Effects of dietary fibre on the human metabolism and metabolome

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EFFECTS OF DIETARY FIBRE ON THE HUMAN METABOLISM AND METABOLOME

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Abstract

It is well-known that dietary fibre can have a positive effect on the development of lifestyle-dependent diseases such as cardiovascular disease and type 2 diabetes. However, the effect may be different for different kinds of fibre and different sub-populations at risk. Therefore, the effects of three different kinds of fibre on postprandial and long-term response in healthy subjects were investigated. Consumption of rye bran, oat powder, sugar beet fibre or a mixture of all three in a meal study, led to lower postprandial glucose levels for all meals except oat powder, although the difference was only significant for rye bran. The outcome seemed to be determined not only by the amount of soluble fibre, but also by the total dietary fibre content. The combined effect of an intake of oat bran, rye bran and sugar beet fibre was also investigated in a 5-week randomised cross-over intervention study in healthy, mildly hypercholesterolaemic subjects. Subjects were given a high-fibre (HF) diet (48 g) and a low-fibre (LF) diet (30 g). Despite the high fibre intake, no significant effects were observed on glucose, insulin, or lipid metabolism. However, low-grade inflammatory response was reduced by the HF diet, as reflected by decreased C-reactive protein and fibrinogen levels. Moreover, markers from the high intake of oat, rye and sugar beet fibre were observed in plasma and 24-h urine samples using an untargeted metabolomic profiling approach. After the HF diet, different benzoxazinoids and their metabolites 2-aminophenol sulphate, HPAA (N-(2-hydroxyphenyl)acetamide) and HHPAA (2-hydroxy-N-(2-hydroxyphenyl)acetamide), together with the alkylresorcinol metabolite DHPPA (3-(3,5-dihydroxyphenyl)-1-propanoic acid), were found to be specific for the rye intake, whereas enterolactone was related to rye and oat fibre intake. Some specific markers for oat intake were found, however, their identity needs further validation. One identified marker, 2,6-DHBA (dihydroxybenzoic acid), has not previously been reported as a marker related to dietary fibre intake. Whether there is a specific marker for sugar beet fibre intake remains unclear. These markers of the intake of specific dietary fibre sources could, if validated, serve as markers in intervention studies and larger observational studies, to provide more accurate data on general dietary fibre intake, apart from the subjects' self-reported values, which are normally used. The effect of a healthy Nordic diet based on the Nordic Nutrition Recommendations, including a high dietary fibre intake, was investigated in obese subjects with metabolic syndrome. This was a randomised, parallel multi-centre study in which the Nordic diet was compared to a control diet. No effects were observed on the glucose and insulin metabolism, however, reductions in lipoproteins were found together with an indication of reduction in the inflammatory response after the intake of the Nordic diet. In conclusion, these studies confirm that a high dietary fibre intake has a beneficial effect on glucose and lipid metabolism. The intervention studies also indicated a reduction in low-grade inflammation markers that are associated with overweight and type 2 diabetes.

Key words arabinoxylan, β-glucan, dietary fibre, inflammation, LC-MS, metabolomics, oat, pectin, postprandial response, randomised controlled trial, rye, sugar beet fibre

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III. **Johansson-Persson A**, Barri T, Ulmius M, Önning G, Dragsted L
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MY CONTRIBUTIONS TO THE PAPERS

Paper I I performed the experimental work, analysed the blood samples, evaluated the results and wrote the manuscript, together with Matilda Ulmius Storm.

Paper II I performed the human study and analysed the blood samples (apart from the cytokine analysis) together with Matilda Ulmius Storm. I evaluated the results and wrote the manuscript.

Paper III I performed the human study together with Matilda Ulmius Storm. I performed the LC-MS analysis together with Thaer Barri. I evaluated the results together with Thaer Barri and Lars O. Dragsted, and I wrote the manuscript.

Paper IV I performed the human study together with Matilda Ulmius Storm. I performed the LC-MS analysis together with Thaer Barri. I evaluated the results together with Thaer Barri, Jan Stanstrup and Lars O. Dragsted, and I wrote the manuscript.

Paper V I took part in planning and conducting the Lund part of this multi-centre human study. I reviewed the manuscript and approved the final version.

ABSTRACT

It is well-known that dietary fibre can have a positive effect on the development of lifestyle-dependent diseases such as cardiovascular disease and type 2 diabetes. However, the effect may be different for different kinds of fibre and different sub-populations at risk. Therefore, the effects of three different kinds of fibre on postprandial and long-term response in healthy subjects were investigated. Consumption of rye bran, oat powder, sugar beet fibre or a mixture of all three in a meal study, led to lower postprandial glucose levels for all meals except oat powder, although the difference was only significant for rye bran. The outcome seemed to be determined not only by the amount of soluble fibre, but also by the total dietary fibre content. The combined effect of an intake of oat bran, rye bran and sugar beet fibre was also investigated in a 5-week randomised cross-over intervention study in healthy, mildly hypercholesterolaemic subjects. Subjects were given a high-fibre (HF) diet (48 g) and a low-fibre (LF) diet (30 g). Despite the high fibre intake, no significant effects were observed on glucose, insulin, or lipid metabolism. However, low-grade inflammatory response was reduced by the HF diet, as reflected by decreased C-reactive protein and fibrinogen levels. Moreover, markers from the high intake of oat, rye and sugar beet fibre were observed in plasma and 24-h urine samples using an untargeted metabolomic profiling approach. After the HF diet, different benzoxazinoids and their metabolites 2-aminophenol sulphate, HPAA (N-(2-hydroxyphenyl)acetamide) and HHPAA (2-hydroxy-N-(2-hydroxyphenyl)acetamide), together with the alkylresorcinol metabolite DHPPA (3-(3,5-dihydroxyphenyl)-1-propanoic acid), were found to be specific for the rye intake, whereas enterolactone was related to rye and oat fibre intake. Some specific markers for oat intake were found, however, their identity needs further validation. One identified marker, 2,6-DHBA (dihydroxybenzoic acid), has not previously been reported as a marker related to dietary fibre intake. Whether there is a specific marker for sugar beet fibre intake remains unclear. These markers of the intake of specific dietary fibre sources could, if validated, serve as markers in intervention studies and larger observational studies, to provide more accurate data on general dietary fibre intake, apart from the subjects' self-reported values, which are normally used. The effect of a healthy Nordic diet based on the Nordic Nutrition Recommendations, including a high dietary fibre intake, was investigated in obese subjects with metabolic syndrome. This was a randomised, parallel multi-centre study in which the Nordic diet was compared to a control diet. No effects were observed on the glucose and insulin metabolism, however, reductions in lipoproteins were found together with an indication of reduction in the inflammatory response after the intake of the Nordic diet. In conclusion, these studies confirm that a high dietary fibre intake has a beneficial effect on glucose and lipid metabolism. The intervention studies also indicated a reduction in low-grade inflammation markers that are associated with overweight and type 2 diabetes.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Kostfiber visar på gynnsamma effekter på blodsockersvar, inflammationsmarkörer och blodfetter utifrån tre humanstudier som genomfördes i detta arbete. I en av studierna analyserades blod och urin med metabolomik. Det är en metod där man analyserar alla små molekyler (metaboliter) som finns i provet med hjälp av vätskekromatografi kopplat till masspektrometri. Med hjälp av denna analys identifierades flera markörer för kostfiberintag, dels nya markörer men även en bekräftelse av markörer som identifierats i tidigare kostfiberstudier.

Kostfiber är växtmaterial som inte kan brytas ner av vårt matspjälkningssystem men som i varierande grad bryts ner av bakterier i tjocktarmen. Kostfiber påskyndar matens transport genom tarmen vilket minskar risken för förstoppning. Sänkning av blodsocker, blodfettnivåer samt den antiinflammatoriska effekten tillskrivs bland annat en långsammare magsäckstömning och upptag av näringsämnen i tarmen efter intag av kostfiber. Kostfiberintaget i Sverige är idag ca 20 g per dag vilket är lägre än de 25-35 g som rekommenderas i de svenska näringsrekommendationerna för att minska risken att drabbas av hjärt- och kärlsjukdomar, övervikt och diabetes typ 2.

I en måltidsstudie undersöktes blodsocker, insulin och triglycerider (blodfett) hos friska försökspersoner efter intag av olika frukostar med tillsatta kostfiber från spraytorkad havredryck, rågkli, sockerbetsfiber eller en blandning av de tre kostfibrerna. Blodprov togs före och sedan varje halvtimme under tre timmar efter måltiden. Alla måltider, förutom den spraytorkade havredrycken, gav en sänkning av blodsockret men endast för måltiden med rågkli var den signifikant. Denna effekt berodde inte bara på andelen lösliga kostfiber i måltiden utan även på totala mängden kostfiber som måltiden innehöll. Kvinnor hade ett lägre blodsockersvar än män när de intog de olika kostfibermåltiderna. Insulin- och triglyceridnivåerna påverkades inte signifikant av kostfiberintaget i denna studien.

I en långtidsstudie undersöktes hur ett högt kostfiberintag påverkade blodsocker, insulin, olika blodfetter och inflammationsmarkörer hos friska försökspersoner med något förhöjt blodkolesterol. Kostfiber från rågkli, havrekli och sockerbetsfiber sattes till en brödbulle, två drycker och en färdigrätt som intogs dagligen under fem veckor. I kontrolldieten användes samma livsmedel men utan tillsatta kostfibrer. Blodprover togs fastande och en sänkning av inflammationsmarkörerna C-reaktivt protein (CRP) och fibrinogen kunde konstateras efter högfiberkosten. Inga skillnader i blodsocker, insulin eller blodfetter uppmättes mellan hög- och lågfiberdieten. Eftersom försökspersonerna fick äta fritt utöver testprodukterna så resulterade det i att kostfiberintaget under lågfiberkosten blev relativt högt, vilket kan vara en anledning till utebliven effekt i dessa markörer.

Urin och blodprover från långtidsstudien analyserades med metabolomik med syftet att hitta och identifiera vilka metaboliter som påverkas av ett högt kostfiberintag samt att bekräfta kända och hitta nya markörer relaterat till kostfiberintag från råg, havre och sockerbetsfiber. En ny markör relaterad till det höga kostfiberintaget identifierades både i urin och blod; 2,6-dihydroxybensoesyra. Ytterligare metaboliter relaterade till råg- och havreintag ökade signifikant både i blod och urin efter det höga kostfiberintaget.

I en nordisk multicenter-studie undersöktes hur intag av en hälsosam nordisk kost under sex månader påverkade blodsockret, insulin, blodfetter och inflammationsmarkörer hos överviktiga försökspersoner med metabolt syndrom. Den hälsosamma nordiska kosten ställdes mot en kontrollkost som återspeglade en typisk kost hos den nordiska befolkningen. Blodfetterna förbättrades signifikant hos de som intog den hälsosamma nordiska kosten. En markör för inflammation ökade successivt och signifikant under hela studien för de som åt kontrollkosten. Ingen effekt på blodsockret eller insulin kunde påvisas.

Sammanfattningsvis bekräftar studierna tidigare visade hälsoeffekter för kostfiber på blodsocker och blodfetter. Interventionsstudierna indikerade också att ett högt kostfiberintag kan ha positiv effekt på låggradig inflammation, ett tillstånd som är associerat med övervikt och diabetes typ 2. Ett flertal potentiella markörer för kostfiberintag från råg och havre kunde påvisas i blod och urin med metabolomik. En del markörer behöver undersökas mer för att se från vilken typ av kostfiber de kommer ifrån. Detta kan utföras genom att undersöka respektive kostfiber i separata studier och även genom att karakterisera de ämnen som finns närvarande i respektive kostfiber. Om de är robusta markörer för en specifik typ av kostfiber kan dessa användas som kontrollmarkörer för att kvantifiera det generella kostfiberintaget i framtida studier.

ABBREVIATIONS

Apo	apolipoproteins
AUC	area under the curve
BCAA	branched-chain amino acid
BMI	body mass index
BOA	1,3-benzoxazol-2-one
CRP	C-reactive protein
CVD	cardiovascular disease
DE	degree of methyl esterification
DHBA	dihydroxybenzoic acid
DHPPA	3-(3,5-dihydroxyphenyl)-1-propanoic acid
DIBOA	2,4-dihydroxy-1,4-benzoxazin-3-one
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionisation
HBOA	hydroxy-1,4-benzoxazin-3-one
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
HF	high fibre
HHPAA	2-hydroxy-*N*-(2-hydroxyphenyl)acetamide
HOMA-IR	homeostasis model assessment score for insulin resistance
HPAA	*N*-(2-hydroxyphenyl)acetamide
IFN-γ	interferon gamma
IL	interleukin
IL-1RA	interleukin 1 receptor antagonist
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol
LF	low fibre
m/z	mass-to-charge ratio
MS	mass spectrometry
MUFA	monounsaturated fatty acids
MW	molecular weight
NEFA	non-esterified fatty acids
NNR	Nordic Nutrition Recommendations
Non-HDL	non-high-density lipoprotein cholesterol
OGTT	oral glucose tolerance test
PUFA	polyunsaturated fatty acids
QTOF/MS	quadrupole time-of-flight mass spectrometry
SCFA	short-chain fatty acid
SFA	saturated fatty acids

SYSDIET	Systems Biology in Controlled Dietary Interventions and Cohort Studies
TC	total cholesterol
TNF-α	tumour necrosis factor alpha
TNF-R	tumour necrosis factor receptor
UPLC	ultra-performance liquid chromatography
VLDL	very-low-density lipoprotein

CONTENTS

1 BACKGROUND

Cardiovascular disease (CVD) and type 2 diabetes are currently two major lifestyle-dependent diseases. CVD is the major cause of death in Europe, and accounts for about 47% of all deaths per year, and 30% of deaths in those aged under 65 [1]. The mortality rate is generally higher in Central and Eastern Europe than in Northern, Southern and Western Europe. However, mortality due to CVD has been decreasing for the past 10-15 years in almost all European countries. In 2009, the total economic losses due to CVD in the European Union (EU) was estimated to be approximately €196 billion per year, including health care costs, loss of productivity, and informal care of people with CVD [1]. The prevalence of diabetes in Europe is also high and has increased over the past ten years; in some countries the increase is more than 50% [1]. It has been estimated that diabetes affects about 8% of the adult population in Europe and that almost 39% live with undiagnosed diabetes [2]. The cost of healthcare related to diabetes in Europe in 2011 was estimated to be about €101 billion [2]. While only small increases in fasting glucose levels have been observed since 1980, the body mass index (BMI) has consistently increased in almost all European countries [1]. The main risk factors associated with CVD are hyperlipidaemia, progression of arteriosclerosis, and hypertension, while type 2 diabetes is more closely related to impaired glucose tolerance, insulin resistance and obesity. The progression of these diseases is largely determined by lifestyle-related factors such as poor diet, physical inactivity and smoking, but also by the genetic susceptibility of the individual. Therefore, changing eating and exercise habits could help prevent these diseases. For instance, an increase in the intake of dietary fibre could reduce the risk of CVD and type 2 diabetes by beneficial effects on blood lipids [3, 4] and insulin sensitivity [5].

The Nordic Nutrition Recommendations (NNR) is the result of a collaborative Nordic initiative with the Nordic Council of Ministers which started in the late 1970s. It is an important resource within the policy arena of food, nutrition and public health, and is also used when formulating national nutrient and dietary recommendations. Today, NNR endorse an intake of 25-35 g dietary fibre per day, and the population goal is 30 g per day [6]. In a national survey in 2010-2011, on the macro- and micronutrient intake in different socioeconomic groups in the Swedish population [7], it was concluded that the intake of dietary fibre by Swedes had increased since the previous national survey in 1997-1998 [8], although the intake was still below the recommended level.

