased risk, while others show protective effects. Diets rich in refined sugars and low in fibre are associated with a higher risk of CMD, whereas diets emphasizing whole grains, lean proteins, and vegetables have demonstrated protective effects (12). Preventive measures, especially those focusing on dietary modifications, increased physical activity and weight management, have proven effective in reducing the risk of T2DM (24-26). Targeted interventions and public health campaigns that highlight healthy lifestyle changes are crucial in offering a pathway to mitigate the rising prevalence of T2DM and its' complications.

Atrial fibrillation

AF, the most common cardiac arrhythmia, affected approximately 33.5 million people worldwide in 2010 (27). It is characterized by irregular and "fast beating" of the chambers of the heart. Beyond causing discomfort and reduced exercise tolerance, AF causes an increased risk of developing heart failure, stroke, CAD, dementia, and premature mortality (27-29). Some adverse events are prevented by cardioversion, antiarrhythmic drugs, ablation, and anticoagulation, but treatments have a significant risk and cost (30).

The cause of AF is complex and not fully understood. It is thought to be a multifactorial combination of structural and electrical remodelling, as well as chronic inflammation (8). The risk factors for AF closely mirror those of CMD, and increased BMI and hypertension are closely linked to both AF and CMD development (6, 31). Strict blood pressure treatment, alcohol abstinence and healthy dietary intake are pillars in AF prevention and management (30).

Traditional nutrition and dietary patterns

Traditional nutrition

Historically, the landscape of dietary research has been predominantly dominated by studies focusing on individual food items or isolated nutrients. For instance, much work was dedicated to exploring the impacts of dietary fats, leading to the discovery of the roles of saturated, unsaturated, and trans fats on cardiovascular health (17). Also, vitamins, minerals, and their function in bodily processes and their

implications for preventing nutrient deficiencies and associated health conditions have been discovered (32, 33). Nutritional research has built a crucial foundation of knowledge which allows for testing in randomized controlled trials (RCTs) comparing potential healthy nutrients with placebo. Many such trials have shown that observational association does not always translate to any health gains in sufficiently powered trials (34, 35).

Dietary patterns

It has gradually become apparent that the study of single nutrients might not fully capture the complexity of human nutrition. In real-world scenarios, food isn't consumed in isolation; rather, it's part of a broader meal where various nutrients and food items interplay and correlate with each other (36). Recognizing these intricate relationships has created a paradigm shift in the research perspective. Instead of merely examining singular food items or nutrients, there's an expanding interest in understanding dietary habits in terms of overall patterns. This holistic approach is not only more representative of how individuals consume food but also provides a more comprehensive insight into the effects of diet on health (37-39). Consequently, numerous guidelines have emerged, emphasizing the importance of healthy eating patterns as a preventive measure against CMD (10, 17, 32). This perspective encourages individuals to consider the entirety of their diet, rather than fixating on specific components, fostering a more balanced and potentially beneficial approach to nutrition (19, 40, 41).

Hypothesis-driven patterns

There are two main ways to define dietary patterns – a priori (hypothesis-driven) or a posteriori (data-driven) (42). In the hypothesis-driven approach dietary indices/scores are created based on previous scientific evidence with a plausible disease protective effect. Examples include Mediterranean diet score (MDS) – with score adherence to Mediterranean diet (MD) (43), Healthy Eating Index (HEI) (44), Healthy Nordic Diet (45), or Dietary Approaches to Stop Hypertension (DASH) (46). The indices are generally scored based on consumption of pre-defined food groups, and the overall score indicate quality of diet in relation to the predefined diet. They have been connected to positive health outcomes with a modest protective effect in observational studies (40).

Data-driven patterns

The data-driven approach is instead based purely on empirical data. With statistical methods like factor analysis or principal component analysis (PCA), patterns existing within a population are discovered. Data-driven methods often discover health-conscious/prudent pattern within a population, showing that the largest variation in dietary intake often is explained by health-conscious food choices. (40,

41, 47). The advantages of data-driven methods are that existing patterns within a population can be discovered. However, the pattern is often unique to the studied population, and comparison between different data-driven patterns are much more difficult than comparing the hypothesis-driven dietary scores (48). Adherence to several of the health-conscious patterns has shown to be associated with a lower risk for CMD (40, 41, 47). The prudent dietary patterns are often similar to the hypothesis-driven dietary patterns that are recommended for T2DM and CVD prevention (32, 40, 41, 47).

A large problem in both data-driven and hypothesis driven dietary research is that the translation from observational data to clinical trials is difficult with dietary patterns. In dietary intervention studies, it's impossible to blind what food participants are eating, and participants' compliance to described diets is difficult to achieve both in trials and in real-life settings. The interventions are also expensive. As of date, the MD stands out as one of the few pre-defined dietary patterns that has a cardiovascular protective effect in a large RCT (49).

Conventional assessment methods and challenges

To measure adherence to both data-driven and pre-defined patterns, reliable dietary data is important. Traditional methods for dietary assessment, such as food diaries, 24-hour recalls, and food frequency questionnaires (FFQs), have been pivotal in our understanding of the relationship between diet and health (50, 51). Among these, multiple-day food records, like the one used in the study Malmö Diet and Cancer (MDC), are often regarded as the gold standard in dietary investigations. However, they pose challenges. For participants, maintaining detailed records is demanding, and for researchers, converting these records into usable data on food compounds and nutrients is time intensive. Moreover, the accuracy of such methods can be compromised due to potential human error or misrecollection (52).

The 24-hour recall, which involves structured interviews designed to capture food and beverage consumption from the latest 24 hours, has its own set of limitations. Specifically, it can overlook foods that are consumed infrequently because it captures a narrow window of intake (53). On the other hand, while the self-administered FFQs are favoured in large cohort studies due to their efficiency, they are susceptible to measurement inaccuracies, often resulting from participants' memory biases or misunderstandings of the questions (54, 55).

Metabolomics

Metabolomics, the measurement of metabolites, represent a powerful approach in the understanding of complex disease states by allowing for comprehensive profiling of small molecules, or metabolites, present within biological systems (56). Metabolites, with typical molecular weight under 1.5 kDa, serve as substrate, intermediates and end products of enzymatic process, and the measurement provide insight into cellular metabolism. The total set of metabolites can be called the metabolome, and the metabolome consist of both endogenous and exogenous metabolites (57). The metabolome reflects the metabolic state of the sample studied, and it is affected by numerous factors like dietary intake, genetic variation, physical activity and gut microbiota metabolism (5). The field of metabolomics aim to systematically study these metabolites. Measurements of the plasma metabolome can provide insight into the metabolic alterations associated with development of diseases like CVD, T2DM, and AF. Utilizing advanced analytical techniques like Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR), metabolomics enables the detection and quantification of up to thousands of metabolites from various biological samples including plasma, serum, saliva, and stool (57-60). Over the years, this rapidly evolving field have shed light on metabolic pathways involved in disease onset and progression, potentially paving the way for targeted therapeutic interventions.

Techniques in metabolomics research

The accurate measurement of metabolites is one of the largest challenges in metabolomics research. The two most predominant techniques employed are mass spectrometry (MS), and nuclear magnetic resonance (NMR) each with its unique advantages (and disadvantages) (61). While NMR is renowned for its reproducibility, MS offers superior sensitivity and resolution, enabling the potential to measure more metabolites than NMR (57).

MS, which is the method used in this thesis, works by ionizing molecules and measuring their mass-to-charge ratio (m/z) generating a mass spectrum, which is a plot of ion signal intensity as function to m/z. Different molecules produce unique mass spectra, enabling their identification and quantification (57). While MS is highly sensitive and capable of detecting a wide concentration range of metabolites, accuracy can be affected by variations in how effective each metabolite is ionized (62). It is important to note that no single tool can capture the entire metabolome.

A key consideration in MS is the choice between untargeted and targeted approaches (5). Untargeted MS aims for a comprehensive analysis, attempting to capture as many metabolites as possible in a single run. This approach is exploratory in nature, often used to uncover novel biomarkers or to gain a holistic understanding of

metabolic changes. The problem is that annotation, the process to confirm the identity of measured features can be very time consuming. Targeted MS, on the other hand, focuses on a predefined set of metabolites. This approach is typically more sensitive and precise, making it well-suited for validation studies. Targeted MS can also offer the possibility to make absolute quantifications, but varying ionization of metabolites means that the ions levels in the mass spectra can suggest widely varying concentrations. To measure the precise concentration of a metabolite, reference standards are required (61). Yet, implementing this for every metabolite in extensive cohorts is expensive and time consuming. Consequently, many MS based studies measure metabolite levels only in relative terms, which in turn complicates cross-cohort comparisons (63).

Nutritional metabolomics

Introduction to nutritional metabolomics

Nutritional metabolomics stands at the forefront of contemporary dietary assessment, with the potential to aid in understanding the complex relationship between diet and health. By addressing the limitations of traditional methodologies like food diaries, 24-hour recalls, and food frequency questionnaires (FFQ), metabolomics introduces a new era of accuracy and reliability in nutritional research (51).

Enhancing accuracy and precision

Numerous circulating metabolites originate directly from the metabolism of dietary components. These metabolites, when considered collectively, are proposed to be termed the "food metabolome" (64). Metabolomics is primarily utilized in nutritional research to discover metabolites that correlate with consumption of specific food groups (64, 65). Extensive validation is needed to transition from observational associations to confirming metabolites as dietary biomarkers. Dragsted et al along with the FoodBall consortium have suggested eight groups of validity criteria for biomarkers of food intake (66). These validated biomarkers of food intake have the potential to increase the accuracy of traditional nutritional assessment methods, augmenting their reliability (51, 65, 67-69). Also, as some dietary-related individual metabolites associate with future disease, they could be singled out as candidate substances for future intervention studies (70, 71).

