Bioavailability of enterolignans and their relation with chronic diseases

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Biobeschikbaarheid van enterolignans en hun relatie met chronische ziekten

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ABSTRACT

Lignans are biphenolic compounds that occur in foods of plant origin. Some of the plant lignans can be converted into the enterolignans, enterodiol and enterolactone, by the microorganisms in the colon. Because of their biological activities, enterolignans may affect the development of chronic diseases. It is not sufficiently known to what extent enterolignans become bioavailable, i.e., are absorbed and used for metabolic processes in the body.

The aim of the present research was to gain further insight in the bioavailability of enterolignans and in their relation with several chronic diseases. To be able to do this, we developed a liquid chromatography-tandem mass spectrometry method using triply ^{13}C-labeled isotopes for the simultaneous quantification of enterodiol and enterolactone in plasma.

Enterodiol and enterolactone absorption started 8 to 10 hours after consumption of secoisolariciresinol diglucoside, an isolated plant lignan, and they were eliminated slowly. A substantial part (~40%) of the enterolignans was excreted in urine, and thus had been available in the blood circulation. Because of the slow elimination, enterolignans will accumulate and reach steady state concentrations in plasma when consumed 2 to 3 times a day. As lignans are present in many foods this is very likely to happen. The bioavailability of lignans from flaxseeds, a high lignan source, improved substantially when whole seeds were replaced by crushed or ground seeds. Independent determinants of plasma concentrations of enterolignans were, besides the intake of plant lignans, use of antibiotic therapy, defecation frequency, and body mass index.

Our data suggest a protective role of enterolignans against colorectal adenomas; the risk reduction was ~2-fold in highest versus lowest quartile of enterolignan plasma concentrations. However, a protective effect could not be confirmed for colorectal carcinomas. Moreover, we observed increased risks (~2.5-fold) in women, especially in postmenopausal women, and in subjects with a high body mass index. This suggests that an estrogen-related hormonal mechanism might be involved. In addition, our data do not support a protective role of enterolignans against the development of nonfatal myocardial infarction.

In conclusion, a substantial part of the enterolignans enters the blood circulation and is subsequently excreted in urine. Enterolignans might protect against colorectal adenomas. We did not find protective associations for colorectal carcinomas and myocardial infarction. At this point, there is not enough evidence to give recommendations regarding the consumption of foods rich in lignans.

Keywords: *Lignans; enterodiol; enterolactone; plasma; flaxseed; bioavailability; liquid chromatography; mass spectrometry; case-control studies; prospective studies; colorectal adenomas; colorectal carcinomas; coronary heart diseases*

CONTENTS

General introduction

Chapter 1

GENERAL INTRODUCTION

Consumption of whole grains, fruits, vegetables, and cereal fiber has been associated with lower risks of colon or colorectal cancer [1-3]. This has traditionally been explained by dietary fiber, although epidemiological data are inconsistent [4]. The beneficial effect might be related to other dietary components, such as lignans, present in whole grains, fruits and vegetables.

Plant lignans are biphenolic compounds, which are constituents of plant cell walls and present in plant roots, stems, leaves, seeds, and fruits. Some of these plant lignans can be converted by intestinal bacteria into the enterolignans, enterodiol and enterolactone [5, 6] (**Figure 1.1**). Enterolignans are present in a range of biological fluids. It has been shown *in vitro* that they influence, among others, the proliferation of breast tumor cells, colon tumor cells, and vascular endothelial cells, and the activity of steroid metabolic enzymes. Furthermore, they possess antioxidant, antigenotoxic, and anti-angiogenic activity. Because of these activities, they may affect the development of cancer and coronary heart disease (reviewed in [7-9]).

In order to evaluate the role of enterolignans in the prevention of chronic diseases, we need information on the exposure to lignans in humans. The exposure to lignans can be measured as the total amount of lignans consumed (external exposure), or as the total amount of enterolignans and plant lignans available for processes inside the body (internal exposure). In this thesis we describe a) studies that evaluate the bioavailability of a major plant lignan and the pharmacokinetic parameters of enterolignans, and b) studies that quantify the associations between internal enterolignan exposure and colorectal adenomas, colorectal cancer, and myocardial infarction. This introduction provides information on the food sources and dietary intake of plant lignans, on current knowledge with regard to absorption, metabolism and excretion of enterolignans, on biological activities, and on potential health effects found in observational studies. Finally, the rationale and outline of this thesis are described.

FOOD SOURCES AND DIETARY INTAKE OF PLANT LIGNANS

One of the richest sources of plant lignans is flaxseed, which contains mainly secoisolariciresinol diglucoside. Other important sources are whole grains, seeds, fruits and vegetables, and beverages, such as coffee and tea [10-15]. Although concentrations in these products are much lower than in flaxseed, they contribute substantially to the dietary intake in western diets. The most important sources of plant lignans consumed in western populations are beverages like tea and coffee, seeds, cereals, berries, fruits and vegetables [16-18]. The estimated daily intake (only secoisolariciresinol and matairesinol) in western diets varies from 100-1100 µg/day [17, 19-22]. Until recently, no information was available on food contents of pinoresinol and lariciresinol, other important precursors of enterolignans, but now Milder et al. have determined the content of plant lignans of more than 100 food samples, including these two precursors [14]. They reported a daily
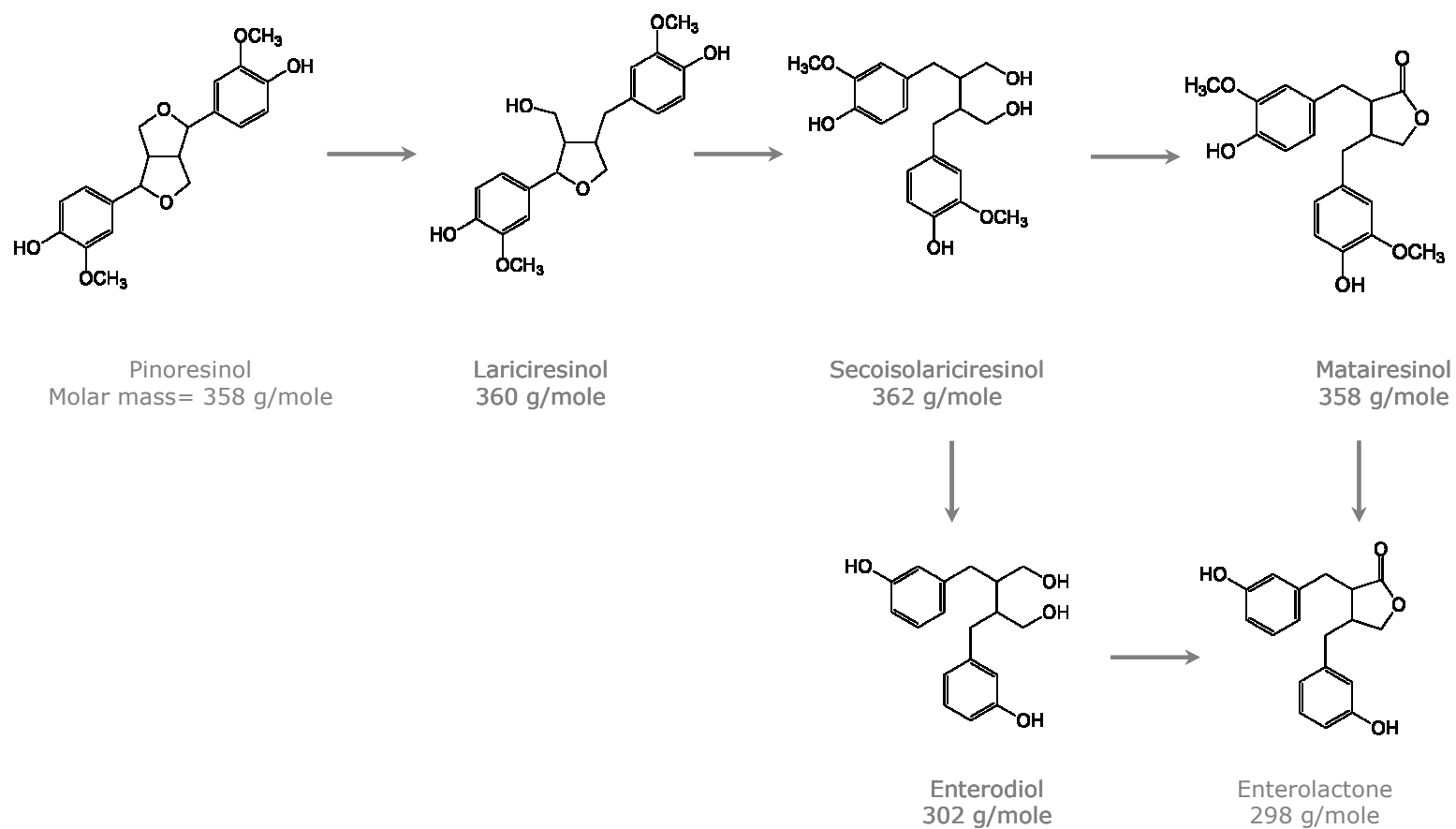
Figure 1.1 Possible metabolic pathways for transformation of plant lignans to enterolignans by colonic microflora. The molar mass of the structures is also given.

Pinoresinol
Molar mass= 358 g/mole

Lariciresinol
360 g/mole

Secoisolariciresinol
362 g/mole

Matairesinol
358 g/mole

Enterodiol
302 g/mole

Enterolactone
298 g/mole

intake of 1241 µg/day in the Dutch population [16]. Pinoresinol and lariciresinol contributed 75% to the total lignan intake.

BIOAVAILABILITY OF LIGNANS

Bioavailability can be defined as the fraction of the ingested plant lignans that is absorbed and that can be used for metabolic processes (internal exposure) and storage in the body. Following metabolism of plant lignans in the human colon, the metabolites of plant lignans, enterodiol and enterolactone, will reach the circulation and target tissues. As metabolism is extensive, enterodiol and enterolactone might be more important for potential health effects than the parent compounds. In this thesis we measure the bioactive enterolignans in plasma instead of the plant lignans to determine the bioavailability. The bioavailability of lignans is determined by several processes (**Figure 1.2**): (1) Conversion of plant lignans to enterolignans in the human colon; (2) Absorption of enterolignans from the colon, which determines whether or not enterolignans become available in the blood circulation; (3) Distribution and metabolism, which determines whether enterolignans reach the target tissues where they can have an effect and whether enterolignans are further metabolized. Finally, (4) enterolignans can be excreted from the body via feces or urine.

Knowledge about the bioavailability of plant lignans and enterolignans is scarce. Factors that might influence lignan bioavailability include, among others, intestinal microflora, antibiotic use, food matrix, type and form (aglycone or conjugate) of plant lignan, chronic exposure, and other host related factors like age and gender [23]. To date, these food and compound related factors have not been studied.

Conversion of plant lignans to enterolignans

After consumption of the plant lignans, a small fraction is absorbed as such in the small intestine [24] and excreted in urine [25, 26]. However, the largest fraction of the plant lignans is transported to the colon where they can be converted (metabolized) into the enterolignans, enterodiol and enterolactone, by intestinal bacteria (Figure 1.1). It has long been assumed that only secoisolariciresinol and matairesinol can be converted into enterolignans. However, in an in vitro experiment using human feces, other enterolignans precursors, i.e., lariciresinol, pinoresinol, acrtigenin, 7-hydroxymatairesinol, syringaresinol, and medioresinol were discovered [27]. The most important precursors of enterolignans were lariciresinol, pinoresinol, secoisolariciresinol, and matairesinol, which had conversion degrees >55%. The other precursors had conversion degrees ≤15%. Secoisolariciresinol, lariciresinol, and pinoresinol can be converted to enterodiol and subsequently to enterolactone by intestinal bacteria [28, 29]. Matairesinol can be converted directly to enterolactone [6].
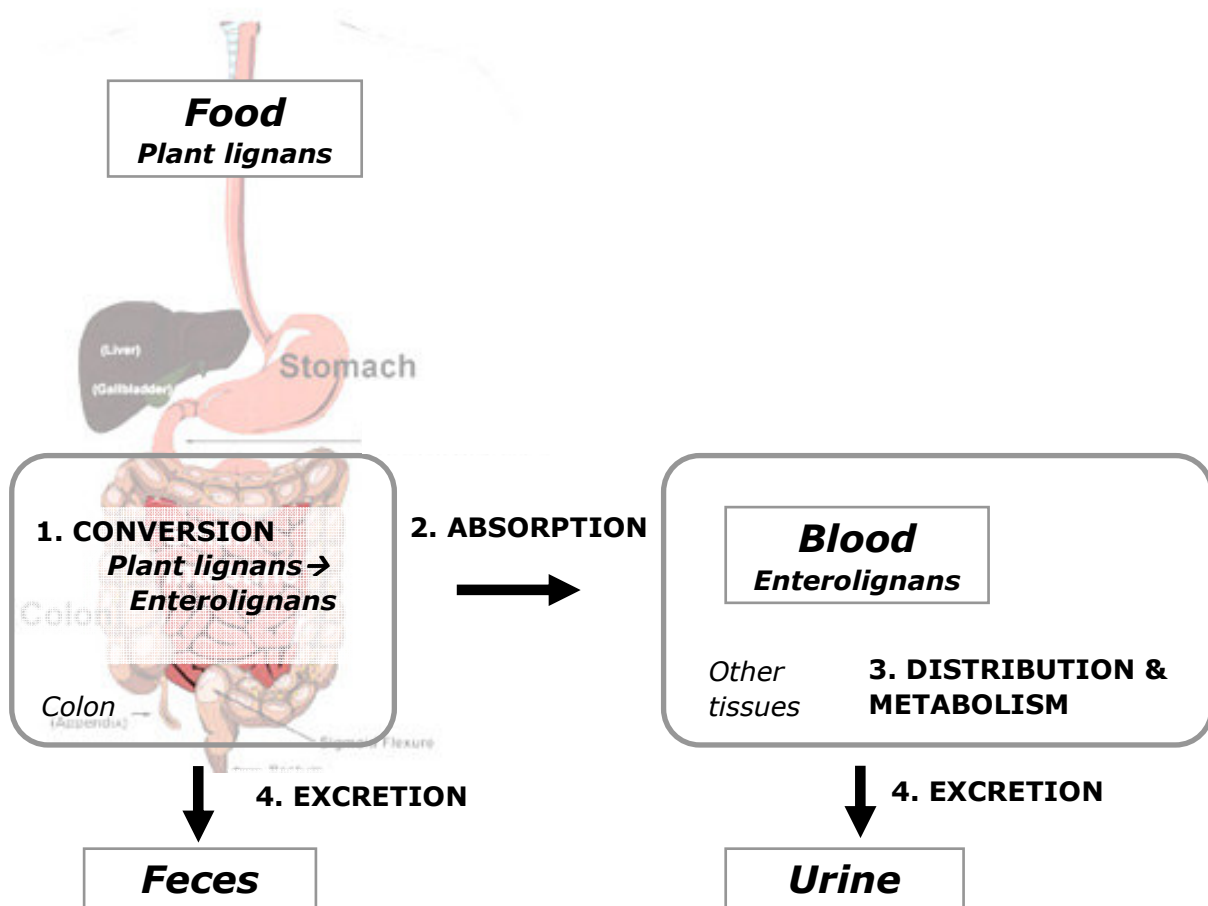
Figure 1.2 Schematic overview of the main bioavailability processes of lignans.

