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Metabolomics and flux analysis by mass spectrometry

Investigations of factors associated with insulin secretion and prostate cancer risk

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Cover:
Dr. Francis William Aston with his third mass spectrograph, *ca* 1937. Aston was a prominent figure in the early days of mass spectrometry who experimentally proved the existence of stable isotopes and discovered no less than 212 naturally occurring isotopes during his career. He was awarded the Nobel Prize in Chemistry in 1922.

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Abstract

Two of the most common diseases associated with the Western lifestyle are type 2 diabetes (T2D) and prostate cancer (PCa). This is in large part due to decreased physical activity, surplus of dietary energy, and an ageing population. T2D and PCa are illnesses that develop during a long period of time and metabolic alterations associated with the diseases are not known in detail. Three studies investigating metabolic alterations associated with the risk of T2D and PCa were performed using liquid chromatography-high-resolution mass spectrometry-based metabolomics and stable isotope labelling.

A human intervention study investigating insulin secretion in response to breads varying in digestibility showed a decreased insulin response when rye bread was ingested in comparison with wheat bread, owing to a reduced rate of glucose appearance in blood, even though the blood glucose levels were unaffected.

Short-term incubation of human EndoC-βH1 cells with glucose and/or palmitic acid *in vitro* showed an increased Krebs cycle activity by glucose and an increased Krebs cycle flux by palmitate co-incubation, possibly mediated via pyruvate carboxylase. Proline containing carbon from the added glucose was also formed and excreted by the cells, uncovering a new fuel excess detoxification process.

An untargeted metabolomics study using 752 case-control pairs of fasting plasma samples matched by age, BMI, and sample storage time from the Northern Sweden Health and Disease Study was conducted in order to look for metabolites prospectively associated with prostate cancer risk. This is the largest prospective untargeted metabolomics study focused on PCa risk to date. Metabolites belonging to different chemical classes *i.a.* aromatic amino acids, several kinds of phospholipids, and free fatty acids were found to be positively associated with the risk of future PCa, while glucose was found to be inversely associated. Stratification by disease aggressiveness and by baseline age showed variations in which metabolites were associated with PCa risk, as well as showing variations in their degrees of association.

Keywords: Metabolomics, metabolic flux, LC-MS, liquid chromatography, mass spectrometry, stable isotopes, type 2 diabetes, prostate cancer, β-cells, NSHDS

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Metabolomik och flödesanalys med masspektrometri
Undersökningar av faktorer associerade med insulinutsöndring och prostatacancerrisk

Sammanfattning
Två av de vanligaste sjukdomarna förknippade med den västerländska livsstilen är typ 2-diabetes (T2D) och prostatacancer (PCa). Detta beror i hög grad på minskad fysisk aktivitet, ett överskott av energirik mat och en åldrande befolkning. T2D och PCa är sjukdomar som utvecklas under lång tid och ämnesomsättningsförändringar associerade med dessa sjukdomar är inte kända i detalj. Tre studier som undersökte ämnesomsättningsförändringar associerade med T2D och PCa utfördes genom metabolomik baserad på kombinerad vätskekromatografi-högupplöst masspektrometri och inmärkning med stabila isotoper.

En interventionsstudie som undersökte insulinfrisättning efter intag av bröd med olika smältbarhet påvisade ett minskat insulinsvar för rågbröd i jämförelse med vetebröd, till följd av att glukos från brödet nådde blodet i en långsammare takt. Glukosnivåerna i blodet var dock oförändrade.

Korttidsbehandling av mänskliga EndoC-βH1 celler med glukos och/eller palmitinsyra *in vitro* påvisade en ökad citronsyracykelaktivitet av glukos och ett ökat citronsyracykelflöde vid sambehandling, möjligtvis förmedlat via pyruvatkarboxylas. Prolin innehållande kolatomer från det tillsatta glukoset bildades även och utsöndrades av cellerna, vilket påvisar existensen av en ny process för avgiftning vid bränsleöverskott.

En oriktad metabolomikstudie på blodplasmaprover tagna efter fasta från kohorten Northern Sweden Health and Disease Study omfattande 752 fall-kontrollpar matchade efter ålder, BMI och provförvaringstid genomfördes för att leta efter metaboliter som uppvisar samband med risk för framtida prostatacancer. Detta är den största oriktade metabolomikstudien med fokus på risk för framtida prostatacancer hittills. Metaboliter tillhörande olika ämnesklasser, bland andra aromatiska aminosyror, flera slag av fosfolipider och fria fettsyror visades ha ett positivt samband med risk för framtida PCa, medan glukos uppvisade ett negativt samband. Stratifiering på sjukdomsaggressivitet och basålder påvisade variationer i graden av samband såväl som i vilka metaboliter som har samband med risk för framtida PCa.

Nyckelord: Metabolomik, metaboliskt flöde, LC-MS, vätskekromatografi, masspektrometri, stabila isotoper, typ 2-diabetes, prostatacancer, β-celler, NSHDS

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Dedication

Till mina farföräldrar
Ingen värderar utbildning högre än de som aldrig fick möjligheten

To my paternal grandparents
No one values education more than those who never got the opportunity

"What could I become with these surroundings? How could my character fail to be influenced by them? Is it to be wondered at if my thoughts were dazed, as my eyes were, when I came out into the natural light from the misty yellow rooms?"

Charles Dickens, *Great Expectations*

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Östman JR.*,** Müllner E., Eriksson J., Kristinsson H., Gustafsson J., Witthöft C., Bergsten P., Moazzami, AA. (2019). Glucose appearance rate rather than the blood glucose concentrations explains differences in postprandial insulin responses between wholemeal rye and refined wheat breads – Results from a cross-over meal study. *Molecular Nutrition & Food Research*, 63(7), e1800959.

II **Östman JR.*,** Müllner E., Moazzami AA. Distinct human β-cell line short-term fuel excess detoxification processes revealed by metabolomics and flux analysis. (Manuscript)

III **Östman JR.*,** Pinto RC., Ebbels TMD., Thysell E., Hallmans G., Moazzami AA. Identification of pre-diagnostic metabolites associated with prostate cancer risk by untargeted mass spectrometry-based metabolomics - A case-control study nested in the Northern Sweden Health and Disease study. (Manuscript)

* Corresponding author

The contribution of Johnny Östman to the papers included in this thesis was as follows:

I Participated in the planning of the analytical work. Performed the LC-MS and NMR analyses. Developed the data processing method. Performed the data processing and statistical analyses. Interpreted the findings and wrote the majority of the manuscript. Responsible for all correspondence with the journal. Corresponding author.

II Participated in the planning of the study. Participated in the cell culture experiments and sample acquisition. Performed the LC-MS and ELISA analyses, data processing and statistical analyses. Interpreted the findings together with co-authors and wrote the majority of the manuscript.

III Participated in the planning of the analytical work. Performed the sample preparation and LC-MS analyses. Performed the data processing and statistical analyses. Interpreted the findings and wrote the manuscript together with the co-authors.

Additional publications

IV Söder J.*, Wernersson S., Dicksved J., Hagman R., **Östman JR.,** Moazzami AA., Höglund K. (2019) Indication of metabolic inflexibility to food intake in spontaneously overweight Labrador Retriever dogs. *BMC Veterinary Research*, 15(1), 96.

V Kortesmäki E.*; **Östman JR.,** Meierjohann A., Brozinski J-M., Eklund P., Kronberg L. (2020) Occurrence of antibiotics in influent and effluent from three major wastewater treatment plants in Finland. *Environmental Toxicology and Chemistry*, 39(9), 1774–1789.

VI Gauchan Prasad D.*, Vélëz H., Acharya A., **Östman JR.,** Lundén K., Elfstrand M., Rosario García-Gil M. (2021) *Annulohypoxylon* sp. strain *MUS1*, an endophytic fungus isolated from *Taxus wallichiana* Zucc., produces taxol and other bioactive metabolites. *3 Biotech*. (Accepted).

VII Kiseleva, A., Nestor, G., **Östman JR.,** Krivoshapkin, P., Krivoshapkina, E.*, Seisenbaeva, GA., Kessler, VG.* (2020) Modulating by solvent treatment the surface properties of the *Linothele fallax* spiderweb – a potentially attractive wound healing material. (Submitted).

List of figures

Abbreviations

ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionisation
ATBC	Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study
ATP	Adenosine triphosphate
AUC	Area under the curve
BCE	Before common era
BMI	Body mass index
BSA	Bovine serum albumin
CE	Common era
CEM	Chain ejection model
CI	Chemical ionisation
CID	Collision induced dissociation
CLR	Conditional logistic regression
COPD	Chronic obstructive pulmonary disease
CRM	Charged-residue model
CV-ANOVA	Cross-validated analysis of variance
DoF	Distance-of-flight
EI	Electron ionisation
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
FAB	Fast atom bombardment
FBA	Flux balance analysis
FDR	False discovery rate
FTICR	Fourier-transform ion cyclotron resonance
FWHM	Full width at half maximum
GC	Gas chromatography
GC-MS	Hyphenated gas chromatography and mass spectrometry
GI	Glycaemic index

GIP	Gastric inhibitory polypeptide
GL	Glycaemic load
GLM	General linear model
GLMM	General linear mixed model
GLP-1	Glucagon-like peptide-1
GLUT2	Glucose transporter type 2
GLUT4	Glucose transporter type 4
GSIS	Glucose-stimulated insulin secretion
HDI	Human development index
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HILIC	Hydrophilic interaction chromatography
HPLC	High-performance liquid chromatograph(y)
ICR	Ion cyclotron resonance
IDF	International Diabetes Federation
IEM	Ion evaporation model
IFG	Impaired fasting glucose
IGF-I	Insulin-like growth factor I
IGT	Impaired glucose tolerance
KRBH	Krebs-Ringer bicarbonate HEPES buffer
LC	Liquid chromatography
LC-MS	Hyphenated liquid chromatography and mass spectrometry
LOESS	Locally estimated scatterplot smoothing
LPCa	Acyl-lysophosphatidylcholine
LSIMS	Liquid secondary ion mass spectrometry
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionisation
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NAD(P)(H)	(Reduced) nicotinamide adenine dinucleotide (phosphate)
NAFLD	Non-alcoholic fatty liver disease
NGT	Normal glucose tolerance
NMR	Nuclear magnetic resonance spectroscopy
NSHDS	Northern Sweden Health and Disease Study
OGTT	75 gram oral glucose tolerance test
OPLS(-DA)	Orthogonal projections to latent structures(-discriminant analysis)
OPLS-EP	Orthogonal projections to latent structures-effect projections
OR	Odds ratio
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCa	Prostate cancer

PCaa	Diacyl-phosphatidylcholine
PCae	Acyl-ether phosphatidylcholine
PCOS	Polycystic ovary syndrome
PD	^{252}Cf-plasma desorption
PDH	Pyruvate dehydrogenase
PEaa	Diacyl-phosphatidylethanolamine
PI3K	Phosphoinositide 3-kinase
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PLS(-DA)	Projections to latent structures(-discriminant analysis)
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homologue
PyC	Pyruvate carboxylase
QC	Quality control
QqToF	Quadrupole-collision cell-time-of-flight hybrid instrument
RaE	Rate of appearance
RF	Radio frequency
RP	Reversed phase
RT	Retention time
SCD-1	Stearoyl-CoA desaturase-1
SEM	Standard error of the mean
SREBP	Sterol regulatory element-binding protein
STD	Sexually transmitted disease
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes mellitus
ToF	Time-of-flight
UHPLC	Ultra-high performance liquid chromatograph(y)
UV-Vis	Ultraviolet-visible light
VIP	Variable importance in projection
WB	Refined wheat bread
WMR	Wholemeal rye bread
XIC	Extracted ion chromatogram

1 Introduction

The global transition towards a Western lifestyle with the accompanying physical inactivity and dietary energy surplus is having a profound impact on the health of people worldwide (Cordain *et al.* 2005). The prevalence of type 2 diabetes mellitus (T2D) in the modern world has taken epidemic proportions, with over 400 million people living with the disease and an incidence that is increasing even further (IDF 2019a).

Besides the westernisation of lifestyle and the negative effects associated with it, the global population is also ageing, which is associated with a decline in overall health (Beard *et al.* 2016). One of the maladies affecting the elderly to a higher degree is cancer (Balducci & Ershler 2005), and for men in Western Europe and America, prostate cancer is the most commonly diagnosed type (Bray *et al.* 2018).

T2D and prostate cancer are both illnesses that develop during a long period of time (Meigs *et al.* 2003; Loeb *et al.* 2012) and an inverse relationship between T2D and the risk of developing prostate cancer has been observed in several studies (Kasper *et al.* 2009; Fall *et al.* 2013; Tsilidis *et al.* 2015; Feng *et al.* 2020; Kincius *et al.* 2020; Peila & Rohan 2020). The biological mechanism underlying this relationship has however not been resolved.

Cell metabolism is a constantly self-regulating system and investigating metabolic alterations induced by external stimuli, such as dietary factors, can provide insight into disease aetiologies (Goodpaster & Sparks 2017). Alterations in metabolism might even be manifested many years prior to disease diagnosis and could be used for risk assessment. Much insight into metabolism can be gained by tracking the fate of the external stimulants as well as by investigating responses elicited by stimuli applied at different rates.

Investigating the nature of metabolic alterations in T2D and prostate cancer might not only yield insight into the disease mechanisms and development but can additionally give insight into metabolic mechanisms underlying their inverse relationship.

2 Aims

The objective of this thesis was to investigate metabolic factors associated with insulin secretion and prostate cancer risk by metabolomics and flux analysis using liquid chromatography-mass spectrometry and stable isotope labelling. In order to attain this objective, the following core questions were addressed:

- Can differences in glucose rate of appearance in blood explain insulin response deviations (Paper I)?

- What metabolic alterations can be seen in a human β-cell line upon hyperglycaemic and hyperlipidaemic treatment (Paper II)?

- What metabolites are prospectively associated with the risk of prostate cancer (Paper III)?

3 Background

"If thou examinest a man for illness in his cardia, whose body shrinks, being altogether bewitched; if thou examinest him and dost not find disease in the belly, but the ḥnwt of the body is like pjt, then thou shalt say to him: it is a decay of thy inside. Thou shalt prepare for him remedies against it: ground dragon's blood from Elephantine, flax-seed, ḏ³rt, are boiled with oil and honey and eaten by the man for 4 mornings, so that his thirst perishes and the decay of his inside may be expelled."

-*Ebers Papyrus* 197 (39, 7–39, 12), as translated by Bendix Ebbell, 1937

3.1 Type 2 diabetes

3.1.1 The nature of type 2 diabetes

Diabetes mellitus, or chronic high blood glucose, is one of the most common ailments affecting humans today, having taken epidemic proportions. According to the International Diabetes Federation's (IDF) 2019 report, 463.0 million adults aged 20–79, corresponding to 9.3% of this age group, are estimated to have diabetes and the prevalence is still expected to increase (IDF 2019a).

Diabetes has been known to humanity for a long time, although the massive incidence observed today is a modern phenomenon. The Ebers Papyrus (Unknown author 1517 BCE), which is the World's oldest preserved medical document, contains passages concerning excess or plentiful urine, which might be diabetic in nature (Nunn 2002), as well as the paragraph presented *vide supra* describing the combination of thirst and possibly halitosis. The earliest certain mention of diabetes is from Vedic India, where the physicians Sushruta (सुश्रुत) and Charaka (चरक) describe patients with sweet urine, excessive urination, extreme thirst, and halitosis (Frank 1957). They also described the hereditary

component of diabetes and differentiated between what we now know as type 1 and type 2 diabetes as lean and corpulent patients sharing similar symptoms, but requiring different treatments (Frank 1957). The knowledge of diabetes seems to have reached Europe around the start of the common era (CE) at the latest, with Aretaeus (Ἀρεταῖος) the Cappadocian making an early and clear description of diabetes in Europe in the late 1st–early 2nd century CE (Aretaeus the Cappadocian 1856)

The glucose concentration in the blood of healthy individuals is kept within a narrow range by interaction of the pancreatic hormones glucagon, which stimulates liver glucose production and promotes breakdown of glycogen to glucose, and insulin which counteracts glucagon in the liver and stimulates glucose uptake by muscles and adipocytes. Both insulin and glucagon are produced in the pancreatic islets of Langerhans, which consist of cells producing different endocrine hormones. The insulin-producing β-cells constitute the majority of the islet, with the glucagon-producing α-cells making up most of the remaining islet volume (Campbell & Newgard 2021). The role of the β-cell is to sense the level of nutrients, mainly glucose, in circulation and respond by releasing an accurate amount of insulin in order to counteract hyperglycaemia, but without risking hypoglycaemia. This sensing is dependent on rapid equilibration of glucose concentrations over the β-cell membrane, via the action of glucose transporters type 1–3 (Thorens 2015). Due to limited fuel storage capacity, the excess glucose needs to be diverted from the β-cell by the means of various pathways for it to be able to maintain its function and be able to respond to a changing glucose environment (Mugabo *et al.* 2017). An excess of fuel substrates can however have detrimental effects on the β-cells, eventually resulting in β-cell death, which is associated with the development of T2D. The biochemistry underlying this effect has not yet been fully elucidated (Prentki *et al.* 2020).

There are three main forms of diabetes mellitus: Type 1 diabetes (T1D), type 2 diabetes, and gestational diabetes. T1D is due to acute loss of insulin-producing β-cells and subsequent inability to maintain normoglycaemia due to an autoimmune reaction (IDF 2019b). Insulin supplementation therapy is thus necessary to avoid complications. T2D accounts for 90% of all diabetes cases and is characterised by increased blood glucose concentrations due to loss of peripheral insulin sensitivity and decreased insulin production (IDF 2019b). T2D is however heterogenous and has recently been stratified into five subgroups with different disease progression and complication risks (Ahlqvist *et al.* 2018). The scope of this thesis concerns T2D, the pathophysiology of which will be discussed in further depth. Gestational diabetes is a diabetic state that affects women during pregnancy. It is similar to T2D, in the respect that insulin

secretion and response is maintained, but impaired. Gestational diabetes affects up to 25% of pregnancies (Sacks *et al.* 2012), but normal glucose tolerance is usually restored post-delivery (IDF 2019b).

Acute complications related to diabetes mellitus can arise from both hypo- and hyperglycaemia as well as from lack of insulin. Hypoglycaemia results in shortage of energy available for cells, mainly affecting the glucose-dependent neurons (Frier 2014), while hyperglycaemia results in high blood osmolarity dehydrating the body (Kitabchi *et al.* 2009). Lack of insulin on the other hand results in the body transitioning into a hyperglycaemic and ketotic state called diabetic ketoacidosis, where the dehydration caused by the hyperglycaemia and the release of ketone bodies to support the body's energy requirements decreases the blood pH to critical levels by depletion of the blood bicarbonate pool (Kitabchi *et al.* 2009). The decrease in blood pH disrupts cell energetics since the activity of the glycolytic enzyme 6-phosphofructokinase is decreased (Ui 1966). Any of these three acute complications can result in diabetic coma and death if left untreated.

The chronically increased blood glucose level associated with diabetes mellitus leads to various chronic complications as well, which can be divided into macro- and microvascular complications. The macrovascular complications are mediated through atherosclerosis, which is increased in diabetes mellitus patients due to increased platelet adhesion, impaired fibrinolysis, and hypercoagulability (Fowler 2008). Increased degree of atherosclerosis is in turn associated with increased risk of cardiovascular disease such as myocardial infarction or stroke. The major microvascular complications associated with diabetes mellitus are retinopathy, nephropathy, and peripheral neuropathy due to thickening of capillary and glomerular basement membranes (Forbes & Cooper 2013). The reason why hyperglycaemia causes microvascular complications is still unknown, but sugar alcohol accumulation, glycoprotein excess, and oxidative stress have been suggested to be involved (Fowler 2008). For a thorough review on the mechanisms of diabetes complications, see the review by Forbes & Cooper (Forbes & Cooper 2013).

Management of T2D strives towards keeping the blood glucose concentration within a normal range by stimulating the peripheral insulin sensitivity or by stimulating insulin secretion. T2D patients commonly retain their insulin production to some degree, since the disease develops progressively over a long period of time with gradual decreases in insulin production and increases in fasting glucose. T2D is associated with obesity, sedentary lifestyle, and unhealthy diet (Basu *et al.* 2013) and lifestyle changes can increase peripheral insulin sensitivity and efficiently reverse and stop people with prediabetes from developing T2D (Tuomilehto *et al.* 2001).

Pharmaceuticals are a common form of T2D therapy, with the gluconeogenesis-suppressing metformin being the most common. Alternative pharmacological agents include *i.a.* sulfonylureas, which trigger insulin secretion, and glucagon-like peptide-1 (GLP-1) analogues, which decrease gluconeogenesis via glucagon suppression (Tan *et al.* 2019).

In manifested T2D the insulin production is compromised or even lost (Sun & Han 2020), and insulin supplementation therapy is then required as in cases of T1D. Before the discovery of insulin (Banting & Best 1922), dietary treatments comprised of high-fat, low-carbohydrate diets were the single treatments which could reduce the risk of hyperglycaemia (Westman *et al.* 2006). T2D is considered incurable once manifested, but bariatric surgery has shown to significantly improve glycaemic control in obese patients (Mingrone *et al.* 2012; Schauer *et al.* 2012) and in a majority of cases even results in T2D remission for periods of at least five years (Arterburn *et al.* 2013). The mechanism underlying this has not been elucidated, but incretins probably play an important part (Karras *et al.* 2019). Islet transplantation (Shapiro *et al.* 2017) and gene therapy for restoration of β-cell mass and induced insulin production show promise for treatment of both T1D and T2D in the future (Tan *et al.* 2019). Astonishingly, a recent report claims to have treated type 2 diabetic mice via modulation of hepatic mitochondria redox homeostasis using static electric and magnetic fields, opening up a whole new angle on T2D treatment (Carter *et al.* 2020).

3.1.2 Progression to type 2 diabetes

In contrast to T1D, T2D is a disease that develops over a long period of time, with T2D being the end point of a gradual decrease in glucose tolerance and insulin production capacity. The transition from normal glucose tolerance (NGT) to T2D goes through a prediabetic state that can be divided into two subgroups called impaired fasting glucose (IFG) and impaired glucose tolerance (IGT).

In IFG the fasting glucose of a patient is elevated, while the insulin response post-meal is still normal. A patient is diagnosed with IFG when the fasting glucose is 6.1–7.0 mmol/L, with the blood sugar levels after a two-hour 75 g oral glucose tolerance test (OGTT) being below 7.8 mmol/L according to IDF criteria (IDF 2019b). Blood glucose concentration is used as a surrogate marker of diabetic status due to ease of measurement in comparison with measuring the insulin sensitivity *per se*, which can be done by hyperinsulinaemic-euglycaemic clamping (Heise *et al.* 2016). When clamping is used for insulin sensitivity

measurements a glucose uptake limit of 37.3 µmol/(kg×min) has been suggested for diagnosis of insulin resistance (ter Horst *et al.* 2015).

A patient is diagnosed with IGT when the blood glucose does not decrease fast enough after an OGTT, showing a blood glucose level between 7.8 and 11 mmol/L, while having a fasting glucose below 7.0 mmol/L. In case the fasting glucose levels are higher than 7.0 mmol/L or the post-OGTT glucose level surpasses 11.1 mmol/L then the patient is diagnosed with T2D (IDF 2019b). T2D can also be diagnosed based on a measurement of glycated haemoglobin exceeding 48 mmol/mol or a random plasma glucose test exceeding 11.1 mmol/L (IDF 2019b). Many countries in the world, *i.a.* Japan, Germany, and the United States, have adopted mg/dL as the standard unit of glucose concentration instead of the mmol/L presented herein. The diagnostic criteria and different diabetic and prediabetic states are summarised in Figure 1.

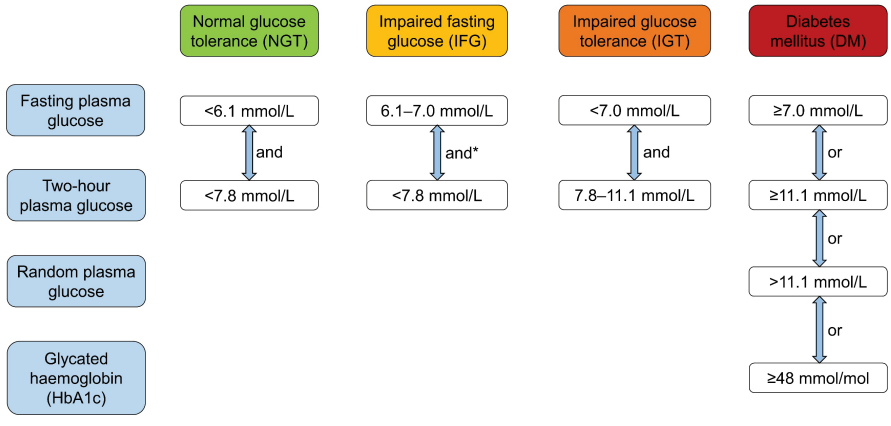

Figure 1. Diagnostic criteria for diabetes and prediabetes according to the International Diabetes Federation (IDF 2019b). The two-hour plasma glucose is measured after a 75 g oral glucose tolerance test. *If measured.