Several observational studies have revealed an inverse relationship between a high fibre intake and the risk of CVD and type 2 diabetes [9-11]; however, confirmation in controlled interventions does not always give the expected results. The reason for this may not be the

lack of an effect, but rather the fact that different kinds of dietary fibre have different physiological effects at different levels. Several health claims regarding specific levels of intake of specific kinds of dietary fibre have been approved to be used in the EU. For instance, it has been claimed that the soluble dietary fibre β-glucan, found in oats and barley, reduces blood cholesterol levels and decreases postprandial glycaemic response; the same health claims have been approved for pectins. Pectins can be found in sugar beet fibre, apples and citrus fruits, although there are differences between the sources in terms of molecular weight (MW), molecular structure and the degree of methyl esterification (DE). Hence, the intake required to achieve a specific biological effect, and the effects that can be expected, could differ for different pectins. Moreover, some intervention studies focus on one specific source of dietary fibre, sometimes using extracted fibre, whereas the effect might only be observed if a combination of fibre from different sources was used. Therefore, it is important to investigate the effects of different fibre-rich foods, and to determine whether a positive effect can be achieved by combining several sources of fibre.

A weakness of observational studies lies in the relative inaccuracy in food and nutrient intake, which is commonly assessed by dietary questionnaires. To increase the accuracy of the data, markers of dietary fibre intake that are unique to specific sources of dietary fibre can be used to determine intake quantitatively [12]. A metabolomic approach can facilitate this, since it allows the detection of all the small-molecule metabolites present in a biological sample. Hence, metabolomics can find new, and confirm known, markers of dietary fibre intake. Moreover, since the exact mechanisms behind the effects of different kinds of dietary fibre have not yet been fully evaluated, metabolomics can provide information on the up- and down-regulation of metabolic pathways after a high dietary fibre intake.

1.1 Risk factors and progression of lifestyle-dependent diseases

Cardiovascular disease

CVD is associated with the progression of arteriosclerosis. Atherogenic lipoproteins, such as low-density lipoprotein (LDL), can enter the arterial cell wall where they are modified by oxidation or enzymatic activity, and undergo phagocytosis by macrophages. The macrophages can be activated by several cytokines, such as interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), interleukin (IL)-4 and IL-13, which initiate the secretion of cytokines such as IL-1β, IL-6 and TNF-α, together with endocytosis of modified LDL [13]. A plaque will start to grow. If the plaque ruptures, a blood clot

(thrombus) could cause blockage of the blood supply or bleeding into the surrounding tissue leading, for example, to a myocardial infarction or stroke. If the plaque does not rupture but continues to grow, the decrease in the lumen diameter could cause a heart attack, angina pectoris or a stroke. The progression of arteriosclerosis is related to the lipoprotein metabolism and inflammation [13].

Several lipoproteins are involved in the transport of lipids in the blood, such as cholesterol and triglycerides. The main lipoproteins are the chylomicrons, very-low-density lipoprotein (VLDL), LDL and high-density lipoprotein (HDL). The main apolipoprotein (Apo) in LDL, VLDL and chylomicrons is ApoB, whereas ApoA is the primary protein component in HDL. Dietary fat is absorbed and transported to peripheral tissues by chylomicrons (large triglyceride-rich lipoproteins). Lipoprotein lipase in tissue hydrolyses triglycerides to enable free fatty acid uptake, and the chylomicron remnants are subsequently taken up by the liver. The liver secretes VLDL, which will eventually form LDL by the continuing hydrolysis of triglycerides by lipoprotein lipase. LDL has a high cholesterol content, and is mainly absorbed by the liver via LDL receptors. The number of LDL receptors on the hepatocyte surface is correlated to the level of LDL in blood. Moreover, blood concentrations of LDL will also influence the hepatic production of VLDL, and increased production of VLDL has been observed in subjects with insulin resistance or type 2 diabetes [13]. HDL is generated in the intestine and the liver by the secretion of ApoA1. ApoA1 stimulates the release of cholesterol from the macrophages and peripheral tissue, while HDL transports the released cholesterol either directly, or via LDL and VLDL, back to the liver, where it can be excreted through the bile [13]. This reversed cholesterol transport is associated with a protective effect on arteriosclerosis.

Measurements of total cholesterol (TC), triglycerides, HDL-C (high-density lipoprotein cholesterol) and LDL-C (low-density lipoprotein cholesterol) are normally made to determine the lipid levels in the blood [14], apart from LDL-C, which is commonly calculated using the Friedewald formula [15]. In some circumstances it could be useful to use non-high-density lipoprotein cholesterol (non-HDL => TC – HDL-C) as certain sub-populations, such as subjects with metabolic syndrome that have low HDL-C, and for healthy women that usually have quite high HDL-C values compared to men [14]. The ratio of ApoB to ApoA1 also provides information on circulating lipoproteins, as VLDL and LDL carry the ApoB protein on their surfaces, whereas HDL carries ApoA1. These factors can also amplify the effects of other risk factors related to CVD, such as hypertension, as well as increasing the risk for type 2 diabetes.

Type 2 diabetes

Both type 2 diabetes and obesity are associated with increased insulin resistance. However, insulin resistance does not predispose a person to develop diabetes per se, rather insulin

release will increase to compensate for the reduced efficiency [16]. Insulin promotes glucose uptake for immediate use, or storage as glycogen in the skeletal muscles and liver. The release of insulin is accompanied by a concomitant decrease in the release of glucose from the liver, by the inhibition of gluconeogenesis, leading to the storage of fatty acids in the adipose tissue through the stimulation of lipoprotein lipase [17]. Moreover, insulin supresses the release of non-esterified fatty acids (NEFA) from the adipose tissue by inhibiting hormone-sensitive lipase, preventing the liver from secreting VLDL [18]. As mentioned above, insulin resistance is manifested by the loss of insulin-mediated signalling by the target cells in skeletal muscles, adipose tissue and the liver. This can cause hyperlipidaemia as insulin is not able to inhibit the NEFA released from the adipose tissue, leading to the overproduction of VLDL in the liver and an increased release into the blood [18].

Metabolic syndrome

Metabolic syndrome is a combination of medical disorders that, when occurring together, increase the risk of developing CVD and type 2 diabetes. The risk factors involved in metabolic syndrome include dyslipidaemia, increased fasting glucose levels, (central) obesity and high blood pressure [19]. It has become evident that low-grade inflammation plays a role, not only in the development of arteriosclerosis, but also in type 2 diabetes and obesity [20]. The adipose tissue is an active organ that influences the homeostasis of body weight, as well as insulin resistance, lipid levels, inflammation and arteriosclerosis, by secreting several hormones (such as leptin and adiponectin) and cytokines (such as IL-6 and TNF-α) [21]. Adiponectin levels are decreased in obese and diabetic (i.e. insulin-resistant) individuals, and it has been suggested that the mechanism involves an increase in the release of TNF-α from accumulated visceral fat [21]. Obesity generally results in higher levels of VLDL, LDL, triglycerides, and the ApoB/ApoA1 ratio, together with reduced levels of HDL.

1.2 DIETARY FIBRE

In Scandinavia, the main sources of dietary fibre from whole grains are wheat, rye and oats [22]. A national survey evaluating the food intake in the Swedish population (*Riksmaten 2010-2011*) showed that the total dietary fibre intake had increased to 20 g per day, on average, although this was still below the recommended level of 25-35 g per day. Bread contributed 28% to the total dietary fibre intake, vegetables 12%, fruits and berries 11%, cereals and porridge 8% and potatoes 6% [7].

The definition of dietary fibre used in the EU [23] is: "*carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the human small intestine and belong to the following categories:*

- *edible carbohydrate polymers naturally occurring in the food as consumed;*
- *edible carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence;*
- *edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence."*

The characteristics of different kinds of dietary fibre are dependent on the source and the physicochemical properties of the fibre. Soluble fibre dissolves in water, where it usually forms a viscous gel, whereas insoluble dietary fibre does not. Examples of soluble dietary fibre are β-glucans, psyllium, pectins and some hemicelluloses, while common kinds of insoluble dietary fibre are cellulose, lignin and some hemicelluloses. However, this definition of soluble and insoluble dietary fibre is not strictly correct, as molecular structure affects solubility. Examples of this will be presented below.

1.2.1 Sources of dietary fibre studied in this work

Oats (*Avena sativa*)

About 20-35% of the oat kernel is hull which contains a high amount of insoluble dietary fibre. The hull is removed and the remaining edible part, the oat groat, has a total dietary fibre content of 6-9% [24]. The insoluble part consists of insoluble arabinoxylans, lignin and cellulose [25], and is located in the outer part of the grain, outside the aleurone layer. β-glucan is a linear polysaccharide with mixed β (1 → 3) and β (1 → 4) linkages between the *D*-glucose units located in the cell walls of the endosperm and subaleurone layer (Figure 1). The β (1 → 3) linkages occur singly between the β (1 → 4) oligosaccharide subunits of *D*-glucose, and contribute to its solubility. Oat bran is produced by grinding and sieving oat groats to separate the starchy part (the endosperm) from the outer layers, providing the oat bran in the coarse fraction. Therefore, the concentration of β-glucans in oat bran is higher, around 5.5-9% [24].

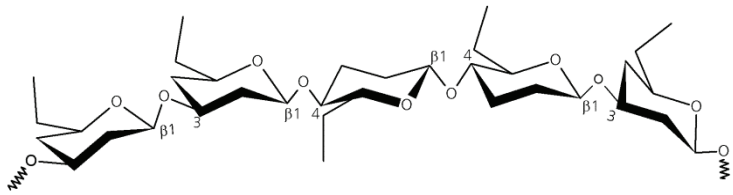


Figure 1. Structure of β-glucan, modified from Vasanthan and Temelli [26].

Rye (*Secale cereale*)

The rye grain contains about 20% total dietary fibre (including fructan), with 9% arabinoxylan as the main fibre component, one-third of which consists of soluble arabinoxylans [27]. The bran fraction used in this work, includes the fruit coat, the seed coat and the aleurone layer, and contains about 50% total dietary fibre (including fructan), of which 23% is arabinoxylans, 5% β-glucans, 6% cellulose and 4% lignin [28]. Arabinoxylan has a xylose backbone to which various numbers of arabinose units are attached (Figure 2). The substituted arabinose units contribute to the solubility of arabinoxylan [29].

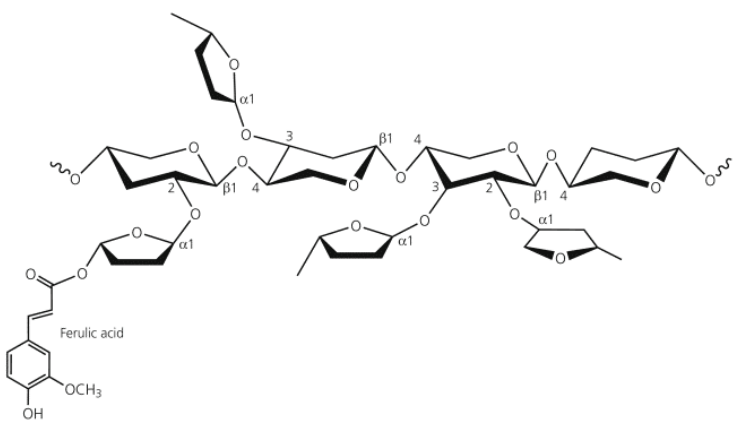


Figure 2. Structure of arabinoxylan, showing the xylose units connected with β (1 → 4) linkages. About half of the xylose residues are monosubstituted with arabinose. Xyloses disubstituted with arabinose occur to a lesser extent. Ferulic acid is attached to the arabinose residues [29].

Sugar beet (*Beta vulgaris)*

Sugar beet is grown commercially for sucrose production. The by-product, sugar beet fibre, is high in dietary fibre (67%), consisting mainly of cellulose (28%), hemicelluloses (42%), pectin (27%) and lignin (3%) [30]. Hemicelluloses and pectin make up the matrix of the cell walls in which cellulose is embedded. The pectin backbone consists of β (1 → 4)-linked *D*-galacturonic acid units, with methylated carboxyl groups and acetylated hydroxyl groups, interrupted by rhamnogalacturonan regions that consist mainly of arabinose, galactose, rhamnose and galacturonic acid (Figure 3). Homogalacturonan occurs in the so-called "smooth regions", whereas rhamnogalacturonan forms the "hairy regions". The pectin in sugar beet has a low viscosity and poor gelling capacity, probably as a result of the higher number of acetyl groups and their relatively low MW, compared with apple and citrus pectin [31]. Pectins extracted from sugar beet pulp have DE ~50 [32].

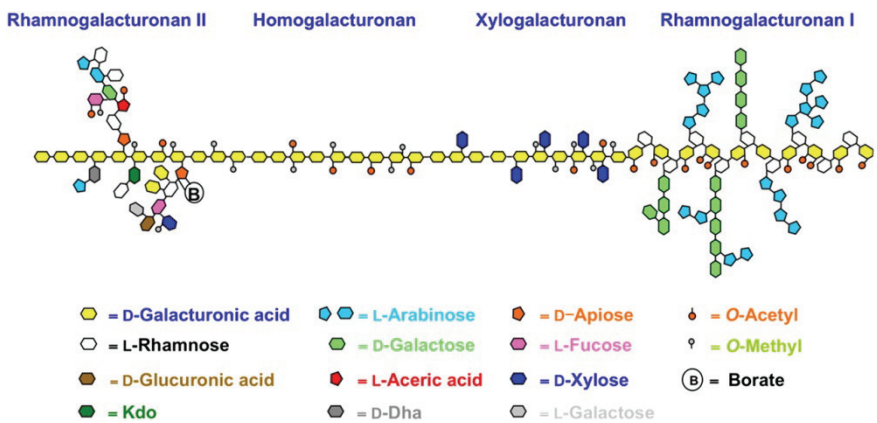


Figure 3. Structure of pectin, showing the smooth (homogalacturonan) and hairy (rhamnogalacturonan) regions. Homogalacturonan and rhamnogalacturonan I are the most abundant of the four polysaccharides [33]. (©American Society of Plant Biologists, reproduced with permission [33]).

1.2.2 Physiological effects of dietary fibre

A large number of studies have been published on oats and rye, regarding their characteristics in terms of nutrient composition and physicochemical properties, and their physiological effects when incorporated into animal and human diets. However, less information is available on sugar beet fibre and its effects on health. Health claims for some kinds of dietary fibre have been approved for use in the EU by the European Commission, based on scientific evaluations by the European Food Safety Authority. An intake of 3 g of β-glucan per day is claimed to be able to reduce blood cholesterol levels [(34)], while 4 g of β-glucans in each 30 g of available carbohydrates per meal is claimed to decrease the postprandial glycaemic response [(35)] and 8 g of arabinoxylan-rich fibre per 100 g of available carbohydrates from wheat can reduce the postprandial glycaemic response [(36)]. However, there are no health claims for rye arabinoxylans. Health claims that sugar beet fibre and oats can increase faecal bulk [(37, 38)] have been approved, and that rye fibre lead to beneficial changes in bowel function, such as reduced transit time, increased faecal bulk and softer stools [(39)]. Sugar beet fibre is rich in the soluble fibre pectin [(40)], and health claims that an intake of 6 g pectin per day can help maintain a normal blood cholesterol level, and that 10 g pectin per meal can decrease the postprandial glycaemic response have been approved [(41)].

Glucose and insulin metabolism

The ability of soluble dietary fibre to form viscous gels, in some cases already at very low concentrations, can delay gastric emptying and increase the viscosity of stomach and intestine content, thereby reducing the rate of starch digestion and glucose absorption [(42, 43)]. Hence, the consumption of soluble dietary fibre is correlated with lower postprandial glucose and insulin responses [(44)], although the effect of insoluble fibre should not be neglected. Insoluble dietary fibre has been shown to reduce the intestinal transit time, and to increase the viscosity of the intestinal content, creating a diffusion barrier which, together with the binding of enzymes (thereby reducing their activity), could affect the rate of digestion and the absorption of nutrients in the small intestine [(42)].

