The role of whole grains and lignans in lifestyle diseases – emphasis on prostate cancer and type 2 diabetes and their risk factors

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Abstract

Whole grains are rich in dietary fibre and bioactive compounds and have consistently been associated to improved health and lower mortality. Whole-grain rye is rich in fermentable fibre and lignans, that are converted to enterodiol and enterolactone by the gut microbiota. Enterolactone has been studied widely for several suggested beneficial health properties. In this thesis, the role of whole grains and lignans in cardio-metabolic health and mortality from type 2 diabetes and prostate cancer was studied.

Whole-grain rye and lignans did not affect glucose response assessed by an oral glucose tolerance test in men with metabolic syndrome after 8 wk intervention. Whole-grain rye improved lipid profile by lowering LDL-cholesterol compared to whole-grain wheat, whereas high-dose lignan supplementation had no effect on any of the investigated cardio-metabolic outcomes. Baseline microbiota enterotype appeared to explain some of the difference in response to treatments. Lipid profile was improved in the *Prevotella* enterotype by whole-grain rye diet and in the *Bacteroides* enterotype by whole-grain wheat diet. Lifestyle intervention with high intake of whole-grain rye and physical activity was feasible to implement in a group of men with non-aggressive prostate cancer. However, a full-scale trial is needed to investigate the potential of whole-grain rye in prostate cancer progression. Lastly, plasma enterolactone was investigated in two observational studies based on data from the population-based Diet, Cancer and Health cohort. Enterolactone concentrations were inversely associated with mortality among persons with type 2 diabetes, whereas no association with mortality was observed in men with prostate cancer.

Overall, the findings from this thesis highlights that whole-grain rye has beneficial health properties related to lipid profile, that long-term dietary interventions with high intake of whole-grains are feasible among middle-aged men with metabolic syndrome or prostate cancer, and that plasma enterolactone is associated with lower mortality among persons with type 2 diabetes. Furthermore, the finding of no association between enterolactone and prostate cancer mortality contributes to the existing evidence from observational studies that does not support the findings of a protective role from cell, animal and a few human interventions.

Keywords: whole-grain rye, lignans, enterolactone, metabolic syndrome, human intervention, type 2 diabetes, prostate cancer, cohort study, prognosis

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Preface

The work for this thesis was conducted in a collaboration between the Danish Cancer Research Center in Copenhagen, Denmark, and the Swedish University of Agricultural Sciences in Uppsala (SLU), Sweden. The PhD project was part of a project "The effects of enterolignans in chronic diseases" (ELIN) (project number 0603-00580B) conducted between 2013 and 2018 and funded by the Innovation Fund Denmark. The aim of the project was to study the role of plant lignans and enterolignans in survival from chronic disease. The project comprised various work packages (WPs): epidemiological studies on chronic diseases (WP1), development of cereal products high in plant lignans (WP2), studies of pharmacokinetics in animals (WP3) and a human intervention study (WP4). The thesis is based on studies in WP1 and WP4, which included one year in Uppsala to conduct the intervention study. The Nordic Lifestyle Intervention Trial on Prostate Cancer Progression (NILS, registered at www.clinicaltrials.gov, NCT01300104), a feasibility study of a diet- and lifestyle intervention among men with non-aggressive prostate cancer, was also part of this PhD project.

Dedication

To Elin

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 Anne Kirstine Eriksen*, Carl Brunius, Mohsen Mazidi, Per Hellström, Ulf Risérus, Kia Nøhr Iversen, Rikard Fristedt, Li Sun, Yi Huang, Knud Erik Bach Knudsen, Natalja Nørskov, Cecilie Kyrø, Kirsten Frederiksen, Anne Tjønneland, Anja Olsen, Johan Dicksved, Rikard Landberg (2019). Effects of whole-grain rye and lignan supplementation on cardio-metabolic risk factors in men with metabolic syndrome are associated with baseline gut microbiota enterotype: a randomized cross-over trial (manuscript)

II Anne Kirstine Eriksen*, Cecilie Kyrø, Natalja Nørskov, Knud Erik Back Knudsen, Kirsten Frederiksen, Kim Overvad, Rikard Landberg, Anne Tjønneland and Anja Olsen (2019). Pre-diagnostic plasma enterolactone concentrations are associated with lower mortality among individuals with diabetes: a case-cohort study in the Diet, Cancer and Health cohort. Accepted 27 February *Diabetologia*

III Anne Kirstine Eriksen*, Rikke Dalgaard Hansen, Michael Borre, Ryan Godsk Larsen, Jeppe Munthe Jensen, Kristian Overgaard, Mette Borre, Cecilie Kyrø, Rikard Landberg, Anja Olsen and Anne Tjønneland (2017). A lifestyle intervention among elderly men on active surveillance for non-aggressive prostate cancer: a randomised feasibility study with whole-grain rye and exercise. *Trials* Jan 13;18(1):20

IV Anne Kirstine Eriksen*, Cecilie Kyrø, Natalja Nørskov, Anne Katrine Bolvig, Jane Christensen, Anne Tjønneland, Kim Overvad, Rikard Landberg and Anja Olsen (2017). Prediagnostic enterolactone concentrations and

mortality among Danish men diagnosed with prostate cancer. *Eur J Clin Nutr:* 71(10), 1235-1240.

Papers *II-IV* are reproduced with the permission of the publishers.

* Corresponding author. Associated papers not included in this doctoral thesis:

V Elin Hålldin, Anne Kirstine Eriksen, Carl Brunius, Andreia Bento da Silva, Maria Bronze, Kati Hanhineva, Anna-Marja Aura and Rikard Landberg (2019). Factors explaining inter-personal variation in plasma enterolactone concentrations in humans. *Mol Nutr Food Res*, Feb 28:e1801159. doi: 10.1002/mnfr.201801159.

The contribution of Anne Kirstine Eriksen to the papers included in this thesis was as follows:

I Designed and planned the study in collaboration with supervisors, recruited participants and performed the study in collaboration with study nurses and students, analysed the results and had the main responsibility for writing the paper

I Had the main responsibility for analysing the data and writing the paper

II Had the main responsibility for analysing the data and writing the paper

III Had the main responsibility for analysing the data and writing the paper

Abbreviations

BMI	body mass index
CI	confidence interval
DCH	Diet, Cancer and Health
EPIC	European Prospective Investigation into Cancer and Nutrition
HR	hazard ratio
LDL	low-density lipoprotein
PSA	prostate-specific antigen
RCT	randomized controlled trial
SCFA	short-chain fatty acid
SDG	secoisolariciresinol diglucoside
SLU	University of Agricultural Sciences in Uppsala
WHO	World Health Organization

1 Introduction

Lifestyle plays an important role in the development of noncommunicable diseases such as type 2 diabetes, cardiovascular disease and several cancers, referred to in this thesis as "lifestyle diseases". According to the World Health Organization (WHO), high blood pressure, tobacco use, high blood glucose, physical inactivity, overweight and obesity are the leading risk factors for mortality and lifestyle diseases, which affect populations worldwide and in all socio-economic groups (WHO, 2009). As diet and physical activity directly affect all these risk factors (except tobacco use) and almost 40% of all deaths worldwide can be ascribed to them, there is potential for prevention (WHO, 2009). Little is known about how diet and other lifestyle factors affect life expectancy among people who already have these diseases, and the potential for secondary prevention may be unexploited. Research in this area is therefore of high priority.

Whole grains are a dietary factor for which there is consistent evidence of beneficial effects against three of the greatest health threats in high-income countries: coronary heart disease (Ye *et al.*, 2012, Mellen *et al.*, 2008, Aune *et al.*, 2016), type 2 diabetes (Aune *et al.*, 2013) and colorectal cancer (WCRF/AIRC, 2017). The underlying mechanisms by which whole grains protect against these threats are not fully elucidated, but whole grains are rich in beneficial components, including dietary fibre and micronutrients. Bioactive components in whole grains have also received attention, including the phytoestrogens lignans, which are converted to enterolignans by gut microbial bacteria. Plant lignans and enterolignans, especially enterolactone, have been studied widely, and beneficial effects have been suggested against hormone-related cancers, with the strongest evidence for improved prognosis after diagnosis of breast cancer (Buck *et al.*, 2011, Seibold *et al.*, 2014) and less clear evidence for prostate cancer (Zhang *et al.*, 2016). Enterolactone concentrations

in urine have also been linked to lower mortality from cardiovascular diseases and all causes (Reger *et al.*, 2016).

1.1 Whole grains

1.1.1 Definition

Whole grains have been part of the human diet since the late Stone Age (Lippi *et al.*, 2015). The most common grains consumed in western nations are wheat, oat, corn/maize, rye and barley, but there are also other species. So-called pseudo-cereals, like wild rice, quinoa, buckwheat and amaranth, although they are strictly speaking not grains, have a similar macronutrient composition and are consumed as grains in some regions. The first definition of whole grains was published in 1999 by the American Association of Cereal Chemists International: "Whole grain shall consist of the intact, ground, cracked or flaked caryopsis whose principal anatomical components – the starchy endosperm, germ and bran – are present in the same relative proportion as they exist in the intact caryopsis" (AACC, 1999). This definition was most recently revised by the HEALTHGRAIN consortium of the European Union, including a suggestion for a world-wide definition of whole grains (Ross *et al.*, 2017). The definitions of whole-grain products vary slightly: that of the American Association of Cereal Chemists International covers products with ≥ 8 g of whole grains for every 30 g of product, whereas that of the US Food and Drug Administration requires ≥ 8 g (dry weight) whole grains per reference serving size and ≥ 51% of the grain ingredients from whole grains (Sawicki *et al.*, 2018). The HEALTHGRAIN Forum recently defined whole-grain products as those containing > 30% whole-grain ingredients in the overall product and more whole-grain than refined-grain ingredients. Further, they recommend that products meet the healthy eating criteria in order to have the whole-grain label (Ross *et al.*, 2017). In Denmark, whole-grain products that contain other ingredients must contain at least 50% whole grain (dry weight) (DTU, 2008).

1.1.2 Recommendations and intake worldwide

Whole grains are included in many national dietary recommendations (Seal *et al.*, 2016). In general, one serving equals 28 g of whole-grain product or 16 g of whole grain (Ross *et al.*, 2015). The highest recommended intakes are those in the Scandinavian countries, which is approximately 75 g of whole grains per 10 MJ (DTU, 2008, Helsedirektoratet, 2016, Livsmedelsverket, 2016). The Dietary

Guidelines for Americans in 2015 recommended consumption of 6 oz-equivalents (28.4 g/oz equivalent) of grain per day (per 2000 kcal = 170 g/2000 kcal/day)), at least half of which should be whole grains (USDA, 2015). Data from nutrition surveys in 266 countries, representing 41% of the world's population, indicate a global mean whole-grain intake of 38 g/day (95% confidence interval [CI]: 1.3–334.3 g/day) (Micha *et al.*, 2015). It was estimated that 7.6% of the global adult population consumed 50 g of whole grain daily, with substantial regional variation.

1.1.3 Whole grains and health

Observational studies provide increasing evidence of the beneficial properties of whole grains in relation to lifestyle diseases. The conclusions of two large meta-analyses published in 2016 were that whole-grain intake is inversely associated with the risks of coronary heart disease, cardiovascular disease and all cancers (Aune *et al.*, 2016) and with mortality from cardiovascular (Zong *et al.*, 2016), respiratory, infectious, diabetes and all non-cardiovascular and non-cancer diseases (Aune *et al.*, 2016). The most recent report from the World Cancer Research Fund indicates that there is *probable evidence* that whole grains decrease the risk for colorectal cancer, the only cancer linked to whole-grain intake in this report (WCRF/AICR, 2018).

The role of whole grains has gained attention during the past decade, and more than 200 intervention studies have been conducted (Sawicki *et al.*, 2018). Most investigated whole-grain wheat and mixed grains in acute (< 1 day) trials, with a focus on glucose and insulin response. Longer trials addressed cardio-metabolic health, appetite and weight. While the acute trails were conducted primarily in healthy individuals, the longer trials involved people who were metabolically at risk (with overweight, type 2 diabetes or metabolic syndrome). The reporting of whole-grain ingredients in these trials is, however, inconsistent, and intake may have varied greatly; this is further complicated by uncertainty about compliance in the dietary interventions. The effects seen in these studies are also expected to differ because of differences in design (duration, parallel or crossover, comparison diet), type of whole-grain diet and the health status of participants. Thus, consistent conclusions cannot yet be drawn from the large body of human interventions. The suggested beneficial effects of interventions with whole grains include lowering of total and low-density lipoprotein (LDL) cholesterol and inflammation, whereas the results are less consistent with regard to other lipids and body weight (Hollaender *et al.*, 2015, Pol *et al.*, 2013, Kelly *et al.*, 2007, Roager *et al.*, 2019). In a meta-analysis of whole-grain interventions and blood lipid changes, whole-grain oats had the strongest effect (Hollaender

et al., 2015). Beta-glucans from oats and barley are currently the only grain constituents for which European Commission-authorized health claims exist for reduction of blood cholesterol and risk of heart disease (EFSA Panel on Dietetic Products, 2011, EFSA Panel on Dietetic Products, 2010).

In summary, there is convincing evidence for a beneficial role of whole-grain intake from observational studies; however, clear distinctions between different types of whole grain can rarely be made. Although specific types of whole grain have been studied in dietary interventions, the evidence is inconsistent, possibly because of variations in study design (Sawicki *et al.*, 2018). In the Nordic countries, where whole-grain rye is part of the traditional diet, its beneficial properties have been studied intensively (Jonsson, 2018).

1.1.4 Dietary fibre and bioactive compounds in whole grains

Several of the proposed mechanisms for the beneficial effects of whole grains include their high fibre content. Dietary fibre can be classified as insoluble (cellulose, hemicellulose) and soluble (e.g. B-glucans, arabinoxylans). Insoluble fibre increases gut transit and faecal bulking, leading to increased satiety. Additional properties include delivery of antioxidant-bound phenolics to the colon and binding of carcinogens (Fardet, 2010). Soluble fibre improves glucose homeostasis by delaying gastric emptying, reducing the rate of glucose absorption. Furthermore, soluble fibre can improve the lipid profile, reduce bile acid reabsorption and increase production of short-chain fatty acids (SCFAs) (Fardet, 2010). SCFAs are the end product of fermentation of undigested fibre and act as an energy source and signalling molecules between gut microbiota and the host (Ganapathy *et al.*, 2013). Positive effects of SCFA on gut integrity, glucose homeostasis, lipid metabolism, appetite regulation and immune function have been proposed (Morrison and Preston, 2016). In both whole-grain wheat and rye, the main dietary fibre component is arabinoxylans. In whole-grain wheat, total dietary fibre constitutes around 13%, of which half (6.5%) is arabinoxylans and 2% cellulose (Andersson *et al.*, 2013). Whole-grain rye contains around 20% dietary fibre, of which arabinoxylan is the main component (8.6%), followed by fructans (4.1%), whereas cellulose accounts for 2.7% (Andersson *et al.*, 2009). Whole-grain rye is therefore expected to have a stronger effect on glucose homeostasis and lipid profile than whole-grain wheat because of its higher arabinoxylan content. Furthermore, the larger amount of fermentable fibre in rye than in wheat generates more SCFAs to stimulate microbial bacteria that are beneficial for insulin sensitivity (Lappi *et al.*, 2014, Bach Knudsen *et al.*, 2005).

1.1.5 Whole grains and gut microbiota

Human gut microbiota are the community of micro-organisms in the gastrointestinal tract (Valdes *et al.*, 2018). Gut microbiota ferments dietary fibre and SCFAs, i.e. acetate, propionate and butyrate, are produced as a result of this process. Dietary fibre constitutes the primary energy source for many microbes in the gut, and fibre has been shown to increase microbiota diversity and SCFA production (David *et al.*, 2014, Flint, 2012, Zhao *et al.*, 2018). Butyrate is the main energy source for human colonocytes and has beneficial effects against colon cancer cells through apoptosis, and on glucose homeostasis through intestinal gluconeogenesis, the latter also a trait of propionate (de Vadder *et al.*, 2014). Acetate is the most abundant SCFA and is essential for the growth of other bacteria. The main role of acetate is in lipid metabolism, but effects on appetite regulation have also been suggested (Frost *et al.*, 2014).