What is driving the progression towards T2D? The progression from IFG or IGT to T2D takes many years (Meigs *et al.* 2003), with a gradually increasing degree of insulin resistance (Gastaldelli *et al.* 2004). Insulin resistance means that cells do not respond normally to an incoming insulin signal. The main cell types that are dependent on insulin signalling for glucose uptake are myocytes and adipocytes, due to the presence of the insulin dependent glucose transporter type 4 (GLUT4) on their surfaces (Huang & Czech 2007). Hepatocytes mainly express glucose transporter type 2 (GLUT2), which is insulin independent (Thorens 2015). As insulin signalling is impaired the entry of glucose into myocytes and adipocytes is disrupted, which keeps plasma glucose levels elevated, resulting in hyperglycaemia (Samuel & Shulman 2016). The insulin

resistance which might follow from chronic hyperglycaemia leads to compensatory increased insulin production by the β-cells, which in turn leads to hyperinsulinaemia. Apart from the hyperglycaemia, T2D patients also exhibit elevated concentrations of circulating free fatty acids (Sobczak *et al.* 2019), which also affects the insulin secretion (Paolisso *et al.* 1995).

The underlying biochemistry on how β-cells handle these circulating nutrients and the nutrient excess has been investigated (Farfari *et al.* 2000; Nolan *et al.* 2006; Lorenz *et al.* 2013; El-Azzouny *et al.* 2014; Mugabo *et al.* 2016; Mugabo *et al.* 2017; Malinowski *et al.* 2020; Spégel & Mulder 2020), but is not yet fully understood (Prentki *et al.* 2020). Most of the previous work in this field has been performed on the murine β-cell line INS-1 832/13 (Spégel & Mulder 2020), but a human β-cell line has recently gained popularity (Ravassard *et al.* 2011; Tsonkova *et al.* 2018; Scharfmann *et al.* 2019) and has the potential to give new insights into how β-cells handle nutrient excess. The metabolic effect of excess glucose on this cell line has been investigated (Andersson *et al.* 2015), but data on the combination of excess glucose and fatty acids are still lacking.

Interestingly, insulin resistance is not only associated with T2D, but with several other diseases as well, such as non-alcoholic fatty liver disease (NAFLD) (Watt *et al.* 2019) and polycystic ovary syndrome (PCOS) (Moghetti & Tosi 2021). Additionally, insulin resistance is associated with low testosterone levels (Ding *et al.* 2006; Grossmann *et al.* 2008) and decreased testosterone production (Pitteloud *et al.* 2005) in men. This association connects insulin resistance to prostate cancer, and patients undergoing androgen deprivation therapy have an increased risk of developing insulin resistance and T2D (Di Sebastiano *et al.* 2018).

The causes underlying insulin resistance have not been fully elucidated, although extensively studied. Insulin resistance is complex and many factors, *e.g.* changes in fatty acid uptake causing lipid accumulation in liver and muscle due to lipoprotein lipase overexpression (Kim *et al.* 2001) and palmitoylation or mutations of GLUT4 in adipocytes (Ren *et al.* 2013; Song *et al.* 2013) have been shown to be involved. The regulating and underlying mechanisms of insulin resistance have been recently and thoroughly reviewed (Petersen & Shulman 2018; Yang *et al.* 2018b; Yaribeygi *et al.* 2019).

Obesity, and abdominal obesity in particular, is a risk factor for developing insulin resistance (Després *et al.* 1990; Lakka *et al.* 2002; Carnethon *et al.* 2003). Obesity is associated with increased insulin secretion in people without insulin resistance as well (van Vliet *et al.* 2020), indicating that increased insulin levels may have a role in the development of insulin resistance. Furthermore, artificial augmentation of plasma insulin induces insulin resistance in lean, healthy people (Rizza *et al.* 1985; Iozzo *et al.* 2001). It is thus not unrealistic to hypothesise the

existence of a link between diet and insulin resistance. Keeping insulin levels chronically elevated by frequent eating or consumption of food items eliciting secretion of large amounts of insulin ought to have a negative impact on insulin sensitivity and might eventually predispose to T2D.

3.1.3 Type 2 diabetes epidemiology and risk factors

T2D is a multifactorial disease with genetic, lifestyle, socioeconomical, psychosocial, environmental, and dietary risk factors being involved in the disease development and progression (Kolb & Martin 2017; Bellou *et al.* 2018; Zheng *et al.* 2018). T2D is a polygenic disease, with a large scale genome-wide association study showing associations of 126 variants at four loci (Fuchsberger *et al.* 2016). Some of these variants might be enriched in some populations, with non-whites being more susceptible to developing T2D at lower body mass index (BMI) (Lee *et al.* 2011; Paul *et al.* 2017). Ethnicity is thus an important genetic T2D risk factor and family history is an additional risk factor of importance, further accentuating the role of hereditary features in T2D (Almgren *et al.* 2011).

T2D is however not primarily a genetic disease, but a lifestyle disease. This is evident from comparative studies of the native American Pima people in Arizona and Mexico, which are genetically similar, but where the Mexican Pima lead more traditional lives and have a much lower T2D prevalence (Schulz *et al.* 2006). The Pima in the United States transitioned into a more sedentary lifestyle than the Mexican Pima during the 20th century and physical activity is strongly associated with decreased T2D risk (Rockette-Wagner *et al.* 2015; Dempsey *et al.* 2016). Sedentary lives are also associated with increases in BMI and adiposity, which are strong T2D risk factors (Hu *et al.* 2001).

Transition to a Western lifestyle is associated with a change in diet and due to the nature of T2D and its relation to blood glucose and insulin response, it is clear that associations between dietary factors and T2D exist. Foodstuff with high glycaemic index (GI) and high glycaemic load (GL) are nutritional components which are clearly associated with an increased T2D risk (Bhupathiraju *et al.* 2014), suggestively due to concomitant high insulin loads (Willett *et al.* 2002). Unsurprisingly, increased intake of whole grain (low GI/GL) has been found to be inversely associated with T2D risk (Aune *et al.* 2013) and the glycaemic quality of food might be more important than the quantity for T2D prevention (Schulze & Hu 2004). Other specific food groups exhibiting positive associations with T2D risk are *i.a.* sugar-sweetened beverages, processed and unprocessed red meat, and fruit juices (Neuenschwander *et al.* 2019). On the other hand coffee, tea, olive oil, and moderate alcohol consumption are negatively associated with an increased T2D

risk (Neuenschwander *et al.* 2019). For further insight into the role of diet and different dietary components in T2D incidence, see the recent meta-analysis review by Neuenschwander *et al.* (Neuenschwander *et al.* 2019). The gut microbiome is shaped by the diet, and the role of the gut microbiome has gained recent interest (Ussar *et al.* 2015) with several bacterial genera having been found to be positively associated with T2D risk (Yang *et al.* 2018a).

Associations between the levels of various metabolites in blood and future T2D risk have also been found by so-called molecular epidemiology for *e.g.* phosphatidylcholines (Floegel *et al.* 2013), sphingolipids (Chew *et al.* 2019), branched chain amino acids (Park *et al.* 2018; Ahola-Olli *et al.* 2019), and oleic acid (Park *et al.* 2018). Whether these alterations stem from genetic, dietary, or other differences is unclear, but knowledge of differences in blood metabolite levels can nevertheless provide insight into risk factors associated with a disease and contribute to the understanding of the metabolic alterations associated with disease progression.

Various other risk factors have been identified as well. Low socioeconomic status is positively associated with T2D risk (Agardh *et al.* 2011), probably mediated through BMI (Sacerdote *et al.* 2012) and an increased propensity of adopting an unfavourable lifestyle. Use of drugs such as statins and β-blockers have a positive association with T2D risk (Anyanwagu *et al.* 2016). Smoking, both active and passive, is also associated with increased T2D risk (Pan *et al.* 2015), maybe as a part of an otherwise unfavourable lifestyle or by smoke-induced insulin resistance (Reaven & Tsao 2003). Air pollution has also been identified as a T2D risk factor, with both particulate matter and NO_2 showing positive associations with T2D risk (Rao *et al.* 2015). Psychosocial factors such as stress (Novak *et al.* 2013) and depression (Graham *et al.* 2020) are associated with T2D risk as well. Gender differences in T2D risk have been thoroughly investigated due to the involvement of sex hormones in energy metabolism and body composition. Pregnancy is associated with additional T2D risk, since gestational diabetes affects up to 25% of all pregnant mothers (Sacks *et al.* 2012), and past gestational diabetes is associated with a seven times higher risk of developing future T2D (Bellamy *et al.* 2009). The role of gender in diabetes risk and complications has been extensively reviewed by Kautzky-Willer *et al.* (Kautzky-Willer *et al.* 2016).

3.2 Prostate cancer

3.2.1 The nature of prostate cancer

The prostate was first described in 1536 (Massa 1536) as follows: "… The neck of the bladder rests upon two glandular carunculae, one on the right and one on the left. … Through these carunculae also pass the seminal vessels, which carry the sperm to the duct of the penis." (Massa 1975). Since the Renaissance we have fortunately gained more detailed knowledge about this organ. The prostate is an exocrine gland in the male reproductive system, the main function of which is secretion of prostatic fluid. The prostatic fluid is a major constituent of the seminal fluid and contains factors that control the ejaculation, impact sperm motility, and effects the semen liquefication process (Verze *et al.* 2016). The prostate is made up of epithelial cells anchored to stromal tissue. The epithelium is histologically divided into three different zones: the peripheral zone (70% of the prostate mass), the central zone (25%), and the transition zone (5%) (McNeal 1988). The prostate epithelium is made up of an array of five different cell types (Henry *et al.* 2018), and it is still unknown from which cell types prostate cancer predominantly arise (Xin 2019).

Prostate cancer is a very common type of cancer, globally being the second most diagnosed cancer in men and the fourth most common cancer overall. Countries with higher human development index (HDI) however have higher incidences of prostate cancer and in many parts of the world, *i.a.* Northern and Western Europe and North and South America, it is the most common cancer in men (Bray *et al.* 2018). Prostate cancer can arise in any of the three histological zones, but the vast majority of cancers originate from the peripheral zone (McNeal *et al.* 1988). Central zone cancers are rare, but aggressive due to a high rate of spreading (Cohen *et al.* 2008). Screening programmes based on prostate-specific antigen (PSA) levels have been tested and they show a degree of mortality decrease, but the screening methods are associated with high risks of overdiagnosis (Schröder *et al.* 2009; Schröder *et al.* 2014) and are currently not in widespread practice.

The prognosis of men with diagnosed prostate cancer and what therapy to use is dependent on the stage of the tumour. The main staging tool nowadays is the tumour-nodes-metastasis system, which groups the cancer into four main groups based on primary tumour size, presence of regional or distant metastases, serum PSA levels, and the tumour's morphological grade score (Fine 2018). A higher grouping level is associated with a more advanced disease. The morphological grade is determined histologically according to the so-called Gleason scale, where decreased glandular differentiation of tumour cells is

associated with a higher score and increased disease severity (Gleason *et al.* 1974; Epstein *et al.* 2016).

Treatment of prostate cancer is dependent on how far the disease has advanced. Prostate cancer treatment is associated with risks of decreased quality of life due to post-treatment complications (Resnick *et al.* 2013). Active surveillance with the goal of initiating therapy only when considered necessary is thus recommended for low-risk cancer cases (Romero-Otero *et al.* 2016). Focal and ablative therapies including *i.a.* cryotherapy, laser ablation, radiofrequency ablation, and high-intensity ultrasound have shown promising results in treatment of intermediate to low-risk prostate cancer (Perera *et al.* 2016; Evans 2018).

For more advanced prostate cancer another palette of treatments is employed, including surgery, radiation therapy, androgen deprivation therapy, or combinations thereof (Teo *et al.* 2019). The most common surgical therapy for treatment of prostate cancer is radical prostatectomy, *i.e.* surgical removal of the prostate, seminal vesicles, and the vas deferens. Radical prostatectomy comes with high risks of unfavourable side effects, such as urinary incontinence and erectile dysfunction, and has thus decreased in use as alternative forms of therapy have matured. It is nevertheless an important intervention for high-risk prostate cancer patients, as it efficiently prevents future metastasis (Costello 2020).

Radiation therapy for prostate cancer treatment is categorised into two main groups of techniques: External beam radiotherapy and brachytherapy. External beam radiotherapy uses an external source of X-ray photons or protons to produce a beam, which is focused on the tumour in order to destroy it. Proton beam therapy utilises more focused beams and does not damage healthy tissue surrounding the cancerous cells to the same extent as photon beams (Podder *et al.* 2018). Brachytherapy involves insertion of radioactive so-called seeds directly into the tumour via long needles. Low-dose brachytherapy involves permanent insertion of seeds containing radioactive isotopes, *e.g.* ^{125}I and ^{103}Pd, which will deliver a more localised dose of radiation for a longer period. High-dose brachytherapy with temporary insertion of radioactive sources can also be performed. Brachytherapy is more suited for low- and intermediate grade tumour treatment, while beam radiotherapy is used for all tumour types. The two types of radiotherapy can also be used in conjunction for treatment of high-grade tumours (Podder *et al.* 2018).

Prostate cancer development is dependent on androgen signalling (Huggins & Hodges 1941) and reducing the levels of circulating androgens through so-called androgen deprivation therapy thus proves an effective prostate cancer treatment. A decrease in androgen levels can be achieved either by surgical

castration or by chemical castration via antiandrogen treatment. Prostate cancer however tends to transform into an androgen-independent or castration resistant state after some time of androgen deprivation therapy. It is thus usually not applied as a treatment for non-advanced prostate cancer (Melissa & Douglas 2017). Androgen deprivation therapy is also associated with a range of adverse effects, which might need additional medical intervention (Nguyen *et al.* 2015).

If the cancer has advanced further to a metastatic or castration/androgen resistant state, chemotherapy is commonly used in addition to the previously mentioned therapies, with a range of pharmaceutical agents being in clinical use (Teo *et al.* 2019). Immunotherapy strategies are currently under active research and might play an important role in treatment of advanced prostate cancer in the future (Boettcher *et al.* 2019; Cha *et al.* 2020).

3.2.2 Prostate cancer origins and risk factors

Prostate cancer is, akin to T2D, a disease that develops over a long period of time and it is frequently discovered in prostate autopsies of men without diagnosed prostate cancer (Haas *et al.* 2007; Bell *et al.* 2015).

Several phenotype changes have been identified in pre-malignant prostate epithelial cells. These are named proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN) and show some similarities to prostate cancer, indicative of a progression towards prostate cancer. PIA is characterised by oxidative stress, inflammation, and increased epithelial cell proliferation. PIA can be a result of prostate inflammation and is associated with prostatitis (Vral *et al.* 2012). Whether PIA can progress to PIN is yet unknown. PIN is phenotypically closer to prostate cancer than PIA, and is often observed in close proximity to cancerous cells (Eminaga *et al.* 2013). There is however no definite proof that PIN progresses to prostate cancer. The molecular and cell biological origin of prostate cancer has been recently reviewed in detail by Packer and Maitland (Packer & Maitland 2016).

Prostate cancer risk factors have recently been reviewed, both overall (Pernar *et al.* 2018) and with focus on diet and lifestyle (Wilson & Mucci 2019). An array of overall prostate cancer risk factors has been identified. Age is an important risk factor and autopsy studies have shown that prostate cancer prevalence almost doubles by each decade of age (Bell *et al.* 2015). Several genetically predisposed risk factors have also been identified. Men of African descent show increased prostate cancer prevalence compared to men of European or Asian ancestry (Rebbeck & Haas 2014), taller men show higher prostate cancer risk (Zuccolo *et al.* 2008), and shared genetic factors also contribute to a higher risk (Hemminki & Czene 2002; Mucci *et al.* 2016). A

history of sexually transmitted infections, primarily gonorrhoea, additionally increases the risk of future prostate cancer (Caini *et al.* 2014), which might be related to the inflammatory response and resulting PIN. Some occupational risk factors have also been identified *i.a.* pesticide exposure and shift work (Krstev & Knutsson 2019). In addition to the overall risk factors, a number of risk factors that are associated with an increased risk of advanced or lethal prostate cancer have been identified, *i.a.* obesity, height (Discacciati *et al.* 2012; Perez-Cornago *et al.* 2017), and smoking (Kenfield *et al.* 2011; Islami *et al.* 2014). For further insight into risk factors with weak or unestablished effects, *i.a.* vitamin D, vitamin E, and selenium, see the aforementioned review articles (Pernar *et al.* 2018; Wilson & Mucci 2019).

A dietary component that has been investigated extensively with regard to its association with prostate cancer risk is dairy. Positive associations between dietary milk (both total and low fat) and cheese with prostate cancer risk have been found in a meta-analysis of 32 studies (Aune *et al.* 2015). Dairy is rich in animal fat, but since the associations are independent of milk fat content, this indicates a different component being involved in the risk increase. Dietary calcium, total calcium, and dairy calcium were all positively associated with prostate cancer risk, but supplemental calcium and non-dairy calcium was not (Aune *et al.* 2015). Insulin-like growth factor I (IGF-I) concentrations in blood are positively associated with both dairy consumption (Qin *et al.* 2009), height (Baron *et al.* 2015), and with prostate cancer risk (Travis *et al.* 2016; Watts *et al.* 2020). IGF-I has been shown to inhibit prostate apoptosis in prostate cancer cell lines, transgenic mice, and xenografts, which is highly indicative of this mechanism playing a part in the association between dairy intake and prostate cancer risk (Wang *et al.* 2019d). The risk factors are presented in Figure 2. Disentangling of causality from epidemiological risk factors is however not a trivial issue, and degrees of uncertainty still exist in this regard (Kazmi *et al.* 2020).

In addition to the risk factors presented above, molecular epidemiology has identified alterations in the concentrations of various blood metabolites, mainly lipid and amino acid related metabolites, as being associated with the risk of developing future prostate cancer (Chavarro *et al.* 2007; Johansson *et al.* 2009; Mondul *et al.* 2014; Mondul *et al.* 2015; Huang *et al.* 2016; Kühn *et al.* 2016; Huang *et al.* 2017; Schmidt *et al.* 2017; Röhnisch *et al.* 2020; Schmidt *et al.* 2020). Alterations in glycerophospholipid levels have been observed in studies across several cohorts (Mondul *et al.* 2015; Röhnisch *et al.* 2020; Schmidt *et al.* 2020), and a possible link between glucose- and fat metabolism and prostate cancer is thus not farfetched.

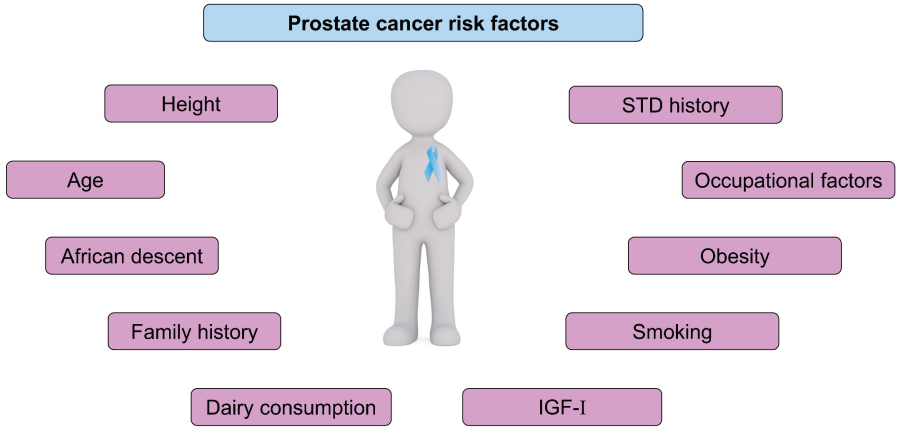

Figure 2. Compilation of risk factors positively associated with overall or aggressive prostate cancer. STD=sexually transmitted disease, IGF-I=insulin-like growth factor I.

Prospective molecular epidemiology studies with lethal prostate cancer as endpoint have also been performed, finding associations between fatal prostate cancer and alterations in steroid metabolites, fatty acid metabolism metabolites, and γ-glutamyl amino acids (Huang *et al.* 2019a; Huang *et al.* 2019b; Wang *et al.* 2021). Additionally, ketone bodies, acyl carnitines, and dicarboxylic acids have been found associated with increased risk of metastatic disease (Huang *et al.* 2019a).

Most of the previous studies have been utilising a so-called targeted metabolomics approach, where a pre-specified set of metabolites have been analysed. An untargeted metabolomics approach, which is analysing all signals generated from a sample in an unbiased way might provide novel insight into the progression to prostate cancer and identify possible new prospective biomarkers. Metabolomics techniques are discussed in more detail in chapter 3.3.

3.2.3 Epidemiological connection to type 2 diabetes

An inverse relationship between T2D and prostate cancer risk has been observed in several cohort studies (Kasper *et al.* 2009; Fall *et al.* 2013; Tsilidis *et al.* 2015; Feng *et al.* 2020; Kincius *et al.* 2020; Peila & Rohan 2020) as well as in several meta-analyses (Kasper & Giovannucci 2006; Zhang *et al.* 2012b; Bansal *et al.* 2013; Jian Gang *et al.* 2015). The inverse relationship is also consistent across populations (Waters *et al.* 2009). In the rarer cases of T2D and prostate cancer comorbidity, mortality has however been shown to increase (Cai *et al.* 2015; Lee *et al.* 2016).

The biology underlying these relationships has been extensively researched but is still unsettled. One clue for unravelling this mechanism has come from the common T2D drug metformin, which reduces hepatic gluconeogenesis, increases liver insulin sensitivity (Rena *et al.* 2017), and additionally shows anticarcinogenic effects in prostate cancer (Whitburn *et al.* 2017). The anticancer effect of metformin appears to be two-sided, with both a direct effect on the cancer cells and an indirect effect propagated through the action of metformin on other tissues. The direct effect involves inhibition of complex I in the electron transport chain, resulting in energy stress (Whitburn *et al.* 2017). The indirect effect is through the effect of metformin on gluconeogenesis. The resulting decrease in circulating insulin downregulates the insulin-phosphoinositide 3-kinase (PI3K) signalling, which is known to be associated with a range of cancers (Hopkins *et al.* 2020). For further insight into the prostate cancer treatment potential of metformin and the molecular targets involved, see the recent review by Zaidi *et al.* (Zaidi *et al.* 2019).

Another potential relationship seems to be through the action of sex hormones and androgen receptor signalling, since diabetic men show lower levels of testosterone (Ding *et al.* 2006; Grossmann *et al.* 2008). Androgens have large impact on both healthy prostate function (Verze *et al.* 2016) and on prostate cancer cell proliferation (Huggins & Hodges 1941; Pandini *et al.* 2005); the rationale behind androgen deprivation therapy. Crosstalk between different signalling pathways is however common in prostate cancer (Yan & Huang 2019) and a reciprocal association between PI3K and androgen receptor signalling has been shown in prostate cancer with the common deletion of the tumour suppressor phosphatase and tensin homologue (PTEN) gene (Carver *et al.* 2011). PTEN is also known to be associated with T2D (Li *et al.* 2020).

Use of statins have been associated with a decreased risk of advanced prostate cancer (Bansal *et al.* 2012) which might be related to the impact of statins on tumour androgen uptake (Harshman *et al.* 2015). Interestingly, statin use is positively associated with T2D risk (Anyanwagu *et al.* 2016), as mentioned previously.

Surprisingly, a recent study by Häggström *et al.* showed no association between T2D and prostate cancer risk after removal of the effect of informative censoring due to competing risks (Häggström *et al.* 2018) and suggests that the extensively observed inverse association might be due to so-called “false protectivity” (Rowley *et al.* 2017). The biological and biochemical relations presented *vide supra* are nevertheless indicative of an existing interplay between T2D and prostate cancer, which clearly warrants further investigation.

3.3 Metabolomics

Technological advances in analytical chemistry and bioinformatics during the last decades have made analysis of biological systems go from foci on individual parts towards taking a holistic approach to the system, which is referred to as systems biology. Holistic investigations of different subsystems have given rise to new subfields of biology, collectively known as '-omics', distinguished by the type of biomolecule under examination. The earliest field was genomics (McKusick & Ruddle 1987), which has been successful in *i.a.* sequencing the human genome (Lander *et al.* 2001). Moving downstream with the flow of biological information we thus find the fields of transcriptomics, proteomics, and more recently metabolomics. The metabolome can be further sub-grouped by chemical similarities into *e.g.* the lipidome. The metabolome is thus closest to the actual cellular function or phenotype and provides a picture of the real-time status of an organism, since genes can be silenced, and enzymes inactivated. The metabolome is influenced by intrinsic factors such as age and genotype, but it is also highly influenced by extrinsic factors such as diet or medications, and perturbations to the metabolic state by such extrinsic factors can be followed by analysing the metabolome. A schematic figure of metabolomics' place in the hierarchy of systems biology is presented in Figure 3.

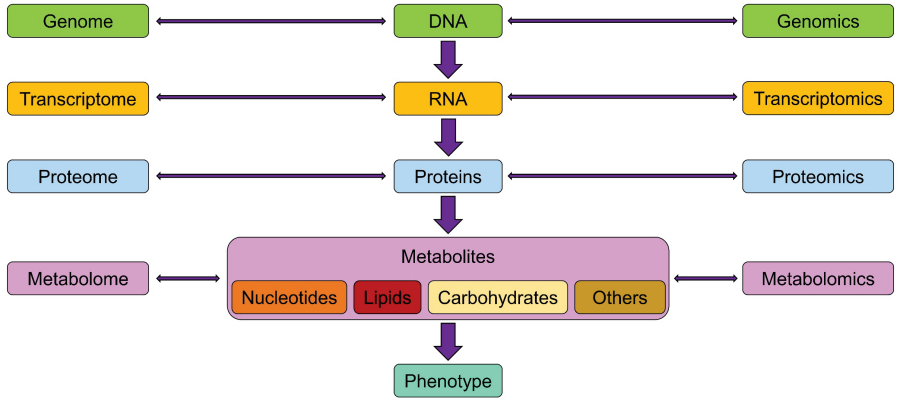

Figure 3. The hierarchy of systems biology.

