ALKYLRESORCINOL METABOLITES AS POTENTIAL BIOMARKERS FOR WHOLE-GRAIN RYE INTAKE AND THE EFFECT OF RYE BREAD INTAKE ON PLASMA LOW-DENSITY LIPOPROTEINS

Päivi Söderholm

ACADEMIC DISSERTATION

To be presented for public examination, with the permission of the Faculty of Medicine of the University of Helsinki, in Small Hall, University Main Building, Fabianinkatu 33, Helsinki, on November 30, 2012, at 12 noon.

Helsinki 2012
To my family
CONTENTS

Contents................................................................................................................................................. 4
Abstract.................................................................................................................................................. 7
Tiivistelmä ............................................................................................................................................ 8
List of original publications................................................................................................................ 10
Abbreviations....................................................................................................................................... 11
1 Introduction .................................................................................................................................... 12
2 Review of the literature ................................................................................................................ 14
   2.1 Alkylresorcinols in dietary sources and estimated intake ................................................. 14
      2.1.1 Alkylresorcinols ................................................................................................................ 14
      2.1.2 Alkylresorcinols in cereals .............................................................................................. 16
         2.1.2.1 Rye ............................................................................................................................... 16
         2.1.2.2 Wheat .......................................................................................................................... 17
         2.1.2.3 Triticale ....................................................................................................................... 18
         2.1.2.4 Barley .......................................................................................................................... 18
         2.1.2.5 Other cereals .............................................................................................................. 19
      2.1.3 Alkylresorcinols in cereal-based foods ......................................................................... 19
      2.1.4 Estimated dietary intake of alkylresorcinols ............................................................. 20
   2.2 Absorption, metabolism, and excretion of alkylresorcinols in humans ............................................. 22
      2.2.1 Absorption of alkylresorcinols and occurrence in human circulation ..................... 22
      2.2.2 Metabolism of alkylresorcinols .................................................................................. 23
      2.2.3 Urinary excretion of alkylresorcinol metabolites .................................................... 24
   2.3 Properties of alkylresorcinols and their metabolites .......................................................... 24
      2.3.1 Alkylresorcinols as a biomarker for intake of whole-grain rye/wheat .................... 24
2.3.2 Alkylresorcinol metabolites as potential biomarkers for the intake of whole-grain rye/wheat ................................................................. 26

2.3.3 Antioxidant activity of alkylresorcinols.............................................. 28
  2.3.3.1 Concept of antioxidants ............................................................... 28
  2.3.3.2 Alkylresorcinols preventing the oxidation of LDL ...................... 29
  2.3.3.3 Alkylresorcinols as hydrogen donators ........................................ 30
  2.3.3.4 Alkylresorcinols and ferric ion reduction .................................... 30
  2.3.3.5 Alkylresorcinols and chemiluminescence inhibition .................. 30
  2.3.3.6 Alkylresorcinols inhibiting oxidation in oil systems ............... 31
  2.3.3.7 Alkylresorcinols as antioxidants in model (liposomes) and biological membranes ...................................................... 31

2.3.4 Other properties of alkylresorcinols .................................................. 32

2.4 Plant sterols in diet .................................................................................. 33

3 Aims of the study .......................................................................................... 35

4 Materials and methods ................................................................................ 36

  4.1 Study designs and subjects .................................................................... 36
    4.1.1 Kinetic study of alkylresorcinol metabolites in plasma and urine (Studies I and II) ................................................................. 36
    4.1.2 Dietary intervention (Studies III and IV) .................................. 37

  4.2 Materials ................................................................................................ 38
    4.2.1 Rye breads .................................................................................. 39
    4.2.2 Solvents, reagents, and chromatographic equipment ............... 39
    4.2.3 Standards .................................................................................. 40
    4.2.4 Instrumentation ........................................................................ 40

  4.3 Methods ................................................................................................. 40
    4.3.1 Sample collection ........................................................................ 40
      4.3.1.1 Kinetic study (Studies I and II) ........................................... 40
      4.3.1.2 Dietary intervention (Studies III and IV) ......................... 41
4.3.2 Isolation of lipoproteins and determination of lipids .......... 41
4.3.3 Determination of serum apolipoproteins ................................ 41
4.3.4 Quantitative analysis of alkylresorcinol metabolites in serum/plasma ......................................................... 41
4.3.5 Quantitative analysis of alkylresorcinol metabolites in urine .. 42
4.3.6 Oxidation of LDL and HDL ............................................... 42
4.3.7 Quantitative analysis of alkylresorcinols in rye breads .........42
4.3.8 Quantitative analysis of alkylresorcinols in LDL fraction ......43
4.3.9 Quantitative analysis of lipophilic (pro)vitamins in serum ....43
4.3.10 Analysis of dietary intake of nutrients ................................. 44
4.3.11 Pharmacokinetic analysis of DHPPA and DHBA ............... 44
4.3.12 Statistical analysis ......................................................... 44
5 Results .................................................................................. 45
5.1 Plasma kinetics of alkylresorcinol metabolites (Study I) .........45
5.2 Urinary kinetics of alkylresorcinol metabolites (Study II) ....... 47
5.3 Oxidation resistance of LDL (Study III) .................................. 48
5.4 Plant sterols and plasma cholesterol (Study IV) ...................... 50
6 Discussion ............................................................................ 53
6.1 Kinetic study of alkylresorcinol metabolites (Studies I and II) ...53
6.2 Oxidation resistance of LDL (Study III) ................................. 55
6.3 Plant sterols and plasma cholesterol (Study IV) ...................... 56
7 Conclusions ........................................................................ 59
8 Acknowledgments .................................................................. 61
9 References ........................................................................... 64
ABSTRACT

Nestled in the research field exploring the protective mechanisms of whole grains against cardiovascular disease (CVD), this thesis focuses on two novel candidate biomarkers for whole-grain rye/wheat intake and the effect of rye bread intake on plasma low-density lipoproteins (LDLs). A special emphasis is on alkylresorcinols, which are rye- and wheat bran-associated phytochemicals. Alkylresorcinols are absorbed in humans, and their plasma concentration is suggested to serve as a biomarker for whole-grain rye/wheat intake. Following absorption, alkylresorcinols are metabolized in the liver to 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) and 3,5-dihydroxybenzoic acid (DHBA), which have been identified in plasma and urine.

Here, we have determined the kinetic parameters for DHPPA and DHBA in plasma and urine from 15 humans after ingesting a single serving of rye bread (6 slices, 198 g). We showed that DHPPA and DHBA possess good biomarker properties with longer half-lives (t_{1/2}) than intact alkylresorcinols. This can result in higher fasting plasma concentrations than those for alkylresorcinols, which is advantageous when blood samples from low-to-moderate whole-grain rye consumers are being analyzed.

Alkylresorcinols possess antioxidant activity in in vitro assays, although human data are lacking. Because LDL particles serve as carriers of alkylresorcinols in human circulation, and oxidative modification of LDL has been linked to atherogenesis, we aimed to study the potential of alkylresorcinols to protect LDL particles against oxidation. We also determined the efficacy of added plant sterols (PSs) in rye bread to lower LDL cholesterol (LDL-C), a known risk factor for CVD.

We explored with a dietary intervention the effects of rye bread intake on plasma LDL by varying the rye bread dose from 0 g/d (baseline) to 99 g/d, and finally to 198 g/d. Rye bread was consumed without (n=31, controls) or with added PSs (n=32). Intake of 198 g/d of rye bread significantly (P < 0.001) increased oxidation resistance and alkylresorcinol contents of LDL from baseline. However, alkylresorcinol concentration did not correlate with the oxidation resistance of LDL. Therefore, alkylresorcinols are presumably not the major causative antioxidants in LDL, and their role remains unclear. Compared with controls, the PS group exhibited similar improvements in the oxidation resistance of LDL, but showed a significant decrease in LDL-C and some other lipid risk factors, suggesting that PS enrichment further increased the health benefits of whole-grain rye.

In summary, this thesis characterizes the kinetics of DHPPA and DHBA and supports their suitability as biomarkers for whole-grain rye intake. Moreover, rye bread intake was shown to significantly increase in vitro oxidation resistance of LDL, and, when enriched with PSs, to also lower plasma LDL-C, providing a promising dietary means for prevention of CVD.

Tässä väitöskirjatyössä määritettiin ensimmäistä kertaa DHPPAn ja DHBAn kineettiset parametrit plasmasta ja virtsasta 15 terveeltä koehenkilöltä ruisleipä-annoksen (6 siivua, 198 g) nauttimisen jälkeen. Saadut tulokset puoltavat DHPPAn ja DHBAn sopivuutta biomarkkereiksi täysjyvärukiin saannista ja lisäksi niiden puoliintumisaika plasmassa on pidempi kuin alkyliresorsinolien. DHPPAn ja DHBAn poistuminen verestä mahdollistaa korkeammat plasmapitoisuudet paastooverinäytteissä, mikä voi olla erityisen hyödyllistä silloin kun tutkitaan vain vähän täysjyväruista nauttivien henkilöiden näytteitä.

Laboratoriokokeissa alkyliresorsinoleilla on havaittu antioksidatiivista aktiivisuutta, mutta vastaavia vaikutuksia ihmisellä ei ole tiedossa. Koska ruokavaliosta saatujen alkyliresorsinoliin yhtenä kuljettimena verenkierrossa toimivat LDL-partikkelit, mahdollinen antioksidanttivaikutus voisi suojata LDL-partikkeleita hapettumiselta. LDL-partikkelien hapettumisella on yhtymäkohtia ateriosklerosin syntyprosessiin ja siksi hapettumisen estäminen on erityisen tärkeää tutkimuksessa ja -tavoite tässä väitöskirjatyössä. Lisäksi tavoitteena olevat määrittää ruisleipään leipomisvaiheessa lisätyn kasvisterolien kyky laskea plasman LDL-kolesterolipitoisuutta, joka on merkittävä sydän- ja verisuonitautien riskitekijä.

Ruisleivän saannin vaikutuksia plasman LDL-kolesterolipitoisuuteen sekä LDL-partikkelien hapettumisherkkyteen tutkittiin 63 terveellä koehenkilöllä kuuden viikon mittaisessa ruisleipä-interventiossa. Intervention aikana koehenkilöiden ruisleipäänannosta vaihdeltiin muun ruokavaliomaisemman pysyvään tunnistamaan. Vertailujakson ruokavaliosta oli poistettu kokonaan ruis- ja täysjyvävehnätuotteet, kun taas ruisjakson aikana ruokavaliot sisälsi ruisleipää päivittäin; ensin 3 siivua (99 g/pv) kahden viikon ajan, ja tämän

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:


These publications have been reprinted with the kind permission of their copyright holders (Study I, American Society for Nutrition; Study II, Cambridge University Press; Studies III and IV, Elsevier).
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA1</td>
<td>apolipoprotein A1</td>
</tr>
<tr>
<td>ApoB</td>
<td>apolipoprotein B</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum plasma concentration</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>C15:0 - C25:0</td>
<td>alkylresorcinol homologues with respective carbon (alkyl) chain lengths</td>
</tr>
<tr>
<td>DHBA</td>
<td>3,5-dihydroxybenzoic acid</td>
</tr>
<tr>
<td>DHPPA</td>
<td>3-(3,5-dihydroxyphenyl)-1-propanoic acid</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>dw</td>
<td>dry weight</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum urinary excretion rate (µmol/h)</td>
</tr>
<tr>
<td>GCMS</td>
<td>gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>HDL-C</td>
<td>high-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HPLC-CEAD</td>
<td>high-performance liquid chromatography with coulometric electrode array detector</td>
</tr>
<tr>
<td>LDL-C</td>
<td>low-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>n.d.</td>
<td>not determined / not detectable</td>
</tr>
<tr>
<td>PS</td>
<td>plant sterol</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>TC</td>
<td>total cholesterol</td>
</tr>
<tr>
<td>TG</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt;</td>
<td>time of reaching maximum plasma concentration or maximum urinary excretion rate</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>half life</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
</tr>
</tbody>
</table>
INTRODUCTION

Epidemiological studies have consistently shown lower risk of cardiovascular disease (CVD) for humans with high habitual intake of whole-grain cereals or cereal fiber [1-4]. Furthermore, a large European prospective study reported that cereal fiber intake is associated with reduced mortality from circulatory diseases [5]. Since CVD causes the majority of premature deaths in Europe at present [6], taking advantage of the protective role of whole grains and cereal fiber and uncovering the underlying mechanism are important research targets. Dietary prevention and treatment of CVD is essentially focused on lowering of plasma low-density lipoprotein cholesterol (LDL-C), a well-established lipid risk factor for CDV [6, 7]. In addition, oxidation of LDL particles has been linked to atherosclerosis and CVD, and therefore, attempts to prevent oxidation of LDL are essential [8, 9]. Noteworthy is, however, that in human circulation, high-density lipoprotein (HDL) has been reported to be able to protect LDL against oxidation, and therefore, the level as well as the functionality of HDL should not be diminished by dietary means or other treatment [10].

In dietary intervention settings, intake of whole grains or cereal fiber over 3 - 4 weeks has induced improvements in cardiovascular risk factors by lowering serum total cholesterol (TC) and LDL-C [11-13]. Soluble fiber of the cereals was considered to cause the observed hypocholesterolemic effect [12] and one suggested mechanism was increased excretion of bile acids [13]. However, in addition to being a source of dietary fiber, whole grains are also a rich source of diverse nutrients and bioactive phytochemicals [14-16], most of which are located within the cereal bran [17]. Therefore, it can be assumed that while cereal fiber plays an important role, bioactive substances also contribute to the overall in vivo effect. This was also noted in a prospective study, as women who consumed cereal fiber from whole grains had lower all-cause mortality than women consuming similar amounts of cereal fiber primarily from refined grains [18]. This finding suggests that the protective effect was due to bran-associated phytochemicals or other nutrients, which were lacking from the refined grain fiber [18].

Alkylresorcinols are rye/wheat bran-associated phytochemicals, which evoke dual interest in human nutrition. Firstly, the intact alkylresorcinols in human plasma samples are suggested to serve as a biomarker for whole-grain rye/wheat intake, e.g., in epidemiological studies when evaluating associations between dietary whole grains and certain diseases [19-21]. Indeed, alkylresorcinols have been studied in this respect for ten years [21], while their less investigated metabolites appear promising candidate biomarkers as well, and therefore call for further examination. Secondly, rye bread, with a reported average consumption of 85 g/d [22], is the main dietary source of whole grains, cereal fiber, and alkylresorcinols in the Finnish population. Many different bioactivities are documented for alkylresorcinols in vitro [23], but these have...
not been studied in humans after dietary intake. Whether alkylresorcinols play a role in the reduction of cardiovascular risk factors among whole-grain consumers is unknown.

Plant sterols (PSs) are natural constituents in vegetable oils, seeds, and grains [24]. In humans, PSs documentedly lower LDL-C [25, 26] and are therefore industrially incorporated into several foods such as dairy products and spreads. Inclusion of PSs in the diet is recommended for cardiovascular risk reduction [27]. Moreover, consumption of PSs have been shown to be safe [28-31], except for rare cases of sitosterolemia [32]. The efficacy of PSs to lower LDL-C can, however, be impaired by the food matrix. It is therefore crucial that the chosen food carrier is suitable for the transport and release of PSs into the intestine, where these substances can compete with dietary and biliary cholesterol for absorption, resulting in lower serum LDL-C [33]. Enrichment of rye bread with PSs is novel and may, in theory, further improve the health-promoting effect of whole-grain rye, but the suitability and efficacy of rye bread as a food carrier for PSs remain to be elucidated.