The diversity of the microbiota seems to be inversely associated with disease: less diversity has been observed in overweight and obese people (Turnbaugh *et al.*, 2009, Frankenfeld *et al.*, 2014), in people with type 2 diabetes (Lambeth *et al.*, 2015) and in men with prostate cancer (Sfanos *et al.*, 2018) than in healthy controls. The association between diversity and disease indicates that a diverse community is more robust towards environmental challenges (Sommer *et al.*, 2017).

It has been suggested, that individuals can be stratified according to enterotype, based on their microbiota characteristics. Enterotypes relates to clusters of species composition of the gut microbiota (Arumugam *et al.*, 2011), and the two most frequently reported enterotypes are enriched in *Prevotella* and *Bacteroides*, respectively. Enterotypes have been associated with long-term dietary patterns; *Prevotella* with plant-based, high-fibre diets, and *Bacteriodes* with high fat/low-fibre diets (Wu *et al.*, 2011b).

Research on the role of whole grains in relation to gut microbiota indicates that it is difficult to make large alterations in gut microbiota and their function with a whole-grain diet (Roager *et al.*, 2019). However, the gut microbiota of the host are of great importance in the metabolic response after whole-grain intake (Hjorth *et al.*, 2017, Kovatcheva-Datchary *et al.*, 2015) and should be accounted for.

Dietary fibre from cereals, as opposed to fibre from fruits and vegetables, has been inversely associated with several lifestyle diseases (Aune *et al.*, 2011, Schulze *et al.*, 2007). This indicates that additional components in whole-grain cereals and perhaps interaction among several factors are important. One of the components of whole grains is lignans, which are naturally occurring bioactive compounds in fibre-rich plant foods. Complete metabolism of lignans requires a consortium of microbes (Rowland *et al.*, 2018) and, hence, a diverse community.

1.2 Plant and enterolignans

Lignans constitute a group of diphenolic plant compounds thought to be involved in the plants' defence against pathogens and pests through antifungal, antimicrobial, antifeedant, antiviral and insecticidal properties (Pan *et al.*, 2009b). The common lignans found in foods are secoisolariciresinol, matairesinol, pinoresinol, medioresinol, lariciresinol, syringaresinol, sesamin, isolariciresinol and 7′-hydroxymatairesinol (Figure 1). After ingestion of lignan-containing foods, plant lignans are metabolized by colonic bacteria to the enterolignans, enterodiol and enterolactone, which can then be absorbed through the colonic barrier. Because of their structural similarity to the female hormone 17-oestradiol (Figure 2), these compounds are categorized as phytoestrogens, i.e. plant-based compounds with weak oestrogenic effects (Rietjens *et al.*, 2017).

Figure 1. Chemical structures of four enterolignans (A: secoisolarisirecinol, B: matairesinol, C: enterodiol, D: enterolactone) in lignan-rich foods (kale, flaxseed, whole-grain rye bread, whole-grain wheat bread and strawberries)

Enterolignans can be measured in blood and urine, where they occur 8–10 h after consumption of lignan-rich foods (Lampe *et al.*, 2006) and circulate in the blood for 5–13 h (Kuijsten *et al.*, 2005b). Enterolactone has been the subject of most of the research on enterolignans and is also the focus of this thesis.

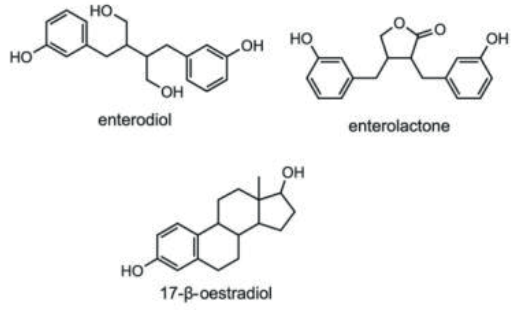

Figure 2. Chemical structures of the enterolignans, enterodiol and enterolactone, and the 17-oestradiol hormone. Modified from Rietjens *et al.* 2017 (Rietjens *et al.*, 2017)

1.2.1 Dietary sources of lignans

Lignans are found in fibre-rich plant foods (Table 1). Oilseeds and flaxseed have especially high contents. Other sources of lignans include whole-grain cereals, of which whole-grain rye has an especially high content, nuts, legumes, fruits and berries, vegetables (especially cabbage and leafy vegetables) and to some extent coffee and tea (Thompson *et al.*, 1991, Thompson *et al.*, 2006, Tetens *et al.*, 2013, Zamora-Ros *et al.*, 2012, Johnsen *et al.*, 2004).

Table 1. Dietary sources of lignans, from the Dutch database of lignans in plant foods (Milder *et al.*, 2005, Tetens *et al.*, 2013)

Source	Lignan content (µg/100 g)
Flaxseed	300 000
Sesame seeds	39 000
Whole-grain rye flour	458
Dark rye bread	320
Whole-grain wheat bread	121
Wheat flour	99
Rolled oats	107
Whole-grain rice	40
Cabbages	600
Strawberry	334
Dark chocolate	44
Coffee	25
Tea	58
Beer	26
Wine	56

1.2.2 Plasma enterolactone concentrations in different populations

In a random subsample from nine European countries (Denmark, France, Germany, Greece, Italy, Spain, Sweden, the Netherlands and UK) in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, the mean enterolactone concentration was 9.0 nmol/L (8.4-9.6). The regional variation was large, ranging from 7.0 nmol/L in northern Italy to 27.5 nmol/L in Denmark (Peeters *et al.*, 2007). Concentrations in the same range as in Denmark were also observed in Swedish (Hedelin *et al.*, 2006) and Finnish (Kilkkinen *et al.*, 2003a) populations. In the American Nurses' Health Study II, a median plasma enterolactone concentration of 11 nmol/L was reported (Xie *et al.*, 2013).

1.2.3 Determinants of plasma enterolactone

Dietary intake of lignans is associated with plasma concentrations of enterolactone, but the content of plant lignans in foods varies substantially (Table 1), and the food matrix and processing can further affect their bioavailability and conversion into enterolignans (Nesbitt *et al.*, 1999, Kuijsten *et al.*, 2005a). Increased enterolactone production after dietary interventions with fruit, vegetables, berries, rye and flaxseed has been observed (Stumpf *et al.*, 2000, Mazur *et al.*, 2000, Juntunen *et al.*, 2000, Knust *et al.*, 2006); however, even in controlled settings, there is large inter-individual variation (Kuijsten *et al.*, 2005b). The main explanation for this variation appears to be the composition and activity of the gut microbiota of the host, and this has been proposed as the main reason for inter-personal variation in enterolactone concentrations (Halldin *et al.*, 2019).

The lignan-rich foods associated with higher enterolactone concentrations are whole-grain and rye products, cabbage, leafy vegetables, roots, berries, coffee and tea (Kilkkinen *et al.*, 2001), while fat consumption has been inversely associated with enterolactone concentrations (Johnsen *et al.*, 2004). The non-dietary factors obesity, smoking and frequent bowel movements have been associated with lower enterolactone concentrations, whereas constipation has been associated with higher levels. Only a small part of inter-individual variation, however, appears to be explained by these factors (Johnsen *et al.*, 2004). Women generally have higher enterolactone concentrations than men (Kilkkinen *et al.*, 2001, Jacobs *et al.*, 2002), possibly because they have higher concentrations of enterodiol- and enterolactone-producing bacteria and a longer gut transit time (Clavel *et al.*, 2005, Lampe *et al.*, 1993).

1.2.4 The role of microbiota and antibiotics

Gut microbiota are essential in the conversion of plant lignans to enterolignans (Wang *et al.*, 2000). As there are large individual differences in the composition of intestinal bacteria, the plasma concentrations and urinary excretion rates of enterodiol and enterolactone may also vary substantially. Four reactions must take place in bacterial conversion: deglycosylation, demethylation, dihydroxylation and dehydrogenation of enterodiol to enterolactone (Clavel *et al.*, 2006). Several bacteria have been identified in these steps (Figure 3).

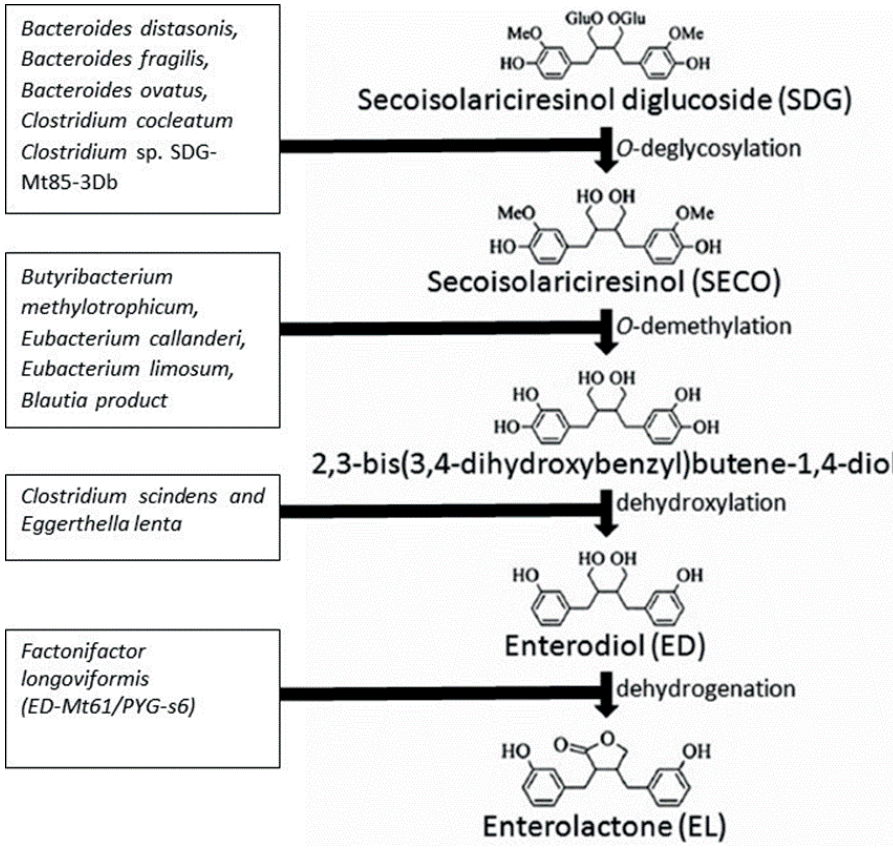

Figure 3. The main steps in bacterial conversion of secoisolariciresinol diglucoside (SDG) to enterolactone and examples of bacteria involved in the different steps. Adopted from Halldin *et al.* 2019.

Use of antibiotic medication has a major impact on the intestinal microbiota (Adgent and Rogan, 2015), as reflected in the lower enterolactone concentrations measured for more than 1 year after antibiotic treatment

(Kilkkinen *et al.*, 2002, Bolvig *et al.*, 2016). The decrease in conversion of enterolactone is probably due to changes in microbiota induced by antibiotics. It is also suspected that enterolactone reabsorption from the gut is decreased as a result of interference with enzymatic hydrolysis of enterolactone conjugates excreted in bile (Kilkkinen *et al.*, 2002).

1.2.5 Biological effects of enterolignans

The mechanisms by which enterolignans elicit their biological activity include anti-oxidation, anti-neo-angiogenesis and growth factor modulation (Kitts *et al.*, 1999, Bergman Jungestrom *et al.*, 2007). These effects indicate a potential role of enterolignans in carcinogenesis. Furthermore, the oestrogen-like structure, similar to 17-β-oestradiol, of enterolactone allows weak binding to oestrogen receptors (Mousavi and Adlercreutz, 1992). Depending on endogenous oestradiol concentrations, enterolactone can act either as an antagonist of oestrogen or as a weak oestrogen (Penttinen *et al.*, 2007). Because of its oestrogen-like structure, its potential role in hormone-dependent cancers, especially of the breast and prostate, has been investigated (Adlercreutz, 2002). Phytoestrogens have also been suggested to enhance the level of sex hormone-binding globulin, the major sex hormone transport protein in plasma. Consequently, a larger proportion of oestrogen is bound, which may subsequently suppress cancer cell growth, as less free oestrogen will be available (Wang, 2002). It has further been proposed that phytoestrogens can decrease free testosterone levels (Martin *et al.*, 1996). Additional benefits of enterolactone include reduced LDL-cholesterol and inflammation (C-reactive protein) (Peterson *et al.*, 2010).

1.2.6 Dietary biomarkers and enterolactone

Dietary biomarkers have been a popular substitute or addition in modern epidemiology to overcome measurement errors that affect dietary assessment (Jenab *et al.*, 2009). Biomarkers were defined by the International Agency for Research on Cancer in 1997 as: “a substance, structure, or process that can be measured in the body or its products and may influence or predict the incidence or outcome of disease” (IARC, 1997). As the limitations of dietary assessment methods can result in both random and systematic measurement errors, biomarkers are widely used to assess the validity and accuracy of exposures of interest in nutritional epidemiology (Jenab *et al.*, 2009).

Dietary biomarkers can be divided into recovery, predictive, concentration or replacement biomarkers. The excretion of recovery biomarkers can be directly

translated to the intake (doubly labelled water) (Bingham, 2002). Predictive biomarkers are similar to those of recovery but have a lower recovery (24-h urinary sucrose) (Tasevska *et al.*, 2005). Concentration biomarkers include vitamins and blood lipids, which cannot be directly measured at the same scale as the intakes, but are correlated with intakes of dietary sources of the exposure of interest (Bingham *et al.*, 2008). Closely related to these are replacement biomarkers, which are used for compounds for which there is no information on content in food databases (including some phytoestrogens) (Grace *et al.*, 2004). The Food Biomarkers Alliance (FOODBALL) has published a new classification of dietary biomarkers, with six classes: food compound intake biomarkers, food or food component intake biomarkers, dietary pattern biomarkers, food compound status biomarkers, effect biomarkers and physiological or health state biomarkers (Gao *et al.*, 2017).

Alkylresorcinols are well-established biomarkers of whole-grain intake (Ross, 2012) in the category of concentration or food component intake biomarkers. They are phenolic lipids present in the bran layer of wheat and rye (and barley) (Landberg *et al.*, 2008) and are therefore good markers of whole-grain intake, as only traces are found in refined cereal products from which the bran has been removed (Ross and Kochhar, 2009). Five homologues of alkyl chain length exist, and the ratio C17:0/C21:0 between homologues reflects whether whole-grain intake is dominated by wheat or rye. The higher the ratio, the higher the rye intake. Whereas alkylresorcinols can be used as biomarkers of whole-grain intake (in observational studies) and of compliance (in human interventions), use of enterolactone as a biomarker is complicated by factors that affect its concentrations, such as intake of lignan-rich foods, body-mass index (BMI), smoking, age, sex, microbiota and antibiotics. These factors are typically of larger importance for the measured concentrations than intake of lignan-rich foods. Therefore, enterolactone may not reflect intake of lignan-rich foods specifically, but rather a healthy lifestyle in general.

1.2.7 Enterolactone and human health

Some of the earlier studies of enterolactone were conducted among postmenopausal women, in which it was found that patients who had undergone surgical removal of breast tumours (Adlercreutz *et al.*, 1982) had significantly lower urinary excretion of enterolactone than controls. Further studies on breast cancer provide less clear evidence of a protective role of lignans in breast cancer incidence than for breast cancer prognosis (Buck *et al.*, 2010, Seibold *et al.*, 2014), but the results are strongest for postmenopausal women for both outcomes. Studies conducted in vitro and in experimental animals have

demonstrated chemopreventive properties, including anti-proliferative effects, of lignans in prostate cancer cells (Saarinen *et al.*, 2010, McCann *et al.*, 2005, McCann *et al.*, 2013). A limited number of dietary interventions conducted among men with prostate cancer support such beneficial properties. High whole-grain rye and bran intake was associated with increased apoptosis (Bylund *et al.*, 2003) and decreased prostate-specific antigen (PSA) concentration (Landberg *et al.*, 2010), respectively, and flaxseed-lignan supplements were associated with a decreased tumour proliferation rate (Demark-Wahnefried *et al.*, 2008). The interventions included high intake of either whole-grain products or lignans from supplements, and it is not known whether the concentrations achievable from actual diets affect prostate cancer progression. Although both mechanistic studies and human interventions indicate effects on prostate tumour cells and in prostate cancer patients, the observational studies conducted so far have focused on incidence rather than prognosis. The evidence for an association between plasma and urinary enterolactone concentrations and risk for prostate cancer is inconsistent (Wallstrom *et al.*, 2017, He *et al.*, 2015, Zhang *et al.*, 2017, Perez-Cornago *et al.*, 2018), and observational research on the association between enterolactone and prostate cancer prognosis is lacking.