The importance of the microflora in the metabolism of lignans is apparent from studies in germ-free rats [30, 31], which have drastically reduced urinary concentrations of enterolignans. Furthermore, in humans use of oral antibiotics, which have a pronounced impact on he intestinal microflora, decreased urinary and plasma concentrations of enterolactone substantially [32, 33]. Although the intestinal bacteria play a crucial role in lignan metabolism, only few studies have been published that identify organisms involved in lignan breakdown. Two strict anaerobes, *Peptostreptococcus* and *Eubacterium*, were able to catalyze the demethylation and dehydroxylation of secoisolariciresinol [28]. A bacterial strain responsible for transformation of pinoresinol to lariciresinol was identified as *Enterococcus faecalis* [29]. Recently, Clavel et al. [34] isolated two organisms *Peptostreptococcus productus* and *Eggerthella lenta,* which were able to demethylate and dehydroxylate secoisolariciresinol and pinoresinol. Although enterolactone concentrations are usually higher than enterodiol concentrations in humans [34], up until now only one bacterial strain was identified that was responsible for transformation of enterodiol to enterolactone (strain ED-Mt61/PYG-s6) [35].

Absorption, metabolism, distribution, and excretion of enterolignans

Once formed, enterolignans are absorbed from the large intestine into the bloodstream or directly excreted via feces. Plasma enterodiol and enterolactone circulate either as glucuronide and sulfate conjugates or as free forms [36]. They are excreted via urine or bile. In urine, enterodiol and enterolactone are excreted in conjugated form; primarily as monoglucuronides (85 and 95%, respectively) with small percentages being excreted as monosulfates (2-10%) and free aglycones (0.3-1%) [37, 38]. Conjugated enterolignans that are excreted via bile, can undergo enterohepatic circulation (i.e. they are excreted through the bile duct into the intestinal tract, further metabolized in the colon, and reabsorbed from the large intestine into the bloodstream) [31, 39]. Due to, among others, different consumption patterns [40], variation in microflora, and use of antibiotics [6, 32], plasma and urinary concentrations of enterodiol and enterolactone vary widely between persons.

Habitual levels in humans

Enterolignans have been quantified in several types of biologic fluids, including urine [41, 42], feces [43, 44], serum and plasma [36, 45], prostatic fluid [46], and amniotic fluid [47]. Enterodiol has been studied less frequently than enterolactone because sufficiently sensitive methods were not available. Thompson et al [9] summarized data on the physiologic ranges of enterolignans in urine and plasma that have been observed in various populations worldwide. Mean urinary excretion ranged from 0.3-6 µmol/ 24 h. Mean plasma lignan concentrations (enterodiol plus enterolactone) appear to be in the range of 10-30 nmol/L in studies where plasma was collected from individuals that consumed their usual diets [9]. In vegetarians enterolignan plasma concentrations and urinary excretion are usually much higher; plasma concentrations up to 1000 nmol/L and urinary excretion up to 400 µmol/24 h have been reported [36]. Usually enterolactone is the lignan in highest concentration, both in plasma [36, 48, 49] and urine [42, 50]. However, higher enterodiol than enterolactone concentrations in plasma and urine have also been reported [51]. Note however, that this was observed after supplementation with high amounts of plant lignans (flaxseed).

Pharmacokinetics of enterolignans

Pharmacokinetics involves the time-dependent change in concentration in body fluids due to absorption, distribution, and elimination (i.e. excretion and metabolism). Pharmacokinetic studies are essential in understanding the relationship between a nutrient and the physiological effect. So far, few studies have examined the rate and extent of absorption of enterolignans in humans. Plasma and urinary concentrations of enterolignans increased after eating flax or flax containing products for several weeks [52-57]. Three studies investigated the absorption and excretion of enterolignans after a single dose of lignan rich foods [51, 58, 59]. Plasma concentrations of enterolactone increased 6-9 h after consumption. Highest plasma concentrations were measured at 24 h; highest urinary excretion was measured between 25-36 h. In these studies no plasma

samples were taken after 24 h. In rats, 48 h after ingestion of ^{3}H-secoisolariciresinol diglucoside, more than 50% of the lignans was excreted via feces, and around 30% was found in urine [60]. Data on pharmacokinetic parameters, such as elimination half-life, time to reach the maximum concentration, and mean residence time, of enterodiol and enterolactone are lacking.

BIOLOGICAL ACTIVITIES OF ENTEROLIGNANS AND PLANT LIGNANS

A large body of experimental studies have been reviewed by Adlercreutz [61], Thompson [9], Wang [62], McCann [7], Magee [63], and Webb [8], and others. There are several mechanisms by which lignans may protect against cardiovascular diseases and cancer.

Due to their chemical structure, enterolignans can bind to estrogen receptors α and ß [64]. But because their efficacy is less than endogenous estrogens, they may actually block or antagonize the effects of estrogen in some tissues [62]. Enterodiol and enterolactone also appear to influence steroid metabolism in vitro, not only by acting on steroid receptors but also by modulating steroid genesis, e.g., sex hormone binding globulin synthesis, 5α-reductase, and 17β-hydroxy-steroid dehydrogenase [65-68]. Lignans have also been shown to have antioxidative effects, which can be another mechanism underlying their potential protective effects on cancer and cardiovascular diseases. The plant lignan secoisolariciresinol and the enterolignans, enterodiol and enterolactone, have demonstrated antioxidant activity in vitro at a concentration range of 10-100 µM [69, 70]. These are extremely high concentrations, which have not been observed in human plasma. Enterodiol was the strongest antioxidant in those studies, followed by enterolactone and secoisolariciresinol. However, in another study enterodiol and enterolactone were not effective in preventing H_2O_2 -induced DNA damage in HT 29 cells and enterolactone did not reduce intracellular oxidative stress at similar concentrations [71].

Enterolactone is capable of stimulating a detoxifying phase II enzyme NADPH: quinone reductase at concentrations between 0.01-1 µmol/L in colonic cells [72]. This suggests another mechanism by which enterolignans may be implicated in cancer chemoprevention.

It should be noted that many of these effects have been shown at very high concentrations (0.5-100 µmol/L) that are not likely to be achieved in plasma of human subject consuming foods containing lignans. In addition, the in vitro studies have been performed with the unconjugated forms of enterolignans, which only constitute a minor fraction, if present at all, inside the body. Conjugation may greatly influence bioactivity.

EPIDEMIOLOGICAL STUDIES

The health effects of lignans can be evaluated in epidemiological studies that use both the intake of plant lignans (external exposure) and plasma or urinary concentrations (internal exposure) of enterodiol and enterolactone as exposure estimates. To data, 28 studies have evaluated the association between lignans and cancer (**Table 1.1**), and 4 studies have evaluated the association

between lignans and cardiovascular diseases (**Table 1.2**).

The association between lignans and **breast cancer** was evaluated in 17 studies. Ten studies used plasma, serum or urinary enterolignan concentrations as exposure measure, 7 studies used dietary intake. All 4 case-controls studies using plasma, serum or urinary concentrations, reported an inverse associations between lignans and breast cancer risk [50, 73-75]. When Dai et al. [76] included only postmenopausal women in their analysis, the inverse association was no longer significant. In the Italian study the inverse association was no longer significant when intracystic fluid concentrations of enterolactone were used instead of serum concentrations [77]. Five out of 6 prospective studies using plasma, serum or urinary enterolignan concentrations, reported no associations between lignans and breast cancer risk [48, 78-81]. Only Olsen et al. reported an inverse association between plasma enterolactone and breast cancer risk, with an OR of 0.55 (95% CI: 0.36-0.85) in the highest quartile versus the second quartile. [82].

Three case-controls studies using dietary intake as exposure measure observed an inverse association between lignans and breast cancer, but only in premenopausal women [83-85]. Two other cases-controls studies showed no association [86, 87]. The 2 prospective studies on breast cancer risk and dietary intake of lignans found no relationship with dietary intake of lignans [19, 88]. Horn-Ross et al. [19] reported that secoisolariciresinol was associated with increased risk but this association was substantially reduced after adjustment for consumption of wine, an important source of secoisolariciresinol.

Few observational studies examined the relation between lignans and **prostate cancer** risk. In none of these studies associations were observed between plasma enterolactone and prostate cancer [18, 49, 89-92]. Significant or borderline significant protective associations were reported for **ovarian cancer** [93], **endometrial cancer** [94], **thyroid cancer** [95], and **lung cancer** [96] but not for **testicular cancer** [97]. A positive association between plasma enterodiol concentrations and **premalignant lesions of the cervix** was reported by Hernandez et al [49]. No studies on lignans and **colorectal cancer** risk are published.

The relationship between lignans and **cardiovascular diseases** has been studied in 3 prospective studies (Table 1.2). One Finnish studies reported that high plasma concentrations of enterolactone are associated with a lower risk of acute coronary events, and cardiovascular disease-related death in men [98, 99]. The risk reduction for acute coronary events was 65% (95% CI 12-86%), 56% (4-80%) for coronary heart disease-related death, and 45% (borderline significant) for cardiovascular disease-related death. However, 2 prospective studies about intake of plant lignans or serum enterolactone concentrations were not associated with cardiovascular diseases [100, 101]. In Finnish male smokers no association was observed between serum enterolactone and nonfatal myocardial infarction or coronary death [100]. Furthermore, dietary intake of plant lignans was not associated with coronary heart disease or cerebrovascular events in women [101]. Note however, that in the latter study only the plant lignans secoisolariciresinol and matairesinol were

included in this study and that the intake was measured using a disputable scoring method (see comment [16, 102]). But all 3 studies show risk estimates below unity.

In summary, observational studies have yielded contradictory results on the relation between lignans and breast cancer risk. No associations were observed with prostate cancers. For the cancers other than breast and prostate no conclusions can be drawn because few studies were performed. No studies on colorectal cancer risk are published. The strong inverse associations found for plasma enterolactone concentrations and the risks of coronary heart diseases are supportive for a beneficial role of lignans, although only 2 out of 4 studies showed a protective effect. More studies are needed to evaluate these potential protective effects of lignans on cardiovascular diseases.

EXPOSURE ASSESSMENT

The quality of the exposure measure is essential when studying the relationship between lignans and disease outcome. Until recently, food composition tables lacked information on the dietary plant lignans, lariciresinol and pinoresinol, which are also extensively converted to enterolignans. These compounds should be included in future evaluations of the health effects of dietary lignans. When concentrations in biological fluids are used as marker of exposure, enterolactone is usually measured and not enterodiol; only 4 out of 18 studies measured enterodiol in addition to enterolactone (Table 1.1 and 1.2). Because enterodiol and enterolactone might have different effects due to their slightly different structures, future studies should include both enterodiol and enterolactone. Time-resolved fluorimmunoassay, a commonly used analytical method, is not available for enterodiol [103, 104]. A simple, rapid, and sensitive analytical method, which is suitable for measuring large numbers of samples, is needed for the quantification of both enterodiol and enterolactone and was developed by us.

Table 1.1 Prospective and retrospective studies on lignan concentrations in body fluids (internal exposure) or dietary lignan intake (external exposure) and incident cancer

Reference	Country	Design	No of cases[1]	Exposure measure	Lignan[2]	Comparison (high vs low)[3]	Adjusted RR (high vs low)[4]	*P* for trend
Breast cancer (n=17 studies)								
Grace, 2004 [48]	UK	Nested-case-control	114 F	Urine	END	Doubling of exp. ($\log_2$)	1.02 (0.84-1.23)	0.87
					ENL	Doubling of exp. ($\log_2$)	0.98 (0.85-1.13)	0.79
			97 F	Serum	END	Doubling of exp. ($\log_2$)	0.91 (0.74-1.13)	0.39
					ENL	Doubling of exp. ($\log_2$)	1.00 (0.82-1.20)	0.96
Kilkkinen, 2004 [78]	Finland	Nested-case-control	206 F	Serum	ENL	>32.3 vs <9.2	1.30 (0.73-2.31)	0.48
Zeleniuch-Jacquotte, 2004 [79]	US	Nested-case-control	189 F, pre-MP	Serum	ENL	>24.1 vs <5.0	1.6 (0.7-3.4)	0.13
			228 F, post-MP			>29.0 vs <5.4	1.0 (0.5-2.1)	0.95
Olsen, 2004 [82]	Denmark	Nested-case-control	381 F	Plasma	ENL	>48.0 vs Q2: 14.5-28.1	0.55 (0.36-0.85)	-
Hulten, 2002 [80]	Sweden	Nested-case-control	248 F	Plasma	ENL	>27.4 vs <10.2	1.1 (0.7-1.7)	-
Den Tonkelaar, 2001 [81]	Netherlands	Nested-case-control	88 F	Urine	ENL	>656 vs <379 µmol/mol creatinine	1.43 (0.79-2.59)	0.25
Boccardo, 2004 [73]	Italy	Case-control	18 F	Serum	ENL	>8.0 vs ≤8.0	0.36 (0.14-0.93)	0.04
Boccardo, 2003 [77]	Italy	Case-control	12 F	Intracystic fluid	ENL	>98.0 vs <98.0	0.7 (0.22-2.27)	0.5
Dai, 2003 [76]	China	Case-control	117 F, post-MP	Urine	END+ENL	-	0.50 (0.23-1.10)	0.09
Dai, 2002 [74]	China	Case-control	250 F	Urine	END	-	0.43 (0.26-0.71)	<0.01
					ENL	-	0.42 (0.25-0.69)	<0.01
					END+ENL	-	0.42 (0.25-0.69)	<0.01

Reference	Country	Design	No of cases[1]	Exposure measure	Lignan[2]	Comparison (high vs low)[3]	Adjusted RR (high vs low)[4]	*P* for trend
Breast cancer (continued)								
Pietinen, 2001 [75]	Finland	Case-control	194 F	Serum	ENL	>34.8 vs <6.2	0.38 (0.18-0.77)	0.03
			68 F, pre-MP			>30.0 vs <5.5	0.42 (0.10-1.77)	0.18
			126 F, post-MP			> 37.7 vs <6.3	0.50 (0.19-1.28)	0.10
Ingram, 1997 [50]	Australia	Case-control	144 F	Urine	END	>480 vs <170 nmol/24h	0.73 (0.33-1.64)	0.29
					ENL	>5250 vs <1460 nmol/24 h	0.36 (0.15-0.86)	0.01
					MAT	>42 vs <17 nmol/24 h	2.18 (0.83-5.76)	0.31
Keinan-Boker, 2004 [88]	Netherlands	Cohort	280 F	Diet	END+ENL[5]	>830 vs <530	0.70 (0.46-1.09)	0.06
Horn-Ross, 2002 [19]	US	Cohort	711 F	Diet	MAT	>33 vs <12	1.1 (0.8-1.4)	0.2
					SECO	>121 vs <48	1.4 (1.0-1.8)	0.02
Linseisen, 2004 [85]	Germany	Case-control	278 F, pre-MP	Diet	MAT	>39 vs <20	0.58 (0.37-0.94)	0.03
					SECO	>1280 vs <274	1.12 (0.73-1.73)	0.65
					SECO+MAT	>1331 vs <298	1.10 (0.72-1.70)	0.84
					END[5]	>582 vs <242	0.61 (0.39-0.98)	0.03
					ENL[5]	>453 vs <227	0.57 (0.35-0.92)	<0.01
					END+ENL[5]	>1164 vs <483	0.61 (0.39-0.98)	0.03
McCann, 2004 [83]	US	Case-control	315 F, pre-MP	Diet	SECO+MAT	>673 vs <329	0.66 (0.44-0.98)	-
			807 F, post-MP			>713 vs <337	0.93 (0.71-1.22)	-
Dos Santos Silva, 2004 [86]	UK	Case-control	240 F	Diet	MAT	>13.3 vs <4.9	0.82 (0.48-1.41)	0.46
					SECO	>225 vs <80	0.69 (0.40-1.18)	0.27
					SECO+MAT	>236 vs <85	0.75 (0.44-1.29)	0.30

(Table 1.1 continued)