The metabolome consists of the array of chemical species with a molecular mass<1,500 Da at any given time point, and the full human metabolome is expected to consist of at least 150,000 different chemical species (Markley *et al.* 2017). The metabolome is defined by the sample type, with *e.g.* plasma, serum, and urine having their own distinct metabolomes. The metabolome of *e.g.* a foodstuff, so-called foodomics can also be defined, which is an important tool in *i.a.* combatting food adulteration (Lioupi *et al.* 2020).

Due to the intrinsic chemical variation of the metabolome, analysing it comes with significant difficulties in comparison to the other fields of -omics, which are all based on analysis of similar types of biomolecules, *e.g.* RNA or proteins. Metabolomics on the other hand requires sampling procedures and methods of data collection that encompass biomolecules of a broad range of sizes, polarities, and function.

3.3.1 Metabolomic analysis

The first step in all kinds of analyses is obtaining the sample. For metabolomics analyses this step requires discretion since a snapshot of the metabolism in the sample at the time of sampling is desired. Effective quenching of the metabolic activity is an essential step in metabolomics sample collection so as not to allow enzymes to continue exerting catalytic effects. A common way of achieving this is by extracting metabolites with cold organic solvents, which both denaturates enzymes and slows down the rates of non-enzymatic reactions (Jang *et al.* 2018). For tissue samples, a clamp cooled by liquid nitrogen can be used to achieve homogenous quenching throughout the tissue sample (Wollenberger *et al.* 1960).

Metabolomics data collection by definition requires simultaneous analysis of many metabolites and the two main analytical techniques employed for metabolomics analyses are mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). MS and NMR both come with their respective strengths and disadvantages. MS is more sensitive, needs less sample, can detect more metabolites, and is more available than NMR. NMR on the other hand shows higher reproducibility, is non-destructive, and is intrinsically quantitative. For more elaborative comparisons of analytical techniques for metabolomics purposes, the reviews by Wishart or Zhang *et al.* can be consulted (Zhang *et al.* 2012a; Wishart 2016).

NMR is an analytical method based on the magnetic moments of atomic nuclei. When in a static magnetic field, the nuclei will align themselves to the magnetic field and precess by their Larmor frequencies. If the alignment is perturbed by applying radiofrequency pulses matching their Larmor frequencies, the nuclei will start to resonate. As the nuclei return to their equilibrium state, they emit exponentially declining radiofrequency radiation, which can be detected. Nuclei with different chemical environments will have slightly different Larmor frequencies and the so-called free induction decay signal can be Fourier transformed to provide the composite resonance frequencies, which form an NMR spectrum, where the frequencies are transformed to a relative scale (chemical shift). The spectrum generated will contain quantitative and qualitative information on the contents of the sample. The most common nuclei

analysed in NMR metabolomics are ^{1}H, ^{13}C, ^{15}N, and ^{31}P. The use of NMR in metabolomics has recently been reviewed (Emwas *et al.* 2019).

Whereas NMR analysis can be performed on samples with minor sample preparation, MS analyses are often preceded by a separation step such as gas chromatography (GC) or liquid chromatography (LC), through so called hyphenated chromatography-mass spectrometry (GC-MS/LC-MS). Mass spectrometry will be discussed in detail in a subsequent chapter, as will liquid chromatography. In brief, molecules in the samples are ionised and the mass-to-charge ratios (*m/z*) of the formed ions are measured, from which the molecular mass of the constituents of the sample can be determined.

3.3.2 Targeted metabolomics

Metabolomics analyses are commonly performed in either a targeted or an untargeted manner (Gorrochategui *et al.* 2016). Targeted metabolomics quantifies a set of pre-chosen and pre-identified metabolites, resulting in a list of metabolite concentrations, which can be used for further statistical analysis and for analysis of differences in metabolic pathway activities. Semi-targeted approaches where a predefined set of metabolite intensities rather than concentrations are determined can also be performed. The targeted approach offers higher precision and is useful for *a priori* hypotheses testing but is limited when it comes to generation of new hypotheses. Due to its specific and quantitative nature, the targeted metabolomics approach requires less data processing than untargeted metabolomics and is often performed by vendor software. Targeted LC-MS metabolomics is often performed using triple quadrupole mass spectrometers which offer very high sensitivity and specificity. Commercial kit solutions that offer quantitation of up to 630 metabolites (MxP® Quant 500 Kit, Biocrates Life Sciences AG, Innsbruck, Austria) have gained increased popularity and a variant with 188 metabolites has been successfully used in large metabolomic studies (Röhnisch *et al.* 2020; Schmidt *et al.* 2020).

Mass spectrometers based on quadrupoles have low *m/z*-resolution, meaning that for any measured *m/z*-value there are many possible permutations of atoms that can give rise to that *m/z*-value. To achieve a high specificity, the ions are fragmented in a second quadrupole working as a collision cell and the fragments are analysed in a third quadrupole coupled in tandem. The combination of parent ion *m/z* and characteristic fragment ion *m/z*-values can then be used for identification with high specificity. A major downside to this is that data can be collected only for a predefined set of *m/z*-values. High-resolution mass spectrometers can measure *m/z*-values with high precision, greatly limiting the number of possible atom permutations which can give rise to the measured *m/z*-

value. Due to this, high-resolution mass spectrometers can also be used for targeted metabolomics (Glauser *et al.* 2016). A great benefit of using high-resolution mass spectrometers for targeted applications is that the same instrument can be used for both targeted and untargeted applications and that the data collected for a targeted analysis can be analysed in an untargeted manner as well. The working principles of different mass analysers are presented in more detail in chapter 3.6.1.

Targeted NMR metabolomics is usually performed using one-dimensional ^{1}H NMR with an internal standard enabling concentration determination. This method is limited by the narrow Larmor frequency range of protons, which leads to severe spectral overlaps when analysing mixtures such as blood plasma or urine, as well as by the lower sensitivity of NMR in comparison with MS. The human NMR plasma coverage is thus limited to around 70 metabolites using an 800 MHz spectrometer (Nagana Gowda & Raftery 2019).

In order to achieve quantitative data from metabolites which have overlapping signals, a deconvolution step is necessary. An example of a software which enables manual deconvolution is Chenomx NMR suite (Chenomx Inc., Edmonton, Canada), in which signal patterns from a spectral library can be stepwise added, adjusted and fitted to the experimental spectrum until a satisfactory fit is achieved (Röhnisch *et al.* 2018).

Since the manual approach is time-consuming, automation of the deconvolution has been attempted by various approaches (Bingol 2018). Notable approaches are the Bayesian modelling based algorithm BATMAN (Hao *et al.* 2012), which iterates over the contents of a spectral library until an acceptable fit of the whole signal pattern is achieved, and AQuA, which is a reductionist approach based on linear combinations of the signals constituting the apex of a selected metabolite reporter signal (Röhnisch *et al.* 2018).

3.3.3 Untargeted metabolomics

The untargeted metabolomics approach differs from the targeted approach in that no *a priori* hypothesis exists and a global profiling of all signals in a sample is performed, with the aim of finding and identifying discriminating features. Untargeted NMR metabolomics is commonly performed by dividing the spectra into narrow bins which are extracted and subjected to multivariate statistical analyses to elucidate which bins differ between treatments. The bottleneck of untargeted NMR metabolomics however lies in the identification of these signals within the complex biological samples. Recent advances has been made to this by applying multi-dimensional and ^{13}C-NMR approaches and databases (Wang *et al.* 2019b; Bruguière *et al.* 2020), by correlating the NMR data with high-

resolution MS data (Hao *et al.* 2016), or by analysing the same sample at different pH-values (Paudel *et al.* 2019). Using hyphenated LC-NMR for separation of the compounds in a complex sample prior to the NMR analysis can also facilitate the identification of unknown signals (Garcia-Perez *et al.* 2020).

The global profiling in untargeted LC-MS metabolomics results in large amounts of data, and bioinformatics tools are paramount for retrieving the biological information therein. Two common open-source software tools for untargeted MS-metabolomics data handling are XCMS (Smith *et al.* 2006) and MZmine2 (Katajamaa *et al.* 2006; Pluskal *et al.* 2010).

Untargeted LC-MS metabolomics pipelines start with data acquisition by high-resolution MS, in which each sample yields a three-dimensional data set of measured *m/z*, chromatographic retention time (RT), and signal intensity. To extract biologically relevant information from this kind of data set it is necessary to first detect the regions of interest, so-called peak picking, and adjust the data for drifts in *m/z* and RT. These drifts appear naturally in LC-MS systems over time and are more prominent during runs with many samples and if samples are run in several batches, as is common in untargeted LC-MS metabolomics. The drifts can *i.a.* be due to differences in ionisation efficiencies between samples, sample stability, temperature fluctuations, or random variations (Smith *et al.* 2013).

A common algorithm for feature detection in untargeted metabolomics is *centWave* (Tautenhahn *et al.* 2008). The *centWave* algorithm is based on detection of so-called regions of interest, which are areas in the three-dimensional space wherein signals coalesce around an *m/z*-value and show an expected intensity distribution around a RT value. The *centWave* algorithm can be tailored by setting values for several parameters, *i.a.* the background noise cut-off, mass accuracy window, and chromatographic peak width. The *centWave* algorithm is implemented in both XCMS and MZmine2. Alternative approaches to feature detection have also been developed (Takahashi *et al.* 2011; Gorrochategui *et al.* 2016).

Drifts in RT and *m/z* will occur between samples, resulting in features originating from the same metabolite yielding different values in different samples. A feature grouping step that accounts for these differences is thus necessary. Two algorithms are the most commonly used for this purpose: *density*, which originates from XCMS (Smith *et al.* 2006) and *nearest*, which is inspired by the alignment algorithm in MZmine (Katajamaa *et al.* 2006). The *density* algorithm creates RT slices and groups features within the same *m/z*-window within a slice into the same feature. The *nearest* algorithm is based on finding the shortest distances in the *m/z* and RT dimensions, appending a feature

to a peak list as a new row in a master peak list in case no fit is found for features already on the list (Katajamaa & Orešič 2005).

Additional correction for RT differences may be applied. A common algorithm for dealing with RT alignment is Ordered Bijective Interpolated Warping (Obiwarp) (Prince & Marcotte 2006), which takes the non-linear variations into account.

While drifts in *m/z* and RT can be handled within the common analysis pipeline, variations in MS signal intensities due to *i.a.* signal suppression by matrix effects, batch differences, and instrument variations require additional consideration. In large-scale metabolomics studies these natural variations can lead to result misinterpretation since comparisons are made between samples analysed in both the beginning and the end of the sample run. The impact of these variations can however be reduced in paired studies if the sample pairs are analysed sequentially in a randomised case-control order (Jonsson *et al.* 2015). For additional details regarding sample normalisation strategies for metabolomics, see the review by Wu & Li (Wu & Li 2016).

The most common way to account for these signal intensity drifts in untargeted chromatography-MS metabolomics studies is the quality control (QC) strategy, where QC samples are prepared by pooling all analytical samples to make sure that the matrix effect differences are small and that all metabolites present in the samples can be found in the QC samples. The QC samples are then analysed regularly throughout the sample run. A locally estimated scatterplot smoothing (LOESS) signal correction that accounts for intensity drifts both within and between batches can then be applied to each feature individually. A cluster-based intra-batch correction together with an inter-batch feature alignment algorithm has been shown to improve the results (Brunius *et al.* 2016). The QC strategy is discussed in further detail in the review by Dunn *et al.* (Dunn *et al.* 2012). It is however important to remember that any kind of correction for systematic errors will add some random variance to the data, which might mask effects with low effect sizes.

After identification of discriminating features, the features need to be identified and validated. The Metabolomics Standards Initiative defines a four-level scale for metabolite identification (Sumner *et al.* 2007), with authentic standard matching using two orthogonal analytical methods in the same laboratory being required for level 1 matching. Levels 2 and 3 correspond to full and partial database matching respectively, and level 4 represents an unidentified metabolite. Examples on important databases for metabolomics are the Human Metabolome Database (Wishart *et al.* 2017), METLIN (Guijas *et al.* 2018), and LipidMaps (Sud *et al.* 2006). Work towards a revision of the metabolite annotation and identification reporting standards is currently under way, with a

seven-degree scale having been recently proposed by the Metabolomics Society's Metabolite Identification Task Group (Rampler *et al.* 2021).

3.4 Flux analysis

Measuring concentrations is the basis of many analytical methods and that holds for metabolomics as well. The amount of a metabolite in a biological system does however not tell the whole story. In biological systems many metabolites are under strict homeostatic control and thus vary only within a strict range. One can imagine a system in which there is continuous consumption of a metabolite under homeostatic control. Within that system the consumption must be compensated by metabolite production or uptake in order to maintain the homeostatic state. In case the consumption increases or decreases, then a concomitant increase or decrease in production or uptake is essential. In any of these cases the concentration of the metabolite in question will stay the same and the change will not be observable by conventional concentration measurements, although clear biological differences might exist. These changes in production or consumption can jointly be called metabolic flux changes. Metabolic reactions are in most cases mediated by enzyme action and the flux through a reaction is dependent on several enzymatic parameters. The concentration of an enzyme forms a capacity basis for the flux, but enzyme activity is also kinetically regulated via presence or absence of post-translational modifications as well as by the concentrations of products, substrate, and potential effectors (Dauner 2010). The concept of metabolic flux is presented in Figure 4.

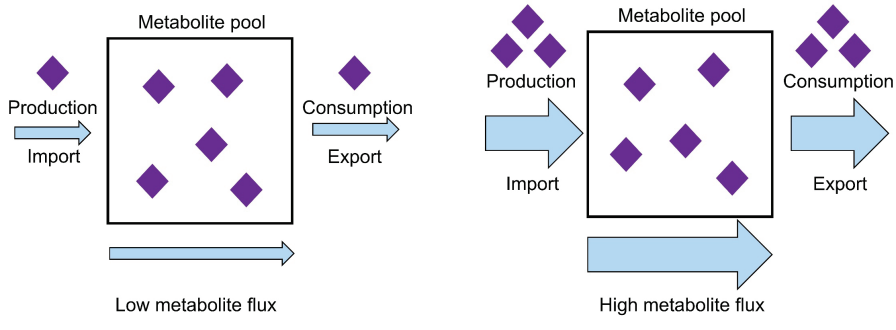

Figure 4. Schematic of a metabolite under homeostatic control with low and high flux. A purple diamond denotes one arbitrary unit of amount of substance.

The metabolic flux of a single reaction can be expressed as in equation 1, by considering a change in concentration over time, where Φ denotes flux, n denotes amount of substance, V denotes volume of the metabolite pool, and Δt

denotes time difference. The flux can be either positive or negative, and in a homeostatic situation it amounts to zero. This shows *e.g.* that an increased consumption can be compensated by an increase in import, production, or a combination of both, without a change in concentration. Flux analysis can also be extended to a system-wide approach by considering many reactions and their reaction rates in a cell simultaneously. This approach is often referred to as fluxomics, analogous to other systems biology approaches.

$$\Phi = \frac{\Delta n_{metabolite}}{V * \Delta t} = \left(\frac{(n_{import} + n_{produced}) - (n_{export} + n_{consumed})}{V * \Delta t} \right) \qquad (1.)$$

Fluxomic analyses can be conducted in two main ways: Stable isotope fluxomics and flux balance analysis (FBA). Flux balance analysis calculates steady-state solutions for linear combinations of fluxes in many pathways by equations like the one presented in equation 1 expanded with constraints such as stoichiometric, thermodynamical, and experimentally measured constraints (Lee *et al.* 2006). Separation of the import factor from the production factor (and export from consumption) in equation 1 is not possible with FBA, but can be investigated by stable isotope fluxomics.

Isotopes are variants of elements that differ only in the number of neutrons in their nuclei. Isotopes can be either stable or unstable, *i.e.* radioactive isotopes. The story of how the nature of isotopes was discovered took place in the first third of the 20th century. Two proposed elements with distinct differences in radioactive behaviour were shown to be completely inseparable by chemical means in 1907 (McCoy & Ross 1907), indirectly proving the existence of radioactive isotopes. The hypothesis that chemical elements are made up of mixtures of entities with different atomic weights came some years later and is often credited to Frederick Soddy (Soddy 1910), even though he was preceded by fellow Nobel laureate Theodor Svedberg (Strömholm & Svedberg 1909).

The first experimental evidence for the existence of stable isotopes was found by Joseph John Thomson in 1912 using the apparatus he called a parabola spectrograph (Thomson 1912) and in 1913 he reported that samples of neon always contained molecules of both atomic weights 20 and 22 (Thomson 1913). The nature of Thomson's unknown gas with atomic weight 22 was questioned for some time. Definite proof of the existence of stable isotopes was given by Francis William Aston (featured on the cover of this thesis) after studies of neon with an improved spectrograph, where he also detected the third stable isotope of neon, *i.e* ^{21}Ne (Aston 1920). After the discovery of the neutron (Chadwick 1932) the nature of isotopes was fully understood.

Different isotopes of a chemical element share chemical behaviour and properties. The main exception being a difference in kinetics, since isotopes with

higher mass react more slowly than lighter isotopes (Washburn & Urey 1932). As the atom number of an element increases, the relative impact of the kinetic isotope effect is decreased, due to the mass of an additional neutron having less relative impact on the total mass of the atom. Because of their identical and non-interfering properties, isotopes are well-suited for following chemical reactions by tracking labelled atoms.

Both stable and unstable isotopes can be used for tracking purposes. The seminal isotopic tracking experiments were performed by labelling with radioactive lead (Hevesy & Paneth 1913; Hevesy 1923), and using *i.a.* ^{3}H and ^{14}C has been crucial for elucidating biochemical pathways and chemical reaction mechanisms (Lappin 2015). Stable isotopes as metabolic tracers were however used already in the 1930s and early 1940s, with works published using labelling with ^{2}H (Schoenheimer & Rittenberg 1935), ^{13}C (Wood *et al.* 1940), and ^{15}N (Schoenheimer *et al.* 1939). Nowadays metabolic tracing experiments utilising stable isotopes are more common than radioactive tracing, owing to a desire to limit researchers' and research subjects' exposure to radioactivity, and as a result of the increased availability, resolution, and sensitivity of mass spectrometers.

Flux measurements can be done by two main techniques, *i.e.* the steady-state and nonsteady-state techniques. For the steady-state technique, a stable isotope tracer is continuously supplied at a given rate, *e.g.* by infusion. The ratio of the stable isotope tracer to the endogenous metabolite of interest (*i.e.* enrichment) will reach a steady state after some time due to consumption and production being equal. The flux can then be calculated based on the infusion rate and the observed enrichment in a sample taken at the steady state (Kim *et al.* 2016).

Calculating kinetics in the nonsteady-state is more demanding, but important for gaining knowledge of the mechanisms involved in dealing with a perturbation, such as exercise or eating. Samples are then taken at different time points before and after the perturbation and various kinetic parameters can be calculated from differences in concentration and enrichment at different time points using the so-called Steele equations (Steele 1959).

Once the isotopically enriched samples have been collected then the fate of these isotopes are probed by analytical techniques such as MS or NMR in order to measure the enrichment. By measuring the degree and nature of incorporation of the stable isotope it is possible not only to calculate the flux, but to discover metabolic pathways and estimate the relative contributions of specific pathways to metabolites as well (Jang *et al.* 2018). Differences in flux can also be probed indirectly without calculated flux values by comparing degrees of enrichment after different treatments (El-Azzouny *et al.* 2014).

The number of atoms in a molecule that have been labelled with a stable isotope can vary from none to all atoms. These variants are called isotopologues

and are commonly denoted by M+X, where X is the number of labelled atoms. Molecules with the same number of incorporated labelled atoms can however differ in the positions of the labelled atoms in the molecule. These variants of isotopically labelled molecules are called isotopomers and can be identified by NMR and by studying MS fragments. Isotopologues are commonly analysed by MS, since they can be discerned even by low-resolution mass spectrometers. A schematic representation of isotopologues and isotopomers are presented in Figure 5.

Examples of software for analysing stable isotope labelled untargeted chromatography-MS metabolomics data are X^{13}CMS (Huang *et al.* 2014) and geoRge (Capellades *et al.* 2016). Targeted stable isotope metabolomics data can *e.g.* be handled using AssayR (Wills *et al.* 2017) or MS-vendor software.

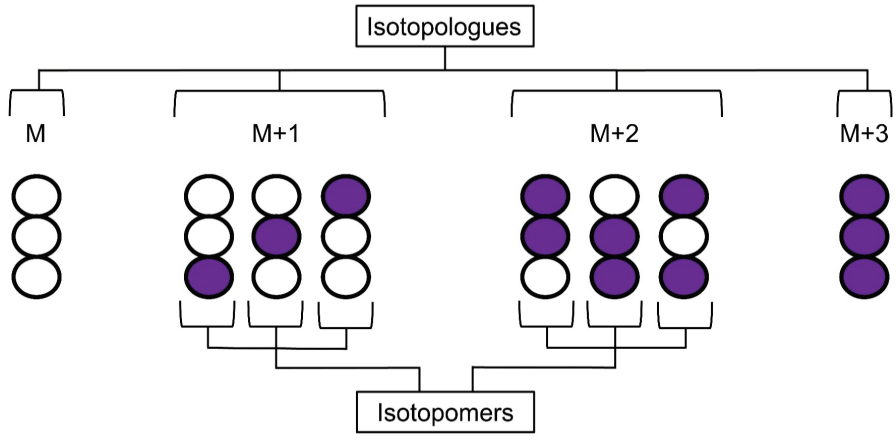

Figure 5. Isotopologues and isotopomers of a three-atom molecule. Labelled atoms are shown in purple.

3.5 Liquid chromatography

It is necessary to separate, or "*Separare necesse est"* as stated on the 2002 medal by the Royal Swedish Academy of Sciences in commemoration of Nobel laureate Arne Tiselius (Voisin 2002). This alludes to the famous quote "*Navigare necesse est, vivere non est*", which is attributed to the Roman general Pompey (Plutarch 1724). Analysis of mixtures is often complicated due to interactions between components, and explicit data for measured variables cannot be considered certain unless it can be determined that the sample has been pure. A separation step in the analysis pipeline might also be necessary in order to find out whether a sample is pure or not.

Separation of components in a mixture can be achieved by various techniques, *e.g.* by extraction, distillation, or filtering. A major technique for separations of components in liquid mixtures, and the one utilised throughout the work of this thesis, is chromatography.

The field of chromatography dates back to the seminal work of Tswett in the early 1900s with his separation of plant pigments dissolved in petroleum ether on a glass column filled with $CaCO_3$ (Tswett 1906). Tswett performed what we now refer to as liquid chromatography, where a liquid mobile phase is passed over a stationary phase, and coined the term chromatography from the Greek roots χρῶμα, "colour", and γράφειν, "to write". The chromatographic separation is based on differences in molecules' affinity to the two phases in the system. Compounds with a high degree of interaction with the stationary phase (through *i.a.* hydrogen bonding or van der Waals forces) will be retained for a longer time on the column and thus separate from other components in the mixture that interact less. Different types of chromatography are named after the mobile phase's state of matter and other important types of chromatography are *i.a.* gas chromatography and supercritical fluid chromatography.

Tswett employed a polar stationary phase and a non-polar mobile phase for his pigment separations (Tswett 1906). This is called normal phase chromatography and is used for separation of components soluble in non-polar solvents. Most biological samples are however water-soluble, and a reversed system with a polar mobile phase and a non-polar stationary phase is commonly employed for these kinds of separations. This is called reversed-phase (RP) chromatography, where less polar components will be retained longer on the column. The stationary phases utilised for RP-chromatography commonly consist of polar silica particles with non-polar alkyl chains bonded to their surfaces. The chromatographic separation can be tailored by choosing columns with different lengths and branching of the bonded chains. The choice of mobile phase will also impact the separation. For further insight into the theoretical frameworks underlying chromatographic separations, see *e.g.* Miller's Chromatography: Concepts and Contrasts (Miller 2005).

Another chromatographic separation method called hydrophilic interaction chromatography (HILIC) is commonly used as a complementary method to RP-chromatography since it is capable of increased separation of polar compounds. HILIC was developed into its modern form in the 1990s (Alpert 1990; Alpert *et al.* 1994). The first report of a HILIC-type separation however dates back to 1950s (Samuelson & Sjöström 1952). Any polar stationary phase can be used for HILIC separations, but bare silica and silica modified with polar groups are commonly used. The mobile phase consists of a polar organic solvent with some degree of aqueous modifier.

The mechanism underlying the HILIC separation is incompletely understood. The mechanism is thought to depend on a water-rich layer of mobile phase immobilised on the stationary phase. Alpert originally suggested that compounds partition between the immobilised layer and the hydrophobic mobile phase, with dipole-dipole interactions playing a part in the partition (Alpert 1990). The existence of this layer has later been proven (Dinh *et al.* 2013). Hydrogen bonding (Berthod *et al.* 1998), electrostatic interactions (Bui *et al.* 2010), and adsorption (Yoshida 2004) have however all been shown to participate in the separation mechanism and HILIC is thus nowadays considered to be a mixed-mode separation technique. For further insight into the HILIC mechanism and stationary phases, see the review by McCalley (McCalley 2017).

Modern analytical chromatography is often performed by pumping the mobile phase over the stationary phase, rather than using gravity flow, in order to decrease analysis time and reduce band broadening effects. The size of the particles in the stationary phase drastically impacts the separation, since packing the column with smaller particles will result in an increased area available for interaction with the compounds in the mixture to be separated. This will reduce the theoretical plate height in accordance with the van Deemter equation (van Deemter *et al.* 1956), which in turn increases the chromatographic resolution. Decreasing the particle size will however result in increased backpressure, which requires additional engineering feats, enabling the pumping system to handle this increased pressure. These kinds of instruments are called high-performance liquid chromatographs (HPLC). In 2004 Waters launched a system tolerating backpressures over 1000 bar and expanded the field into what is nowadays called ultra-high performance liquid chromatography (UHPLC), with columns using particle sizes down to 1.5 µm (Thermo Scientific Accucore Vanquish) and instruments capable of handling up to 1500 bar backpressure (Thermo Scientific Vanquish Horizon) being commercially available nowadays.