The role of lignans and enterolignans in cardio-metabolic disease remains an interesting research topic. A number of randomized controlled trials with lignans showed beneficial effects on blood pressure, C-reactive protein (Peterson *et al.*, 2010) and cholesterol levels (Pan *et al.*, 2009a). Data from observational studies on cardiovascular disease are inconclusive, and no association has been found between lignan intake and cardiovascular disease risk (van der Schouw *et al.*, 2005) or mortality (Milder *et al.*, 2006). Enterolactone concentrations have, however, been inversely associated with death from coronary heart disease and cardiovascular disease (Vanharanta *et al.*, 2003).

A few animal studies have found enterolactone to decrease insulin resistance, and in lignan-supplemented rats, type 2 diabetes development was retarded (Zhou *et al.*, 2017, Prasad, 2001). Effects on glycaemia control of flaxssed lignan (SDG) supplementation indicate a potential role in diabetes management; 360 mg/d lowered HbA1c levels (Pan *et al.*, 2007), though, without affecting fasting glucose or insulin concentrations, while 600 mg/d lowered plasma glucose in subjects with high cholesterol and pre-diabetic glucose concentrations (Zhang *et al.*, 2008). However, observational studies are lacking. The association between urinary excretion of enterolactone (Talaei *et al.*, 2016, Sun *et al.*, 2014) or lignan intake (Zamora-Ros *et al.*, 2013) and incident type 2 diabetes is inconsistent and the role of lignans and enterolignans in diabetes management and mortality remains unknown.

1.3 Metabolic syndrome

Metabolic syndrome is characterized by the concurrent presence of metabolic abnormalities, including glucose intolerance, insulin resistance, central obesity, dyslipidaemia and hypertension, that affect cardio-metabolic health and increase the risks for cardiovascular disease and type 2 diabetes (Grundy *et al.*, 2005). The syndrome was first defined in the late 1980s (Reaven, 1988), but descriptions of common coexistence of the components dates back almost 100 years (Sarafidis and Nilsson, 2006). It is important to note that metabolic syndrome is a syndrome and not a disease and therefore not a common diagnosis.

WHO, the US National Cholesterol Education Program Panel and several other health organizations have separately presented definitions of metabolic syndrome (Vinluan *et al.*, 2012). First in 2005 and later in 2009, consensus groups attempted to agree on one definition (Alberti *et al.*, 2005, Alberti *et al.*, 2009). Today, the modified definition is abdominal obesity with any two of the following abnormalities: raised triglycerides, reduced high-density lipoprotein cholesterol, raised blood pressure and raised fasting blood glucose (Table 2) (Alberti *et al.*, 2009).

Table 2. *Risk factors and cut-off values for the joint definition of metabolic syndrome. Adapted from Alberti et al. 2009 *Population- and country-specific definitions shown for whites.*

Risk factor	Cut-off
Waist circumference*	Increased risk: Men ≥ 94 cm, women ≥ 80 cm
	Still higher risk: Men ≥ 102 cm, women ≥ 88 cm
Triglycerides	≥ 1.7 mmol/L or treatment for abnormal triglyceride levels
High-density lipoprotein cholesterol	< 1.03 mmol/L for males, < 1.29 mmol/L for females or treatment for abnormal high-density lipoprotein levels
Blood pressure	Systolic ≥ 130 or diastolic ≥ 85 mm Hg or hypertensive treatment
Fasting plasma glucose	≥ 5.6 mmol/L or previously diagnosed type 2 diabetes

Co-existence of these risk factors can be regarded as an intermediate step in the development of chronic lifestyle diseases. Prevalent metabolic syndrome is associated with a twofold increase in the risk of cardiovascular events and a fivefold increase in the risk of type 2 diabetes (Grundy *et al.*, 2004a, Grundy *et al.*, 2004b). Metabolic syndrome is a huge burden for health care systems worldwide, and, as an increasing fraction of the population is overweight and obese (Figure 4), the burden is expected to increase.

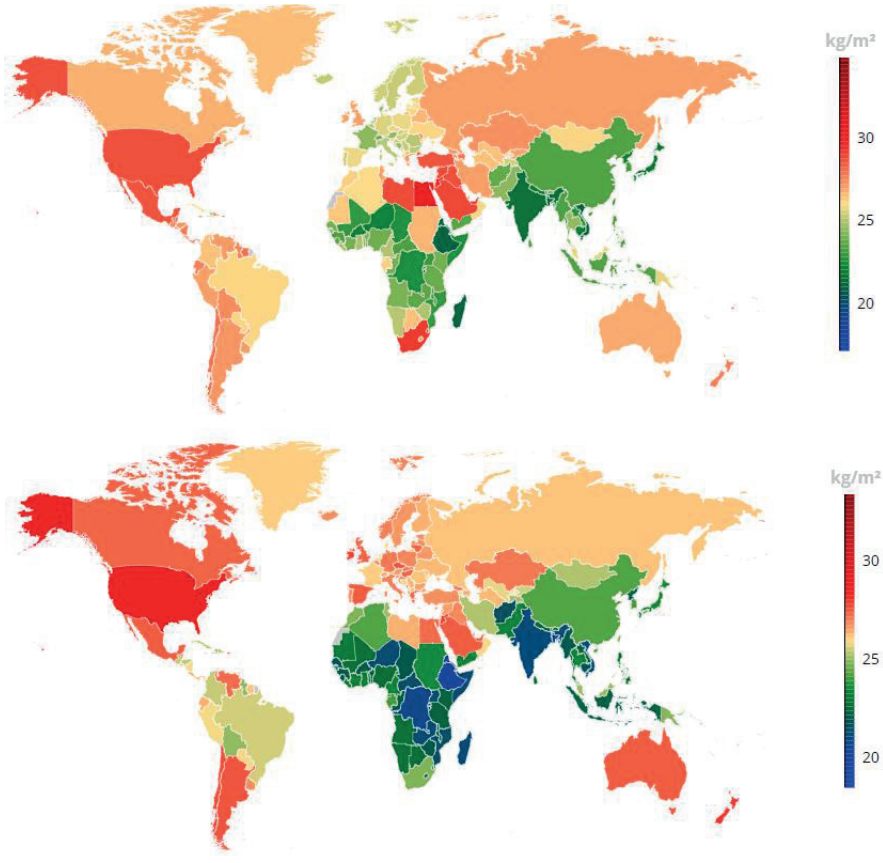

Figure 4. World map of age-standardized mean BMI (kg/m^2) for women (top) and men (bottom) aged > 18 years in 2014. Source: NCDrisk.org

1.4 Type 2 diabetes

Type 2 diabetes is characterized by insulin deficiency caused by β-cell dysfunction and insulin resistance in target organs (Chatterjee *et al.*, 2017). A steep rise in incidence has been observed over the past few decades (NCD-risC, 2016) (Figure 5), probably as a consequence of increasing trends in obesity and unhealthy lifestyle. As type 2 diabetes can remain undiagnosed for years, elevated blood glucose levels might already have caused damage to the body at the time of diagnosis (Harris *et al.*, 1992). The complications arising from high blood glucose levels include cardiovascular disease, kidney failure, limb amputation, vision loss and nerve damage, and people with type 2 diabetes are

at increased risk of premature death from such complications. In general, they are at excess risk of morbidity and mortality, the excess mortality being linked especially to cardiovascular disease (Tancredi *et al.*, 2015).

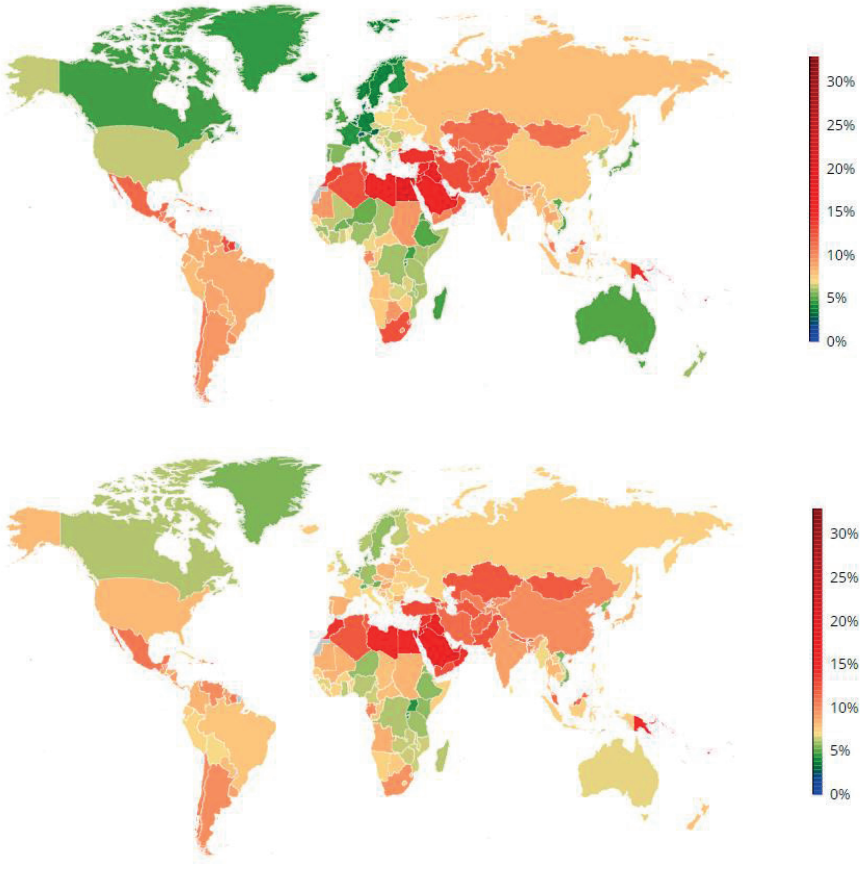

Figure 5. World map of age-standardized prevalence of diabetes (fasting plasma glucose ≥ 7.0 mmol/L, a history of diagnosis of diabetes, use of insulin or oral hypoglycaemic drugs); women (top) and men (bottom) aged >18 years in 2014. Source: NCDrisk.org

Correct diagnosis of diabetes requires laboratory tests; therefore, separate estimates for types 1 and 2 diabetes are rarely available. In high-income countries with available data, about 90% of diabetes is type 2 diabetes (WHO, 2016). The diagnostic criteria for diabetes and prediabetic conditions are shown in Table 3.

Table 3. *Diagnostic criteria for diabetes. Adapted from American Diabetes Association (ADA, 2015)*

Test	Limit	Description
Glycated haemoglobin	≥ 6.5%	Reflects the average plasma glucose concentration for the past 3 months
Fasting plasma glucose	≥ 7.0 mmol/L (126 mg/dL)	Glucose concentration in plasma after fasting for at least 8 h
2-h plasma glucose	≥ 11.1 mmol/L (200 mg/dL)	2-h glucose concentration in plasma after an oral glucose tolerance test: glucose load of 75 g anhydrous glucose dissolved in water
Random plasma glucose	≥ 11.1 mmol/L (200 mg/dL)	In patients with classic symptoms of hyperglycaemia
Fasting plasma glucose	5.6-6.9 mmol/L (100-125 mg/dL)	Impaired fasting glucose
2-h plasma glucose	7.8-11.0 mmol/L (140-199 mg/dL)	Impaired glucose tolerance

Risk factors for type 2 diabetes

Lifestyle factors, including physical inactivity and poor nutrition (and excess body weight), play a major role in the development of type 2 diabetes. According to WHO, tobacco use, high blood pressure, high blood glucose, physical inactivity and overweight and obesity are the five leading risk factors for mortality worldwide (WHO, 2009). These are also either components, consequences or risk factors of diabetes, indicating the massive impact on health of this disease and also the immense potential for prevention.

Foods that have been included in successful diabetes management by improving glycaemic control and blood lipids include whole grains, fruits, vegetables, legumes and nuts, whereas refined grains, red and processed meats and sugar-sweetened beverages are associated with poor management of diabetes (Ley *et al.*, 2014). Additionally, diets with a low glycaemic index or load result in better glucose control (Thomas and Elliott, 2010). In the Danish national plan for fighting diabetes, one of the four initiatives is specific dietary recommendations for fibre, whole grains and fibre-rich vegetables for people with diabetes (Danish Ministry of Health, 2017).

Diabetes has consistently been associated with increased risks for several cancers (Carstensen *et al.*, 2014, Giovannucci *et al.*, 2010). The situation with regard to prostate cancer, however, is not straightforward. People with diabetes appear to have a lower incidence of prostate cancer (Bansal *et al.*, 2013, Tsilidis *et al.*, 2015), while obesity, which is strongly associated with type 2 diabetes (Bhupathiraju and Hu, 2016), has been linked to an increased risk for aggressive

prostate cancer (Allott *et al.*, 2013). This contradictory picture may be due to the fact that the association with diabetes depends on the clinical grade of the prostate tumour, so that the inverse association concerns only low- to moderate-grade and not high-grade tumours (Dankner *et al.*, 2016). Low PSA concentrations in obese men have been reported to result in lower referral rates for prostate biopsy and therefore more aggressive disease at the time of delayed diagnosis (Muller *et al.*, 2009, Dankner *et al.*, 2016). In a study in which biopsies were conducted regardless of PSA levels, no association was found between diabetes and prostate cancer (Wu *et al.*, 2011a). Antidiabetic medication, and especially insulin (Fall *et al.*, 2013, Haggstrom *et al.*, 2017), has also been suggested as an explanation for an association with prostate cancer. Insulin is prescribed to patients with long-term diabetes and high levels of glycated haemoglobin (Ekstrom *et al.*, 2012), both of which have been associated with a decreased risk of prostate cancer. The relation is complex, but mortality from prostate cancer is increased in the presence of diabetes (Bensimon *et al.*, 2014).

1.5 Prostate cancer

Prostate cancer is a heterogeneous disease, ranging from small, confined tumours to high-grade, aggressive tumours that are likely to metastasize to the surrounding tissue (Scardino *et al.*, 1992). Indolent disease and disease that progresses to an aggressive state constitute entirely different patterns of treatment, prognoses and quality of life (Abbas and Scardino, 1997, Borre *et al.*, 1997). Classification of prostate cancer stage is based on the TNM staging system (T: size and extent of primary tumour, N: whether it has spread to nearby lymph nodes, and M: whether it has metastasized), on Gleason score grading of the (ab)normality of tissue from prostate biopsies under the microscope and PSA levels in blood. PSA is a protein produced by the prostate gland that can be measured in blood, the level of which tends to be higher in men with prostate cancer. PSA may also be increased in other conditions, and levels increase naturally with age (National Collaboration Centre for Cancer, 2014). The European Association of Urology classifies prostate cancer risk as low, intermediate or high (Heidenreich *et al.*, 2014), as shown in Table 4.

Table 4. *Classification of prostate cancer disease, adapted from the European Association of Urology*

Risk classification	TNM	PSA	Gleason score
Low	T1-2a and	< 10 ng/mL and	< 7
Intermediary	T2b or	10–20 ng/mL or	7
High	T2c-3 or	> 20 ng/mL or	> 7

The major treatment options for prostate cancer are surgery, radiation, medical therapy and active surveillance. Invasive treatment can have unwanted side-effects, especially on urination and erectile function. The conservative approach of active surveillance, in which disease progression is monitored by regular PSA testing and prostate biopsies, is used for men with early-stage, indolent prostate cancer to avoid unnecessary side-effects. This conservative approach can, however, cause distress among patients and relatives due to lack of active treatment.