Reference	Country	Design	No of cases[1]	Exposure measure	Lignan[2]	Comparison (high vs low)[3]	Adjusted RR (high vs low)[4]	*P* for trend
Breast cancer (continued)								
McCann, 2002 [84]	US	Case-control	301 F, pre-MP	Diet	END+ENL[5]	>2480 vs <460	0.49 (0.32-0.75)	-
			439 F, post-MP				0.72 (0.51-1.02)	-
Horn-Ross, 2001 [87]	US	Case-control	1272 F	Diet	MAT	>50 vs <18	1.1 (0.9-1.5)	-
					SECO	>176 vs <75	1.3 (1.0-1.6)	-
					SECO+MAT	>224 vs <104	1.3 (1.0-1.6)	-
Prostate cancer (n=5 studies)								
Stattin, 2004 [89]	Sweden	Nested-case-control	265 M	plasma	ENL	>28.3 vs <9.4	1.05 (0.65-1.69)	-
Kilkkinen, 2003 [91]	Finland	Nested-case-control	214 M	serum	ENL	>24.4 vs <5.9	0.71 (0.42-1.21)	0.37
Stattin, 2002 [90]	Scandinavia	Nested-case-control	794 M	plasma, serum	ENL	>15.6 vs <4.3	1.08 (0.83-1.39)	-
Hedelin, 2006 [92]	Sweden	Case-control	209 M	serum	ENL	>37.8 vs <15.2	0.74 (0.41-1.32)	0.82
			1431 M	diet	MAT	>4.0 vs <2.1 μg/d MJ	0.86 (0.63-1.16)	0.4
					SECO	>13.6 vs 7.6 μg/d MJ	1.04 (0.77-1.41)	0.7
					SECO, MAT, PINO, LARI, SYR, MED[6]	>213 vs <114 μg/d MJ	0.85 (0.65-1.12)	0.3
Strom, 1999 [18]	US	Case-control	83 M	diet	MAT	>46 vs <46	0.89 (0.47-1.66)	0.71
					SECO	>483 vs <483	1.20 (0.65-2.21)	0.55

Reference	Country	Design	No of cases[1]	Exposure measure	Lignan[2]	Comparison (high vs low)[3]	Adjusted RR (high vs low)[4]	*P* for trend
Testicular cancer								
Walcott, 2002 [97]	US	Case-control	159 M	diet	SECO+MAT	>1416 vs <275 µg/d 1000 kcal	0.96 (0.11-8.09)	0.27
					END+ENL[5]	>302 vs <170 µg/d 1000 kcal	0.73 (0.21-2.56)	0.09
Ovarian cancer								
McCann, 2003 [93]	US	Case-control	124 F	diet	SECO+MAT	>708 vs <304	0.43 (0.21-0.85)	-
Endometrial cancer								
Horn-Ross, 2003 [94]	US	Case-control	482 F	diet	MAT	>49 vs <18	1.60 (0.99-2.40)	0.07
					SECO	>197 vs <87	0.63 (0.40-0.98)	<0.01
					SECO+MAT	>239 vs <121	0.68 (0.44-1.10)	0.03
			94 F, pre-MP		SECO+MAT	>239 vs <121	0.77 (0.26-2.30)	0.42
			362 F, post-MP		SECO+MAT	>239 vs <121	0.57 (0.34-0.97)	0.02
Thyroid cancer								
Horn-Ross, 2002 [95]	US	Case-control	590 F	diet	MAT	>57 vs <18	0.72 (0.46-1.10)	0.49
					SECO	>107 vs <42	0.56 (0.35-0.89)	<0.01
					SECO+MAT	>161 vs <65	0.68 (0.43-1.10)	0.07
Lung cancer								
Schabath, 2005 [96]	US	Case-control	1674	diet	SECO+MAT	>9116 vs <3413	0.73 (0.59-0.90)	<0.01
					END+ENL[5]	>478 vs <252	0.67 (0.53-0.84)	<0.01

(Table 1.1 continued)

Reference	Country	Design	No of cases[1]	Exposure measure	Lignan[2]	Comparison (high vs low)[3]	Adjusted RR (high vs low)[4]	*P* for trend
Schabath (continued)			774 F		SECO+MAT	>8402 vs < 2941	0.89 (0.65-1.22)	0.51
					END+ENL[5]	>459 vs <242	0.59 (0.43-0.82)	<0.01
			900 M		SECO+MAT	>9698 vs <3673	0.73 (0.54-0.98)	0.02
					END+ENL[5]	>499 vs <261	0.75 (0.54-1.04)	0.02
Premalignant lesion of the cervix								
Hernandez, 2004 [49]	Hawaii	Case-control	122 F	plasma	END	>2.5 vs <0.1	2.7 (1.1-6.3)	0.01
					ENL	>17.3 vs <2.1	2.4 (1.0-5.8)	0.06

[1] pre-MP, premenopausal women; post-MP, postmenopausal women.

[2] ENL, enterolactone; END, enterodiol; MAT, matairesinol; SECO, secoisolariciresinol; LARI, lariciresinol; PINO, pinoresinol; SYR, syringaresinol; MED, medioresinol; - no data provided.

[3] Category cutoff value or as indicated; plasma and serum values in nmol/L, urine values as indicated, intake levels in µg/d or as indicated.

[4] 95% CI in parentheses.

[5] Dietary intake based on END and ENL production from foods determined by in vitro fermentation with human fecal microflora.

[6] Sum of plant lignans multiplied by conversion factors (SECO, 0.72; MAT, 0.62; LARI, 1.01; PINO, 0.55; SYR, 0.04; MED, 0.80).

Table 1.2 Prospective studies on serum lignan concentrations or dietary lignan intake and cardiovascular diseases[1]

Reference	Country	Outcome	No of cases	Exposure measure	Lignan[2]	Comparison (high vs low)[3]	Adjusted RR (high vs low)[4]	P for trend
Kilkkinen, 2006 [100]	Finland	nonfatal myocardial infarction	205 M	serum	ENL	> 28.2 vs <5.0	0.67 (0.37-1.23)	0.10
		coronary death	135 M				0.57 (0.26-1.25)	0.18
Vanharanta, 2003 [99]	Finland	CVD death	103 M	serum	ENL	>23.9 vs <6.9	0.55 (0.29-1.01)	0.04
		CHD death	70 M				0.44 (0.20-0.96)	0.03
Vanharanta, 1999 [98]	Finland	CHD events	167 M	serum	ENL	>30.1 vs <7.2	0.35 (0.14-0.88)	0.03
van der Schouw, 2005 [101]	Netherlands	CHD events	372 F	diet	SECO+MAT	>1392 vs <738	0.92 (0.65-1.29)	-
		CBV events	147 F				0.80 (0.45-1.42)	-
		Total events	519 F				0.89 (0.66-1.19)	-

[1] CVD, cardiovascular diseases (ICD-9 390-459); CHD, coronary heart diseases (ICD-9 410-414, and 427.5); CBV, cerebrovascular events (ICD-9 430-438) ; - no data provided.

[2] ENL, enterolactone; END, enterodiol; MAT, matairesinol; SECO, secoisolariciresinol.

[3] Mean, median or category cutoff value; plasma and serum values in nmol/L or as indicated, urine values as indicated, intake levels in µg/d.

[4] 95% CI in parentheses.

RATIONALE AND OUTLINE OF THIS THESIS

The objectives of this thesis are a) to determine the bioavailability of major plant lignans in humans and b) to quantify the associations between plasma enterolignans and several chronic diseases in observational studies (**Figure 1.3**). First of all, we developed and validated an analytical method to quantify enterodiol and enterolactone in plasma and urine because sufficiently sensitive methods were not available, especially for plasma enterodiol. Secondly, we carried out single and multiple dose studies to understand some aspects of the link between external and internal lignan exposure (bioavailability). Furthermore, we studied the use of plasma enterolignans as a biomarker of dietary intake. Finally, we studied the association between enterolignans and colorectal adenomas, colorectal cancer and myocardial infarction. Epidemiological studies on the relation between plasma enterodiol and enterolactone and these outcome measures have not been published. The main findings from these studies are summarized and discussed in the general discussion (*Chapter 9*).
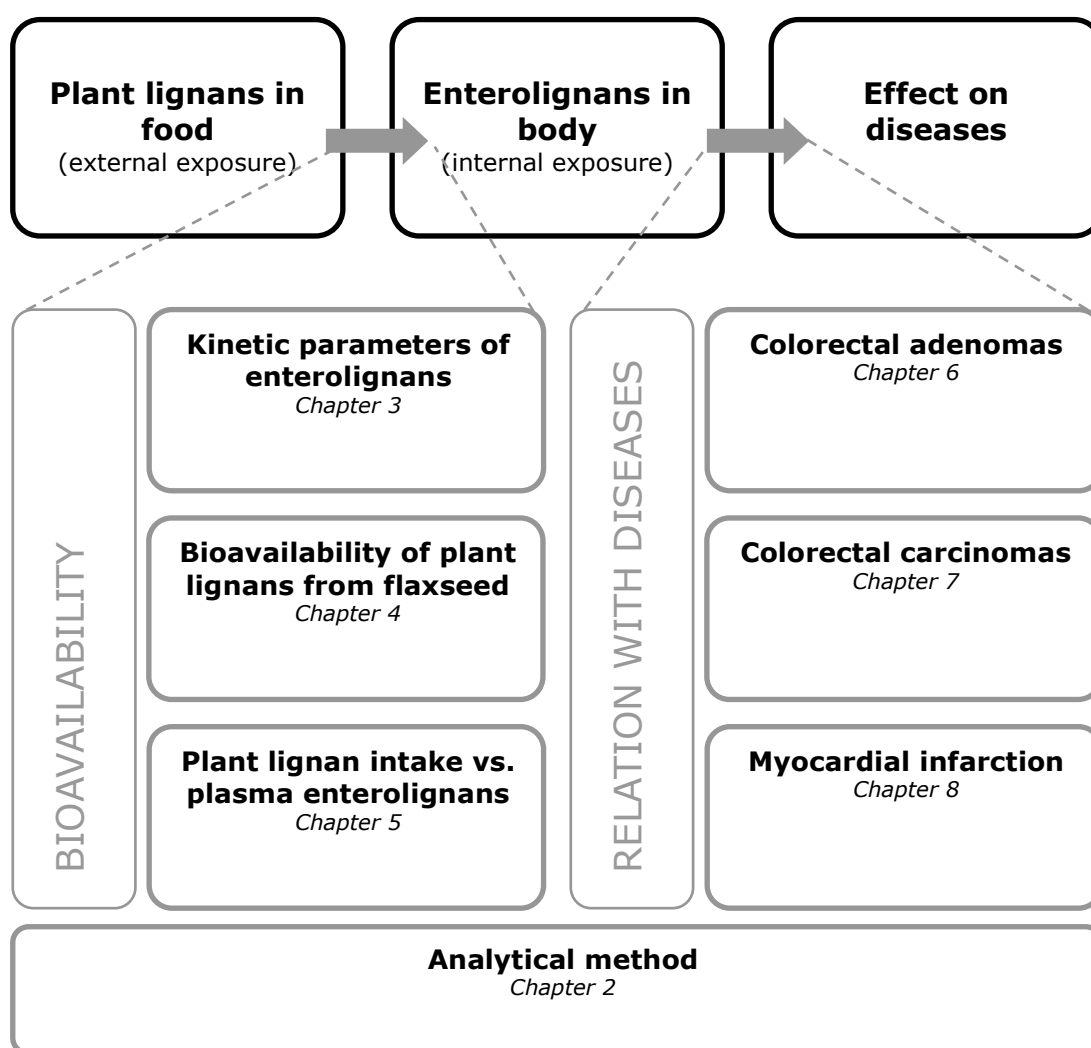

Figure 1.3 Schematic overview of the bioavailability and epidemiological studies described in this thesis

Analytical method for the quantification of enterolignans in plasma and urine

In order to quantify enterodiol and enterolactone in plasma, we developed and validated a liquid chromatography-tandem mass spectrometry method with electrospray ionization using triply ^{13}C-labeled isotopes. Our aim was to obtain a specific, simple, straightforward and robust method with sufficient sensitivity to measure low concentrations (>0.5 nmol/L) of both enterolignans applicable to the analysis of large numbers of samples (*Chapter 2*). The analytical method for the quantification of enterolignans in urine is described in the method section of *Chapter 3*.

A. BIOAVAILABILITY OF ENTEROLIGNANS

Bioavailability of enterolignans in single and multiple dose studies in humans

In order to evaluate the internal exposure to enterolignans, data on the absorption, distribution, and excretion of enterolignans are needed. So far, no studies have been carried out with isolated lignans in humans. The pharmacokinetic parameters of enterolignans, which describe absorption, distribution and elimination processes, and the urinary excretion of enterodiol and enterolactone, were evaluated after consumption of the purified plant lignan, secoisolariciresinol diglucoside (*Chapter 3*). Twelve healthy volunteers ingested a single dose of purified secoisolariciresinol diglucoside. This study was performed with isolated secoisolariciresinol diglucoside, one of the major plant lignans that can be converted to enterolignans. To avoid the influence of the food matrix it was given in purified form. Plasma and urine were collected and analyzed.

To determine the influence of the food matrix on the relative bioavailability of enterolignans from flaxseed we carried out a crossover study with multiple doses of flaxseed. The bioavailability is important to estimate the internal exposure when only data on external exposure are available. One of the richest sources of lignans is flaxseed, which is a small hard-coated seed increasingly used in food products or as a supplement. Whole seeds are used in breads, whereas most supplements consist of crushed seeds. We questioned whether lignans in whole flaxseeds are accessible to bacteria in the colon. We expected that milling or crushing could substantially enhance the accessibility of the bacteria to the plant lignans, and as a result, could improve their conversion into enterolignans. In *Chapter 4* we describe whether milling and crushing enhances the bioavailability of enterolignans from flaxseed. In a randomized crossover study twelve healthy subjects supplemented their diet with whole, crushed, or ground flaxseed for a number of days. Blood samples were collected and plasma enterodiol and enterolactone were measured using the newly developed LC-MS/MS method.

Plasma enterolignans as biomarker of dietary intake (external exposure) or as biomarker of internal exposure

We studied the relation between plasma enterolignans and intake of plant lignans in a population based study to evaluate the use of plasma enterolignans as biomarkers of dietary intake of plant

lignans (*Chapter 5*). In this study we determined also important determinants of plasma enterolignans, other than the major plant lignans. Understanding the sources of variation that modulate the internal exposure is important to be able to use plasma enterolignans as a measure of dietary intake or as biomarker of internal exposure.

B. ENTEROLIGNANS AND THEIR RELATION WITH DISEASES

Associations between enterolignans and colorectal adenomas, colorectal cancer and myocardial infarction in epidemiological studies

Consumption of lignan containing products, such as grains, nuts and seeds, fruits, vegetables, and tea has been associated with lower risks of colorectal cancer. However, no epidemiological studies have examined the associations between plasma enterolignans and colorectal cancer. We evaluated the relation between plasma enterodiol and enterolactone and colorectal adenomas, a precursor of colorectal cancer, in a case-control study (*Chapter 6*). Additionally, we determined the association between plasma enterodiol and enterolactone and colorectal cancer in a prospective study (*Chapter 7*). In the latter study we used a nested case-control design.

Recent epidemiological studies suggest that high enterolactone plasma concentrations are associated with a lower risk of acute coronary events. The association between plasma enterodiol and cardiovascular diseases has not been published. In *Chapter 8*, we describe the relation between plasma enterodiol and enterolactone and myocardial infarction in a cohort study.

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ABSTRACT

Enterolactone and enterodiol are phytoestrogens with structural similarity to endogenous estrogens. Because of their biological activities, they may affect the development of several diseases. To quantify enterodiol and enterolactone in plasma, we developed and validated a liquid chromatography-tandem mass spectrometry method with electrospray ionization using $^{13}C_3$ labeled isotopes. The method consists of a simple enzymatic hydrolysis and ether extraction followed by a rapid liquid chromatography separation (run-time of 11 min). Detection limits as low as 0.15 nmol/L for enterodiol and 0.55 nmol/L for enterolactone were achieved. The within-run coefficient of variation (CV) ranges from 3-6% and the between-run CV ranges from 10-14% for both enterolignans. This method allows simple, rapid, and sensitive quantification, and is suitable for measuring large numbers of samples.