Recently, an orthogonal separation method named ion mobility separation has matured and gained usage in tandem with chromatography. Ion mobility separation takes place on the millisecond scale in contrast to the second scale of chromatography and the microsecond scale of the common mass spectrometer detectors and is as such well suited for tandem use between chromatography and mass spectrometry. The technique itself dates back to the late 19th century (Thomson & Rutherford 1896) and ions are separated by their differences in mobility in an electric field during collisions with an inert gas (Dodds & Baker 2019). Ion mobility separations have proven beneficial *e.g.* in lipidomics for separation of lipid isomers (Hinz *et al.* 2019).

3.6 Mass spectrometry

After separation of components in a mixture by chromatography, a detector is required for evaluation of the separation success and for further analysis. Various types of detectors coupled to chromatographic systems based on different phenomena exist. For example, ultraviolet-visible light (UV-Vis) detectors that detect changes in absorbance of ultraviolet or visible light are common in LC-systems and GC-systems are frequently equipped with flame ionisation detectors, which detect changes in current produced when compounds passing through a flame are ionised.

These detectors are limited in the sense that they do not provide much information on the structure of the compound detected. Other types of detectors, called mass spectrometers, which can provide this information, can thus often be found coupled to chromatographic systems. An alternative detector which can be hyphenated with liquid chromatographic systems and provide structural information is NMR (Garcia-Perez *et al.* 2020).

The basis of mass spectrometry is the separation of compounds based on their *m/z* and subsequent qualitative and quantitative detection. A mass spectrometer can be schematically constructed from the following parts: an inlet for sample introduction, an ion source for ionising molecules in the introduced sample, a mass analyser for separation of the ions by *m/z*, a detector for conversion of the ions into electric signals, and a data system for processing these output signals. A mass spectrum is then created by plotting the signal intensities relative to their corresponding *m/z*-values. A more detailed account of different ionisation techniques and mass analysers will be given in the consecutive subchapters.

The resolving power/resolution of a mass analyser is a metric commonly used to compare different mass analysers and mass spectrometers. Consider two peaks in a mass spectrum. These peaks are by definition considered resolved if the signal intensity in the valley between them is 10% of the apex intensity of the lower peak. The resolution (R) is then calculated as stated in equation 2 with $\Delta(m/z)$ being the *m/z*-differences of the peak apices. One can also define resolution based on a single peak by relating the peak full width at half maximum (FWHM) to the measured *m/z*-value as stated in equation 3. The method by which the $\Delta(m/z)$ has been defined needs to be reported (Kermit *et al.* 2013).

$$R = \frac{m/z}{\Delta(m/z)} \tag{2.}$$

$$R = \frac{m/z}{FWHM} \tag{3.}$$

The definitions of resolution and values they are based on are presented schematically in Figure 6. Noteworthy is that the same mass analyser has different resolving power at different *m/z*-values.

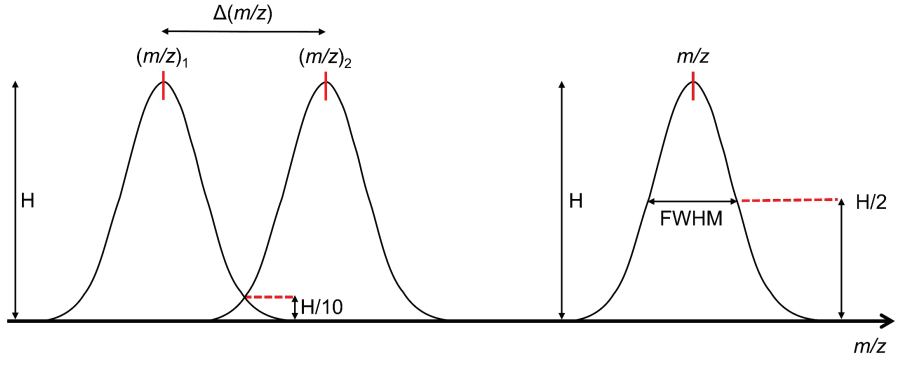

Figure 6. Resolving power determination in a mass spectrum using two adjacent peaks or the full width at half maximum (FWHM).

3.6.1 Mass analysers

The separation of ions based on their *m/z* can be achieved in various ways, and an array of different mass analysers that separate ions based on different principles have been developed.

The first separation of ions based on their *m/z* was achieved by Thomson, where beams of positive ions were deflected differently by an electric field depending on their mass (Thomson 1907). This instrument was further developed into the first mass spectrometer, called parabola spectrograph, by which the ions reaching the detector could be chosen and the signal intensities could be plotted against the corresponding *m/z* values, *i.e.* what we today know as mass spectra (Thomson 1912). This instrument was later developed further by Aston into his mass spectrograph (Aston 1919), by which he proved the existence of stable isotopes through analyses of neon (Aston 1920). Aston later improved the mass spectrograph to reach a resolving power of around 2,000 (Aston 1937). This mass spectrograph is featured on the cover of this thesis.

The second kind of mass analyser was developed by Arthur Jeffrey Dempster in 1918. This magnetic sector instrument separates ions in a static magnetic field based on their curvature radius, which is dependent on the ions' charge and momentum (Dempster 1918). The magnetic sector mass analysers were subsequently improved by applying both electric and magnetic fields for improved ion beam control (Mattauch & Herzog 1934; Johnson & Nier 1953). For many decades the magnetic sector instruments were common due to their high resolution and GC-interfacing possibility. They have since become rarer,

due to that tabletop instruments providing the same resolution have become available.

The principle of the time-of-flight (ToF) mass analyser was presented in 1946 (Stephens 1946) and a working instrument was presented in 1948 (Cameron & Eggers 1948). As all mass spectrometry data presented in this thesis has been collected on a ToF-instrument, this will be discussed more in depth in a consecutive subchapter.

Ion cyclotron resonance (ICR) mass analysers (Sommer *et al.* 1951) and later Fourier-transform ICR (FTICR) mass analysers (Comisarow & Marshall 1974) separate ions by bringing them to resonate as they are rotating in a magnetic field and detecting the image current that form as they deviate from the overall motion. FTICR mass analysers can provide the highest resolution of the currently available instrumentation. The ultra-high resolution of over 2,000,000 (Hendrickson *et al.* 2015) is however not attainable with scan speeds in the chromatographic peak time scale.

The next mass analyser to be invented was the quadrupole mass analyser, which was presented in 1953 (Paul & Steinwedel 1953). It consists of two pairs of parallel electrodes, upon which potentials made up of a static and a radio frequency (RF) component are applied, with oscillating opposite signs between the electrode pairs. Ions entering the electric field within the quadrupole will be drawn towards a rod of opposite charge, and if the sign of the electrode potential changes before the ion touches the rod it will propagate through the quadrupole in a spiralling motion. The stability of ions passing through this kind of electric field is governed by the Mathieu equation, which states that ions will be able to pass through the field without touching the rods only at a set ratio between the two component potentials. At a set combination of the potential components only ions within a specific *m/z*-range will be able to pass through the quadrupole. By adjusting the magnitudes of the two potential components, while maintaining a constant ratio between them, one can scan a range of *m/z*-values and create a mass spectrum.

Quadrupoles without a static potential applied to them can focus ion beams and act as ion guides, since all ions above a *m/z*-threshold governed by the magnitude of the radio frequency potential will be stable. RF-only quadrupoles are ubiquitous for this purpose in modern mass spectrometers. They can also be used for storing ions by trapping them between potentials of the same sign at both ends of the quadrupole. When used for this purpose they are usually called linear ion traps.

When filled with gas, an energy can be applied to radio frequency-only quadrupoles and they will act as a collision cell, causing collision induced dissociation (CID) (Jennings 1968) of analytes. Helium, argon, or high-purity

nitrogen is often used for this purpose. Quadrupoles can further be connected in tandem with an RF-only quadrupole as a collision cell in between, forming the triple quadrupole mass spectrometer (Yost & Enke 1978), which makes performing analyses with high selectivity as well as studying of ion fragmentation possible. Quadrupoles offer limited resolution and mass range, but their high scan speeds, high transmission, and low size have made them a common type of analyser in mass spectrometers.

Together with the original patent filing for the quadrupole mass spectrometer an alternative electrode geometry was also presented (Paul & Steinwedel 1956). This quadrupole ion trap can confine ions within a three-dimensional space using three electrodes. Ions with different *m/z*-values can be released from the trap by altering the potentials and a mass spectrum can be created. A unique feature of the ion trap is that a collision gas can be let into the trap and CID can be performed inside the trap. A specific ion can then be kept in the trap when releasing other ions and further CID can be performed on the confined ion, enabling tandem MS (MS/MS) in time for fragmentation analysis or for structure identification.

The orbitrap is a recently developed mass analyser which can achieve very high resolution at a chromatographic time scale. The working principle was initially presented by Makarov (Makarov 2000) and a working instrument was described and commercialised in 2005 (Hu *et al.* 2005). The orbitrap is based on the Kingdon trap (Kingdon 1923) and traps ions in a static electric field between a central spindle shaped electrode and two outer electrodes. The ions will then oscillate around the central electrode with different frequencies based on their *m/z*-value and separate into *m/z*-packets. As they oscillate around the electrode an image current is induced, detected, and Fourier transformed to create the mass spectrum from the individual oscillation frequencies of the ions inside the trap. Resolutions of 1,000,000 at *m/z* 200 with a 0.5 Hz scan speed are specified by the manufacturer Thermo Fisher Scientific for the current flagship models and a resolution of 1,700,000 has been reported at *m/z* 86.909 (Hoegg *et al.* 2019).

The newest addition to the mass analyser family is the distance-of-flight (DoF) analyser (Graham *et al.* 2011), which utilises position-sensitive detection in a flight tube after the ions have been accelerated. No commercial instruments are available at the time of writing, but the technique might find applications in MS/MS and in mass spectrometry analysis of very high mass analytes (Dennis *et al.* 2016).

Additionally, one can often find mass spectrometers containing several types of mass analysers. These so-called hybrid instruments can provide more information than their individual counterparts and thus provide analysis versatility with a limited laboratory footprint. Examples of common hybrid

instruments are linear ion trap-orbitraps and quadrupole-collision cell-time-of-flight hybrid instrument (QqToF). All mass spectrometry data presented in this thesis were collected on a QqToF, enabling isolation of a precursor ion, subsequent fragmentation and accurate mass measurement of the fragments formed. This feature was utilised in papers II and III to aid in metabolite identification. For further insight into mass analysers and their working principles consult *e.g.* the textbook by Gross (Gross 2017).

Time-of-flight mass spectrometry

A time-of-flight mass analyser is based on the fact that heavy ions take longer time to traverse a set distance than lighter ions having the same kinetic energy. When accelerated in an electric field with a potential U, an ion of mass m will gain kinetic energy equal to the product of the potential, the charge of the ion (z), and the elementary charge (e). It then follows that the time (t) it takes for the ion to drift through a flight tube of a set distance (s) is proportional to its m/z-value as shown in equation 4.

$$t = \frac{s}{\sqrt{2eU}}\sqrt{\frac{m}{z}} \tag{4.}$$

In order to achieve high resolution in a ToF mass analyser the ions must not lose any kinetic energy through collisions as they traverse the drift tube. ToF mass spectrometers thus require high vacuum conditions in the flight tube. Since the dispersion in time is dependent on the ions' total kinetic energy, high resolution is only attainable if the ions have the same kinetic energy post-acceleration. They will receive the same amount of energy in the acceleration stage, but there will be a spread in kinetic energy stemming from the ionisation event.

Delayed extraction is one method used to improve resolution by accounting for these differences (Wiley & McLaren 1955). Delayed extraction decouples the ionisation event from the ion acceleration by allowing the formed ions to disperse in space before applying the acceleration voltage. This will result in ions with high initial kinetic energy moving further away from the point of ionisation, and thus spend less time in the accelerating field, in practice experiencing a lower acceleration potential than ions with lower initial kinetic energy. The acceleration can also be applied in two stages with the second acceleration stage giving the same kinetic energy to ions, no matter their initial kinetic energy.

The original instruments were equipped with linear flight tubes with the sample and ionisation event at one end of the tube and the detector at the other end. The resolution of ToF mass analysers was significantly improved by the

invention of the reflectron (Mamyrin *et al.* 1973), which corrects for differences in the ions' kinetic energy as well as enabling elongated flight paths without drastically increasing the instrument footprint. The reflectron consists of a stack of ring-shaped electrodes located at the end of the flight tube. The electrodes are set at a potential opposite to the acceleration potential. Ions entering the reflectron field will thus penetrate the reflectron further if they have higher kinetic energy. The ions will then be reflected out from the reflectron field, retaining the same kinetic energy they had upon entering, but having become corrected for initial energy differences by differences in time spent in the reflector field. The use of a reflectron however causes a loss in sensitivity and instruments often have the option of being operated in either linear or reflectron mode.

All ions in the flight tube need to reach detector before the next ionisation event in order to be able to accurately measure the flight time. The ToF is thus a well-suited analyser for pulsed ionisation techniques such as matrix-assisted laser desorption/ionisation (MALDI). This limitation however poses an issue for coupling of continuous ion sources to ToF analysers. An alternative geometry called orthogonal ToF utilising an orthogonal accelerator for operation with a continuous ion source has been developed for this purpose (Dawson & Guilhaus 1989). The orthogonal accelerator works like the two-stage accelerator used post-ionisation, but is rotated 90°, giving the ions an orthogonal velocity component. The accelerator is filled with ions which are accelerated into the flight tube. The accelerator is re-filled with ions during the flight time of the previous ion package and these ions are accelerated into the flight tube as soon as all ions from the previous package have reached the detector, resulting in a pulsed injection of ions into the flight tube. A part of the continuous ion beam will be discarded due to mismatches in flight time and accelerator filling times, which has some impact on the sensitivity. The orthogonal acceleration will however have a beneficial impact on the resolution, since the ion beam entering the accelerator will be narrow and well-defined in the direction of entry. The velocity component responsible for the separation in the flight tube will thus be strictly controlled by the pulsed accelerator, with low initial spread. A schematic layout of an orthogonal ToF equipped with a reflectron is presented in Figure 7.

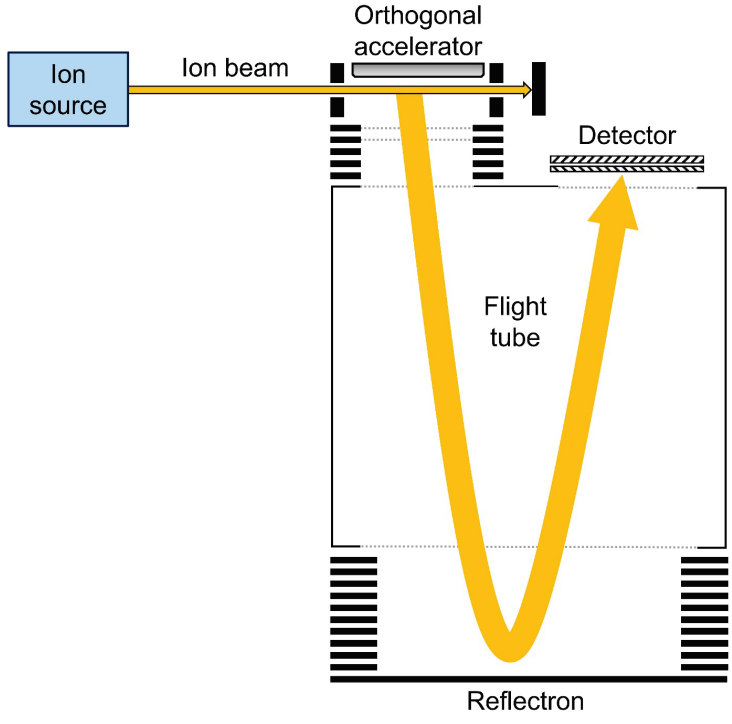

Figure 7. Schematic representation of an orthogonal time-of-flight mass spectrometer equipped with a reflectron.

3.6.2 Ionisation methods and ion sources

The ions analysed by Thomson in the original experiments were formed via irradiation of gases by anode rays (Thomson 1907; Thomson 1913). In 1918 electron bombardment for ion generation was reported by Dempster (Dempster 1918), and this ion source was developed further (Bleakney 1929) into a form where the energy of the ionising electrons could be controlled, which in turn made it possible to produce highly reproducible mass spectra and build spectral libraries (Stein 2012). The mechanism of electron ionisation (EI) is based on the beam electrons exciting the sample, which can lead to an electron being expelled. EI is to this day the prevailing mode of ionisation in GC-MS applications.

EI is a so-called hard ionisation technique, which results in high degrees of fragmentation. In order to circumvent this and enable analysis of compounds with higher molecular mass and detection of the unfragmented molecular ion for identification purposes, much focus have been directed towards development of softer ionisation techniques. An early soft ionisation technique which became widespread was chemical ionisation (CI), where a reagent gas, *e.g.* methane, is

ionised and the analyte is ionised through interaction with the reagent gas (Munson & Field 1966).

CI is nevertheless a rather hard ionisation technique and mass spectrometric analysis of biomacromolecules was elusive for a long time, since the available ionisation methods were insufficient for ionisation of highly polar molecules with high molecular mass. In the 1970s and 1980s the techniques fast atom bombardment (FAB) (Barber *et al.* 1981), liquid secondary ion mass spectrometry (LSIMS) (Aberth *et al.* 1982), and ^{252}Cf-plasma desorption (PD) (Macfarlane & Torgerson 1976) were developed and used for analysis of medium-large biomolecules (Roepstorff 1989; Das & Pramanik 1998), but did not reach widespread use in life science applications.

In the late 1980s ionisation methods which radically facilitated the analysis of biomacromolecules and brought mass spectrometry into life sciences were developed. Soft laser desorption was presented in 1988, showing ionisation of chicken lysozyme heptamer with an *m/z* of 100,872 by mixing the sample with fine cobalt powder and irradiating the sample with a nitrogen laser (Tanaka *et al.* 1988). This method did however not achieve wide usage, since a similar method called MALDI was presented around the same time (Karas *et al.* 1987), and this method proved superior because of higher sensitivity and the possibility to control the ionisation by changing the organic matrix used for the co-crystallisation. The ion formation mechanism in MALDI is still under debate, but there is some degree of consensus that ionising reactions resulting in analyte adducts take place both in the initial irradiation phase as well as in the gas phase ablation plume (Knochenmuss 2006). MALDI ionisation is commonly used with ToF-MS because of its large mass range and compatibility with pulsed ionisation.

While MALDI proved useful for analysis of purified samples of proteins and peptides, another ionisation method called electrospray ionisation (ESI) which is LC-compatible and as such useful for analyses of mixtures was developed around the same time (Fenn *et al.* 1989). ESI also produces multiply charged ions to a higher degree than MALDI and is thus compatible with a wider range of mass analysers. ESI is the ionisation method that has been used throughout this thesis work and will thus be discussed in further detail below.

Atmospheric pressure chemical ionisation (APCI) is another LC-compatible ionisation method, where ions are formed by a corona discharge on heated vaporised droplets (Horning *et al.* 1974; Carroll *et al.* 1975). APCI is complementary to ESI, since it is suitable for ionisation of smaller, less polar compounds. A field where APCI has proven especially useful is in analysis of steroids (Shackleton *et al.* 2018). For further insight into different ionisation methods and their mechanisms, consult *e.g.* the textbook by Gross (Gross 2017).

Electrospray ionisation

The concept of the electrospray was originally presented in the 1960s (Dole *et al.* 1968), but the first spectra from a working ESI-MS were not presented until 1989 (Fenn *et al.* 1989). In ESI the liquid is pumped through a capillary under high voltage. At a solvent specific voltage limit a so-called Taylor cone will form at the capillary tip due to charge separation in the solution (Zeleny 1917; Taylor 1964), from which highly charged droplets will be ejected when the electric forces surpass the surface tension (Wilm & Mann 1994). Elongation of the droplets moving in the electric field will additionally induce formation of Taylor cones in the droplets and further disintegration by droplet jet fission will occur (Gomez & Tang 1994). In current instrumentation the desolvation of the droplets are additionally supported by assisting evaporation of the solvent with a flow of heated gas in the ion source and heated ion transfer capillaries. The ions formed in ESI are adducts, with $[M\pm H]^{\pm}$ being the most common adducts depending on the ionisation mode. Other commonly observed adducts are $[M+Na]^{+}$, $[M+K]^{+}$ and $[M+NH_4]^{+}$ in positive mode and $[M+HCOO]^{-}$ and $[M+Cl]^{-}$ in negative mode. A schematic figure of an ESI-ion source is presented in Figure 8.

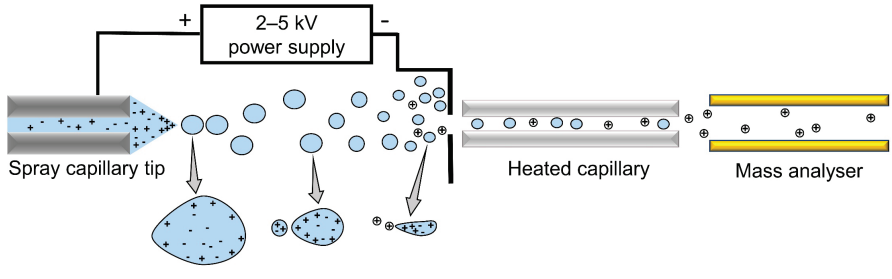

Figure 8. Schematic representation of the working principle of an electrospray ionisation (ESI) ion source operating in positive ionisation mode.

The model for production of gas-phase ions in ESI is not fully clear. Three explanatory models exist: the charged-residue model (CRM) (Dole *et al.* 1968), the ion evaporation model (IEM) (Iribarne & Thomson 1976), and the chain ejection model (CEM) (Ahadi & Konermann 2012).

The CRM was presented together with the initial electrospray by Dole *et al.*, and postulates that droplet radii decrease until only a single molecule remains in the droplet. The charges present in this droplet will thus be transferred to the analyte and the gas phase ion will form. According to the IEM, analyte ions already preformed in the droplets evaporate from the droplets due to energy provided by the electric field inside. The CEM is similar to the IEM, but gives additional explanation concerning the ionisation of large chain-formed molecules. According to the CEM, one end of a large chain-formed molecule

migrates to the surface of a droplet and moves into the gas phase in an IEM manner, whereas the rest of the molecule is sequentially pulled out into the gas phase. No single model explains the mode of ionisation for all kinds of molecules in ESI. The CRM seems to be applicable for explanation of globular protein ionisation by ESI (McAllister *et al.* 2015), the CEM better explains the ionisation of unfolded proteins (Metwally *et al.* 2018), and the IEM seems to be applicable for small analytes (Ahadi & Konermann 2011).

3.7 Statistical methods

3.7.1 Univariate statistics

Statistics in its most basic form is what is called descriptive statistics, when characteristics of datasets are summarised by measures such as the mean, standard deviation, and standard error of the mean (SEM). Once these measures have been calculated for two or more datasets, questions might arise regarding whether they are actually different from each other, or if observed differences stem from variations within the same population. Statistics relating to answering these questions belong to the field of statistical inference.

The start of modern statistical inference and hypothesis testing is often credited to the seminal paper by Student (*nom de plume* of Guinness head brewer William Sealy Gosset), in which the *t*-distribution and the *t*-test was introduced (Student 1908). Student's findings were later developed further and formalised by Ronald Fisher (Fisher 1925). The *t*-test can be used to analyse whether samples from an unknown population is different from a known mean (so-called one-sample *t*-test) or for comparing samples from two populations with each other (two-sample *t*-test).

The core of the *t*-distribution is that estimations of the mean and standard deviation of an unknown (albeit normally distributed) population by random sampling will converge towards a normal distribution with known mean and standard deviation as the sample size increases. The *t*-distribution is thus actually a collection of probability distributions with different so-called degrees of freedom, which in the case of the *t*-distribution are equal to the sample size minus one (Tu 2007). To perform a one-sample *t*-test, a so-called *t*-statistic is calculated based on the sample size, known mean, and the mean and standard deviation of the sampled population. Analogously, a *t*-statistic can be calculated for the two-sample *t*-test based on the sample sizes, means, and standard deviations of the two sampled populations. The *t*-statistic is then compared with tabulated values that tell the probability of the mean of the sampled population

being different from the known mean in the case of a one-sample *t*-test (Tu 2007) or the probability of the populations having the same mean (*i.e.* if they are actually from the same population) in the case of a two-sample *t*-test (Berman 2007). The probability level at which one considers the groups to be different is called the significance level, often denoted by α. A common significance level used is 0.05, meaning that there is a 5% probability that the means of the groups are not different.

In many cases it will be of interest to compare more than two groups with each other. This can be achieved by analysis of variance (ANOVA) testing, introduced by Fisher (Fisher 1921). An ANOVA test compares several groups with each other by separately estimating the variances within and between groups. The core of the ANOVA is the *F*-distribution, which has two degrees of freedom based on the number of analysed groups and the number of samples. Akin to the *t*-statistic, an *F*-statistic is calculated based on the degrees of freedom, the means of sampled populations, and the sum of squared differences both within and between groups. The *F*-statistic is then compared to tabulated values giving the probability of the sampled groups all belonging to the same population. The *F*-statistic in the case of two groups (*i.e.* between-group degree of freedom=1) is equal to the *t*-statistic squared, meaning that ANOVA can be used for two-group comparisons as well, and can be considered a generalisation of the *t*-test (Berman 2007).