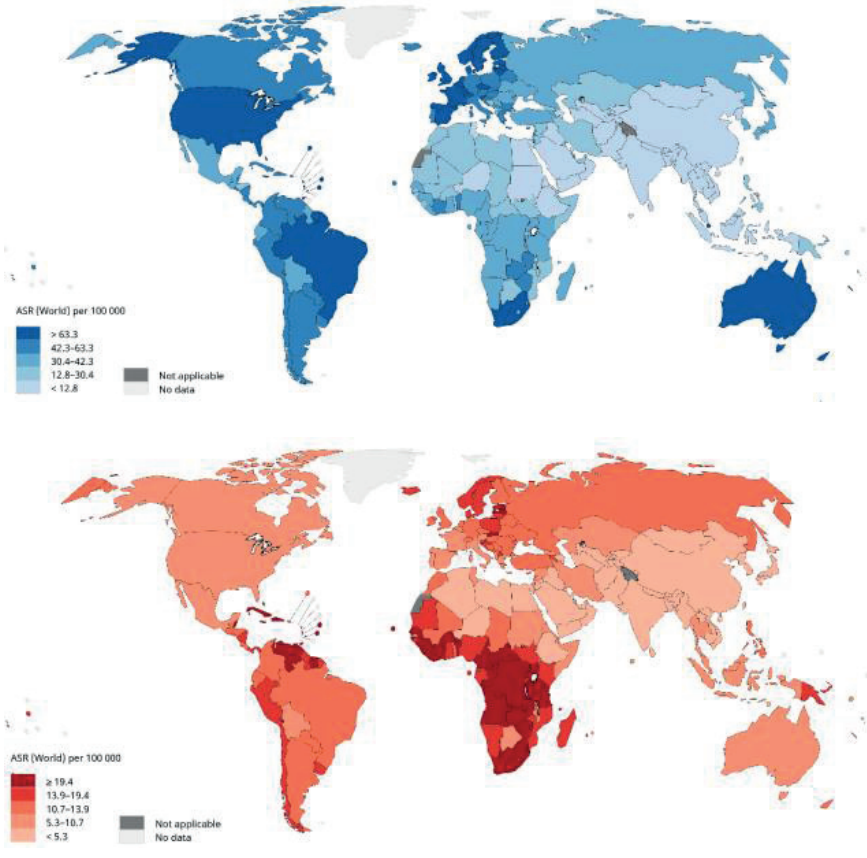

Figure 6. Age-standardized rates of prostate cancer incidence (blue) and mortality (red) in the world (2018). Source: GLOBOCAN 2018, International Agency for Research on Cancer (Ferlay J, 2013).

Prostate cancer is the second most commonly diagnosed male cancer and the fifth leading cause of cancer-related deaths worldwide. Largely because of the practice of PSA testing, prosperous regions of the world account for almost 70% of cases of diagnosed prostate cancer (Ferlay *et al.*, 2010). Although large

differences in incidence are seen worldwide (Ferlay J, 2013), less variation is seen in mortality rates (Figure 6), which indicates that the higher incidence due to PSA screening adds mainly to the number of cases of indolent, not aggressive, prostate cancers. The rates of PSA screening have declined in Denmark during the past decade, probably as a result of updated recommendations from urologists (DAPROCA, 2014) and in the USA, from the national task force (Carlsson and Roobol, 2016).

In Denmark, the disease is diagnosed in almost 4500 men every year, and nearly 1200 die from this cause, making it the most common cancer among Danish men and the second most common cause of cancer death. The 5-year relative survival is 87% (Engholm *et al.*, 2010).

Risk factors for prostate cancer

The only established risk factors for prostate cancer are advanced age, ethnic group and heredity (Cuzick *et al.*, 2014). Most clinically detected prostate cancers appear in men over 60 years of age, after which the incidence rises steeply, while diagnosis before the age of 50 is rare (1%). For this thesis, prostate cancer only in white men was studied, although the mortality rates are higher in other ethnic groups (WHO, 2014). The risk for prostate cancer is two to three times higher among men with a first-degree relative with prostate cancer than among men with no family history of the disease (Johns and Houlston, 2003).

Of the lifestyle factors, smoking, lack of physical activity and obesity have been investigated as risk factors for prostate cancer. The World Cancer Research Fund update in 2014 concluded that there is strong evidence for an increased risk of advanced prostate cancer in overweight and obese men (WCRF/AIRC, 2014). Although diet and nutritional risk factors, including beta-carotene, dairy products, calcium, vitamin E and selenium, have been investigated, the evidence is limited. Physical activity has also been studied in relation to prostate cancer progression and the quality of life of patients. A recent meta-analysis found no effect of exercise interventions on disease progression but indicated improved cancer-specific quality of life, including less fatigue and better fitness and function (Bourke *et al.*, 2016).

No specific dietary recommendations have been made for prostate cancer patients. A beneficial effect of whole-grain rye on prostate cancer has been suggested (Landberg *et al.*, 2010, Bylund *et al.*, 2003), but no specific recommendations have been made to increase whole-grain rye intake.

1.6 Summary of diseases

Together, metabolic syndrome, type 2 diabetes and prostate cancer constitute an enormous health burden. In Denmark alone, there are currently more than 38 000 men living with prostate cancer and 225 000 people with a diagnosis of type 2 diabetes; presumably, about 20–30% of the middle-aged population has metabolic syndrome. As more than half the population over 55 years of age has cardiovascular disease, which is the greatest contributor to death among people with type 2 diabetes, the health burden is considerable. Similarly, overweight and obesity are important risk factors for these diseases, whereas increased physical activity and dietary changes, including whole grains, could improve or even reverse morbidity and mortality.

2 Objectives

The overall aim of this thesis was to investigate the effects of whole grains and dietary lignans and their metabolite enterolactone on cardio-metabolic risk factors and to investigate the association between plasma enterolactone and the prognoses of type 2 diabetes and prostate cancer. These diseases share several risk factors, which are present in metabolic syndrome. Therefore, the whole grain and lignan intervention was conducted in a population of men with metabolic syndrome.

The specific objectives included:

- to evaluate the differential effect of a whole-grain lignan-rich rye diet vs a whole-grain low-lignan wheat diet on cardio-metabolic risk factors in men with (or with signs of) metabolic syndrome in a crossover intervention study;

- to study the association between pre-diagnostic enterolactone concentrations and mortality among people with type 2 diabetes in the Diet, Cancer and Health (DCH) cohort;

- to investigate, in a pilot study, the feasibility of a lifestyle intervention with whole-grain rye and physical activity among men with non-aggressive prostate cancer; and

- to study the association between pre-diagnostic enterolactone concentrations and mortality among men with prostate cancer in the DCH cohort.

The hypotheses on which the thesis is based are that:

1 Whole-grain rye with a high lignan content has a greater beneficial effect on cardio-metabolic risk factors such as glucose homeostasis and lipids than whole-grain wheat with a low lignan content.
2 Enterolactone concentrations are associated with lower mortality rates among people with type 2 diabetes.
3 High intake of whole-grain rye and physical activity are feasible for men with non-aggressive prostate cancer. Such lifestyle changes may improve clinical and prognostic markers in the intervention group as compared with the control group.
4 Enterolactone concentrations are associated with lower mortality rates in men with prostate cancer.

3 Materials and Methods

Two human interventions and two cohort studies within the ELIN project formed the basis for the thesis (Table 5**)**:

- *Papers I* and *III* describe human interventions: the first, with a cross-over design, was a dietary intervention among men with or with signs of metabolic syndrome, and the second, with a parallel design, was a lifestyle intervention among men with non-aggressive prostate cancer.

- *Papers II* and *IV* are based on observational prospective studies: *paper II* describes a case–cohort study among people with diabetes, and *paper IV* describes a traditional cohort study among men with prostate cancer.

Table 5. *Studies in this thesis by disease and design*

	Metabolic syndrome and diabetes	Prostate cancer
Intervention studies	*Paper I*:	*Paper II*:
	The ELIN cross-over intervention study	The NILS feasibility intervention study
	Population: middle-aged Swedish men with metabolic syndrome	Population: middle-aged Danish men with non-aggressive prostate cancer
	n=40	n=21
Observational studies	*Paper III*:	*Paper IV*:
	Case-cohort study among people with diabetes in the DCH cohort	Cohort study among men with prostate cancer in the DCH cohort
	n=1183	n=1390

3.1 Study populations and designs

3.1.1 Human interventions

The ELIN intervention study

Participants and recruitment

Of 107 men who showed interest in participating in the study, 49 were eligible, enrolled and randomized to the study, of whom 40 completed the intervention (Figure 7). The channels for recruitment included local newspapers, Facebook, a clinical study website and an established cohort study database (Lind *et al.*, 2013). We advertised for men aged 40–75 years with a BMI > 25 kg/m^2 and a waist circumference > 102 cm living in Uppsala County, Sweden. Enrolment was conducted continuously between June 2015 and March 2016. Before screening, a brief introduction to the study was given over the telephone or by e-mail, and the inclusion criteria were confirmed as fulfilled.

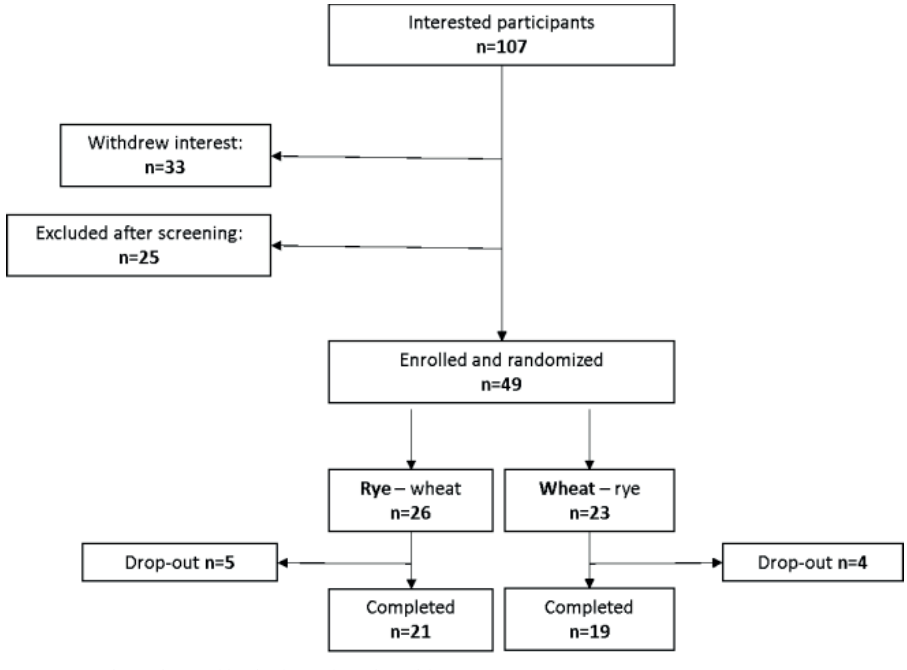

Figure 7. Flow chart of inclusion of study subjects

The inclusion criterion of waist circumference > 102 cm was complemented by any of the other risk factors for metabolic syndrome. Most of the participants

were included with additionally elevated blood pressure and/or fasting glucose (Table 6).

Table 6. *Overview of metabolic syndrome criteria and numbers of participants who met them*

Criteria for metabolic syndrome	n
Waist circumference > 102 cm	39
Systolic blood pressure ≥ 130 mm Hg or diastolic blood pressure ≥ 85 mm Hg	35
High-density lipoprotein cholesterol ≤ 1.03 mmol/L	11
Triglycerides ≥ 1.70 mmol/L	7
Fasting glucose ≥ 5.6 mmol/L	38
Met the international definition of metabolic syndrome	33

Intervention

The intervention lasted 24 weeks and included six study visits, at baseline, at 4 weeks, at 8 weeks, at 16 weeks (after 8 weeks of wash-out), at 20 weeks and at 24 weeks (end of intervention) (Figure 8). One intervention arm was given whole-grain rye products for 8 weeks, with lignan capsules for the last 4 weeks. The other intervention arm was given whole-grain wheat products for 8 weeks with no supplement. The two intervention periods were separated by an 8-week wash-out period during which the subjects ate their habitual diet with no restrictions.

Before each study visit, participants kept 3-day weighted dietary records and collected 24-h urine and a faecal sample that was brought to the study clinic on the day of examination.

Biological samples

Blood samples (38 mL in total) were spun, divided into plasma, erythrocytes and buffy coat and frozen at –20 °C for approximately 1 week before they were moved to a –80 °C freezer until analysis. Urine was aliquoted into five 1-mL tubes and stored similarly to the blood samples. Faecal samples were kept in a cooling bag and frozen immediately upon arrival at the study clinic.

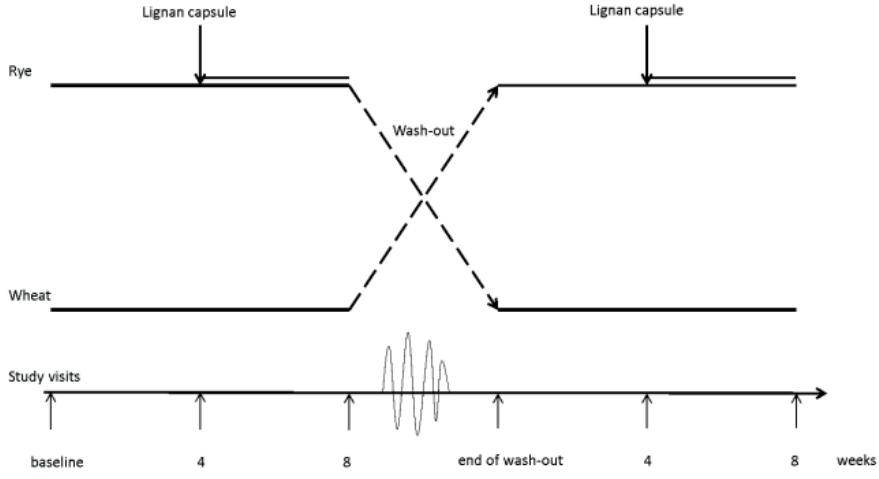

Figure 8. Cross-over study design of the ELIN intervention

The NILS feasibility study

Participants and recruitment

A total of 26 men (aged 53–72 years) with non-aggressive prostate cancer were enrolled in the NILS study between March 2011 and November 2012. The participants were recruited from the Department of Urology, Aarhus University Hospital, Denmark, at the time of diagnosis or at regular visits for active surveillance, which was offered to men with non-aggressive prostate cancer at the hospital. Detailed information on the inclusion and exclusion criteria is provided in *paper I* (Eriksen *et al.*, 2017).

Intervention

Participants were randomly allocated to the intervention or the control group. The control group received prostate cancer management according to conventional guidelines, and the intervention group was assigned to 135 min of vigorous physical activity, encouraged to walk 10 000 steps per day and to consume 1200 g of whole-grain rye per week. The intervention lasted 6 months, comprising study visits at baseline, after 3 months and after 6 months (end of intervention). A final visit was scheduled 12 months after baseline (see Figure 9).

Biological samples

At each study visit, participants were examined after an overnight fast. A blood sample was drawn (26 mL), spun, divided into plasma, serum, erythrocytes and

buffy coat and stored at –80 °C. At baseline and at the 6-month visit, prostate biopsy samples were taken to evaluate prostate cancer progression.

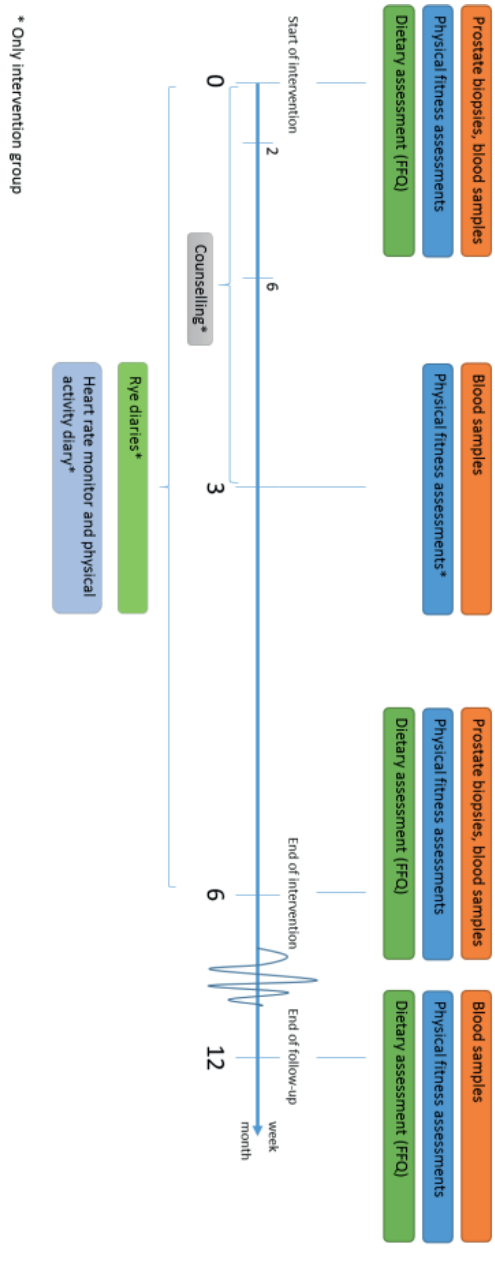

Figure 9. Design of the NILS feasibility study

3.1.2 Observational studies

The Diet, Cancer and Health cohort

The observational studies were based on data from the Danish DCH cohort, which was established in the mid-1990s to investigate the relations between diet and lifestyle and the incidence of cancer. Between 1993 and 1997, 160 725 Danish men and women were invited to participate. Of these, 57 053 were enrolled, corresponding to a participation rate of 34% for men and 37% for women (Tjonneland *et al.*, 2007). Invited participants were Danish citizens living in the Copenhagen and Aarhus regions of Denmark, aged 50–64 years and with no registered cancer diagnosis. Physical examinations and blood sampling were conducted by trained study staff.