Biomarkers for dietary patterns

Endogenous plasma metabolites and metabolites from the food metabolome have been shown to correlate with dietary patterns in cohort studies (65, 72). While single metabolites correlate with dietary patterns, using a metabolite pattern has been

suggested to better reflect overall adherence to dietary patterns, rather than reducing the complexity of the pattern to one metabolite (38). Metabolite pattern-based biomarkers also have the potential to capture individual variation in endogenous- and microbiota metabolism of food components (58). It has been shown that metabolite measurements could classify individuals into groups based on their dietary habits (38, 67, 73, 74). By using these dietary pattern biomarkers, associations can then be made between dietary habits and reduction of CMD incidence (37, 70). This has the potential to identify subgroups with unfavourable dietary habits and increased risk for CMD and test the effectiveness of dietary interventions on these subgroups to decrease CMD risk.

Such intervention could then also use metabolite measurements to assess dietary changes during the intervention (75, 76). In clinical practice in Sweden, one commonly used biomarker is phosphatidylethanol (PEth), a phospholipid group formed only in the presence of ethanol, to assess alcohol intake. PEth is both used in clinical practice to discover individuals at high risk for alcohol related complications, but also to tailor and adjust interventional strategies depending on follow-up measurements.

Metabolites as Predictors

Circulating metabolites have been shown to associate with future incident T2DM (77), premature mortality (78), CAD (79) and AF (80). By understanding the correlation between specific metabolites and emergent diseases, researchers can pinpoint the metabolic irregularities preceding disease onset. These findings are significant as they not only suggest the potential for developing screening programs, that leverage lifestyle, dietary, and weight-loss interventions to mitigate disease risk, but it also illuminates new avenues for drug research by offering deeper insights into the pathogenesis of CMD**.**

In the context of atrial fibrillation, despite the promise, there are only a few large cohort studies utilizing metabolomics to study new-onset AF that had been published (80-82). While groundbreaking, these studies show conflicting result, highlighting a need for future research in this area. The close link between the development of AF and conditions such as obesity and T2DM, which are also associated with specific metabolites underscores the potential to discover novel metabolic changes and predisposed individuals to AF (31, 83).

Aims

Paper I

To identify metabolites associated with incident AF to highlight metabolic changes related to AF development.

Paper II

To discover metabolites associated with both a healthy dietary pattern and with future cardiometabolic disease.

Paper III

To create a multivariate metabolic signature of a healthy dietary pattern and to investigate the association of this signature with CAD and T2DM in two cohorts.

Paper IV

To investigate if the healthy dietary metabolic signature is modifiable by a six-day Mediterranean diet intervention.

Methodology

Author's contribution to the papers

Paper I – IV

Conceptualization: The author initiated and shaped the foundational ideas for paper I-III. In paper IV, the author was only partly involved in the design and implementation of the intervention.

Pre-processing: Prepared a large part of the plasma samples.

Mass Spectrometry: Had a large part in the operation of the mass spectrometer.

Post-processing: Refined and organized the collected data.

Statistical Analysis: Performed all the statistical analysis.

Interpretation: Contributed significantly to the interpretation of results.

Writing/publishing: Throughout the projects, the author maintained active collaboration with fellow researchers, was the corresponding author for each project and drafted all manuscripts.

The author did not contribute to the gathering of clinical data, dietary data, or the longitudinal data. He did not have a part in the discovery of the dietary patterns used in papers II-IV. He was not involved in the design of the Malmö cohorts.

Cohorts

In paper I and II, a sample of participants from the Malmö Diet and Cancer Study (MDC) was used. For paper III, data from MDC was used as well as from the Malmö Preventive Project (MPP) and the Malmö Offspring Study (MOS). In paper IV, data from the Cilento dietary intervention (CDI) is used. Anthropometric baseline data, basic lab work and plasma for metabolomics analysis were collected in MOS, MPP, MDC, and CDI using consistent and standardized methods across all cohorts.

MDC

The Malmö Diet and Cancer Study (MDC) is a prospective cohort study based in Malmö, initiated in the 1990s with the primary aim of investigating the associations between dietary factors and cancer incidence (84). An invitation was extended to 74,138 individuals, resulting in the participation of 30,447 individuals, which corresponds to a participation rate of 41%. At the outset, half of the participants were randomly chosen for inclusion in the Malmö Diet and Cancer – Cardiovascular Cohort (MDC-CC), which aimed to further phenotype participants to explore the epidemiology of carotid artery disease (85). Within the MDC-CC, a subset of 6,103 participants provided samples of fasted citrate plasma, which were preserved at -80°C until the analysis was performed in 2018. Our laboratory analysed 3,833 of these samples, and the metabolite data derived from these analyses were utilized in Papers I, II, and III (5). The baseline age of participants was around 58 years in the sample used in this thesis.

In MDC, an exhaustive dietary sampling was conducted as described previously (86-88). Briefly, the assessment deployed a modified dietary history method, integrating of a 7-day food diary with a comprehensive diet history questionnaire supplemented by an interview. Over a period of seven consecutive days, participants documented their intake of prepared meals, cold beverages, pharmaceuticals, and dietary supplements. The diet history questionnaire was designed to examine the habitual meal pattern, including the frequency and portion sizes of food consumed on a regular basis. Subsequently, during the interview, a review was conducted to identify any duplications between the 7-day food diary and the questionnaire.

The dietary information gathered was quantitatively transformed into estimates of energy and nutrient consumptions using the MDC-nutrient database. The database contained information mostly from the PC-KOST2-93 provided by National Food Agency of Sweden. Adjustments for energy intake were made in the analysis of food group consumption, employing regression techniques to standardize intakes to total non-alcoholic energy consumption. Estimations of alcohol consumption were derived from the 7-day menu records.

MPP

The Malmö Preventive Project (MPP) represents another population-based prospective cohort study conducted in Malmö (89). This study initially comprised 33,346 participants who underwent a baseline examination during the period from 1974 to 1992. From 2002 to 2006, surviving participants were invited for a re-examination, which is considered the baseline for the current study, encompassing 18,240 individuals aged between 65 and 80 years (79). In a prior publication, we detailed the formation of a case-control cohort (79). In summary, from an initial random sample of 5,386 individuals, we excluded those with a history of type 2 diabetes mellitus (T2DM), coronary artery disease (CAD), stroke, or incomplete covariate data, resulting in 1,406 exclusions. From the remaining cohort, we identified individuals who developed CAD (n = 382) or T2DM (n = 203) during the follow-up period, along with a randomly selected control group of 498 individuals. In comparison to the Malmö Diet and Cancer Study (MDC), the average age in MPP was higher, at 69.5 years, compared to 57.4 years in MDC. Unlike MDC, no dietary assessment was conducted within the MPP. Baseline plasma samples in MPP were collected using EDTA as an anticoagulant.

MOS

The Malmö Offspring Study (MOS) is a third cohort within Malmö, extending invitations to the children and grandchildren of participants from the MDC-CC to partake in this investigation, initiated in 2013 with the objective of identifying risk factors for chronic diseases (90). As in MPP, baseline, plasma sampling was done using EDTA as an anticoagulant. The dietary assessment methodology employed a 4-day online food diary, known as "Riksmaten 2010," alongside a brief food frequency questionnaire, both of which have undergone validation (91). For the purposes of Paper III, a subset of 1,538 participants was selected, characterized by the availability of plasma samples and data pertaining to adherence to a published health-conscious food pattern (HCFP) (92). The average age of these participants at the baseline assessment was 40 years.

CDI

In Paper IV, the Cilento Dietary Intervention (CDI) was designed to test the metabolic effects of a short dietary intervention. Sixty Swedish individuals were recruited by an ad in a newspaper. In collaboration with a travel agency, participants took part in a leisure travel from Sweden to Italy. During the stay in Italy, participants were served all meals, which were cooked with local ingredients and recipes to reflect the local MD. There was a selection of several dishes served during lunch and dinner. The intervention lasted for 6 days, and blood samples

(anticoagulated with EDTA plasma) were taken in the morning of the first day of the intervention, and on the morning after the intervention. There was no dietary sampling before, during or after the intervention.

Health-conscious food patterns

Data from previous publications by Ericson and co-authors was used in paper II and III. Ericson et al. used principal component analysis to derive food patterns in over 20000 individuals in MDC (47). The data driven pattern that explained the most variance (7%) was characterized by high intake of fibre-rich bread, breakfast cereals, fruit, vegetables, fish, and low-fat yoghurt and by low intake of low fibre bread. The pattern aligned with previous publications that health-conscious/prudent dietary patterns have shown to explain the most variance in dietary intake (93). The health-conscious food pattern (HCFP) was associated with lower incidence of T2DM, and CAD, and in men also ischemic stroke (47). This HCFP in MDC was used in paper II to discover metabolites that associated with it, and in paper III to compare with the model created there.

Ericson et al also identified data driven food patterns in MOS using similar methods (92). The food pattern that explained the most variance (also 7%) had similar loadings compared to the HCFP in MDC, despite the almost 30-year gap between data collection. This pattern was associated with a lower prevalence of prediabetes and higher levels of butyrate producing gut bacteria (92). The HCFP from MOS was used to discover the healthy dietary metabolic signature (HDMS) that was used in paper III and IV as described below.

Longitudinal data and validation of endpoints

In both MDC and MPP, incident disease data was procured by associating the Swedish personal number with various registers. Primarily, this was achieved through the Swedish Hospital Discharge Register and the Swedish Cause of Death Register (94).

AF: AF was characterized as persistent or recurring atrial fibrillation or atrial flutter. Within the MDC, this endpoint demonstrated an accuracy range of 95-97% (31).