Our curiosity towards the new candidate biomarkers for whole-grain rye/wheat intake together with our interest in determining whether alkylresorcinols induce health effects in humans inspired us to examine the kinetics of alkylresorcinol metabolites and to conduct a dietary intervention with special emphasis on the effects of rye bread intake on plasma LDL; these studies together comprise the present thesis.
2 REVIEW OF THE LITERATURE

2.1 ALKYLRESORCINOLS IN DIETARY SOURCES AND ESTIMATED INTAKE

2.1.1 ALKYLRESORCINOLS

Essential dietary intake of alkylresorcinols comes from foods containing whole-grain rye/wheat and consists of 5-alkylresorcinols [20, 34], to which the term alkylresorcinols refers throughout the study. Even though our expectations for potential effects of alkylresorcinols on humans are positive, the initial interest towards alkylresorcinols emerged decades ago from the worrisome observation that alkylresorcinols in cattle feed caused inhibition of growth and decrease of appetite in animals; a rapid determination method for alkylresorcinols was therefore considered desirable [35, 36].

Alkylresorcinols belong to a large group of phenolic lipids. Structurally, they consist of a 1,3-dihydroxybenzene ring (resorcinol), which is alkylated at position 5 by a saturated, odd-numbered hydrocarbon side-chain consisting of 13 to 27 carbon atoms (C13:0 - C27:0) (Figure 1). The existence of water-soluble and -insoluble groups in the molecule give alkylresorcinols an amphiphilic nature [23]. The octanol/water partition coefficient (log P<sub>o/w</sub>) values of alkylresorcinols are between 7.0 and 13.4, the hydrophobicity increasing with increasing chain length [20, 37]. In cereals, alkylresorcinols are present as mixtures of homologs with different chain lengths.

![Chemical structure of alkylresorcinol. R = alkyl chain consisting of 15 - 25 carbons.](image)

Alkylresorcinols are formed by type III polyketide synthases [38]. In cereals, they are detected already during kernel development, and thereafter, the amounts decrease as the grain matures [39-41]. One explanation for the decrease is a dilution effect due to an increase in starch and protein proportions in a growing grain [39]. Alkylresorcinols are suggested to serve as phytoanticipins in the cereal kernels, thus being present for defensive purposes. This viewpoint is supported by the <em>in vitro</em> antimicrobial properties [23, 42-44] as well as by the specific bran-associated accumulation of alkylresorcinols in the
outer layers, protecting the seed [39, 45]. The exact location of alkylresorcinols in a cereal kernel has been elucidated to be in the intermediate layer between the testa and pericarp, containing 99% of the alkylresorcinols [46]; see Figure 2, which illustrates the structure of a rye kernel.

For the quantitative determination of alkylresorcinols, different extraction methods preceding the chromatographic analysis [47] are required for unprocessed vs. processed cereals. The extractability of alkylresorcinols is equivalent with acetone, ethyl acetate, and methanol in unprocessed cereal samples [45], but the non-polar acetone and ethyl acetate are not suitable for extracting alkylresorcinols from bread [34]. Previously, the baking process was thought to destroy or reduce alkylresorcinols, as only low amounts were detected in bread [40, 48]. However, later experiments revealed that alkylresorcinols remain stable during food processing [34, 49, 50], but probably
form complexes with amylose, and therefore a complete recovery can be obtained by using hot 1-propanol:water (3:1, v/v) [34]. Interestingly, alkylresorcinols can, in turn, affect the baking of bread. High amounts of added alkylresorcinols inhibit the activity of baker’s yeast, resulting in a lower volume and more compact structure of wheat bread than that of other breads with lower levels of added alkylresorcinols [51]. However, addition of rye bran, which contains natural levels of alkylresorcinols, does not inhibit the activity of baker’s yeast [51]. Other properties of alkylresorcinols will be discussed in Section 2.3.

### 2.1.2 ALKYLRESORCINOLS IN CEREALS

#### 2.1.2.1 Rye

The alkylresorcinol contents of rye grains (*Secale cereale* L.) vary according to the cultivar and environmental factors, reported values on a dry weight basis (dw) being 360 - 3200 µg/g [20] and most often around 700 - 1100 µg/g [20, 45, 48, 52-56]. If calculated without the starchy endosperm, rye bran alone possesses a very high alkylresorcinol content, 2034 - 2753 µg/g [55, 56]. Alkylresorcinol homologs, consisting of carbon chain lengths C15:0 - C25:0, are present in rye, and of these C19:0 is the most abundant [20]. Characteristic to rye, the C17:0/C21:0 ratio is near 1.0 [57], although higher values like 1.5 - 1.9 can be detected as well [48, 55]. Treatment with herbicides at nonoptimal growing temperatures has been shown to increase total alkylresorcinol concentration and decrease the proportion of long-chain (C23:0 - C25:0) homologs [58]. An example of a typical distribution of alkylresorcinol homologs in rye [34] is shown in Figure 3.

![Figure 3](image)  
**Figure 3.** Typical percentage distribution of alkylresorcinol homologs C15:0 - C25:0 in rye. Modified from [34].
2.1.2.2 Wheat

Wheat (*Triticum aestivum* L.) has been reported to contain alkylresorcinols in amounts (per dw) of 317 - 1430 µg/g [20, 52, 56, 59]. Wheat bran contains 2388 - 3630 µg/g of alkylresorcinols [55, 56], while refined white flour contains only 20 - 50 µg/g [60]. Alkylresorcinol contents were found to possess a significant, negative correlation with 1000-kernel weight, which is a measure of kernel mass [59]. This association is suggested to be due to smaller kernels after a dry growth season, and thus, proportionally higher alkylresorcinol contents. Factors affecting alkylresorcinol contents and homolog composition were listed to be year, location, weather condition, wheat variety, and to a lesser extent soil composition, fertilization, and pesticide treatment [59]. On the other hand, no correlation was found between alkylresorcinol contents in wheat and weather conditions (temperature, \( P = 0.792 \); precipitation, \( P = 0.939 \)) in another analysis using the same samples [61]. In wheat, the most abundant alkylresorcinol homolog is C21:0, and the C17:0/C21:0 ratio is about 0.10 [34, 56, 57, 59, 62]. An example of alkylresorcinol homolog distribution in wheat [55] is shown in Figure 4.

![Figure 4](https://example.com/image4.png)

**Figure 4.** Typical percentage distribution of alkylresorcinol homologs C15:0 - C25:0 in wheat. Modified from [55].

Of the other wheat species, durum wheat (*Triticum durum* L.) and spelt wheat (*Triticum spelta* L.) have been reported to contain alkylresorcinols in amounts of 194-687 and 490-819 µg/g of dw, respectively, the homolog C21:0 being the most abundant in both species [34, 50, 62]. In durum wheat, the homolog C17:0 could not be detected [34] or was found only in minute amounts, with the C17:0/C21:0 ratio being as low as 0.01 [62]. Figure 5 illustrates an alkylresorcinol homolog distribution of durum wheat based on two reports [50, 62]. For spelt wheat, the alkylresorcinol homolog distribution was similar to that of wheat [34, 59, 62].
Review of the literature

Figure 5. Typical percentage distribution of alkylresorcinol homologs C15:0 - C25:0 in durum wheat. Modified from [50, 62].

2.1.2.3 Triticale

Triticale (x Triticosecale) is a hybrid cereal of wheat and rye. The alkylresorcinol content (per dw) has been reported to be 439 - 647 µg/g [34], and the homologs C19:0 and C21:0 to equally comprise the majority of alkylresorcinols, while C15:0 could not be detected. An example of alkylresorcinol homolog distribution in triticale [34] is shown in Figure 6.

Figure 6. Typical percentage distribution of alkylresorcinol homologs C15:0 - C25:0 in triticale. Modified from [34].

2.1.2.4 Barley

Only small amounts of alkylresorcinols (per dw) are found in barley (Hordeum vulgare L.), 32 - 103 µg/g [34, 52, 63]. We have determined the alkylresorcinol
contents of a commercial barley bran product in our laboratory and obtained a value of 100 µg/g (unpublished results). Interestingly, the alkylresorcinol content in barley has been reported to correlate positively with 1000-kernel weight [63], contrary to in wheat [59]. The alkylresorcinol homolog distribution (Figure 7) differs from other cereals in C25:0 generally being the most abundant homolog of those detected (C17:0-C25:0) [34, 63]. However, in one report barley was not found to contain C25:0 at all [52], and in another report C21:0 was the major alkylresorcinol homolog, followed by C19:0 and C25:0 [64]. In our analysis of barley samples, C25:0 was the most abundant homolog (data not shown), in agreement with two other reports [34, 63].

Figure 7. Typical percentage distribution of alkylresorcinol homologs C17:0 - C25:0 in barley. Modified from [34, 63].

2.1.2.5 Other cereals

Edible parts of oat, rice, millet, and corn are reported not to contain alkylresorcinols [34, 52, 65], while buckwheat may contain small amounts [52] or none [34].

2.1.3 ALKYLRESORCINOLS IN CEREAL-BASED FOODS

Rye bread is a particularly rich dietary source of alkylresorcinols, and, because of its high consumption (85 g/d [22]), also the most relevant food source of alkylresorcinols in Finland. Depending on the amount of whole-grain flour and/or added rye bran used for baking, the alkylresorcinol content (per dw) may vary widely, being 197 - 707 µg/g in soft rye breads [34, 55, 57], and 490 - 1007 µg/g in crisp breads [34, 57]. Furthermore, pasta containing rye bran has been reported to provide an alkylresorcinol content of 262 - 402 µg/g [34, 57], the value for breakfast rye cereals being 721 µg/g [55]. Rolled rye grains are
comparable with whole-grain rye and possess an alkylresorcinol content of 698 µg/g [34].

Whole-grain wheat is another important dietary source of alkylresorcinols. The consumption of wheat in Finland is threefold that of rye (46.4 vs. 16.1 kg per capita in 2010, respectively)[66], but in contrast to rye, wheat is usually not consumed in whole-grain form. Instead, foods from refined wheat flour are mostly available, and special food products made of wheat bran are sold separately. The alkylresorcinol contents (per dw) of soft wheat breads vary from nondetectable amounts to 332 µg/g, while those of crisp breads and digestive-type biscuits are 58 - 420 µg/g [34, 57]. A sample of spaghetti has been found to provide 154 µg/g of alkylresorcinols [57]. The highest alkylresorcinol contents among wheat products are found in wheat bran-based breakfast cereals, the reported values ranging from 558 µg/g (Weetabix) to 1784 µg/g (All Bran) [34]. Of other relevant food sources, alkylresorcinol content in muesli is 124 - 299 µg/g [55, 57] and spelt wheat flakes 657 - 789 µg/g [56]. The actual importance of a certain food as a dietary source of alkylresorcinols depends on individual food consumption patterns. Barley and triticale normally comprise a negligible portion of dietary cereal consumption (1.4 kg per capita in 2010 for barley [66]), thus not being essential dietary sources of alkylresorcinols. The alkylresorcinol contents of some cereal foods are listed in Table 1.

Table 1. Alkylresorcinols in cereal foods.

<table>
<thead>
<tr>
<th>Food product</th>
<th>Alkylresorcinol content µg/g dry weight</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rye bread, soft</td>
<td>197 - 707</td>
<td>[34, 57]</td>
</tr>
<tr>
<td>Rye crisp bread</td>
<td>490 - 1007</td>
<td>[34, 57]</td>
</tr>
<tr>
<td>Pasta containing rye bran</td>
<td>262 - 402</td>
<td>[34, 57]</td>
</tr>
<tr>
<td>Breakfast cereals from rye</td>
<td>721</td>
<td>[55]</td>
</tr>
<tr>
<td>Rolled rye grains</td>
<td>698</td>
<td>[34]</td>
</tr>
<tr>
<td>Wheat bread, soft</td>
<td>n.d. - 332</td>
<td>[34, 57]</td>
</tr>
<tr>
<td>Wheat crisp bread and biscuits</td>
<td>58 - 420</td>
<td>[34, 57]</td>
</tr>
<tr>
<td>Spaghetti containing whole-grain wheat</td>
<td>154</td>
<td>[57]</td>
</tr>
<tr>
<td>Wheat bran-based breakfast cereals</td>
<td>558 - 1784</td>
<td>[34]</td>
</tr>
<tr>
<td>Muesli</td>
<td>124 - 299</td>
<td>[55, 57]</td>
</tr>
<tr>
<td>Spelt wheat flakes</td>
<td>657 - 789</td>
<td>[56]</td>
</tr>
<tr>
<td>Whole-grain pasta from durum wheat</td>
<td>215 - 270</td>
<td>[50]</td>
</tr>
</tbody>
</table>

2.1.4 ESTIMATED DIETARY INTAKE OF ALKYLRESORCINOLS

The intake of alkylresorcinols is highest in Northern and Eastern Europe because rye bread is frequently consumed in these areas. The average per capita intake of alkylresorcinols has been estimated by using food supply data or food consumption data [67]. Food supply data were obtained by converting first the
amounts of milled rye/wheat (during a certain year) to per capita amounts and then multiplying this value by the average alkylresorcinol content of that cereal. According to this method, the intake of alkylresorcinols was highest in Finland, 39.8 mg/d; followed by Denmark, 37.1 mg/d; Norway, 18.5 mg/d; Sweden, 17.5 mg/d; and the UK, 11.9 mg/d [67]. In the alternative method utilizing food consumption data, the individual food records (in Sweden) or the household shopping data (in the UK) were supplemented by data of average alkylresorcinol concentrations based on a typical recipe for the food in question. According to this data, the mean intake of alkylresorcinols in Sweden was 22.9 mg/d and in the UK 11.8 mg/d, while the highest individual intakes were above 100 mg/d in both countries [67]. About half of the UK subjects and 3% of the Swedish subjects did not consume alkylresorcinol-containing foods at all. The Swedish data were further analyzed and the intake of alkylresorcinols was found to be higher in males than females (26.4 vs. 19.6 mg/d), which was suggested to indicate higher energy intake. On the other hand, alkylresorcinol intake was significantly higher in the older age groups in both genders, which, in turn, was probably not due to energy intake, but rather to higher typical consumption of cereals among older people [67]. The estimated alkylresorcinol intakes in different countries are shown in Figure 8.

**Figure 8.** The estimated mean alkylresorcinol intakes in the UK, Sweden, Norway, Denmark, and Finland. Modified from [67].
2.2 ABSORPTION, METABOLISM, AND EXCRETION OF ALKYLRESORCINOLS IN HUMANS

2.2.1 ABSORPTION OF ALKYLRESORCINOLS AND OCCURRENCE IN HUMAN CIRCULATION

Ileal absorption of alkylresorcinols begins rapidly after dietary intake [68] and has been noted to be reasonably stable regardless of the dose [69, 70]. Approximately 60% of the ingested dose is absorbed in the intestine [69]. The absorbed alkylresorcinols are transported in chylomicrons via the lymphatic system to the circulation and liver [71]. Plasma levels of alkylresorcinols vary widely even at steady state, the mean overnight fasting levels being 37.8 - 388 nmol/L with a moderate to high rye intake [19, 72, 73], and 943 nmol/L with an extremely high (483 g/d) rye intake [74]. Intake of a high single dose of rye bran (120 g) induces mean postprandial plasma alkylresorcinol levels of 3365 nmol/L [68]. The majority of the plasma alkylresorcinols are carried by lipoproteins VLDL, HDL, and LDL, with respective shares of 46%, 33%, and 20% [71]. In the circulation, alkylresorcinols can also be incorporated into erythrocyte membranes, the average concentrations being 62.5 - 69.6 nmol/L of packed erythrocytes during constant, moderate intake of whole-grain rye/wheat [72]. The longer chain alkylresorcinol homologs (C23:0 - C25:0) tend to accumulate based on observations that they are present in higher proportions in plasma and erythrocyte membranes than in rye bread [68, 72, 73].