Assessment of dietary habits and lifestyle

Information on dietary habits was collected at baseline from replies to a validated 192-item food frequency questionnaire (Tjonneland *et al.*, 1991) that was posted to all participants before their examination. Participants were asked to report their average intake of a wide range of foods and beverages during the previous 12 months. The questionnaire allowed 12 categories, ranging from never to eight times a day or more. With the software program Foodcalc (Lauritsen, 1998), standardized recipes and portion sizes were used to calculate the intake of specific foods and nutrients by each participant. Additionally, all participants filled in a questionnaire on lifestyle risk factors for cancer.

Biological samples

A total of 30 mL of blood (non-fasting) was drawn from each participant. Blood samples were spun and separated into plasma, serum, erythrocytes and buffy coat and frozen within 2 h at – 20 °C. Later the same day, all samples were moved to a biobank, where they are stored in liquid nitrogen vapor (– 150 °C).

Registries

For the studies described in *papers II* and *IV*, information was obtained from several Danish registries (Table 7).

Table 7. *Danish registries from which information was obtained for the studies in papers II and IV*

Registry	Information
Danish Cancer Registry (Storm *et al.*, 1997)	Cancer diagnosis
Danish Diabetes Register (Carstensen *et al.*, 2008)	Diabetes diagnosis
Danish Civil Registration System (Pedersen, 2011)	Vital status and date of death or emigration
Danish Register of Causes of Death (Helweg-Larsen, 2011)	Cause of death
Danish National Prescription Registry (Kildemoes *et al.*, 2011)	Use of antibiotic medication

Follow-up

In the studies in both *papers II* and *IV*, prospective cohort designs were used to examine the association between pre-diagnostic enterolactone concentrations and mortality among people with type 2 diabetes (*paper II*) and among men with prostate cancer (*paper IV*) from the DCH cohort. All cases of diabetes and cancer from the DCH cohort diagnosed between baseline and the end of 2009 were included (Figure 10). These cases were then followed from the date of diagnosis until date of death, emigration or end of follow-up at 31 December 2013 (*paper IV*) or 1 February 2016 (*paper II*).

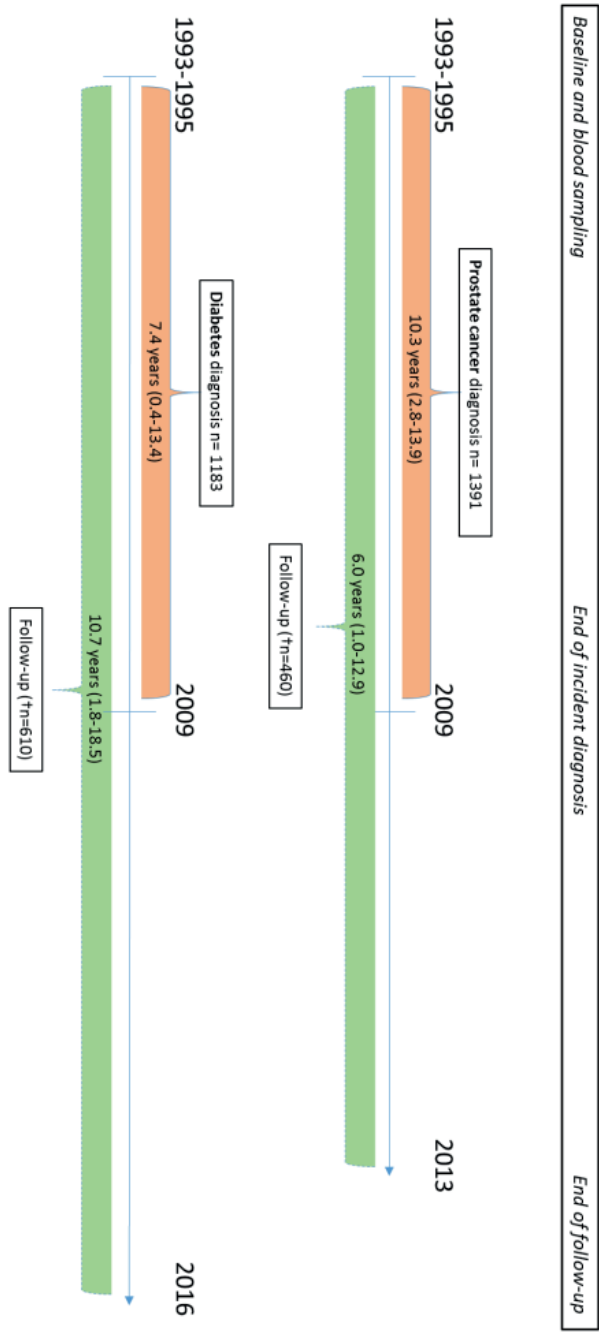

Figure 10. Timeline of cohort study design for papers II and IV

3.2 Laboratory analyses

The practical laboratory analyses were not part of the current PhD project and are described only briefly. An optimized method for enterolactone quantification was developed as part of the ELIN project (Norskov *et al.*, 2016), which was a high-throughput liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for quantification of glucuronidated, sulfated and free enterolactone. This method was used for the studies in *papers II* and *IV*. For those in *papers I* and *III*, enterolactone in plasma and urine was analysed with a slightly different method (Norskov *et al.*, 2015), involving enzymatic hydrolysis. Plant lignans in intervention products were also analysed by LC-MS/MS (Norskov and Knudsen, 2016). All analyses were conducted by the same person at the Department of Animal Science, Aarhus University.

Alkylresorcinols were analysed at the SLU for both intervention studies by a rapid gas chromatography–mass spectrometry method (Landberg *et al.*, 2009). Clinical biomarkers in samples from the ELIN and NILS interventions were analysed at the certified laboratory of the Department of Clinical Chemistry at Uppsala University Hospital, Sweden. Total PSA was analysed at the Biochemical Laboratory, Aarhus University Hospital, Denmark, for the NILS study. The microbiome of faecal samples from the ELIN intervention was analysed at the Department of Animal Nutrition and Management at SLU by sequencing 16S rRNA gene amplicons with Illumina MiSeq (Kampmann *et al.*, 2016). Table 8 gives an overview of the analyses performed for this thesis.

Table 8. *Analyses conducted for the studies in this thesis*

Analyte	ELIN	NILS	DCH cohort
Plasma enterolactone	X	X	X
Urine enterolactone	X	X	X
Plasma enterodiol	X	X	
Urine enterodiol	X	X	
Alkylresorcinols	X	X	
Lipids	X	X	X
Glucose/insulin	X	X	X
PSA		X	
Faecal microbiome	X		
Plant lignans	X		

3.3 Statistical analyses

All statistical analyses were conducted with SAS® statistical software, release 9.3/9.4 (SAS Institute, Cary, NC) for Windows.

3.3.1 Human interventions

The ELIN intervention study

The ELIN intervention was a cross-over design in which participants acted as their own controls. This design was preferred because there is wide inter-individual variation in enterolactone concentrations. The primary outcome was the difference in change in plasma glucose after a 2-h oral glucose tolerance test after the two interventions. The secondary outcomes included differences in fasting blood lipid concentrations and blood pressure between the interventions after 4 and 8 weeks and differences between diets in gut microbiota, plant and enteroligans in plasma and SCFA in plasma and faecal samples. Sample size was calculated on the basis of effects estimated by means of a paired two-sided *t* test, with a significance level of $\alpha = 0.05$ on the assumption of no cross-over effect or interaction among participants, treatments and periods.

The effect of the intervention diets on glucose homeostasis, lipid profile, blood pressure and anthropometrics was evaluated in linear mixed models (PROC MIXED). Diet, period and a diet x period interaction term were included as fixed effects and subject, as a random factor allowed to depend on diet in the models.

The NILS feasibility study

The study was planned as a pilot for a future, larger study. Therefore, only 26 men were recruited, of whom 21 completed the intervention. The statistical analyses are based on those who completed the study. The feasibility of the intervention was evaluated from reported whole-grain rye intake, alkylresorcinol concentrations, physical activity diaries and heart rate monitoring during the intervention period, which are presented as means (± SD). The effects of the intervention were evaluated by linear multiple regression analyses (least squares means) from differences in mean group changes between baseline and the end of the intervention, with adjustment for the baseline level of the outcome variable.

Because of the feasibility aspect of the NILS intervention, the statistical power was limited.

3.3.2 Observational studies

The statistical analyses for the observational studies were based on the Cox proportional hazards model, with follow-up time as the underlying time scale. To account for age as a risk factor for mortality, 5-year bands were made to separate underlying hazards in each age group. The assumption of linearity of the associations was evaluated graphically by linear splines.

Type 2 diabetes mortality: case–cohort design

Between baseline and the end of 2009, incident diabetes was diagnosed in 4483 DCH participants. During follow-up, 1402 died. A random sub-cohort of 850 people was sampled from the total of 4483, and a random group of 349 deceased people was sampled from the total of 1402 deceased. After exclusions, 841 people with diabetes in the sub-cohort and 342 from the sampled deceased group were included in the study reported in *paper III* (Figure 11). Of the sub-cohort, 268 died during follow-up. The total case–cohort study population was therefore 1183 people with diabetes and 610 deaths (342+268). The primary outcome of interest was all-cause mortality. Secondarily, death from diabetes, cardiovascular disease, cancer, respiratory disease and other causes was evaluated.

Had the study for *paper II* been conducted with a traditional cohort design, as that for *paper IV*, the number of deceased people in a random subsample from the DCH cohort would have been approximately 268 out of 841 (32%). In the case–cohort design, the proportion of deceased people in the study almost doubled (610/1183 = 52%). This design was chosen to ensure sufficient statistical power by the possibility of oversampling deceased cases, as people with type 2 diabetes often live for many years with their disease.

The Cox proportional hazards model was applied, adjusted by special weighting for the case group (the deceased case group). The subsample entered the Cox model as in the study in *paper II*. The sampled deceased group entered the analyses slightly differently, as these cases contributed follow-up time only from 0.5 day before their date of death. To account for potential sex differences and due to problems with the proportional hazards assumption for smoking, additional strata for sex and smoking were added in this model. Two levels of covariate adjustment and a number of sensitivity analyses were conducted (details described in *paper II*).

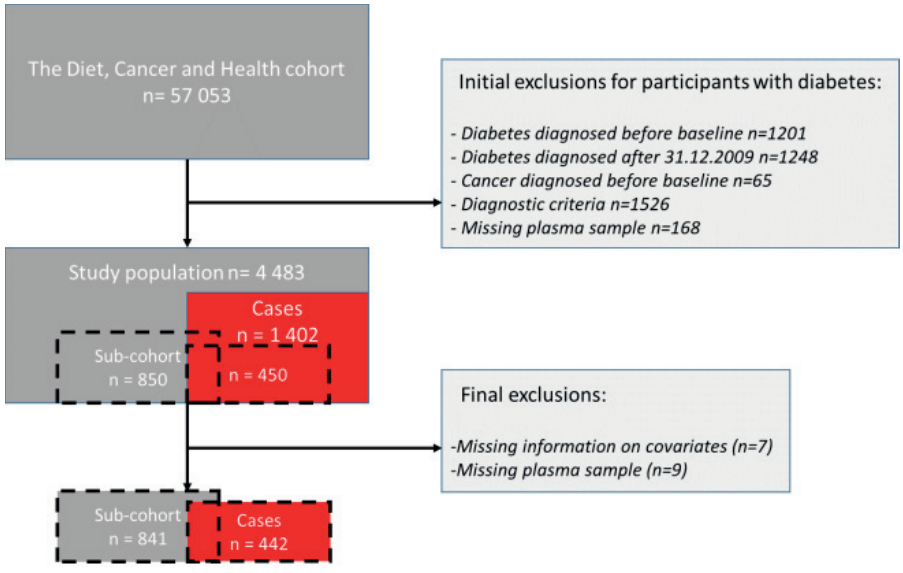

Figure 11. Selection of participants for the case-cohort study (paper II)

Prostate cancer mortality: prospective cohort design

Between baseline and end of 2009, prostate cancer was diagnosed in 1431 male participants in the DCH cohort. After exclusions, 1391 prostate cancer cases were included in the study in *paper IV*. The outcomes of interest were overall mortality and mortality from prostate cancer. During the follow-up period, 460 men with prostate cancer died, of whom 301 died specifically from prostate cancer. The Cox model allows thorough adjustment of co-variates, and two levels of adjustment were evaluated to test the robustness of the results by including relevant potential confounding factors (see paragraph below). Furthermore, disease aggressiveness and effect modification by BMI, smoking and physical activity were evaluated.

Confounding factors

Tobacco use, physical inactivity, high blood pressure, high blood glucose and overweight and obesity are the leading risk factors for mortality, according to WHO (WHO, 2009). We chose not to include blood pressure and blood glucose, as both are metabolic markers of health affected by smoking, physical activity and obesity. These three risk factors were therefore included in both papers in this thesis that evaluated mortality. Other factors were considered as potential confounders but not included in the final models. Adjustment for use of antibiotics was considered relevant and included in both papers because of their

known effect on microbiota and thereby enterolactone production. In the study described in *paper II*, a stratified analysis was conducted among non-users of antibiotics.

4 Results and study-specific discussions

4.1 Lignan intake and enterolactone concentrations

Enterolactone concentrations are considered to be the result of habitual dietary intake of lignan-containing foods in combination with lifestyle factors and the microbiota that are known to affect the production of enterolactone. In the study for *paper I*, the intervention diet contributed approximately 220 g/day of whole-grain rye or whole-grain wheat, providing 12.8 and 4.7 mg of total plant lignans, respectively. The lignan capsules given in the last 4 weeks of rye treatment provided a daily dose of 300 mg of secoisolariciresinol diglucoside (SDG). Thereby, the difference in lignan content of the diets increased from 1:3 to 1:64. In the study described in *paper III*, the intervention diet contributed 170 g of whole-grain rye products per day. The lignan content in the NILS study was not determined but is assumed to have been approximately 10 mg/day. In comparison, the mean dietary lignan intake in the European EPIC cohort was estimated to be 2.5 mg/day for men and 3.6 mg/day for women (Zamora-Ros *et al.*, 2016).

The enterolactone concentrations at baseline in the two interventions were 17 nmol/L (7–73) in the ELIN study (*paper I*) and 36 nmol/L (3–143) in the NILS study (*paper III*). In the ELIN study, a modest increase was observed after 4 weeks of whole-grain rye intake, whereas the concentrations observed after 8 weeks approached 200 nmol/L (Table 9). Other intervention studies with lignan supplementation of 300–600 mg/day have resulted in enterolactone concentrations of the same magnitude, 183–561 nmol/L (Hallund *et al.*, 2006, Zhang *et al.*, 2008), or 11-fold increases when only urinary excretion was determined (Pan *et al.*, 2007).