Ischemic Stroke: Validation of ischemic stroke was conducted via computed tomography or autopsy. Recent validations in MDC indicated a diagnostic confirmation rate of 89% (95). For the purpose of paper I, only those ischemic stroke instances that either preceded or were concurrent (within a month) with an AF

diagnosis were taken into account to pinpoint potential cardioembolic stroke occurrences.

CAD: For CAD data, the research leaned on the Swedish Coronary Angiography and Angioplasty Register (SCAAR) and the Swedish classification system of surgical procedures. This system registers percutaneous coronary interventions and coronary artery bypass grafts. An MDC validation confirmed acute myocardial infarction in 96% of cases (95).

Cause of death: Upon a death occurrence in Sweden, the declaring physician is mandated to create a certificate containing the assumed primary and contributing causes of death which is dispatched to Socialstyrelsen. In paper II, both general mortality and CVD-mortality were utilized as endpoints. CVD-mortality is defined by codes 390–459 from ICD version 9 and I00 – I99 from ICD version 10, covering diseases of the circulatory system. A validation study by Bergwall et al. confirmed CVD mortality in 94% of instances (96).

T2DM: To comprehensively capture incident T2DM cases, the study integrated data from regional registers, the Swedish National Diabetes Register, the Swedish Hospital Discharge Register, and the Swedish Cause of Death Register as previously described (97).

Follow-up Dates: The concluding follow-up date was set at 2016-12-31 for MDC and 2013-12-31 for MPP.

Metabolite measurements

Liquid chromatography – mass spectrometry

In our laboratory, the assessment of metabolites was conducted using MS coupled to liquid chromatography (LC-MS) following a method developed by Dr Filip Ottosson (5). Papers I-III utilized a 1290 LC system, together with a 6550 mass spectrometer, Agilent Technologies Santa Clara, CA, USA and paper IV used an Agilent Technologies 1290 LC system, coupled with a 6546 mass spectrometer. Both used mass spectrometers were quadrupole-time-off-flight (QTOF) instruments. LC-MS separate analytes on a chromatographic column, ionize them at an ion source, and then separate them by a mass analyser before the abundance of ions is measured on a detector (5). This technique enables the quantification of relative metabolite abundance across different samples and aids in determining their molecular weight (57). In our procedure, the same sample preparation and LC-MS methodology were applied across all projects, with minor adjustments to the metabolite library and sample run order utilized for each specific study.

Sample Preparation

Plasma samples were stored at -80°C after an overnight fast and thawed on ice prior to analysis. For each sample, 120 µl of an extraction solution 80:20 methanol/water extraction solution was added to 20 µl of plasma. Thes samples were then incubated at 4°C with a 1250 rpm agitation for 1 hour followed by a 14,000g centrifugation for 15 min. The supernatant, containing the metabolites of interest was transferred to a glass vial for measurement (61). It is noteworthy that the choice of anticoagulant used during plasma collection can affect the analysis. In MOS, MPP and CDI, EDTA was used, versus citrate in MDC. Both anticoagulants are acceptable, they do exhibit different suppression effects on the measurement of specific metabolites (98).

Liquid Chromatography

The samples were separated using an Acquity UPLC BEH Amide column (1.7 µm, 2.1 × 100 mm; Waters Corporation, Milford, MA, USA) before being analysed in positive ion mode MS. The column is a hydrophilic interaction liquid chromatography column (HILIC) which separates polar metabolites.

Quality control

Samples were processed in batches of 180. To ensure analytic consistency, quality control (QC) samples were injected initially and subsequently every six to eight samples. These QC samples consisted of a pooled plasma sample unique to each cohort. Throughout the analysis of each cohort, the same QC sample was consistently used. This approach served two purposes, firstly QC samples were integral to the normalisation process, and to assess analytical variation, both of which are described more in detail in later section. The QC samples are needed to mitigate analytic drift, both within individual batch analysis and across different batches.

Inhouse library

The analyses in the project in this thesis used an in-house library which contained metabolites with annotated identities. Most metabolites in the in-house library had their annotation confirmed by comparison to a synthetic standard that was run through the same system using tandem mass spectrometry. For some metabolites, mostly acylcarnitines, the identity was putative based the mass-over-charge ratio (m/z), chromatographic retention times and spectral fragmentation and comparing with fragmentation data from public metabolite libraries (99).

The library is a “living document” continually revised and updated between each project to ensure the most accurate and up-to-date information is available. The Agilent Profinder B.06.00 software was used for integrating the peak areas of the metabolites.

Internal standard normalization

In paper 1 and 2, a part of the measured metabolites had corresponding synthetic standard labelled with heavy isotopes added to each sample in known concentrations, called internal standards. The internal standard share structure and polarity with the metabolite of interest and have the same retention time, but because of different isotopes (usually due to deuterium) they have a different m/z. By comparing the abundance of the internal standard and the metabolite of interest, a simple normalization can be done which removes the effect of variation in metabolite extraction and ionization efficiency, as the internal standard goes through the same process as the metabolite of interest.

LOESS curve normalization

For the metabolites without corresponding internal standards, a different normalisation method had to be applied, due to the inherent variability over time in LC-MS analyses. For each separate cohort, quality control samples were injected in pre-determined intervals in the whole cohort analysis. The quality control remained identical for each separate cohort. Using the QC samples, which have the same concentration of metabolites throughout the analysis, a correction curve with locally estimated scatterplot smoothing (LOESS) can be applied. With cubic splines, the correction curve can be interpolated to the entire analytical run (5, 100). In paper 1 and 2, all annotated metabolites without internal standards were normalized using this method. In paper III and IV, we switched to normalising all metabolites, including those with added synthetic internal standards, with this method.

The coefficient of variation (CV) for each metabolite was calculated after normalization, using:

$$CV = \frac{QC\ Standard\ deviation}{QC\ mean}.$$

The standard deviation and mean were calculated using measurements of the repeated injections of the above-described QC samples. Features with CV over 20% were excluded and for each metabolite, a CV curve was inspected visually to capture potential batch variation that had not been correctly normalized.

The technical intraclass correlations (ICCs) were calculated using:

$$ICC = 1 - \frac{\text{technical variance}}{\text{total variance}}.$$

The technical variance was quantified as the standard deviation in the QC samples while the total variance was quantified as the standard variation in all measured samples, including QC samples.

Statistical methods

Multiple test correction

With every independent statistical test conducted, the probability of encountering false positives increases. This is particularly evident in fields like metabolomics and other omics research, where a large number of statistical tests are common. Relying solely on the conventional significance threshold of $p < 0.05$ can in the cast of many tests lead to an increase in false positives (type 1 errors) (101).

To counteract this, multiple testing correction methods are commonly implemented in metabolomic studies. Paper II in this thesis employs the Bonferroni correction, where the significance level is divided by the total number of tests conducted. It is viewed as a rigorous method; its stringency can sometimes inflate the risk of false negatives (type 2 errors). This risk is accentuated in metabolomics due to the correlation amongst many plasma metabolites. Consequently, the performed statistical tests aren't truly independent, and Bonferroni might be too conservative.

In contrast, the false discovery rate (FDR) correction was adopted in papers I and IV to offer a balance between type 1 and type 2 errors, focusing on controlling the expected proportion of false discoveries (101). However, while FDR is less conservative than Bonferroni, it can still fail to detect genuine effects in the presence of a vast number of comparisons (type 2 errors).

An effective strategy to circumvent the pitfalls of multiple testing is to adopt a multivariate approach. This method capitalizes on the collinearity of plasma metabolites, presenting a comprehensive view of the data. Paper III integrates this holistic approach, offering a potentially more insightful analysis of the intricate relationships amongst metabolites. The overall summary of statistical analysis per paper is presented in table 1.

Table 1: A summary of the main statistical analysis conducted in paper I-IV.
AF: Atrial fibrillation. CDI: Cilento Dietary Intervention. CMD: Cardiometabolic disease, HCFP: Health-conscious food pattern. HDMS: Healthy dietary metabolic signature. MDC: Malmö Diet and Cancer. MOS: Malmö Offspring Study. MPP: Malmö Prentive Project. PLS: Partial Least Square regression.

	Paper I	**Paper II**	**Paper III**	**Paper IV**
1st Analysis				
Population	MDC	MDC	MOS	CDI
Statistical tool	Cox model	Linear regression	Partial least square regression	Paired T-test
Variables	Metabolites-Incident AF	Metabolites - HCFP	Metabolites - HCFP	HDMS-change
2nd Analysis				
Population	MDC	MDC	MDC	Paired T-test
Statistical tool	Cox model	Cox model	Cox model	Paired T-test
Variables	Caffeine quartiles - AF	Metabolites - CMD, mortality	HDMS - CMD	Single metabolite changes
3rd analysis				
Population			MPP	
Statistical tool			Logistic regression	
Variables			HDMS – CMD	

Metabolite-dietary pattern associations

Linear regression models

Two different approaches to discover metabolites associated with dietary patterns are applied, a linear regression in paper II and a partial least squares (PLS) model in paper III.

In paper II, linear regression models were applied in MDC to investigate the relationship between circulating metabolites and the previously published HCFP (47). The regression models were adjusted for multiple risk factors for CMD. While this has the potential to eliminate some metabolite-dietary patterns association confounded by risk factors, it is also an approach with the risk of type 2 errors. In combination with the Bonferroni multiple correction method, the risk of false positive finding should be low.

Partial Least Squares (PLS) model

For the development of Paper III and the subsequent Paper IV, a multivariate Partial Least Squares (PLS) analysis was employed (102), also referred to as Projection to Latent Structures. PLS operates as a supervised statistical method, designed to identify variations within a dataset (X) that most significantly explain variations in

another set (Y). This is achieved by projecting both X and Y datasets into new dimensions to establish linear regression models (102).