In case measurements are made on the same object both before and after treatment, or at two different time points, then the measurements are considered to be paired. The statistical power can then be increased by analysing the difference between the two paired samples, which reduces the influence of variation between the sampled individuals. When samples being compared have been chosen in order to account for factors that might add variation, *e.g.* age and gender, the term matching is used. In the case of three or more measurements, the term repeated measures is often used, *e.g.* in the case of a time series. The paired *t*-test and repeated measures ANOVA are two common variants of the traditional tests used for analysing paired and matched data (Keselman *et al.* 2001; Berman 2007).

The classical tests for statistical inference have an underlying assumption that the data are normally distributed. When they are not however, so-called non-parametric counterparts can be used instead. The Wilcoxon rank-sum test, also known as the Mann-Whitney test, and the Wilcoxon signed-rank test (Wilcoxon 1945) are non-parametric counterparts of the unpaired and paired Student's *t*-test, respectively. The common counterparts to the parametric ANOVA tests are the Kruskal-Wallis test for standard ANOVA (Kruskal & Wallis 1952) and Friedman test for repeated measures ANOVA (Friedman 1937).

The *t*-test and ANOVA have later been shown to be special cases of a more general kind of model called general linear model (GLM) (Ip 2007). GLM:s are based on multiple linear regression and can simultaneously model responses based on one or more variables, which can be either continuous or categorical. These explanatory variables, the impacts of which on a response are of interest to analyse, are commonly called fixed factors. GLM:s can also contain covariates and interaction effects between variables.

By incorporating so-called random factors, *e.g.* sample matching or repeated measures factors, into the model apart from the fixed factors, these models can be extended to so-called general linear mixed models (GLMM:s) (Oberg & Mahoney 2007). GLM:s and GLMM:s are based on the underlying assumption that the model residuals are normally distributed (Ip 2007). They can however be extended to an even more general form called generalised linear mixed models, which can handle variables with a range of different distributions as well as both fixed and random factors (Nelder & Wedderburn 1972; Breslow & Clayton 1993).

In metabolomics studies it is common to measure the intensity or concentrations of many metabolites, *i.e.* response variables, at once. When performing many statistical tests using *e.g.* the common significance level of $\alpha=0.05$, there is a 5% probability of getting a false positive test result by each test performed. This implies that the probability of getting one or more false positive results increases by increasing the number of tests performed. In order to account for this and ensure that whatever significant effect that is found is not purely because of probability, different methods are used to account for the multiple testing. Examples of common methods for accounting for this issue are the more conservative Bonferroni correction (Dunn 1961) and the more liberal Benjamini-Hochberg procedure, which is also known as false-discovery rate (FDR) (Benjamini & Hochberg 1995).

3.7.2 Multivariate statistics

When many response variables are measured simultaneously, as is often the case in metabolomics, multivariate statistical methods have shown to be useful for aiding data interpretation and visualisation. Multivariate statistical tools can roughly be divided into unsupervised and supervised methods. Principal component analysis (PCA) is the most common unsupervised multivariate statistical method and has been called the “workhorse of chemometrics” (Trygg *et al.* 2007).

PCA originates from the beginning of the 1900s when Pearson tried to find “lines and planes of closest fit to systems of points in space” (Pearson 1901).

PCA decomposes a matrix of X-variables (measured variables) into a fewer number of variables, called components, which are all perpendicular to each other. This is done without any prior assumption about the data, hence the term "unsupervised" method. As more components are fitted, more of the variation in the data is explained. The first components will be the ones explaining most of the variation however, and at some point, only noise will be modelled by additional components. The decomposing yields so-called scores and loadings for each component. By plotting the scores of two components against each other one can see which samples cluster together (*i.e* share variation), giving insight into relationships between individual samples. The directions of the score and loading plots are corresponding to each other, meaning that one can see which X-variables are responsible for the clustering by plotting the loadings of the same two components and see which loadings are close to the position of the corresponding cluster in the score plot.

A common supervised method is projection to latent structures (PLS) (Wold *et al.* 2001). PLS is based on multivariate linear regression, hence "supervised", because the method is told to find and model the relationships between responses and variables. PLS finds the relationships between an X-matrix of variables (as in PCA) and one or several responses (Y-vector/matrix) and gives components based on covariance between the matrices. Like in PCA, the resulting PLS-model has scores and loadings which can be interpreted for evaluation of which effect different variables have on different components. An extension of PLS called orthogonal PLS (OPLS) has also been developed (Trygg & Wold 2002). OPLS decomposes the variation in the X-variables into components associated with the Y-matrix (so-called predictive variation) and into components containing variation which is not related to the Y-matrix (so-called orthogonal variation). OPLS can as such make it easier to interpret the model, since the variation relevant to the responses are collected in their own components.

PLS and OPLS models can be extended to so-called PLS-discriminant analysis (PLS-DA) (Ståhle & Wold 1987) and OPLS-DA (Bylesjö *et al.* 2006) models for classification purposes when the Y-matrix contains qualitative data. This is beneficial for finding which X-variables are associated with *e.g.* specific treatments or other categorical variables. An additional extension to OPLS called OPLS-effect projections (OPLS-EP) which can handle the benefits of matched and paired samples have also been developed (Jonsson *et al.* 2015). An additional benefit of the OPLS-EP approach is that variations due to analytical drift in the data collection step can be reduced by analysing paired or matched samples after each other (Jonsson *et al.* 2015).

The handling of outliers is of importance in multivariate statistics, since outliers can have a strong impact on the fitted components. A common metric

for outlier detection is Hotelling's T^2 (Hotelling 1931), which is a multivariate extension of the *t*-statistic in two dimensions. By calculating Hotelling's T^2 a two-dimensional confidence interval space is produced, wherein observations lying outside the space can be considered outliers.

After a multivariate model has been fitted, measures have to be taken to ensure its reliability and make sure that the components model real variation in the data instead of noise. For PCA models each component gets an R^2-value which tells how much of the variation in the model (in percent) that is represented by that component. When the addition of a new component does not have big impact on the cumulative R^2-value, it is a sign that it might not be of importance for explaining any more variation in the data. The Q^2-value is a metric on the predictive ability of a PLS or OPLS model. Adding components will always increase the R^2-value, since more variation is included, but not necessarily increase the predictive ability of the model, making the Q^2 a metric which can be used to determine the optimal number of components for a model. For evaluation of PLS and OPLS models two other tests are also commonly used. These are cross-validated analysis of variance (CV-ANOVA) (Eriksson *et al.* 2008) and response permutation testing (Eriksson *et al.* 2013). To assess the importance of a specific X-variable on a PLS or OPLS component, a metric known as the variable importance on projection (VIP)-value can be calculated and interpreted for each X-variable in each component (Galindo-Prieto *et al.* 2014). The average VIP-value in a component is one, meaning that variables with a VIP>1 have a larger contribution to the component. For a more thorough overview on the multivariate statistical methods used in metabolomics see *e.g.* the book chapter by Pinto (Pinto 2017).

3.7.3 Statistics in observational studies

Molecular epidemiology, which relates metabolite levels to certain outcomes, is an important means of discovering new risk factors, as exemplified *vide supra* for both T2D and prostate cancer. Observational studies are an important source of data for epidemiological studies and they can be performed as cohort studies, case-control studies, or cross-sectional studies (Song & Chung 2010), which require their own kind of statistical methods (Filardo *et al.* 2011). Since a case-control study has been conducted within the scope of the thesis, the statistical methods applicable to that will be discussed in more detail.

An important metric for investigating case-control data is the odds ratio (OR). For calculating the OR, all cases and controls are counted and classified into exposed ($n_{\text{case,exposed}}$ and $n_{\text{control,exposed}}$) and unexposed ($n_{\text{case,unexposed}}$ and $n_{\text{control,unexposed}}$), respectively. The OR is then calculated as the ratio of the odds

of exposure in cases to the odds of exposure in controls, as presented in equation 5. A variable with an OR>1 is thus positively associated with the outcome, meaning that an increase in that variable is associated with an increase in outcome risk and *vice versa* (Filardo *et al.* 2011).

$$OR = \frac{n_{case,exposed}/n_{case,unexposed}}{n_{control,exposed}/n_{control,unexposed}} = \frac{n_{case,exposed}n_{control,unexposed}}{n_{case,unexposed}n_{control,exposed}} \quad (5.)$$

Logistic regression models (Berkson 1944) can be used to relate the levels of different X-variables, *e.g.* metabolite concentrations, to a binary outcome, as is the intention with a case-control study, and provide an OR to that relationship (Nick & Campbell 2007). The relationship between a continuous variable, such as a metabolite concentration, and the probability of the outcome risk is often S-shaped. Logit-transformation, *i.e.* the logarithm of the ratio of the probability and opposite probability, is thus commonly applied on the probability to make this relationship approximately linear (Nick & Campbell 2007). By *e.g.* $\log_2$-transforming the X-variables one can ease the interpretation of the OR:s, since a doubling of a metabolite concentration would thus imply a relative increase or decrease of risk of the outcome according to the OR. Logistic regression can also be extended for taking pairing and matching into account by so-called conditional logistic regression (CLR) (Breslow *et al.* 1978).

4 Methodology

4.1 Human intervention study (Paper I)

Ingestion of rye bread leads to lower postprandial plasma insulin levels than wheat bread containing the same amount of available carbohydrates, while the postprandial plasma glucose levels do not differ (Leinonen *et al.* 1999). To investigate whether observed deviations in postprandial patterns could be explained by differences in gut-to-blood glucose rate of appearance (RaE) a human intervention study with a two-treatment crossover design and three-week washout was conducted.

Thirteen men of ages 18–40 with a BMI of 22–25.5 kg/m^2 were recruited for screening prior to the study. Exclusion criteria were regular use of nicotine, regular medication intake, food intolerance, recurrent extensive exercise, and a history of T2D in the family. Two of the screened participants were excluded for not meeting the exercise criterion. One participant chose to leave the study post-screening and one left after the first study visit.

The remaining nine participants consumed a breakfast of either refined wheat bread (WB) or wholemeal rye bread (WMR) containing 50 g available carbohydrates while being under infusion of a D-[6,6-$^2H_2$]-glucose solution at 0.40 µmol/(kg×min). Blood samples for analysis of glucose, insulin, and gastric inhibitory polypeptide (GIP) concentrations, as well as for measurement of D-[6,6-$^2H_2$]-glucose enrichment were drawn at different time points before and after the breakfast. The study protocol was approved by the Regional Ethical Review Board in Uppsala, Sweden (Dnr 2014/290) and all participants gave informed written consent upon participation.

The glucose kinetics were studied by calculating glucose RaE between two time points using the non-steady state, one-pool Steele equation (Steele 1959)

modified for stable isotopes (Schenk *et al.* 2003). The RaE equation is presented in equation 6.

$$\mathrm{RaE}(t_i, t_{i+1}) = \frac{2 \times F - V_d \times (C_{t_{i+1}} + C_{t_i}) \times \frac{(E_{t_{i+1}} - E_{t_i})}{(t_{i+1} - t_i)}}{(E_{t_{i+1}} + E_{t_i})} \quad (6.)$$

F denotes the rate of D-[6,6-$^2H_2$]-glucose infusion, V_d is the effective volume of distribution of 150 mL/kg (Schenk *et al.* 2003), C is the blood glucose concentration, E is the enrichment of D-[6,6-$^2H_2$]-glucose to monoisotopic glucose, and t is the time of sampling relative to the start of the breakfast.

4.2 β-Cell culture study (Paper II)

The human β-cell line EndoC-βH1 (Ravassard *et al.* 2011; Tsonkova *et al.* 2018) was used to investigate metabolic changes in β-cells upon short-term treatment with glucose and palmitate. The cells were cultured with a 50:50 (vol:vol) mixture of Dulbecco's Modified Eagle Medium and Ham's F12 (Krizhanovskii *et al.* 2017) with 5.5 mmol/L glucose and necessary supplements as previously described (Ravassard *et al.* 2011). The cells were seeded and grown on extracellular matrix/fibronectin-coated 24-well plates in a 5% CO_2 atmosphere until a cell number of approximately 10^6 cells per well was obtained.

In preparation for the short-term incubation study, the cells were washed with phosphate-buffered saline (PBS) and incubated for one hour with priming conditions of the same constitution as the treatment conditions. The media was then removed, washed with PBS and the treatment media was added. The six treatment conditions were 0, 5.5, and 15 mmol/L of D-glucose, with or without 0.5 mmol/L palmitate in Krebs-Ringer bicarbonate 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (KRBH) with 0.5% bovine serum albumin (BSA). Triplicate wells of each condition and three different cell passages were used. The experiment was further repeated with ^{13}C-labelled D-glucose (U-$^{13}C_6$-D-glucose) and natural palmitate as well as with natural D-glucose and ^{13}C-labelled palmitate, for a total of three experiments and nine passages. Insulin content in the media post-treatment was measured by enzyme-linked immunosorbent assay (ELISA).

The media was collected and cells were washed with PBS and extracted with a -20 °C solution of methanol:water:chloroform (8:1:1). The cell extract was dried down and reconstituted in methanol increasing the concentration tenfold. The media samples were extracted in methanol (99 times the sample volume) and 90% was collected after centrifugation. The supernatant was dried down and reconstituted with methanol volumes increasing the concentration tenfold.

4.3 Biobank study (Paper III)

Blood plasma samples and baseline examination data from the Northern Sweden Health and Disease Study (NSHDS) (Hallmans *et al.* 2003) were used in the study. The study design has recently been described elsewhere (Röhnisch *et al.* 2020). In brief, 778 case-control pairs were selected using the following inclusion criteria: No baseline T2D, samples taken after overnight fasting, no previous cancer diagnoses, and at least five years between cohort enrolment and prostate cancer diagnosis. The long minimum follow-up time of at least five years was used to minimise variation from potential subclinical prostate cancer at baseline. The case-control pairs were matched by age (95% within 183 days, 99% within 256 days), sample storage time (95% within 127 days, 99% within 202 days), and BMI (95% ±0.4 kg/m^2, 99% ±0.6 kg/m^2). In the end 752 out of the 778 case-control pairs were used, due to insufficient sample availability.

All cases were classified into aggressive or non-aggressive. Fulfilment of at least one of the following criteria was sufficient for inclusion into the aggressive subgroup: Poor tumour differentiation (World health organisation grade 3 or Gleason score 8–10), non-localised tumour (T3–4), presence of lymph node or bone metastases (N1 or M1), serum PSA levels >50 ng/mL at time of diagnosis, or fatal prostate cancer by January 2017. 165 cases were classified as aggressive, while 587 were classified as non-aggressive.

The cases were additionally classified by age at baseline. The subgroups of 40 and 50 years of age at baseline were pooled together into a subgroup of 326 pairs (45+281) and the subgroup of 60-year olds contained 426 pairs. The study was approved by the Research Ethics Committee of Umeå University Hospital and by the Regional Ethical Review Board in Uppsala, Sweden (2013/124).

4.4 Liquid chromatography

The chromatographic separations of the samples analysed in this thesis were carried out on two different chromatographs. In papers I & II the separation was carried out using an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA). In paper III the separation was performed on an Agilent 1290 Infinity II UHPLC (Agilent Technologies). Four different chromatographic methods were used during this thesis work: A positive mode HILIC (Paper II), a negative mode HILIC (Papers I and II), and positive and negative mode reversed-phase methods (Paper III).

Positive mode HILIC (paper II)

A Waters Atlantis HILIC Silica column (3 µm, 2.1×150 mm) (Waters, Milford, MA, USA) equipped with a 10 mm guard column was used for the separations.

The following elution gradient was used (%-organic phase): 0 min 95%, 0.5 min 95%, 10.5 min 40%, 15 min 40%, 17 min 95%, and 32 min 95%. The aqueous phase A consisted of 10 mmol/L ammonium formate with 0.1% (v:v) formic acid. The organic phase B consisted of acetonitrile with 0.1% formic acid (v:v). The column oven was kept at 30 °C and the flow rate was 0.25 mL/min. The injection volume was 10 µL.

Negative mode HILIC (papers I and II)
A Waters Xbridge BEH amide column (3.5 µm, 4.6×100 mm) equipped with a 20 mm guard column was used for the separations. The following gradient program was used (%-organic phase): 0 min 85%, 3 min 30%, 12 min 2%, 15 min 2%, 16 min 85%, and 23 min 85%. The aqueous mobile phase A consisted of 95:5 (vol:vol) water:acetonitrile with addition of 20 mmol/L ammonium acetate and 20 mmol/L ammonium hydroxide. Mobile phase B was 100% acetonitrile. The flow rate was 0.40 mL/min and the column was kept at 30 °C. The injection volume was 10 µL.

Reversed phase methods (paper III)
The chromatographic separations were carried out on polarity-dedicated Waters BEH C18 (1.7 µm, 2.1×100 mm) columns. The column was kept at 60 °C, and the flow rate was 0.35 mL/min. The injection volume was 5 µL. The following gradient program was used in both positive and negative mode (%-organic phase): 2% to 70% in 4 min, 70–98% in 1.5 min, 98% for 2.9 min, 2% in 1 min, 2% for 2.6 min. For positive mode separations 0.1% formic acid in water and 0.1% formic acid in methanol were used as aqueous and organic eluents respectively. For the negative mode separations 6.5 mM ammonium bicarbonate in water and in methanol (soluble in methanol upon sonication) were used as aqueous and organic eluents respectively.

4.5 Mass spectrometry

All mass spectrometry data presented in this thesis were collected on a Bruker maXis Impact (Bruker Daltonics, Bremen, Germany) quadrupole orthogonal time-of-flight hybrid mass spectrometer equipped with an electrospray ion source.

For all the works presented in this thesis, the mass spectrometer was operated at a capillary voltage of ±4 kV and a plate offset voltage of ±500 V depending on the analysis mode. Desolvation was carried out using 200 °C nitrogen gas at 8 L/min. The nebuliser pressure was set to 2 bar. The digitiser sample rate was set at 4 GHz. Profile spectra were collected at a rate of 1 Hz when coupled to

HPLC methods (Papers I and II) or 5 Hz for both the MS and MS/MS UHPLC methods (Paper III). MS/MS fragmentation was achieved via CID using helium as collision gas (Papers II and III).

4.6 Data analysis

Paper I

The data was processed using Compass DataAnalysis 4.1 (Bruker Daltonics, Bremen, Germany). Extracted ion chromatograms (XIC) for *m/z* 179.0561, corresponding to monoisotopic glucose and *m/z* 181.0687, corresponding to D-[6,6-$^{2}H_2$]-glucose were generated with an extraction width of ±0.01 *m/z* and smoothed with one cycle of Gaussian smoothing with a 2 second width. The average mass spectrum over the *m/z* 179.0561 chromatographic peak was determined and the ratio of the profile mass spectrum signal areas was calculated, after subtraction of the influence of natural isotopes to the *m/z* 181.0687 signal.

The standard method of using chromatogram areas was deemed invalid at the low enrichment levels observed in the study. Every data point on an extracted ion chromatogram corresponds to the mass spectral peak intensity of the extracted ion in the corresponding mass spectrum. When two mass spectral signals are not sufficiently resolved, the signal peak will broaden while the apex intensity is unchanged. The change in mass spectral signal areas will thus reflect the increase in enrichment while the corresponding chromatogram area will not change. The result of this effect is presented in Figure 9 showing calibration curve data from the same samples using either the XIC area or the profile mass spectral peak area. The plateauing effect is clearly visible for the XIC data below the level of the natural abundance of the M+2 isotopologue of glucose (1.43%), while the linearity is preserved for the mass spectral area-based data.

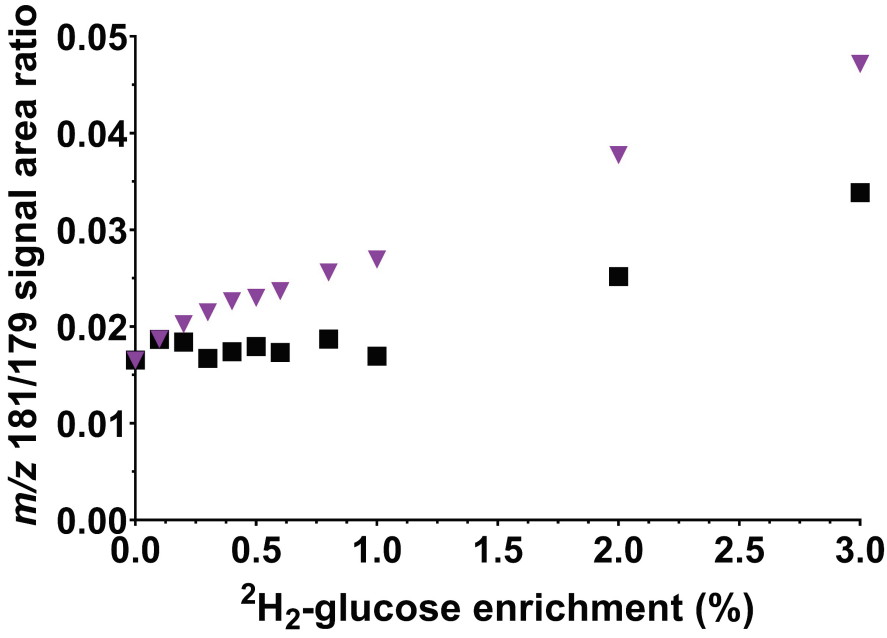

Figure 9. Comparison of extracted ion chromatogram (XIC) area and profile mass spectral area ratios for preparation of low-level enrichment calibration curves in situations of insufficient resolution. Black squares, XIC area ratios; purple triangles, profile mass spectral area ratios. Note the loss of linearity and plateauing for the XIC curve below the natural abundance of M+2 glucose (1.43%).

Paper II

Lists of metabolites for targeted screening encompassing a wide variety of pathways based on the accurate masses of the metabolite $[M\pm H]^{\pm}$ adducts were compiled. XIC:s of these features were plotted with an extraction width of ±0.01 *m/z* for the data from the experiment without ^{13}C-labelling. Gaussian peaks with an apex signal-to-noise ratio>3 detected in the respective XIC:s were assigned an RT and transferred to a list of detected features.

The lists of detected features were expanded with the accurate masses of all ^{13}C-isotopologues of the metabolites and used to investigate the data from the experiments with U-$^{13}C_6$-D-glucose and U-$^{13}C_{16}$-palmitate. All data were processed using Bruker Compass DataAnalysis 4.1 and chromatogram areas were used for statistical analysis. Features found discriminating by statistical analysis were validated by authentic standards based on accurate mass, RT, and MS/MS spectra comparison. Discriminating phospholipids were cross-referenced to authentic standards sharing the same head group and fragmentation pattern (Godzien *et al.* 2015).

Paper III

The data was processed by the R-package XCMS (Smith *et al.* 2006), version 3.4.2, including peak picking, grouping, and filling of zero intensity features. The peak picking was performed with the '*centWave*' algorithm (Tautenhahn *et al.* 2008). Grouping was performed by the '*density*' method and zero-intensity filling by the '*fillPeaks*' algorithm and '*chrom*' method.

Batch effects could be observed, and the number and identities of the detected features differed between batches. To account for this, the dataset was reduced to containing only the features present in all batches. This reduction was achieved by matching features using an in-house algorithm based on polarity-wise similarities in *m/z* and RT. The matching was performed in three steps as follows: 1) All possible matches within adequately defined median RT and *m/z*-thresholds were identified batchwise. This resulted in clusters including features from the four batches, and in inter-batch alignment of both RT and *m/z*. 2) In each cluster, only features that matched features in all other batches were allowed (*i.e.* cliques), while the rest were deleted. 3) In case of match multiplicity, a penalisation score was created to decide the best match. This score was defined after batch-to-batch alignment as the Euclidean distance of the RT and *m/z*-differences (both normalised) between each of the features in the match. Correction for intra-batch instrumental drift was not performed, since all statistical comparisons were performed pairwise with the paired samples analysed consequentially in random order.

Features of interest were annotated based on the measured accurate mass and detected MS/MS fragments. The databases Human Metabolome Database (Wishart *et al.* 2017), Lipidmaps (Sud *et al.* 2006), METLIN (Guijas *et al.* 2018), mzCloud, and MassBank were used to assist in the annotation. The annotation of phospholipid features was additionally assisted by previously presented guidelines, consisting of lists of ions characteristic for phospholipid class identification for different adducts and ionisation modes, common fragmentation patterns, and lists of fragments characteristic for specific side chains (Godzien *et al.* 2015). The relative positions (when applicable) of the phospholipid side chains were determined based on the accurate mass and signal strength of detected lysophosphatidylcholine and lysosphingomyelin CID-fragments. Secondary carbocations formed by loss of the side chain at the *sn*-2 position of the glycerol or serinol backbone are more stable than the primary carbocations formed by loss at the *sn*-1 position, resulting in stronger signals. Based on the signal strengths of the two potential lyso-fragments and the differences between their accurate masses and the parent ion mass, the phospholipid side chain lengths, degrees of unsaturation, and relative position can thus be annotated.

4.7 Statistical analysis

Paper I

General linear mixed models were used for investigating differences in glucose, insulin, and GIP concentrations. The baseline concentrations at the initiation of breakfast ingestion were used as covariates and the participants were set as a random factor in order to account for the crossover design. Time point, treatment, and time×treatment interaction were used as fixed factors. Differences in glucose RaE were analysed using time interval, treatment, and interval×treatment interaction as fixed factors with participants as a random factor. When a significant fixed factor was observed, the individual time points or intervals were investigated by GLMM:s using the treatment as a fixed factor and the participants as a random factor. The analyses were performed using Minitab version 18 (Minitab Inc., State College, PA) and p-values<0.05 were considered significant.