Keywords: *lignans; enterodiol; enterolactone; plasma; liquid chromatography; mass spectrometry*

A validated method for the quantification of enterodiol and enterolactone in plasma using isotope dilution liquid chromatography with tandem mass spectrometry

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Chapter 2

INTRODUCTION

Enterolactone and enterodiol **(Figure 2.1)**, also called enterolignans, are phytoestrogens with structural similarity to endogenous estrogens. Enterolignans have demonstrated antioxidant [1, 2], and weak (anti-) estrogenic effects [3, 4]. They are capable of induction of NADPH: quinone reductase (phase II enzymes) [5] and can inhibit enzymes involved in the metabolism of sex hormones (e.g. sex hormone binding globulin, 5α-reductase, and 17β-hydroxy-steroid dehydrogenase) [6-8]. Because of these activities, they may affect the development of several diseases. Epidemiological studies suggest that high serum concentrations of enterolactone are associated with a lower risk of acute coronary events [9, 10]. Associations between enterolignans and cancer are unclear. Inverse associations for breast or prostate cancer were reported only in case-control studies [11-13], whereas no associations were found in three prospective studies [14-16] (reviewed by Arts and Hollman [17]).

Enterolignans are products of bacterial conversion of the plant lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol in the human colon [18]. Plant lignans are present in flax, grains, seeds, fruits and vegetables, olive oil, and beverages, such as tea, coffee, and wine [19-23]. After consumption of these plant lignans, enterolactone and enterodiol are found as glucuronide and sulfate conjugates in human plasma, urine, and feces [24]. Due to different consumption patterns and variation in microflora, plasma concentrations of enterodiol and enterolactone vary widely between persons. For example, Kilkkinen et al [25] reported enterolactone concentrations between 0 and 100 nmol/L in men (n = 1168) and 0 and 180 nmol/L in women (n = 1212); Adlercreutz et al [24] found higher concentrations in vegetarians (up to 1000 nmol/L; n = 14).

Techniques that have been used to measure enterolignans are time-resolved fluorescence immunoassay, gas chromatography-mass spectrometry (GC-MS), and liquid chromatography (LC) in combination with ultra violet, diode array, mass spectrometry (MS), or electrochemical detection. For routine measurements in human biological fluids, the immunoassay combines the
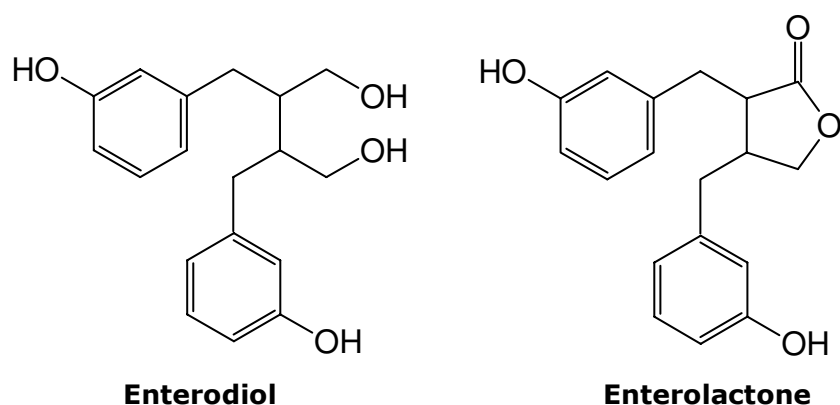

Enterodiol   **Enterolactone**

Figure 2.1 Chemical structures of enterodiol and enterolactone

advantage of high sensitivity with low costs. A major drawback of this method is that enterodiol, one of the two enterolignans, cannot be measured. Various aspects of method performance as well as benefits and limitations of the above mentioned techniques have been discussed by Wilkinson et al [26].

Recently, Grace et al [27] developed a liquid chromatography-tandem mass spectrometry method (LC-MS/MS) for isoflavones and lignans using $^{13}C_3$ labeled isotopes. A range of these phytoestrogens can be measured simultaneously with this method. We set out to adapt this method specifically for the quantification of enterodiol and enterolactone in human plasma. Our objective was to obtain a simple, straightforward and robust method applicable to the analysis of large numbers of samples, for instance from epidemiological studies. In this paper, we describe the results of the optimization of hydrolysis, extraction, and chromatographic conditions and evaluate the method's performance. The method described here is suitable for analyzing large numbers of samples due to a simple sample treatment, a short chromatographic run time, and simultaneous detection of both compounds.

EXPERIMENTAL

Materials

Pure standards of enterodiol and enterolactone were obtained from Fluka Chemie GmbH (Buchs, Switzerland). The internal standards, $^{13}C_3$-enterodiol and $^{13}C_3$-enterolactone (purity >97%; refers to the isotopic purity of the sum of all labeled C atoms, with C_0 <0.4%), were purchased form Dr. Botting (University of St Andrews, Scotland). β-Glucuronidase-sulfatase (EC 3.2.1.31) from *Helix Pomatia* (G7017, G1512) and β-glucuronidase from bovine liver (G0501) were obtained from Sigma (St. Louis, MO, USA). Another β-glucuronidase-sulfatase from *H. Pomatia* (cat. no. 104114) was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and water was purified with a Milli-Q system.

Human test samples

Human plasma samples were obtained from two men and one woman aged 28 to 53 years. Plasma samples with relatively high concentrations of enterolignans (mean concentration of enterodiol: 7.0 nmol/L; enterolactone: 39.2 nmol/L), hereafter called 'high lignan plasma', were obtained after these subjects had consumed 25 g crushed flaxseed per day for 3 successive days. Plasma samples with low concentrations of enterolignans (mean concentration of enterodiol: 0.6 nmol/L; enterolactone 5.4 nmol/L), hereafter called 'low lignan plasma', were obtained after the same subjects had followed a diet poor in lignans for 3 successive days: they were not allowed to eat whole grain products, seeds, nuts and some specified fruits and vegetables. Plasma was prepared from venous blood samples drawn into vacuum tubes containing EDTA. The samples were centrifuged within 30 min at 1187 x g for 10 min at 4°C.

Subsequently, the plasma samples of these three subjects were pooled and homogenized. Samples were stored at -80°C until analysis.

Hydrolysis and extraction procedure

Total enterodiol and enterolactone concentrations were measured after hydrolysis of the conjugates using a freshly prepared enzyme mixture of β-glucuronidase-sulfatase from *H. Pomatia* (G1512) in sodium acetate buffer (0.5 M, pH 5.0). First, 10 µL of a mixture of $^{13}C_3$-labeled enterodiol and enterolactone (500 nmol/L) was added into 4 mL vials. Subsequently, 300 µL plasma, 300 µL sodium acetate buffer (0.1 M, pH 5.0), and 60 µL enzyme mixture (2600 units β-glucuronidase) were added. The samples were incubated at 37°C for 4 h and subsequently extracted twice with 1.5 mL diethyl ether. The samples were shaken with a Vortex mixer for 5 s and centrifuged after each extraction (2300 x *g*, 10°C, 10 min). The two ether fractions were combined and transferred into tubes containing 500 µL 40% methanol/water (v/v). The ether fraction was evaporated under a gentle stream of nitrogen at 30°C with a Turbovap evaporator (Zymark, Hopkinton, MA, USA), after which the tubes were shaken with a Vortex mixer for 5 s. Prior to analysis, extracts were filtered through acrodiscs containing a 0.45-µm hydrophilic polyvinylidene fluoride membrane (Pall Corporation, Ann Arbor, MI, USA), transferred into vials, and injected into the LC-MS/MS system.

Chromatography and detection conditions

A Waters Alliance chromatography separation module 2690 (Milford, MA, USA) was used, which consisted of a chromatographic system equipped with a binary pump, and an auto sampler with a cooled sample tray kept at 10°C. Separations were performed on an XTerra MS C_{18} column (50 x 3.0 mm, 5 µm; Waters, Milford, MA, USA), which was placed into a column oven set at 40°C. The mobile phase consisted of a mixture of water and methanol and was run at a flow rate of 0.4 mL/min. The gradient, starting at 10% methanol for 1 min, was increased linearly to 80% methanol in 6 min, which composition was kept for 0.5 min. Returning to the starting conditions in 0.5 min, the column was allowed to equilibrate in 3 min. The total run time was 11 min. The sample injection volume was 100 µL. The LC eluate was introduced into the mass spectrometer at 0.2 mL/min after a 50:50 (MS/waste) split. The divert valve was programmed to allow flow into the mass spectrometer from 4 to 9 min of each run.

Mass spectrometry

Detection was performed with a Micromass Quatro Ultima triple quadrupole mass spectrometer (Waters-Micromass, Manchester, UK) equipped with an electrospray probe. The mass spectrometer was operated in negative ion electrospray mode, with the capillary voltage at 2.5

Table 2.1 Precursor and product ion combinations of enterodiol, enterolactone, and their internal standards.

Compound	Precursor ion	Product ion	Confirmation ion
Enterolignans			
Enterodiol	301.1	253.1	106.2
Enterolactone	297.1	253.1	107.2
Internal standards			
$^{13}C_3$-enterodiol	304.1	256.1	
$^{13}C_3$- enterolactone	300.1	255.1	

kV. Nitrogen was used as desolvation gas and cone gas. Desolvation gas was used at a flow rate of 550 L/h and cone gas at a flow rate of 50 L/h. Source and desolvation gas temperatures were set at 120 and 350°C, respectively. Dwell time was set at 0.5 s for each transition. Product ions were formed by collision-induced dissociation with argon as collision gas at a pressure of 2.3×10^{-3} mbar, and a collision energy ranging from 20-36 eV. **Table 2.1** summarizes the characteristic precursor and product ions used for determination of enterodiol, enterolactone, and their internal standards. The most abundant fragment ion was used for quantification, while a second, less abundant, ion was used for confirmation by means of the observed ratio. In samples containing low concentrations of enterolignans confirmation was not always possible due to lack of sensitivity. Integration of peak areas was performed using MassLynx software (Waters-Micromass, Manchester, UK).

Calibration curves

Calibration curves were constructed in low lignan plasma (enterodiol 0.6 nmol/L, enterolactone 5.4 nmol/L). Calibration standards were freshly prepared for each series of analyses. Plasma samples were spiked with known concentrations of a standard mixture of enterodiol and enterolactone (0, 0.3, 1, 3, 10, 30, and 100 nmol/L), and with 10μL of a mixture of triply ^{13}C labeled enterodiol and enterolactone (500 nmol/L). Subsequently, the plasma samples were hydrolyzed and extracted as described above. Calibration standards were injected at the start and at the end of each series of analyses. The average of the calibration standards within each series was used to obtain the calibration curve. Calibration curves were constructed by plotting the response factor (area enterolignan/area internal standard) against the concentration of the calibration standard. Because the plasma samples used for calibration contained small amounts of enterolignans, the response factor of a non-fortified plasma sample was subtracted from the response factor of the individual calibration samples. The calibration curves were forced through the origin.

Optimization experiments

To find optimal conditions for hydrolysis and extraction of enterolignans in plasma, we used an HPLC method with electrochemical detection (coulometric), which had been developed to measure enterolignans in cell culture medium and intracellular fluids [28]. For quantification of both enterolignans, carbon working and reference electrodes were used, and the cell potential was increased to 650 mV. Four types of *β*-glucuronidase-sulfatases from *H. Pomatia,* and one type of *β*-glucuronidase from bovine liver were evaluated for the hydrolysis of the enterolignans. The amount of enzyme (range 120-3500 units), the incubation time (0-6.5 h), and temperature (37 and 50°C) were varied in order to obtain complete hydrolysis. High lignan plasma was used in these experiments. When plasma samples with very low concentrations of enterolignans were analyzed with electrochemical detection, the resolution from interfering matrix components proved to be insufficient. To improve the resolution, we tested several columns: Chromolith (100 x 4.6 mm, Merck, Darmstadt, Germany), Symmetry (250 x 4.6 mm, 5 μm) and Nova-Pak (250 x 4.6 mm, 4 μm; 250 x 3.9 mm, 4 μm) from Waters (Dublin, Ireland), Discovery (250 x 4 mm, 5 μm, Supelco, Bellefonte, PA, USA), and Inertsil ODS 3 (250 x 4.6, 5 μm, Alltech, Deerfield, IL, USA). In addition, we varied the pH and type of buffer of the mobile phases (phosphate buffer pH 2.4, citrate buffer pH 3.7, and sodium acetate buffer pH 4.8-5.4) and tested several gradients (methanol, acetonitrile) for optimal separation of enterodiol and enterolactone from interfering peaks in plasma samples. Minor improvements in separation from matrix peaks were obtained. Therefore, HPLC with MS/MS detection, which has excellent specificity, was chosen instead as the preferred method.

Limits of detection

Limit of detection was determined by injection of 100 μL of five plasma samples with low amounts of enterodiol and enterolactone (range: 0.1-5.0 nmol/L) on 10 different days. The limit of detection was defined as the amount of enterodiol or enterolactone that resulted in a peak height three times the standard deviation of the baseline noise. The signal to noise ratio was calculated using MassLynx 4.0.

Recovery, within-run and between-run variability

To calculate the recovery, low lignan plasma samples (enterodiol 0.6 nmol/L, enterolactone 5.4 nmol/L) were spiked with standard solutions of enterolignans (10, 30 and 100 nmol/L). To assess the within-run variation, six identical high lignan plasma samples (concentration of enterodiol: 7.0 nmol/L; enterolactone: 39.2 nmol/L) were analyzed in one run. To assess the between-run variation, high and low lignan plasma samples were analyzed in duplicate on 22 separate days within a period of 3 months.
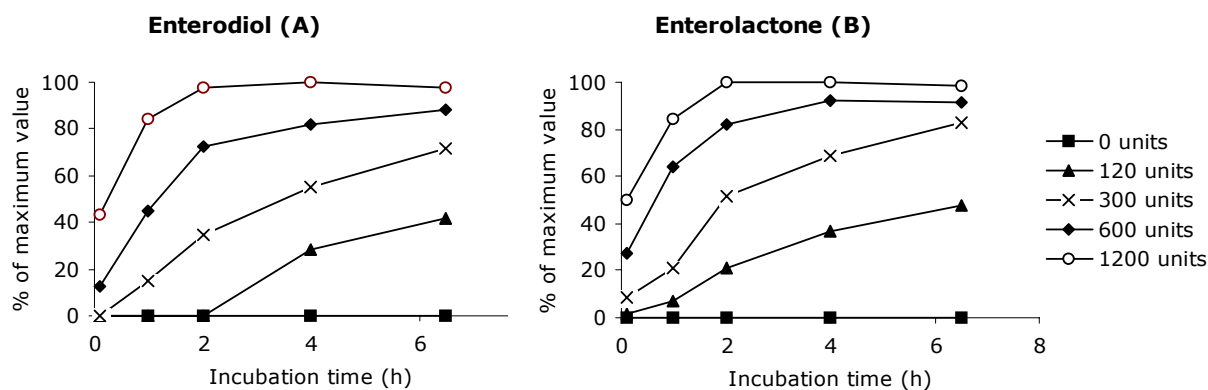
Figure 2.2 Effect of incubation time and amount of β-glucuronidase-sulfatase from *H. Pomatia* (G1512) on enterolactone (A) and enterodiol (B) release from plasma at 37°C; values expressed as percentages of maximum value. The optimal hydrolysis conditions consisted of 4 h incubation with 2600 units enzyme per 300 μL plasma.

Stability of sample extracts

The stability at -80°C of enterodiol and enterolactone in sample extracts was evaluated, to enable storage before analysis on LC-MS/MS. Ten different plasma samples were extracted and analyzed without and after storage at -80°C for 9 days. The concentrations were compared using the paired student *t*-test for statistical analysis.