In the context of Paper III, the PLS model was trained on a training dataset comprising 80% of the participants from the Malmö Offspring Study (MOS) cohort with available data. This training utilized metabolomics data as the predictor variables (X) and the HCFP previously described (92) as the response variable (Y), employing the MixOmics package in R for statistical analysis (103). The model's performance was evaluated through 7-fold cross-validation, calculating both the predicted (Q2) and explained (R2) variations. The inclusion of components within the model was based on a predefined Q2 threshold of greater than 0.0975 (104). The model was then validated using the remaining 20% of the MOS cohort, predicting participants' healthy dietary scores and correlating these predictions with the HCFP scores via Pearson correlation. This PLS model was designated as the healthy dietary metabolic signature (HDMS).

For external validation, the HDMS was applied to the MDC cohort using metabolite data to assess the correlation between the HDMS (as a proxy for healthy dietary intake) and the HCFP within the MDC. Additionally, the HDMS was calculated for the Malmö Preventive Project (MPP) cohort using metabolite measurements.

Despite the existence of various multivariate analytical methods, each with their unique advantages and limitations, PLS demonstrates comparable predictive accuracy in metabolomics analyses relative to more contemporary approaches, such as random forests, support vector machines, and artificial neural networks (105).

Survival analysis

Kaplan Meier

In paper III and supplement of paper II, Kaplan Meier is applied as a survival analysis (106). The main strength of the model lies in the clear visualization of survival probabilities, which in our papers are quintile split. The main disadvantage is the inability to account for covariates (107).

Cox regression

In papers I, II, and III we employed the Cox Proportional Hazard models to analyse the associations between metabolite levels or the HDMS and the onset of diseases. The Cox proportional hazard model, also known as the “Cox model” is a commonly used statistical method to investigate the associations between predictor variables and survival time (108). These models were estimated using the “Survival” package in R (109). A crucial assumption of the Cox model is the proportionality of hazards, which means that the hazard ratios for the predictors should remain constant over time (108). This assumption is vital for the validity of the model's conclusions. To

ensure the proportionality assumption holds, Schoenfeld residuals was employed in all cases. This diagnostic method allowed us to test and visually assess the proportionality of hazards for all Cox models created.

In MPP, the case-cohort design made the use of a cox model inappropriate as the hazard ratio cannot be estimated with the selected population. Instead, logistic regression models were created to estimate the odds ratios between the HDMS and incident disease.

Adjustment strategies

In our survival analyses, we employed consistent covariate adjustment strategies across all analyses. Paper III exemplifies our approach, conducting three separate analyses for each endpoint and cohort to examine the relationship between the HDMS and incident disease. The first is an unadjusted analysis, testing the basic existence and statistical significance of the association, though any significance detected could be fully attributable to a confounder—a variable linked to both the independent variable and the endpoint, potentially creating a misleading association. To address this, a second analysis adjusting for confounders was performed; in paper III, these adjustments included smoking, age, sex, alcohol intake, and physical activity, selected using a directed acyclic graph. While these two analyses are often sufficient, many publications mandate a "full adjustment" analysis, accounting for numerous known risk factors. In paper III, a third analysis adjusted for both the confounders and additional variables (LDL- and HDL-cholesterol, glucose, triglycerides, BMI, systolic blood pressure, and anti-hypertensive medication), identified as potential mediators in the directed acyclic graph. These mediators might influence the relationship between the metabolic signature and incident disease; for example, a metabolic change from healthy eating could reduce blood pressure, subsequently lowering CAD incidence and mediating the HDMS-CAD association. However, full adjustment carries risks of misinterpretation and over-adjustment; a non-significant association in the third analysis doesn't invalidate the importance of the second model, which can still identify high-risk individuals.

CDI statistics

To test potential changes in clinical parameters, the HDMS or individual metabolites, paired T-test was conducted in the CDI. As the levels of these factors are paired to the same individual pre- and post-intervention values, confounder or mediator adjustments were not done.

Ethical considerations

The cohorts MPP (EPN Lund 2009/633), MDC (LU 51–90), MOS (DNR 2012/594) and CDI (Dnr 2019-06108) all had ethical approval, and all participants provided written informed consent.

In our project, we handle sensitive personal data related to health, genetics, and biometrics, such as metabolite data. All study participants are assigned a coded ID, with a separate storing of the decoding key. All data processing occurs on a protected server or computer. Only the lead researchers have access to the material. Data is reported only at the group level, ensuring individual participants cannot be identified. We believe that with these measures, we've significantly reduced the risks to participants concerning the handling of their sensitive personal information.

Consent has been obtained to research risk factors for a variety of diseases. Participants have also agreed to allow access to diagnostic registries and to store blood samples for future analyses. However, it's challenging for both the study participants and researchers to anticipate which analyses might become possible in the future, given the long duration over which the cohort is studied. In other projects, separate from mine, whole-genome sequencing has been performed, making the entire genetic makeup available to researchers. This data could be used to identify individuals.

Additionally, there exists a broader concern outside of the immediate research setting: the potential for the public to misinterpret or overinterpret our findings. Given the complexity in scientific research, there is always a possibility that the public, media outlets, or even other researchers, might draw broad conclusions from preliminary data points. As an example, paper II has been dubiously cited by news articles and videos that incorrectly claim that we have found a causative metabolite in CVD development and even recommending specific food to be consumed. To combat this, we must be careful what conclusions we make but also how we communicate our results with the public.

In conclusion, given the prospective benefits of our research—potentially uncovering insights that could lower the risks of CVD and T2DM—I believe that the advantages considerably surpass the potential risks faced by participants.

Summary of results

The complete results are present in the papers in the end of this thesis, the most important findings are summarised in this short section.

Paper I summary

Paper I, from the 3,770 MDC participants analysed, 650 developed incident AF over a median follow-up duration of 23.1 years. Utilizing sex- and age-adjusted Cox proportional hazards models, 15 metabolites were identified as being significantly associated with either an increased or decreased risk of AF development.

Following additional adjustments for variables including body mass index (BMI), baseline smoking status, systolic blood pressure, alcohol consumption, antihypertensive medication use, levels of N-terminal prohormone of brain natriuretic peptide (NT-proBNP), and the presence of diabetes mellitus, heart failure, and ischemic heart disease, 11 of these metabolites retained their statistical significance. Notably, caffeine exhibited the strongest association with an elevated risk of AF (hazard ratio (HR) for each standard deviation increase in caffeine, HR 1.17; 95% confidence interval (95% CI), 1.06–1.28, P=0.001), followed by acylcarnitine 16:1 (HR 1.13; 95% CI, 1.04-1.23, P=0.004) in the fully adjusted model. The metabolites most significantly associated with AF were primarily medium- and long-chain acylcarnitines.

Caffeine levels were undetectable in 16% of the samples, prompting further analysis. When including all samples and dividing them into quintiles, the fifth quintile showed a significant association with AF incidence compared to the first quintile in sex- and age-adjusted models. However, no significant differences were observed between the first quintile and the remaining quintiles in the fully adjusted models.

The investigation into the relationship between AF-associated metabolites and the incidence of cardioembolic stroke yielded non-significant results.

Paper II summary

Linear regression analyses identified ergothioneine, proline betaine, methylproline, acetylornithine, and pantothenic acid as metabolites positively associated with the health-conscious food pattern HCFP in MDC while urobilin was negatively associated, achieving statistical significance at a Bonferroni-corrected threshold of $P < 0.00044$.

In Cox proportional hazards models, adjusted for sex and age, exploring the relationship between metabolites associated with the HCFP and future CMD, CVD-mortality, and all-cause mortality, ergothioneine, acetylornithine, proline betaine, and methylproline demonstrated significant inverse associations with at least one CMD outcome. Conversely, urobilin was linked to an increased risk of type 2 diabetes mellitus (T2DM), cardiovascular mortality, and all-cause mortality.

Further analyses employing Cox proportional hazards models, which additionally adjusted for traditional risk factors (including age, sex, BMI, fasting glucose levels, LDL-cholesterol, HDL-cholesterol, triglycerides, systolic blood pressure, antihypertensive medication use, alcohol consumption, and smoking status), revealed that ergothioneine consistently showed a significant association with reduced incidences of CAD, CVD-mortality, and all-cause mortality. Acetylornithine was linked to decreased incidence of cardiovascular mortality, all-cause mortality, and stroke. Proline betaine was associated with a reduced incidence of CAD and all-cause mortality, while methylproline was significantly associated with a lower risk of all-cause mortality.

In these comprehensively adjusted models, urobilin continued to be significantly associated with an elevated risk of T2DM and all-cause mortality.

Paper III summary

In MOS, a PLS model was developed using the previously published HCFP as the dependent variable and metabolite data as predictors. This model exhibited a predictive ability (Q2) of 0.29 and an explained variance (R2) of 0.28 within the MOS training dataset. The correlation between the healthy dietary metabolic signature (HDMS) and the HCFP in the MOS validation subset was robust (Spearman's $\rho = 0.52$, 95% CI 0.44-0.60, $p < 0.0001$), with an R2 of 0.27 in the validation set.

The HDMS was characterized by positive loadings predominantly from metabolites previously associated with dietary intake, with the most significant positive contributors being beta-carotene, C4:OH-acylcarnitine, ergothioneine, homostachydrine, C13:0 acylcarnitine, acetylornithine, and hippurate. Conversely,

the most notable negative loadings included proline, dimethylguanidino valerate (DMGV), isoleucine, leucine, 4-trimethylaminoiobutonate, and urobilin.

Within MOS, the HDMS demonstrated positive correlations with the intake of fruits and berries, non-legume vegetables, tea, legumes, and nuts and seeds, and negative correlations with the consumption of low-fibre bread, sugar-sweetened beverages, and both red non-processed and processed meats.

When applied to MDC using participant metabolite levels, the HDMS showed a moderate correlation with the HCFP in MDC (Spearman's $\rho = 0.20$, 95% CI 0.16–0.24, $p < 0.0001$).