Additionally, area under the curve (AUC) calculations were performed for glucose, insulin, and glucose RaE. The AUC-values were compared by GLMM with treatment as fixed factor and participants as a random factor. The AUC calculations were carried out using GraphPad Prism 5.03 (GraphPad Software, La Jolla, CA).

Paper II

Multivariate data analysis was performed on the cell extract data using SIMCA 16 (Sartorius Stedim Data Analytics, Umeå, Sweden) on the signal areas of the XIC:s from the experiment using unlabelled D-glucose and palmitate in order to find pathways and metabolites influenced by the treatments. PLS-DA was applied using the glucose levels, palmitate treatment, and their combination as class variables, respectively. Inter-passage variation was accounted for by normalising the signal area of a metabolite to the total signal area of the metabolite within the passage. Outliers were identified by PCA and Hotelling's T^2 (95% confidence interval) and subsequently excluded. The models' significances were ascertained via CV-ANOVA, with a p-value<0.05 considered significant (Eriksson *et al.* 2008). Features with a VIP≥1.0 and VIP-95% confidence interval>0 in at least one component, in one or several of the three models, were considered tentatively discriminating.

To evaluate the effects of the treatments, the treatment levels, and their interaction on the features found tentatively discriminating by the multivariate analysis, GLM:s with palmitate concentrations (0 or 0.5 mmol/L), glucose concentrations (0, 5.5, or 15 mmol/L), and palmitate×glucose interaction as

fixed factors were used. Features with at least one significant fixed factor were considered discriminating. These features were further investigated in the culture media by GLM:s using the same fixed factors. The insulin secretion was analysed by a GLMM using the same fixed factors and cell passage number as a random factor. The Bonferroni method was used for pairwise comparisons within the fixed factors.

For evaluating the effect of the treatments on the flux of glucose carbons individual GLMM:s were built for each isotopologue, with glucose level, palmitate level, and palmitate×glucose as fixed factors and cell passage number as a random factor. The 0 mmol/L glucose treatments were excluded from analysis for all isotopologues except the monoisotopic isotopologue, due to the inconceivability of ^{13}C-enriched isotopologue detection when the natural distribution has been subtracted and no ^{13}C has been added. The contributions of the natural isotopes were subtracted and Bonferroni correction was used to account for multiple testing of the fixed factors according to the number of detected isotopologues. Missing values were imputed by feature-wise half minimum (Wei *et al.* 2018) for both the experiments with and without ^{13}C-labelling, and values were $\log_{10}$-transformed when necessary to achieve normality of the model residuals. The univariate analyses were performed using Minitab version 19 and p-values<0.05 were considered significant.

Paper III

A multi- and univariate approach was taken to analyse the untargeted metabolomics data and to identify features discriminating between the cases and controls. The prospective associations of the discriminating features with prostate cancer risk were furthermore investigated by conditional logistic regression.

The orthogonal projections to latent structures approach for paired samples called OPLS-EP was used (Jonsson *et al.* 2015). Differences in signal intensities for each feature between the paired samples were calculated, the data was unit variance scaled and modelled towards an all-ones y-vector. Fifteen models were built in total, with three models each being built for the full set, the non-aggressive only subset, the aggressive only subset, subset with participants of 40 and 50 years of age at baseline, and participants with 60 years of age at baseline. Model 1 contained all pairs, model 2 excluded the extreme 1% of cases based on Hotelling's T^2 range, and the third model excluded the extreme 5% of the pairs. This three-model method was performed in order to encompass all features possibly associated with prostate cancer risk, while also accounting for potential outliers. The significances of the models were analysed by CV-ANOVA (Eriksson *et al.* 2008) and p-values<0.05 were considered significant. Features

with a VIP≥1.5 and VIP-95% confidence interval>0 were considered discriminating between cases and controls. The multivariate statistics were performed using SIMCA 16.

A conservative univariate analysis was additionally conducted. The two-sided Wilcoxon signed-rank test was used, with the threshold for statistical significance being p-values<4.55×10^{-5}, based on Bonferroni correction for 1100 tests (the total number of detected features). The univariate statistics were performed using R 3.5.1.

A four-step filtering procedure was used to identify which significantly discriminating features that are prospectively associated with prostate cancer risk. The associations of the discriminating features with prostate cancer risk were evaluated by CLR for calculation of OR:s. Log_2-transformed signal intensities were used as explanatory variables with the case-control pairs as strata. A p-value<0.05 was considered significant for features of interest by the multivariate or univariate approach. For features yielding CLR models with significant p-values<0.05, without having been significant in the multivariate and univariate analysis, Bonferroni correction was applied. This did however not result in any additional features. The CLR analysis was performed in SAS, version 9.4 (SAS Institute, Cary, NC).

The features of interest were further tested for covariance through the variables age, BMI, smoking status, and alcohol consumption. Features were excluded if the CLR p-value exceeded 0.05, or if the OR changed more than 10% after the covariates were included in the model. Features originating from the same metabolite (^{13}C isotopes, Na^+-adducts, and in-source H_2O or NH_3-loss) were manually removed and as a final filtering step the XIC:s were plotted by the R package peakPantheR (v 1.0.0) (Wolfer & Correia 2020) and visually inspected to ensure a Gaussian nature of the features and to validate the results of the peak picking process. The workflow and the effect of the different filtering steps on the number of remaining features is presented in Figure 10.

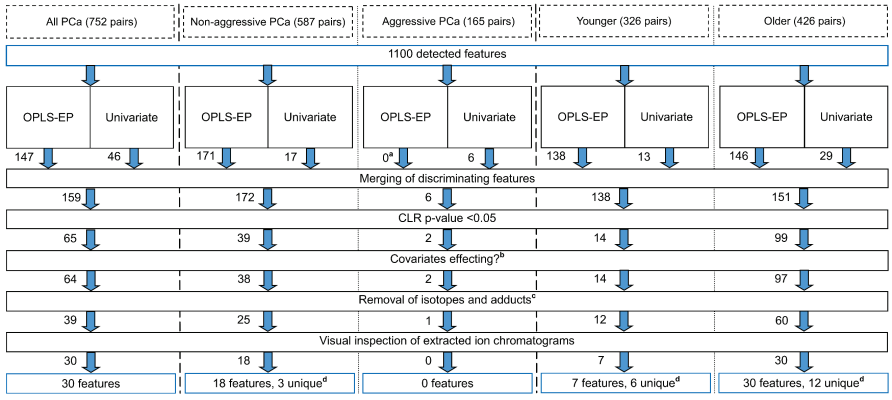

Figure 10. Flowchart presenting the workflow for extracting the metabolite features associated with overall risk of future prostate cancer (PCa) as well as after stratification by disease aggressiveness or baseline age (younger, 40 and 50 years; older, 60 years). [a]Orthogonal projections to latent structures-effect projections (OPLS-EP) models not significant via cross-validated analysis of variance (CV-ANOVA), $p>0.05$. [b]Features were excluded if the conditional logistic regression (CLR) p-value rose above 0.05 or if the odds ratio changed more than 10% after the covariates exact age, body mass index, alcohol consumption (<10, 10–19, 20–39, ≥40 g/day), and smoking status (no, past, current, unknown) were included in the CLR-model. [c]Isotope and adduct filtering was made by assessing if features with the same retention time differed in *m/z* corresponding to ^{13}C isotope differences ($\Delta=n\times1.0033$), Na^+-adducts ($\Delta=21.9819$), or in-source H_2O ($\Delta=18.0153$) or NH_3 ($\Delta=17.0266$)-loss, retaining (by decreasing priority) the $[M\pm H]^{\pm}$ adduct, the lowest *m/z* isotopologue, or an in-source fragment rather than sodium adduct. [d]Unique features are features which were not found significantly associated in the full dataset of 752 pairs.

5 Results and discussion

Can differences in glucose rate of appearance in blood explain insulin response deviations (Paper I)?

In accordance with observations in previous studies (Leinonen *et al.* 1999; Juntunen *et al.* 2003), deviations in the postprandial glucose and insulin patterns were observed. No statistically significant treatment or time×treatment differences were observed in the plasma glucose concentrations. Neither was there any observed difference between treatments at individual time points (Figure 11A), nor a difference in 90 min AUC (Figure 11B). A significant treatment effect but no significant time×treatment difference was observed for insulin. Additionally, the insulin responses at 60 and 75 minutes after ingestion of WMR were significantly lower than after intake of WB ($p<0.05$) (Figure 11C). The 90 min insulin AUC was significantly lower after WMR ingestion as well ($p<0.05$) (Figure 11D). A significant treatment effect was also observed for GIP ($p<0.001$), with significantly lower GIP responses at 60 and 90 minutes after WMR ingestion (Figure 11E).

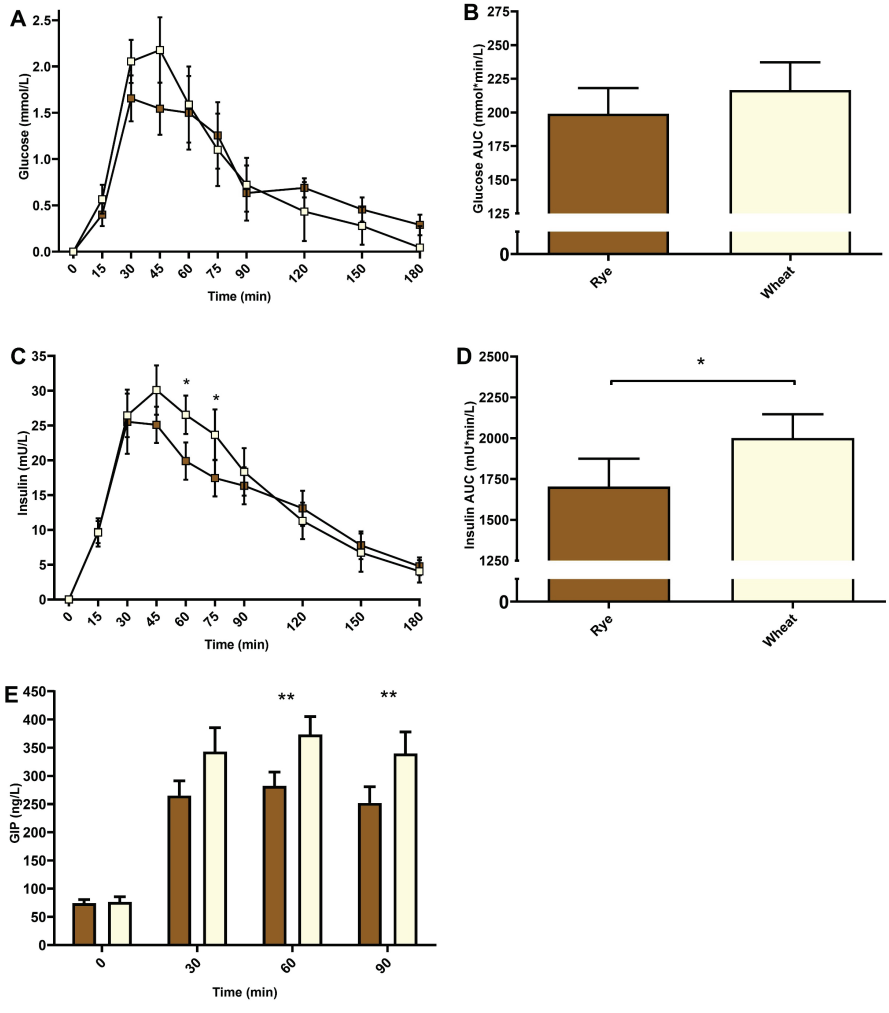

Figure 11. Glucose and insulin responses after ingestion of 50 g of available carbohydrates in the form of wholemeal rye or refined wheat bread. Postprandial blood glucose (A) and insulin (C) concentrations and corresponding 90 min areas-under the curve (AUC:s) (B, D). Postprandial gastric inhibitory peptide (GIP) concentrations (E). Wholemeal rye (brown). Refined wheat (cream). Mean±SEM (n=9). *Significant treatment difference, $p<0.05$. **$p<0.01$.

The glucose RaE in blood was measured using a single tracer stable isotope approach. A significant treatment effect ($p<0.05$) was observed, as well as a significantly lower RaE for the interval 0–15 min after WMR ingestion ($p<0.05$) (Figure 12A). Additionally, the glucose RaE 90 min AUC was significantly lower after ingestion of the WMR breakfast ($p<0.01$) (Figure 12B).

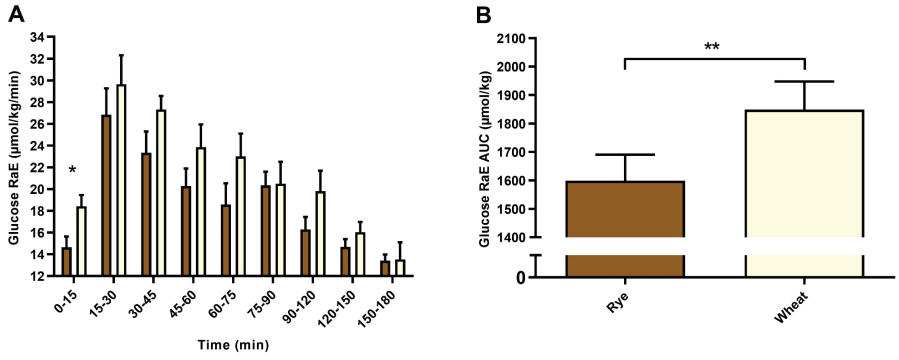

Figure 12. Rate of glucose appearance in blood plasma after ingestion of 50 g of available carbohydrates in the form of wholemeal rye or refined wheat bread. Rate of glucose appearance (A) and corresponding 90-min AUC (B). Wholemeal rye (brown). Refined wheat (cream). Mean±SEM (*n*=9). The global treatment and interval×treatment effects, as well as treatment effects at each interval and for the AUC were analysed by general linear mixed models with participant as a random factor. *Significant treatment difference, $p<0.05$. **$p<0.01$.

The discrepancy in insulin response might thus, at least in part, originate from differences in intestinal glucose uptake kinetics rather than from differences in glucose concentrations. Previously published micrographs of breads similar to the ones used in the current study revealed a more compact structure with higher botanical integrity in the WMR, and showed a slower *in vitro* hydrolysis of the WMR (Juntunen *et al.* 2003). The higher botanical integrity of the WMR would thus mean lower substrate availability for digestive enzymes in the gut. This would lead to a slower rate of release of glucose from the bread substrate, a concomitantly lower gut uptake, and lower glucose RaE in the blood, yielding a lower insulin response. Strong correlations between glucose RaE, insulin, and GIP have been shown previously for meals of varying digestibility (Wachters-Hagedoorn *et al.* 2006) and the observed differences in GIP indicate that the effect of glucose RaE on insulin could be mediated by GIP.

These observations suggest that foodstuff microstructure and the rate of digestion have profound impact on biological responses and that altering the microstructure can change the responses, as has been previously observed (Johansson *et al.* 2015). A recent meta-analysis assessing several kinds of foodstuff has also found that reduction of glucose RaE results in significantly lower postprandial insulin responses (Boers *et al.* 2019). The role of RaE of amino acids from intact and hydrolysed protein sources on insulin and glucagon responses has also been shown previously, albeit without a stable-isotope tracer approach (Calbet & MacLean 2002).

The primary goal of the study was to investigate whether the discrepancy in postprandial insulin responses after rye and wheat bread ingestion, called the

"rye factor", could be explained by differences in glucose kinetics. The current results indicate that differences in glucose kinetics might indeed be sufficient to explain the observed phenomenon.

The rate at which a stimulant is applied, and not only the amount of stimulant, impacts the degree of the exerted biological response in the present study. This phenomenon might seem trivial but appears to have been overlooked. The effect of rate contra concentration has previously been observed for nerve potentials induced by olfactory events as well (Han *et al.* 2018), and it might be highly applicable in physiological and medical settings yet unknown.

What metabolic alterations can be seen in a human β-cell line upon hyperglycaemic and hyperlipidaemic treatment (Paper II)?

An array of metabolites from several different pathways *i.a.* the Krebs cycle, non-essential amino acids, and phospholipids, were found to be associated with glucose and/or palmitate incubation by multi- and univariate statistical analysis. The p-values presented correspond to the GLM and GLMM fixed factor effects.

The Krebs cycle intermediates citrate, α-ketoglutarate, succinate, fumarate, and malate were detected and increased by glucose and palmitate to various degrees. No significant fixed factor effects were observed for oxaloacetate (Figure 13).

Culture with U-$^{13}C_6$-D-glucose led to observed incorporation of ^{13}C into the isotopologues of the Krebs cycle intermediates with the exception of succinate and oxaloacetate. A significant effect of palmitate on the degree of incorporation was observed for the M+2 isotopologue of α-ketoglutarate, with palmitate treatment decreasing the degree of ^{13}C-incorporation into the isotopologue ($p<0.05$). For the M+3–4 isotopologues of fumarate and malate, palmitate increased the degree of ^{13}C-incorporation, ($p<0.0001$) (Figure 14). Incubation with U-$^{13}C_{16}$-palmitate caused no detectable incorporation of ^{13}C into the Krebs cycle intermediates. Treatment of the β-cells with glucose and palmitate affected the insulin secretion. Insulin secretion exhibited a significant increase by glucose, palmitate, and glucose×palmitate interaction ($p<0.0001$, $p<0.0001$, and $p<0.05$, respectively) (Figure 15).

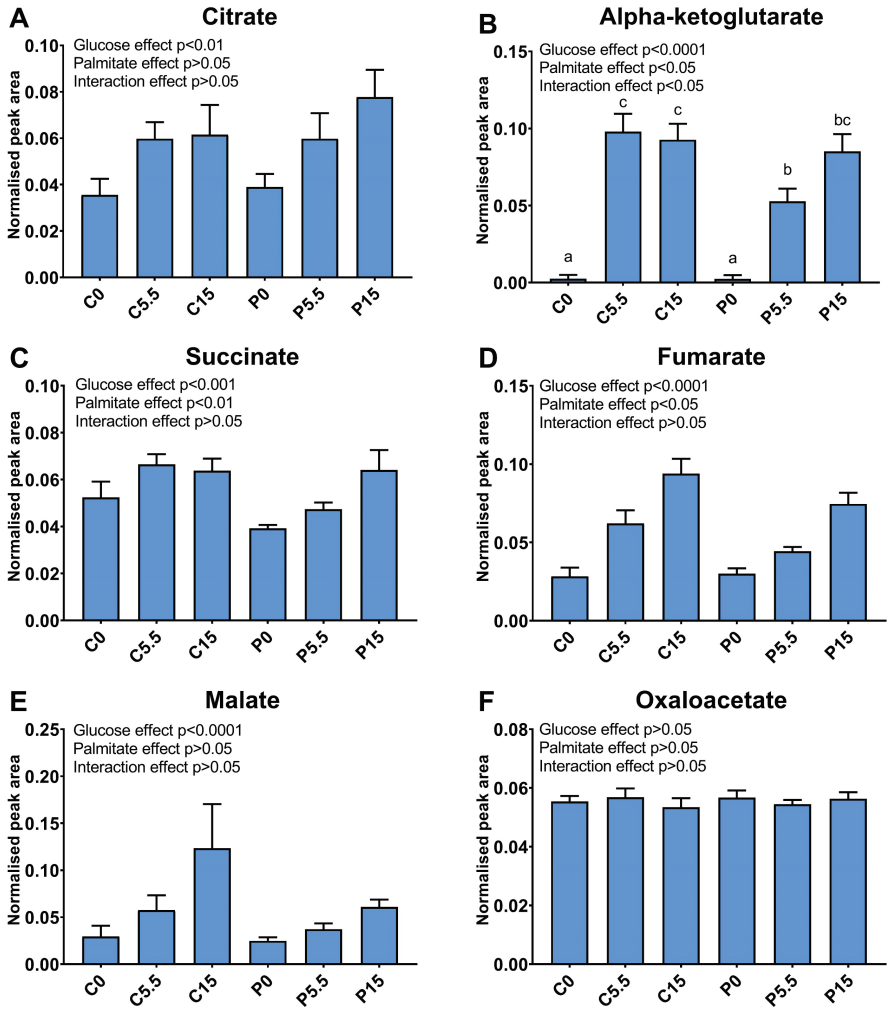

Figure 13. Responses of Krebs cycle intermediates after treatment of EndoC-βH1 cells with D-glucose and palmitate. Cells were incubated with 0, 5.5, or 15 mmol/L D-glucose with (P) or without (C) 0.5 mmol/L palmitate. The signals were normalised to the total signal area of proline within the passage to account for inter-passage variation. General linear models (GLM:s) with palmitate level, glucose level, and glucose×palmitate as fixed factors were used. Groups not sharing a common letter were significantly different (Bonferroni pairwise comparisons, $p<0.05$). Mean±SEM (n=9).

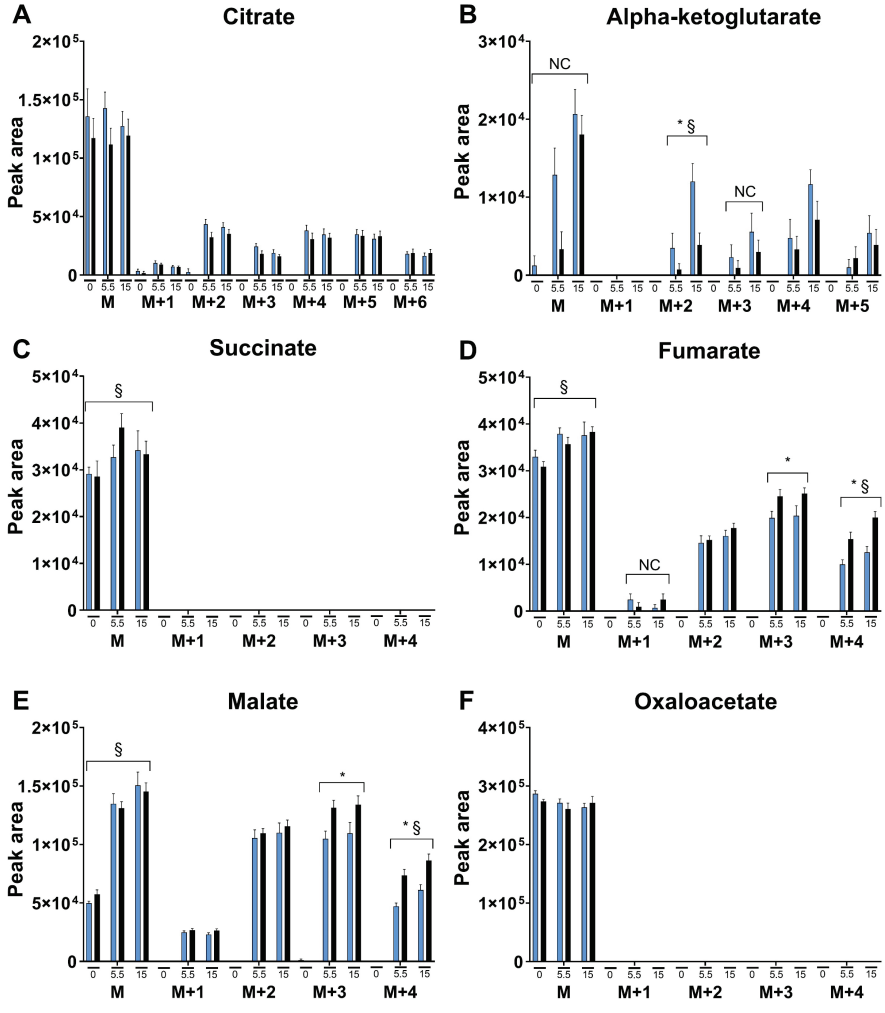

Figure 14. Responses of Krebs cycle intermediate isotopologues after treatment of EndoC-βH1 cells with U-$^{13}C_6$-D-glucose and palmitate. Cells were incubated with 0, 5.5, or 15 mmol/L U-$^{13}C_6$-D-glucose with (black) or without (blue) 0.5 mmol/L palmitate. Each isotopologue was analysed individually by a general linear mixed model (GLMM), with Bonferroni correction for multiple testing being applied for the fixed factors according to the number of detected isotopologues. The 0 mmol/L glucose treatments were excluded from analysis for all isotopologues except the monoisotopic isotopologue, due to the inconceivability of ^{13}C-enriched isotopologue detection when the natural distribution has been subtracted and no ^{13}C has been added. NC=non-normal residuals, model not considered. *=palmitate fixed factor effect, $p<0.05$. §=glucose fixed factor effect, $p<0.05$. Mean±SEM (n=9).

Because of the β-cells' glucose sensing role, they rapidly equilibrate their internal glucose levels to the level in their surroundings and rapidly metabolise it for adenosine triphosphate (ATP) production and subsequent insulin

exocytosis (Lewandowski *et al.* 2020). Whereas the glucose taken up during sensing exceeds the β-cells' basic needs, the excess must be diverted into other pathways in order to enable further detection of changes in levels of circulating glucose, since β-cells are unable to block glucose uptake (Mugabo *et al.* 2017). The potentiating effect of palmitate on the higher isotopologues of fumarate and malate indicates that co-incubation causes increased mitochondrial metabolism with a high flux through the Krebs cycle.