Determination of kinetic parameters for plasma alkylresorcinols indicates two peak concentrations, the lower of which occurs at 2.8 h and the higher at 6.6 h after rye intake [68]. The terminal elimination $t_{1/2}$ of plasma alkylresorcinols is relatively short, 4.8 h, indicating fairly rapid clearance of alkylresorcinols from the circulation. Most of a single dose is excreted within 24 h of ingestion [68].

Plasma alkylresorcinols hardly ever reach zero, even after avoiding whole-grain products for a certain time (wash-out period) in trials. This is at least partly due to the storage of dietary alkylresorcinols in adipose tissue and liberation during low or no intake [75], in line with an earlier suggestion [71].

Table 2 summarizes the essential findings on the absorption of alkylresorcinols and their occurrence in human circulation.
Table 2. Summary of essential findings on the absorption of alkylresorcinols and their occurrence in human circulation.

<table>
<thead>
<tr>
<th>Issue</th>
<th>Year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylresorcinols are present in human plasma</td>
<td>2002</td>
<td>[19]</td>
</tr>
<tr>
<td>Dietary alkylresorcinols are absorbed by humans, absorption rate is not affected by dose or meal frequency</td>
<td>2003</td>
<td>[69]</td>
</tr>
<tr>
<td>Alkylresorcinols are present in human erythrocyte membranes</td>
<td>2005</td>
<td>[72]</td>
</tr>
<tr>
<td>Plasma alkylresorcinols correlate with rye bread intake</td>
<td>2005</td>
<td>[73]</td>
</tr>
<tr>
<td>Determination of alkylresorcinol kinetics; half-life 4.8 h</td>
<td>2006</td>
<td>[68]</td>
</tr>
<tr>
<td>Alkylresorcinols are carried by lipoproteins</td>
<td>2007</td>
<td>[71]</td>
</tr>
<tr>
<td>Clear dose-response of plasma alkylresorcinols to dietary intake, suggesting fairly stable intestinal absorption regardless of dose</td>
<td>2006</td>
<td>[70]</td>
</tr>
<tr>
<td>Alkylresorcinols are stored in adipocytes</td>
<td>2007</td>
<td>[75]</td>
</tr>
</tbody>
</table>

2.2.2 METABOLISM OF ALKYLRESORCINOLS

Based on the literature and structural similarities with tocopherols, the hepatic metabolism of alkylresorcinols is expected to include ω-hydroxylation of the side-chain, oxidation to yield carboxylic acid, and β-oxidation to shorten the alkyl tail and yield water-soluble compounds [20, 76]. The reported ability of alkylresorcinols to interfere with the metabolism of γ-tocopherol in cultured hepatocytes and in rats supports this idea [77]. In humans, two alkylresorcinol metabolites, 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) and 3,5-dihydroxybenzoic acid (DHBA), were identified in urine [78] and in plasma [79]. Both free and conjugated forms (glucuronide or sulfate) of the metabolites are present in both of these body fluids [78, 79]. The chemical structure of the two alkylresorcinol metabolites DHPPA and DHBA is shown in Figure 9.

![Figure 9. Chemical structure of the alkylresorcinol metabolites. 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) on the left; 3,5-dihydroxybenzoic acid (DHBA) on the right. Reproduced with permission from Sigma-Aldrich Co.](image-url)
2.2.3 URINARY EXCRETION OF ALKYLRESORCINOL METABOLITES

DHPPA and DHBA are the major urinary excretion forms of the ingested alkylresorcinols, although small amounts of intact alkylresorcinols can be found in urine after a high single intake (150 mg) of alkylresorcinols [78].

Increasing the dose of ingested alkylresorcinols has been shown to increase the urinary excretion of DHPPA and DHBA significantly (P < 0.001), albeit the total urinary recovery/24 h of these metabolites is significantly (P < 0.020) decreased simultaneously, from 89.0% to 44.6% following alkylresorcinol doses of 85 µmol and 342 µmol, respectively [70]. Whether a high alkylresorcinol dose can cause a change in the elimination route from urinary to biliary excretion or is incompletely excreted during a 24-h urine collection is unclear [70].

Conjugation of metabolites increases their water solubility for urinary excretion [78, 80]. Conjugation of DHPPA and DHBA with sulfates accounts for 15% for both metabolites, while conjugation with glucuronides comprises 39% for DHPPA and 18% for DHBA, following a daily intake of 170 µmol (66 mg) of alkylresorcinols [81]. Therefore, the excretion of the less hydrophilic DHPPA is more dependent on conjugation than that of the more hydrophilic DHBA [81]. The balance between conjugation with sulfates vs. glucuronides may be affected by the ingested dose of alkylresorcinols [81], similarly to some other compounds [82], or by the lipid solubility of the substrate or even by the substrate specificity of the transferases involved [83].

2.3 PROPERTIES OF ALKYLRESORCINOLS AND THEIR METABOLITES

2.3.1 ALKYLRESORCINOLS AS A BIOMARKER FOR INTAKE OF WHOLE- GRAIN RYE/WHEAT

During the past 10 years alkylresorcinols in plasma have been suggested to be a suitable biomarker for whole-grain rye/wheat intake [19-21, 84]. Ideally, alkylresorcinols as a biomarker could be utilized as an objective indicator of whole-grain rye/wheat intake when studying the effects of these foods on risk of certain diseases in large populations. A reliable biomarker could additionally overcome the potential bias associated with self-reported dietary intakes, those being under-/overestimation of the consumed amounts or intake frequencies [85, 86], as well as incorrect identification of whole-grain products in one's diet [84, 87]. Furthermore, one study explored the potential of alkylresorcinols as surrogate markers by testing the association between plasma alkylresorcinols and endometrial cancer, but found no correlation [88].

The described quantification methods for plasma alkylresorcinols include GC/MS and LC/MS/MS analysis after varying extents of sample preparation [19, 89, 90]. In plasma samples, alkylresorcinol homologs can reflect the cereal source of alkylresorcinols. The C17:0/C21:0 ratio is characteristically around 0.6 - 0.8 after rye bread intake and 0.1 - 0.5 after whole-grain wheat intake [71, 73].
The suitability of alkylresorcinols as a biomarker for whole-grain rye/wheat intake is supported by several studies reporting correlation coefficients between plasma alkylresorcinols and cereal-based food items, these being summarized in Table 3. In these studies, correlations between plasma alkylresorcinols and intake of cereal fiber [91-93] or rye/wheat whole grains [91, 93] have been observed in humans consuming their habitual diet. Also, under dietary intervention settings plasma alkylresorcinols have been found to correlate with the intake of rye bread and insoluble fiber [73] or rye/wheat fiber as well as total dietary fiber [94] and whole-grain wheat and total whole grains [95]. In a prospective study using nonfasting plasma samples, a modest correlation was found between the estimated intake or rye bread and plasma alkylresorcinols [96]. Some other studies have noted a response in plasma alkylresorcinols to whole-grain rye/wheat intake [68, 70-72, 74].

**Table 3.** Correlation studies on plasma alkylresorcinols and the intake of cereal-based food components.

<table>
<thead>
<tr>
<th>Food component correlating with plasma alkylresorcinols</th>
<th>Dietary intake g/d</th>
<th>Diet/study type</th>
<th>Dietary assessment method</th>
<th>n</th>
<th>Plasma AR nmol/L</th>
<th>r</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole-grain rye</td>
<td>29</td>
<td>Habitual</td>
<td>FR</td>
<td>51</td>
<td>73</td>
<td>0.28*</td>
<td>[91]</td>
</tr>
<tr>
<td>Whole-grain rye + wheat</td>
<td>49</td>
<td>Habitual</td>
<td>FR</td>
<td>51</td>
<td>73</td>
<td>0.53***</td>
<td>[91]</td>
</tr>
<tr>
<td>Cereal fiber</td>
<td>11</td>
<td>Habitual</td>
<td>FFQ</td>
<td>31</td>
<td>55.8</td>
<td>0.37*</td>
<td>[91]</td>
</tr>
<tr>
<td>Cereal fiber</td>
<td>10</td>
<td>Habitual</td>
<td>FFQ</td>
<td>31</td>
<td>55.8</td>
<td>0.47*</td>
<td>[91]</td>
</tr>
<tr>
<td>Whole-grain cereals</td>
<td>26</td>
<td>Habitual</td>
<td>FR</td>
<td>31</td>
<td>55.8</td>
<td>0.57*</td>
<td>[91]</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>Habitual</td>
<td>FFQ</td>
<td>31</td>
<td>55.8</td>
<td>0.47*</td>
<td>[91]</td>
</tr>
<tr>
<td>Whole-grain wheat</td>
<td>17</td>
<td>Habitual</td>
<td>FFQ</td>
<td>31</td>
<td>55.8</td>
<td>0.47*</td>
<td>[91]</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>Habitual</td>
<td>FFQ</td>
<td>31</td>
<td>55.8</td>
<td>0.47*</td>
<td>[91]</td>
</tr>
<tr>
<td>Cereal fiber</td>
<td>9</td>
<td>Habitual</td>
<td>FFQ</td>
<td>31</td>
<td>55.8</td>
<td>0.37*</td>
<td>[91]</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Habitual</td>
<td>FFQ</td>
<td>31</td>
<td>55.8</td>
<td>0.56*</td>
<td>[91]</td>
</tr>
<tr>
<td>Rye bread</td>
<td>214</td>
<td>Intervention</td>
<td>FR</td>
<td>39</td>
<td>352</td>
<td>0.34*</td>
<td>[73]</td>
</tr>
<tr>
<td>Insoluble fiber</td>
<td>30</td>
<td>Intervention</td>
<td>FR</td>
<td>39</td>
<td>352</td>
<td>0.39*</td>
<td>[73]</td>
</tr>
<tr>
<td>Rye/wheat fiber</td>
<td>18</td>
<td>Intervention</td>
<td>FR</td>
<td>28</td>
<td>202</td>
<td>0.47**</td>
<td>[94]</td>
</tr>
<tr>
<td>Whole-grain wheat</td>
<td>-</td>
<td>Intervention</td>
<td>FFQ</td>
<td>252</td>
<td>-</td>
<td>0.39***</td>
<td>[94]</td>
</tr>
<tr>
<td>Whole-grains</td>
<td>-</td>
<td>Intervention</td>
<td>FFQ</td>
<td>252</td>
<td>-</td>
<td>0.32***</td>
<td>[94]</td>
</tr>
<tr>
<td>Rye bread</td>
<td>62.5</td>
<td>Habitual</td>
<td>FFQ</td>
<td>360</td>
<td>78</td>
<td>0.25***</td>
<td>[96]</td>
</tr>
</tbody>
</table>

* P < 0.05
** P < 0.01
*** P < 0.001
1 r value is based on combined data from baseline, refined grain and whole-grain -periods
2 non-fasting plasma samples
AR, alkylresorcinols; r, correlation coefficient; FR, food record; FFQ, food frequency questionnaire; -, data missing
Besides these studies, other facts supporting the suitability of alkylresorcinols as a biomarker for whole-grain rye/wheat intake are the lack of these compounds in other foods, stability during cooking, successful absorption in humans in response to intake, and detectability in grains, foods, and biological samples (see 2.1. and 2.2.). However, limiting the usage are the short elimination t_{1/2} (4.8 h) of the plasma alkylresorcinols and the vast interindividual variation in plasma levels even after similar intakes (see Section 2.2.1.), potentially resulting in a lack of correlation between dietary whole-grains and plasma alkylresorcinols and also hindering quantitative estimation of individual whole-grain rye/wheat intakes.

Reproducibility studies evaluate whether a single measurement of a biomarker can reflect average levels over a longer time period [97]. Plasma alkylresorcinols have been reported to possess poor to moderate reproducibility over a 2- to 4-month period in individuals consuming their habitual diet [91, 95, 98]. In two of these studies, alkylresorcinols correlated with whole-grain rye/wheat intake [91, 95]. Good reproducibility for plasma alkylresorcinols has been reported only in a 6-week intervention setting with extremely high daily intake (483 g/d) of rye bran/whole-grain rye products [74].

In conclusion, the suitability of alkylresorcinols as a biomarker for whole-grain rye/wheat intake has been shown in many studies, and at present it appears to be the best option. However, the short t_{1/2}, the large inter-individual variation in plasma levels, and the poor to moderate reproducibility as a longer-term biomarker limit their usefulness. Nevertheless, plasma alkylresorcinols could be used to distinguish between high and very low consumers of whole-grain rye/wheat in epidemiological studies, or as a compliance marker in dietary interventions, including whole-grain rye/wheat intake.

### 2.3.2 ALKYLRESORCINOL METABOLITES AS POTENTIAL BIOMARKERS FOR THE INTAKE OF WHOLE-GRAIN RYE/WHEAT

Alkylresorcinol metabolites DHPPA and DHBA in plasma and urine are suggested to serve as biomarkers for whole-grain rye/wheat intake [78, 99, 100], although they have been less extensively investigated than the intact alkylresorcinols. Furthermore, in a small study, these metabolites were proposed as potential surrogate markers for breast cancer risk, [101] but this has not been confirmed in a larger study. The analysis methods for urinary metabolites include a rapid and simple HPLC-CEAD [102] as well as a more laborious GC-MS method [81], these two being considered methodologically equal [103]. Furthermore, for plasma samples an HPLC-CEAD method has been described [79].

In one of the first studies on alkylresorcinol metabolites in plasma, a correlation between the sum of DHPPA + DHBA and intact alkylresorcinols was found (P < 0.001, r = 0.686) [79]. Thereafter, a few studies have presented correlation coefficients between the intake of cereal-based food items and plasma DHPPA/DHBA, these being summarized in Table 4. In plasma samples
from subjects consuming a habitual diet, DHPPA and DHBA were found to correlate with cereal fiber and rye intake, estimated from 5-day food records [99, 104]. When the study subjects were categorized into three levels according to their habitual intake of rye, at the highest intake level (68 g/d) plasma DHPPA was 44% higher than at the lowest intake level (23 g/d) and 27% higher than at the medium intake level (44 g/d), (P < 0.05 for both), while plasma DHBA did not differ significantly between the intake levels [104].

Some studies have reported correlation coefficients between the intake of cereal-based food items and urinary DHPPA/DHBA; these are summarized in Table 4. DHPPA and DHBA, in a 72-h urine collection, have been reported to have significant correlations with habitual cereal fiber intake, estimated from 5-d food records, and according to the author, an intake of 10 g of cereal fiber induced urinary excretion of 40.2 and 26.8 µmol/24 h for DHPPA and DHBA, respectively [92]. Furthermore these metabolites showed good response to different rye intake levels; at the highest level (68 g/d of rye), DHPPA and DHBA increased by 50% and 52% relative to the lowest level (23 g/d), and 25% and 29% relative to the medium level (44 g/d), (P < 0.05, for all) [104]. In American population with none-to-moderate habitual intakes of whole-grains (0 - 3 servings/d), and less than 10% of the participants consuming rye, 12-h urinary DHPPA excretion (1.3 - 99.4 µmol/12 h) was increased by 67% (P < 0.0001) by increasing the intake of whole-grains by one serving/d [100]. A very recent study on American women consuming habitual diets showed a correlation between whole-grain intake (mean 15.9 g/d) and urinary DHPPA, even in spot urine samples [105]. In addition, an intervention study revealed a clear dose-response relationship between the intake of alkylresorcinols and urinary excretion of DHPPA and DHBA, confirming that these metabolites are solely derived from alkylresorcinols [70].