Beneficial effects have been found in several studies on oat β-glucans on fasting and postprandial glucose and insulin metabolism in healthy subjects [(45)], type 2 diabetic subjects [(46, 47)] and hypercholesterolaemic subjects [(48)]. Arabinoxylans have been shown to reduce the postprandial glucose and insulin responses in healthy subjects [(49)], in type 2 diabetic subjects [(50)] and in subjects with impaired glucose tolerance [(51)]. Only a limited number of studies have been performed on the effects of sugar beet fibre, some of which have shown effects on postprandial glucose and insulin levels in healthy subjects [(52, 53)].

Lipid metabolism

Soluble dietary fibre has been shown to have a hypolipidaemic effect, although the mechanism is not fully understood. One suggestion is that the soluble fibre increases the excretion of bile acids, probably by binding. The reabsorption of bile acids by the enterohepatic circulation is therefore reduced, leading to upregulation of the liver enzyme 7-α-hydroxylase and increased production of bile acids. This will lead to a reduction in the cholesterol pool in the liver, and an increase in the expression of LDL receptors on the hepatocytes, which will in turn result in an increase in the uptake of LDL-C from the blood [18]. Several studies on oats have shown significant reductions in TC, LDL-C and, in some cases, also non-HDL-C in obese subjects [54], type 2 diabetic subjects [47] and hypercholesterolaemic subjects [48, 55-58]. Fasting triglyceride concentrations were found to be reduced after arabinoxylan intake in subjects with impaired glucose tolerance [59], but the effect was not observed in type 2 diabetic subjects with arabinoxylans extracted from wheat [50]. It has been suggested that sugar beet fibre does not increase bile acid excretion; rather the hypocholesterolaemic effect is thought to be caused by interference with lipid absorption, thereby increasing the excretion of cholesterol [60]. This was observed in a study on healthy subjects given sugar beet fibre as part of their diet [61]. Although TC and LDL-C were significantly reduced, no changes in bile acid excretion were observed. A reduction in TC was observed in healthy subjects given 20 g sugar beet fibre per day for 3 weeks [62], while another 3-week intervention study showed reductions in both TC and LDL-C [53]. Pectin has been shown to reduce TC and LDL-C [63], and more recently sugar beet pectin was shown to increase HDL-C and to decrease the TC/HDL-C ratio in subjects with impaired glucose metabolism [64].

Immune function

Dietary fibre appears to have anti-inflammatory effects, and certain diets or specific food components could thus help prevent diseases that are related to low-grade inflammation. The mechanism behind this is not clear, but a decrease in lipid oxidation [65], induced body weight loss, a reduction in postprandial glucose response and the production of the short-chain fatty acid (SCFA) butyrate in the intestine by the gut microbiota [43], have been suggested to influence low-grade inflammatory responses. Fibrinogen is a marker of inflammation and a main coagulation protein. A long-term increase in fibrinogen of 1 g/L increases the risk of CVD [66], and dietary fibre has been shown to decrease fibrinogen in the EPIC-Norfolk cohort [67]. Moreover, dietary fibre intake has also been inversely associated with C-reactive protein (CRP) levels in the NHANES cross-sectional study [65, 68]. CRP is an acute-phase protein produced in response to inflammation and infection, and minor elevations have been observed in low-grade inflammatory states. Cytokines such as IL-1, IL-6 and IL-17A stimulate the production of CRP [20]. An observational study

showed that total, soluble and insoluble dietary fibre intake were inversely related to levels of IL-6 and the tumour necrosis factor receptor (TNF-R), but no significant reduction in CRP was seen [69]. Human intervention studies on dietary fibre intake have demonstrated effects on inflammatory markers such as IL-6 and TNF-α in obese women [70] and IL-1β in subjects with metabolic syndrome [71]. However, findings regarding the ability of dietary fibre to modulate cytokine levels are inconsistent [72, 73].

Gut health

The intake of insoluble fibre in the diet, which is incompletely or slowly fermented in the large intestine, can promote normal laxation, increase the faecal bulk, and improve intestinal disorders [43]. Soluble fibre can function as a prebiotic, stimulating the growth and activation of specific gut microbiota (such as bifidobacteria and lactobacilli), which can enhance health [44]. Lignin is not fermented at all by the gut microbiota, while 30 to 50% of cellulose is fermented, 50 to 80% of hemicelluloses and almost all (90 to 100%) pectins, gums and oligosaccharides (e.g. fructooligosaccharides) [74]. The fermentation of prebiotics by the colonic bacteria results in the formation of SCFAs, mainly acetate, propionate and butyrate, which are absorbed in the colon. Acetate serves as an energy source for muscle tissue, while propionate may inhibit cholesterol synthesis and provide a substrate for gluconeogenesis in the liver, thereby inhibiting the gluconeogenetic pathways of pyruvate and amino acids [75]. Butyrate serves as an energy source for colonocytes, and may also offer protection against colon cancer by inducing apoptosis, and could thus be beneficial for patients with inflammatory bowel diseases [75]. A reduction in pH as a consequence of SCFAs production could also enhance the uptake of minerals such as calcium and magnesium in the gut, as well as creating an unfavourable environment for pH-sensitive pathogens [75].

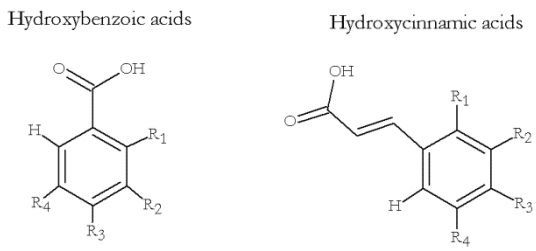
1.2.3 Components in different sources of dietary fibre

Other dietary components associated with dietary fibre can be used as markers for dietary fibre intake. Some of these compounds are presented below.

Phenolic acids

Phenolic acids are hydroxylated products of benzoic and cinnamic acids (Figure 4), where hydroxycinnamic acids are more common [76]. The main phenolic acid in oats, combining both free and bound forms, is ferulic acid (~58%), followed by *p*-coumaric acid (~34%)

and sinapic acid (~6%) [77]. About 97% of the phenolic acids in oats are in the bound form, mainly via ester linkages to the cell wall polysaccharides. The main free phenolic acids in oats are ferulic acid, syringic acid, vanillic acid and sinapic acid, in decreasing order [77]. Rye contains several phenolic acids; about 63% in bound form, 33% in conjugated form and 3% as free phenolic acids. The main phenolic acids in rye in bound form are ferulic acid (~ 74%), followed by 2,4-dihydroxybenzoic acid (DHBA) (9%), sinapic acid (~8%) and *p*-coumaric acid (~4%) [76]. Most of the ferulic acid is present in the rye bran fraction, primarily ester-linked to the arabinose units in arabinoxylan, which allows cross-linking between arabinoxylans [29]. The free phenolic acids in rye are, in decreasing order, sinapic acid, ferulic acid and caffeic acid, while the main conjugated forms are 2,4-DHBA, sinapic acid, and ferulic acid [76]. Ferulic acid-4-*O*-sulphate has been identified as a marker of rye bread intake in human urine [78]. Oats contain about five times more total phenolic acids than rye, although the amount of free phenolic acids is somewhat higher in rye. Sugar beet consists mainly of ferulic acid and *p*-coumaric acid, both of which are ester-linked to arabinose and galactose in the hairy regions of pectin [31]. This enables cross-linking between the pectins in sugar beet.



Hydroxybenzoic acids	R1	R2	R3	R4
Vanillic acid	H	OCH_3	OH	H
Syringic acid	H	OCH_3	OH	OCH_3
2,4-DHBA	OH	H	OH	H
3,5-DHBA	H	OH	H	OH
Hydroxycinnamic acids	**R1**	**R2**	**R3**	**R4**
p-Coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H
Caffeic acid	H	OH	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3

Figure 4. Structures of some phenolic acids. (Modified from Irakli et al. [77])

Alkylresorcinols

Alkylresorcinols are phenolic lipids found in the bran layer of rye and wheat grain [79], with an odd-numbered alkyl chain from C15:0 to C25:0 attached to 1,3-dihydroxybenzene. The relative abundance of the alkylresorcinol homologues differs between rye and wheat. The C17:0/C21:0 ratio can be used to distinguish between wheat and rye alkylresorcinols since the ratio is 1 in rye and 0.1 in wheat [80]. About 60% of the alkylresorcinols are absorbed or converted in the small intestine [81], and they are mainly transported in lipoproteins [82] and erythrocyte membranes [83]. The metabolism of alkylresorcinols has been suggested to be by β-oxidation of the alkyl tail, in a similar way to tocopherols, followed by glucuronide or sulphate conjugation at the hydroxyl groups on the phenolic ring [84]. Two metabolites of alkylresorcinol metabolism have been identified: 3,5-DHBA and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) [85]. Both the sulphate and glucuronide conjugates of DHPPA have been identified in human urine after intake of rye bread [78].

Lignans

Rye grain is rich in lignans, which are concentrated in the outer layers of the kernel [79]. The most abundant lignans in rye are syringaresinol, pinoresinol, lariciresinol, medioresinol, secoisolariciresinol and matairesinol [86]. Syringaresinol accounts for more than half of the total lignan content in rye. The total lignan content in oats is less than half of that in rye. The occurrence of lignans in oats is, in decreasing order, syringaresinol, pinoresinol, lariciresinol, matairesinol, medioresinol and secoisolariciresinol [87]. In the gut, the intestinal microbiota converts plant lignans into the mammalian derivatives, enterodiol and enterolactone [88], which mainly exist conjugated to glucuronic acid and, to a lesser extent, sulphate [89].

Benzoxazinoids

Benzoxazinoids are part of the plants' defence against pathogens. There are three common chemical structures of benzoxazinoids: hydroxamic acids, lactams and benzoxazolinones (Figure 5). Hydroxamic acids can be spontaneously degraded into benzoxazolinones [90]. Several benzoxazinoids have been identified in rye: DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one), DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), HBOA (2-hydroxy-1,4-benzoxazin-3-one) and BOA (1,3-benzoxazol-2-one) [91]. The benzoxazinoid content in wheat, oats and barley has been analysed, and their presence was found only in wheat, although at lower amounts than in rye.

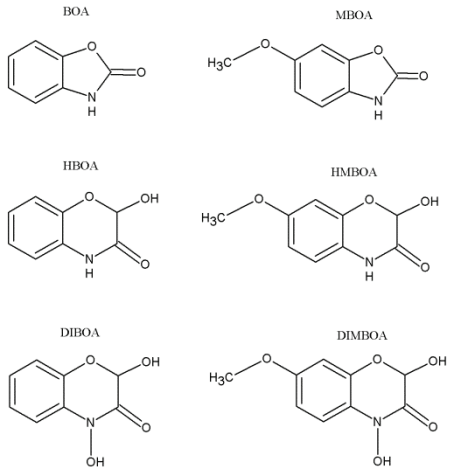


Figure 5. Structures of common benzoxazinoids: BOA and MBOA are benzoxazolinones, HBOA and HMBOA are lactams and DIBOA and DIMBOA are hydroxamic acids. (Modified from Hanhineva et al. [91])

Saponins

Saponins are divided into triterpenoids and steroid glycosides. Several triterpenoid saponins, betavulgarosides, have been identified in sugar beet [92, 93]. Some of these saponins will remain in the sugar beet fibre after the extraction of sucrose [94]. Betavulgarosides consist of a steroid backbone of oleanolic acid with up to four sugar moieties attached. Triterpenoid saponins (avenacins) and steroidal saponins (avenacosides) are present in the roots and leaves, respectively, of oats [95]. The steroidal saponins, avenacosides A and B, have been observed in oatmeal [96] and in the grain and husk of oat [97]. The backbone in avenacosides consists of the furospirostanol, nuatigenin with four or five sugar moieties attached (one rhamnose and three/four glucose moieties). A new sulphated nuatigenin-type steroidal saponin has also been identified in oats [98]. Avenacosides A and B form the defence system in oats, and are activated in the plant through hydrolysis of the 26-C glucose moiety by avenacosidase to form 26-desglucoavenacoside [99]. The presence of 26-desglucoavenacoside in oat grain and husk has been demonstrated [97]. Avenacins A1, A2, B1 and B2 have avenestergenins as a steroidal backbone [100] with three sugar moieties attached, and are found in the oat root [95]. The structures of these saponins are shown in Figure 6.

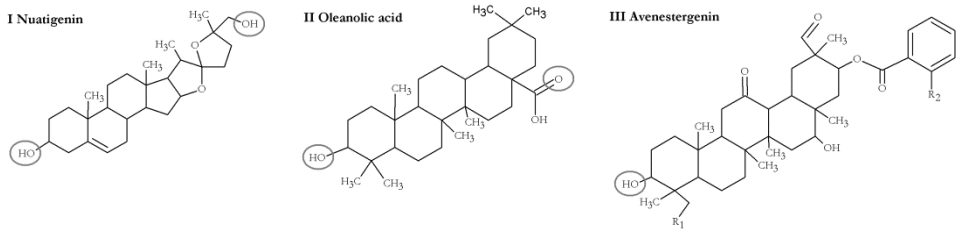


Figure 6. Three backbone structures in different saponins. The circles in each structure indicate where the sugar moieties are attached. Nuatigenin is the backbone structure in avenacosides A and B, oleanolic acid is the backbone structure in the betavulgarosides, and avenestergenin A1, A2, B1 and B2 are the backbone structures in avenacin A1, A2, B1 and B2.

1.3 NUTRIGENOMICS

Metabolomics, used in the present work, is one of the techniques included in the term nutrigenomics. Nutrigenomics also includes the analysis of genomics, transcriptomics and proteomics (Figure 7). These techniques are applied exploratively in nutritional intervention studies to identify potential nutritional biomarkers, to explain the metabolic pathways of metabolites or an observed health effect, and to help understand the individual genetic variability in response to nutrients or diets. Nutritional intervention studies in humans generally produce subtle changes in the *-omes* compared with interventions with drugs. Any effect of diet may be masked by inter-individual variation, short study duration, too healthy subjects, or the way in which subjects are standardised during the intervention and prior to sampling.

Transcriptomics is used to observe the up- and down-regulation of genes by analysing the mRNA expression in blood or tissues following an intervention. Expression analysis is carried out on microarray chips, on which mRNA is hybridised to its complementary probe; each probe representing one gene. The number of publications on nutritional human intervention studies using transcriptomics is increasing [101]. This technique was used in a sub-study of the meal study presented in this work, published by Ulmius et al.[102], and it was found that the postprandial response to the oat bran meal down-regulated genes related to insulin signalling in healthy subjects. A similar observation has been made in the adipose tissue of subjects with metabolic syndrome after a rye/pasta diet [103].

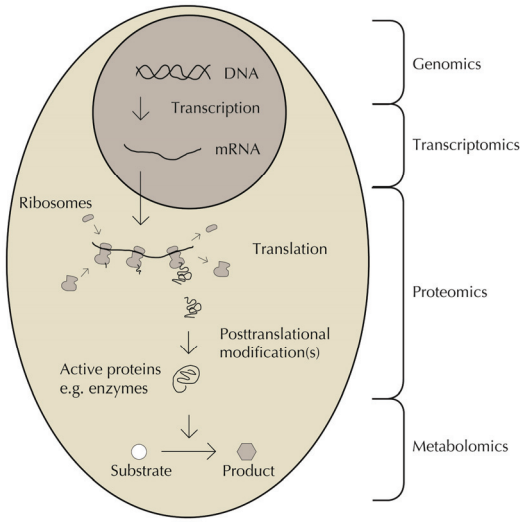


Figure 7. An overview of the nutrigenomic levels within a cell.