Using the HDMS in a Kaplan-Meier analysis revealed the HDMS to be significantly associated with decreased incidence of T2DM and CAD. As a further analysis, cox regression analysis was performed in MDC and logistic regression performed in MPP associating the HDMS with CAD and T2DM.

In MDC and MPP, the HDMS was associated with a lower incidence of T2DM and CAD in unadjusted models in MDC. In full adjustment, the HDMS remained significantly associated with T2DM in both MDC (hazard ratio 0.73 per 1SD, 95% CI 0.63-0.83, p = 3E-6) and MPP (odds ratio 0.70 per 1SD, 95% CI 0.55-0.88, p = 0.003). The previously significant negative associations between the HDMS and CAD was attenuated by full adjustment and was not significant in MDC nor MPP.

Paper IV summary

In the CDI, 59 individuals completed the intervention. The mean age was 69 years old, and the mean BMI was 24. There were no statistically significant differences in body weight, BMI, or blood pressure after the intervention. Most routine lab were unaffected, there was however a significant decrease in urea levels by 10mg/dl (95% CI -8.6 – 11.9, p =4E-16).

The intervention saw an overall large improvement in the HDMS, the change was statistically significant (mean SD increase 1.3, 95% CI 1.1-1.4, p = 6E-25) and each participant saw a nominal increase.

Out of 109 measured metabolites, 66 were significantly altered (p fdr < 0.05). Among the most significant increases were pipecolate, hippurate, caffeine, homostachydrine, acylcarnitine C11:0, beta-carotene, 7-methylguanine, acylcarnitine c14:1 and paraxanthine. The most significant decreases were seen in piperine and 3-methylhistidine.

Combined results paper II-IV

Out of the 5 metabolites found to be associated with the HCFP in paper II, ergothioneine, acetylornithine and proline betaine all had a large contribution to the HDMS in paper III (loading ≥ 0.15), while the contributions of methylproline and pantothenic acid were small (table 2). Urobilin, the only significantly negatively associated metabolite to the HCFP in paper II, had a large negative contribution to the HDMS (loading ≤ -0.15) (table 3).

There were 10 metabolites that either were significantly positively associated with the HCFP in paper II or had a loading ≥ 0.15 in the HDMS in paper III. Among these 10 metabolites, all but ergothioneine and methylproline had a significant increase with the intervention in paper IV (table 2). Beta-carotene and acylcarnitine C13:0 had both a large contribution to the HDMS and were significantly altered by the CDI, but they were not measured in the time of publication of paper II, so their associations with the HCFP in MDC are unknown.

In contrast, there were 6 metabolites that were either associated negatively with the HCFP in paper II or had a loading ≤ -0.15 in the HDMS in paper III. Out of these 6, only urobilin had a significant alteration in the CDI (-0.4 SD decrease, p fdr = 0.04). Proline, DMGV; isoleucine, 4-trimethylaminobutanoic acid and leucine were unaltered in paper IV (table 3).

Table 2 – Positively associated metabolites

Metabolites with either loading over 0.15 in the HDMS paper in III or significant positive association with the HCFP in paper II. AHEI: Alterantive Healthy Eating Index. CDI: Cilento Dietary Intervention. DASH: Dietary Advances to Stop Hypertension. HCFP:Health-Conscious food pattern. HEI: Healthy Eating Index. HDMS: Healthy Dietary Metabolic signature. MED: Medieterrenean Diet. NOR: Nordic Diet. SD: Standard deviation. Non sig: non significant. Significant associations are marked with either + as positive or – as negative.

Metabolite	Paper III HDMS loading	Paper II HCFP association	Paper IV change (SD, fdr p)	Known dietary pattern associations
Beta-carotene	0.36	NA	0.85, 5,5E-10	HEI, MED (110, 111)
Acylcarnitine C4:0-OH	0.24	Non sig	0.47, 8,5E-05	
Ergothioneine	0.23	+	-0.01, 1	HEI, AHEI, MED (38)
Homostachydrine	0.21	Non sig	1, 1,8E-13	HEI (73)
Acylcarnitine C13:0	0.21	NA	0.81, 9,9E-09	NOR (37)
Acetylornithine	0.20	+	0.68, 2,0E-10	HEI, DASH, NOR. MED (37, 38)
Hippurate	0.17	Non sig	1.26, 8,8E-17	AHEI, DASH, MED (38)
Proline betaine	0.15	+	0.48, 6,9E-04	DASH (38)
N-methylproline	0.07	+	0.28, 0.07	HEI, DASH (38)
Panothenic acid	0.03	+	0.30, 8E-4	DASH, HEI (37, 38)

Table 3 – Negativly associated metabolites

Metabolites with either loading under -0.15 in the HDMS in paper III or significant negative association with the HCFP in paper II. AHEI: Alternative Healthy Eating Index. CDI: Cilento Dietary Intervention. DASH: Dietary Advances to Stop Hypertension. HCFP:Health-Conscious food pattern. HEI: Healthy Eating Index. HDMS: Healthy Dietary Metabolic signature. MED: Medieterrenean Diet. NOR: Nordic Diet. SD: Standard deviation. Non sig: non significant. Significant associations are marked with either + as positive or – as negative

Metabolite	Paper III HDMS loading	Paper II HCFP association	Paper IV change (SD, fdr p)	Known dietary pattern associations
Proline	-0.20	Non sig	-0.04, 1	Negative: DASH HEI (37)
DMGV	-0.18	Non sig	0.18, 0,2	
Isoleucine	-0.17	Non sig	0.07, 0.8	Negative: HEI MED (37)
Leucine	-0.16	Non sig	0.09, 0,7	
4-Trimethyl-ammoniobutanoic acid	-0.15	Non sig	0.03, 1	
Urobilin	-0.15	-	-0.4 0,04	Negative: DASH (112) (urine)

Discussion

Summary

In this thesis, the author identified novel metabolites linked to the incidence of atrial fibrillation. Additionally, there is a detailed exploration of metabolites associated with a healthy dietary pattern observed in the Malmö diet and cancer study. These metabolites further exhibit a connection to the onset of CMD. The research also unveils a distinctive metabolic signature of a healthy diet, which is associated with reduced incidence of CMD across two separate cohorts. Significantly, a brief intervention with a Mediterranean diet was found to modify this healthy dietary metabolic signature across all study participants. Together, these insights underscore the intricate relationship between diet, metabolic markers, and cardiometabolic health.

Atrial fibrillation

Altered acylcarnitine metabolism

In paper I, 10 metabolites associated significantly with incident AF. Most of the significantly associated metabolites were acylcarnitines, surprisingly, caffeine levels had the most significant association to AF. In the time of the publication of paper I, three larger cohort studies investigating the link between plasma metabolites and incident atrial fibrillation had been conducted, in Atherosclerosis risk in communities (ARIC) (80), Framingham heart Study (82) and in Measurement to Understand Reclassification of Disease of Cabarrus/Kannapolis (MURDOCK) Horizon 1 CV Study (81).

In MURDOCK, using principal component analysis, the authors discovered several metabolite factors composed of medium- and long chained acylcarnitines to associate with incident AF (81). The population studied was patients that had undergone coronary angiography, and had a high incidence of AF, around 12% during 2.8 years of follow up. With our finding of association between acylcarnitines and AF, we replicate previous MURDOCK finding in a general population.

The ARIC study measured metabolites in 3922 participants and discovered 3 metabolites, glycochenodeoxycholate, acisoga, and pseudouridine to associate positively, and 1 (uridine) negatively associating with incident AF. The study measured some acylcarnitines and acylcarnitine C:12:0 was associated with a higher incident of AF in fully adjusted models, but after Bonferroni correction, the association was not significant. (80). Framingham Heart Study saw no plasma metabolites to be associated with atrial fibrillation during 10 years of follow-up in 2458 subjects (82). The study did not measure any of the metabolites found to associate with AF incidence in paper I.

Shortly after publication, another study in JAHA was published in 3 independent population-based samples, with a combined pool of 3950 individuals (113). A meta-analysis of two of the three cohorts revealed acylcarnitine C10:1, bilirubin and acylcarnitine C8:0 to be associated with AF. In validation in the third cohort, only acylcarnitine C10:1 remained significantly associated with AF in multivariate adjusted analysis. Acylcarnitine C10:1 was also related to size of the left atrium at echocardiography, but mendelian randomization analysis did not support a causal role for circulating acylcarnitine C10:1 in the development of incident AF (113).

Neither acylcarnitine C10:1 nor acylcarnitine C8:0 was associated with incident AF in paper I.

To summarize, if paper I is included, three out of five AF-metabolomic studies discovered some acylcarnitines or acylcarnitine based components to associate with incident AF. Out of the two others, one found weak and non-statistically significant associations with acylcarnitines and AF, and the last study did not measure any acylcarnitines. Together, these findings suggest that the finding between long- and medium chained acylcarnitines displayed in paper I could be generalizable. The study design in paper I is not designed to test causality, and a potential mechanism of action is speculative. Altered levels of acylcarnitines have been associated with increased risk of CVD and CVD-related mortality (114, 115). Some speculations can be made about accumulation of acylcarnitines in the cytoplasm could cause membrane instability by inhibiting the exchange of sodium and calcium ions in the sarcolemma, thus leading to arrhythmia (116). It has been shown that stressed myocardial cells change their metabolism from fatty acid oxidation to glycolysis, thus causing acylcarnitine build up (117). Also, the longed chained acylcarnitine C18:1 has been shown to increase arrhythmia susceptibility in the atrial myocardium (118). The authors welcome replication of our studies but also integrations of genetics to utilize mendelian randomization as a proxy for causality.