The reproducibility of urinary DHPPA and DHBA measurements over 1 - 3 years was reported to be poor to modest in spot urine samples, indicating that long-term intake of whole-grain cereals cannot be estimated based on a single sample [105].

In summary, additional data are needed concerning DHPPA and DHBA and their occurrence in plasma and urine before these compounds can be applied as biomarkers. However, relative to determination of alkylresorcinols, a smaller sample volume and less sample pretreatment are required.
Table 4.  
**Correlation studies on plasma/urinary DHPPA and DHBA and intake of cereal-based food components.**

<table>
<thead>
<tr>
<th>Food component correlating with alkylresorcinol metabolites</th>
<th>Dietary intake g/d</th>
<th>Diet/ study type</th>
<th>Dietary assessment method</th>
<th>DHPPA/DHBA in plasma/urine</th>
<th>n</th>
<th>r</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cereal fiber</td>
<td>10</td>
<td>Habitual</td>
<td>FR</td>
<td>90&lt;sup&gt;1&lt;/sup&gt; 94&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5</td>
<td>0.463*** 0.411**</td>
<td>[99]</td>
</tr>
<tr>
<td>Rye</td>
<td>-</td>
<td>Habitual</td>
<td>FR</td>
<td>.&lt;sup&gt;1&lt;/sup&gt; .&lt;sup&gt;2&lt;/sup&gt; .&lt;sup&gt;3&lt;/sup&gt; .&lt;sup&gt;4&lt;/sup&gt;</td>
<td>6</td>
<td>0.397** 0.299* 0.438*** 0.524***</td>
<td>[104]</td>
</tr>
<tr>
<td>Cereal fiber</td>
<td>10</td>
<td>Habitual</td>
<td>FR</td>
<td>40.2&lt;sup&gt;3&lt;/sup&gt; 26.7&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5</td>
<td>0.402** 0.359**</td>
<td>[92]</td>
</tr>
<tr>
<td>Whole-grains Cereal fiber</td>
<td>15.9 5.8</td>
<td>Habitual</td>
<td>FFQ</td>
<td>8.4&lt;sup&gt;5&lt;/sup&gt; 3.3&lt;sup&gt;5&lt;/sup&gt;</td>
<td>9</td>
<td>0.37* 0.33*</td>
<td>[105]</td>
</tr>
</tbody>
</table>

* P < 0.05  
** P < 0.01  
*** P < 0.001  
<sup>1</sup> plasma DHPPA (3-(3,5-dihydroxyphenyl)-1-propanoic acid) nmol/L  
<sup>2</sup> plasma DHBA (3,5-dihydroxybenzoic acid) nmol/L  
<sup>3</sup> urinary DHPPA µmol/24h urine collection  
<sup>4</sup> urinary DHBA µmol/24h urine collection  
<sup>5</sup> urinary DHPPA µmol/l in spot urine samples  
r, correlation coefficient; FR, food record; FFQ, food frequency questionnaire; -, data missing

### 2.3.3 ANTIOXIDANT ACTIVITY OF ALKYLRESORCINOLS

#### 2.3.3.1 Concept of antioxidants

Antioxidants are substances that 'when present at low concentrations compared to those of an oxidizable substrate, significantly delay or prevent oxidation of that substrate' [106]. The oxidizable substrates include lipids, proteins, and DNA in biological systems and almost any molecules in food [107]. Antioxidant activities include inhibition of formation of reactive oxygen species (ROS) and scavenging of free radicals of endogenous/environmental origin [106, 107].  

Vitamin E is a well-established antioxidant, and because of the structural and metabolic similarities between tocopherols and alkylresorcinols, the latter has been anticipated to have some antioxidant activity as well [20, 77, 108, 109], inspired a variety of *in vitro* studies, which are discussed below.
2.3.3.2 **Alkylresorcinols preventing the oxidation of LDL**

An LDL particle contains approximately 1300 molecules of polyunsaturated fatty acids (PUFAs), these being susceptible to oxidative modification [110]. Considering that oxidatively modified LDL is involved in atherosclerosis [8, 111] and that LDL is one of the carriers of alkylresorcinols in human circulation [71], the potential of alkylresorcinols to protect LDL from oxidation is an important issue.

An *in vitro* method by Esterbauer has been widely used to investigate LDL oxidation [112]. In this assay, isolated LDL particles are exposed to Cu²⁺ ions, which initiate oxidation of the PUFAs in LDL and cause depletion of endogenous antioxidants. This phase is called the lag time and its duration determines the oxidation resistance of LDL. Antioxidants are important contributors to the duration of lag time, although individual variation is large [110]. After the endogenous antioxidants of LDL are depleted, the lag time ends and the propagation phase (oxidation of the PUFAs in LDL) begins. The oxidation process in the Esterbauer assay is monitored at 234 nm, a wavelength characteristically absorbed by conjugated dienes [112]. When evaluating the contribution of a certain antioxidant to the oxidation resistance of LDL, the compound in question can either be added directly to the assay system or consumed orally before blood sampling and isolation of LDL.

Vitamin E has been described to be the major endogenous antioxidant in LDL, also being associated with the oxidation resistance of these particles [110]. *In vitro* enrichment of the assay system with α-tocopherol led to a linear increase in the oxidation resistance of LDL [113], as did dietary supplementation [110]. Oral consumption of other antioxidants or antioxidant-rich foods has also been used to improve oxidation resistance of LDL [114, 115].

Recently, alkylresorcinol homologs C15:0 and C17:0 were found to increase the resistance of isolated LDL particles to copper-induced oxidation when added as pure compounds to the assay system [116]. The tested concentrations were 2.5 and 25 µmol/L, the former of which could be normally achievable as postprandial plasma concentration of alkylresorcinols (C15:0 - C25:0) after a high dietary intake of rye (120 g of rye bran flakes) [68], and also as an extreme fasting plasma concentration during a habitual diet [91]. It is worth noting, however, that rye is not a substantial source of C15:0 alkylresorcinols [20], and the effect of the longer chain homologs C19:0 and C21:0 was not tested because of solubility problems [116].

In summary, vitamin E is the major antioxidant in LDL, with an established *in vitro* effect on LDL oxidation resistance. Alkylresorcinols as pure compounds have shown some antioxidant activity in LDL oxidation assay, but whether dietary consumption of alkylresorcinols improves the oxidation resistance of LDL, thus far remains obscure.
2.3.3.3 Alkylresorcinols as hydrogen donators

The ease of hydrogen donation of a compound (a measure of antioxidant activity) can be examined in vitro using a relatively stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and monitoring the absorbance decrease at 515 nm as a result of this radical being reduced by the examined antioxidant [117].

The hydrogen donation ability of C15:0 - C21:0 alkylresorcinols is lower than that observed for ferulic acid or vitamin E [116, 118, 119], which are also present in rye [120-122]. The observed relatively poor scavenging activity of alkylresorcinols is in line with another report [123], and considering the structure of alkylresorcinols, also expected. Phenols with para- and ortho-dihydroxylic groups are the most effective antioxidants [124], while alkylresorcinols contain the two hydroxyl groups at meta-position. Furthermore, when extracts from whole-grain bread extracts were studied, the observed DPPH-radical reduction was not proportional to the alkylresorcinol content of these samples [119], and therefore, the possibility for other compound(s) in the samples to contribute to DPPH reduction cannot be ruled out. The hydrogen donation ability of the different alkylresorcinol homologs (C15:0 - C25:0) was relatively equal [119], which is also expected, as the length of the alkyl chain has been reported to affect the lipophility of alkylresorcinols, playing a role in the incorporation of alkylresorcinols into the biological membranes [125].

Overall, the hydrogen donation ability of alkylresorcinols is quite poor, suggesting that this mechanism of action is more likely for some other compounds in rye than alkylresorcinols.

2.3.3.4 Alkylresorcinols and ferric ion reduction

The ferric-reducing ability of plasma is considered a measure of the 'antioxidant power' of a compound to reduce ferric ions (Fe$^{3+}$) to ferrous (Fe$^{2+}$) form, which, at low pH and in the presence of tripyridyltriazine complex, results in an increase in absorbance at 593 nm [126]. Alkylresorcinol homologs C15:0 - C21:0 showed 10-fold lower ferric-reducing ability than ferulic acid [116], suggesting that the potential antioxidant activity of alkylresorcinols would not be mediated via this mechanism.

2.3.3.5 Alkylresorcinols and chemiluminescence inhibition

In the chemiluminescence method, a radical-luminol system produces emission of light, which is measured by a luminometer. An antioxidant acts by inhibiting the luminescence. Alkylresorcinols were only weak antioxidants compared with tocopherols, and the inhibition of luminescence was not stable [119]. Furthermore, when studying extracts from whole-grain rye bread and mixed cereal products, the reduction of luminescence was not proportional to the
alkylresorcinol contents of the samples, and therefore, it is quite possible that the observed antioxidant activity of the bread samples was produced by compounds other than alkylresorcinols [119].

2.3.3.6 Alkylresorcinols inhibiting oxidation in oil systems
Antioxidant activity of alkylresorcinols has been studied in different oil systems [109, 118, 127, 128]. Alkylresorcinol homolog C17:0, isolated from rye grains and used at high concentrations (2.6-20% of total lipids in the assay), inhibited 50-90% of the linolenic acid oxidation induced by ferrous ions [109].

In another study, oils from corn, olive, safflower, sunflower, and soybean were incubated at different temperatures with and without added unpurified C15:0 alkylresorcinols at concentrations of 0.025%, 0.05% and 0.075% [127]. The oil samples with the added alkylresorcinols were found to resist the Cu²⁺-induced oxidation of the lipids [127]. However, in later experiments by others, purified C15:0 alkylresorcinols were added at concentrations of 0.01%, 0.1% and 0.5% to sunflower triacylglycerols (TGs), and the oxidation experiments showed only negligible inhibitory effects, while α-tocopherol was highly effective at concentrations of 0.1% and 0.01% [118]. The authors explained the discrepancy between the latter and the previously reported [127] study by the impurity of the previously used C15:0 alkylresorcinols (declared to also contain 4-methyl-5-pentadecylresorcin), as they also found the unpurified C15:0 alkylresorcinols to possess a significant antioxidant effect on sunflower oil [118].

Purified C15:0 alkylresorcinols have, however, in an earlier study shown antioxidant activity by inhibiting oxidation of linoleic and linolenic acid at concentrations higher than 2.4%, and 1.2%, respectively [128]. The concentrations required for the antioxidant activity are higher than those occurring naturally in foods or in the human circulation, and therefore, this finding may not translate into a practical benefit.

All in all, these studies do not support a very active role for alkylresorcinols as peroxyl radical scavengers. This is consistent with the observation that the redox potential of alkylresorcinols is higher than that of PUFAs, and therefore, the former cannot compete with the latter for hydrogen donation to peroxyl radicals [118].

2.3.3.7 Alkylresorcinols as antioxidants in model (liposomes) and biological membranes
Liposomes are cell membrane-resembling, artificially made vesicles consisting of a phospholipid bilayer. The membrane of LDL shares similarities with liposomal and biological membranes, but consists of a phospholipid monolayer and free cholesterol. Alkylresorcinols, with a reportedly high octanol/water partition coefficient and an amphiphilic nature, are able to become incorporated
Review of the literature

into liposomal and biological membranes, thus existing near the PUFAs attached to phospholipids [125, 129].

Alkylresorcinols (C5:0, C15:0, C17:0) were found to reduce the amount of ferrous ion-induced peroxidation products in a liposomal lipid bilayer membrane [109, 129]. The effective concentrations for 87-100% inhibition in the assays were, however, high: 75-85µM for C15:0 and C5:0 [129]. The resorcinolic ring (without the carbon chain) was found to be less effective [129]. Although the mechanism of antioxidant activity remained somewhat obscure, hydrogen donation [129] or stabilization of the liposome membrane [109] by alkylresorcinols was suggested to play a role, the latter via restricting contacts between PUFA double bonds and the oxidizing agent.

In another experiment, alkylresorcinol homologs C15:0, C19:0, and C23:0 were able to inhibit hydrogen peroxide-initiated oxidation of the erythrocyte membrane lipids, while C5:0 was not [108]. The antioxidant activity was observed at concentrations of 6-60µM, and the effect was dependent on alkylresorcinol homolog chain length, test temperature, and preincubation time of erythrocytes with alkylresorcinols prior to addition of the oxidizing agent. The lack of effect for the shortest homolog C5:0 was thought to be due to inefficient incorporation/anchoring of the very short-chain alkylresorcinols into the lipid bilayers. On the other hand, the longest homolog C23:0 was the next weakest of the tested ones, and this was hypothesized to result from the phenolic group being located too far away from the membrane surface. As another potential mechanism of protection, the authors suggested that hydrogen bonds between alkylresorcinols and phospholipids served as a stabilizing factor for the membrane [108].

In line with previous findings, a recent study showed that oxidation resistance of liposome lipids was improved by alkylresorcinol addition during liposome preparation [130].

In summary, the antioxidant activity of alkylresorcinols on different membranes depends on the alkyl chain length and presumably involves stabilization of the membrane bilayer and/or hydrogen donation by alkylresorcinols; the required effective concentrations are high (micromolar).

2.3.4 OTHER PROPERTIES OF ALKYLRESORCINOLS

A variety of in vitro bioactivities have been documented for alkylresorcinols. Among other activities, these substances are capable of binding to the hydrophobic regions of some proteins and modulating their enzymatic activity [131]. Medium-chain alkylresorcinol homologs (C15:0 - C19:0) have been reported to inhibit the activity of glycerol-3-phosphate dehydrogenase at micromolar concentrations [132, 133], while data concerning other homologs are missing. This finding could be of biological importance, as the studied enzyme is crucial for TG synthesis in adipose tissue and its activity is enhanced in obese humans [134]. Additionally, alkylresorcinol homologs C15:0 - C21:0 were able to prevent accumulation of TGs in cultured adipocytes, the efficacy
being associated with the alkyl chain length [132]. A potential explanation offered by the authors is incorporation of alkylresorcinols into bilayer membranes and interaction with glycerol-3-phosphate dehydrogenase [132]. In another study, alkylresorcinols inhibited oxidative activity of soybean lipoygenase on linoleic acid and arachidonic acid, again the activity being stronger for longer chain homologs (C21:0 - C23:0) [135]. The suggested mechanisms were chain length-dependent penetration of alkylresorcinols into the membrane and alkylresorcinol-induced alteration of substrate properties. Interaction of alkylresorcinols with biological and liposomal membranes is reportedly a complicated property for alkylresorcinols, affecting the shape, rigidity, stability, and permeability of the membranes, depending on the way alkylresorcinols are added to the system [130, 136-138].

Preincubation of cultured human colon cancer cells with alkylresorcinols was demonstrated to improve self-protection of these cells against DNA damage [116]. The protective effect was observed against hydrogen peroxide as well as genotoxic fecal water, and it was inversely related to alkyl chain length (C15:0 - C23:0). However, homolog C15:0 at 100 µmol/L showed cytotoxicity against these cultured cells [116].

Lastly, many reports describe alkylresorcinols to possess antimicrobial activity in vitro, in line with their protective function in plants as well as their outermost location in the cereal kernel [139-142].