Proteomics can not only be used to investigate the quantity of proteins, but it also allows the identification of protein interactions and any post-translational modifications. The use of proteomics in human nutrition research is still limited, and the most common method used is to compare the protein expression profiles from groups subjected to different interventions. So far, it has mainly been used to help understand the effects of a specific food item and/or to search for biomarkers related to disease [(101)]. Blood is often used, which requires some pretreatment to remove highly abundant proteins, to enable the detection of the less abundant proteins.

The aim of metabolomics is to identify and (semi-)quantify the metabolome, i.e. "all" metabolites with molecular weights below 1000-1500 Da that are present in a biological sample. The metabolome is dynamic. Different metabolomic models have shown that less than 30% of the metabolites are involved in two reactions, about 12% in over 10 reactions and about 4% in over 20 reactions [(104)]. Moreover, most reactions involve more than one substrate and one product. However, some metabolites will remain unchanged in the body as they are not involved in any metabolic reactions. Progress in high-throughput analytical and bioinformatics tools has allowed the simultaneous analysis of hundreds or more metabolites in the metabolome of biofluids or tissues. Two main approaches are commonly used: untargeted and targeted metabolomics. Untargeted metabolomics is used to explore changes within the system of interest to discover new markers of dietary intake and/or effect markers related to a dietary intake, and can be seen as a hypothesis-generating approach. The targeted approach is aimed at quantifying already known metabolites, or

metabolites in a specific pathway, and is usually used to test a specific hypothesis rather than to generate one.

1.3.1 Metabolomics in nutrition

A number of metabolomic studies on dietary fibre have resulted in the discovery of new markers related to fibre intake. Several of these studies have been performed with rye as the source of dietary fibre. The intake of rye bread/pasta has been shown to increase the n-3 fatty acid docosahexaenoic acid (DHA), and decrease the branched-chain amino acid (BCAA) isoleucine in a group of subjects with metabolic syndrome, as a result of a decrease in the intake of protein [105]. An oat/wheat bread/potato diet increased pro-inflammatory lysophosphatidylcholines [105]. This effect was also observed in the concurrent transcriptomic analysis; stress-related pathways were upregulated in the oat/wheat/potato group, while pathways associated with stress, insulin signalling and energy metabolism were downregulated in the rye bread/pasta group [105]. Metabolomic profiles after 8 weeks' consumption of rye bread revealed that ribitol and ribonic acid increased, while no effect was observed after wheat bread consumption [106]. Both ribitol and ribonic acid are precursors of tryptophan, and a positive association was observed between ribonic acid and tryptophan. Since tryptophan is a precursor in the synthesis of serotonin, which depresses hunger, the authors hypothesized that this could be one mechanism behind the increase in satiety following rye intake. An increase in ribitol postprandially has also been reported after rye bread intake in another study [107]. The BCAAs leucine and isoleucine have also been found to decrease in fasting serum samples after the intake of rye bread [108], as has been observed previously [105]. A decrease in BCAAs is thought to reduce the risk of developing type 2 diabetes. Moreover, betaine and *N,N*-dimethylglycine (involved in the betaine-homocysteine methyl transferase reaction) have been found to increase after an 8-week period on a rye bread diet [108], which has also been reported previously [109], indicating a protective effect against CVD.

There are limited publications on oats. A comparison has been performed of the postprandial response in humans and pigs after the intake of different kinds of bread (rye, wheat, arabinoxylan-enriched and β-glucan-enriched) [110], where it was concluded that the metabolic response to the breads was similar. No metabolomic studies have been performed on sugar beet fibre or sugar beet pectin. Several metabolites were identified in a metabolomic study on urine from rats after feeding them raw apple or apple-pectin [111]. The effect marker after apple intake was hippuric acid, while quinic acid, m-coumaric acid and (-)-epicatechin were identified as exposure markers. Intake of apple pectin led to pyrrole-2-carboxylic acid and 2-furoylglycine being identified as exposure markers, while 2-piperidinone was considered an effect marker.

These markers are either endogenous effect markers or exogenous markers related to the dietary intake. Effect markers could provide more insight into the mechanisms behind observed effects, whereas markers from different fibre sources can be used to quantify specific or total dietary fibre intake. This is useful in larger observational studies, in which food intake may be under- or overestimated as it is based on the subjects' compliance in recording their food intake, as well as their ability to recall and describe food intake accurately. Food records for a limited number of days or weeks or 24-h recalls, are commonly used as validation of the results from the questionnaire in a subset of the study population. Some bias still exists [112], and the use of dietary intake markers could provide more accurate data. However, the correlation between a specific marker and a food intake questionnaire could be weak. Factors influencing the correlation, which are not related to intake, include absorption, postabsorptive metabolism and/or physiological regulation of nutrient levels [112], and it is therefore important to thoroughly investigate and validate the dietary marker of interest.

2 OBJECTIVES

The aims of the work presented in this thesis were to evaluate whether specific sources of dietary fibre can have positive effects on known risk markers of CVD and type 2 diabetes. This was done using different study designs and sub-populations at different levels of risk. As well as studying traditional biomarkers of risk, an untargeted metabolomic approach was used to search for dietary markers of oat, rye and sugar beet fibre intake, and effect markers related to the intake of these dietary fibre sources in plasma and urine. This was accomplished in the following studies:

- Evaluation of the response of postprandial glucose, insulin and triglyceride in healthy subjects after the intake of different breakfasts containing oat powder, rye bran and sugar beet fibre, or a mixture of them (the meal study, Paper I)

- Investigation of the effects of a 5-week high-fibre diet containing oat bran, rye bran and sugar beet fibre, on the glucose and lipid metabolism, and on the inflammatory response in healthy, but mildly hypercholesterolaemic, subjects (the intervention study, Paper II)

- Investigation of the changes in metabolome profile in plasma and urine following a high-fibre diet, and attempts to identify new and verify known dietary markers of oat, rye and sugar beet fibre intake, and dietary-fibre-related response markers, in mildly hypercholesterolaemic subjects (the intervention study, Papers III and IV)

- Evaluation of whether the intake of a healthy Nordic diet with a high dietary fibre content for 18 or 24 weeks could induce beneficial effects on glucose tolerance, insulin resistance, blood lipids or inflammatory markers in subjects with metabolic syndrome (the SYSDIET study, Paper V)

3 SUBJECTS AND METHODS

The characteristics of the subjects included in all the studies presented in this thesis are given in Table 1. The subjects in each study and the design of the studies are described in more detail below.

Table 1. Baseline characteristics of the subjects included in these studies (means and SD)

	The meal study	The intervention study	The SYSDIET study	
			Healthy Nordic diet	Control diet
Total number	18	30	104	96
Dropouts/Excluded	4/1	4/1	8	26
Women/Men	8/10	18/12	69/30	57/33
Age (y)	23.4 (2.9)	59.3 (5.5)	54.0 (8.5)	54.9 (8.6)
BMI (kg/m^2)	23.1 (2.5)	26.6 (2.4)	31.6 (3.5)	31.7 (2.8)
Systolic blood pressure (mmHg)	n.a.	132 (16)	130 (15)	130 (16)
Diastolic blood pressure (mmHg)	n.a.	82 (8)	82 (10)	82 (11)
Glucose 0 min (mmol/L)	5.3 (0.5)	5.6 (0.8)	5.8 (0.6)	5.7 (0.6)
OGTT 120 min (mmol/L)	n.a.	n.a.	6.2 (1.6)	6.8 (2.1)
Insulin (mU/L)	5.3 (2.4)	8.4 (6.8)	n.r.	n.r.
TG (mmol/L)	1.13 (0.51)	1.58 (0.79)	1.52 (0.75)	1.61 (0.80)
TC (mmol/L)	n.a.	5.90 (0.64)	5.30 (0.88)	5.24 (0.98)
LDL-C (mmol/L)	n.a.	3.74 (0.55)	3.25 (0.80)	3.21 (0.89)
HDL-C (mmol/L)	n.a.	1.46 (0.40)	1.36 (0.33)	1.33 (0.41)
ApoA1 (g/L)	n.a.	n.a.	1.44 (0.20)	1.40 (0.24)
ApoB (g/L)	n.a.	n.a.	1.06 (0.26)	1.05 (0.26)

Apo = apolipoprotein, BMI = body mass index, HDL-C = high-density lipoprotein cholesterol, LDL-C = low-density lipoprotein cholesterol, n.a. = not analysed, n.r. = not reported, OGTT = oral glucose tolerance test, TC = total cholesterol, TG = triglycerides

3.1 THE MEAL STUDY (PAPER I)

The meal study was designed as a randomised cross-over study in which each visit was separated by a week. Blood samples were drawn before the meal intake and every 30 min up to 180 min postprandially. During each visit, subjects were randomly assigned one of

the five different breakfast meals: a control meal without added fibre, or one of the four meals containing dietary fibre from different sources: oat powder (produced from spray-dried oat beverage; Oatly AB, Landskrona, Sweden), rye bran (Lantmännen Food R/D, Järna, Sweden), sugar beet fibre (Fibrex, Nordic Sugar AB, Malmö, Sweden) or a mixture of all three kinds of fibre. The fibre was added to blackcurrant juice with pulp. The nutrient contents of the test meals are presented in Table 2. The amount of lipids was adjusted to 7.9 g in all the meals (including the control meal) using rapeseed oil, and dextrose powder and white bread were used to adjust the amount of carbohydrates in all the meals to 75.0 g. The protein content was not adjusted due to the lack of an appropriate vegetable protein source. This study is described more in detail in Paper I.

Table 2. The nutrient contents of the different test breakfast meals. The amounts of lipids and carbohydrates in each meal were adjusted to 7.9 g and 75.0 g, respectively

	Energy (kJ)	Proteins (g)	Total fibre (g)	Soluble fibre from added fibre/total soluble fibre (g)
Control meal	1640	5.4	1.4	0.0/0.3
Oat powder meal	1665	6.3	3.3	2.7[1]/2.7
Rye bran meal	1695	8.5	13.0	1.7[2]/2.0
Sugar beet fibre meal	1664	6.7	13.2	5.0[3]/5.3
Mixed meal	1725	10.0	18.3	5.0[4]/5.2

[1]Amount of soluble β-glucan is estimated to be 2.5 g [(113)]

[2]Amount of soluble arabinoxylan is estimated to be 0.9 g [(27)]

[3]Amount of soluble pectin is estimated to be 3.7 g [(60)]

[4]Amount of soluble β-glucan is estimated to be 1.5 g [(113)], arabinoxylan 0.9 g [(27)] and pectin 1.2 g [(60)]

3.2 THE INTERVENTION STUDY (PAPERS II-IV)

This study was a 5-week single-blinded randomised cross-over intervention study, separated by a 3-week washout period. Fasting blood samples were collected at the beginning and end of each intervention period. Samples of 24-h urine were collected at the end of each intervention period for non-targeted metabolomic analysis. Subjects were randomly assigned to start with a high-fibre (HF) or a low-fibre (LF) diet. A bread roll (Lantmännen), one ready meal (Findus Sverige AB, Bjuv, Sweden) and two beverages (Oatly) were consumed daily in both the HF and LF diet. For the HF diet, rye bran (Lantmännen) and sugar beet fibre (Fibrex, Nordic Sugar) were added to the bread roll (22.5 g rye bran; 3.2 g sugar beet fibre) and three different ready meals (7-12 g rye bran;

3-5 g sugar beet fibre). No dietary fibre was added to the LF food products. The beverage in the HF diet was made from oat bran (β-glucan MW 0.1×10^6 g/mol [114]), while rice was used in the beverage in the LF diet.

Subjects were allowed to ingest other foods and drinks besides the test food products, *ad libitum*, only plant-sterol-containing foods were prohibited. The subjects were instructed to maintain their physical activity, weight and on-going nutritional supplementation. To standardise the nutrient intake on the day before blood sampling, all subjects consumed a spaghetti Bolognese ready meal as their last evening meal. Compliance with the diets was assessed with daily food frequency questionnaires to monitor the test food product intake. A record of the intake of all food and drinks was assessed on three consecutive days (two weekdays and a weekend day) in the middle of each intervention period. This study is described in more detail in Papers II, III and IV.

3.3 THE SYSDIET STUDY (PAPER V)

In 2007, NordForsk (an organisation under the Nordic Council of Ministers that provides funding for Nordic research) launched the project SYSDIET (Systems Biology in Controlled Dietary Interventions and Cohort Studies) within the Nordic Centre of Excellence programme on Food, Nutrition and Health [115]. The overall aim of the SYSDIET project was to identify novel ways in which healthy Nordic foods and diets could increase health and prevent diseases associated with metabolic syndrome. Twelve research groups, representing all five Nordic countries, were involved in SYSDIET.

The multi-centre SYSDIET intervention study included six centres, in Aarhus (Denmark), Kuopio and Oulu (Finland), Lund and Uppsala (Sweden) and Reykjavik (Iceland). The objective of the study was to investigate whether an isocaloric healthy Nordic diet, based on the NNR, could help bring about improvements in insulin sensitivity, blood lipids, blood pressure and inflammatory biomarkers in subjects with metabolic syndrome. Originally, 213 subjects were enrolled in the study, but 13 of them dropped out before the intervention started. Thus, 200 subjects started the intervention, 104 in the Nordic diet group and 96 in the control diet group (Table 1). The inclusion criteria were age 30-65 years, BMI 27-38 kg/m^2 and two other criteria of the metabolic syndrome as identified by the International Diabetes Federation [19]; i.e., elevated triglycerides, low HDL, hypertension or elevated blood glucose. In all, 166 subjects completed the study: 96 in the Nordic diet group and 70 in the control diet group. Data from 11 subjects were excluded from the calculations due to an increase in weight exceeding 4 kg.

The study was a randomised controlled parallel study carried out over 18 or 24 weeks with a 4-week run-in period before the start. In Lund and Kuopio the study continued for 24 weeks, while in Aarhus, Oulu, Reykjavik and Uppsala the duration was 18 weeks. During the run-in period, the subjects were instructed to maintain their habitual diet, but to cease taking any fish or vegetable oil supplements and foods with added plant-sterols. The subjects were randomly assigned to a healthy Nordic diet or a control diet after the run-in period, and blood samples and 24-h urine samples were collected at the start, and on three occasions during the intervention. At additional visits, body weight and blood pressure were measured, and diet counselling was provided by a clinical nutritionist or dietician.

The main differences between the Nordic diet and the control diet were higher dietary fibre intake, limited sucrose intake, higher fruit, berry and vegetable intake and a lower fat intake, with focus on reduced saturated fatty acids (SFA) and increased monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). Some key food items, such as bread, cereals, spreadable margarines and oils, were provided in both diet groups to ensure compliance. Important key food items in the Nordic diet were berries, fish, spreadable margarine/oil and whole grain bread, cereals, etc. The control diet was based on the habitual diets in the Nordic countries. To monitor diet compliance, food records were kept during four consecutive days (including one weekend day) during the run-in period and on three occasions during the intervention. A daily food intake record of some of the key food items was also used regularly to check the subjects' adherence to the diet. In this thesis, the methodological part in Lund is reported, whereas the results are reported for all centres included. This study is presented in Paper V.

3.4 METHODS

3.4.1 Biochemical analyses

Plasma glucose was analysed with a glucose-oxidase-based method and insulin, and high-MW adiponectin using an enzyme-linked immunosorbent assay (ELISA). Cytokines were measured in blood serum using a Bio-Plex instrument (the intervention study) or ELISA (the SYSDIET study). The score from the homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as (fasting insulin × fasting glucose)/22.5 [116], were a value ≥2.5 indicates insulin resistance. The oral glucose tolerance test (OGTT) was performed by drawing blood after overnight fasting and 30 and 120 min after drinking 75 g *D*-glucose dissolved in 300 mL water.