Plasma caffeine and atrial fibrillation

We were surprised at the strong association between plasma caffeine and incident AF in paper I. The previously mentioned AF study in ARIC measured caffeine, but there was no significant association found with AF incidence (80). In a larger meta-analysis, caffeine exposure has not been shown to associate with increased risk of AF, and caffeine has even been suggested to have a protective effect (119). The subsequent analysis that revealed quintile 5 to have higher incidence than quintile 1 also revealed a non-linear relationship between caffeine levels and AF. This is in direct contradiction to previous findings that high coffee consumption has been showed to associate with lower incident AF (120, 121).

Our finding suggests that there is a need for more studies with plasma levels of caffeine rather than intake levels to explain the associations in paper I. The paper did not include genetic data and, our first hypothesis was that "slow metabolisers" of caffeine have increased levels of caffeine and therefore increased AF incidence, but previous research have shown that addition of genetic polymorphism of the main metaboliser of caffeine, CYP1A2, does not improve the AF risk prediction (121).

Other AF-metabolite findings

Two metabolites that were associated with AF incidence in sex-and age adjusted analysis but non-significant after full adjustment were ergothioneine and n2n2-dimethylguanosine. As shown in paper II, ergothioneine is associated with a decreased risk of CAD, CVD-mortality, and overall mortality. The association with AF incidence could perhaps be explained by the strong correlation between ergothioneine and alcohol intake (shown in paper II), as alcohol intake is a well-known risk factor for the development of AF (122). We have previously shown n2n2-dimethylguanosine to associate with incident T2DM, which may explain the link between the metabolite and AF discovered in paper I (123).

In the published paper, we made an error and falsely cited that a previous report showed a positive association between beta-carotene and incident AF. Instead, the previous report showed that beta-carotene was associated with a decreased AF incidence, the same as in our study (124). The association could be explained by an association with beta carotene and heart failure, as heart failure has closely aligned risk factors and pathogenesis with AF (125, 126). One could speculate that the association between beta-carotene also could be confounded by healthy dietary intake as beta-carotene is an established marker for fruit and vegetable intake (91, 127). As discussed later, the HDMS in paper III had beta-carotene as the strongest loading for the model. The evidence regarding dietary changes for AF risk reduction is sparse, and healthy dietary biomarkers (ergothioneine and beta-carotene) show different directionality in our study (128). We propose that the application of healthy dietary biomarkers show greater potential in predicting CMD than in AF prediction.

Nutritional metabolite alterations

Dietary associated metabolites and their changes

Out of the 10 metabolites that either associated positively with the HCFP in MDC or had a loading over 0.15 in the HDMS in MOS all but one have been shown in other publications to be associated with healthy dietary patterns. Also, most of these 10 metabolites were significantly altered by the intervention in paper IV. Many of these metabolites exist in numerous dietary items and are non-specific biomarkers, with a few exceptions. While this makes them ineffective in determining the exact intake of specific food items, alone or together, they could be further developed as biomarkers of healthy diet, as exemplified in paper II and III. The associations between the dietary biomarkers and food groups are discussed below while the disease related associations found in paper II and III are discussed later.

Beta-carotene

Beta-carotene, a carotenoid, was the top loading in the healthy dietary metabolic signature and has been shown to associate with the Healthy Eating Index (HEI) and with MD (110, 111). It found in fruits and vegetables and is known to associate with their intake (91, 127). The high contribution to the HDMS is expected as the health-conscious food pattern consisted of high intake of fruits and vegetables, and so is the increase after the CDI.

Ergothioneine

Ergothioneine, which exists in many dietary sources like vegetables (129), has previously associated with HEI, Alternative Healthy Eating Index (aHEI), and MD (38, 73). Interestingly, ergothioneine is also associated with alcohol intake in paper II and other cohorts (130, 131), while being undetectable in wine and beer (132). The association between ergothioneine and alcohol intake that we replicated is counterintuitive and unexplained. Ergothioneine was one of the few metabolites with a positive association to the healthy dietary pattern that were not significantly altered by the CDI. A previous intervention with ergothioneine supplementation displayed a delay in plasma level increase by around 7 days, which might explain the non-change in ergothioneine (133).

Proline Betaine

Proline Betaine is also known as stachydrine is a known biomarker for citrus fruit intake (73, 134). It has also been associated with DASH (38). In our studies, it had the largest nominal association with the health-conscious dietary pattern in MDC, was the 13th most contributor to the healthy dietary metabolic signature and had a significant increase after the intervention. Citrus fruits were not a sub-group in the original health-conscious food pattern publications in MDC and MOS (47, 92).

Acylcarnitine C4:0-OH (3-Hydroxybutyrylcarnitine)

Acylcarnitine C4:0-OH is a metabolite that contributed positively to the metabolic signature and is known to be associated with 3-hydroxyacyl-CoA dehydrogenase deficiency (135) as well as hyperinsulinism (136) and hypoglycemia (137). In the context of dietary intake acylcarnitine C4:0-OH has to our knowledge not been associated with any specific dietary intake or dietary pattern before. We know that levels increase during fasting (138). It is surprising that it is the second highest loading in the metabolic signature, something that we cannot explain with previous knowledge.

Homostachydrine

Homostachydrine is the homologue of proline betaine and has previously been associated with the consumption of citrus fruit, legumes, rye bread and coffee (139). It has also been shown to associate with HEI (73). The contribution to the HDMS and the large change observed in the CDI is in line with previous findings.

Acetylornithine

Acetylornithine is a metabolite involved in the urea cycle and the arginine biosynthesis. It has been connected to vegetable intake, bean intake (140) and many different healthy food pattern, for example HEI, DASH, Nordic diet and MD (37, 38). As with the known associations with healthy dietary patterns, acetylornithine had consistent results with being associated to the healthy dietary pattern in paper II, having a large loading influence in the signature in paper 3 and being significantly increased in paper IV.

Hippurate

Hippurate (hippuric acid) is a carboxylic acid formed by the combination of benzoic acid and glycine. The benzoic acid is a conversion from phenols and consumption of polyphenolic containing foods such as fruit, berries, and coffee are linked to higher hippurate concentrations (127, 141). Hippurate has also been showed to associate with the AHEI, DASH and MD (38). The previous findings are consistent with the positive contribution that hippurate displayed in the metabolic signature. In paper II, the association with the healthy dietary pattern was just barely nonsignificant after Bonferroni adjustment.

Methylproline

Methylproline was also significantly connected to the healthy dietary pattern in MDC, and its contribution to the healthy dietary pattern in MOS was moderate/low. Like proline betaine, methylproline is associated with citrus fruit intake (142). Methylproline had been positively associated with HEI and DASH (38, 112) previously.

Pipecolate

Pipecolate, deserves as special mention even though the contribution to the HDMS was lower (0.10) than the other metabolites discussed. It has the greatest increase in the CDI and is a promising marker for dry bean intake (68, 143). As pulses are recommended a significant part of a healthy diet in the Nordic nutrition recommendations, pipecolate might be important as a marker for adherence to the recommendations (32).

Summary positive metabolites

Together, we have discovered many metabolites to be associated to health-conscious dietary patterns in MDC and MOS. Most of these metabolites are known or candidate biomarkers of other healthy dietary patterns. Their robustness seems to be true over decades, and all but ergothioneine shift considerably during the CDI. We argue for plasma metabolomics as a possible tool for estimating the healthiness of dietary intake, at least at a population level.

Metabolites with inverse HDMS associations

Urobilin

Urobilin stands out as the only negatively associated metabolites to the HCFP in MDC and was also a large negative contributor (loading -0.15) to the HDMS in MOS. Urobilin levels were also significantly reduced in the CDI. To our knowledge, there has been no study connecting urobilin in plasma with dietary intake. Urobilin is produced through gut microbiota metabolism and microbiota composition has been shown to influence the concentration of urobilin in faeces (144). It has been speculated that dietary changes could alter the microbiota composition in a way that influences the urobilin concentration, but this has been yet to be confirmed (144).

Other negatively associated metabolites

As of the other metabolites that had a significant (≤-0.15) negative loading in the HDMS, the previously shown connections to dietary patterns were not as obvious. Many of the metabolites demonstrating negative loadings, such as DMGV and the branched chain amino acids isoleucine and leucine, have been shown to associate with cardiometabolic disease development (77, 145, 146). With the exception of Urobilin, the principal negative metabolites were unaffected by the CDI. It suggests that a more extended period of intervention may be necessary to induce alterations in these pivotal metabolites. The CDI, as implemented in Project IV, led to a reduction in specific metabolites, notably 3-methylhistidine, recognized as an indicator of chicken consumption (68), and piperine, a candidate biomarker for processed meat intake (68). These metabolites however, did not associate with the HCFP in paper II or had a significant loading within the HDMS as reported in paper III.

Within the scope of this thesis, greater emphasis is placed on discussing metabolites that contribute positively to the HDMS, given that the potential for modulating their levels through dietary adjustments suggests significant implications for future research and applications, a topic that will be explored in subsequent sections.

Dietary related metabolites and cardiometabolic disease

All associations between dietary-associated metabolites and incident cardiometabolic disease presented in paper II were to the best of our knowledge novel at the time of publication.

Ergothioneine

The association between ergothioneine and reduced incidence of CAD, overall mortality and CVD-mortality was highlighted in paper II. While this specific finding has yet to be replicated, a RCT has been planned to test the possible causality of ergothioneine affecting cardiovascular risk factors (71). In animal studies conducted after publication of paper II, ergothioneine has shown to improve the clinical characteristics of preeclampsia (147) and myocardial remodelling and heart function after acute myocardial infarction (148). The potential causal effect of ergothioneine in CMD protection has been suggested to be via its anti-inflammatory effect (149). I happily encourage the clinical trial already started and advise caution to over-interpret our own and others' results as casual.