Taken together, alkylresorcinols possess multifaceted in vitro bioactivities, but whether these compounds contribute to the whole-grain rye/wheat-induced health effects in humans remains unknown.

2.4 PLANT STEROLS IN DIET

PSs are cholesterol-resembling compounds of plant origin. In the human diet, the richest natural sources of PSs constitute vegetable oils, cereal products, and nuts [24]. In the intestine, PSs inhibit equally the absorption of dietary and biliary cholesterol, and the reduction in serum cholesterol is equal to that observed with plant stanols at doses around 2 g/d [26, 143, 144]. The hypocholesterolemic mechanism is based on the ability of PSs to compete with dietary and biliary cholesterol for uptake into mixed micelles in the intestinal lumen, after which these compounds are transferred to enterocytes [33, 145]. From the enterocytes, most of the PSs are pumped back into the intestinal lumen by ATP-binding cassette transporter proteins [33] and only 0.51-1.89%, depending on the molecular structure, are normally absorbed [146]. However, in patients with a rare lipid disorder called sitosterolemia, PS excretion by these transporter proteins is impaired, and the subsequent accumulation is associated with premature atherosclerosis [32].

The estimated habitual intake of PSs has been reported to be around 160-400mg/d in different populations [147]. In a large European study, habitual PS intake – calculated from food frequency questionnaires – varied between 38 and
749 mg/d, being inversely associated with serum TC and LDL-C in these individuals [148]. Because of the cholesterol-lowering properties of PSs, they are also industrially incorporated into various foods like spreads or yoghurts. These special foods assist dietary treatment of elevated cholesterol and may contribute markedly to cardiovascular risk reduction. While consumption of these foods can multiply the intake of PSs, no adverse effects have been reported in healthy humans, except for the lowering of serum β-carotene [28, 29, 143]. The food matrix plays an important role in transporting and releasing PSs into the intestine for the cholesterol-lowering action [149], and this efficacy must be evaluated in clinical studies. To our knowledge, PSs have not been incorporated into rye bread before, and therefore, no reports exist on the suitability and efficacy of rye bread as a food carrier for PSs. Also unknown is whether the health-promoting effects of whole-grain rye can be further improved by PS enrichment.
3 AIMS OF THE STUDY

The aims of this study were as follows:

1. To explore the kinetics of the alkylresorcinol metabolites DHPPA and DHBA in plasma and in urine after the intake of a single dose of rye bread containing a known amount of alkylresorcinols, in order to estimate the potential of these metabolites as biomarkers for the intake of whole-grain rye (Studies I and II).

2. To investigate with a dietary intervention the effect of rye bread intake on the oxidation resistance of isolated LDL particles, and to evaluate the potential role of the rye-derived alkylresorcinols as antioxidants within these LDL particles (Study III).

3. To examine with a dietary intervention the cholesterol-lowering efficacy of PSs, which are incorporated into a novel food carrier, rye bread, in order to estimate whether rye bread is a suitable matrix for the transport and release of PSs into the intestine, where they are known to inhibit the absorption of cholesterol (Study IV).

4. To assess the utilization of alkylresorcinol metabolites as biomarkers for dietary compliance (rye bread intake) in a dietary intervention trial (Studies III and IV).
4 MATERIALS AND METHODS

4.1 STUDY DESIGNS AND SUBJECTS

4.1.1 KINETIC STUDY OF ALKYLRESORCINOL METABOLITES IN PLASMA AND URINE (STUDIES I AND II)

The kinetic study was designed to monitor the appearance of alkylresorcinol metabolites DHBA and DHPPA in plasma and urine after a two-day low-alkylresorcinol diet and overnight fasting, and thereafter following ingestion of a single dose of rye bread (198 g) containing 100 mg of alkylresorcinols. Plasma samples and all urine were collected at the following time-points: before ingestion of rye bread dose (0 h), and at 3, 4, 5, 6, 7, 8, 10, 12, 14, 16 and 25 h after ingestion. Additionally, all urine between 16 and 24 h was collected in a container. During the study the subjects were provided standardized meals and drinks and instructed to avoid consuming any other foods.

Fifteen healthy subjects volunteered for the kinetic study. The demographic characteristics of the study participants are shown in Table 5. All volunteers were provided with written and oral information about the study, and they attended a blood screening test. As exclusion criteria, we specified low hemoglobin values; < 125 g/L for females or < 135 g/L for males, and the use of antibiotics within the past three months. Informed consent was obtained from all subjects. The study protocol was approved by the Ethics Committee of Helsinki University Central Hospital, Helsinki, Finland, and is registered in www.ClinicalTrials.gov under the study ID no. NCT00953836.

All 15 subjects completed the study and were included in the statistical analyses.

Table 5. Demographic characteristics of the study subjects participating in the kinetic study of alkylresorcinol metabolites. Modified from Studies I and II.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>15</td>
</tr>
<tr>
<td>Gender (females/males)</td>
<td>8/7</td>
</tr>
<tr>
<td>Age (y)</td>
<td>24.3 (5.3)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.3 (10.9)</td>
</tr>
<tr>
<td>BMI</td>
<td>22.1 (2.0)</td>
</tr>
</tbody>
</table>
4.1.2 DIETARY INTERVENTION (STUDIES III AND IV)

The dietary intervention study was a randomized, double-blind, placebo-controlled trial designed to test the effect of null versus high intake of rye bread (without or with added PSs) on plasma lipids and oxidation resistance of isolated LDL particles in healthy humans. The suitability of rye bread to act as novel food carrier for added PSs for cholesterol-lowering purposes was also evaluated. Apart from the specifications regarding the intake of rye bread and some other cereals (see details below), the habitual diet was followed throughout the trial. Compliance was monitored by utilizing 3-day food diaries and by determining plasma DHBA concentration as a biomarker for rye bread intake.

The duration of this trial was six weeks; this consisted of a one-week habitual diet period, followed by a one-week baseline period with null intake of rye bread, a two-week rye period with 3 slices (99 g/d) of rye bread intake, and finally another two-week rye period with a doubled dose (6 slices, 198 g/g) of rye bread (Figure 10). A stepwise increase in the rye bread dose was applied to diminish abdominal discomfort, which is typical for suddenly introduced high doses of rye bread. After the habitual diet period, the subjects were instructed to exclude all other sources of rye, oat, barley, and whole-grain wheat in their diet until the end of the trial. In their place, all subjects were provided with suitable cereal food items based on refined wheat, rice, and corn. At the end of each dietary period, fasting blood samples were drawn before moving on to the next dietary period.

![Figure 10. Study design for the 6-week dietary intervention (Studies III and IV). Arrows indicate collection of overnight fasting blood samples. Modified from Study IV.](image)

Sixty-eight healthy volunteers were recruited for the dietary intervention. The volunteers were given written and oral information about the trial in advance, after which they completed an eligibility questionnaire and attended a blood screening test. Exclusion criteria included hemoglobin < 125 g/L for females or < 135 g/L for males, serum TC > 6.5 mmol/L or TG > 4.0 mmol/L, medication affecting serum lipids or gastrointestinal function, and use of antibiotics within the past three months, which might interfere with intestinal absorption by
Materials and methods

altering gut microbiota. Additionally, the use of vitamin/fish oil supplements was forbidden from one month before to the end of the trial. The subjects were instructed to maintain their body weight, physical activity, and possible medication constant during the trial. All subjects signed an informed consent. The study protocol was approved by the Ethics Committee of Helsinki University Central Hospital, Helsinki, Finland, and is registered in www.ClinicalTrials.gov under the study ID no. NCT01084226.

The subjects were randomly allocated into two equal groups according to TC concentration and gender. The subjects in the PS group were provided with rye bread enriched with PSs, while the other half of the subjects received identical rye bread but without added PSs (control) in a double-blinded manner. The dose of the added PSs was 2 g/d during the first two weeks of the rye bread period and 4 g/d during the second two weeks of the rye bread period.

All subjects were instructed to keep dietary records during the last three days of each dietary period. In addition, the amount and type of cereal foods were documented daily.

Of the 68 subjects, three dropped out for personal reason or because of time schedule conflicts. Moreover, one subject was excluded after the trial for not returning the food diary and for also reporting not having consumed the rye bread during the trial. Another subject was excluded for changing her hormonal medication during the trial. Ultimately, the data from 63 subjects were included in the statistical analyses (see Table 6 for demographic characteristics).

Table 6. Demographic characteristics of the study subjects participating in the dietary intervention. Values are presented as mean (±SD). Modified from Studies III and IV.

<table>
<thead>
<tr>
<th>Variable</th>
<th>All</th>
<th>PS group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>63</td>
<td>32</td>
<td>31</td>
</tr>
<tr>
<td>Gender (females/males)</td>
<td>47/16</td>
<td>24/8</td>
<td>23/8</td>
</tr>
<tr>
<td>Age (y)</td>
<td>35.8 (12)</td>
<td>34.6 (11.7)</td>
<td>37.1 (12.4)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.8 (13.6)</td>
<td>67.8 (13.5)</td>
<td>69.8 (13.8)</td>
</tr>
<tr>
<td>BMI</td>
<td>24.1 (3.2)</td>
<td>24.0 (3.3)</td>
<td>24.1 (3.2)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.07 (0.85)</td>
<td>5.06 (0.77)</td>
<td>5.08 (0.94)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3.09 (0.74)</td>
<td>3.07 (0.75)</td>
<td>3.11 (0.75)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.63 (0.46)</td>
<td>1.63 (0.48)</td>
<td>1.63 (0.45)</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L)</td>
<td>1.13 (0.51)</td>
<td>1.17 (0.53)</td>
<td>1.08 (0.48)</td>
</tr>
<tr>
<td>Total energy intake (MJ)</td>
<td>7.84 (1.86)</td>
<td>7.83 (1.70)</td>
<td>7.85 (2.04)</td>
</tr>
<tr>
<td>Cereal fiber intake (g/d)</td>
<td>11.70 (4.17)</td>
<td>11.66 (4.64)</td>
<td>11.74 (3.70)</td>
</tr>
</tbody>
</table>

In the investigation of the effect of rye bread intake on the oxidation resistance of isolated LDL particles (Study III), the subjects served as their own controls, as the oxidation resistance of LDL after the second rye period was compared with that obtained at baseline (with null rye intake). In the evaluation of the suitability of rye bread to function as a food carrier for added PSs (Study IV), the decrease in plasma lipids and serum apolipoproteins (TC, LDL-C, apoB/apoA1,
and TC/HDL-C ratios) within the PS group was compared with that of the control group.

4.2 MATERIALS

4.2.1 RYE BREADS
Two kinds of rye breads were used in the study. For the PS group in the dietary intervention, a rye bread enriched with PSs [150] (‘Ruisihme’, Fazer Bakeries, Lahti, Finland) was used. The bread was sourdough-processed and yeast-leavened and consisted of whole-grain rye (68.3%), rye bran (8.3%), water, refined wheat flour (20%), added PSs, barley and rye malt extract, mineral salt, magnesium sulfate, potassium chloride, and yeast. All ingredients are reported on a dry weight basis. The fiber and alkylresorcinol contents of the bread were 9.4 g/100 g and 50.5 mg/100 g, respectively, and the amount of the added PSs was 2.0 g/100 g (on a wet weight basis). For the control group of the dietary intervention trial, an identical rye bread without PS enrichment was specially baked by the same bakery. In the kinetic study, the latter rye bread was solely used.

4.2.2 SOLVENTS, REAGENTS, AND CHROMATOGRAPHIC EQUIPMENT
Acetonitrile, hexane, and methanol (MeOH) were obtained from Rathburn Chemicals Ltd. (Walkenburg, Scotland, UK) and ortho-phosphoric acid from Riedel-de Haën (Seelze, Germany). Silanizing agents trimethylchlorosilane and hexamethyldisilazane were from Pierce (Rockford, IL, USA) and pyridine from Romil (Waterbeach, Cambridge, UK). Acetic acid, diethylether, sodium acetate, potassium dihydrogen phosphate, ascorbic acid, and copper sulfate were obtained from Merck GmbH (Darmstadt, Germany) and β-glucuronidase from Roche Diagnostics GmbH (Mannheim, Germany). Ethylenediaminetetraacetic acid (EDTA) and sulfatase were from Sigma-Aldrich Co. (St. Louis, MO, USA), and ethanol was from Altia Oyj (Rajamäki, Finland). Commercial kits (Konelab 60i) for the determination of serum lipids and apolipoproteins were from Thermo Scientific Oy (Vantaa, Finland). DEAE-Sephadex and sephadex G-25 column were obtained from Pharmacia Biotech, (Uppsala, Sweden). BP-1 and BP-5 columns were from SGE (SGE Analytical Science Pty Ltd., Ringwood, Victoria, Australia). Inertsil ODS-3 column was from GL Sciences Inc. (Tokyo, Japan) and Quick Release RP-18 column from Upchurch Scientific Inc. (Oak Harbor, WA, USA).
4.2.3 STANDARDS
Standards for alkylresorcinols C15:0, C17:0, C19:0, C21:0, and C23:0 were kindly provided by Professor Kristiina Wähälä, or purchased from ReseaChem Life Science (Burgdorf, Switzerland). The internal standard: alkylresorcinol C20:0 was purchased from Dr. Nawaf Al-Maharik, University of St. Andrews (St. Andrews, UK). The standard for DHPPA was from Aldrich (Steinheim, Germany) and for DHBA from IsoSep AB (Tollinge, Sweden). Syringic acid (Syr A) was from Sigma-Aldrich Co. (St. Louis, MO, USA).

4.2.4 INSTRUMENTATION
Ultracentrifugation for separating the lipoprotein fractions was performed with a Beckman Optima LE-80K ultracentrifuge with a Ti 50.4 rotor.

The analytical instruments used for the commercial kits for serum/plasma lipid and apolipoprotein determination were by Thermo Scientific (Vantaa, Finland).

Alkylresorcinols were quantified with gas chromatography-mass spectrometry (GC-MS) (Fisons Instruments, Manchester, UK), consisting of a GC 8000 gas chromatograph and an MD 1000 quadrupole mass spectrometer.

DHPPA and DHBA were analyzed using a high-performance liquid chromatography (HPLC) system by ESA Biosciences, Inc. (Chelmsford, MA, USA) equipped with a model 540 autosampler, two model 580 solvent pumps, and a model 5600 coulometric electrode array detector (CEAD) with eight electrode pairs.

The oxidation resistance of LDL was monitored by UV-spectrophotometer (Safire Scientific, India) with Magellan data analysis software (TECAN, Austria GmbH).

4.3 METHODS

4.3.1 SAMPLE COLLECTION

4.3.1.1 Kinetic study (Studies I and II)
For the blood sampling, a catheter was placed in the cubital vein to avoid numerous needle punctures (altogether 12). All blood samples were drawn in EDTA-coated tubes, cooled to room temperature, centrifuged, and plasma was stored at -20°C until analysis. The subjects collected all urine at the same time-points as blood samples were drawn. Additionally, between the time-points 16 and 24 h the subjects were advised to collect all urine in a container. The volumes of the urine samples were measured, and 10 ml of each sample was stored at -20°C until analysis.
4.3.1.2 Dietary intervention (Studies III and IV)

Blood samples were collected by venipuncture after overnight fasting. Samples were drawn in either EDTA-coated tubes or serum tubes and set aside to cool to room temperature. Samples were then centrifuged to separate plasma and erythrocytes before deep-freezing (-80°C) or further treatment on the same day. The subjects collected overnight (12 h) urine in a container (containing 2 g of ascorbic acid), on the last night of the baseline period as well as during the second two-week rye period. Urine volumes were measured and a 10-mL sample was stored at -20°C until analysis.