RESULTS AND DISCUSSION

Optimization of hydrolysis

Several enzymes with β-glucuronidase and/or sulfatase activity were tested in 300 μL high lignan plasma samples (enterodiol: 7.0 nmol/L; enterolactone: 39.2 nmol/L). β-Glucuronidase-sulfatase from *H. Pomatia* (G1512) had the highest response per unit of activity (data not shown). With this enzyme, we varied the amount of enzyme, and incubation time (**Figure 2.2**). The highest yield was obtained with 1200 units β-glucuronidase between 2-6 h. In additional experiments the amount of enzyme was further increased (range 1300-3500 units) at 2, 3, 4, 5, and 6 h. It showed that a 4 h incubation period at 37°C was sufficient. The yield of both enterolignans increased approximately 20% when 2600 units enzyme were used for hydrolysis. When the amount of enzyme was increased further to 3500 units in a separate experiment the yield did not increase significantly (2600 units: enterodiol 31±2.2 nmol/L, enterolactone: 56±2.0 nmol/L; 3500 units: enterodiol: 31±3.2 nmol/L, enterolactone: 59±3.3 nmol/L). The final amount of enzyme (867 units/100 μL plasma) in our study is comparable with the amount of enzyme used by Grace et al. [27] (1205 units/100 μL serum). Other studies [29, 30] reported lower amounts of enzyme (<150 units/100 μL plasma), or are not comparable because they used enzymes with differently defined activities. Most studies used an overnight incubation for the hydrolysis of enterolignans in plasma

[27, 29-33]. Of those studies, only Valentin-Blasini et al [30] reported the optimization of hydrolysis. They showed that incubation with β-glucuronidase-sulfatase for 8 h is sufficient for complete deconjugation of daidzein. They also stated that the other analytes, including enterodiol and enterolactone, showed a similar deconjugation rate. In our study no differences were observed between incubation at 37 and 50°C. Without enzyme, no enterolignan aglycones were detected in plasma (Figure 2.2), which strongly suggests that all enterolignans in plasma are conjugated with glucuronic acid or sulfate. A similar observation has been reported by Smeds et al [29], where in pooled unhydrolysed plasma only traces of enterolignans (below the quantification limit) were found.

Optimization of extraction

To optimize extraction conditions, plasma samples were spiked with known concentrations of a standard mixture of enterodiol and enterolactone, and the recovery was determined. Diethyl ether was used as an efficient and low-boiling extraction solvent. By varying the number of extractions and the volume of the diethyl ether, the optimal extraction conditions were determined. Extraction with 1.5 mL diethyl ether twice was as efficient as extraction with 1 mL diethyl ether three times (data not shown). After extraction, diethyl ether has to be removed. However, when the diethyl ether fraction was evaporated to dryness and the residue re-dissolved in 50% methanol/sodium acetate buffer (0.1 mol/L, pH 5.0) (v/v), more than 75% of the enterolignans was lost. To prevent this loss, the ether fractions were transferred to tubes containing a solvent capable of readily dissolving enterolignans, and immiscible with ether. In this way, the lignans were kept in solution. When sodium acetate buffer (0.1 mol/L, pH 5.0) was used alone as immiscible solvent, the recovery of enterodiol and enterolactone was 54% and 15%, respectively. Several other solvents were tested to improve the recovery. The highest recovery (88% for enterodiol, and 104% for enterolactone) was obtained with a mixture of 50% methanol/sodium acetate buffer (0.1 mol/L; pH 5.0; v/v). A mixture of 50% acetonitrile/sodium acetate buffer (v/v) was not appropriate because it produced heavily tailing peaks in the chromatograms.

Optimization of chromatography and detection

Several column types and chromatographic conditions were tested in order to develop a short, though robust and sensitive analytical method. A short (50 x 3.0 mm) Xterra column run with a methanol/water gradient was selected, providing the best compromise between selectivity and speed of analysis. The overall analysis time was only 11 min. The use of ammonium acetate buffer, described in the method of Grace et al.[27], was abandoned as this led to a reduced sensitivity of the enterolignans by mass spectrometric detection. MS conditions were optimized for the detection of two product ions for both enterolignans and of one product ion for the $^{13}C_3$ labeled analogs. The most abundant product ion was used for quantification and the second

product ion for confirmation of the identity of the analyte. In this way the risk of 'over'-quantification due to co-eluting matrix components could be greatly reduced. In low lignan containing plasma samples, however, confirmation was not always possible.

The solvent composition of the injected extracts was found to be a critical parameter. The injection of samples containing 50% methanol/water (v/v) resulted in asymmetric and poor peak shapes. This was due to the fact that the sample is injected in mobile phase containing only 10% methanol. It was decided to reduce the methanol content of the sample. The peak shapes improved when the methanol content in the sample was reduced to 40%. This had little effect on the observed recovery of the enterolignans. Furthermore, because we used internal standards for enterodiol and enterolactone, possible losses during extraction and re-dissolution steps were
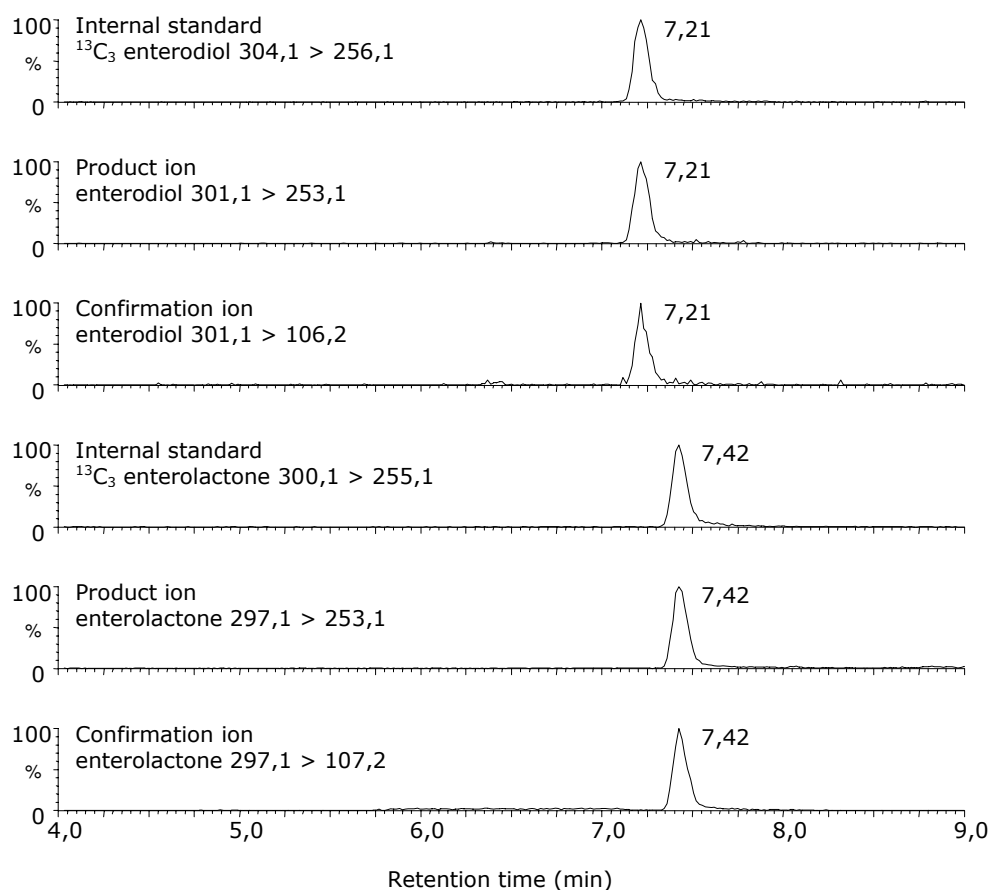
Figure 2.3 Chromatograms (unsmoothed) of enterodiol, enterolactone, and their internal standards in a quality control sample (high lignan plasma). The chromatograms show the MRM transitions that were used for quantification and confirmation. From bottom to top: internal standard $^{13}C_3$ enterodiol (304.1>256.1), product ion enterodiol (301.1>253.1), confirmation ion enterodiol (301.1>106.2), internal standard $^{13}C_3$ enterolactone (300.1>255.1), product ion enterolactone (297.1>253.1), and confirmation ion enterolactone (297.1>107.2).

corrected for automatically. Representative chromatograms of enterodiol and enterolactone with their internal standards are shown in **Figure 2.3.**

We compared the slopes of calibration curves constructed 40% methanol/water (v/v) (n = 5) with calibration curves constructed in plasma (n = 5), analyzed within the same run. For enterodiol calibration curves constructed in 40% methanol/water (v/v) had 0-30% (mean 15%) higher response factors (area enterolignan/area internal standard) than calibration curves constructed in plasma, and for enterolactone calibration curves constructed in 40% methanol/water (v/v) had 0-15% (mean 10%) higher response factors . Although triply ^{13}C labeled internal standards were used, we observed that plasma matrix ion suppression was not sufficiently corrected for when using the calibration curve in methanol/water. Therefore, standard curves in blank human plasma are preferred. Valentin-Blasini et al [30] observed no significant matrix effect of serum on calibration curves. However, they used fetal bovine serum as matrix, whereas we used a homogenized mixture of plasma from three humans. Furthermore, their analytical procedure differed from ours. This might explain the lack of matrix effect. The calibration curves for enterodiol and enterolactone were found to be linear over the concentration range used, with correlation coefficients ≥ 0.99. The slope of the calibration curve of enterodiol was 0.237 ± 0.042 and that of enterolactone 0.096 ± 0.015. The limit of detection determined in plasma samples was 0.15 nmol/L for enterodiol and 0.55 nmol/L for enterolactone (**Table 2.2**). Comparable or higher detection limits have been reported for time-resolved fluorescence immunoassay detection of enterolactone in plasma (0.35 nmol/L) [32], for GC with MS detection of enterodiol and enterolactone in plasma (0.2-1.0 nmol/L) [34], for LC with MS detection of enterodiol and enterolactone in plasma or serum (0.4-0.5 nmol/L [27], 0.3-3.6 nmol/L [30]), and for LC with electrochemical detection (1.9-2.1 nmol/L) [33]. Smeds et al [29] and Grace et al [27] reported much lower detection limits for LC-MS (<30 pmol/L), but these detection limits were determined in the absence of plasma.

Table 2.2 Detection limits, within- and between-run variation for enterolignans in plasma.

	n	Enterodiol	Enterolactone
Limit of detection (S/N = 3; nmol/L)[1]	10	0.15 ± 0.1	0.55 ± 0.4
Within-run CV (%)	6	5.8	3.2
Between-run CV (%) high[2]	22	14	10
Between-run CV (%) low[3]	22	21	13

[1] Mean ± SD.

[2] High lignan plasma (enterodiol: 7.0 nmol/L; enterolactone: 39.2 nmol/L)

[3] Low lignan plasma (enterodiol: 0.6 nmol/L; enterolactone: 5.4 nmol/L)

Table 2.3 Recovery of enterolignans in plasma

| Spike (nmol/L) | n | Recovery (%) [1] | |
		Enterodiol	Enterolactone
10	13	99 ± 11	103 ± 20
30	13	107 ± 7	107 ± 11
100	10	97 ± 4	99 ± 2

[1] Mean ± SD.

contained high (enterodiol: 7.0 nmol/L; enterolactone: 39.2 nmol/L) and low (enterodiol: 0.6 nmol/L; enterolactone 5.4 nmol/L) concentrations of enterolignans. Recovery of enterolignans added prior to the hydrolysis procedure varied from 97 to 107% (**Table 2.3**). These data are in accordance with recoveries reported elsewhere [24, 27, 30, 32]. Lower recoveries (68-85%) of enterolignans in plasma were reported using LC with electrochemical detection [33]. The within-run variation of our method was quite satisfactory. The coefficient of variation (CV), at a concentration of 7.0 nmol/L for enterodiol and 39.2 nmol/L for enterolactone, ranged from 3 to 6% (Table 2.2). Comparable CVs are reported by other authors [27, 35]. Nurmi et al [33] reported a high within-run variation for enterodiol (42%) but similar variation for enterolactone (1.5%). Slightly higher variations (9-20%) were reported by Smeds et al [29]. The between-run variation of our method was relatively high; the CV in high enterolignan plasma (enterodiol: 7.0 nmol/L; enterolactone 39.2 nmol/L) ranged from 10 to 14% for both enterolignans, and from 13 to 21% in low enterolignan plasma (enterodiol: 0.6 nmol/L; enterolactone 5.4 nmol/L). Lower between-run variations (3.7% for enterodiol; 3.3% for enterolactone) were reported by Grace et al [27] using LC-MS/MS at concentrations of >30 nmol/L, and by Valentin-Blasini et al [30] (5.1% for enterodiol; 4.5% for enterolactone) at concentrations of >15 nmol/L. Both authors constructed their calibration curves in the absence of plasma. When calibration curves of enterodiol and enterolactone are constructed in plasma the CVs of the slopes are around 2-fold higher than in the absence of plasma [29]. The use of calibration curves constructed in plasma might explain the relatively high CVs of the quality control samples of our method.

Stability of sample extracts

When sample extracts could not be analyzed immediately, they were stored at -80°C. Stability tests indicated that after 9 days of storage at -80°C the enterodiol concentration in plasma decreased by 3 ± 10% (n = 10), whereas that of enterolactone did not change 0 ± 6% (n = 10). The decrease in enterodiol was not statistically significant (paired student *t* test; *P* = 0.95 for enterodiol). Therefore, it was concluded that sample extracts can be stored for 9 days at -80°C without affecting the concentration of both enterolignans.

Levels of enterolignans in plasma samples

The LC-MS/MS method described in this paper was successfully applied to quantify enterolignans in plasma after supplementation of purified secoisolariciresinol diglucoside to healthy men and women [36]. Furthermore, this method is currently being used to quantify concentrations of enterolignans in epidemiological studies. Preliminary results showed that the median plasma concentration for enterolactone was 9.2 nmol/L, and for enterodiol 1.0 nmol/L (n = 807). The range of enterolignan concentrations was very wide. The three highest concentrations observed for enterolactone were 687, 564, and 298 nmol/L, and for enterodiol 184, 86, and 78 nmol/L. The distribution of plasma enterolignans appeared skewed to higher values. The lowest concentrations observed were below the detection limits for both compounds.

CONCLUSIONS

We developed and validated a LC-MS/MS method using $^{13}C_3$ labeled isotopes for the simultaneous quantification of total enterodiol and enterolactone concentrations in plasma. This method allows detection and quantification of nanomolar concentrations of these enterolignans. By optimizing the hydrolysis and extraction specifically for enterodiol and enterolactone, an efficient and adequate method was developed. Our method proved itself to be useful for the analyses of large numbers of plasma samples in a wide range of concentrations and is currently used for the analysis of enterolignans in plasma samples collected from large epidemiological studies.

ACKNOWLEDGEMENTS

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PART A

Bioavailability of enterolignans

ABSTRACT

High concentrations of enterolignans in plasma are associated with a lower risk of acute coronary events. However, little is known about the absorption and excretion of enterolignans. The pharmacokinetic parameters and urinary excretion of enterodiol and enterolactone were evaluated after consumption of their purified plant precursor, secoisolariciresinol diglucoside (SDG). Twelve healthy volunteers ingested a single dose of purified SDG (1.31 µmol/kg bodyweight). Enterolignans appeared in plasma 8-10 h after ingestion of the purified SDG. Enterodiol reached its maximum plasma concentration 14.8 ± 5.1 h (mean ± SD) after ingestion of SDG, whereas enterolactone reached its maximum 19.7 ± 6.2 h after ingestion. The mean elimination half-life of enterodiol (4.4 ± 1.3 h) was shorter than that of enterolactone (12.6 ± 5.6 h). The mean area under the curve of enterolactone (1762 ± 1117 nmol/L·h) was twice as large as that of enterodiol (966 ± 639 nmol/L·h). The mean residence time for enterodiol was 20.6 ± 5.9 h and for enterolactone 35.8 ± 10.6 h. Within 3 days, up to 40% of the ingested SDG was excreted as enterolignans via urine, with the majority (58%) as enterolactone. In conclusion, a substantial part of enterolignans becomes available in the blood circulation and is subsequently excreted. The measured mean residence times and elimination half-lives indicate that enterolignans accumulate in plasma when consumed 2-3 times a day and reach steady state. Therefore, plasma enterolignan concentrations are expected to be good biomarkers of dietary lignan exposure and can be used to evaluate the effects of lignans.