Proline Betaine

The found association between proline betaine, decreased CAD incidence, and decreased all-cause mortality was novel. Servillo et al investigated proline betaine addition to cells grown in glucotoxic environments and found an ameliorative effect (150). In vivo, proline betaine reduced infarct volume and alleviated neurological impairment in rats with induced stroke (151). However, there was no significant association between proline betaine and incident stroke in our material in MDC. Multiple factors could explain this non-finding, the most important one being that the protective effect in an animal study does not translate to any significant effect in humans in a real-world setting.

Urobilin

Urobilin has been associated with BMI and adiposity in obese men and women, and with insulin resistance in women (152). and heart failure (153). Our finding associating urobilin with higher incidence of T2DM is novel, and while some of the found association might be mediated via BMI, the analysis was still significant after risk factor adjustment. The cause of the association between T2DM and increased T2DM incidence remains unclear.

Non causality

The previous discussion highlights potential pathways through which dietary metabolites may influence the risk of CMD, yet it's crucial to recognize the limitations of our study design in establishing causality. These observations should be interpreted with caution, as they do not definitively suggest causal relationships. A plausible mechanism of action is important for justifying further research into the metabolite-disease interactions. Absence of such mechanisms should prompt a reconsideration of the research direction.

Mendelian randomization offers a viable approach for investigating the causative aspects of these associations without significant modifications to the study design. (154). This technique can provide insights into the potential causality underlying observed correlations. Nonetheless, RCTs remain the golden standard for assessing clinical relevance and establishing causality, particularly when investigating the effects of single metabolites on disease incidence. In such trials, the metabolite of interest could be administered as a supplement, allowing for a high level of scientific rigor, and blinding. However, when examining dietary patterns, the feasibility of blinding diminishes, complicating the trial design and increasing the scope of the study.

Large and long RCTs carry inherent risks to study participants and financial implications for the research group. Therefore, they should be based on robust observational data, evidence of potential causal pathways, and indications of causality from Mendelian randomization studies. Undertaking such trials without this foundational evidence could lead to inefficient use of resources and unnecessary exposure of participants to potential risks.

The HDMS and its' alteration

Proxy for healthy dietary intake

Cross-validation within the MOS training set and correlation analysis in the MOS validation set produced nearly identical results, suggesting generalizability of the model. The correlation with food groups were also similar with the HDMS and the HCFP. A moderate correlation was observed between the HDMS and the HCFP in MDC. The moderate correlation was expected, given the differences in dietary sampling methods used to construct the food pattern, the subtle variations in their loadings, and the fact that the plasma collection for the two studies was separated by over two decades. Associations between the HDMS and dietary intake could not be tested in MPP due to there being no dietary data available. Overall, our findings suggest that the HDMS could be used to estimate healthy dietary intake on a population level.

HDMS and incident T2DM and CAD

In both MPP and MDC, the HDMS was associated with a reduced incidence of CAD and T2DM. Those with a low metabolic signature demonstrated a more unfavourable risk profile for CMD. Intriguingly, even after adjusting for known risk factors, the relationship between the metabolic signature and a decreased T2DM risk remained substantial in both groups. Our approach shows promise in pinpointing populations more susceptible to T2DM, potentially due to poor dietary choices. Utilizing plasma metabolomics in the form of the HDMS as a proxy for healthy dietary intake in MDC and MPP could be considered a proof of concept for future study designs. A recent study by Tessier et al showed that metabolomics has the potential to both measure adherence to a healthy lifestyle and predict mortality as well as longevity (155).

The alteration of HDMS

Given the short nature of the CDI, and that the dietary intake differed for each participant as they had a selection of meals provided, the large alteration in HDMS was surprising. The study might resemble a real-life situation more than a tightly controlled study. Still, it would be valuable to have information about both the intake during the study, but also data on the habitual diet of the participant. Without that information, the number of dietary changes that happened are hard to realise. Some of the changes might come from the use of local ingredients, as nutrient composition in vegetables and fruit differ from country to country. That the top changes observed almost all were in metabolites known to associate with dietary intake is reassuring that the metabolite alterations stem from a change in dietary intake.

We see that the largest changes in the metabolites contributing most to the patterns is in the positive loading, i.e.," healthy" metabolites, that are already known biomarkers of healthy foods. The unchanged negative metabolites, especially the branched chain amino acids are known to be associated with negative health outcomes, and part of the association between the HDMS and incident CMD could be explained by their contribution. It might be that the intervention was too short to capture any changes in endogenous negative metabolite, and a different study design with longer intervention time might be beneficial.

A word of caution is that we must note that there is no evidence that alteration of the HDMS leads to changed risk for developing T2DM or CVD. Still, the survival models in paper III still significantly inversely associated with T2DM in both MDC and MPP after adjustment for potential mediators and confounders.

Largest Limitations

Many of the limitations of the papers in the included study are discussed in various sections in the methodology and discussion. For clarity, I will shortly discuss what I believe are our largest limitations.

Where is the microbiota and genetic data?

The human plasma metabolome exhibits considerable inter-individual variability, influenced significantly by dietary intake. However, the roles of the microbiome and genetics are also substantial and cannot be overlooked (156). To better understand the effect and to improve the prediction of disease risk, an integrated approach that encompasses all these factors could be considered. The associations between metabolites and disease risk identified in this thesis may be influenced by genetic variations or may be mediated through the composition of the gut microbiota (156). This underscores the complexity of metabolite-disease relationships and highlights the necessity of a multifaceted analysis to disentangle the contributions of diet, genetics, and the microbiome to the plasma metabolome and its association with disease risk. The thesis is therefore limited by the lack of integration of other omics- data other than metabolomics.

Dietary sampling in the CDI

The flexibility of the CDI, while allowing for a varied Mediterranean diet, came without stringent dietary tracking. This absence of precise dietary data limits our ability to directly link specific dietary changes to the observed shifts in the plasma metabolome. Despite these constraints, the positive outcomes—significant alterations in the dietary metabolome—highlight the potential of plasma metabolites to reflect dietary changes.

However, the lack of individual dietary records prevents a detailed analysis of how specific dietary components influence metabolomic profiles. This limitation underscores the need for future studies to incorporate detailed dietary tracking to better understand the relationship between diet and metabolome. Despite this, the study demonstrates the utility of metabolomic analysis in detecting dietary modifications, suggesting a promising direction for future dietary intervention research.

Transferability

As previously discussed, the HCFP in MDC and the HCFP in MOS are both created using data driven methods. The specificity of our metabolite library, confined to our laboratory's unique collection, and the reliance on relative concentration measurements, present significant challenges to the HDMS's applicability beyond our immediate datasets. Current constraints, including Swedish legal frameworks and ethical considerations, further restrict the open sharing of our data, complicating external validation efforts. There is a lot of potential improvements in the construction of the HDMS that would make the transferability to potentially validate our results easier. Some of these potential improvements and future challenges are discussed below in the “future perspectives” section.

Conclusion and further perspectives

Future perspectives

Given our findings of noticeable changes in each participant after a brief six-day period, we believe that HDMS, or comparable methodologies, could play a vital role in tracking the progress of dietary counselling. Witnessing metabolic changes in such a short timeframe can serve as a powerful motivator for individuals, encouraging them to stick more closely to recommended diets by visually demonstrating the benefits of their nutritional choices. With clear links established between HDMS results, CVD, and T2DM, leveraging this information in discussions could be particularly persuasive and impactful. Hence, the HDMS has the potential to act not just as an assessment tool but also as a source of motivation, closing the gap between dietary advice and concrete health results.

Integrating this approach into routine health check-ups could provide patients with a richer, more detailed understanding of their health, potentially leading to better engagement and improved outcomes over time. However, there are hurdles to overcome before this can become a reality.

To create a model adaptable to various settings, it would be necessary to develop a scoring system based on absolute metabolite concentrations rather than relative values. To implement this in a routine lab environment, simplification of the model by reduction of the number of metabolites involved would also be beneficial. Future development of the clinical implementation of the HDMS would have to balance the cost of analysis for each included metabolite against the loss of precision by excluding them from the measurement.

Furthermore, for practical application in a clinical environment, dietitians might find it easier to work with a score aligned with established nutritional guidelines, rather than one based on a dietary health-conscious pattern. I propose the development of a metabolite model that could assess and grade adherence to national nutritional recommendations, offering clear and actionable feedback to patients.

If such a scoring system could be consistently reproduced, show similar inverse associations with CMD as the HDMS, and demonstrate rapid alterations during interventions comparable to the HDMS, we could be on the brink of a significant transformation in clinical nutrition.

Conclusion

Through this thesis, we have identified significant associations between specific plasma metabolites and AF, suggesting a potential link between acylcarnitine metabolism and the risk of developing AF. Our investigation into plasma metabolites has also highlighted their role in relation to CMD, with certain metabolites inversely associated with the risk of these conditions. Notably, our analysis suggests that dietary habits, as mirrored by metabolic profiles, can influence the risk of CMD, with healthy dietary patterns being associated with a beneficial metabolic signature. Furthermore, our research indicates that even short-term adherence to a Mediterranean diet can lead to detectable metabolic changes.

These findings contribute to the growing body of evidence on the importance of metabolomics in understanding the relationship between diet and disease. They suggest that metabolic profiling could potentially enhance dietary assessment methods and provide insights into the effectiveness of dietary interventions. However, the implications of these findings for clinical practice and public health strategies require further investigation. Future research should aim to expand our understanding of the complex interactions between diet, metabolism, and disease risk, potentially leading to more personalized dietary recommendations and interventions.

Populärvetenskaplig sammanfattning

WHO rankar dåliga matvanor, tobaksrökning och högt blodtryck som de främsta riskfaktorerna för ohälsa och för tidig död i Sverige. De senaste decennierna har det skett stora framsteg i blodtrycksbehandling och rökningen minskar. Däremot har inte våra matvanor förbättrats i samma takt. Kaloririk mat är billig och lättillgänglig och resulterande övervikt ökar risken för både hjärt-kärlsjukdom och typ 2 diabetes, speciellt för dem med hög genetisk risk. Livsmedelsverket har vetenskapligt belagda rekommendationer för vad man ska äta för att minska risken för att bli sjuk, men i sociala medier, bokhandlar och tidningar florerar det flertalet råd med tveksam vetenskaplig grund.