4.3.2 ISOLATION OF LIPOPROTEINS AND DETERMINATION OF LIPIDS

Plasma lipoprotein fractions were isolated by sequential ultracentrifugation [151]. The density for very low-density lipoprotein (VLDL) was 1.006 g/mL, the range for LDL 1.019 – 1.063 g/mL, and the range for HDL 1.063 – 1.21 g/mL. Cholesterol and TG concentrations in the lipoprotein fractions were determined by enzymatic methods using commercial kits.

4.3.3 DETERMINATION OF SERUM APOLIPOPROTEINS

Serum concentrations of apoB and apoA1 were determined by immunoturbidimetric methods using commercial kits.

4.3.4 QUANTITATIVE ANALYSIS OF ALKYLRESORCINOL METABOLITES IN SERUM/PLASMA

Plasma (100 µL) together with 20 ng of the internal standard SyrA in 2 µL of MeOH were hydrolyzed overnight at 37°C with a hydrolysis solution (0.1 mol/L Na-acetate buffer pH 5, 2 U/mL sulfatase, and 0.2 U/mL β-glucuronidase). Thereafter, the sample was acidified (pH 3) using 10 µL of glacial acetic acid and extracted with 2 mL of diethylether by vigorously shaking for 2 min. The organic phase was collected and extraction repeated three times. The organic phases were combined, evaporated to dryness under nitrogen, and resuspended with 50 µL of MeOH and 100 µL of HPLC mobile phase (20% phase B / 80% phase A). The mobile phases consisted of 50 mM phosphate buffer (pH 2.3) - MeOH (90:10, v/v) (phase A) and 50 mM phosphate buffer (pH 2.3) - MeOH - acetonitrile (40:40:20, by volume) (phase B). The sample was filtered through a Gelman GHP 0.2-µm filter, and 10 µL was analyzed with HPLC-CEAD. For the analysis, we used a 25-min linear gradient from 0 to 100% phase B, thereafter 12 min 100% phase B, followed by re-equilibration of the column with 0% phase B for 15 min, with a total flow of 0.3 mL/min. The analytical column was an Inertsil ODS-3, 3 x 150 mm with particle size 3 µm, connected to a Quick Release RP-18, 3 x 10 mm guard column. DHPPA was quantified at 570 mV, DHBA at 670 mV, and syringic acid at 380 mV.
4.3.5 QUANTITATIVE ANALYSIS OF ALKYLRESORCINOL METABOLITES IN URINE

A 25-µL sample of the urine together with 25 µL of hydrolysis solution (0.1 mM-sodium acetate buffer (pH 5), 2 U/mL sulfatase, and 0.2 U/mL β-glucuronidase) were incubated overnight at 37°C. Thereafter, 318.5 ng of the internal standard, SyrA (in 50 µL of MeOH and 650 µL of HPLC mobile phase (20% phase B / 80% phase A, for compositions, see Section 4.3.4.), was added, and 10 µL was analyzed with HPLC-CEAD as described above (see Section 4.3.4.).

4.3.6 OXIDATION OF LDL AND HDL

The oxidation resistance of isolated LDL and HDL against copper-induced oxidation was determined using the method by Esterbauer [112]. The lipoprotein fractions were first purified from EDTA using Sephadex G-25 gel filtration, column 1 x 20 cm. EDTA-free LDL or HDL in a concentration of 100 µg protein/mL, in 0.01 M phosphate-buffered saline was exposed to 1.66 µM of freshly prepared copper sulfate (CuSO₄) to initiate oxidation of PUFAs in LDL or HDL. The subsequent formation of conjugated dienes was monitored by UV-spectrophotometer at 234 nm wavelength at room temperature every 3 min for 10 h. The lag time – occurring prior to the formation of the conjugated dienes (propagation phase) – was defined graphically as the time (min) to the intercept of the tangents of the baseline and propagation phase. The slope of the propagation tangent was defined as the velocity of the conjugated diene production (nmol dienes/min mg LDL or HDL protein).

4.3.7 QUANTITATIVE ANALYSIS OF ALKYLRESORCINOLS IN RYE BREADS

The rye breads used in the study were analyzed for their alkylresorcinol contents using a method reported by Ross [34], with slight modifications. Briefly, bread samples were dried and homogenized and thereafter 25 mg was extracted three times with 2 mL of fresh 1-propanol - water (10 mL, 3:1 v/v) on a heating block (2 x 2 h and 1 x 1 h). The combined extracts were evaporated to dryness and reconstituted with 1 mL of ethyl acetate. To 20 µL of the sample, 50 µL of the internal standard, alkylresorcinol C20:0, was added (97.5 ng/mL) and thereafter evaporated to dryness. The silanizing agent (100 µL) (trimethylchlorosilane - hexamethyldisilazane - pyridine 9/3/1 by volume) was added, and the samples were analyzed by GC-MS [45], with slight modifications. For the separation and quantification, we used a BP-1 capillary column. The temperature program was as follows: starting with 150°C for 1 min, increasing by 40°C/min to 230°C, then increasing by 9°C/min to 300°C, holding for 15 min. The temperatures of the injector and interface heating were 300°C and 250°C, respectively. The flow rate was 1 mL/min.
4.3.8 QUANTITATIVE ANALYSIS OF ALKYLRESORCINOLS IN LDL FRACTION

Alkylresorcinols in LDL were quantified using a modification of a plasma method described by Linko et al. [19, 71]. Briefly, to 500 µL of LDL, 39 ng of the internal standard, alkylresorcinol C20:0, in 20 µL of MeOH, and 500 µL of water were added and incubated at 37°C overnight. After incubation, the sample was allowed to cool to room temperature and extracted four times with 4 mL of diethyl ether. The organic phases were combined and evaporated to dryness. The sample was reconstituted with 300 µL of MeOH and thereafter purified using DEAE-Sephadex in free base form. The gel was packed in MeOH in a 0.5 x 1.5 cm column, the sample applied to the column in 300 µL of MeOH, and the tube rinsed with 300 µL of MeOH, then again applied to the column. The column was first eluted with 6 mL of MeOH and discarded. Alkylresorcinols were eluted further with 6 mL of 0.1 M acetic acid in MeOH. This fraction was evaporated to dryness and the sample was derivatized by incubating for 30 min at room temperature with 100 µL of silanizing agent pyridine - hexamethyldisilazane - trimethylchlorosilane 9:3:1 (vol/vol/vol), and then analyzed by GC-MS. The column was a 12.5 m BP-1 with film thickness of 0.25 µm and internal diameter of 0.22 mm. Injection volume of the sample was 1 µL. The carrier gas was helium and the flow rate 1 mL/min. The column temperature was set to 150°C for 1 min, then raised by 40°C/min to 230°C, followed by an increase of 9°C/min to 290°C, kept for 2 min, then increased further by 12°C/min to 300°C and kept for 13 min. The injector temperature was 280°C and that of the ion source 250°C. Ionization energy was 70 eV. Differing from the method by Linko et al. [19], we used ion m/z 268 for quantifying alkylresorcinols.

4.3.9 QUANTITATIVE ANALYSIS OF LIPOPHILIC (PRO)VITAMINS IN SERUM

The analysis of α- and γ-tocopherols as well as α- and β-carotenes was performed by Professor Georg Alfthan at the National Institute of Health and Welfare, Helsinki, Finland. Briefly, 50 µL of serum sample was mixed with 800 µL of 50% ethanolic solution containing ascorbic acid, butylated hydroxytoluol, echinenone (internal standard for carotenoids), and tocol (internal standard for tocopherols), followed by extraction with 1 mL of hexane. A 0.8-mL hexane aliquot was evaporated to dryness under nitrogen and resuspended with 120 µL of MeOH. For the separation of α- and γ-tocopherols, an Inertsil ODS-3 column (2.1 x 100 mm, with film thickness of 3 µm) was used. Injection volume of the sample was 5 µL. The mobile phase was MeOH and the flow rate 0.3 mL/min. The tocopherols were detected by their fluorescence at 292/324 nm using vitamin/tocol peak height ratios. For the separation of α- and β-carotenes, a NovaPak column (3.9 x 150 mm, with film thickness of 4 µm) was used. Injection volume of the sample was 25 µL. The mobile phase was acetonitrile - dichlormethane - MeOH (7:2:1, by volume) and the flow rate 1.2 mL/min. The
Materials and methods

carotenoids were detected at 450 nm using carotenoid/echinenone peak height ratios.

4.3.10 ANALYSIS OF DIETARY INTAKE OF NUTRIENTS
The dietary data were analyzed using Diet32 software, version 1.4.4.2., by Aivo Finland Oy (Turku, Finland), which is based on the food composition database (Fineli) of the National Institute of Health and Welfare (Helsinki, Finland).

4.3.11 PHARMACOKINETIC ANALYSIS OF DHPPA AND DHBA
The kinetic parameters were calculated by Dr. Johan Lundin at the Institute for Molecular Medicine Finland, Helsinki, Finland. The parameters for DHPPA and DHBA were maximum plasma concentration ($C_{\text{max}}$), maximum urinary excretion rate (µmol/h)(ER$_{\text{max}}$), time of reaching $C_{\text{max}}$/ER$_{\text{max}}$ ($t_{\text{max}}$), and $t_{1/2}$, at which the plasma concentration/urinary excretion rate has decreased to half of $C_{\text{max}}$/ER$_{\text{max}}$, and finally the area under the kinetic curve (AUC). Comparisons of the pharmacokinetic parameters between the two metabolites or between genders were carried out by independent or paired $t$-tests. All pharmacokinetic analyses were conducted using STATA software (version 10.0 StataCorp, College Station, TX, USA).

4.3.12 STATISTICAL ANALYSIS
The data obtained in the dietary intervention trial were tested for normality of distribution using the Kolmogorov-Smirnov test. For normally distributed variables, paired $t$-test and independent $t$-test were used to uncover differences within and between groups, respectively. For non-normally distributed variables, Wilcoxon Signed-Rank test or Mann-Whitney U-test was performed. Correlation analysis was conducted using Spearman’s rho. All statistical analyses were conducted using SPSS for Windows, version 15.0 (SPSS, Chicago, IL, USA). P-values < 0.05 were considered significant. The results are presented as means ($\pm$ SD) unless otherwise stated.
5 RESULTS

5.1 PLASMA KINETICS OF ALKYLRESORCINOL METABOLITES (STUDY I)

We determined plasma DHPPA and DHBA concentrations (nmol/L) after a two-day low-alkylresorcinol diet and overnight fasting (0 h), and thereafter following the intake of a single dose of rye bread containing 100 mg (257.6 µmol) of alkylresorcinols, at 11 time-points (3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 25 h), using HPLC-CEAD. Based on the resulting concentrations, pharmacokinetic parameters were calculated and statistical analyses conducted (n = 15). The kinetic curves are drawn according to the mean plasma concentrations of DHPPA and DHBA at each sampling time-point, thus only illustrating the average appearance of these metabolites in the circulation (Figure 11).

![Figure 11](image-url)

**Figure 11.** Mean (± SEM) plasma concentrations of alkylresorcinol metabolites DHPPA and DHBA in 15 individuals before (0 h) and 3 - 25 h after the intake of a single dose of rye bread containing 100 mg (257.6 µmol) of alkylresorcinols. Modified from Study I.

The pharmacokinetic calculations are based on individual parameters (C<sub>max</sub>, t<sub>max</sub>, t<sub>1/2</sub>, and AUC). According to these calculations, C<sub>max</sub> was 350.5 (± 29.7) nmol/L for DHPPA vs. 570.3 (± 45.7) nmol/L for DHBA, (P < 0.0001). Despite these differences, the respective t<sub>max</sub> values were rather similar, 6.4 (± 0.7) and 6.1 (± 0.5) h for DHPPA and DHBA, respectively. Furthermore, a significant (P = 0.0002) difference was present in t<sub>1/2</sub> between DHPPA and DHBA, 16.3 (± 1.8)
Results

and 10.1 (± 0.8) h, respectively. At 25 h, both metabolites appeared in significantly higher concentrations than at 0 h (P = 0.001 for both), suggesting that the elimination from the circulation was not yet completed. Finally, AUC also differed significantly (P < 0.0001) between DHPPA [4269 (± 244) nmol · h/L] and DHBA [6631 (± 389) nmol · h/L].

Plasma concentrations and AUCs were significantly higher in females than in males for both metabolites (P = 0.01 for DHPPA and p = 0.03 for DHBA), although all subjects received an equal dose of alkylresorcinols (Figure 12). However, when adjusted for body weight, the difference disappeared (data not shown). Based on this, we speculated that the metabolic rate is quite similar for both genders, but due to the smaller body weight (and thus, blood volume) of females, their postprandial plasma concentrations appear higher, even though the total amount of the metabolites is quite similar in all subjects.

![Figure 12](image-url)

**Figure 12.** Mean plasma concentrations of alkylresorcinol metabolites DHPPA and DHBA in 8 females and 7 males before (0 h) and 3 - 25 h after the intake of a single dose of rye bread containing 100 mg (257.6 µmol) of alkylresorcinols. Values are not adjusted for body weight.
5.2 **URINARY KINETICS OF ALKYLRESORCINOL METABOLITES (STUDY II)**

DHPPA and DHBA were determined in urine samples collected after a two-day low-alkylresorcinol diet and overnight fasting (0 h), and thereafter following the intake of a single dose of rye bread containing 100 mg (257.6 µmol) of alkylresorcinols at 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 25 h. Furthermore, an overnight (16-24 h) urine sample was collected in a container. The analysis was performed using HPLC-CEAD. The obtained values at each time-point were first corrected by the respective urine volumes and then divided by the number of hours elapsed since the previous collection time-point. The results are presented as urinary excretion rates (ERs) per hour. The kinetic curves of the mean ER of DHPPA and DHBA at each collection time-point are shown in **Figure 13**.

![Figure 13](image)

**Figure 13.** Mean (± SEM) urinary excretion of alkylresorcinol metabolites DHPPA and DHBA in 15 individuals before (at 0 h) and at 3 - 25 h after ingestion a single dose of rye bread containing 100 mg (257.6 µmol) of alkylresorcinols. Modified from Study II.

According to the pharmacokinetic calculations, ER$_{\text{max}}$ for DHPPA was 8.15 µmol/h and for DHBA 4.46 µmol/h (P < 0.0001). However, these distinct ER$_{\text{max}}$ values were reached fairly simultaneously, t$_{\text{max}}$ being 5.40 h for DHPPA and 6.00 h for DHBA (P = 0.167). The respective t$_{1/2}$ values for these metabolites were 11.89 and 9.93 h (P = 0.142, n = 12). Three subjects generated multiple peaks, and therefore, the t$_{1/2}$ could not be determined for them. At 25 h, the ERs for DHPPA and DHBA were still significantly greater than those at 0 h (P = 0.001 for both), indicating an incomplete elimination of these metabolites. The AUC for DHPPA was 67.3 µmol/25 h, which accounts for 26.1% of recovery from the ingested dose of alkylresorcinols (257.6 µmol). This is significantly higher (P
< 0.0001) than the AUC for DHBA, 44.7 µmol/25 h, which accounts for 17.3% of recovery. The total (DHPPA + DHBA) mean urinary recovery was 43.4% of the ingested dose of alkylresorcinols over 25 h. Females and males did not differ significantly in any of the pharmacokinetic parameters (P > 0.168 for all).