To evaluate insulin sensitivity from the OGTT, the Matsuda insulin sensitivity index [117, 118] was calculated (Eq. 1), where a value ≤2.5 indicates insulin resistance.

$$\frac{10{,}000}{\sqrt{glu_0 \cdot ins_0 \cdot glu_{30} \cdot ins_{30} \cdot glu_{120} \cdot ins_{120}}} \qquad \text{(Eq. 1)}$$

The glucose and insulin incremental area under the curve (AUC) were calculated geometrically using the trapezoid rule [119, 120]. Accredited analyses of triglycerides, TC, HDL-C, ApoA1 and B, γ-glutamyltransferase, alanine-aminotransferase, alkaline-phosphatase, fibrinogen and CRP were performed at Skåne University Hospital, Sweden.

3.4.2 Non-targeted metabolomic profiling

Non-targeted metabolomic profiling was performed on plasma and 24-h urine samples collected in the intervention study.

Sampling, storage and sample preparation

Vacutainers from the same batch were used to collect blood plasma for the metabolomic analysis, to avoid any possible variation resulting from the use of vacutainers from differences batches [121]. The proteins in the plasma samples must be removed before analytical analysis, and the method most commonly used is precipitation of the protein using methanol [122, 123], followed by repeated centrifugation and washing steps. In the present work, filtration was used instead as this has been shown to improve the metabolite coverage [124]. The deproteinised plasma was evaporated to dryness and stored at -80°C. Milli-Q water was added to each sample before analysis with ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS). During the identification process, some plasma samples were hydrolysed by β-glucuronidase and sulphatase to release and confirm any glucuronic acid and sulphate conjugates from the metabolites of interest.

In this work, 24-h urine samples were collected to obtain better coverage of the excreted metabolites, since differences have been observed between spot samples collected at 2-h intervals, and to limit intra-individual variation due to diurnal variation [121]. During collection, the urine was kept cool to minimise changes in the metabolome, and was then stored at -80°C prior to analysis. The urine samples were thawed on ice, centrifuged, and Milli-Q water was added prior to analysis.

Both plasma and urine samples were analysed in duplicate. Samples were randomised using a computerised list for each plate and for each batch. However, all samples from one subject were analysed in the same batch to avoid batch variation effects on the results from one subject.

Data acquisition

Several separation methods are used in metabolomics, such as gas chromatography, capillary electrophoresis and liquid chromatography. In the present work, UPLC was used, as this method provides better separation, together with reduced analysis time, compared with high-performance liquid chromatography [125]. In metabolomics, it is not possible to detect all the metabolites in a complex sample such as blood or urine, using only a separation technique, and mass spectrometry (MS) or a similar technique must be included. MS determines the mass-to-charge ratio (*m/z*) of metabolites or compounds that are charged by an ion source. (These are referred to as features until they have been identified). In this work, QTOF/MS with electrospray ionisation (ESI), operating in both positive and negative ionisation modes, was used. Matrix effects, which could cause a reduction in sensitivity and discrimination, due to reduced ion intensity of some features, or sometimes even a total loss of some features, are of major concern in ESI. In complex samples such as blood and urine, some compounds are more efficiently ionised than others, which may suppress the ionisation of others. In positive ESI (ESI+) mode, more adducts are formed, such as sodium and potassium adducts. Negative ESI (ESI-) is advantageous as less adducts are formed, and there are fewer matrix problems than in ESI+. The normal settings used in QTOF/MS in this study provided relatively soft ionisation, with only limited fragmentation. In the identification process, some plasma samples were re-analysed with MS^E, a method that will increase the fragmentation by increasing the collision energy from 10 to 50 eV in each scan. Some specific features in plasma were further analysed by MS/MS, using the same parameters as in the normal run, apart from a stepwise increase in the voltage from 10 to 50 eV. The difference between MS^E and MS/MS is that in MS/MS a specific *m/z* ratio is chosen in a specified retention time (RT) interval, while in MS^E all *m/z* ratios at all RTs are included, i.e. all the compounds will become fragmented. MS/MS analysis confirms that all the fragments obtained originate from the parent ion (i.e. the chosen *m/z* ratio).

Data preprocessing and analysis

The raw mass spectrum is often called the continuum mass spectrum, and is usually converted to centroid data by determining the position and the height or area of the peaks, usually normalised to the highest peak in the spectrum. The raw LC-MS centroid data were extracted and aligned in MarkerLynx™ v. 4.1 (Waters, Milford, MA, USA). Each

feature, assigned from the data processing in MarkerLynx is defined in terms of RT and *m/z*. The intensity of each feature reflects the semi-quantitative amount of that marker in each sample. Further noise reduction was then applied to the MarkerLynx features, as described by Bijlsma et al. [126]. An exploratory analysis using principal component analysis was used to evaluate any discrepancies between batches (for plasma samples), the time of sampling, gender and diet. No discrepancies were observed, and therefore a univariate statistical approach was chosen.

Mean values of intensity were calculated for each plasma feature and subject at the four different sampling times during the HF and LF diets. The final intensity values for each feature were subtracted from the initial values. For the urine samples, the final mean intensity of each feature was determined for the HF diet and the LF diet. The changes in values shown by those on the HF diet (ΔHF) were compared with the corresponding changes shown by those on the LF diet (ΔLF) using a two-tailed paired samples *t* test. A *p*-value <0.01 was used to define a significant difference, which means that in the dataset tested, 1% of the significant variables could be false positives by chance. This is a problem in *–omics* since large datasets, i.e. a large number of variables obtained from a small number of samples, will give many false positives. Therefore, *q*-values of the false discovery rate were calculated [127] (Bioinformatics Toolbox™, Mathworks). The *q*-values were used to test the significant *p*-values to determine the number of false positives among the significant variables. Using a value of $q < 0.05$ as a cut-off means that 5% of the significant variables will be truly null variables, i.e. false positives. Features with $p < 0.01$ and $q < 0.05$ were considered significant in the metabolomic datasets.

Marker identification

The detected features processed in MarkerLynx were identified using the four levels of certainty defined by Sumner et al. [128], with slight modifications. The identification levels for the urine samples differed somewhat from those in plasma as the urine samples were not analysed by MS^E and MS/MS due to limits on time;

Level I *Identified marker:* when confirmed by an authentic standard run separately or spiked in a sample (Paper III), or confirmed by an authentic standard from an in-house database based on RT and mass similarity (Paper IV).

Level II *Putatively identified marker:* based on elemental composition and/or identified via literature and/or public databases and/or MassFragment (Waters) analysis using MS^E and MS/MS spectra (MassFragment analysis of MS^E and MS/MS spectra was not possible for urine).

Level III *Putatively characterized compound class:* based on elemental composition and similarity with a certain metabolite class in public databases and/or MassFragment analysis of MSE and MS/MS spectra (MassFragment analysis of MS^E and MS/MS spectra was not possible for urine).

Level IV *Unknown compound.*

Searches were made for significantly increased or decreased features in the Human Metabolome Database (http://www.hmdb.ca/) [(129)], Metlin (http://metlin.scripps.edu/) [(130)], Phenol Explorer (http://phenol-explorer.eu/) [(131)] and an in-house database based on authentic standards for potential identity. When an authentic standard was not available for the compound of interest, a molfile was retrieved from the databases or produced in ACD/ChemSketch (http://www.acdlabs.com/), and the fragmentation pattern of any candidate compound was analysed in MassFragment. The molfile format includes information on the atoms, bonds, connectivity and coordinates of a molecule. MassFragment compares the fragments in the MS/MS spectra for the feature of interest with the structure in the molfile of the suggested compound, and scores the different fragmentation candidates to identify the most likely fragments. Elemental composition analysis was used in MassLynx™ to assign each parent ion and each fragment to a probable molecular formula. For the urine features, a correlation analysis [(132)] was applied to all detected features in MarkerLynx. This method enhances the identification process without MS/MS fragmentation. In some cases, significant features originated from the same metabolite but as adducts or fragments of the parent ion; in other cases correlating features that were discarded during the data extraction process could be added as fragment/adduct information to the significant features.

4 RESULTS

4.1 NUTRIENT INTAKE AND DIETARY COMPLIANCE

In the meal study, the breakfasts were served at the study centre, and therefore compliance was monitored on-site. The data from one subject had to be excluded from the calculations, due to illness and poor compliance. Compliance with the diets in the intervention study was satisfactory: 96.9% in the HF diet and 95.0% in the LF diet. A significantly higher total dietary fibre intake was observed during the HF diet, together with a significant decrease in carbohydrate intake. The intake of dietary fibre during the HF diet was 48 g, compared to 30 g in the LF diet. The test food products contributed 30.2 g fibre in the HF diet and 10.5 g fibre in the LF diet, of the total dietary fibre intake per day. The soluble fibre in the test food products of the HF diet was estimated to consist of 2.7 g β-glucan, 1.5 g arabinoxylan and 2.4 g pectin, per day. Estimates of the specific soluble dietary fibre were calculated from the reported intake of test food products in the HF diet, based on reported values [27, 60, 114] and, the fact that about 1/3 of the arabinoxylan in rye bran is soluble [27]. The total energy intake was on average 10 MJ per day, and the protein intake was between 16 and18 E% in both diet groups. The intake of lipids in both groups was about 29 E%, including 11 E% MUFA, 5 E% PUFA and 10 E% SFA.

In the SYSDIET study, there was no significant difference in the energy intake between the different diet groups. The energy and nutrient intake for the respective diet groups are presented in Table 3. Significantly higher intakes of dietary fibre, carbohydrates, protein, PUFA, α-linolenic acid, β-carotene, vitamins C, D and E, potassium and magnesium were observed in the Nordic diet group. The intake of dietary fibre increased to 35 g per day in those on the Nordic diet, compared to 16 g in the control diet group. Additionally, significant increases in plasma alkylresorcinols were observed for the Nordic diet compared to the control diet [133]. Significant concurrent decreases in total intake of fat, SFA, cholesterol, salt and sodium were observed in those on the Nordic diet, compared to the control group.

Table 3. Energy and nutrient intake (mean and SD) calculated from the 4-day food records during the SYSDIET study

	Before the Nordic diet	During the Nordic diet[1]	Goals in the Nordic diet	Before the control diet	During the control diet[1]	Goals in the control diet	*p* (between diets)
Energy (MJ)	8.5(2.0)	8.6 (1.8)	Isocaloric	8.4 (2.1)	8.5 (1.9)	Isocaloric	0.72
Carbohydrates (E%)	45.7 (6.0)	46.8 (6.4)	45-52 E%	46.1 (6.7)	44.6 (6.5)	45-47 E%	0.002
Sucrose (g)	39.6 (23.6)	34.7 (18.4)	≤50 g	38.7 (17.6)	35.7 (19.8)	No restrictions	0.40
Protein (E%)	16.6 (2.8)	17.5 (3.2)	18-20 E%	16.9 (2.3)	16.2 (2.3)	18-20 E%	<0.001
Fat (E%)	33.2 (6.3)	31.7 (5.3)	30-35 E%	32.9 (5.9)	35.2 (5.2)	35 E%	<0.001
SFA (E%)	12.6 (3.3)	10.1 (2.5)	< 10 E%	13.1 (3.1)	14.8 (2.9)	15 E%	<0.001
MUFA (E%)	11.3 (2.7)	11.6 (2.5)	20-24 E%[3]	11.1 (2.3)	12.1 (2.2)	15 E%	0.058
PUFA (E%)	4.9 (1.7)	6.8 (2.0)		4.6 (1.4)	4.4 (1.2)	5 E%	<0.001
Dietary fibre (g)	22.4 (7.1)	34.7 (10.2)	≥35 g	20.3 (6.1)	15.9 (4.5)	15-20 g	<0.001
Salt[2] (g)	7.4 (2.2)	6.5 (2.2)	6-7 g	7.2 (2.5)	7.1 (2.3)	≤10 g	0.009

MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, SFA = saturated fatty acids

[1]The values represent the mean values (and SD) from the three food records during the intervention

[2]Sodium intake from food was converted to g salt by multiplying by 2.54

[3]MUFA and PUFA should account for at least 2/3 of the total fat intake

4.2 BLOOD GLUCOSE, INSULIN AND LIPIDS

In the meal study, all the breakfast meals with added fibre tended to reduce peak glucose concentration in the blood (at 30 min), compared to the control meal; although this reduction was only statistically significant for rye bran (Figure 8). Additionally, subjects eating the breakfasts with rye bran and sugar beet fibre showed significantly lower peak glucose response than those eating the meal containing oat powder. No significant differences in glucose AUC were seen between any of the meals. All breakfasts, except that containing oat powder, tended to lead to a reduction in glucose AUC, compared to the control meal, although this was not statistically significant. The women showed a lower incremental glucose response than men after the intake of all the fibre-amended meals (Figure 8). No significant differences in peak insulin or insulin AUC were observed between any of the fibre-amended breakfasts and the control meal. However, significantly lower insulin response was observed 60 min after the ingestion of meals containing rye bran, sugar beet fibre and the mixture, compared to the oat powder meal. The largest insulin AUC was observed following the oat powder meal, and subjects eating the rye bran meal and the sugar beet fibre meal showed significantly lower values of insulin AUC than those eating the oat powder meal. All the fibre-amended breakfasts tended to lead to an *increase* in the postprandial triglyceride response, although this was only significant for the oat powder meal and the meal containing the mixture of fibre, after 60 min.

In the intervention study, no significant differences were observed between the diets regarding fasting glucose, insulin or blood lipid levels, except for a small, but statistically significant, *reduction* in TC and LDL-C in those on the LF diet.

During the SYSDIET study, no significant differences were found in variables related to glucose metabolism. Significant reductions in non-HDL-C, the LDL-C/HDL-C ratio and the ApoB/ApoA1 ratio were observed in the Nordic diet group, compared to the control diet group. Moreover, tendencies were seen towards reductions in LDL-C and ApoB, together with an increase in HDL-C, in the Nordic diet group, compared to the control diet group, although these were not statistically significant. It appears that the duration of the study influenced the outcome of some variables (Figure 9).

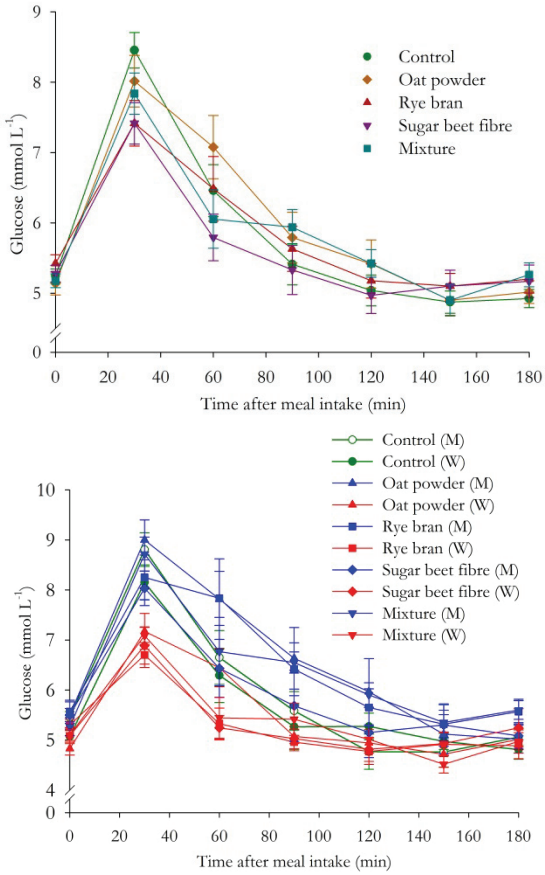


Figure 8. Blood glucose responses in the meal study: (above) after ingestion of breakfasts containing different kinds of dietary fibre, and (below) the responses in men (M; blue) and women (W; red) separately. Data pertaining to control meals are shown in green.