Keywords: enterodiol; enterolactone; lignans; secoisolariciresinol diglucoside; bioavailability

Pharmacokinetics of enterolignans in healthy men and women consuming a single dose of secoisolariciresinol diglucoside

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Chapter 3

INTRODUCTION

Enterolactone and enterodiol, also called enterolignans, are phytoestrogens with structural similarity to endogenous estrogens. Enterolignans have demonstrated antioxidant and weak (anti-) estrogenic effects [1-4]. They are capable of induction of NADPH: quinone reductase (phase II enzymes) [5], and can inhibit enzymes involved in the metabolism of sex hormones (e.g., SHBG, 5α-reductase, and 17β-hydroxysteroid dehydrogenase) [6-8]. Because of these activities, enterolignans may affect the development of chronic diseases. Epidemiological studies suggest that high plasma concentrations of enterolactone are associated with a lower risk of acute coronary events [9, 10]. Associations between enterolignans and cancer are unclear. Inverse associations were reported only for case-control studies [11-13], whereas no associations between enterolignans and breast or prostate cancer were found in 3 prospective studies [14-16] (reviewed by Arts and Hollman [17]). Enterolactone and enterodiol are products of bacterial conversion of plant lignans in the human colon [18-20]. Plant lignans are naturally occurring compounds that have a polyphenolic structure. Dietary sources of plant lignans are flax, grains, seeds, fruits and vegetables, olive oil, and beverages such as tea, coffee, and wine [21-28].

Secoisolariciresinol, one of the plant lignans, is converted to enterodiol and subsequently to enterolactone by intestinal bacteria [29] **(Figure 3.1)**. Matairesinol, another plant lignan, is converted directly to enterolactone. Other precursors of enterolignans are pinoresinol, lariciresinol, isolariciresinol, and syringaresinol [30]. Enterodiol and enterolactone are absorbed from the large intestine. They are mainly present as glucuronide and sulfate conjugates in body fluids and are excreted via urine [31]. Due to different consumption patterns [32], variation in microflora, and use of antibiotics [19, 33], plasma concentrations of enterodiol and enterolactone
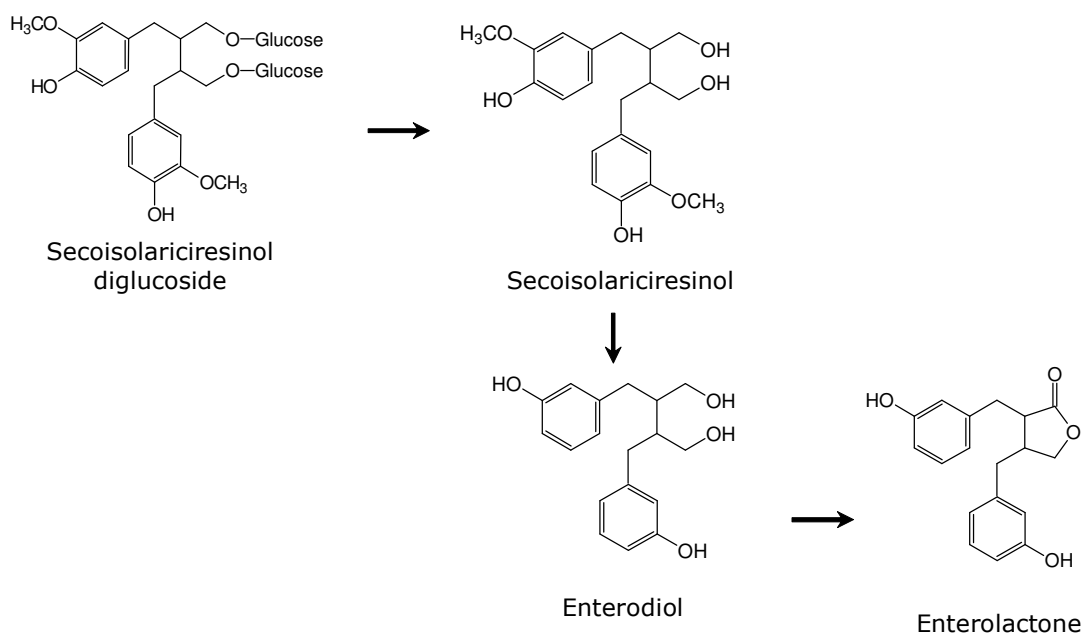

Figure 3.1 Conversion of secoisolariciresinol diglucoside by bacteria in the colon.

vary widely among people. We know little about the kinetics of absorption and distribution of enterodiol and enterolactone in the body.

To evaluate the exposure to enterolignans, data on the absorption, distribution, and excretion of enterolignans are needed. So far, no studies have been carried out with isolated lignans in humans, but several studies used lignan rich foods. Plasma and urinary concentrations of enterolignans increased after eating flax or flax-containing products for several weeks [34-39]. Two studies investigated the absorption and excretion of enterolignans after a single dose of lignan-rich foods. Nesbitt et al [40] found a dose-dependent urinary excretion of enterodiol and enterolactone after consumption of 5, 15, or 25 g ground flax. Plasma concentrations of enterolignans started to increase 9 h after intake, and were still higher than baseline values after 12 and 24 h. In a study carried out by Mazur et al [24], enterolactone plasma concentrations started to increase 8 h after consumption of 500 g of strawberries. Urinary excretion of enterolactone increased also, with the highest excretion between 25 and 36 h. In both studies no plasma samples were taken after 24 h, when enterolignan concentrations are probably still high. Furthermore, the investigators used foods that contain various precursors of enterolignans. They were not able to calculate pharmacokinetic parameters, such as the maximum concentration and mean residence time.

In the present study, we investigated the absorption and excretion of enterodiol and enterolactone in healthy men and women consuming a single dose of secoisolariciresinol diglucoside (SDG), which is the major lignan in flax. Based on a pilot study we designed an optimal sampling schedule, which covered the increase in plasma and urine concentrations of enterolignans and their return to baseline. This is the first report describing the pharmacokinetics of enterodiol and enterolactone.

MATERIALS AND METHODS

Subjects

The Medical Ethical Committee of the Department of Human Nutrition at Wageningen University approved the study, and all subjects gave their informed consent. Six men and 6 women participated in this study. The participants ranged from 18 to 25 years old. None of the subjects had diarrhea or had used antibiotics or other medication in the past three months, except for oral contraceptives or painkillers. All subjects were generally healthy (self-reported). The weight of the men was 73.0 ± 6.8 kg (mean ± SD), and the mean weight of the women was 67.6 ± 4.5 kg. The mean body mass index in men was 21.5 ± 1.3 kg/m^2, and 23.5 ± 1.6 kg/m^2 in women. Subjects were excluded if their hemoglobin concentration was low (<7.5 mmol/L for women and <8.5 mmol/L for men) or if their urine contained traces of glucose or protein (test strip for rapid determination of protein and glucose in urine, Macherey-Nagel, Düren, Germany). Vegans, vegetarians (defined as persons who consume fish or meat less than once a week), and people

consuming flax-containing supplements were excluded, as were pregnant or lactating women.

Diet

To avoid interference from other dietary sources of lignans, the participants started a diet poor in lignans seven days prior to the study and followed it throughout the experiment. The participants were given a list of lignan-containing foods and beverages and were asked to avoid them. They avoided dried fruits, berries, several vegetables (e.g., asparagus, broccoli, and zucchini), legumes, seeds and nuts (e.g. flax, sesame, and peanut), breakfast cereals, cereal and muesli bars, whole-grain products (e.g., rye bread, whole grain bread, and brown rice), olives, virgin olive oil, herbal tea, grape juice, and orange juice. Furthermore, they limited their consumption of black tea and coffee to a maximum of 2 cups (500 mL) a day. Consumption of selected wheat products (white bread, pasta), white rice, milk products (milk, yogurt, cheese), meat and fish, several fruits (e.g., apple, pear) and vegetables (e.g., cucumber, tomato, paprika, cabbage) was allowed so that, in principle, the intake of micro- and macronutrients was adequate. To ensure an adequate fiber intake, wheat bread with a low lignan concentration (370 nmol lignans/100 g bread) was supplied daily. Bread is an important source of fiber in the Netherlands. Every day a standard breakfast (low lignans) was provided at the Division of Human Nutrition (Wageningen University, The Netherlands). Lunch and dinner were also provided on the first two days of the study.

Lignan supplement

On day 1 of the study after a 12-h overnight fast, the subjects consumed 1.31 µmol SDG/kg bodyweight (0.9 mg SDG/kg bodyweight) in water, just before having their breakfast at around 0800 h. SDG was obtained from the Institute of Food Chemistry, Technical University of Braunschweig. SDG was isolated from a natural source, i.e., flax (*Linum usitatissimum* L.) [41]. For isolation, extraction, and purification of SDG only p.a. quality solvents (food grade) were used. In order to remove remaining traces of solvents the SDG extract was freeze-dried. The purity was above 93%. One day before consumption, the supplement was weighed, dissolved in 50 mL water, and then kept at -20°C. The supplement was thawed 1 h before consumption.

Collection of samples

Venous blood samples were taken into vacuum tubes containing EDTA immediately before the intake of SDG (0 h), every 3 h over the next 36 h, and at time points 48, 72, and 96 h. Samples were centrifuged within 30 min at 1187 x *g* for 10 min at 4°C, and plasma was stored at -80°C until analysis.

Urine samples were collected from 24 h prior to the intake of SDG until 72 h after the intake of SDG. The participants stored each bottle on dry ice immediately after voiding. Each day, urine samples were transported from the participants' homes to the laboratory where they were kept at

-20°C. At the laboratory, urine samples were thawed and homogenized. To obtain 24-h urine samples, the samples of 1 day were pooled per subject.

Logistics

During the study, blood samples t = 0-12 h and t = 24-96 h were taken at the Division of Human Nutrition at Wageningen University. The samples t = 15-21 h, which were collected during the night, were drawn at the hospital Gelderse Vallei in Ede, where the subjects stayed overnight. Volunteers were transported between the 2 sites under supervision of a research nurse.

Analytical method

Total enterodiol and enterolactone concentrations were measured in plasma and urine after hydrolysis of conjugates using a freshly prepared enzyme mixture of β-glucuronidase-sulfatase from *Helix Pomatia* (G1512, Sigma, St. Louis, USA) in sodium acetate buffer (0.5 mol/L, pH 5.0). Quantification of enterodiol and enterolactone in **urine** was performed by HPLC with electrochemical detection as described previously [42]. The original method was slightly modified. Briefly, 200 µL urine was mixed with an equal amount of buffer, and 40 µL enzymes (50 g/L). Subsequently, samples were incubated at 37°C for 2 h and extracted twice with diethyl ether. Prior to the analysis, extracts were filtered, transferred into vials, and injected into the HPLC system. To separate the enterolignans from other compounds, we used a binary gradient. Mobile phase A consisted of 15% acetonitrile in 50 mmol/L sodium acetate buffer (pH 5.0). Mobile phase B consisted of 60% acetonitrile in 50 mmol/L sodium acetate buffer (pH 5.0). A total of 100 µL extract was injected onto 2 Chromolith columns (100 x 4.6 mm each; Merck) in series. The gradient at a flow rate of 2.5 mL/min was as follows: 0-8.5 min, linear from 0 to 15% mobile phase B; 8.5-14.5 min, linear from 15 to 38% B; 14.5-14.7 min linear from 38 to 100% B; 14.7-16.7 min, isocratic at 100% B; 16.7-17.0 min, linear return from 100 to 0% B; 17.0-21.0 min, isocratic at 0% B to equilibrate. For detection and quantification of enterolignans, we used a 4-channel Coularray HPLC detection system. Enterodiol and enterolactone were quantified at 650 mV. Determinations in urine were carried out in duplicate. The limit of detection of enterodiol and enterolactone was 3 nmol/L. The recovery of enterodiol and enterolactone was 115 ± 20% (n = 2). The within-run CV of enterodiol was 3% and of enterolactone 4% (n = 6). The between-run CV was of both enterolignans was 13% (n = 4).

Plasma samples contained much lower concentrations of enterolignans than urine samples. When plasma samples were measured with electrochemical detection, the resolution proved not to be adequate. Thus HPLC with MS/MS detection, which has excellent specificity, was chosen instead. The sample preparation as describe above was adjusted for plasma. First, we added 10 µL of a mixture of $^{13}C_3$ labeled enterodiol and enterolactone (500 nmol/L each; purchased from

Dr. Botting, University of St Andrews) into vials. Subsequently, 300 µL sodium acetate buffer (0.1 mol/L, pH 5.0), 60 µL enzyme mixture (100 g/L), and 300 µL plasma were added. Plasma samples were incubated at 37°C for 4 h, and subsequently extracted twice with 1.5 mL diethyl ether. The mixtures were shaken with a Vortex mixer for 5 s and centrifuged after each extraction (2300 x g, 10 min, 10°C). The 2 ether fractions were combined and transferred into new tubes containing 500 µL of 40% methanol/water, (v/v). The ether fraction was evaporated under a gentle stream of nitrogen at 30°C with a Turbovap evaporator. The tubes were shaken with a Vortex mixer for 10 s. Prior to the analysis, extracts were filtered, transferred into vials, and injected into the LC-MS system. We used a Waters Alliance 2690 HPLC pump (Millford, MA, USA), which consisted of a chromatographic system, and an auto-injector with a cooled sample tray set at 10°C. Separations were performed with an XTerra C_{18} column (3.0 x 50 mm, 5 µm, Waters, Millford, MA, USA), which was placed into a thermostatic column chamber set at 40°C. Mobile phases A and B consisted of water and methanol, respectively. The gradient at a flow rate of 0.4 mL/min was as follows: 0-1.0 min, isocratic at 10% mobile phase B; 1.0-7.0 min, linear from 10 to 80% B; 7.0-7.5 min isocratic at 80% B; 7.5-8.0 min, linear return from 80 to 10% B; 7.0-11.0 min, isocratic at 10% B to equilibrate. The total run time for each sample was 11 min. The sample injection volume was 100 µL. The divert valve was programmed to allow flow into the mass spectrometer from 4-9 min of each run. Detection was performed with a Micromass Quatro Ultima MS (Micromass, Manchester, UK) equipped with an electrospray probe in negative ion mode, with the capillary voltage at 2.5 kV. We used nitrogen at a flow rate of 550 L/h as

Table 3.1. Pharmacokinetic parameters of enterodiol and enterolactone in healthy men and women consuming a single dose of SDG[1, 2]

	Enterodiol			Enterolactone		
Parameter	Total	males	females	Total	males	females
n	12	6	6	12	6	6
t_{lag}, h	10.1 ± 4.3	12.5 ± 4.4	7.8 ± 2.9	8.3 ± 4.0	9.2 ± 5.0	7.3 ± 2.8
Absorption						
Half-life, $t_{1/2}$ (abs), h	2.8 ± 1.7	3.4 ± 1.8	2.2 ± 1.5	6.4 ± 4.3	8.4 ± 4.7	4.4 ± 2.9
C_{max}, nmol/L	73 ± 40	65 ± 33	81 ± 47	56 ± 30	42 ± 22	70 ± 32
t_{max}, h	14.8 ± 5.1	17.8 ± 4.1	11.7 ± 4.2*	19.7 ± 6.2	24.1 ± 4.5	15.4 ± 4.2*
Elimination						
Half-life, $t_{1/2}$, h	4.4 ± 1.3	4.6 ± 1.1	4.1± 1.5	12.6 ± 5.6	15.1 ± 6.7	10.2 ± 3.2
AUC, nmol/L·h	966 ± 639	1019 ± 794	914 ± 511	1762 ± 1117	1706 ± 1217	1817 ± 1120
MRT, h	20.6 ± 5.9	23.9 ± 4.7	17.3 ± 5.4*	35.8 ± 10.6	43.2 ± 9.3	28.4 ± 2.2*

[1] All values are means ± SD. *different from males, $P \leq 0.05$.