Table 9. *Intervention studies with lignans from supplements of SDG or whole grains*

Study	Sample	Intervention	Enterolactone concentration (nmol/L)
		High-dose SDG	
(Hallund *et al.*, 2006)	n=22 postmenopausal women	500 mg/day SDG for 6 weeks	46 (8) –> 385 (67)*
(Zhang *et al.*, 2008)	n=18/20 hypercholesterolaemic	300 and 600 mg/day SDG for 8 weeks	300 mg/day: 43 (58) –> 183 (246) 600 mg/d: 28 (62) –> 561 (1340)
(Pan *et al.*, 2007)	n=73 with type 2 diabetes	360 mg/day SDG for 12 weeks	Only urinary enterolactone determined: 6 (12)–> 71 (11) µmol/mL
(Cornish *et al.*, 2009)	n=50/50 older adults (metabolic syndrome)	543 mg/day SDG for 6 months	Not reported Effect on diastolic blood pressure and z score
(Eriksen *et al.*, unpublished)	n=40 middle-aged men (metabolic syndrome)	300 mg/day SDG for 4 weeks	29 (5–86)–> 197 (27–573)
		Lignans from dietary interventions	
(Jacobs *et al.*, 2002)	n=11 overweight, hyperinsulinaemic	30% of total energy intake from Whole-grain diet based on wheat, rice and oats	10.6 nmol/L–> ≈19 nmol/L
(Juntunen *et al.*, 2000)	n=18 men/21 women	20% of daily energy from rye bread	Men: 28.1 (3.8)–> 25.6 (4.7)* Women: 39.3 (4.4)–> 39.7 (6.5)
(Landberg *et al.*, 2010)	n=17 men	485 g/day of rye whole-grain and bran products (≈12 mg/day of lignans)	9.0 (43)–> 18 (95% CI: 12,28) (geometric mean)
(Eriksen *et al.*, unpublished)	n=40 middle-aged men (metabolic syndrome)	220 g of whole-grain rye with 12.8 mg/day of lignans	17 nmol/L (7–73)–> 29 (5–86)
(Eriksen *et al.*, 2017)	n=14 middle-aged men (prostate cancer)	170 g of whole-grain rye products with ≈10 mg/day of lignans	33 (0.1–195)–> 50 (0.1–231)

*Mean ± SEM, otherwise mean ± SD or median (5th–95th percentiles)

The enterolactone concentrations reported in this thesis, based on the Danish DCH cohort, ranged from 9.8 mol/L (1–51) for men and 12.1 (1–74) nmol/L for women with type 2 diabetes to 18.6 (3–89) nmol/L for men with prostate cancer. A marked difference in enterolactone concentrations was found for cohort participants who later were found to have prostate cancer and type 2 diabetes in data from the same source population and an identical method of enterolactone

determination. It has been shown that the gut microbiota of people with type 2 diabetes has a less favourable enterolactone-producing composition (Larsen *et al.*, 2010, Roncaglia *et al.*, 2011), which may affect the conversion of enterodiol to enterolactone. Unfortunately, enterodiol was not determined in the study in *paper II*.

The enterolactone concentration in people with type 2 diabetes was lower than the average Danish concentration but similar to those reported in populations in France, Germany, Italy, the Netherlands and the UK (Table 10). The differences from those in the Nordic countries may be a result of higher intake of whole grains (Kyro *et al.*, 2014), less use of antibiotic medication or general gut microbiota health.

Table 10. *Plasma enterolactone concentrations in [a] the EPIC study (Peeters et al., 2007), [b] the DCH cohort (Olsen et al., 2011), [c]Cancer of the Prostate in Sweden (CAPS) (Hedelin et al., 2006), [d]a Finnish study (Kilkkinen et al., 2001), [e] a second Finnish study (Kilkkinen et al., 2003b) and [f] the Nurses Health Study II (Xie et al., 2013)*

	Mean plasma enterolactone concentration (nmol/L) (SD) or median (IQR/P5–P95)	
Country	Population/Control	Cancer
Denmark	27.5 (16.8-45.9) [a]	Breast (alive/deceased): 29 (1–110)/19 (2–89)[b]
Sweden, Umeå	15.1 (9.4-23.8) [a]	
Sweden (CAPS study)	23.9 (3.9-79.3) [c]	Prostate: 21.1 (1.5–79.3) [c]
Finland	13.8 (0-95.6), ♂ [d]	
	16.6 (0-182.6), ♀ [d]	
	16.9 (±14.9) [e]	Prostate: 15.9 (±15.2) [e]
United Kingdom, Cambridge	8.7 (6.4-11.4) [a]	
Netherlands	9.4 (7.0-12.4) [a]	
Germany, Potsdam	10.4 (7.7-13.7) [a]	
France	11.4 (7.4-17.4) [a]	
Italy, Naples	8.0 (6.0-10.7) [a]	
Greece	15.1 (11.1-20.1) [a]	
USA (NHS II)	11 (1-40) [f]	Breast: 11 (1–38) [i]

The median enterolactone concentration among 21 Danish men with non-aggressive prostate cancer was the highest (35.6 nmol/L) found in the studies in this thesis. In the ELIN intervention study, the median concentration was 17.0 nmol/L, which is similar to that observed in the study for *paper IV*. Metabolic syndrome may be regarded as an intermediate step towards type 2 diabetes. Thus, low enterolactone concentrations might have been expected at a pre-

diabetic stage; however, this was not the case. The difference in enterolactone concentrations between the ELIN and NILS trials might indicate marked differences in lifestyle between men who develop type 2 diabetes rather than prostate cancer, although differences in habitual lignan intake between Danish and Swedish men may also have contributed.

4.2 Compliance in dietary intervention studies

Good compliance is crucial to obtain relevant information from intervention studies. In the ELIN intervention (*paper I*), the median baseline total alkylresorcinol concentration was 47 nmol/L (5th–95th percentiles: 18–147), and the alkylresorcinol C17:0/C21:0 ratio was 0.33 (0.11–0.83), reflecting a relatively low intake of mixed whole grains, somewhat dominated by wheat. An increase in total alkylresorcinols was observed in both groups after 4 and 8 weeks of treatment, and the C17:0/C21:0 ratio increased significantly in the rye treatment arm and decreased significantly in the wheat treatment arm (Table 11), confirming high intakes of whole-grain rye and whole-grain wheat, respectively. The alkylresorcinol concentrations indicate good compliance in both treatment arms.

In the NILS intervention (*paper III*), the baseline alkylresorcinol concentration was 74 nmol/L (17–180), which increased after 3 and 6 months of the intervention. The C17:C21 ratio indicates higher intake of whole-grain rye in the intervention group than in the controls, but the difference was smaller than in the ELIN intervention. This might have been due to poorer compliance in the NILS study owing to its long duration. In both interventions, the increase in alkylresorcinols was generally followed by smaller increases in enterolactone concentrations, except after lignan supplementation in the ELIN trial, where a steep increase in enterolactone was found.

Table 11. *Alkylresorcinols (total and C17:0/C21:0 ratio) and enterolactone concentrations in the ELIN and NILS interventions*

ELIN intervention	Baseline	4 weeks	8 weeks
	Median (5th–95th percentile)		
Rye (n=40)			
Total alkylresorcinol (nmol/L)	47 (18-147)	170 (90-432)	193 (73-484)
Alkylresorcinol C17:0/21:0	0.33 (0.11-0.83)	0.64 (0.33-1.22)	0.60 (0.30-1.03)
Enterolactone (nmol/L)	17 (7-73)	29 (5-86)	197 (27-573)
Wheat (n=40)			
Total alkylresorcinol (nmol/L)	47 (18-147)	165 (71-498)	162 (63-410)
Alkylresorcinol C17:0/21:0	0.33 (0.11-0.83)	0.08 (0.04-0.64)	0.08 (0.03-0.26)
Enterolactone (nmol/L)	17 (7-73)	21 (7-74)	25 (4-107)
NILS intervention	Baseline	3 months	6 months
	Median (5th–95th percentile)		
Intervention (n=14)			
Total alkylresorcinol (nmol/L)	83 (17-414)	194 (62-412)	176 (43-608)
Alkylresorcinol C17:0/21:0	0.36 (0.16-0.75)	0.46 (0.16-0.66)	0.37 (0.06-0.80)
Enterolactone (nmol/L)	33 (0.1-195)	37 (0.2-315)	50 (0.1-231)
Control (n=7)			
Total alkylresorcinol (nmol/L)	73 (13-128)	84 (32-230)	94 (25-227)
Alkylresorcinol C17:0/21:0	0.30 (0.16-0.43)	0.29 (0.17-0.49)	0.27 (0.22-0.40)
Enterolactone (nmol/L)	37 (3-143)	49 (11-105)	30 (19-121)

4.3 Effects of whole-grain rye and lignans on cardio-metabolic risk factors

The hypothesis that whole-grain rye and high lignan intake have beneficial effects against risk factors such as glucose homeostasis and lipid profile was tested in the study for *paper I*, but the results of the study in *paper III* are also relevant for evaluating the effects of whole-grain rye on cardio-metabolic risk factors. In view of the feasibility aspect of the NILS study (*paper III*), however, its statistical power was not adequate to test the effects of the intervention, and the results should be interpreted with caution.

4.3.1 Glucose homeostasis

Despite apparently good compliance with the intervention diet (*paper I*), with high intake of whole-grain rye products supplemented by lignan capsules for a period considered to be adequate to obtain the proposed health benefits, no

difference was found in the primary outcome, glucose and insulin response to an oral glucose tolerance test. We hypothesized that the high fibre content and lignans in the whole-grain rye diet would have a beneficial effect on glucose homeostasis due to improved insulin sensitivity, as it has been suggested that insulin sensitivity is a consequence of improved glycaemia control inherent to the structural properties of whole-grain food (Juntunen *et al.*, 2002). For example, starch in whole-kernel rye bread has been found to be less accessible for hydrolysis than that in wheat bread (Juntunen *et al.*, 2003). A lower incretin hormone response has also been observed after consumption of rye rather than wheat bread, possibly due to the higher content of soluble fibres in rye (Juntunen *et al.*, 2003), which may affect long-term glucose metabolism and insulin sensitivity.

In the ELIN study, we did not include soft bread products for logistic reasons. The diets were standardized with similar 100% whole-grain crisp bread and breakfast cereal products. Consequently, the differences between groups were mainly in the structure of the products. The diets were further standardized on fibre content, although the fibre quality differed somewhat, with more soluble and fermentable fibre in rye, which may account for its beneficial effects.

The higher level of fermentable fibre in rye than in wheat might generate more SCFAs to stimulate beneficial microbial bacteria related to improved insulin sensitivity. Nevertheless, a remarkably small effect was found on gut microbiota in the ELIN intervention, consistent with the results of two other studies (Lappi *et al.*, 2013, Korem *et al.*, 2017), which showed no effect of a whole-grain diet on gut microbiota or glucose response. The baseline individual composition of the microbiota has been shown to determine glycaemic response (Korem *et al.*, 2017), and, more specifically, a greater abundance of *Prevotella* has been linked to improved glucose metabolism (Kovatcheva-Datchary *et al.*, 2015). In the study for *paper II*, we found that baseline microbiota enterotype dominated by *Prevotella* was associated with an improved lipid profile after the whole-grain rye intervention but no differential effect on glucose homeostasis by enterotype.

Only limited data are available on the relation between lignans and glucose homeostasis, but a few interventions with flaxseed have resulted in a lower glucose response (Lemay *et al.*, 2002, Cunnane *et al.*, 1993) and modestly lowered glycated haemoglobin levels (Pan *et al.*, 2007). Fasting plasma glucose was lowered in a group of hypercholesterolaemic people after consumption of 600 mg/day of SDG, but this finding was confined to participants with baseline glucose values ≥ 5.83 mmol/L (Zhang *et al.*, 2008). Furthermore, flaxseed supplementation resulted in improved insulin sensitivity in obese and glucose-intolerant people (Rhee and Brunt, 2011). Flaxseed-derived lignans in these

studies represented 120–600 mg/day of SDG. The proposed mechanisms for the effects of lignans on glucose homeostasis include effects on oxidative stress of decreased lipid peroxidation and increased antioxidant activity of flaxseed lignans. As oxidative stress may suppress insulin sensitivity through insulin receptor activity or translocation of GLUT4 (Rudich *et al.*, 1998), the antioxidant properties of lignans may be beneficial.

In the NILS intervention (*paper III*), no effect on glucose or insulin was observed, but the statistical power was insufficient for such evaluation.

4.3.2 Blood lipids

In the study in *paper I*, both total cholesterol and LDL cholesterol were lower after the first 4 weeks of the intervention with rye than with wheat; however, this effect was attenuated after 8 weeks. Because of the study design, we do not know whether the attenuation was due to the introduction of lignan capsules, poorer compliance or adaptation to the high whole-grain lignan intake after 4 weeks. The alkylresorcinol concentrations indicate that compliance was similar between weeks 4 and 8.

According to the findings from studies in both experimental animals (Prasad, 1999, Prasad, 2008) and humans (Pan *et al.*, 2009a), introduction of lignans was hypothesized to reduce plasma cholesterol levels. The evidence from human studies is strongest for pre-menopausal women with high initial cholesterol concentrations. Hallund *et al.* reported no effect on blood lipids of 500 mg/day of SDG (Hallund *et al.*, 2006), whereas Zhang *et al.* (Zhang *et al.*, 2008) found that 300 mg/day was associated with lower total and LDL cholesterol. The Danish women in the study by Hallund *et al.* were postmenopausal and normocholesterolaemic, whereas those in the study of Zhang *et al.* were hypercholesterolaemic. In a meta-analysis with stratification on an initial concentration of ≥–< 5.7 mmol/L total cholesterol and ≥–< 3.4 mmol/L LDL cholesterol, a cholesterol-lowering effect was observed only among people with high initial concentrations (Pan *et al.*, 2009a). The median initial concentrations in the ELIN study were 5.5 mmol/L (4.4–6.8) total cholesterol and 3.1 mmol/L (2.3–4.4) LDL cholesterol. A possible explanation for the attenuation of effect after 8 weeks is that lignans have an effect when cholesterol levels are elevated; hence, after the decrease in cholesterol levels after 4 weeks, any additional effect was diminished.

The cholesterol-lowering properties of whole-grain rye remain to be established, as few studies have been published so far. One study of an intervention with rye bread as compared with refined wheat bread resulted in lower total and LDL cholesterol among men but not among women (Leinonen

et al., 2000). In an unpublished study among Chinese men and women (Landberg *et al.*, unpublished results), fermented rye bran lowered LDL cholesterol by 6% when compared with refined wheat. The high levels of certain soluble arabinoxylans in rye, which increase its viscosity, like beta-glucans in oats, are proposed to account for its lipid-lowering effect, but a higher production of SCFA, particularly propionate may also contribute (Berggren *et al.*, 2007). Attenuation of the cholesterol-lowering effect after 8 weeks in the ELIN study might be due to adaptation to regular high consumption of whole-grain rye during the study. This effect was not, however, found in the unpublished Chinese study after 12 weeks or in a 4-week Finnish intervention (Leinonen *et al.*, 2000).

In the NILS study (*paper III*), weak lowering of total and LDL cholesterol was observed after the rye and physical activity intervention, by 0.3 mmol/L (-0.7, - 0.0) for LDL cholesterol and 0.4 mmol/L (-0.8 - 0.1) for total cholesterol. It is unclear whether this was due to the high whole-grain rye intake or increased physical activity or a combination. The results nevertheless indicate a cholesterol-lowering effect.

The observed magnitude of change in LDL cholesterol in the interventions described in this thesis with lignans in whole-grain products is modest (–0.2 to –0.3 mmol/L), yet changes in this range have been estimated to reduce all-cause mortality by 3% and coronary heart disease morbidity and mortality by ~5% (Gould *et al.*, 2007). Statins are widely recommended as safe, efficient treatment for hypercholesterolaemia and hyperlipidaemia (Piepoli *et al.*, 2016); however, muscle symptoms, dysglycaemia and, although very rare, liver injury have been observed (Mach *et al.*, 2018). Inclusion of whole grains in the habitual diet is recommended worldwide and could replace statins in modestly lowering cholesterol.

4.3.3 Correlations between enterolactone and cardio-metabolic risk factors

Correlations were investigated to further evaluate the specific role of enterolactone in risk factor alteration (Table 12). Enterolactone was not significantly correlated with any of the intervention outcomes in the ELIN study.