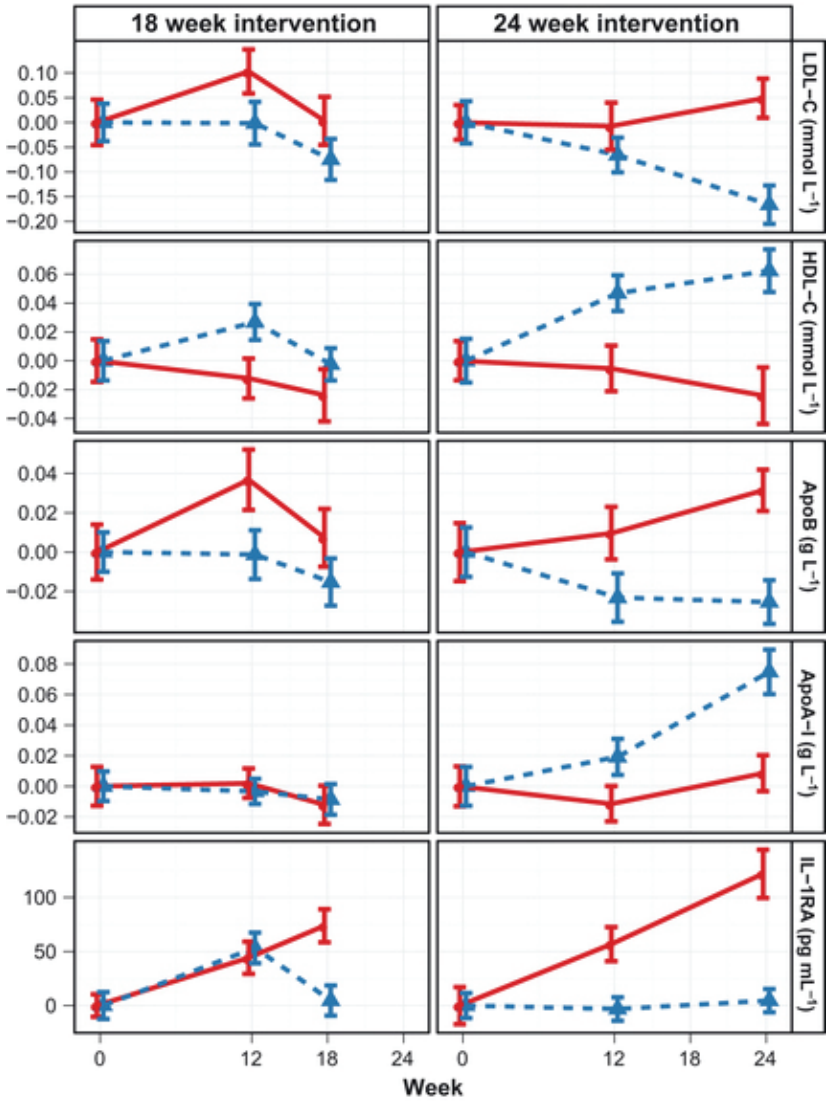


Figure 9. Changes in LDL-C, HDL-C, ApoB, ApoA1 and IL-1RA (IL-1 receptor antagonist) during the SYSDIET study in subjects on the Nordic diet (dotted line) and the control diet (solid line). The results from the studies with a duration of 18 weeks (left) and 24 weeks (right) are shown separately.

4.3 INFLAMMATORY MARKERS

There was a significant reduction in CRP, of 21%, in the subjects on the HF diet compared to those on the LF diet, during the intervention study. Moreover, a significant decrease in fibrinogen was seen in the group of subjects on the HF diet. No other changes were observed in the circulating cytokines analysed, apart from a tendency towards a decrease in IL-17A in the HF diet group, with a concomitant increase in the LF diet group.

During the SYSDIET study, a significant increase in IL-1 receptor antagonist (IL-1RA) of 27% was observed in the control diet group, while the level remained unchanged in the Nordic diet group (Figure 9).

4.4 NON-TARGETED METABOLOMIC PROFILING

After the preprocessing steps, the number of detected features in plasma samples from the intervention study was 3,035 using ESI+ and 1,818 using ESI- (Paper III). Of these, 6 features with ESI+ and 14 features with ESI- showed significant differences after the HF diet compared to the LF diet. Several of these significant features were glucuronide or sulphate conjugates, observed in the mass spectra as a neutral loss of 176 Da and 80 Da, respectively (Figure 10). After hydrolysis of some of the samples with β-glucuronidase, a decrease in intensity was observed. This confirmed that the glucuronide was present in the feature.

A total of 2,579 features in ESI+ and 1,927 features in ESI- were found in 24-h urine samples from the intervention study, and of these, 135 features in ESI+ and 173 features in ESI- were significantly different between the HF and the LF diets (Paper IV). To reduce the number of significant features, a fold change cut-off value was set at 2.5, which narrowed the search to only include features that were increased at least 2.5 times after the HF diet compared to the LF diet. The number of remaining features was reduced to 21 in ESI+ and 65 in ESI-, some of them being adducts or fragments of the same metabolite, giving 35 metabolites detected as significantly increased by the intake of the HF diet.

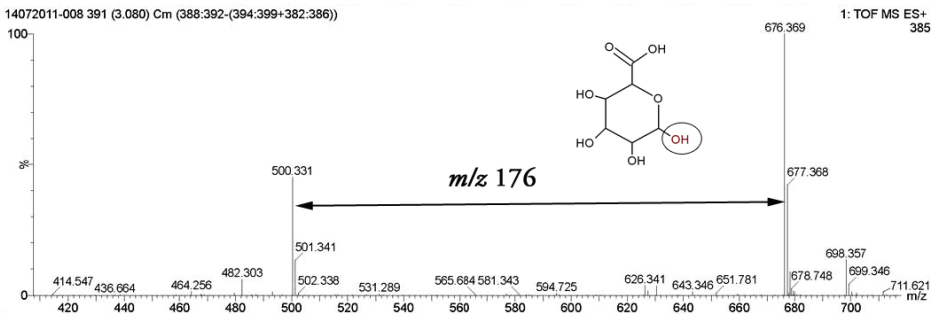


Figure 10. An example of the neutral loss of glucuronide observed in the mass spectrum for the feature identified at level III as belonging to the steroid compound class as a glucuronide conjugate. Glucuronic acid has a mass of 194 Da, but when glucuronide conjugation of the compound occurs, one water molecule is eliminated (the marked hydroxy group) and therefore the neutral loss is 176 instead of 194.

From the plasma and urine samples in the intervention study, 5 metabolites could be identified at level I; three being specific to urine, whereas the remaining two were observed in both biofluids (Table 4). The metabolites identified at level I were confirmed by similarity in RT and *m/z* with an authentic standard, used to spike the plasma samples. Eleven features were identified at level II; however, one of them was identified only by mass similarity with a metabolite in public databases. Due to time constraints, no MS/MS analysis was performed on the urine samples and, therefore, no comparison of mass fragmentation patterns was possible. In plasma, the level II identification was based on similarity in fragmentation patterns obtained from the MS/MS analysis and with fragments from the *in silico* fragmentation of the feature of interest.

2,6-DHBA has not previously been described in relation to dietary fibre intake, and was found to increase in both plasma and urine after the intake of the HF diet (Figure 11). It is unknown from which of the fibre source(s) it originates. Enterolactone glucuronide is a breakdown product of lignans, and this metabolite could, therefore, originate from both rye and oat since both these fibre sources contain lignans. An oat-related saponin metabolite was identified in both plasma and urine as the furospirostanol nuatigenin that was hydroxylated and conjugated to glucuronide. The didehydrodeoxy- or oxo-nuatigenin form could only be observed in plasma. Two additional metabolites specific to oat were tentatively identified in urine, 26-desglucoavenacoside A and avenestergenin A2. However, the identity of these two metabolites is less certain since their identification is based only on mass similarity with metabolites in public databases; their identity therefore remains on level III, and they were classified as belonging to the saponin compound class. Avenestergenin is the backbone of avenacins, which are saponins that have only been reported in oat roots. Therefore, it is less likely that avenestergenin will be present in the

urine. Saponins should preferably be extracted from oats and analysed by LC-MS to determine the presence of different avenacins, followed by MS/MS to obtain mass fragmentation patterns.

Most of the urine metabolites were related to the intake of rye. DHPPA (as the sulphate and glucuronide conjugates) and 3,5-dihydroxyhydrocinnamic acid sulphate are products of the endogenous metabolism of alkylresorcinols. Several benzoxazinoid metabolites were identified, often as conjugates to sulphate or glucuronic acid. DIBOA sulphate has been observed previously in human urine following the intake of rye bread [78]. The presence of HBOA glycoside and glucuronide has also been confirmed in rye [91], and in human plasma [134] and urine [135] following rye bread intake. BOA has previously been reported in human plasma after rye intake [134] and by characterisation of rye [91]. 2-aminophenol sulphate is a degradation product of BOA [136] and was found to be significantly increased after the HF diet in both plasma (Figure 11) and urine samples in the intervention study. The non-conjugated form was also significantly increased in urine. 2-aminophenol sulphate has been detected previously in urine after the intake of rye [78], but this is the first time increased levels have been detected in human plasma following a HF diet. The phenylacetamides HPAA (*N*-(2-hydroxyphenyl)acetamide) and HHPAA (2-hydroxy-*N*-(2-hydroxyphenyl)acetamide) are suggested to be breakdown products of DIBOA [137, 138]. These phenylacetamides were not observed in plasma, which is probably due to their relatively short half-lives, as shown in 24-h fasting plasma samples following an intake of rye bread [134].

Additional metabolites, identified at level II in the urine samples are presented in Paper IV, however their identity are less certain. The metabolites identified at III and IV in the plasma and urine samples can be found in Papers III and IV, respectively.

Table 4. Markers identified in plasma (P) and urine (U) that were significantly increased after the intake of the HF diet in the intervention study

	Biofluid	Fibre source	ID level
2,6-DHBA	P, U	Unknown	I
26-desglucoavenacoside A	U	Oat	III[1]
2-aminophenol	U	Rye	II[1]
2-aminophenol sulphate	P, U	Rye	I
3,5-dihydroxyhydrocinnamic acid sulphate	U	Rye	II
Avenestergenin A	U	Oat	III[1]
BOA	U	Rye	II
DHPPA glucuronide	U	Rye	I
DHPPA sulphate	U	Rye	I
DIBOA sulphate	U	Rye	II
Didehydrodeoxy- or oxo-nuatigenin	P	Oat	II
Enterolactone glucuronide	U	Oat, rye	I
HBOA glucuronide	U	Rye	II
HBOA glycoside	U	Rye	II
HHPAA glucuronide	U	Rye	II
HHPAA sulphate	U	Rye	II
HPAA sulphate	U	Rye	II
Hydroxylated and glucuronidated nuatigenin	P, U	Oat	II

BOA = 1,3-benzoxazol-2-one, DHBA = dihydroxybenzoic acid, DHPPA = 3-(3,5-dihydroxyphenyl)-1-propanoic acid, DIBOA = 2,4-dihydroxy-1,4-benzoxazin-3-one, HBOA = hydroxy-1,4-benzoxazin-3-one, HHPAA = 2-hydroxy-*N*-(2-hydroxyphenyl)acetamide, HPAA = *N*-(2-hydroxyphenyl)acetamide

[1]Identification based only on similarity in mass with the suggested metabolite from public databases

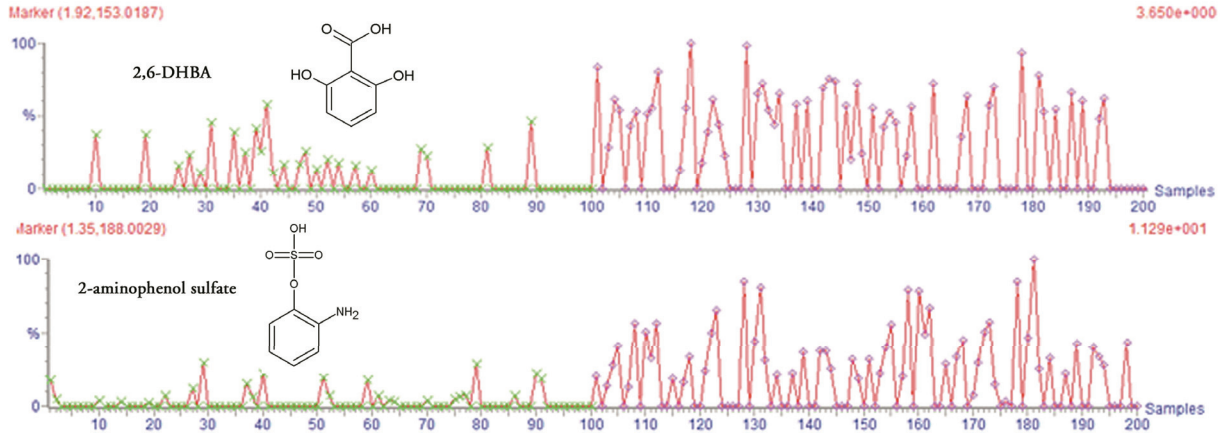


Figure 11. Relative intensity of 2,6-DHBA (upper trace) and 2-aminophenol sulphate (lower trace) in plasma samples (green crosses = samples from subjects on the LF diet, purple squares = samples from subjects on the HF diet).

5 DISCUSSION

5.1 THE EFFECTS OF DIETARY FIBRE ON HUMAN HEALTH

5.1.1 Glucose and insulin metabolism

The aim of the meal study was to investigate how 5 g of soluble dietary fibre from different sources influenced the postprandial glucose, insulin and triglyceride response in healthy subjects. The meal study was conducted as a challenge test, but instead of using 75 g glucose as in the OGTT, we aimed to reach 75 g of total available carbohydrates in each meal. This resulted in the addition of different amounts of carbohydrates, as each fibre source contained different levels of carbohydrates. White wheat bread and dextrose were used to adjust the carbohydrate levels. Dextrose is a fine powder and will be absorbed relatively quickly, while white bread is more solid and will take longer to absorb. No carbohydrates were added to the oat powder meal since oat powder contains a high amount of carbohydrates, and the whole meal was ingested as a liquid. The high carbohydrate level also resulted in a lower amount of soluble fibre (2.7 g) and total dietary fibre (3.3 g), and this meal resulted in higher postprandial glucose and insulin levels than expected, sometimes even higher than the control meal.

The rye bran meal caused a significantly reduced glucose peak compared to the control meal, but no effect was seen on postprandial insulin. The total dietary fibre content in the rye bran meal was 13 g, of which 1.7 g was soluble. This finding is in contrast to two other studies [107, 139] that included subjects with similar characteristics and a similar total dietary fibre content from rye bread. In these previous studies, the postprandial insulin levels were significantly decreased, but not the postprandial glucose response [107, 139]. The sugar beet fibre meal decreased the postprandial glucose and insulin responses, although the decrease did not reach statistical significance. A previous postprandial study showed improvements in both glucose and insulin metabolism in healthy men when including 5 g total dietary fibre from sugar beet (3.7 g soluble) [52]. In the meal study in the present work, the amount of total and soluble dietary fibre was twice as high, but no significant effects on glucose and insulin could be detected.

The mixed meal provided 5 g of soluble fibre, with equal amounts from each of the three fibre sources (1.7 g/fibre source). This meal resulted in the highest amount of total dietary fibre (18 g) with only small amounts of white bread added. The effect of mixing oat powder in this meal seemed to reduce the effects of rye bran and sugar beet fibre. The reason for the counteracting effect of oat powder could be the high amount of carbohydrates, which is the reason for the small amount of bread added. A solid matrix

will reduce the rate of digestion, and hence the glucose uptake, compared to a liquid matrix. Another reason for the counteracting effect could be the amino acid composition of the oat powder. Oats contain relatively high levels of protein, especially rich in BCAAs, compared to the other fibre sources used in this study. The BCAAs could have stimulated the secretion of insulin, as has been shown previously [140]. In a transcriptomic sub-study of the meal study [102], an oat bran meal was found to significantly reduce the insulin response. Oat bran and oat powder should contain similar amino acids, but the insulin response differed between the meals. Therefore, it can be hypothesized that although the amino acid composition could influence the postprandial insulin response following the oat-containing meals, the impact of the total amount of dietary fibre as well as the difference in food matrix, probably explain the difference in the postprandial response between the different oat meals.