[2] AUC and C_{max} are corrected for baseline values. t_{lag} is time to reach first appearance in plasma; $t_{1/2 \, abs}$ is absorption half-life; C_{max} is maximum plasma concentration; t_{max} is time to reach C_{max}; $t_{1/2}$ is elimination half-life; AUC is area under the curve; MRT is mean residence time.

desolvation gas. Source and desolvation gas temperatures were set at 120 and 350°C, respectively. Daughter ions were formed by collision-induced dissociation with argon as collision gas at a pressure of 2.3 x 10 $^{-3}$ mbar, with collision energy ranging from 20-36 eV. Multiple reaction monitoring was performed with the following precursor/product combinations: enterodiol (301.1/253.1), and enterolactone (297.1/253.1) with $^{13}C_3$ labeled internal standards enterodiol (304.1/256.1), and enterolactone (300.1/255.1). Integration of peak areas was performed using Mass Lynx (Micromass). The limit of detection, i.e., the concentration producing a peak height 3 times the standard deviation of the baseline noise, was 0.2 nmol/L for enterodiol and 0.6 nmol/L for enterolactone. The recovery of 10 nmol/L enterodiol and enterolactone aglycone was 98 ± 16% (mean ± SD; n = 6). The within run coefficient of variation (CV) was 6% for enterodiol and 3% for enterolactone (n = 6), and the between-run CV was 16-18% for both enterolignans (n = 12).

Pharmacokinetic analysis

A 1-compartmental pharmacokinetic model was used to describe the absorption and disposition of lignans (MW/Pharm, Mediware, Groningen) [43]. The area under the curve (AUC) for plasma was calculated using the trapezoidal rule. When participants followed a diet poor in lignans for 7 days, total enterolignan concentrations in plasma were reduced by half (data not shown). However, due to the abundance of lignans in foods, plasma concentrations of enterodiol were not zero at the start of the study. Baseline plasma concentrations of enterodiol were fluctuating between 0.3 and 12 nmol/L (mean 3.4 nmol/L), and concentrations of enterolactone fluctuated between 3.3 and 15 nmol/L (mean 7.2 nmol/L). To calculate the AUC and maximum concentration, baseline values for each person were subtracted from the crude pharmacokinetic parameters.

Statistical analysis

An independent *t* test was used to study sex differences. Two-sided Pearson correlation coefficients were calculated. In all tests, differences were considered statistically significant at *P* ≤ 0.05. Differences between enterodiol and enterolactone were not tested because the performed with SPSS statistical software package (version 10.0, Chicago, IL, USA). Data are presented as means ± SD, unless stated differently.

RESULTS

Pharmacokinetic analysis of the plasma curves showed that it takes ~8-10 h **(Table 3.1)** before both enterolignans appear in plasma. Although the maximum plasma concentration of enterodiol (73 ± 40 nmol/L), corrected for baseline, exceeded the maximum plasma concentration of enterolactone (56 ± 30 nmol/L), the AUC of enterolactone (1762 nmol/L·h) was approximately twice that of enterodiol (966 nmol/L·h). As expected from this, we found clear differences in the plasma concentration-time course for enterodiol and enterolactone. The maximum concentration
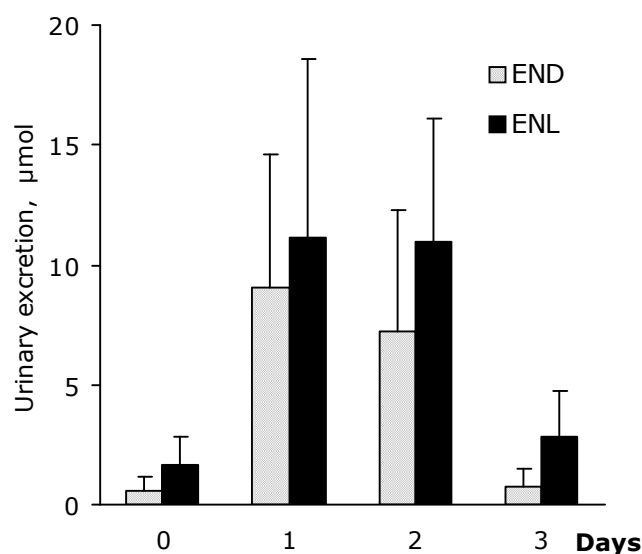
Figure 3.2 24-h urinary excretion of enterodiol (END) and enterolactone (ENL) from twelve healthy subjects consuming a single dose of SDG at around 0800 h of day 1. Values are means ± SD.

of enterodiol was reached 14.8 ± 5.1 h after consumption of SDG, while the maximum concentration of enterolactone was reached 19.7 ± 6.2 h post dose. In addition, the elimination half-life of enterodiol (4.4 ± 1.3 h) was much shorter than that of enterolactone (12.6 ± 5.6 h). The mean residence time of enterodiol was ~21 h, and that of enterolactone ~36 h.

Most of the enterolignans were excreted in urine within the first 2 days (**Figure 3.2**). Within 3 days, up to 40% of the ingested SDG was excreted via urine, with the majority (58%) as enterolactone (**Figure 3.3**). The urinary excretion of enterolactone correlated very well with the amount of enterolactone in plasma, based on the AUCs (r = 0.673, *P* = 0.016). The correlation between the urinary excretion of enterodiol and the amount of enterodiol in plasma was not significant, but tended to be positive (r = 0.432, *P* = 0.16).

Although all subjects ingested the same dose of SDG per kilogram of bodyweight, there was a substantial variation among subjects in plasma concentrations and urinary excretion of enterodiol and enterolactone (Figure 3.2 and **3.4**). In 5 subjects the AUC of enterolactone was more than twice as large as that of enterodiol (Figure 3.4A and B). In 5 other subjects the AUC of enterolactone was only 1-2 times as large as the AUC of enterodiol (Figure 3.4C). In 2 subjects the AUC of enterodiol exceeded the AUC of enterolactone. In 1 of those subjects enterolactone concentrations hardly increased at all (Figure 3.4D).

When data for men and women were analyzed separately, some pharmacokinetic parameters differed (Table 3.1). The onset of the plasma curve (t_{lag}) of both enterolignans tended to be earlier in women than in men (enterodiol, *P* = 0.06; enterolactone, *P* = 0.42). This was in agreement with the time to reach the maximum concentration, which was earlier in women. Furthermore, the
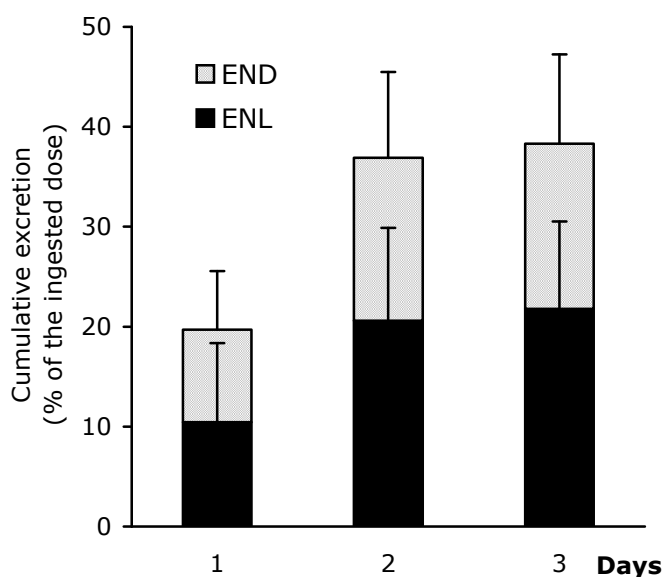
Figure 3.3 Cumulative urinary excretion of enterodiol (END) and enterolactone (ENL) from 12 healthy subjects consuming a single dose of SDG. Values are corrected for baseline concentrations and expressed as percentages of the ingested dose, means ± SD.

maximum concentrations of both enterolignans tended to be higher in women than in men (enterodiol, P = 0.52; enterolactone, P = 0.11). Additionally, the residence time of enterodiol and enterolactone was shorter in women than in men. The AUC and elimination half-life of both enterolignans did not differ between men and women.

DISCUSSION

Our study is the first pharmacokinetic study on enterodiol and enterolactone in humans consuming a single dose of purified SDG. A substantial part (at least 40%) of the metabolites of SDG, enterodiol and enterolactone, becomes available in the blood circulation and is subsequently excreted. Enterodiol and enterolactone are absorbed 8-10 h after consumption of SDG, and eliminated slowly. The systemic exposure to enterolactone, as computed from the mean AUC, was approximately 2 times the exposure to enterodiol. This difference in systemic exposure might be explained by enterohepatic circulation. This causes enterodiol to reach the colon for a second time, where it is available for oxidation into enterolactone. As a result, predominantly enterolactone is absorbed. This is evidenced by the second peak of enterolactone, which was seen in 5 out of 12 subjects in this study. We did not observe a clear second peak of enterodiol in plasma in any of the subjects. Evidence for enterohepatic circulation of enterolignans has been observed in rats [44] and pigs [45]. Alternatively, the difference in systemic exposure between enterodiol and enterolactone might be explained by an efficient enterolactone production from enterodiol immediately after it is formed from SDG. A third option could be that enterolactone is more efficiently absorbed. However the absorption half-life of enterolactone is longer than that of
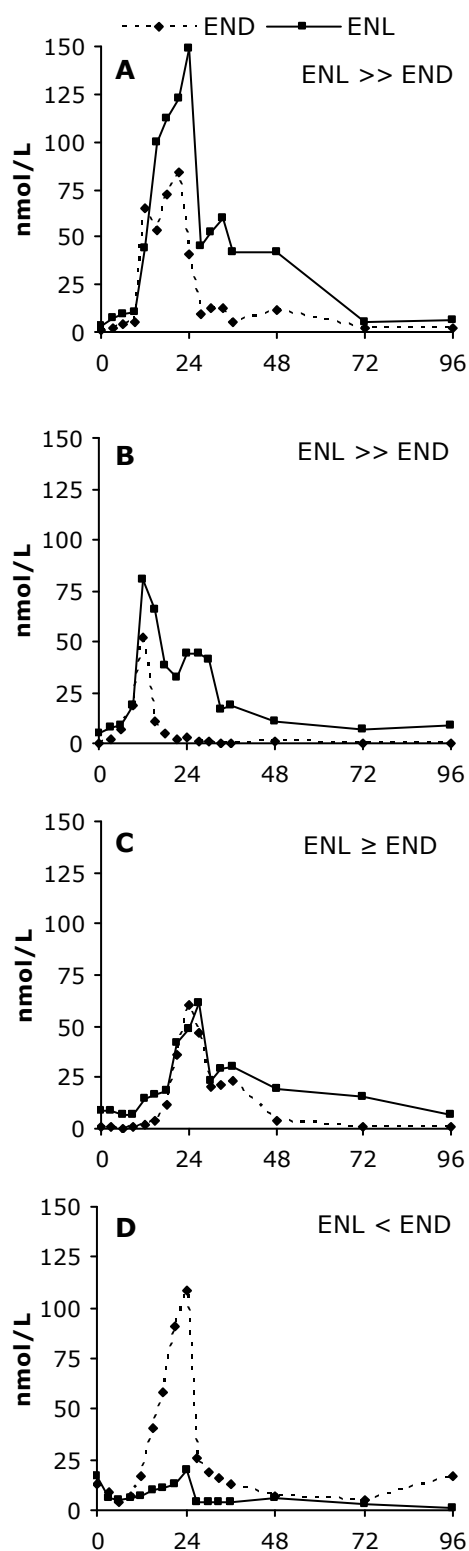
Figure 3.4. Plasma curves of enterodiol and enterolactone in four healthy individuals consuming a single dose of SDG. **A and B:** represent subjects in whom the total amount of enterolactone, which is based on the AUC, is much higher than the total amount of enterodiol (n = 5). **C:** represents subjects in whom the total amount of enterolactone is slightly higher or equal to the amount of enterodiol (n = 5). **D:** represents subjects in whom the total amount of enterolactone is smaller than the total amount of enterodiol (n = 2).

enterodiol, thus suggesting the opposite.

The delayed appearance of enterodiol and enterolactone in plasma indicates that absorption of lignans occurs in the colon. Other studies observed the same delayed appearance of 8-9 h with lignan-rich products [24, 40], suggesting that the food matrix did not play an important role in the release of enterolignans.

The difference in time to reach the maximum plasma concentration between enterodiol and enterolactone might be overestimated. Data points from the enterohepatic circulation (second peak) were used in the 1-compartmental model, and thus the time to reach the maximum plasma concentration might be overestimated, especially for enterolactone. A similar problem may have influenced the absorption and elimination half-lives. A specific kinetic model, which takes into account enterohepatic circulation, may lead to more precise kinetic parameters. However, this is only feasible when there are enough data points to calculate the enterohepatic contribution. This kind of experiment would impose a considerable burden to the volunteers involved. The order in which SDG is converted, SDG>> enterodiol>> enterolactone, is consistent with the difference in absorption half-life between enterodiol and enterolactone. In a number of subjects the absorption and elimination half-lives were identical for both compounds. This means that the absorption governs the elimination, that the intrinsic elimination of the compound is faster than measured here, and that the observed elimination half-life is apparent.

The difference in time to reach the maximum plasma concentration that we found between men and women might be explained by the smaller blood volume in women, even when adjusted for bodyweight [46], because the enterolignans are confined to the blood compartment. When this volume is smaller, enterolignans will reach maximum concentrations earlier, and maximum plasma concentrations will be higher.

In our study the percentage of enterolignans excreted via urine is higher than in animal studies. In a study with rats 28-32% of the ingested ^{3}H-SDG was excreted in urine within 48 h [47]. Knudsen et al [45] found that 24% of the ingested lignans were excreted as enterolignans via urine when pigs were fed a low-lignan wheat bread diet. However, only 14% of the ingested lignans were excreted via urine in rats fed a high- lignan diet. In humans, the excretion of enterolignans via urine was 47.3 µmol/day after consumption of flaxseed powder (10 g/day)[37]. Unfortunately, the percentage of the ingested dose excreted via urine could not be calculated because the authors did not report the amount of lignans in the flaxseed powder.

We did not measure other metabolites of SDG other than enterodiol and enterolactone or the plant lignan itself. Jacobs et al [48] detected 9 hydroxylated metabolites of enterodiol and enterolactone in urine of 4 humans ingesting flax for 5 days. These metabolites accounted for <5% of the total urinary lignan excretion. Additionally, enterodiol and enterolactone accounted for 82% of the total amount of lignans excreted in urine of humans consuming their habitual diet (unpublished results, Tarja Nurmi, University of Kuopio, Finland). Thus, we expect enterodiol and

enterolactone to be the main metabolites.

In 1 subject plasma concentrations of enterolactone did not increase after consumption of SDG, while plasma enterodiol concentrations did increase. The habitual concentrations of enterolactone, measured before starting the lignan-poor diet was begun, were also exceptionally low in this subject (3 nmol/L) compared to others (29 ± 7 nmol/L). The urinary enterolactone excretion after consumption of SDG was also low, only 4% of the ingested dose, whereas the total amount of enterolignans excreted was approximately the same as in other subjects. This suggests that this person was not able to convert enterodiol to enterolactone, likely due to the absence of specific bacteria in the colon that are responsible for the oxidation of enterodiol. The enterolactone present was likely formed from other lignan precursors in the diet, such as matairesinol, which can be directly converted to enterolactone. A similar observation was made by Nesbitt et al. [40], who found that 2 out of 9 subjects produced little or no enterolactone during flaxseed supplementation for 7 days.

As demonstrated in other studies [37, 40, 49], we observed a wide variation in both urinary excretion and plasma concentrations of enterolignans among subjects. The variation is most likely due to differences in microflora between subjects. Other factors that could explain variation, such as background diet and age, were controlled for in our study. SDG was consumed purified; therefore, the food matrix could not have contributed to the variation either.