Table 12. *Spearman correlations between enterolactone and cardio-metabolic risk factors*

Entero-lactone concen-tration	Risk factor, correlation coefficient (*P*)									
	BMI	waist	BP, diastolic	BP, systolic	Total C	LDL-C	HDL-C	TG	Plasma glucose	Plasma insulin
Baseline	-0.19 (0.25)	-0.35 (0.03)	0.29 (0.07)	0.10 (0.54)	0.14 (0.39)	0.13 (0.43)	0.19 (0.23)	-0.06 (0.73)	0.13 (0.41)	-0.16 (0.33)
Rye 4 weeks	-0.09 (0.59)	-0.13 (0.44)	-0.25 (0.12)	-0.23 (0.16)	-0.25 (0.13)	-0.12 (0.48)	-0.07 (0.69)	-0.17 (0.30)	0.12 (0.47)	0.05 (0.74)
Rye 8 weeks	0.13 (0.44)	0.06 (0.72)	-0.21 (0.19)	0.00 (0.99)	-0.23 (0.15)	-0.20 (0.22)	-0.02 (0.89)	0.05 (0.75)	0.19 (0.22)	-0.03 (0.87)
Wheat 4 weeks	-0.10 (0.54)	-0.13 (0.42)	0.16 (0.34)	0.23 (0.16)	-0.15 (0.34)	-0.12 (0.46)	-0.22 (0.18)	0.01 (0.95)	0.25 (0.12)	0.00 (0.999)
Wheat 8 weeks	-0.06 (0.69)	-0.09 (0.59)	0.22 (0.18)	0.17 (0.30)	-0.15 (0.36)	-0.10 (0.52)	0.26 (0.11)	-0.17 (0.30)	0.23 (0.16)	0.08 (0.61)

BP, blood pressure; C, cholesterol; HDL, high-density lipoprotein; TG, triglycerides

4.3.4 Gut microbiota

The ELIN intervention (*paper I*) contributes to the existing evidence on whole-grain and cardio-metabolic health, providing valuable information from faecal samples collected at six times by each participant. In this study, small differences in gut microbiota abundance of certain species were found between whole-grain diets. More striking though, was that the response to intervention diets depended on the baseline enterotype, especially the lipid profile response.

In both interventions described in this thesis (*papers I* and *III*), the study populations were highly homogeneous; however, although they received standardized diets, large differences in enterolactone response were observed. This indicates that inter-individual differences in the composition and activity of the microbiota are important. Surprisingly, we did not find any association between gut microbiota and plasma or urinary enterodiol or enterolactone concentrations in our study (*paper I*). This may suggest that the conversion capacity of the gut microbiota is already high, potentially due to a high habitual whole-grain intake among participants. In the NILS intervention, the participants received antibiotics during the standard biopsy procedure; however, the fact that antibiotics were given to all participants at approximately the same time might have attenuated any possible effect.

In a Danish cross-over intervention study of a mixed whole-grain diet and a refined grain diet, no alteration in gut microbiota was observed with the whole-grain diet; however, the gut microbiota composition before the intervention

played a major role in weight loss (Hjorth *et al.*, 2017), as also reported earlier for glycaemic response (Zeevi *et al.*, 2015, Kovatcheva-Datchary *et al.*, 2015).

The wide inter-individual variation in enterolactone concentrations is probably due to a complex interaction between the microbiota of the host and external and internal factors (Figure 12) and may also affect health outcomes (Lampe *et al.*, 2006). As the production of enterolactone depends on several factors other than dietary lignan intake, it may in fact reflect gut microbial health or differences in enterolactone producing bacteria and therefore health outcomes. However, we did not find any support for that in our study.

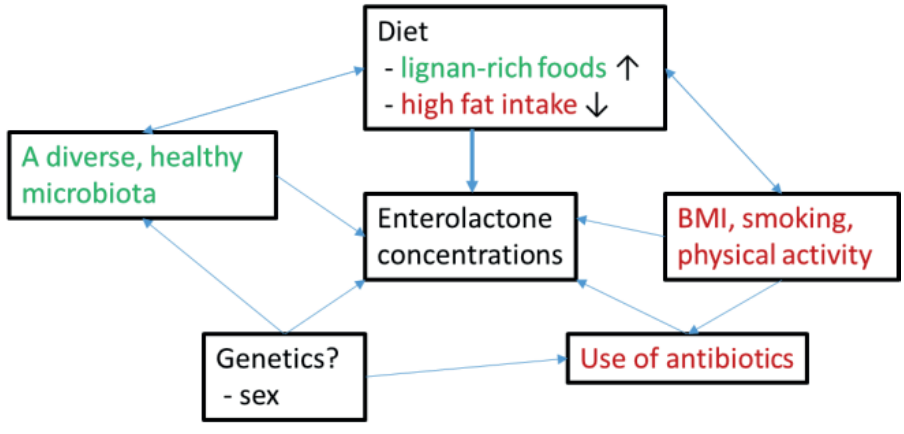

Figure 12. The complex interactions between enterolactone, diet and lifestyle factors

4.4 Enterolactone and mortality in people with type 2 diabetes

In the study described in *paper II*, we investigated the association between pre-diagnostic enterolactone concentrations and mortality among people with diabetes. We found an inverse association between plasma enterolactone concentration and all-cause mortality, with a hazard ratio (HR) of 0.91 (95% CI: 0.85, 0.96), which remained robust after testing for potential effect modification by health behaviour. All-cause mortality was the primary outcome, but, despite low numbers, an inverse association was also observed for diabetes-related mortality, with an HR of 0.75 (95% CI: 0.64, 0.86). Cardiovascular disease is the most common cause of death among people with diabetes, and lowering of LDL cholesterol by 1 mmol/L has been associated with a reduction of 20% in major vascular events, including coronary death, myocardial infarction and stroke (Baigent *et al.*, 2005). Therefore, the hypothesis that the cholesterol-

lowering effect of enterolactone is related to lower mortality among people with diabetes is plausible. In the study in *paper I*, however, an effect on cholesterol was apparent only with the whole-grain rye treatment and disappeared after introduction of lignan capsules, even though they resulted in very high enterolactone concentrations. The results of the studies in *papers I* and *III* indicate that whole-grain rye and not enterolactone improved the lipid profile.

In order to explain the difference in enterolactone concentrations between people with diabetes and the cancer cases in the DCH cohort, we evaluated lifestyle factors that differed for the two diseases. The most striking differences between participants in the highest quartile of enterolactone concentration in the study in *paper II* and in the previous studies are in BMI and level of education. The difference in BMI is not surprising, as a high BMI is one of the most important risk factors for type 2 diabetes. The World Cancer Research Fund has grouped overweight and obesity as a risk factor with "convincing" evidence for colorectal cancer, breast cancer (postmenopausal) and prostate cancer (aggressive). In meta-analyses, the risks for breast and prostate cancer increased linearly by 8–12% per 5 kg/m^2. For colorectal cancer, the association is non-linear, with estimates of a 15–34% increased risk due to overweight and obesity. The degree of overweight and obesity is expected to be more pronounced among people with type 2 diabetes, in whom the increased risk for type 2 diabetes is 3-fold for overweight and 7-fold for obesity (Abdullah *et al.*, 2010). A significant difference was found with education, as only 13% of cases of type 2 diabetes and 22–34% of cancer cases in previous studies had higher education. Other studies have shown that education plays a crucial role in lifestyle behaviour (Nordahl, 2014). In the study reported in *paper II*, no notable differences were found for alcohol intake, participation in sports, smoking status or whole-grain intake. As socioeconomic class reflects several unfavourable lifestyle choices, it could explain some of the observed difference and the gradient within the group of people with type 2 diabetes, whereby people who died from diabetes-related complications, indicating poorly managed disease, also had an unhealthy lifestyle and a low level of education.

A beneficial role of enterolactone has been indicated in intervention studies with people with type 2 diabetes and in cohort studies on cardiovascular mortality. The study in *paper II* contributes to existing evidence by showing a beneficial role of enterolactone in the management of type 2 diabetes or at least as an indicator of disease management. The guidelines for people with type 2 diabetes include lifestyle advice on physical activity and diet; emphasizing intake of whole-grain cereals and vegetables could be added to these recommendations.

4.5 Enterolactone and mortality among men with prostate cancer

We found no association between enterolactone concentrations and mortality from all causes (HR, 0.95; 0.68 , 1.02) or from prostate cancer (HR, 0.98; 0.92 , 1.05) when assessed linearly by 20 nmol/L increments in enterolactone concentrations (*paper IV*). The aggressiveness of disease did not alter the main conclusion, nor did adjustment for type 2 diabetes (not included in the paper). However, only 23 people were found to have diabetes at baseline, and this could be a confounding factor for enterolactone concentrations because of dietary changes after diagnosis and also for mortality, by increasing morbidity and mortality from diabetes. Furthermore, sensitivity analyses of people with diabetes diagnosed before or a maximum of 6 months after diagnosis of prostate cancer (n=129) and of those in whom diabetes was not diagnosed before or a maximum of 6 months after diagnosis of prostate cancer (n=1262) showed little deviation (HR, 0.92; 95% CI, 0.71 , 1.19 vs. HR, 0.96; 95% CI, 0.90 , 1.02).

The highly heterogeneous nature of prostate cancer makes it unlikely that a single experimental model or pathway can fully explain its etiology. In the study in *paper IV*, the sample comprised a diverse group of prostate cancer patients, of whom 57% were defined as having aggressive disease. For almost 90% of these participants, prostate cancer was the cause of death. Lifestyle may be expected to affect the prognosis of localized prostate cancer, whereas substances like enterolactone may have little effect in more aggressive disease. When aggressive and non-aggressive disease were analysed separately, enterolactone had a more pronounced effect in non-aggressive cases. These results should, however, be interpreted with caution because of the very low number of deaths in this group.

Localized or indolent prostate cancer can develop into aggressive disease, although it is difficult to predict which ones will do so. Men with indolent prostate cancer on active surveillance may therefore experience anxiety and stress that their disease will develop. Prognostic approaches for this group are therefore highly relevant. It has been proposed that a better prognosis is associated with decreased intake of dairy products (Downer *et al.*, 2017), indicating that lifestyle can affect prognosis; however, the finding was confined to men with localized disease. Interventions with physical activity and diet are being investigated for effects on the prognosis of men with indolent prostate cancer (Focht *et al.*, 2014, Hackshaw-McGeagh *et al.*, 2016).

In studies in which the anti-proliferative and tumour apoptotic properties of lignans have been demonstrated in vitro, the lignan concentrations used (> 10 μM) clearly exceeded those in humans (Saarinen *et al.*, 2010). High-dose lignan supplements of 300–600 mg/day have been tested in interventions (not limited

to prostate cancer), and in interventions with whole-grain rye, the amount of lignans (~12 mg/day) also exceeds that in the general diet (Landberg *et al.*, 2010). As the lignan intakes in these interventions were 4–200 times those obtained from habitual diets, direct comparisons with observational studies are difficult. Our finding of no association between enterolactone and prostate cancer mortality in the study in *paper IV* is therefore hardly conflicting.

Because of the complex interplay between type 2 diabetes and prostate cancer, adjustment for diabetes at baseline was evaluated in a sensitivity analysis in the study in *paper IV*. No indication of confounding by type 2 diabetes was observed.

4.6 Lifestyle changes in middle-aged men with lifestyle diseases

In the study in *paper III*, 21 of 26 enrolled prostate cancer patients completed the 6 months of the intervention. Compliance with the whole-grain rye diet was good, although the mean intake reported in diaries was lower than the target (170 g/day), at 146 (SD: 19) g/day between baseline and 3 months and 125 (SD: 40) g/day between 3 months and 6 months of the intervention. Similar compliance was observed for physical activity at an intensity of > 70% of the maximum monitored heart rate. The median for the first 3 months of the intervention was 91 (P5–P95: 17–193) min/week and for the last 3 months of the intervention, 66 (P5–P95: 13–259) min/week, somewhat below the target of 135 min/week. A positive trend was observed in physical fitness, assessed by a cycle-ergometer test-generated VO^2 peak, in the intervention group. The improved peak can be regarded as a measure of compliance to physical activity and furthermore suggests an effect of the intervention. Owing to the feasibility aspect of the NILS study, its statistical power was not adequate to test effects of the intervention; hence, these results should be interpreted as suggestive. A secondary aim was to evaluate the effect of the intervention on prostate cancer progression by changes in PSA levels. No clear pattern was observed (Figure 13), also due to the limited statistical power of the study.

Lifestyle changes in a similar group of middle-aged, metabolically unhealthy men in the ELIN study (*paper I*) were also successful, indicating that intensive lifestyle change could be used as a secondary prevention strategy in highly motivated individuals at high risk for cardiovascular disease.

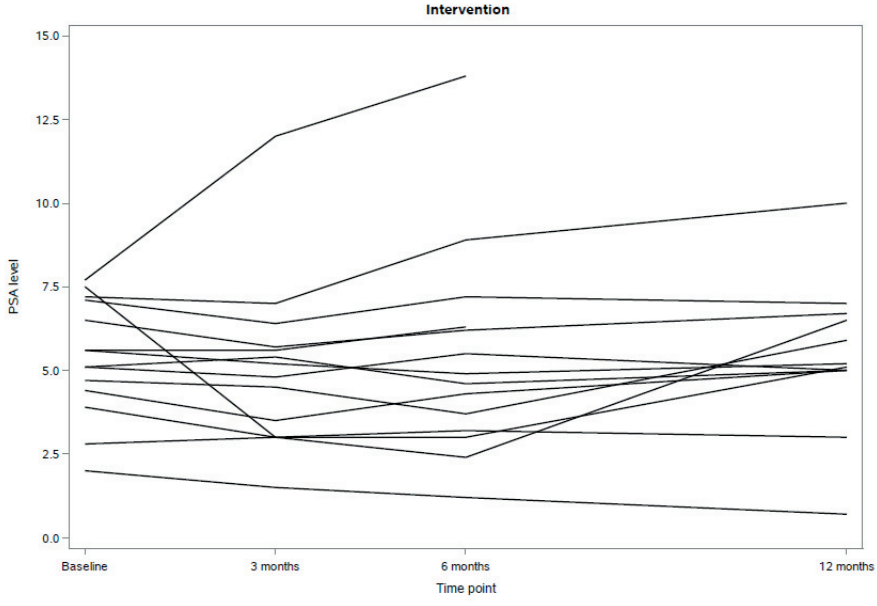

Figure 13. Individual PSA concentrations in the intervention group in the NILS study (*paper III*)

5 General discussion

5.1 Summary of findings

The overall aim of this thesis was to determine the association between consumption of whole grains and lignans and progression of metabolic syndrome and enterolactone concentrations and the prognoses of type 2 diabetes and prostate cancer. The main findings are as follows:

1 Among men with metabolic syndrome, whole grains or lignans had no effect on glucose homeostasis, but a lipid-lowering potential of whole-grain rye was noted (*paper I*). Gut microbiota enterotype was associated with response to treatment and appears to play an important role in cardio-metabolic outcomes in whole-grain interventions.

2 Pre-diagnostic enterolactone concentrations were inversely associated with mortality among people with type 2 diabetes (*paper II*).

3 In a group of men with non-aggressive prostate cancer, comprehensive lifestyle changes, with high intakes of whole-grain rye and physical activity during 24 weeks, were found to be feasible (*paper III*).

4 Pre-diagnostic enterolactone concentrations were not associated with mortality from prostate cancer (*paper IV*).

5.2 Study designs and populations

In epidemiology, the quality of evidence depends on the type of study used to investigate a research question. As illustrated by the evidence pyramid (Figure 14), randomized controlled trials (RCTs) are regarded as the highest level of evidence for causality and are often referred to as the "gold standard" (Schulz *et al.*, 2010). Randomization of participants limits the risk of confounding, as the risk factors that predict the outcome of interest are equally randomly distributed. Drop-out can, however, lead to selection bias and introduce confounding; furthermore, the results of RCTs are relevant to only a highly controlled, limited study population, exposure and time. Just below RCTs are cohort studies, which have a strong design because of their prospective nature. This thesis is based on two randomized human intervention studies and two cohort studies of the role of lignans and enterolactone in lifestyle diseases.

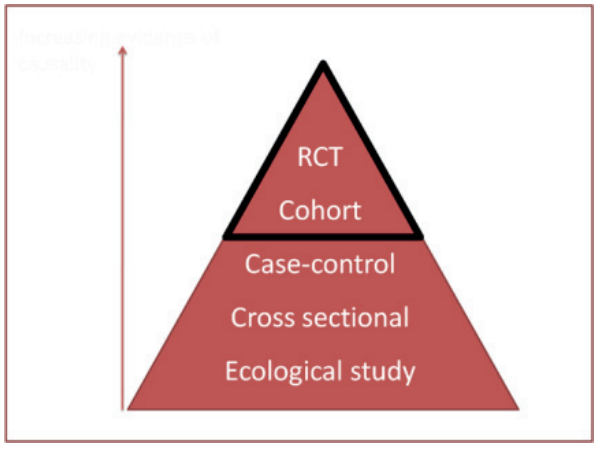

Figure 14. The evidence pyramid.