The oat bran meal in the sub-study of the meal study contained 5 g soluble fibre (12.6 g total dietary fibre), and had a tendency to lower the postprandial glucose response, although statistical significance was not reached [102]. However, the oat bran meal had a significant lowering effect on insulin and insulin AUC. Significant decreases in postprandial glucose and insulin levels have been reported previously following the ingestion of 5 g β-glucans by hypercholesterolaemic subjects [48] and 4 g β-glucans by healthy subjects [45]. Therefore, it was decided to use oat bran instead of oat powder when testing a mixture of different kinds of fibre in the intervention study. The aim of the HF test food products was to provide an intake of >30 g/day, whereas the LF test food products was intended to give less than 10-15 g/day. However, the LF diet group also had a rather high total fibre intake during the intervention, averaging 30 g/day. The HF diet group had a greater intake of dietary fibre (48 g/day on average), however, glucose and insulin levels remained unchanged in this group. In the LF diet, 10 g of the dietary fibre originated from rice and the endosperm of wheat, whereas the remaining 20 g could have come from rye, wheat or oats, since these cereals are common food staples in the Nordic countries. The subjects had normal fasting glucose and insulin values. In a previous study of hypercholesterolaemic subjects, significant improvements in fasting insulin and HOMA-IR were observed after 6 weeks on a Nordic diet providing 54 g total dietary fibre, compared to a control diet containing 30 g of total dietary fibre [141]. These dietary fibre intakes are comparable to those in the present intervention study, but the improvements in insulin sensitivity became non-significant when correcting for the significant weight reduction in the Nordic diet group; an effect probably caused by the significantly lower total energy intake in that group. In the intervention study in the present work, the subjects' weight and energy intake remained unchanged throughout the study. It was also concluded in a review of the beneficial effects of β-glucans on metabolic syndrome [44] that the postprandial effect on glucose and insulin in hypertensive and hypercholesterolaemic subjects was not so apparent as in type 2 diabetic subjects, probably due to relatively normal blood glucose levels in these populations, compared to diabetic subjects.

An OGTT that challenges the system might have been needed to detect any systemic beneficial effects in the intervention study. An OGTT was included in the SYSDIET study since two of the main outcomes were glucose tolerance and insulin sensitivity in subjects with metabolic syndrome. However, no changes, within or between groups, were observed in glucose and insulin metabolism at the end of the study. The ratio of the plasma alkylresorcinols C17:0/C21:0 was recently shown to be inversely correlated with fasting insulin levels, and positively correlated to the Matsuda insulin sensitivity index [142]. This ratio is used to distinguish between whole grain rye and wheat intake, as the ratio is 0.1 for wheat and 1.0 for rye [82]. From this it could be concluded that the intake of rye seemed to be correlated to increased insulin sensitivity in the subjects with metabolic syndrome.

Comparison with health claims regarding glycaemic response

There is an EU-approved health claim that an intake of 4 g of β-glucans for each 30 g available carbohydrates per meal could decrease the postprandial glycaemic response [35]. In the meal study, only 3 g of β-glucans per 75 g of carbohydrates was used in the oat powder meal (Paper I). This could be an additional reason for the lack of postprandial reductions in glucose and insulin responses after the oat powder meal. However, in a review on β-glucans from oats and barley [143] investigating their ability to lower postprandial glucose, it was concluded that in studies with ≥4 g β-glucans, 76% of the treatments significantly reduced the postprandial glucose response, regardless of the carbohydrate content. In the intervention study, the two oat bran beverages were estimated to provide almost 3 g β-glucans per 32 g carbohydrates (Paper II), and this is more in line with the dose in the EU health claim. However, the postprandial glucose and insulin levels were not measured, only the fasting levels. It could be useful to include a postprandial challenge in future studies, since the long-term effect of decreased postprandial glucose and insulin responses might be beneficial regarding glucose tolerance and insulin sensitivity. However, the OGTT in the SYSDIET study did not show any improvements in glucose levels or insulin sensitivity in subjects with metabolic syndrome that had consumed the Nordic diet (Paper V). The Nordic diet provided, on average, 35 g total dietary fibre daily, but no data were available on the content of β-glucans, or other specific fibre sources in the diet. However, the subjects were encouraged to increase their intake of oats and barley.

A health claim regarding arabinoxylan has also been EU-approved, in which at least 8 g arabinoxylan-rich fibre (at least 60% arabinoxylan by weight; i.e. ≥4.8 g arabinoxylans) per 100 g of available carbohydrates is believed to bring about a decrease in postprandial glycaemic response [36]. This health claim applies to wheat but not rye. The estimated arabinoxylan intake in the meal study was lower, 0.9 g per 75 g carbohydrates (Paper I). However, we still observed a significantly reduced postprandial peak glucose response after

30 minutes compared with the control meal, probably due to the relatively high content of total dietary fibre (13 g) in the meal.

A health claim regarding the ability of pectins to reduce postprandial glycaemic response is approved when 10 g of pectin is incorporated into a meal [41]. The breakfast meal with sugar beet fibre provided 3.7 g pectin in 75 g of carbohydrates, and there were tendencies towards reductions in the glucose peak and AUC, although these were not statistically significant. In the intervention study, the estimated daily amounts of pectin were 1.5 g in the ready-made meals and 0.9 g in the bread. These food products also contained arabinoxylan (1.1 g and 0.4 g in the bread and the ready-made meals, respectively). The combination of several fibre sources in one meal were thought to induce an additive effect. Despite this, no effects were observed on fasting glucose or insulin; however, the postprandial responses were not measured.

5.1.2 Lipid and lipoprotein metabolism

In the meal study, a postprandial increase was seen in the triglycerides after all fibre-rich meals, whereas a decrease in triglycerides was seen following the control meal. It has been suggested that the decrease in rate of digestion and absorption of nutrients due to the viscosity of dietary fibre causes slower clearance of glucose and triglycerides as a result of lower insulin levels [144]. However, this hypothesis does not explain why the triglyceride levels increased after the oat powder meal; this meal led to the highest postprandial insulin response of all the meals, including the control meal.

No significant changes in triglycerides or lipoproteins were observed between the HF and LF diets in the intervention study. This could be due to the subjects' relatively high dietary fibre intake during the LF diet period (30 g total dietary fibre per day). The subjects included in the SYSDIET study had lower average values of TC and LDL-C than the subjects in the intervention study; however, subjects using statins were included in the SYSDIET study (18% in the Nordic diet group, 29% in the control group) but not in the intervention study. The subjects in the SYSDIET study were overweight or obese with at least two more components of the metabolic syndrome. Significant reductions were observed in non-HDL-C, and the LDL-C/HDL-C and ApoB/ApoA1ratios in the Nordic diet group, compared with the control group. LDL-C had a tendency to decrease, whereas HDL-C tended to increase in the Nordic diet group, although not statistically significantly. Two previous studies have shown significant reductions in TC and LDL-C in healthy, moderately hypercholesterolaemic subjects after the intake of 5 g β-glucans for 3 and 5 weeks [48, 145]. However, the subjects included in these two previous studies had higher baseline values of TC (6.2 and 7.5 mmol/L) than the present subjects (5.7 and 5.9 mmol/L), possibly explaining why no significant reductions were observed in the intervention study in the present work. The NORDIET study [141] investigated the health

effects of a Nordic diet on LDL-C, blood pressure and insulin sensitivity. The study had a parallel design and the subjects had similar age and BMI to those in the intervention study. Moreover, the dietary fibre intake was also comparable: 53 g in the Nordic diet versus 30 g in the control diet. Significant reductions in TC, LDL-C, HDL-C, LDL-C/HDL-C and the ApoB/ApoA1 ratio were observed in the NORDIET study after 6 weeks, which remained significant even after adjusting for the weight reduction of 4%. The subjects' baseline values were similar to the present ones, although the HOMA-IR was even lower. Two variables that differed compared to the present values at baseline were TC (NORDIET: 6.2 and 6.4 mmol/L; the intervention study: 5.7 and 5.9 mmol/L) and LDL-C (NORDIET: 4.0 and 4.2 mmol/L; the intervention study: 3.6 and 3.8 mmol/L). Similar results were also observed in a randomised cross-over study in which a diet containing barley products, beans and chickpeas was compared to a wheat-based control diet [146]. Both diets generated relatively high dietary fibre intakes (>40 g/day), and TC, LDL-C and HDL-C were significantly reduced. The barley/beans/chickpeas diet led to greater reductions in TC and LDL-C , together with a significant reduction in ApoB. Since the dietary fibre intake was similar between the diets, this shows that the source of dietary fibre determines its effect. Some baseline values were higher in this study (TC: 6.37 and 6.42 mmol/L, LDL-C: 4.01 and 4.03 mmol/L) than in the present intervention study. However, the diet of the subjects in the study given barley/beans/chickpeas food products was controlled to a higher degree than in the present intervention study, and followed a 14-day rotating menu, which probably contributed to the beneficial results observed after the relatively short time of 4 weeks.

These results provide further evidence that in order to observe a cholesterol-lowering effect of a fibre-rich diet, the baseline values of TC and/or LDL-C must be relatively high, preferably LDL-C exceeding 4.3 mmol/L [3], together with a relatively low value of HDL-C. The subjects in the present intervention study had relatively high values of HDL-C, as indicated by the TC/HDL-C and LDL-C/HDL-C ratios. Therefore, it might be of value to consider HDL-C and LDL-C values as inclusion criteria when studying the cholesterol-lowering effects of dietary fibre.

Comparison with health claims regarding blood cholesterol

A health claim that 3 g β-glucan per day from oats can reduce the blood cholesterol level has been approved to be used in the EU [34]. This amount was used in the oat bran beverage in the intervention study, in which no reduction in blood cholesterol was observed. However, the MW of the β-glucans in the oat bran beverage was relatively low (about 100,000 g/mol [114]). It was also found that the TC and LDL-C were not lowered in another study using 4 g β-glucans per day during 5 weeks in which the MW of the β-glucans was reported to be 80,000 g/mol [147]. The MW of the β-glucans has been shown to be of importance when reducing LDL-C in a randomised parallel study [148]. This study included subjects with elevated LDL-C (3.7-3.9 mmol/L) divided into four

groups; 3 g high-MW β-glucans (2,210,000 g/mol), 3 g or 4 g medium-MW β-glucans (530,000 g/mol), and 4 g low-MW β-glucans (210,000 g/mol). After 4 weeks, a significant reduction in LDL-C, of 5-6%, was seen in all groups, except the low-MW group. The wide MW distribution in commercially available foods is mentioned in the positive health claim on β-glucans, although no clear recommendation is given regarding the MW in relation to cholesterol-lowering properties [34].

The estimated amount of pectin from sugar beet fibre in the intervention study was 2.4 g per day. This is only 1/3 of the dose recommended by the EU health claim (6 g pectin per day) to induce a cholesterol-lowering effect. Therefore, further investigations are required on ways of incorporating higher amounts of sugar beet fibre into food products without affecting their palatability. Moreover, the molecular structure of pectin differs between different sources. Seven different pectins were tested in a cross-over study: three citrus pectins with different degrees of methyl esterification (DE-0, DE-35, DE-70), two apple pectins (DE-35, DE-70), an orange pulp fibre (DE-70), and a low-MW pectin (DE-70) [149]. The dose was 15 g per day, incorporated into foods, for 4 weeks. The most effective pectins were found to be the apple pectin with DE-70, and the citrus pectins with DE-35 and DE-70, which significantly reduced LDL-C by 7-10%, and reduced levels of TC. Citrus pectin with DE-0, the orange pulp fibre pectin with DE-70, and the low-MW DE-70 pectin had no effect. A second cross-over study was performed in the same study, with three groups receiving either a cellulose control, citrus pectin DE-70, or high-MW citrus pectin DE-70, to investigate the impact of MW. The dose was 6 g pectin per day, and each intervention period was 3 weeks. Both citrus pectins lowered LDL-C to a similar level; however, the reduction was only statistically significant for the high-MW citrus pectin. Based on the results obtained in these studies, the authors concluded that the health claim of 6 g pectin per day could not be applied to all pectins. The claim should, therefore, include information on the characteristics of the pectins, such as MW and DE, to ensure a cholesterol-reducing effect.

5.1.3 Inflammatory response

The HF diet, containing 48 g fibre per day, significantly reduced CRP by 21%, from 1.9 g/L to 1.5 g/L, in the intervention study (Paper II). The clinical significance of this reduction can be debated, but the American Heart Association concluded that the relative risk of CVD is low when the level of CRP is below1 mg/L, average for CRP levels of 1-3 mg/L and high when the CRP is above 3 mg/L [150]. Also, the small, but significant, reduction seen in fibrinogen following the HF diet could be indicative of a combined CVD protective effect. Similar results have been reported in a randomised controlled cross-over study on an intake of 28 g total dietary fibre per day in a HF group, eating according to the DASH diet [151], and in another study on a daily intake of 26 g total dietary fibre in a psyllium-supplemented group [152], showing reductions in CRP of 13.7%

and 18.1%, respectively. Pro-inflammatory cytokines such as IL-1, IL-6 and IL-17A stimulate the production of CRP [20], but no changes were found in these cytokines in the intervention study. However, a significant reduction in CRP, but not in IL-6, has also been observed previously after a HF intake [153].

Interestingly no effects were seen on inflammatory markers following the LF diet during the intervention study, despite the relatively high fibre intake during this period. This could be because the source of dietary fibre may be important in obtaining an anti-inflammatory effect. A significant increase in IL-1RA was reported in the control diet group in the SYSDIET study, whereas the value remained the same following the Nordic diet. The increase was consistent throughout the study, and could be attributed to the worsening effect of consuming the control diet. However, the other cytokines investigated did not change significantly between the groups.

The reason for the lack of effect on cytokine levels in the intervention study might be explained by too low a power to detect significant differences using multiplex analysis. Some samples had to be excluded from the analysis owing to extreme values (>3 times the interquartile range) and the values for some cytokines were below the detection limit (especially IL-1β and IL-1RA). Similar results have been obtained when different multiplex methods were compared for the analysis of cytokines in human serum samples [154]. The proportion of samples below the detection limit was ~75% for IL-1β (cf. 72% in the present study), ~30% for IL-6 (48%), ~20% for IFN-γ (24%) and~5% for TNF-α (12%). The high proportion of samples below the detection limit for some of the cytokines demonstrates that the methods lack sensitivity for some cytokines. Several multiplex kits for cytokine measurements are commercially available, and the Luminex-based kits using magnetics beads, used in the intervention study, are considered to perform better than kits using polystyrene beads [155]. The advantages of using multiplex methods are that they are time-saving and only a small sample volume is needed. However, if this technique is to be used in future studies, the cytokines of interest included in a specific kit should be evaluated before use. An alternative is to analyse each cytokine separately, for example, with ELISA. The ELISA method was chosen in the SYSDIET study to measure the cytokines IL-1β, IL-1RA, IL-6, IL-10 and TNF-R. However, the number samples below the detection limit was 70% for IL-1β and 40% for IL-10, so this problem appears to exist for some cytokines, even when using separate ELISA assays.