Att pseudovetenskapliga kostråd är utbredda beror nog till stor del på de kunskapsluckor som finns om hur kosten påverkar vår hälsa samtidigt som intresset för vad man ska äta är stort. Orsakssamband mellan livsmedel och sjukdom är svåra att belägga med nuvarande metoder, delvis beroende på att det är svårt att med träffsäkerhet ta reda på vad någon äter. Insamlad data baserar sig på de enskilda individernas minne och noggrannhet i att fylla i en kostdagbok eller svara på ett formulär. En persons kost består dessutom av flera måltider, som i sin tur består av flera olika ingredienser. Att isolera hälsoeffekten av varje enskild komponent i kosten är i praktiken närmast omöjligt. Därför fokuserar rekommendationer från myndigheter på mer övergripande råd; att man ska ha ett mönster av hälsosamt ätande i stället för att fokusera på enskilda nyttiga ingredienser.

För att öka kunskapen om hur kosten påverkar hälsan har ett forskningsfält utvecklats där man mäter små nedbrytningsprodukter, metaboliter, i blodet. Det finns tusentals metaboliter i blodet, både från kroppens egen produktion, men också från nedbrytning av det vi äter. I genombrottstudier har man kunnat visa att redan flera decennier innan man utvecklar hjärt-kärlsjukdom eller typ 2 diabetes kan man mäta förändrade nivåer i metaboliterna som ett tecken på att kroppen inte mår bra. Den här kunskapen kan användas för att identifiera de personer som behöver extra insatser för att minska risken för att bli sjuk i typ 2 diabetes och hjärtkärlsjukdom. Vi forskare får med hjälp av metabolitdatan en ökad förståelse för hur dessa sjukdomar utvecklas.

Som författare till denna avhandling blev jag involverad i metabolitforskning 2016 som sommarjobb under läkarprogrammet. Jag har sedan dess ägnat mig åt forskning vid sidan av mina studier och mitt jobb som läkare. Jag började intressera mig för

kost och de kostrelaterade metaboliterna. Min framtidsvision är att man kommer kunna använda ett blodprov för att mäta hur nyttigt man äter och hur det påverkar ens framtida risk för typ 2 diabetes, hjärtinfarkt och för tidig död. Ett sådant blodprov hade kunnat möjliggöra individualiserade förebyggande åtgärder för att undvika framtida sjukdom.

I avhandlingen presenteras fyra arbeten baserade på metabolitmätningar. Metaboliterna är uppmätta i olika forskningsprojekt i Malmö där blodprover har hållits frysta, vissa sedan tidigt 90-tal. Proverna har sedan tinats och analyserats i en masspektrometer där nivåer av över 100 metaboliter per blodprov har kunnat mätas. Masspektrometern utnyttjar att molekylerna har en unik vikt och atomstruktur och kan med förprogrammerade inställningar mäta nivåerna av varje metabolit i ett blodprov. I avhandlingen presenteras mätningar från över 6000 olika blodprov.

Metaboliter och förmaksflimmer

Första arbetet undersöker kopplingen mellan förmaksflimmer och metaboliter. Förmaksflimmer är den vanligaste rytmrubbningen i hjärtat, och leder till en oregelbunden puls. Förutom sänkt fysisk prestationsförmåga kan förmaksflimmer leda till allvarliga konsekvenser som stroke och tidig utveckling av demens. I våra mätningar hittar vi kopplingar mellan en grupp molekyler och utvecklingen av förmaksflimmer. Våra fynd antyder att man kan mäta att cellerna i hjärtat är stressade långt innan man utvecklar oregelbunden puls och får diagnosen förmaksflimmer. Vår studie är en av få gjorda studier på förmaksflimmer, men de som är gjorda har liknande resultat.

Metaboliter och hälsosam kost

I det andra arbetet fördjupar sig avhandlingen i kopplingen mellan kost, metaboliter och risken för framtida sjukdom. Projektet bygger på information från över 3000 personer med blodprov tagna på 90-talet. I början av studien gjordes noggranna undersökningar om vad varje person åt. Tack vare dessa undersökningar har andra forskare från Lunds universitet kunnat visa att det som förklarar mest skillnad mellan hur vi äter är huruvida vi har ett nyttigt kostmönster eller ej. En person som till exempel dricker mycket sötade drycker äter sannolikt mindre grönsaker och mer rött kött. Man visade i studien att personerna som åt nyttigast hade en lägre risk att utveckla typ 2 diabetes och hjärtinfarkt.

Vi undersökte vilka metaboliter som var kopplade till detta mönster av hälsosamt ätande. Av de över hundra metaboliter vi mätte stack fem ut som starkt kopplade till det hälsosamma mönstret och en kopplad till ohälsosamt ätande. Vidare analyser visade att de flesta av de kostkopplade metaboliterna associerade till en minskad risk för framtida sjukdom. I den publicerade artikeln lyfts en metabolit, ergothioneine, fram som särskilt viktig. Ergothioneine var starkast kopplad till hälsosam kost, men också till en minskad risk för hjärtinfarkt och för tidig död. Ergothioneine finns i många olika typer av grönsaker. Studien var inte designad för

att ta reda på om intag av ergothioneine minskar risken för sjukdom. Det kan vara en generell markör för hälsosam kost, utan att ha någon egen effekt på framtida sjukdomsrisk. Lyckligtvis har flera andra projekt startats upp efter vår publikation för att undersöka närmre hur ergothioneinetillskott påverkar hälsan.

En modell för hälsosam kost

I det tredje projektet använder vi metabolitdata från tre stora studier, inkluderande över 5000 personer, för att skapa en metabolitmodell för hälsosamt ätande. Modellen är en slags maskininlärningsmodell, där datorn utnyttjar data från samtliga metabolitmätningar, och försöker gissa hur nyttigt någon äter. Gissningen visar sig vara ganska nära sanningen. Genom att gissa vad alla 5000 personerna har ätit kan vi senare se om gissningen är kopplad till ökad risk för framtida sjukdom.

Det visade sig att vår modell var starkt kopplad till både minskad risk för hjärtinfarkt och typ 2 diabetes. Kopplingen till typ 2 diabetes var oberoende av andra saker som vi vet ökar risken, som till exempel övervikt. Vi kunde alltså med hjälp av endast ett blodprov avgöra om någon åt nyttigt eller ej, och om de hade en förhöjd risk för typ 2 diabetes och hjärtinfarkt.

Metaboliterna kan ändras – en resa till Italien

I sista projektet gjordes en interventionsstudie. Deltagarna, ca 60 personer från Sverige, reste till Italien i en vecka. I Italien fick de bo i ett område som heter Cilento, där man har sett att chansen att leva ett långt och hälsosamt liv är högre, en så kallad blå zon. Deltagarna blev bjudna på italiensk mat i en vecka, då tidigare forskning visar att medelhavskost kan vara effektiv för förebyggande av hjärtkärlsjukdom. De fick lämna blodprover innan och efter studien, och vi mätte metaboliter och undersökte om dagarna i Italien hade förändrat något. Vi såg en förändring i över hälften av alla metaboliter, och den största förändringen sågs i de metaboliter som datorn i förra projektet hade markerat som extra viktiga för hälsosamt ätande. Vi kunde alltså visa en markant förbättring i metabolitmönstret, bara efter en vecka.

Framtiden

Vår förhoppning är att dessa fyra projekt ska integreras i den vetenskapliga kunskap som redan finns. Vi tror att det finns en stor potential i att mäta metaboliter för att kunna göra en uppskattning av hur nyttig ens kost är. Eftersom vi med den italienska studien visar att man snabbt kan ändra sina metaboliter genom att byta kost så tror vi att det finns en potential i att mäta metaboliter för att följa upp och motivera de som har fått kostråd av sjukvården. Vi planerar framtida studier där vi ska undersöka hur mycket kostrådgivning påverkar ens metaboliter, och om information om hur metabolitnivåerna ligger påverkar motivationen. Vår förhoppning är att färre personer ska behöva uppleva det lidande det innebär att bli sjuk i typ 2 diabetes och hjärtinfarkt och att färre dör i förtid.

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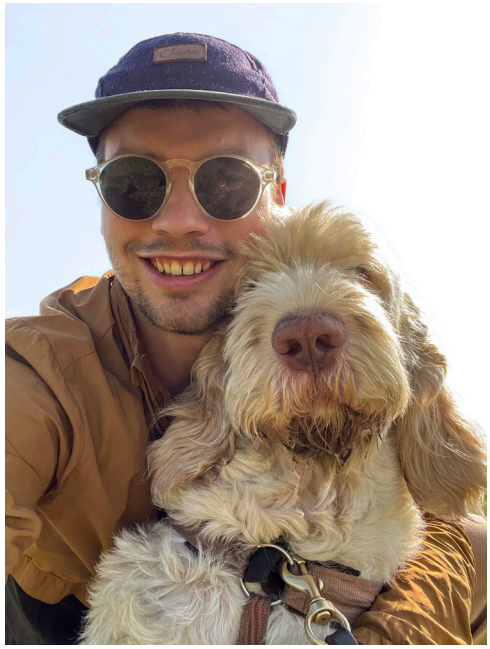

EINAR SMITH is a resident in internal medicine at Skåne University Hospital, Malmö. Engaged in research since 2016 under the tutoring of Olle Melander, Céline Fernandez and Filip Ottosson, his work delves into plasma metabolites and their impact on cardiometabolic diseases. His research particularly emphasizes the role of nutrition-related metabolites.

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