5.3 OXIDATION RESISTANCE OF LDL (STUDY III)

In the dietary intervention, we determined the oxidation resistance of isolated LDL particles [112] at baseline (0 g/d of rye intake) and at the end of the second two-week rye period (6 slices, 198 g/d of rye bread). The main outcomes were the duration of lag time and the slope of the propagation phase. We observed a significant prolongation in lag time (14.4%, P < 0.001) as well as a decrease in the slope of the propagation phase (4.5%, P = 0.048) from baseline to the end of the rye period (Figure 14). The observed prolongation in lag time did not differ significantly between subjects who did or did not receive added PSs in rye bread (P = 0.429), nor did the decrease in the slope of the propagation phase (P = 0.261).

Concurrently, the concentration of alkylresorcinols in the LDL fraction (nmol/L plasma) was increased by 8-fold, (P < 0.001), but we were unable to show any correlation between oxidation lag time and alkylresorcinols (P = 0.407 for total alkylresorcinols, P = 0.294 – 0.546 for the separate alkylresorcinol homologs).
The slope of the propagation phase did not correlate with either total alkylresorcinols or separate homologs (P = 0.950 for total alkylresorcinols, P = 0.188 – 0.960 for the separate alkylresorcinol homologs). The concentrations of the alkylresorcinol homologs in LDL samples of the study subjects at baseline and at the end of the second two-week rye period are shown in Figure 15.

![Figure 15](image.png)

**Figure 15.** Concentrations of the alkylresorcinol homologs C15:0 - C25:0 in LDL samples of study subjects at baseline and at the end of the second two-week rye period (with 198 g/d of rye bread intake).

The serum concentrations of lipophilic (pro)vitamins did not change from baseline to the end of the second two-week rye period (P > 0.14 for all). However, TC-adjusted α- and γ-tocopherols correlated positively with the lag time at baseline (r = 0.287, P = 0.024, and r = 0.400, P = 0.001, respectively) and following the rye period (r = 0.329, P = 0.012, and r = 0.456, P < 0.001, respectively).

We observed no significant change in the oxidation resistance (lag time) of HDL (P = 0.319) from baseline to the end of the second two-week rye period. **Table 7** summarizes the results observed in the oxidation study.
Results

Table 7. Summary of results of the LDL oxidation resistance study (III).

<table>
<thead>
<tr>
<th>Determined values</th>
<th>Baseline</th>
<th>Rye period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time in LDL oxidation assay (min)</td>
<td>192.0</td>
<td>219.1 *</td>
</tr>
<tr>
<td>Slope of propagation phase in LDL oxidation assay</td>
<td>1.62</td>
<td>1.55 **</td>
</tr>
<tr>
<td>(nmol dienes/min mg LDL protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkylresorcinol intake from rye bread (mg/d)</td>
<td>0</td>
<td>97.9 *</td>
</tr>
<tr>
<td>Alkylresorcinol concentration in LDL (nmol/L plasma)</td>
<td>6.1</td>
<td>48.0 *</td>
</tr>
<tr>
<td>Serum concentration of α-tocopherol (µmol/L)</td>
<td>22.6</td>
<td>22.8</td>
</tr>
<tr>
<td>Serum concentration of γ-tocopherol (µmol/L)</td>
<td>2.02</td>
<td>1.89</td>
</tr>
<tr>
<td>Serum concentration of α-carotene (µmol/L)</td>
<td>0.44</td>
<td>0.43</td>
</tr>
<tr>
<td>Serum concentration of β-carotene (µmol/L)</td>
<td>0.76</td>
<td>0.75</td>
</tr>
<tr>
<td>Lag time in HDL oxidation assay (min)</td>
<td>73.9</td>
<td>71.9</td>
</tr>
</tbody>
</table>

* P < 0.001 compared with baseline

** P < 0.05 compared with baseline

5.4 PLANT STEROLS AND PLASMA CHOLESTEROL (STUDY IV)

In this part of the dietary intervention, we evaluated the suitability of rye bread to act as a food carrier for added PSs by measuring plasma lipids and serum apolipoproteins in 63 healthy subjects. The subjects in the PS group (n = 32) received 2 g/d of added PSs in the rye bread during the first two-week rye period. The dose was doubled for the second two-week rye period. The control group (n = 31) received identical rye bread, but without the added PSs. The main outcomes were %-changes in LDL-C concentration and apoB/apoA1 ratio.

During the first two weeks of the rye period (3 slices, 99 g/d of rye bread) LDL-C decreased by 8.1% (0.20 mmol/L) and apoB/apoA1 ratio by 8.3% in the PS group compared with controls, both differences being statistically significant (P = 0.021 and P = 0.006, respectively). Accordingly, at the end of the second two-week rye period with a doubled dose of rye bread (6 slices, 198 g/d), the reductions in the PS group were 10.4% (0.33 mmol/L) for LDL-C and 5.5% for the apoB/apoA1 ratio (P = 0.001 and P = 0.043, respectively) compared with controls (Figure 16). The reduction in the apoB/apoA1 ratio was due to a decrease in apoB (8.8% at 2 g/d of PSs and 5.9% at 4 g/d of PSs, compared with controls), while apoA1 remained stable. In the PS group, also TC and TC/HDL-C ratio were reduced following the first two-week rye period (5.1%, P = 0.026, and 7.2%, P = 0.015, respectively), and TC was diminished further during the second two-week rye period (6.5%, P = 0.002) compared with controls. No changes occurred in the HDL-C, VLDL-C, TG, α- and γ-tocopherol, or α- and β-carotene concentrations in study subjects from baseline to the end of the second two-week rye period.
Figure 16. Percentage changes of plasma LDL-C concentration and serum apoB/apoA1 ratio for the PS (n=32) and control (n=31) groups from baseline to the end of the first two-week rye period with 99 g/d of rye bread intake (containing 2 g/d of added PSs for the PS group), and to the end of the second two-week rye period with 198 g/d of rye bread intake (containing 4 g/d of added PSs for the PS group). All %-changes in the PS group differ significantly from those in the control group (P < 0.05). Furthermore, within the PS group, the reductions in LDL-C and apoB/apoA1 ratio from baseline to the end of the second rye period (198 g/d of rye bread with 4 g/d of PSs) were significant (P < 0.05). Modified from Study IV.

Besides documentation in three-day food diaries, we monitored subjects' treatment compliance (intake of rye bread) by determining plasma DHBA concentration (Figure 17). DHBA was chosen as a biomarker rather than DHPPA because it produces higher plasma concentrations. In general, dietary compliance was good and plasma DHBA levels increased following the larger rye bread dose. Furthermore, we observed that plasma DHBA concentration during the first two-week rye period was similar to that in the habitual diet (data not shown), suggesting that Finns commonly consume relatively large amounts of whole-grain rye/wheat foods.
Results

Figure 17. Plasma concentrations of the alkylresorcinol metabolite DHBA, utilized as a biomarker for rye bread intake, at baseline (0 g/d of rye bread intake), after the first two-week rye period with 99 g/d of rye bread intake, and after the second two-week rye period with 198 g/d of rye bread intake (n = 63). In the boxplot presentation, the length of the box is the variable’s interquartile range, containing 50% of the scores. The whiskers extend to the variables’ smallest and largest values, except for the outliers, which are indicated with circles and an asterisk. The horizontal line inside the box indicates the median value. Modified from Study IV.
6 DISCUSSION

6.1 KINETIC STUDY OF ALKYLRESORCINOL METABOLITES (STUDIES I AND II)

The kinetic parameters of DHPPA and DHBA are determined here for the first time. These data are of importance in characterizing the biomarker properties of these compounds concerning whole-grain rye intake. Even though we observed a relatively large interindividual variation in the kinetic parameters, the data indicate that both metabolites possess good biomarker potential in both plasma and urine.

The study subjects had been instructed to avoid whole-grain rye- and wheat-containing foods for two days before entering the study. At baseline, we observed very low plasma and urinary concentrations of DHPPA and DHBA, indicating that the two-day wash-out period was both successful and sufficient. Thereafter, ingestion of the single dose of rye bread triggered a rapid increase in the metabolite concentrations, indicating efficient metabolism and excretion of alkylresorcinols. We observed DHPPA both peaking and possessing t\(_{1/2}\) first in urine and later in plasma, while DHBA peaked simultaneously in plasma and urine and also had a similar t\(_{1/2}\) in both body fluids. DHPPA peaking earlier in urine than in plasma could presumably be explained by its higher glucuronidization rate (39%) than that for DHBA (18%), as reported recently [81]. The hepatic glucuronidization increases the water solubility of DHPPA, thereafter becoming quickly filtrated into urine by the kidneys.

The sampling of blood was interrupted for the night while the collection of urine samples was continued overnight using a container. The lower urinary ER discovered in the overnight urine collection (compared with the preceding or the following ones) suggests a slight slow-down of alkylresorcinol metabolism/excretion while sleeping. The samplings were finished on the following morning at 25 h, after over-night fasting. At this time-point, the final plasma concentrations and urinary ER for the metabolites were still significantly higher than the corresponding baseline values, indicating that even these single samples could potentially distinguish whether whole-grain rye has been consumed by the subject. On the other hand, at 25 h the kinetic curves indicated that the metabolism of the given dose of alkylresorcinols (Figure 11) and the urinary excretion of the formed metabolites (Figure 13) were almost completed. This observation is also supported by calculations according to which the urinary excretion of DHPPA and DHBA during the last hour accounted for only about 2.5% of the total AUC\(_{0-25h}\).

The total AUC\(_{0-25h}\) of the urinary metabolites was also used to calculate recovery, which was altogether 43% of the ingested amount of alkylresorcinols. This is in line with the literature, as the urinary recovery of DHPPA and DHBA was quite recently reported to be 45% after 132 mg/d intake of alkylresorcinols.
The recovery calculations are also supported by the reported absorption of alkylresorcinols, which is around 60% of the ingested amount [69]. Additionally, according to a recent study, it is also possible that a small proportion of the ingested alkylresorcinols has been stored in adipose tissue, therefore escaping detection [75]. The possibility of intact alkylresorcinols being delayed in the circulation as such is minor, as the kinetic data of alkylresorcinols have also described low plasma concentrations at 24 h after ingestion, and furthermore, estimated that AUC_{24-∞} would not exceed 10% of the AUC_{0-∞} [68]. Hence, we considered that the 25-h study period was sufficient.

Females possessed higher plasma concentrations of DHPPA and DHBA than males, presumably due to their smaller body weight (and blood volume). We therefore adjusted the values for body weight. However, as the plasma concentrations of these candidate biomarkers cannot be used for estimating dietary intake (serving size) of whole-grain rye/wheat, it remains unclear whether this kind of correction is essential.

Research among whole-grain cereals or cereal fiber is likely to increase since these food items show health-protecting effects, although the mechanisms have remained partly unclear. Presumably, also remaining samples from previous studies can be explored and relations between whole-grain/fiber exposure and incidence of certain diseases can be analyzed post hoc. Dietary data may not be available for samples or it may have become distorted by identification and quantification problems concerning whole-grain intake. In these cases, a reliable biomarker is a valuable tool. Alternatively, these candidate biomarkers can also be utilized in whole-grain rye-based dietary trials when monitoring treatment compliance. With the data here, we provide support for the use of plasma or urinary DHPPA and DHBA as biomarkers for the intake of whole-grain rye. Plasma DHBA appeared in higher concentrations than DHPPA, but it is worth noting that the dose of alkylresorcinols can play a role in the proportional distribution of these metabolites and a definitive recommendation for the choice of either metabolite as a plasma biomarker of whole-grain rye intake cannot yet be made. However, in urine samples, DHPPA appeared a more promising choice because it had higher concentrations over the whole 25-h period.

Here, we analyzed DHPPA and DHBA using the methods developed in our laboratory [79, 102]. The methods are fast and simple to conduct and are also routinely performed in our laboratory. Compared with a method for alkylresorcinols [19], fewer steps are required. In addition, higher plasma concentrations of DHPPA and DHBA have been reported than those of alkylresorcinols in the same samples [92, 99]. This can be due to the longer t_{1/2} for the metabolites and be an advantage when analyzing samples from subjects with low whole-grain rye/wheat intakes.

Taken together, the present kinetic data indicate that DHPPA and DHBA in both plasma and urine samples possess good biomarker properties concerning whole-grain rye intake. Firstly, a two-day elimination of whole-grain rye/wheat foods from the diet reduced the quantified amounts effectively. Secondly, these
compounds responded rapidly to the ingestion of rye bread, and even at 25 h the levels were significantly higher than at baseline. Finally, after adjusting the plasma concentrations for body weight, no gender differences were present in the pharmacokinetic parameters.

6.2 OXIDATION RESISTANCE OF LDL (STUDY III)

The dietary intervention (Study III) reports a significant increase in the oxidation resistance of LDL from baseline (0 g/d of rye bread) to the end of the second two-week rye period (6 slices, 198 g/d of rye bread intake). The improved oxidation resistance was defined by significantly prolonged lag time as well as by reduced oxidation rate in the Esterbauer assay [112]. Additionally, rye bread consumption induced a significant increase in the LDL alkylresorcinol concentration, and particularly the longer chain homologs C23:0 and C25:0 were accumulated in LDL, which is in line with previous observations [72, 73]. This can be due to the increased lipophility of the longer chain alkylresorcinols, playing a role in the incorporation of alkylresorcinols into the biological membranes [125].

Before Study III, we presumed that alkylresorcinols would play a role as antioxidants in the LDL particles, somewhat similarly to α-tocopherols, the main antioxidants in LDL [113]. This idea was supported by several facts. Firstly, the structure of alkylresorcinols resembles that of α-tocopherols, and both compounds can be found in LDL, although the concentration of α-tocopherols is considerably higher than that of alkylresorcinols. Secondly, according to a recent report, alkylresorcinols (C15:0 and C17:0) have been found to prolong the oxidation lag time in the Esterbauer assay when added directly to the LDL solution [116]. Finally, our own laboratory experiments confirmed the improvement of oxidation resistance of LDL by addition of C17:0 alkylresorcinols to the assay system (data not shown). The results here, did not, however, support our hypothesis, as the increased LDL alkylresorcinol concentration did not correlate positively with the observed oxidation resistance of these particles. Rather, these results were in line with an earlier report indicating poor antioxidant activity for alkylresorcinols [118]. Despite the negative observation, we cannot totally exclude some role of alkylresorcinols in the oxidation resistance of LDL. Alkylresorcinols have been suggested to stabilize the bilayer membranes of erythrocytes by decreasing mobility of the phospholipids [108], but whether this could occur on the surface monolayer of LDL and affect the oxidation resistance of this particle is unknown. Another potential bioactivity for alkylresorcinols concerns their phenolic ring, which in theory could be involved in regenerating α-tocopherols, those being oxidized during the lag time in the Esterbauer assay [112]. An activity like this has been reported for a few other phenols [152]. Moreover, it is possible that other rye-originating phenols or phytochemicals (or their metabolites in humans) are able to regenerate α-tocopherols or possess another kind of antioxidant activity.
We determined the oxidation resistance of isolated HDL particles [112] to evaluate whether these particles could affect the oxidation resistance of LDL. Because the oxidation lag time of HDL did not change significantly following rye bread intake, we assumed that HDL particles did not contribute to the increased oxidation resistance of LDL. Instead, we found moderate correlations between the oxidation lag time of LDL and both tocopherols (α- and γ-) at baseline and at the end of the second two-week rye period, but because these vitamins remained stable during the trial, they are not likely to explain the significant improvement.