Glucose carbons can enter the Krebs cycle via the action of either pyruvate carboxylase (PyC) or pyruvate dehydrogenase (PDH). The flux through both pathways, as well as the PyC/PDH flux ratio increase with glucose, and during incubation in 9 mmol/L glucose the PyC arm accounts for circa 20% of the carbon flux in INS-1 832/13-cells (Alves *et al.* 2015). Taken together these findings indicate that changes in flux might be involved in regulation of GSIS. The regulating role of PyC in maintaining GSIS through involvement in several pathways has been reviewed previously (Jitrapakdee *et al.* 2010) and increased glucose-stimulated insulin secretion (GSIS) by higher glucose has been shown to correlate with pyruvate cycling rather than PDH-conversion in murine β-cell lines (Lu *et al.* 2002). Pyruvate cycling in β-cells consists of two main cycles, the pyruvate-citrate cycle and the pyruvate-malate cycle, which both transport the corresponding Krebs cycle intermediates into the cytoplasm where they are metabolised to pyruvate which can re-enter the Krebs cycle (Lu *et al.* 2002). Whether palmitate also increases the flux through the pyruvate cycles could not be derived from the current data, but might be discernible by utilising isotopomer analysis.

The detected Krebs cycle intermediates could be divided into two groups based on the observed signal intensity patterns. The responses of all intermediates except oxaloacetate were increased by glucose (Figure 13). However, during incubation with U-$^{13}C_6$-D-glucose it could be observed that there was a significantly higher enrichment of ^{13}C in the M+3–4 isotopologues of fumarate and malate upon glucose and palmitate co-incubation compared to the other intermediates (Figures 14D and 14E). Fumarate and malate can be reversibly interconverted and are affected more by the PyC/PDH flux ratio due to the close-to-equilibrium between malate and oxaloacetate, which is in part formed by PyC-action. This is indicative of a preference of PyC-shuttling during glucose and palmitate co-incubation. The other parts of the Krebs cycle do not exhibit the same enrichment pattern and might thus be dominated by the activity of PDH. The current data thus suggests that palmitate co-incubation with glucose might increase the PyC/PDH activity ratio further than high glucose alone (Alves *et al.* 2015) and amplify the GSIS even more, as was observed (Figure 15).

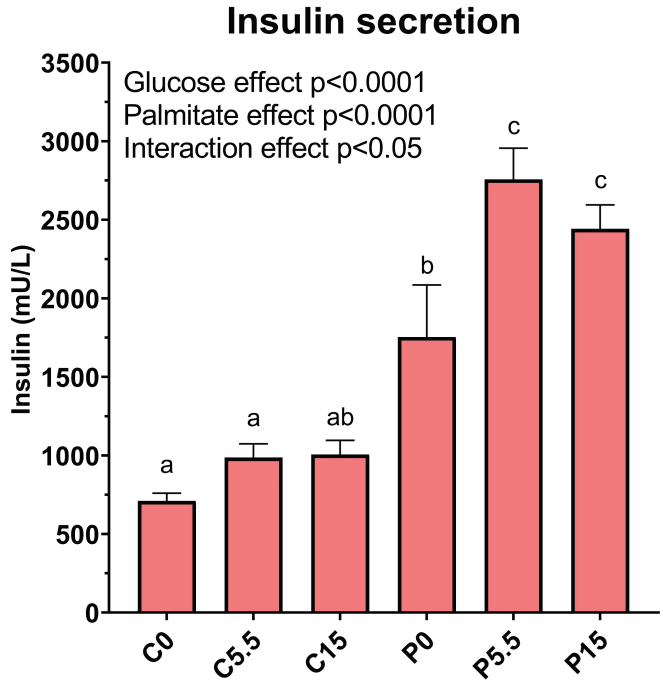

Figure 15. Insulin secretion as measured by ELISA after treatment of EndoC-βH1 cells with glucose and palmitic acid. Cells were incubated with 0, 5.5, or 15 mmol/L glucose (unlabeled and ^{13}C-labeled) with (P) or without (C) 0.5 mmol/L palmitate (unlabeled and ^{13}C-labeled). Groups not sharing a common lowercase letter were significantly different by a general linear mixed model (GLMM) with cell passage number as a random factor. For the glucose treatment level as fixed factor, the 5.5 and 15 mmol/L treatments were significantly different than the 0 mmol/L treatment. Pairwise comparisons were carried out using the Bonferroni method, $p<0.05$. Bars represent mean±SEM (n=27).

Furthermore, the fate of the ^{13}C incorporated into the Krebs cycle intermediates was investigated. The metabolomic screening indicated that intracellular proline levels were affected by the treatments. Highly significant glucose and palmitate effects were detected ($p<0.0001$) as well as a significant interaction effect ($p<0.05$), all of which increasing the proline response (Figure 16A). Incubation of the cells with U-$^{13}C_6$-D-glucose caused incorporation of ^{13}C from the media glucose, with palmitate treatment significantly increasing the incorporation into the heavier isotopologues M+4 and M+5 ($p<0.05$) (Figure 16B). The *de novo* synthesised proline contained carbon both from the media glucose and from another internal carbon pool (possibly glutamate) (Figure 16C). Incubation with U-$^{13}C_{16}$-palmitate yielded no detectable ^{13}C-incorporation into proline.

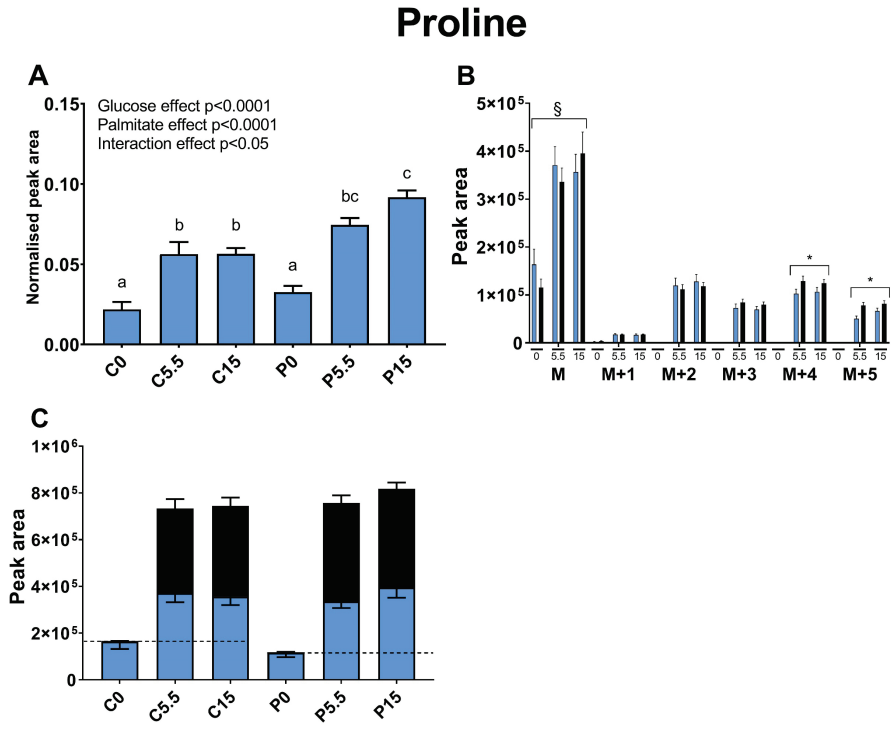

Figure 16. Proline responses after treatment of EndoC-βH1 cells with D-glucose or U-$^{13}C_6$-D-glucose. (A) Cells were incubated with 0, 5.5, or 15 mmol/L glucose with (P) or without (C) 0.5 mmol/L palmitate. The signals were normalised to the total signal area of proline within the passage to account for inter-passage variation. Data analysed by a general linear model (GLM). Groups not sharing a common letter were significantly different ($p<0.05$). (B) Incubation with U-$^{13}C_6$-D-glucose showed incorporation of ^{13}C from glucose in media into proline. Incorporation of ^{13}C into the M+4–5 isotopologues was significantly higher with palmitate treatment. Control (blue), palmitate (black). Each isotopologue was analysed by an individual general linear mixed model (GLMM) with cell passage number as a random factor. Bonferroni correction for multiple testing was applied for the fixed factors according to the number of detected isotopologues. The 0 mmol/L glucose treatments were excluded from analysis for all isotopologues except the monoisotopic isotopologue, due to the inconceivability of ^{13}C-enriched isotopologue detection when the natural distribution has been subtracted and no ^{13}C has been added. *=palmitate fixed factor effect, $p<0.05$. §=glucose fixed factor effect, $p<0.05$. (C) Addition of glucose causes production of proline from other carbon sources than media glucose. Monoisotopic proline (blue). Sum of proline M+1–5 isotopologues (black). The dashed line indicates the levels of internal carbon recruitment. Mean±SEM (n=9).

A glucose-dependent increase in media proline was detected ($p<0.01$), but no significant palmitate or interaction effects were detected for the media proline (Figure 17A). Additionally, culture with U-$^{13}C_6$-D-glucose showed that the carbon backbone of the excreted proline is to a large extent formed from carbon originating from glucose in the culture media (Figure 17B), indicating efflux of *de novo* synthesised proline.

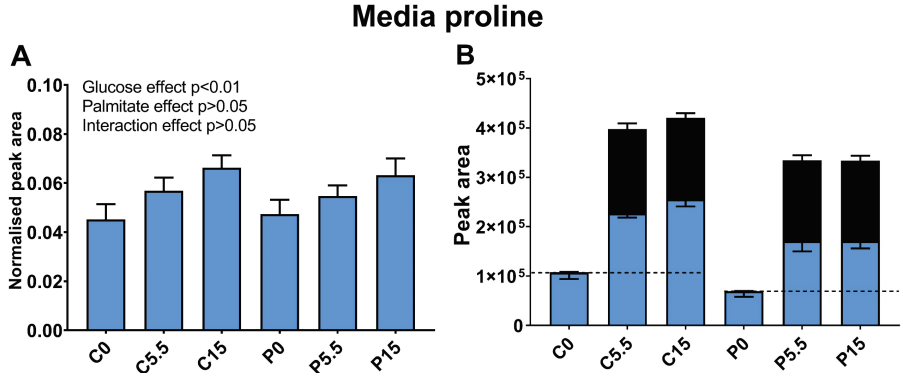

Figure 17. Proline responses in incubation media after treatment of EndoC-βH1 cells in D-glucose or U-$^{13}C_6$-D-glucose. (A) Cells were incubated with 0, 5.5, or 15 mmol/L glucose with (P) or without (C) 0.5 mmol/L palmitate. The signals were normalised to the total signal area of proline within the passage to account for inter-passage variation. Data analysed by a general linear model (GLM). (B) Media glucose carbons are excreted into media in the form of proline. Monoisotopic proline (blue), sum of proline M+1–5 isotopologues (black). The dashed line indicates the levels of internal carbon recruitment. Mean±SEM (n=9).

De novo synthesised proline using the carbon from the media glucose could be detected both in the cells (Figure 16C), as well as in the incubation media post-treatment (Figure 17B). The production and excretion of proline might be a means of carbon detoxification which has not been shown previously in β-cells. Re-routing of carbon towards proline maintains flux into the Krebs cycle, while reducing the production of ATP, reducing power, and harmful reactive oxygen species. Proline biosynthesis via reduction of 1-pyrroline-5-carboxylate by reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) has been shown to be important for maintaining flux through the glycolysis and for supplying pyridine nucleotides via the oxidative arm of the pentose phosphate pathway in cancer cells (Liu *et al.* 2015). This has not been observed previously in β-cells, and it is unknown whether it fills the same purpose in β-cells. In order to produce proline from glutamate originating from the Krebs cycle, two oxidoreductases with preference for NADPH are required (Sellés Vidal *et al.* 2018). NADPH is produced *i.a.* via pyruvate cycling, which has been shown to be associated with GSIS (Lu *et al.* 2002). In the case of high pyruvate cycling

activity, NADPH is formed in surplus and if palmitate increases the flux through the pyruvate cycle, then the proline formation might work as an NADPH-sink. Production of proline by the β-cells might be a way to maintain flux through glycolysis and keep up the glucose sensing capabilities, while disposing of excess carbon, ATP, and reducing power produced in the fuel sensing process. The proposed impact of glucose and palmitate on the flux of Krebs cycle and proline is summarised in Figure 18.

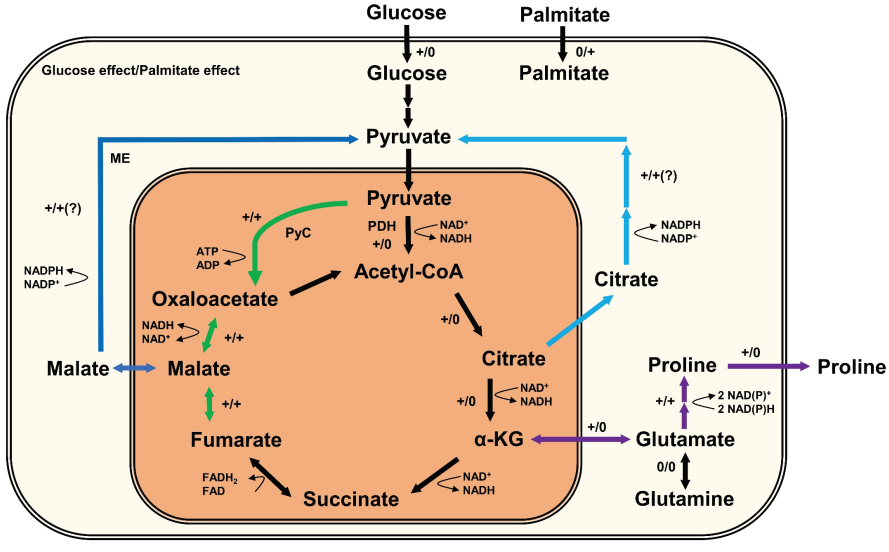

Figure 18. Proposed effect of glucose and palmitate on the Krebs cycle flux in β-cells. The pyruvate malate-cycle is highlighted in dark blue, and the pyruvate citrate cycle in light blue. The proline production pathway is presented in purple and the pyruvate carboxylase affected part of the Krebs cycle in green. α-KG=alpha-ketoglutarate, ADP=adenosine diphosphate, ATP=adenosine triphosphate, CoA=Coenzyme A, FAD(H_2)=(reduced) flavin adenine dinucleotide, ME=malic enzyme, PDH=pyruvate dehydrogenase, NAD(H)=(reduced) nicotinamide adenine dinucleotide, NADP(H)=(reduced) nicotinamide adenine dinucleotide phosphate, PyC=pyruvate carboxylase.

The fate of the palmitate in the incubation media was additionally investigated by looking at phospholipids with C16:0 side chains, *i.a.* diacyl-phosphatidylcholine (PCaa) (16:0/16:0) and diacyl-phosphatidylethanolamine (PEaa) (16:0/16:0). Treatment of EndoC-βH1 cells with glucose and palmitate was shown to change the phospholipid content of the cells. Significant glucose ($p<0.0001$) and palmitate ($p<0.0001$) effects on increasing the responses of *i.a.* PCaa(16:0/16:0) and PEaa(16:0/16:0) was observed. Potentiating interaction effects were also observed for both PCaa(16:0/16:0) ($p<0.05$) and PEaa(16:0/16:0) ($p<0.0001$) (Figures 19A and 19C).

Culture with U-$^{13}C_6$-D-glucose and unlabelled palmitate indicated that the detected phospholipids PCaa(16:0/16:0) and PEaa(16:0/16:0) were *de novo*

synthesised. The M+3 isotopologue of PCaa(16:0/16:0) and the M+3 and M+5 isotopologues of PEaa(16:0/16:0), corresponding to incorporation of a $^{13}C_3$-glycerol moiety for PCaa(16:0/16:0) with or without an additional $^{13}C_2$-ethanolamine moiety for PEaa (16:0/16:0) were detected. For PCaa(16:0/16:0) the degree of incorporation into the M+3 isotopologue increased by glucose, palmitate, and by co-incubation ($p<0.01$, $p<0.0001$, and $p<0.05$ respectively) (Figure 19B). The M+5 isotopologue of PEaa(16:0/16:0) exhibited significantly increased ^{13}C-incorpoation by palmitate ($p<0.0001$) as well as a potentiating effect of glucose and palmitate co-incubation ($p<0.05$) (Figure 19D). Furthermore, culture with U-$^{13}C_{16}$-palmitate showed incorporation of the fatty acid moiety from the incubation media into PCaa and PEaa(16:0/16:0), indicating that these phospholipids were *de novo* synthesised from both glucose and fatty acids present in the incubation media (Figure 20).

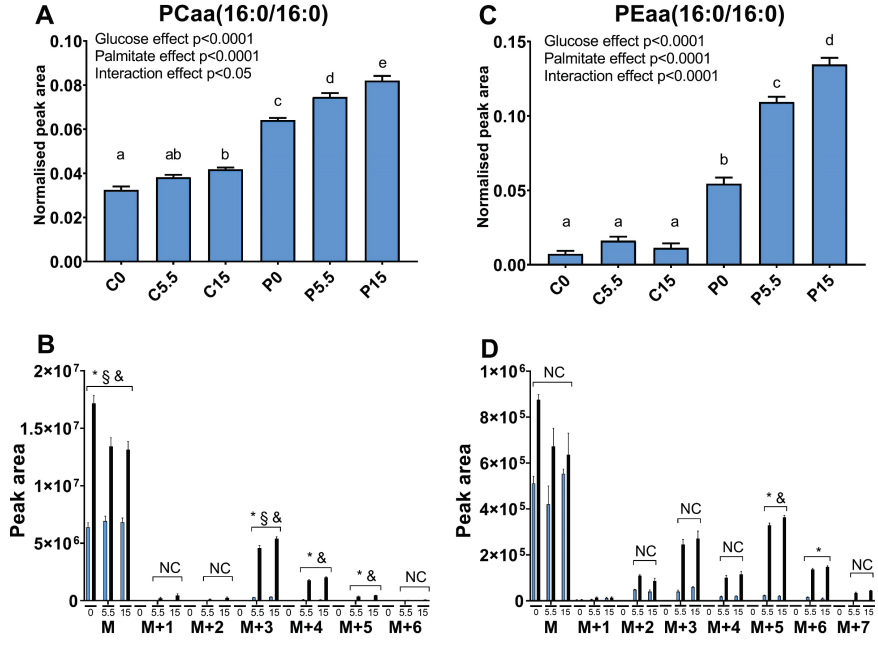

Figure 19. Responses of PCaa(16:0/16:0) and PEaa(16:0/16:0) after treatment of EndoC-βH1 cells with D-glucose or U-$^{13}C_6$-D-glucose. PCaa(16:0/16:0) (A–B) and PEaa(16:0/16:0) (C–D). Cells were incubated with 0, 5.5, or 15 mmol/L D-glucose (A, C) or U-$^{13}C_6$-D-glucose (B, D) with (P) or without (C) 0.5 mmol/L palmitate. (A, C) The signals were normalised to the total signal area of the metabolite within the passage to account for inter-passage variation. Groups not sharing a common lowercase letter were significantly different by a general linear model (GLM). Pairwise comparisons were carried out using the Bonferroni method, $p<0.05$. Culture with U-$^{13}C_6$-D-glucose (B, D) showed incorporation of ^{13}C into the glycerol moiety of PCaa(16:0/16:0) (B), and the glycerol and ethanolamine moieties of PEaa(16:0/16:0) (D). Each isotopologue was analysed by an individual general linear mixed model (GLMM) with cell passage number as a random factor. Bonferroni correction for multiple testing was applied for the fixed factors according to the number of detected isotopologues, $p<0.05$. The 0 mmol/L glucose treatments were excluded from analysis for all isotopologues except the monoisotopic isotopologue, due to the inconceivability of ^{13}C-enriched isotopologue detection when the natural distribution has been subtracted and no ^{13}C has been added. Control (blue), palmitate (black). NC=non-normal residuals, model not considered. *=palmitate fixed factor effect, $p<0.05$. §=glucose fixed factor effect, $p<0.05$. &=interaction fixed factor effect, $p<0.05$. Mean±SEM (n=9).

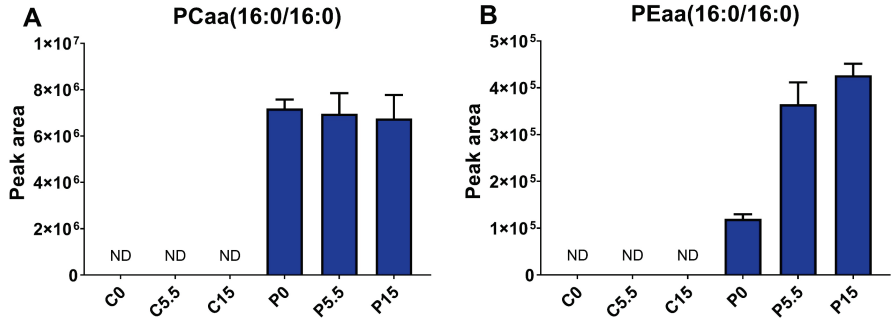

Figure 20. Responses of the M+32 isotopologues of diacyl-phosphatidylcholine (PCaa) (16:0/16:0) and diacyl-phosphatidylethanolamine (PEaa) (16:0/16:0). EndoC-βH1 cells were incubated with 0, 5.5, or 15 mmol/L glucose with (P) or without (C) 0.5 mmol/L U-$^{13}C_{16}$-palmitate. (A) PCaa(16:0/16:0). (B) PEaa(16:0/16:0). Bars represent mean±SEM (*n*=9). ND=not detected.

Addition of palmitate to the culture media significantly increased the intracellular levels of a set of phospholipids with palmitate side chains. Co-incubation of glucose and palmitate increased the flux of carbon from media glucose to the phospholipid glycerol backbone, a hitherto unpresented phenomenon in human cells, but previously observed in the rat cell line INS-1 832/3 (El-Azzouny *et al.* 2014). The increased flux into the ethanolamine moiety of PEaa has to the author's knowledge not been observed previously.

The current data shows that glucose addition stimulates production of a range of phospholipids and that palmitate co-incubation augments the effect. This indicates that the produced phospholipids might be a means of fuel excess detoxification rather than being produced as building blocks. Production of phospholipids would be a mechanism that decouples both glucose carbon and free fatty acids from energy-producing pathways and other potentially harmful detoxification pathways, as is suggested to be the case with proline production. The produced phospholipids were not excreted into the culture media however, which indicates that they might be involved in cell membrane remodelling as previously shown (Cohen *et al.* 2015). Production of glycerol from glyceraldehyde-3-phosphate for the use in the phospholipid backbone additionally requires reducing power (Sellés Vidal *et al.* 2018) and production of phospholipids might serve as a way of handling both excess carbon and reducing power at the same time, analogous to the suggested detoxification by proline production.

How β-cells deal with the excess of reducing power produced during the continuous flux through the glycolysis and Krebs cycle has not been fully resolved (Malinowski *et al.* 2020). Taken together, the glycerophospholipid and proline production pathways constitute routes of fuel excess detoxification with

common features, including the use of reducing power, which could partly explain both the handling of excess reducing power and excess carbon.

The current *in vitro* study clearly shows that both high glucose, high palmitate, and glucose-palmitate co-incubation cause metabolic alterations in a human β-cell line. Whether β-cells actually are exposed to these concentrations of free fatty acids *in vivo* has not been elucidated and the concept of lipotoxicity and accentuated glucolipotoxicity has been recently questioned, since the concentration in circulation might not be the same as the ones sensed at the membrane surface of the β-cells (Weir 2020). The nature of acute *in vivo* lipo- and glucolipotoxicity warrants further investigation.

What metabolites are prospectively associated with the risk of prostate cancer (Paper III)?

The statistical analysis and feature filtering procedure of the 1100 detected features resulted in 30 features prospectively associated with prostate cancer risk for the full set of 752 cases and 18 features for the non-aggressive only set of 587 cases. No features were retained for the aggressive only set of 165 cases. Of the subgroup of 326 cases with 40 and 50 years of age at baseline, seven features were retained. For the subgroup of cases with 60 years of age at baseline 30 features were retained. The OR:s for the final features found to be significantly associated with the risk of future prostate cancer for the full set, non-aggressive subgroup, younger subgroup, and older subgroup are graphically summarised in Figure 21. All the significant features were positively associated with the risk of future prostate cancer, except glucose (OR 0.54) which was found significant in the older subgroup.

For the 30 features found to have significant associations in the full dataset, the OR:s were between 1.68 and 1.13. For the 18 significantly associated features in the non-aggressive only dataset, the OR:s were between 1.53 and 1.21. Seven features were found significant in the subset of cases with 40–50 years at baseline, with OR:s ranging from between 1.23 and 5.49. For the 30 features found significant in the subset of cases with 60 years at baseline, the OR:s for the 29 positively associated features were between 1.14 and 2.27.

The discriminating features were annotated by MS/MS and databases. Significant associations with prostate cancer risk were found for *i.a.* PCaa:s with both odd and even side chain lengths, sphingomyelins, tryptophan, tyrosine, free fatty acids, and uric acid. These metabolites will be discussed further. There were differences between which features were found significantly associated in overall prostate cancer and in the subgroups. The parent ion *m/z* errors of identification for all features were 2.69±1.76 ppm (mean±95% confidence interval).