5.2 METABOLOMIC MARKERS OF DIETARY FIBRE INTAKE

Overall, more features could be detected in urine, and several metabolites were only observed in this biofluid. Two markers, namely 2,6-DHBA and 2-aminophenol sulphate, could be identified as significantly increased after the HF diet in both plasma and urine in the intervention study (Papers III and IV). These are probably markers related to the intake of rye, oat and sugar beet fibre, however, 2,6-DHBA has not previously been reported as a marker of dietary fibre intake, and very limited information is available on this metabolite. It is not possible from this study design to determine from which of the three fibre sources 2,6-DHBA originated. It is probably a marker related to the intake of oat and/or sugar beet fibre, since it has not been reported in any of the previously published intervention studies on rye [78, 106-109, 134, 135]. 2,6-DHBA may be an endogenous conversion product of other compound(s) present in oat and/or sugar beet fibre. To investigate this, samples from each fibre source could be fermented in vitro by human faecal microbiota, as has been described previously for rye bran [156].

Most of the markers identified originate from rye since most work has been done on this source of dietary fibre. Several benzoxazinoids were detected in urine after the HF diet in the intervention study, and are most certainly markers of the rye bran intake since they do not exist in oats [91], although the benzoxazinoid content of sugar beet fibre has not been analysed. The identified benzoxazinoids (DIBOA, HBOA and BOA) and their related degradation products (2-aminophenol, HPAA and HHPAA) confirm previously reported findings in human intervention studies on rye fibre intake [78, 134, 135] and findings from the characterisation of different rye grain fractions and breads [91, 134, 135, 157].

The metabolism of these benzoxazinoids has been investigated in an *in vitro* colonic model [156]. Rye bran was fermented, and the fractions were analysed using an untargeted metabolomic approach. It was shown that the sugar moieties on HBOA and DIBOA were hydrolysed with a concordant increase in HBOA in the faecal extractable fraction. The phenylacetamides HPAA and HHPAA have recently been reported to be elevated in plasma [134] and urine [78, 135] in rye intervention studies. Their presence in rye bread has also been demonstrated [134, 135]. A proposed pathway for the benzoxazinoid metabolism has recently been published, which included proposed mechanisms in both the bread-making process and in metabolism in humans [135]. DIBOA can be degraded to HBOA and BOA. HBOA can be further metabolised to HHPAA and 2-aminophenol, whereas BOA will be converted to HPAA and 2-aminophenol [137, 158]. In the present work, HPAA was conjugated to sulphate only, whereas HHPAA was conjugated to sulphate or glucuronide, which is in agreement with previously published observations in urine [135]. The fermentation and baking of bread was shown to reduce the content of DIBOA, with a concordant increase in BOA and HPAA[135], which implies that some of the transformations start before consumption. None of these phenylacetamides was observed in the fasting plasma samples in the present intervention study, which is probably due to

their short half-lives. A previous postprandial study showed that the levels of HPAA and HHPAA could not be detected in plasma 24 h after rye bread intake [134].

Enterolactone glucuronide, the glucuronide and sulphate conjugates of DHPPA, and 3,5-dihydroxyhydrocinnamic acid sulphate were increased in urine following the HF diet, as previously reported after rye bread intake [78]. Enterolactone glucuronide is an endogenous lignan metabolite converted from plant lignans by the gut microbiota. This marker probably reflects both rye and oat intake since both contain plant lignans, although the total amounts are higher in rye [79, 87]. In a previous study on the intake of rye and wheat breads [159], the enterolactone content in urine was found to be significantly higher during the rye bread period; however, neither the enterolactone concentration nor the daily enterolactone excretion was correlated to the consumption of rye bread. It was suggested that a plateau was reached after the intake of 70-90 g rye bread per day. Significant amounts of alkylresorcinols are found in rye but not in oats [160, 161]. The endogenous metabolites of alkylresorcinols are DHPPA and 3,5-DHBA, but only DHPPA was observed in urine after the HF diet in the present work. A compound with the monomeric mass of 3,5-DHBA was observed in plasma, although it was filtered out during the data extraction process. The half-life of 3,5-DHBA and DHPPA in plasma has been determined to about 10 h and 16 h, respectively [162], which could be the reason why no significant increases were detected in 3,5-DHBA. Also, the concentration of DHPPA has been reported to be higher than 3,5-DHBA in both plasma and 24-h urine [162, 163]. The reason for the absence of alkylresorcinols in plasma is probably that the samples were collected after fasting, since their half-life has been reported to be 5 h [164].

Several metabolites were putatively identified as oat specific. Oats contain the saponins avenacoside A and B, which consist of the furospirostanol nuatigenin as a backbone with three or four sugar moieties attached to it. Hydroxylated and glucuronidated nuatigenin were identified in plasma and urine, and the non-conjugated nuatigenin in plasma, and if it indeed originates from oat avenacosides, the sugar residues would have to be hydrolysed in the gut and the nuatigenin further hydroxylated and conjugated after its absorption.

The identified food metabolomic markers related to oat and rye intake in the present work demonstrate the potential of non-targeted metabolomic profiling to verify known markers and to find new source-specific markers of dietary fibre intake. However, a large number of metabolites remain unidentified, demonstrating the need for an imporved identification process.

5.3 DIETARY FIBRE IN A HEALTHY DIET

Attention has previously been focused on the Mediterranean diet as a healthy choice. This diet originates from observed food patterns in Greece, particularly Crete, and southern Italy, in the early 1960s [165], with high intakes of fruit, vegetables, bread, potatoes, grains, nuts, beans and legumes. However, there is a growing interest in the healthy Nordic diet based on foods with health-promoting properties, and this diet may be as beneficial as the Mediterranean diet.

Olive oil is the main source of fat in the Mediterranean diet, and cheese and yoghurt are the main dairy products consumed daily. This diet is also characterised by moderate intakes of fish, poultry, eggs and sweets, and by a low intake of red meat [165]. The Mediterranean diet has also been associated with a reduced risk of developing diseases such as CVD, diabetes and cancer [166-168]. However, implementing the Mediterranean diet in a Nordic population could be challenging for several reasons; some food items cannot easily be grown locally, which lead to long-distance transportation, and specific regions would have often developed their own regional foods based on cultural traditions and their availability within the region.

A healthy, more regional, Nordic diet, with similarities to the Mediterranean diet, has been proposed including six key food items: berries, cabbage, fish and shellfish, game, rapeseed oil, and the grains oats, barley and rye [169]. An increased intake of regionally grown or produced foods could create a sustainable effect for local producers, apart from a reduction in transportation cost and carbon dioxide emissions. A number of initiatives have been undertaken to promote a healthy Nordic diet. Three of them directly target a new healthy Nordic diet: SYSDIET, OPUS (Optimal well-being, development and health for Danish children through a healthy New Nordic Diet) and NORDIET. The common factor in these projects is that the diets are based on the NNR [6] with slight modifications, and the key foods included in the diets are very similar, as can be seen in Table 5.

The healthy Nordic diet in SYSDIET focuses on whole grain products (mainly from rye, barley and oats), rapeseed oil, low-fat dairy products, lean meat (chicken, lamb, game, pork and beef), and a high intake of berries (bilberries, strawberries, blueberries, blackberries, raspberries, black/red/white currants, gooseberries, cherries, elderberries and cloudberries), fruits (apples, pears, oranges, bananas and quince), vegetables (tomatoes, leaks, lettuce, carrots, rhubarb, sweet beets, beetroot, chives, brassicas, celery root and fresh herbs), and nuts (hazelnuts and sunflower seeds), fish, and a low salt intake.

Table 5. Comparison of the nutrient composition in the Nordic diets proposed in different projects

	SYSDIET Healthy Nordic diet[1]	OPUS New Nordic Diet [170]	NORDIET Healthy Nordic diet [141]	NNR [6]
Protein (E%)	18-20	17	10-20	10-20
Fat (E%)	30-35	32	25-35	25-35
SFA (E%)	<10	10	<8	≤10
MUFA (E%)	Minimum 2/3 of fat intake	13	11	10-15
PUFA (E%)		8	5-10	5-10
Carbohydrates (E%)	45-52	51	45-60	50-60
Dietary fibre	≥35 g/day or 4 g/MJ	41	>3 g/MJ	25-35/day or 3 g/MJ
Refined sugar (E%)	≤10 (max 50 g/d)	4	-	≤10
Alcohol (E%)	not defined	1	2	≤5
Salt (g/day)	6 g women, 7 g men	not defined	<5.5[2]	6 g women, 7 g men

MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, SFA = saturated fatty acids

[1]Paper V

[2]Sodium was converted to salt (g) by multiplying by 2.54

In a prospective cohort study in Denmark, a healthy Nordic food index was constructed based on traditional health-promoting Nordic foods [171]. A higher score was found to be significantly associated with a lower rate of mortality. When studying the different food groups, rye bread was the food most related to lower mortality among men, while cabbage and root vegetables were significantly associated with lower mortality among women. Good adherence to the Nordic diet, as assessed by the Nordic food index, showed a 35% lower risk of developing colorectal cancer among women [172]. The FINRISK study, a cross-sectional study on the Finnish population, was used to assess a Baltic Sea Diet Score, based on similar food items to other healthy Nordic food diets [173]. The intake of cereals (rye, oats and barley) was found to be inversely correlated to waist circumference. Another study, SYSDIMET, showed that a healthy diet including fatty fish (≥3 times per week), bilberries and whole grain products, reduced markers of inflammation (CRP) and endothelial dysfunction (E-selectin) in subjects with impaired glucose tolerance [174]. Interestingly, it was observed that the higher the increase in dietary fibre, the greater the reduction in E-selectin. Moreover, a higher intake of rye bread was significantly associated with a decrease in CRP. The SYSDIMET study also showed improvements in 2-h glucose response and the glucose AUC in the healthy diet group [175]. It can be concluded from these findings that one of the key food components in sustaining human health is cereal and whole grain products from oats, rye and barley.

The improvement of public health is an important aim of the NNR. An intervention programme was initiated in Sweden in 1985, as the prevalence of CVD was among the highest in the world at that time [176]. The effect of the intervention programme was followed in a cohort study on 140,000 men and women living in northern Sweden. Data on the subjects' food and nutrient intake were collected, together with measurements of blood cholesterol levels and BMI, during the years 1986 to 2010. This initiative led to a decrease in total fat intake over time, however, the trend started to revert after 2004. An increase in blood cholesterol levels coincided with an increase in fat intake, especially the increased intake of SFA. It was found that the increase in fat intake and blood cholesterol levels occurred simultaneously with the increased popularity of low-carbohydrate high-fat diets. A reduction in carbohydrate intake was also observed as fat intake began to rise. This is worrying, since dietary fibre originates from foods that usually contain carbohydrates. Unfortunately, no information on dietary fibre intake was recorded in this study, so it is not known whether the dietary fibre intake also decreased. Since national food intake surveys in Sweden have shown that the Swedish population does not reach the recommended daily intake of dietary fibre, a further decrease could have a negative effect on the prevention of CVD and type 2 diabetes.

6 CONCLUSIONS

In the present work, different study designs and different sub-populations at risk were used to investigate whether a high dietary fibre intake could have positive effects on known risk markers for CVD and type 2 diabetes, and to confirm known markers and find new ones related to the intake of oat, rye and sugar beet fibre using a metabolomic approach. The main conclusions are summarised below.

- Postprandial differences in response to a meal containing oat powder, rye bran, sugar beet fibre, or a mixture of all three, were observed in healthy subjects. Rye bran had the most pronounced effect on postprandial glucose, and the effect was determined not only by the soluble fibre content, but also by the total dietary fibre content (Paper I).

- Diets including fibre from oat bran, rye bran, and sugar beet may have the potential to reduce inflammatory markers such as fibrinogen and CRP in healthy, mildly hypercholesterolaemic subjects. The source of dietary fibres may be crucial as the low-fibre diet, which still provided a relatively high fibre intake, did not have any effect on inflammatory markers (Paper II).

- Previously described source-specific markers of dietary fibre intake and new potential markers for the intake of oat bran, rye bran and/or sugar beet fibre were identified in plasma and urine using an untargeted metabolomic approach. The alkylresorcinol metabolites and benzoxazinoids in urine could be ascribed to the intake of rye bran, whereas enterolactone could originate from oat and/or rye. Additionally, 2,6-DHBA and a tentatively identified oat nuatigenin metabolite, could have the potential of being biomarkers of dietary fibre intake since these could be observed in both plasma and urine (Papers III and IV). However, it is not known from which fibre source(s) 2,6-DHBA originates, and this requires further evaluation.

- A healthy Nordic diet improved the lipoprotein profiles and reduced low-grade inflammation in overweight and obese subjects with metabolic syndrome. The multi-centre setting showed some diversity in effect between centres, and the duration was found to affect the amount by which some of the biomarkers were lowered (Paper V).

7 FUTURE PERSPECTIVES

There is limited knowledge on how well the Nordic population adheres to the NNR, although the SYSDIET study has provided such data on the Nordic population with metabolic syndrome. In this study, the subjects' energy and nutrient intake at baseline (i.e. the run-in period) was compared with the NNR [177]. The most discriminating nutrient variable was found to be the intake of dietary fibre. The NNR stipulate an intake of 25-35 g dietary fibre per day (≥3 g/MJ), and this was only met by 20% of the men and 26% of the women. The mean daily intake of dietary fibre was 22 g for men and 21 g for women. When comparing the energy-adjusted fibre intake (g/MJ), the percentage that met the criteria was even lower for men (13%), but slightly improved in women (30%), with an average of 2.1 g/MJ and 2.6 g/MJ for men and women, respectively. During the SYSDIET intervention, the intake of dietary fibre was increased to 35 g per day (~4.1 g/MJ) in the Nordic diet group whereas the intake in the control diet group was reduced to 16 g per day (~1.9 g/MJ). These findings show that it is important to further emphasize the intake of healthy Nordic food components, especially from whole grain sources such as oats, rye, barley and sugar beet fibre, to increase the dietary fibre intake in the Nordic population, in order to prevent the development of CVD, overweight and type 2 diabetes.

Metabolomic approaches have become more common in nutritional intervention studies to investigate human health status and/or to relate metabolomic profiles to food intake [101]. Until know, non-targeted approaches have been more commonly used in nutritional metabolomics. Bearing in mind the large number of metabolites that the methods can yield, the results so far are rather modest. One limitation in metabolomics is that it is often time consuming, with the identification process as the main bottleneck. Moreover, the lack of available commercial standards requires the synthesis of the proposed metabolite in order to correctly confirm its identity. Authentic standards must be synthesized for all tentatively identified markers in the present work to confirm their identity. Furthermore, quantification of several compounds in dietary fibre from different sources is needed. Analysis of known fibre fractions using an untargeted metabolomic approach, especially sugar beet fibre, could also identify new source-specific markers for better prediction of which compounds should be expected in plasma and urine. The number of metabolites in available databases is increasing, but this process that takes time.

The use of different biofluids (blood, urine, faeces) in intervention studies to determine which metabolites are found in which samples, as well as the combination of different platforms (i.e. liquid chromatography-MS, gas chromatography-MS, nuclear magnetic resonance) to cover different kinds of metabolites, could increase our knowledge on the presence and amounts of effect markers and dietary intake markers in different

compartments in the body following the intake of various diets. It is also important to further elucidate the absorption, metabolism and excretion of these markers. Metabolites present in foods are often transformed in the body, for instance by the gut microbiota and by conjugation. Therefore, the fibre or food product of interest could be fermented with human faecal microbiota *in vitro* to enable identification of transformation products. These challenges have been discussed within the food and nutritional metabolomic community and suggestions have been made to move the field forward [178]. Apart from the needs described above, the development of software tools that could predict endogenous metabolite conversion (conjugations and microbiota fermentation) would be helpful during the identification process. A marker of interest could be validated by dose-response studies, together with identification of the fibre source(s) associated with the marker. It has also been suggested that a database including existing human samples from different intervention studies could be used for marker validation [178]. Validated markers of dietary fibre intake from different fibre sources could also be used to quantify the general dietary fibre intake in future studies. Metabolomics is still in its infancy, and the number of metabolites (not yet identified) demonstrates the huge potential for elucidating the pathways involved (i.e. effect markers produced as a result of dietary intake) and markers of fibre intake (i.e. dietary intake markers).

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