The samples from the present dietary intervention (see Section 4.1.2.) were also used for Study IV (Plant sterols and plasma cholesterol). Therefore, half of the subjects received rye bread enriched with PSs, while the other half received rye bread without added PSs. No significant differences were observed in oxidation resistance of LDL between subjects who did and those who did not receive added PSs.

The results from this trial are in contrast to a previous study reporting no improvements in the oxidation resistance of LDL after supplemented rye bran or purified wheat fiber intake compared with the habitual diet [153]. The reason for the lack of effect remained unknown, and furthermore, this study did not report or correlate the obtained results with alkylresorcinols. Noteworthy is, however, that differing from our method, EDTA was present in the oxidation assay [153], and EDTA has the potential to interfere with copper-induced LDL oxidation [110].

Overall, the present dietary intervention shows that rye bread intake (3 slices, 99 g/d during two weeks + 6 slices, 198 g/d during two weeks), compared with baseline diet (0 g/d of rye), caused a significant improvement in the oxidation resistance of LDL. The LDL alkylresorcinol concentration increased significantly following rye bread intake, but was not associated with the oxidation resistance, suggesting that alkylresorcinols are not the major causative antioxidants behind this phenomenon. The oxidation resistance of HDL was not affected by rye bread consumption, suggesting that HDL is also not likely to play a role in the oxidation resistance of LDL. Although α- and γ-tocopherols correlated with the oxidation lag time of LDL at baseline, and also following the second two-week rye period, they are not likely to explain the significant improvement because their concentrations remained stable. Finally, we cannot exclude that alkylresorcinols are involved to some degree in mediating the beneficial effect of rye bread intake on the oxidation resistance of LDL, but it seems likely that some other phytochemical(s) in rye are also involved.

6.3 PLANT STEROLS AND PLASMA CHOLESTEROL (STUDY IV)

The dietary intervention trial (Study IV) demonstrates that intake of rye bread enriched with PSs (2 g/d during two weeks), compared with control rye bread
(without added PSs), significantly reduced several risk factors for CVD. Additional positive changes were induced in some risk factors by doubling the dose of PSs for the following two-week period.

The subjects chosen for this trial were normocholesterolemic or mildly hypercholesterolemic, and therefore, the baseline LDL-C was relatively low (mean 3.1 mmol/L). For the evaluation of PS function, we also included risk factors besides lipids, such as the apoB/apoA1 ratio, which reflects a balance between pro-atherogenic and anti-atherogenic lipoproteins in circulation. This ratio has additionally been suggested to provide more predictive power than the conventional lipid risk factors, particularly in a population with relatively low LDL-C levels [154, 155].

In our study, the PS group, relative to controls, showed significant reductions in TC, LDL-C, apoB/apoA1, and TC/HDL-C ratios. These results indicate that the PS treatment reduced several known risk factors for CVD, besides lipids also the pro-atherogenic lipoprotein apoB, without depleting the anti-atherogenic apoA1. We observed slight, albeit insignificant, increase in these risk factors in the control group during the intervention, but the reason for this remains unknown.

To our knowledge, rye bread has not been used as a food carrier for PSs before, and therefore, the cholesterol-lowering efficacy of PSs in this novel food matrix was of specific interest. The ultimate 10.4% (0.33 mmol/L) reduction in LDL-C in our trial is in agreement with a recent meta-analysis [26] reporting a maximal mean LDL-C reduction of 8.3% (0.32 mmol/L) for PS treatment. This indicates that the action of PSs had not been diminished by the components of rye bread. In other words, rye bread turned out to be a suitable food carrier for PSs, and the incorporation of PSs into rye bread may further enhance the health-protecting effect of rye bread.

PS or plant stanol treatments have been associated with potential reduction in serum carotenoids in some [31, 156], but not all [157, 158], studies. In the present trial we determined the serum concentrations of α- and γ-tocopherols as well as α- and β-carotenes at baseline and after the second two-week rye period (6 slices/d of rye bread containing 4 g/d of PSs for the PS group), and observed no changes in these values.

In the present intervention (Studies III and IV), the recommended intakes of rye bread were 0 g/d (baseline), 3 slices/d (99 g/d), or 6 slices/d (198 g/d). Treatment compliance of study subjects was good. This was monitored by analyzing the three-day food records and also by determining plasma DHBA concentration as a biomarker for rye bread intake. We preferred to utilize DHBA over DHPPA because the former shows a higher plasma concentration according to the results of Study I. Furthermore, we observed that plasma DHBA concentrations after the two rye doses (99 g/d or 198 g/d) differed from each other more clearly than those of DHPPA. We noted that the plasma DHBA concentrations were in line with the rye bread intakes reported in the three-day food diaries, confirming that subjects had followed the given instructions.
Discussion

addition, we analyzed the overall nutrient intakes from the food diaries and observed these to be fairly stable throughout the trial, except for fiber.

A limitation of the study is the relatively short PS treatment periods (two weeks each). Although we assume that a steady state of serum lipids was achieved before blood sampling, it remains unclear whether we would have observed further changes had the dietary periods been longer. On the other hand, based on another study using plant stanol treatment, the major reductions in serum lipids were seen on day 8, with no further reduction occurring between days 8 and 15 [159]. Also, taking into account the fairly high doses of rye bread in our trial (99 and 198 g/d), we were concerned about potential drop-out rates if longer treatment periods had been applied. Another limitation of the study was that we did not synchronize the blood samplings with the day of the menstrual cycle in premenopausal women, which might have affected cholesterol levels in women [160].

In summary, in the present dietary intervention, study subjects received rye bread without (control) or with added PSs (2 - 4 g/d) during 2 + 2 weeks. Compared with controls, the PS group demonstrated a significant reduction in cardiovascular risk factors such as LDL-C, TC, apoB/apoA1, and TC/HDL-C ratios. The results indicate that rye bread is a suitable food carrier for PSs, not interfering with the beneficial action of these dietary supplements. Additionally, serum lipophilic (pro)vitamins were unaffected by PSs. Finally, we have introduced the utilization of plasma DHBA as a biomarker for treatment compliance (rye bread intake) and consider it to be a promising tool for future research.
This thesis explores rye bran-associated alkylresorcinols and their metabolites in healthy humans. We aimed to evaluate the suitability of alkylresorcinol metabolites DHPPA and DHBA as biomarkers for whole-grain rye intake, and also the potential antioxidant activity and cholesterol-lowering effect of rye bread intake on plasma LDL. We presumed that the antioxidant activity would be induced by alkylresorcinols in rye bread, while the cholesterol-lowering effect was expected to be caused by PSs, which were incorporated in rye bread for half of the study subjects. All data presented here were obtained from a kinetic study (Studies I and II) and a dietary intervention (Studies III and IV) using rye bread as a source of alkylresorcinols. The conclusions are as follows:

1) The kinetic parameters for DHPPA and DHBA in human plasma (Study I) and urine (Study II) support the suitability of these substances as biomarkers for whole-grain rye intake. Some gender differences were present in the plasma parameters, but these disappeared after adjusting for body weight. However, whether this adjustment is needed remains unclear. Despite the identical alkylresorcinol dose for all subjects, large interindividual variation in metabolite concentrations was observed. This suggests that quantitative estimations of ingested rye servings cannot be done accurately. Instead, DHPPA and DHBA can provide useful tools in epidemiological studies exploring the association between habitual intake of whole-grain rye and chronic diseases, even when dietary food records are not available. Ultimately, DHPPA and DHBA possess longer plasma $t_{1/2}$ than alkylresorcinols, which could be an advantage when whole-grain intakes are low.

2) Rye bread intake significantly improves in vitro oxidation resistance of LDL (Study III). In addition, a significant increase in the LDL alkylresorcinol concentration was observed following rye bread consumption, but it did not correlate with the oxidation resistance of LDL. The lack of correlation suggests that alkylresorcinols were not the major causative antioxidants, although we cannot exclude the possibility that alkylresorcinols contribute to the oxidation resistance of LDL in some way. Vitamin E ($\alpha$- and $\gamma$-tocopherols) was associated with the oxidation resistance of LDL, but as the level of vitamin E remained stable throughout the trial, it does not explain the significant improvement in resistance. Further studies should be conducted to clarify which substances in rye were involved in the significant improvement in the oxidation resistance of LDL and what mechanism underlies this beneficial change. Of special interest would be to elucidate whether this improvement contributes to the known cardiovascular protection in vivo.
Conclusions

3) Rye bread enriched with PSs significantly lowers LDL-C and apoB/apoA1 ratio, compared with control rye bread (Study IV). The observed reduction in LDL-C is consistent with results in other PS trials, indicating that the efficacy of PSs in our trial was not diminished by the components of rye bread. We can therefore conclude that rye bread is a suitable food carrier for PSs, and at the same time PS enrichment can further potentiate the beneficial health effects of whole-grain rye bread. Ultimately, combining the results from the dietary intervention (Studies III and IV), whole-grain rye bread enriched with PSs can provide dietary protection against CVD, being able to both reduce LDL-C and improve the *in vitro* oxidation resistance of these lipoprotein particles.

4) Plasma DHBA in overnight fasting blood samples served as a suitable biomarker for rye bread intake (study compliance) in our dietary intervention. Plasma DHPPA could also be utilized, but here DHBA showed higher concentrations and responded more clearly to none vs. moderate vs. high intakes of rye bread than did DHPPA.
ACKNOWLEDGMENTS

This thesis project was carried out in the Preventive Medicine Research Program of the Folkhälsan Research Center during 2008-2011. All study participants are thanked for enthusiastic and committed attendance and for making this study possible. I acknowledge the Head of the Folkhälsan Research Institute, Anna-Elina Lehesjoki, for providing excellent research facilities and a positive working environment in Biomedicum Helsinki.

This study was financially supported by the Folkhälsan Research Center, the Sigrid Jusélius Foundation, Fazer Bakeries, the Biomedicum Helsinki Foundation, and Oscar Öflunds Stiftelse. I am sincerely grateful to all contributors for the trust that enabled the study as well as the opportunity to attend international conferences during this project.

My deepest gratitude is owed to my supervisors for giving me the opportunity to join their excellent research group and collaborate on a most fascinating project. Professor Emeritus Matti J. Tikkanen is gratefully acknowledged for his vast expertise and approachable guidance in the complicated field of cardiovascular medicine. The numerous rapid comments on my written output as well as encouragement during the writing of the thesis are most appreciated. Professor Emeritus Herman Adlercreutz is sincerely acknowledged for his open-minded and pioneering ideas in nutrition that inspire research. I am grateful for the many interesting conversations about nutritional topics, which helped me to clarify the importance of nutrition in human health.

Coauthors Professor Georg Alfthan and Docent Johan Lundin are warmly thanked for expertise and generous analytical help, which were crucial for completion of this thesis. Collaborators Sanna-Maria Hongisto, Sampsa Haarasilta, and Jussi Loponen are gratefully acknowledged for their enthusiastic approach towards this project and for all of the support over the years. Sanna is also thanked for being such a joyful travel companion.

I am sincerely grateful to the official referees of my thesis, Professor Emerita Helena Gylling and Docent Marjukka Kolehmainen, for the careful review and valuable comments that improved this thesis immensely.

Carol Ann Pelli is sincerely thanked for revising the language of this thesis.

I have been privileged to work in an experienced and highly skilled group. My first steps in this group and in the laboratory were guided by Inga Wiik, who is warmly acknowledged for the kindness and support that helped me to manage the first challenges and believe in my chances! I am indebted to Päivi Ihamuotila and Päivi Ruha for excellent work with the blood samples - from drawing the samples to the numerous assays, not to mention the troublesome Esterbauer method! Your well-organized and positive approach towards this project made my work a lot easier and more fun. I miss our breakfasts on the 'bridge' on Wednesdays after blood samplings as well as the inspirational all-round
discussions in the relaxed atmosphere that you created around you. Thank you for always making me feel welcome in your lab! Anja Koskela and Adile Samaletdin are warmly acknowledged for outstanding skills in HPLC-CEAD and GC-MS methods and for quantifying alkylresorcinols, their metabolites, and many, many other compounds in food, plasma, and urine samples. Thank you also for sharing your extensive knowledge and giving valuable advice to me over the years! Post-doctoral research fellow Veera Vihma is sincerely thanked for wise and encouraging guidance, warm empathy, and numerous morning briefings, which I treasure. Doctoral students Feng Wang and Hanna Paatela are thanked for helping me with diverse issues and for sharing joyful chats! In such an outstanding research group, happy moments were worth celebrating and sad feelings easier to carry.

I thank Kirsti Räsänen, Paula Kokko, and Merja Lahtinen for carrying out blood samplings when we needed backup or extra pairs of skilled hands.

The secretaries and office staff of the Folkhälso Center; Marjatta Valkama, Madeleine Avellan, Solveig Halonen, Stephan Keskinen, Jaana Welin-Haapamäki, and Nina Forss are thanked for all the practical help during these years. Marjatta, special thanks for the cheerful moments that often alleviated my troubles and also widened my perspective.

I am truly grateful to Docent Anu Turpeinen for guiding me with great expertise in the paperwork and practical aspects of a dietary intervention trial.

Teemu Masalin and Jaakko Vartia are thanked for the technical support that helped me to enjoy writing this thesis with my Mac!

My deepest gratitude goes to all previous and present coworkers at the Folkhälso Center for the nice lunch and coffee breaks on the 'bridge'! Mervi Kuronen, Aila Ahola, Anna Naukkarinen, Anne Juvonen, Vilma-Lotta Lehtokari, Tarja Joensuu, and Outi Kopra are thanked for encouragement and providing valuable advice on the thesis, Ann-Liz Träskelin, Eira Leinonen, Maria Sandbacka, Tuula Soppela, and Hanna Hellgren for the cheerful spirit and kind care, to mention but a few! Maija Wessman is warmly thanked for giving me good advice and determinedly pushing me towards this destination. Catharina Sarkkola, Sabina Simola, Veera Seppänen, Kaarina Penttilä, and the ever-growing FinnHit-group are thanked for warm support and joyful lunch conversations. Catharina is also thanked for hearty friendship outside of work.

All of the people around the 4th floor coffee table in room C415a are warmly thanked for memorable occasions as well as numerous morning coffees in nice and joyful company!

Irma Isotalo is gratefully acknowledged for her inspiring attitude and for help during the application process for this position. I also thank Endla Lipre for supporting my efforts ever since my undergraduate years at Viikki. I am happy that our paths still cross!

Outside work, friends of our family as well as hobby mates are thanked for support and joyful times. Marja-Liisa is warmly thanked for encouragement and sparring! Benita and Outi are especially thanked for long-lasting friendship! A
special thanks is reserved for the members of RaKo, my colleagues in nutrition, for numerous, delightful, and sparkling chats over dinners around the town.

I owe my dearest thanks to my family and relatives. My parents Pirjo and Erkki are thanked for the love, support, and infinite faith shown in me, as well as for taking care of our household and children, whenever working days became too long. My sister Nina and her family, and my brother-in-law Ludde are thanked for unflagging support and for being an important part of my life. My parents-in-law Kitten and Unna are thanked for always being there for us, for helping and caring, and for sharing their cheerful spirit. Maisi is thanked for kind support and positivity. Finally, I thank my beloved husband Jens and our sweet children Ella and Rasmus for unconditional love, curiosity towards my work, and lots of patience during this long project.

Helsinki, November 2012
9 REFERENCES


References


References


References


but showed low to modest reproducibility over one to three years in U.S. women. J Nutr 2012;5:872-7.


References