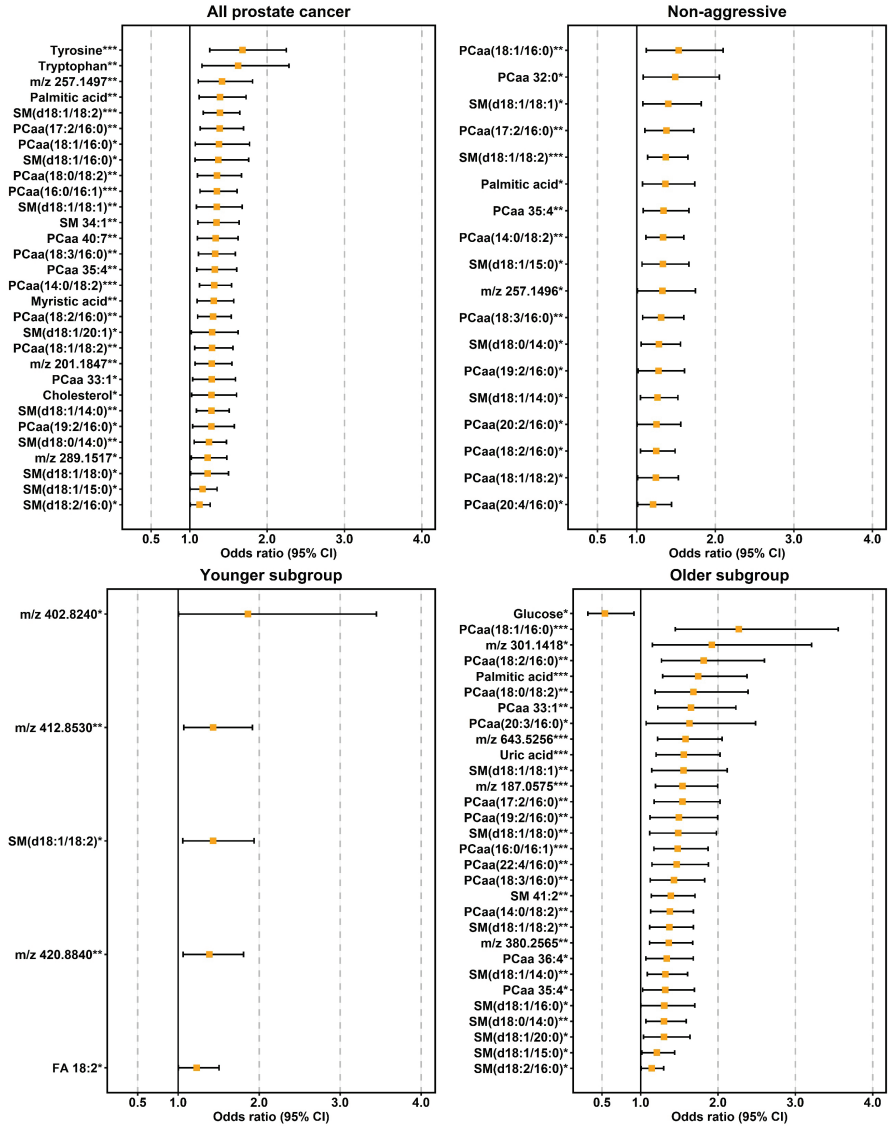

Figure 21. Odds ratios for prostate cancer risk by $\log_2$ of feature signal intensities for all cases and after stratification by disease aggressiveness or baseline age. The odds ratios and 95% confidence intervals are derived from a conditional logistic regression, *p<0.05, **p<0.01, ***p<0.001. The upper left panel encompasses all 752 matched case-control pairs, the upper right panel presents the results from the 587 pair non-aggressive subset, the lower left presents the results of the younger subset of 326 pairs with a baseline age of 40 and 50 years, and the lower right panel shows the results of the older subset of 426 pairs with a baseline age of 60 years. Two unannotated features (*m/z* 216.9227** and *m/z* 206.8938*) were excluded from the younger subgroup panel due to large confidence intervals. Note the linear x-axes. FA, fatty acid; PCaa, diacyl-phosphatidylcholine; SM, sphingomyelin.

Eight other prospective studies investigating the association of metabolite levels with prostate cancer risk have previously been published (Mondul *et al.* 2014; Mondul *et al.* 2015; Huang *et al.* 2016; Kühn *et al.* 2016; Huang *et al.* 2017; Schmidt *et al.* 2017; Röhnisch *et al.* 2020; Schmidt *et al.* 2020). The studies differed in the cohorts and methodologies used; some applied a kit-based targeted metabolomics approach while some applied an untargeted metabolomics approach. The metabolites found to have an association with the risk of prostate cancer also differed between the previous studies. In an earlier targeted metabolomics study performed on the same samples from the same cohort, positive associations between several phospholipids, *i.e.* acyl-ether phosphatidylcholines (PCae:s) and acyl-lysophosphatidylcholines (LPCa:s), particularly LPCa 17:0, and risk of prostate cancer were found (Röhnisch *et al.* 2020).

These specific metabolites were not found discriminating in the present study, maybe due to differences in sensitivity between the previous kit-based triple quadrupole MS approach in comparison with the current QqToF method and due to specificity issues with the targeted metabolomics kit used in the earlier study. For example, the feature annotated as PCaa(18:0/18:2) in the present study would overlap with phospholipids with the same amount of total side chain carbon and degrees of unsaturation, since individual side chain lengths and degrees of unsaturation are not analysed by that method. Additionally, due to lower resolution, what is reported as *e.g.* PCaa(17:2/16:0) in the present study would also overlap with what was annotated as PCae 34:2 in the previously used kit. The specificity issue and degrees of overlap in this commercial kit have been investigated before (Quell *et al.* 2019). Several other phospholipids with odd-carbon side chains, *e.g.* PCaa(19:2/16:0) and SM(d18:1/15:0), were found in the present study as well, and these are not included in the list of phospholipids analysed by the kit from the earlier study.

The largest prospective prostate cancer study to date is a 3,057 pair case-control study nested in the EPIC-multicentre cohort where the same targeted metabolomics kit approach was used (Schmidt *et al.* 2020). Therein, two phosphatidylcholines (PCaa 36:3 and PCaa 38:3) with an inverse association with prostate cancer risk were found significant after correction for multiple testing. In the present study a significant, albeit positive, association between PCaa 36:3 in the form of PCaa(18:1/18:2) could be observed in the overall and non-aggressive subgroup.

The EPIC-multicentre study included only non-localised tumours (T3–4/N1–3/M1) in the advanced-stage prostate cancer group, while in the present study, the aggressive disease subtype included both high grade (Gleason sum score≥8) and non-localised tumours, which might partly explain the observed differences.

Additionally, in the present study only overnight fasting plasma samples were used. The EPIC-multicentre study on the other hand matched case-control sets based on categories of time since last meal. The extent of variation in the concentrations of some metabolites, phosphatidylcholines and lysophosphatidylcholines included, within the ranges of the time categories used is however known to be large (Shrestha *et al.* 2017). This might thus lead to lower pre-analytical variation in the present study in comparison with the EPIC-multicentre study, which may partly account for the more pronounced association found between metabolites and risk of overall prostate cancer in the present study.

In the present study several sphingomyelins were found to be positively associated with prostate cancer risk in both the overall set and in the subgroups. The EPIC studies observed associations for some sphingomyelins, but none of the sphingomyelins in the EPIC-multicentre studies were found discriminating in the present study (Schmidt *et al.* 2017; Schmidt *et al.* 2020). One of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC) studies found a positive association between SM(d18:1/18:0) and the risk of T3 prostate cancer (Huang *et al.* 2017) and this SM was also found to be associated with prostate cancer risk in the overall and older subgroup of the present study. The association in the ATBC study was however not significant after correction for multiple testing.

The aromatic amino acids tryptophan and tyrosine were found to have the strongest positive associations with the risk of overall prostate cancer. Inverse associations between tryptophan and prostate cancer risk have been observed before, but the findings have not been significant after correction for multiple testing (Huang *et al.* 2016; Schmidt *et al.* 2020). The current findings suggest that alterations in tryptophan metabolism might be prostate cancer risk factor, and elevations of tryptophan metabolism and tryptophan concentrations have recently been shown in a study on South Korean prostate cancer patients (Khan *et al.* 2019).

Palmitic acid was found to have a significant positive association with prostate cancer risk in both the overall, non-aggressive, and the older subgroup. Palmitate has not been found significantly associated with prostate cancer risk after correction for multiple testing in the previous studies, but conflicting trends have been observed (Mondul *et al.* 2014; Mondul *et al.* 2015). Several of the phospholipids found to be associated with prostate cancer risk in the present study contained palmitate side chains. Associations between the amount of palmitate in phospholipids and prostate cancer have been observed previously (Dahm *et al.* 2012), although higher degrees of palmitic acid in phospholipids are not necessarily reflecting the concentrations of free palmitate in plasma.

Higher plasma palmitate might however be considered a biomarker of dietary palmitate intake (Jenab *et al.* 2009) or biosynthesis (Saadatian-Elahi *et al.* 2009).

A positive association between prostate cancer risk and uric acid was found in the older subgroup. This has not been observed in any of the other eight previous prospective metabolomics studies. Other studies have shown conflicting results. A positive association with prostate cancer risk was found in a prospective cohort of Japanese men in Hawaii (Kolonel *et al.* 1994), as well as in a small Swedish study (Hammarsten & Högstedt 2004), while other studies show no association (Kühn *et al.* 2017; Wang *et al.* 2019a). Another study found increased levels of uric acid to be associated with overall cancer, but surprisingly not with prostate cancer (Strasak *et al.* 2009). Uric acid is associated with the metabolic syndrome and its potential association with prostate cancer and other cancers is of interest for future research.

6 General discussion

A change in metabolic flux can be explained by several factors, as previously mentioned in chapter 3.4. Enzyme concentration, activity, and the concentrations of substrates, products, and effectors are all involved to different degrees. In a case where a cell is continuously presented with a large amount of substrate, the cell might respond to the excess by increasing the activity or the synthesis of enzymes necessary for handling the substrate, resulting in an increased flux. In the case of β-cells in a hyperglycaemic environment, higher glucose flux implies an increased overall mitochondrial activity, as observed in paper II. This would result in increased production of reactive oxygen species, which is associated with β-cell dysfunction and death (Elksnis *et al.* 2019). A chronic supply of fuel substrates to the β-cells would thus lead to a chronic increase in metabolic flux, which could be associated with progression towards β-cell dysfunction.

Changes in metabolic flux have been shown to be associated with other diseases as well. Chronic obstructive pulmonary disease (COPD) has been shown to be associated with upregulation of endogenous arginine production due to higher arginine utilisation, without differences in blood arginine concentrations (Jonker *et al.* 2016). The Epstein-Barr-Virus is associated with B cell lymphomas and it has been shown *in vitro* that it induces an increased uptake of serine and increases the flux through the one-carbon metabolism for B cells during progression toward lymphoma (Wang *et al.* 2019c).

The time scale is important to consider regarding metabolic alterations associated with disease. A short-term increase in *e.g.* mitochondrial metabolism, as observed in paper II, will not result in mitochondrial dysfunction, but chronic elevation over the better part of a lifetime sums up to a large amount of reactive oxygen species produced, every molecule of which having the possibility to cause critical damage to the mitochondrial DNA, which might lead to dysfunction. The same holds for the COPD case, where chronic inflammation of the lung tissue often is an associated factor (Brightling & Greening 2019). Risk factors such as smoking and dietary habits which affect the body on a long-term

scale could thus be modified at any time to achieve a positive impact on health and disease risk.

An inverse relationship between plasma glucose and prostate cancer risk was observed in paper III, and an inverse relationship between T2D and prostate cancer risk has been observed in several previous studies from different cohorts (Kasper *et al.* 2009; Fall *et al.* 2013; Tsilidis *et al.* 2015; Feng *et al.* 2020; Kincius *et al.* 2020; Peila & Rohan 2020). At the crossroads between T2D and prostate cancer we find the tumour suppressor PTEN, as previously mentioned in chapter 3.2.3. PTEN is a suppressor of the cell cycle-regulating PI3K/AKT pathway, which can be activated by insulin (Li *et al.* 2020). PTEN loss has been shown to be associated with increased risk of pathological prostate cancer (Gao *et al.* 2016) and PTEN knockout increases prostate cancer tumourigenesis (Wu *et al.* 2019) as well as glucose tolerance and insulin sensitivity (Li *et al.* 2020).

Environmental factors and epigenetics related to nutrition have also been found to have great impact on the levels of PTEN expression (Venniyoor 2020). A hypothesis has been presented which suggests that low PTEN expression primes the organism for efficient handling of nutrients, which would be beneficial in a nutrition-poor environment (Venniyoor 2020). The Westernisation of diet and lifestyle could according to this hypothesis have a larger impact on the health of people with either PTEN over- or underexpression, predisposing them to either T2D or cancer. The inverse relationship between T2D and prostate cancer risk might thus be related to differences in the expression of PTEN. A hypothesis and a suggestion for further analyses of this relationship are presented in the consecutive paragraphs.

Lower PTEN expression is associated with higher fatty acid biosynthesis (Yi *et al.* 2020). The increased PI3K/AKT-activation by PTEN downregulation causes activation of sterol regulatory element-binding protein (SREBP) (Yue *et al.* 2014), upregulating stearoyl-CoA desaturase-1 (SCD-1), which in turn produces monounsaturated fatty acids (Yi *et al.* 2020). Several phospholipids with monounsaturated side chains were found to be associated with prostate cancer risk in paper III (Figure 21), and this increase in fatty acid biosynthesis might be involved in explaining the associations with prostate cancer risk observed for several phospholipids in paper III. Consistent with this, it has been observed that overweight PTEN-deficient individuals (Cowden syndrome) have a higher degree of insulin sensitivity while having similar plasma lipid levels as their BMI-matched controls (Pal *et al.* 2012), although patients with higher insulin sensitivity are expected to have lower plasma lipid levels.

Lower expression of PTEN seems to be associated with aggressive prostate cancer as well, since tumours exhibiting PTEN loss tend to develop into an aggressive phenotype (Jamaspishvili *et al.* 2018). Whether the risk of getting

aggressive prostate cancer could be related to PTEN expression *per se* has to the best of the author's knowledge not been clarified. Interestingly in relation to paper III, a trend for higher intake of daily energy and macronutrients was observed for the subgroup of aggressive cancer cases compared to the controls, albeit without a concomitant difference in BMI (data not shown). This trend was not statistically significant and requires validation using a larger sample size. One potential explanatory connection between this trend and prostate cancer aggressiveness could be differences in the metabolic rate, where a higher metabolic rate could be associated with *e.g.* higher production of reactive oxygen species, which can have adverse effects.

The thyroid hormones thyroxine and triiodothyronine are important regulators of metabolic rate and several studies both *in vitro* and *in vivo* have shown that thyroid hormones can affect different aspects of cancer (Krashin *et al.* 2019) and insulin resistance (Martinez & Ortiz 2017). A prospective study has shown that higher free thyroxine was associated with increased overall prostate cancer risk (Yi *et al.* 2017) and higher levels of thyroid hormones have also been shown to be associated with increased migration of prostate cancer cells (Delgado-González *et al.* 2016), a hallmark of aggressive disease status. The aromatic amino acid tyrosine is a precursor of the thyroid hormones, and interestingly in paper III tyrosine was the feature found to have the highest association with prostate cancer risk in the full dataset (Figure 21).

Future studies investigating whether an association exists between baseline levels of thyroid hormones and the risks of developing more aggressive cancer forms or T2D could yield important insight into the role of thyroid hormones on prostate cancer and insulin resistance.

7 Concluding remarks and future perspectives

Within the scope of this thesis, metabolomics and flux analysis were used for investigations of topics related to T2D and prostate cancer. A human intervention study (paper I), an *in vitro* study (paper II), and a prospective biobank study (paper III) were conducted.

The results from paper I show a lower insulin response when rye bread was ingested in comparison with wheat bread owing to a lower rate of glucose appearance in blood, highlighting the importance of considering the rates of stimulus application when investigating biological responses. Future research ought to take this factor into account, especially in pharmacological settings since the impact on the biological response can be considerable.

Paper II yielded insight into the pathways utilised by pancreatic β-cells when subjected to a short-term glucose and fatty acid fuel excess environment. The activity of the Krebs cycle was increased, and a new fuel excess detoxification pathway comprised of proline production and exocytosis was observed. Co-incubation of β-cells with both glucose and fatty acid caused a strong increase in the production of phospholipids incorporating both incubation species into their structure. Future research on this matter should be directed towards investigating whether the observed pathways are implicated during long-term incubation as well, and whether the current observations from a human β-cell line can be replicated in pancreatic islets of Langerhans.

In paper III a range of metabolites, including many phospholipids, were found to be prospectively associated with prostate cancer risk in the largest untargeted metabolomics study on prostate cancer risk to date. Since untargeted metabolomics comprise only an initial discovery step, further investigation into the current findings by quantitative methods and mechanistic studies is warranted in order to ascertain the current findings and investigate their causality.

The mechanistic relationship between prostate cancer and T2D is still elusive, but a body of evidence is accumulating indicating that the PTEN-PI3K/AKT pathway is implicated in both diseases. Further research oriented towards understanding and intervening in the PI3K/AKT pathway could possibly help humanity in mitigating two major illnesses at once.

On a more general note, metabolomics shows great promise for giving new insight into biological systems and for discovering new disease biomarkers. As the field of metabolomics has matured, it might be time for it to take the step towards investigation of dynamic systems as well, with integration of fluxomics into commonplace workflows and commercial kits. The accessibility of mass spectrometers suitable for analysis of isotopically labelled material (high-resolution or tandem instruments) and the catalogue of commercially available stable isotope labelled chemicals is continuously expanding, facilitating tailored flux experiments and even paving the way for the discovery of completely new metabolic pathways.

Since metabolite flux has historically been arduous to measure and has been largely overlooked when it comes to disease, there are probably many disease mechanisms associated with flux changes yet to be identified. Identification of these changes could also uncover routes to new treatments, since flux is mediated by enzymes which can be characterised and pharmacologically inhibited.

Above all, what we strive for when researching the mechanisms of different diseases are, apart from doing it purely out of scientific interest, is to find potential treatments and cures for the diseases in order to increase the lifespan and quality of life of afflicted patients. Metabolomics and flux analyses will certainly play an important part in many life-changing discoveries to come.

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Popular science summary

The Western lifestyle is associated with a lack of exercise and an over-consumption of energy-rich food of low nutritional quality. This has led to a large increase in the number of people suffering from type 2 diabetes. People of today also live longer, which increases the number of people suffering from diseases that mainly affect the elderly. One of these diseases is cancer and the most diagnosed cancer form in Western European and American men is prostate cancer. Patients with type 2 diabetes seem to have a reduced risk of developing prostate cancer but the details of this connection are still unknown. Changes in metabolism however seem to be involved.

In this thesis, two main techniques were used to investigate changes in metabolism associated with these two diseases. The first one is called metabolomics, which is a combination of analytical and statistical methods that enable simultaneous measurements of many kinds of molecules in for example blood. The main analytical technique used in this thesis is called mass spectrometry, which distinguishes different substances by their molecular weight.

The second one is called stable isotope labelling, which uses molecules containing atoms that are heavier than normal to track metabolism. For example, by feeding glucose that has had its six carbon atoms replaced with heavier carbon atoms to a cell culture, one can see where these heavier carbon atoms end up when the glucose molecules are metabolised and use that information to find out how the metabolism works.

High blood glucose, also known as high blood sugar, and decreased insulin production are characteristic of type 2 diabetes. Glucose and insulin levels are affected by diet. The first of the three studies included in this thesis examined how fast blood sugar increases after eating wheat or rye bread and how the body responds to these two kinds of bread through insulin release. It could be seen that more insulin was released when glucose released from the bread enters the blood faster, which was the case with wheat bread. The blood sugar levels did

however not differ after consumption of the two types of bread. These findings are important for understanding how different kinds of food affect the body and can be used to develop healthier food products.

Insulin is produced in the pancreas by a type of cells called β-cells, which can be found in the Islets of Langerhans. The second study examined how the metabolism of these insulin-producing β-cells changed when they were short-term treated with different concentrations of glucose and with a fatty acid. This was done to simulate the environment to which the cells are exposed in both healthy people and diabetics. The activity of the mitochondria, which are the parts of the cells responsible for supplying them with energy, was increased by the treatment. The cells also transformed the added glucose into the amino acid proline and released it from the cell, which has not been seen before.

The cells also formed different kinds of molecules, so-called phospholipids which are important cell membrane building blocks, from glucose and the fatty acid they were treated with. These findings are important for understanding how the β-cells work in detail and for understanding what makes them fail to do so as is the case with type 2 diabetes.

Prostate cancer develops under a long period of time and it can take many years before any symptoms can be observed. The third study compared the presence of different substances in blood samples from two groups of healthy men with the same body mass index and age (40, 50, or 60 years). One group later developed prostate cancer while the other did not. Differences between the groups were observed for different phospholipids, glucose, amino acids, and fatty acids, among others. The substances that were associated with prostate cancer risk differed between different age groups and between those who developed an aggressive or non-aggressive form of prostate cancer. The results included substances that were not previously associated with the risk of developing prostate cancer and may provide new insight into how the disease develops.

The findings in this thesis can help in understanding how type 2 diabetes and prostate cancer develop and work, which is necessary for being able to develop the tools and medical techniques for curing them, or even prevent them from developing at all.

Populärvetenskaplig sammanfattning

Den västerländska livsstilen är förknippad med brist på motion och ett överintag av energirik mat av låg näringsmässig kvalitet. Detta har lett till en stor ökning av antalet personer som lider av typ 2-diabetes. Människor lever också längre idag, vilket ökar antalet personer som lider av sjukdomar som främst drabbar äldre. En av dessa sjukdomar är cancer, varav prostatacancer är den mest diagnosticerade cancerformen hos västeuropeiska och amerikanska män. Patienter med typ 2-diabetes tycks ha en minskad risk för att utveckla prostatacancer, men det är fortfarande okänt hur denna koppling fungerar i detalj. Ämnesomsättningsförändringar verkar dock vara inblandade.

I den här avhandlingen användes två huvudtekniker för att undersöka ämnesomsättningsförändringar förknippade med dessa två sjukdomar. Den första kallas metabolomik, vilken är en kombination av analytiska och statistiska metoder som möjliggör samtidiga mätningar av många olika typer av molekyler i till exempel blod. Den analytiska teknik som främst använts i denna avhandling kallas masspektrometri, med vars hjälp man kan särskilja olika ämnen på basis av deras molekylvikt.

Den andra tekniken kallas inmärkning med stabila isotoper, genom vilken man använder molekyler innehållande atomer som är tyngre än vanligt för att följa ämnesomsättningen. Till exempel, genom att ge glukos som har fått sina sex kolatomer utbytta mot tyngre kolatomer till en cellodling kan man se vart dessa tunga kolatomer tar vägen då glukosmolekylerna ombildas under ämnesomsättningen. Denna information kan då användas för att få reda på hur ämnesomsättningen fungerar.

Höga nivåer av glukos i blodet, även kallas högt blodsocker, och minskad insulinproduktion är utmärkande för typ 2-diabetes. Glukos- och insulinnivåer påverkas av kosten. Den första av de tre studierna i denna avhandling undersökte hur snabbt blodsockret ökar efter intag av råg- eller vetebröd och hur kroppen svarar på detta genom insulinfrisättning. Det kunde observeras att mer insulin frisätts då glukos som frisatts från brödet tas upp i blodet i en snabbare takt,

vilket skedde med vetebrödet. Blodsockernivåerna var dock desamma efter intag av de två brödtyperna. Dessa upptäckter är viktiga för att kunna förstå hur olika sorters mat påverkar kroppen och kan användas för att utveckla mer hälsosamma livsmedel.

Insulin produceras av en typ av celler som kallas β-celler som hittas i bukspottskörtelns Langerhanska öar. Den andra studien undersökte hur ämnesomsättningen förändrades i dessa insulinproducerande β-celler då de korttidsbehandlades med olika koncentrationer av glukos och en fettsyra. Detta gjordes för att simulera miljön som cellerna utsätts för hos både friska personer och diabetiker. Behandlingen ökade aktiviteten hos mitokondrierna – den del av cellerna som ansvarar för cellernas energiförsörjning. Cellerna omvandlade också tillförd glukos till aminosyran prolin och släppte ut den ur cellerna, vilket inte observerats tidigare. Cellerna skapade även olika sorters molekyler, så kallade fosfolipider, som är viktiga byggstenar i cellernas membran, från glukos och den fettsyra de behandlats med. Dessa upptäckter är viktiga för att kunna förstå hur β-celler fungerar i detalj och för att förstå vad det är som gör att de slutar fungera, vilket är fallet i typ 2-diabetes.

Prostatacancer utvecklas under lång tid och det kan dröja många år innan man kan observera några symptom. I den tredje studien jämfördes förekomsten av olika ämnen i blodprover från två grupper av friska män med samma kroppsmasseindex och ålder (40, 50 eller 60 år). Den ena gruppen utvecklade senare prostatacancer medan den andra inte gjorde det. Skillnader mellan grupperna kunde observeras för bland annat glukos, aminosyror, fettsyror och olika fosfolipider. Vilka ämnen som uppvisade ett samband med prostatacancerrisk skilde sig mellan olika åldersgrupper och mellan dem som utvecklade en aggressiv eller icke-aggressiv form av prostatacancer. Resultaten innefattade ämnen som inte tidigare förknippats med risken att utveckla prostatacancer och kan ge ny insikt i hur sjukdomen utvecklas.

Upptäckterna i denna avhandling kan öka insikten om hur typ 2-diabetes och prostatacancer utvecklas och fungerar, vilket är nödvändigt för att utveckla verktyg och medicinska tekniker för att bota dem, eller till och med hindra dem från att överhuvudtaget uppkomma.

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Acta Universitatis Agriculturae Sueciae

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Metabolic factors associated with insulin secretion and prostate cancer risk were investigated by liquid chromatography-mass spectrometry-based metabolomics, stable isotope labelling, and flux analysis. The insulin secretion in response to rye and wheat breads was investigated *in vivo* and investigations of β-cells incubated with glucose and palmitic acid *in vitro* revealed new fuel excess detoxification pathways. Furthermore, new plasma metabolites prospectively associated with prostate cancer risk were identified in a Swedish cohort by untargeted metabolomics.

Johnny Östman received his graduate education at the Department of Molecular Sciences, SLU, Uppsala. He attained his B.Sc. degree in Biology and M.Sc. degree in Chemistry at Åbo Akademi University, Finland.

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