5.2.1 Human interventions

Neither of the interventions in this thesis can be regarded as an RCT, and they were therefore not expected to fulfil the Cochrane Collaboration requirements for RCTs. The domains of potential bias related to RCTs may nevertheless be relevant. These are: random sequence generation, allocation sequence concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data and selective outcome reporting (Cochrane Collaboration, 2011).

Blinding of participants and personnel is recommended to reduce the risk that awareness of the intervention treatment can bias assessment of the effect. If one

treatment is expected to have a beneficial effect, the subject or the study personnel may be predisposed to perceive or detect an effect of this treatment in favour of the control or another treatment (Cochrane Collaboration, 2011). Blinding was not possible in the interventions described in this thesis. In the ELIN intervention, the type of whole-grain diet was evident; furthermore, placebo capsules were not given during the last 4 weeks of wheat treatment whereas lignan capsules were given at the end of the rye treatment. This may have limited observation of a placebo effect, whereby the act of taking a capsule can in itself benefit patients if they believe they have taken treatment (Altman, 1991). Likewise, the NILS intervention had a parallel design, and placebo treatment for whole-grain rye and physical activity was impossible. As the outcomes of both interventions were objective (based primarily on blood samples), the effect of blinding is not expected to affect the outcomes of interest considerably.

As participants who dropped out of both the NILS and the ELIN study were not invited for further study visits, the approach of intention-to-treat was not possible. However, an imputation approach could have been considered.

Randomization in crossover designs is used to determine the order of treatments, not to randomize subjects. Furthermore, as the treatment comparison is within instead of between subjects, only participants who completed both (or all) treatments can contribute data for the analysis. The possibility of drop-out due to side-effects of the first treatment is a concern, and treatment periods should be kept short to minimize this problem (Altman, 1991). The ELIN intervention was 8 weeks long, which was considered necessary to show stable potential effects on metabolic outcomes, but was relatively long for participants with regard to side-effects of the intervention, such as flatulence due to the high fibre content and becoming tired of eating the same products in the same amounts. The reasons for drop-out in ELIN included stomach ache, the large amounts and the boring diet. Another concern in the crossover study is carry-over effects among treatments, whereby an observed difference between treatments depends on the order in which the treatments are received. To address this potential problem, an 8-week washout period was included in the ELIN study. No carry-over effect was expected after this time, on the basis of similar studies.

We learnt from the NILS study that there is substantial individual variation in enterolactone concentrations, even with standardized diets (additional results not included in *paper III*). Furthermore, the effect of antibiotic treatment on enterolactone concentrations is seen for more than 1 year after administration (Kilkkinen *et al.*, 2002). As prostate cancer patients automatically enter a biopsy

regime as part of disease monitoring, all participants in the NILS study inevitably received antibiotics before or during the intervention, which may have diluted the potential effect on and of enterolactone concentrations in the NILS study. Enterolactone was not, however, related to the main objectives of the study and was therefore not considered during planning. These lessons were valuable for planning the ELIN intervention, for the choice of a crossover intervention design, in which participants act as their own controls, and for including antibiotic in exclusion criteria.

5.2.2 Observational studies

When it is difficult to achieve the gold standard of a perfectly conducted RCT in nutritional epidemiology, the next level of evidence can be obtained in the prospective cohort study, which is also a strong design for investigating associations because of their prospective nature. Moreover, RCTs are often highly challenged when it comes to study development of disease and death due to the (often) long timeframe for such endpoints. For the two remaining studies of the thesis (*papers II* and *IV*), cohort designs were used to study mortality.

In epidemiology, the final estimate depends on the study design, conduct and data analysis. The validity of an estimate depends on the degree of error, which can be either random or systematic (bias). A study with little systematic error is highly valid, internal validity referring to the inference of the estimate for the study population and external validity referring to the inference of the estimate outside that population (generalizability) (Rothman and Greenland, 1998). Internal validity is most often violated by confounding, selection bias and information bias, which are discussed below in relation to *papers II* and *IV*.

Selection bias

Selection bias can be introduced by the recruitment approach or by factors that influence study participation. It occurs if the relation between exposure and disease is different among study participants and eligible people who do not participate in the study (Rothman, 2002). Generally, in prospective studies, selection bias is of little concern.

The type of selection bias commonly referred to as the “obesity paradox” (Preston and Stokes, 2014) may be relevant in studies of prognosis. The obesity paradox was introduced after several studies reported lower mortality from several illnesses among obese than normal weight patients. The paradox has been described in particular in studies of older people with type 2 diabetes. In prognostic studies, patients with the relevant disease are selected into the study and therefore must be alive until a certain age. Significant selection is introduced

in studies of elderly people, as the most severe cases will have died from their disease and therefore cannot be enrolled into the study. Possibly, obese people who live for years with their disease are significantly different from obese people who die early from their disease. It is therefore crucial to adjust for factors associated with obesity and mortality properly.

Smoking is associated with lower BMI and higher mortality. If it is not accounted for, the higher proportion of smokers among lean people who are at increased risk of dying from smoking will incorrectly indicate an inverse association between obesity and mortality. An example of such potential bias was considered in the study in *paper II*, in which people with type 2 diabetes were selected as the study population and deceased cases were selected into the case group. The low enterolactone concentrations, especially among the deceased, indicated selection of people with low enterolactone concentrations into the case group. People with low enterolactone concentrations may consequently have developed diabetes, whereas people with high enterolactone concentrations developed the disease for other reasons. Therefore, it will appear as low enterolactone is associated with lower mortality. This could attenuate the results if diabetes is diagnosed in people with high enterolactone concentrations, for example if they are genetically disposed and have a more aggressive type, in which lifestyle factors have a minimal effect on progression.

Information problems – misclassification and bias

Information bias can arise when information is either collected or reported erroneously, leading to differential or non-differential misclassification of participants. Exposure misclassification is non-differential if it is unrelated to the occurrence or presence of disease and is differential if it is related. Similarly, misclassification of disease outcome is non-differential when it is unrelated to exposure and differential when it is related (Rothman, 2002). While differential misclassification results in bias, non-differential misclassification causes only random error and attenuation of the true association.

Neither misclassification of disease diagnoses, mortality outcomes or enterolactone concentrations can be excluded in the cohort studies in this thesis. Misclassification is unlikely to be differential, as potential flaws in registration or ICD coding of disease diagnoses and death certificates are unrelated to enterolactone concentrations. Determination of enterolactone is not affected by the vital status of the participants, and the people responsible for enterolactone analyses were blinded towards the outcome.

Recall bias is a type of information bias that is problematic primarily in case–control studies if cases remember and report exposure differently from controls. People with a disease often tend to seek explanations for why they have it,

leading to this type of bias (Rothman and Greenland, 1998). In a prospective study design, this should not be a problem, as information on lifestyle and diet is collected at baseline, when the participants were healthy. There could, however, be systematic differences in reporting of dietary habits according to BMI, as overweight and obese people tend to underreport their dietary intake and especially that of unhealthy foods (Ferrari *et al.*, 2002, Mendez *et al.*, 2004). Smoking and inadequate participation in sports were considered important risk factors in the studies described in *papers II* and *IV*; as both factors were self-reported, however, misclassification could have been introduced, although this would introduce only some random error.

In survival studies, exposure can be measured before, at the time of and after diagnosis. The timing of measurement affects interpretation of the results and can cause exposure misclassification. Pre-diagnostic exposure is presumably (depending on lag time and type of disease) not affected by disease status, as it is measured when the study participants are free of disease. Cohort studies have the advantage of long follow-up, and the lag time between measurement of exposure and disease or event occurrence may be long (10–20 years). During this time, it is not known in most studies whether lifestyle habits and exposure have changed. Exposure measured at time of diagnosis is often affected by the disease, and this is also the case for post-diagnostic measurement. In an optimal survival study of enterolactone, a plasma sample taken at time of diagnosis and randomization to high or low lignan intake until death or end of follow-up would provide an unbiased view of the effect of lignans on disease progression and mortality. In such a design, the problems related to the obesity paradox would be eliminated.

We were able to account for use of antibiotic medication by linkage to the Danish Prescription Registry. As use of antibiotics can affect enterolactone production for more than 1 year after administration (Bolvig *et al.*, 2016), its inclusion was expected to increase the accuracy of the estimated association by eliminating noise from the effect of antibiotics on enterolactone. Different approaches were applied in the studies in *papers II* and *IV*; in the study in *paper II*, the analysis was restricted to people with baseline use of antibiotics after 1996 (from information in the Prescription Registry), whereas in that in *paper IV*, people with baseline use before 1996 were categorized as "missing".

Confounding

Confounding is an inherent systematic error in cohort studies. A confounding factor must be associated with the disease or outcome of interest, be associated with the exposure and not be an effect of the exposure or an intermediate step in the causal pathway between exposure and outcome (Rothman, 2002).

Comprehensive, detailed information on lifestyle and diet collected at baseline in the DCH cohort study enabled us to thoroughly adjust for potential confounders in the studies in *papers II* and *IV*; however, not all relevant confounders were included, such as family history of disease, which is an important risk factor for both type 2 diabetes and prostate cancer. Inherent prostate cancer is often aggressive with a poor prognosis, and a potential role of enterolactone would be expected to be minimal; an effect of family history of prostate cancer on dietary habits and lignans is speculative. Family history might also play a role in type 2 diabetes, through lifestyle patterns introduced in childhood that lead to overweight and thereby type 2 diabetes. Conversely, family history could also predispose to a health-conscious lifestyle with high lignan intake.

Obesity and type 2 diabetes may be confounding factors for mortality from prostate cancer. As discussed earlier, lower PSA levels in obese people may be related to a lower probability of detecting prostate cancer at an early stage. If more obese people than non-obese people with prostate cancer receive a diagnosis of aggressive disease (due to a delay in detection), their prognosis will be affected. An additional analysis conducted with adjustment for type 2 diabetes diagnosed before or soon after the prostate cancer diagnosis showed associations similar to those in the unadjusted model. As obesity and stage of disease were accounted for in the study (*paper IV*), the association between enterolactone and prostate cancer does not appear to be confounded by obesity or type 2 diabetes.

As there is no clear evidence about the confounders in prognostic studies on type 2 diabetes or prostate cancer, the most important risk factors for overall mortality were addressed. Smoking is a significant risk factor for early death and was therefore included in both studies a priori. In the study in *paper II*, smoking did not affect the estimates significantly, whereas, in the study described in *paper IV*, the observed association in model 1 became nonsignificant after adjustment for smoking. This indicates that general health and lifestyle are crucial in determining death from prostate cancer, while enterolactone does not seem to play a role. For type 2 diabetes, the association between enterolactone and mortality remained robust after adjustment for lifestyle behaviour.

Effect modification

A biological interaction between two causes is mechanistic, whereby the effect of one cause depends on that of the other (Rothman, 2002). In people with type 2 diabetes or prostate cancer, factors such as obesity, physical inactivity and smoking are known risk factors and predictors of mortality. It is not known whether the biological effects of enterolactone differ according to any of these

factors. Therefore, assessment of potential effect modification by health behaviour was considered in elucidating the association and role of enterolactone.

Effect modification was evaluated in the studies in *papers II* and *IV* to determine whether the association between enterolactone and mortality differed according to obesity, physical inactivity and smoking. The associations were similar, indicating that enterolactone has additional effects (in *paper II*) apart from being related to a healthy lifestyle.

In summary, the internal validity of the observational studies in this thesis was considered to be good.

External validity

Internal validity, as discussed above, is considered a prerequisite for external validity. The external validity or generalizability of a study is important for extending findings and conclusions to a wider or general population. The population-based cohort studies and randomized human interventions conducted for this thesis were generally of high quality. They included people with prostate cancer, type 2 diabetes or metabolic syndrome, and the results can therefore be extrapolated to these groups.

Recruitment for the DCH cohort study was population-based, and nearly all eligible people were invited (19% of the entire Danish population aged 50–64 years); however, only 34% of the invited men and 37% of women agreed to participate. Participation was related to socioeconomic factors, including education and marital status (Tjonneland *et al.*, 2007). The people with type 2 diabetes and prostate cancer in this population also represented a higher socioeconomic level than the general population of people with these diseases. The biological mechanisms of the association between enterolactone and mortality are not, however, expected to differ by socioeconomic status, and therefore the findings are generalizable to other populations of the same age with these diseases.

5.3 Contribution to existing evidence

Enterolactone has been studied for the past 30 years, and numerous beneficial properties have been proposed (Adlercreutz, 2007, Peterson *et al.*, 2010). This thesis has filled some of the remaining gaps in information.

In the study described in *paper I*, we found that whole-grain rye caused lower total and LDL cholesterol. This was a novel finding, as the effect was significant in comparison with that of whole-grain wheat with similar fibre and whole-grain

content. Furthermore, although the enterolactone concentration was 200 nmol/L after SDG lignan supplements, no effect on any cardio-metabolic outcome was observed. The information on faecal microbiota constitutes a valuable resource for further studies and demonstrates that gut microbial composition plays a crucial role in the response to intervention diets.

The study in *paper II* contributes to a gap in research on the role of enterolactone in management of and mortality from type 2 diabetes, as, to the best of my knowledge, this is the only observational study on these aspects. Further studies are required to support our finding of an inverse relation between enterolactone and mortality among people with type 2 diabetes

Likewise, the results of the study in *paper IV* contribute to the unexplored area of enterolactone and prostate cancer. The rationale for beneficial effects of enterolactone in prostate cancer progression appears to be plausible from mechanistic studies. The current study adds information to this research area, indicating that the beneficial properties of enterolactone in cell and animal models are difficult to replicate in human observational studies, and the levels of enterolactone in habitual diets may not be clinically relevant. The finding of no association should be confirmed in further studies.

The NILS intervention was a feasibility study, but *paper III* clearly showed that a long-term lifestyle modification with whole-grain rye and physical activity is feasible among men with prostate cancer for whom the potential for improving quality of life is relevant. This has been shown previously but only with regard to either whole-grain rye (Landberg *et al.*, 2010, Bylund *et al.*, 2003) or physical activity (Bourke *et al.*, 2016) and during considerably shorter time-periods. The studies of combined diet and physical activity were based on caloric restriction (500–1000 kcal/day) (Demark-Wahnefried *et al.*, 2016, Focht *et al.*, 2014), reduced fat intake (Focht *et al.*, 2014) or lycopene/fruit and vegetable/no dairy diets (Hackshaw-McGeagh *et al.*, 2016). The evidence that prostate cancer patients are willing to adapt to interventions is increasing, but it is uncertain whether the effects go beyond improved quality of life. Nevertheless, this can be important for the large patient group of interest.

5.4 Perspectives for future research

As mentioned above, the work presented in this thesis has filled some knowledge gaps in research, but has also led to new questions about the role of fibre-rich foods and lignans in relation to health and disease progression. The following research is therefore proposed:

In order to assess causality between whole-grain rye (or any specific component of rye) and lipid-lowering effects caused by enterotypes, a crossover intervention study is warranted where subjects with *Prevotella* and *Bacteroides* dominated enterotypes are randomised to treatments with rye or specific fibre fractions of interest.

If future research can further document that whole-grain rye has health effects beyond those generally seen for whole grain, then specific health claims on rye may be seen in the future. Furthermore, future dietary intervention trials in which microbiota is properly accounted for holds interesting perspectives in regards to personalised nutrition.

Based on the findings from the feasibility study (*paper III*), a full-scale trial is warranted to investigate the effect of lifestyle intervention on prognosis among men with non-aggressive prostate cancer.

Our finding of an inverse association between enterolactone and mortality in people with type 2 diabetes (*paper II*) is novel and should be replicated in other studies. A study where subjects are randomised to either a high- or low-lignan diet at time of diagnosis would provide new knowledge that could be used when formulating dietary advice in this patient group.

In order to address causality in future observational studies of enterolactone, it would be interesting to use Mendelian randomisation if a suitable design for this could be found.

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