The health implications of the higher systemic exposure to enterolactone than enterodiol are not clear. Thus far, most studies investigated only the effect of enterolactone. A few studies compared the effects of enterolactone and enterodiol, and showed that they have similar antioxidant activities [1, 2]. However, enterolactone had a greater ability than enterodiol, to inhibit the binding of estradiol and testosterone to sex steroid binding protein [50] or to inhibit human aromatase in vitro [51]. Further studies are necessary to determine whether physiological effects of enterodiol and enterolactone are different. Therefore, investigators must quantify concentrations of enterodiol and enterolactone in experimental and epidemiological studies to understand the metabolism and effect of both compounds. Furthermore, bioavailability studies for other important dietary enterolignan precursors, such as pinoresinol and lariciresinol, are needed. Whether the absorption, distribution, and elimination are influenced by other factors, such as food matrix, is also of interest.

Our data show that at least 40% of the ingested SDG is available for the body. The measured residence time, and elimination half-life indicate that enterolignans will accumulate in plasma when consumed 2-3 times a day. Thus steady state plasma concentrations of enterodiol and enterolactone are likely to be achieved because plant lignans are present in many foods and beverages [21-26]. As a result, enterolignan plasma concentrations are expected to be suitable biomarkers of lignan exposure and may be used to evaluate the effects of lignans.

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ABSTRACT

Flaxseed is one of the richest sources of lignans and is increasingly used in food products or as a supplement. Plant lignans can be converted by intestinal bacteria into the so-called enterolignans, enterodiol and enterolactone. For a proper evaluation of potential health effects of enterolignans, information on their bioavailability is essential. The aim of this study was to investigate whether crushing and milling of flaxseed enhances the bioavailability of enterolignans in plasma. In a randomized, crossover study, 12 healthy subjects supplemented their diet with 0.3 g whole, crushed, or ground flaxseed/ kg body weight per day. Each subject consumed flaxseed for 10 successive days separated by 11-days run-in/wash-out periods, in which the subjects followed a diet poor in lignans. Blood samples were collected at the end of each run-in/wash-out period, and at the end of each supplement period. Plasma enterodiol and enterolactone were measured using liquid chromatography with tandem mass spectrometry. The mean relative bioavailability of enterolignans from whole compared with ground flaxseed was 28% ($P \leq 0.01$), whereas that of crushed compared with ground flaxseed was 43% ($P \leq 0.01$). Crushing and milling of flaxseed substantially improve the bioavailability of the enterolignans.

Keywords: *bioavailability; enterodiol; enterolactone; lignans; flaxseed*

The relative bioavailability of enterolignans is enhanced by milling and crushing of flaxseed

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Chapter 4

INTRODUCTION

Lignans are biphenolic compounds that occur in foods of plant origin. Some plant lignans can be converted into the enterolignans, enterodiol and enterolactone, through a series of reactions mediated by the bacterial flora in the colon [1, 2]. Enterolignans become available in the blood circulation 8-10 h after ingestion of plant lignans [3-5]. They are eliminated slowly [5] and excreted via urine and feces [3-12].

Two epidemiological studies suggested that high serum concentrations of enterolactone are associated with a lower risk of acute coronary events [13, 14]. Associations between enterolignans and cancer are less clear. Inverse associations for breast cancer were reported in case-control studies, and one prospective study [15-18], whereas no associations were found in most prospective studies [19-23].

Among foods consumed by humans, flaxseed contains the highest concentration of enterolignan precursors, mainly secoisolariciresinol diglucoside. Other seeds, nuts, whole grains, fruits and vegetables, and beverages such as coffee and tea contain smaller amounts [24]. The most important sources of lignan precursors in western diets are beverages such as tea and coffee, seeds, cereals, berries, fruits and vegetables [25, 26]. Flaxseed is a relatively minor dietary component in most countries, but because of its potential health benefits (flaxseed also contains a high quantity of (n-3) fatty acids as well as dietary fiber), it is increasingly being incorporated into a variety of food products, such as bread, muesli bars and breakfast cereals, or used as a supplement. For example, in the Netherlands whole flaxseed is used in commercial breads (up to 3.5 g flaxseed/100 g bread); therefore is an important potential source of dietary lignans. In a case-control study carried out in Texas, Strom et al [27] found that flaxseed bread was one of the main food sources of lignans.

Flaxseed is a small hard-coated seed. Whole seeds are used in breads, whereas most supplements consist of crushed seeds. We questioned whether lignans in whole flaxseeds are accessible to bacteria in the colon. We expected that milling or crushing would substantially enhance the accessibility of the bacteria to the plant lignans, and as a result, would improve their conversion into enterolignans. In the present study, we investigated whether milling and crushing enhanced the bioavailability of enterolignans from flaxseed.

SUBJECTS AND METHODS

Subjects

Six men and 6 women, with a mean age of 25 (range 18-64) years, participated in this study. Subjects were recruited in Wageningen by advertisement in the university newspaper, by flyers, and by posters. All subjects were generally healthy (self-reported). None of the subjects had diarrhea, or had used antibiotics or other medication in the past 3 months, except for oral contraceptives or analgesics. Height and weight were measured with the participants wearing

indoor clothing and no shoes. The mean (± SD) weight of the men was 72.3 ± 5.2 kg, and the mean weight of the women was 59.2 ± 6.7 kg. The mean body mass index in men was 22.0 ± 1.7 kg/m², and 19.9 ± 1.2 kg/m² in women. During the study, subjects did not loose or gain weight. Subjects were excluded if their hemoglobin concentration was low (< 120 g/L for women and < 140 g/L for men) or if their urine contained traces of glucose or protein (test strip for rapid determination of glucose and protein in urine, Macherey-Nagel). Vegans, vegetarians (defined as persons who consume fish or meat < 1 time/week), and people consuming flax-containing supplements were excluded, as were pregnant or lactating women. The Medical Ethical Committee of the Department of Human Nutrition at Wageningen University (The Netherlands) approved the study, and all subjects gave their informed consent. All subjects completed the study.

Experimental Design

This study was designed as a randomized, crossover trial consisting of three 10-days supplement periods preceded by three 11-days run-in/wash-out periods. During the supplement periods, subjects consumed whole, crushed or ground flaxseed provided by us. To avoid interference from other dietary sources of lignans, volunteers consumed a diet poor in lignans (see below) throughout the entire study. On day 10 of the supplement periods and day 11 of the run-in/wash-out periods, 3 venous blood samples were drawn from each subject. Men and women were randomly allocated to 1 of 6 treatment order groups so that each order group consisted of 1 man and 1 woman.

Diet

To avoid interference from other dietary sources of lignans, the participants consumed a diet poor in lignans (contributing <5% of the intake from the flaxseed supplements) throughout the entire study. Food products were classified (allowed, limited, or to be avoided) according to the amount consumed, and to the lignan content of the foods [24]. The participants were given a list with the classified food products. They avoided several fruits (e.g., dried fruits, berries, peach, pear, nectarine, and apricot), a few vegetables (e.g., cabbage, broccoli, and haricot beans), seeds and nuts (e.g., flaxseed, sesame seed, and peanut), breakfast cereals, cereal and muesli bars, whole-grain products (e.g., rye bread and whole grain bread), and herbal tea. Furthermore, they limited their daily consumption of several fruits (e.g., kiwi, orange, and cherries) to 100-150 g, tea to 300 mL or coffee to 800 mL, fruit juices to 660 mL, and beer to 800 mL or wine to a maximum of 300 mL. Consumption of selected wheat products (white bread, pasta), rice, milk products (milk, yogurt, and cheese), meat and fish, several fruits (e.g., apple, grapes, and banana) and vegetables (e.g., cucumber, tomatoes, leek, and spinach) was allowed so that, in principle, the intake of micro- and macronutrients was adequate. To ensure an adequate fiber intake, wheat

bread with low lignan content (370 nmol lignans/100 g bread) was supplied daily. Bread is an important source of fiber in the Netherlands. When subjects accidentally consumed too much of a specific food product they were instructed to report the time of eating and the amount eaten of this specific product in a diary.

Supplements

Flaxseed was industrially pretreated with steam to reduce microbiological contamination. Whole and crushed flaxseeds were prepared from the same batch and were provided by a commercial retailer (Weller-Verhoef). To obtain ground flaxseed, we milled part of the whole flaxseed to a coarse texture using a household blender (Waring) for 10 s 4 times and homogenized the particles. To determine the lignan contents of the flaxseed supplements, secoisolariciresinol, matairesinol, pinoresinol, and lariciresinol were measured by LC-MS/MS [28]. The flaxseed contained 3.6 mg lignans/g (> 98% consisted of secoisolariciresinol); the whole, crushed and ground flaxseeds did not differ.

During the supplement periods, subjects consumed 0.3 g flaxseed/kg body weight per day (3.0 µmol lignans/kg body weight). They consumed the flaxseed supplements in 2 servings, at breakfast and dinner, mixed with applesauce or custard sauce. On day 10 of the supplement periods, when blood samples were taken, the subjects consumed only 1 serving of flaxseed at breakfast. Before the study, each flaxseed serving was weighed, packed individually, and stored at -20°C. Subjects received the packages twice a week, with instructions to keep the flaxseed frozen until consumption. Subjects were instructed to consume the entire contents of the individual packages. Furthermore, subjects were not allowed to cook or microwave the flaxseed. Used packages were collected to monitor compliance. In addition, subjects were asked to record at what time they consumed each flaxseed supplement.

Collection of samples

On day 10 of each supplement period and on day 11 of each run-in/wash-out period, 3 venous blood samples were taken into vacuum tubes containing EDTA. The first sample was taken after a 12-h fast between 0700 and 0800 h. The second sample was taken immediately before lunch between 1200 and 1300 h, and the third sample was taken immediately before dinner between 1700 and 1800 h. Samples were centrifuged within 30 min at 1187g for 10 min at 4°C and stored at -80°C until analysis.

Analytical methods

The concentrations of enterodiol and enterolactone in plasma were measured by LC-MS/MS using triply ^{13}C-labeled isotopes [29]. Briefly, enterolignans were measured in 300 µL plasma after hydrolysis of conjugates using a freshly prepared enzyme mixture of *β*-glucuronidase-sulfatase

from *Helix Pomatia* at 37°C for 4 h. Subsequently, samples were extracted twice with diethyl ether. The ether fractions were combined and transferred into new tubes containing 40% methanol/water (v/v). The ether fraction was evaporated under a gentle stream of nitrogen at 30°C. Before the analysis, extracts were filtered, transferred into vials, and injected into the LC-MS system. The limit of detection was 0.15 nmol/L for enterodiol and 0.55 nmol/L for enterolactone. All plasma samples from 1 subject were analyzed in one assay. Lab technicians were unaware of a subject's treatment group.

Statistical analysis

The individual mean enterolignan plasma concentrations on each day were based on the 3 blood samples taken. The relative bioavailability for each individual was defined as the ratio of the mean plasma enterolignan concentration after the supplement periods and the mean plasma enterolignan concentration after supplementation with ground flaxseed (reference) multiplied by 100. The plasma enterolignan concentrations at the end of each supplement period were corrected for the baseline value, which was based on the mean concentration of all run-in/wash-out periods. The differences between the supplement periods were tested by paired *t* tests. The effects of treatment order per supplementation group (whole, crushed, or ground flaxseed) were tested with 1-way ANOVA. Differences were considered statistically significant at $P \leq 0.05$, unless stated differently. Estimates of within-subject, and between-subject variances for each supplement period were obtained by the SPSS reliability command using 1-way ANOVA. The within-subject and between-subject coefficient of variation (CV) were calculated as the square root of the variance component estimates (σ^2_b and σ^2_w) divided by the overall mean multiplied by

Table 4.1 Plasma enterolignan concentrations in healthy subjects after consumption of whole, crushed, and ground flaxseed (0.3 g/kg body weight per day) for 10 days. [1,2]

	Enterodiol	Enterolactone	Total
	nmol/L		
Whole flaxseed	20 ± 5[a,b,c]	65 ± 16[b,c]	85 ± 19[a,b,c]
Crushed flaxseed	37 ± 11[b,c]	85 ± 17[b,c]	122 ± 21[b,c]
Ground Flaxseed	103 ± 31[c]	167 ± 25[c]	270 ± 34[c]
Baseline[3]	1.9 ± 0.5	9.5 ± 1.1	11.4 ± 1.6

[1] Values are means ± SEM (n = 12), not corrected for baseline concentrations. [a] Different from crushed flaxseed, ($P \leq 0.05$); [b] different from ground flaxseed, ($P \leq 0.01$); [c] different from baseline ($P \leq 0.01$).

[2] For each subject, the enterolignan concentrations at the end of each supplement and run-in/wash-out period are based on 3 plasma samples.

[3] The baseline value is calculated by taking the mean of the enterolignan concentrations at the end of all run-in/wash-out periods.

100, where σ^2_b is the between-subject variance component and σ^2_w the within-subject variance component. The reproducibility of measurements in plasma, an important characteristic for a biomarker, was evaluated using the intraclass correlation coefficient (ICC). The ICC represents the proportion of variance in the measure explained by the between-subject variation (i.e. ICC= σ^2_b/ (σ^2_b + σ^2_w). High ICC values (close to 1) represent excellent reproducibility. The ICC of a single measurement was estimated assuming a 1-way random effects model. All statistical analyses were performed with SPSS statistical software package (version 10.0). Data are presented as means ± SEM.

RESULTS

The highest concentrations of enterolignans were reached after supplementation with ground flaxseed (range 122-539 nmol/L) **(Table 4.1)**. Plasma enterolignan concentrations after supplementation with crushed (22-277 nmol/L), and whole flaxseed (29-262 nmol/L) were lower. We found no significant effect of treatment order. The relative bioavailability of enterolignans from whole flaxseed was 28% (range 14-78%), whereas the relative bioavailability of enterolignans from crushed flaxseed was 43% (14-83%; **Figure 4.1**); both differed significantly from ground flaxseed and from one another. Men and women did not differ in relative bioavailability. Because the plasma concentrations of enterolignans within subjects did not differ among the 3 run-in/wash-out periods, we used the mean concentration of all 3 run-in/wash-out periods per subject to calculate their mean baseline concentration. One of the subjects had an unexplained high
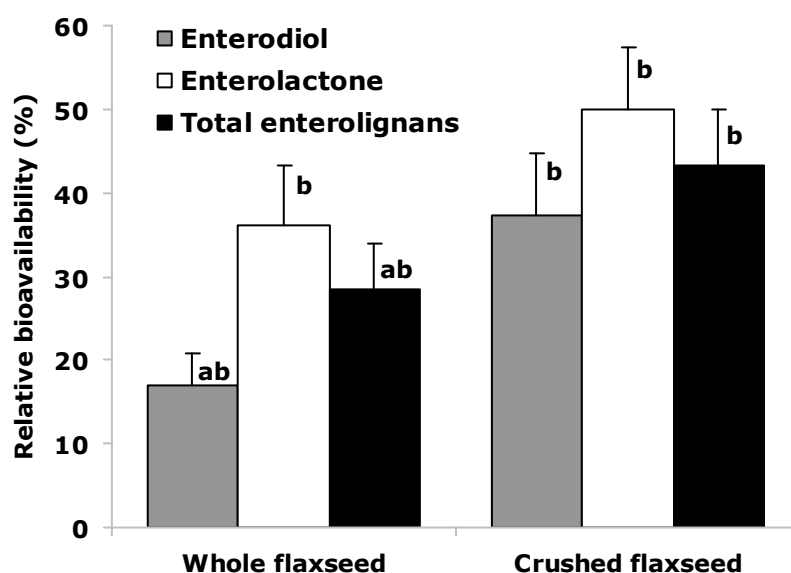

Figure 4.1 Relative bioavailability in human subjects of lignans from whole and crushed flaxseed compared with ground flaxseed (set at 100%). Values are means ± SEM, n = 12.
[a] Different from crushed flaxseed ($P \leq 0.05$); [b] different from ground flaxseed ($P \leq 0.01$).
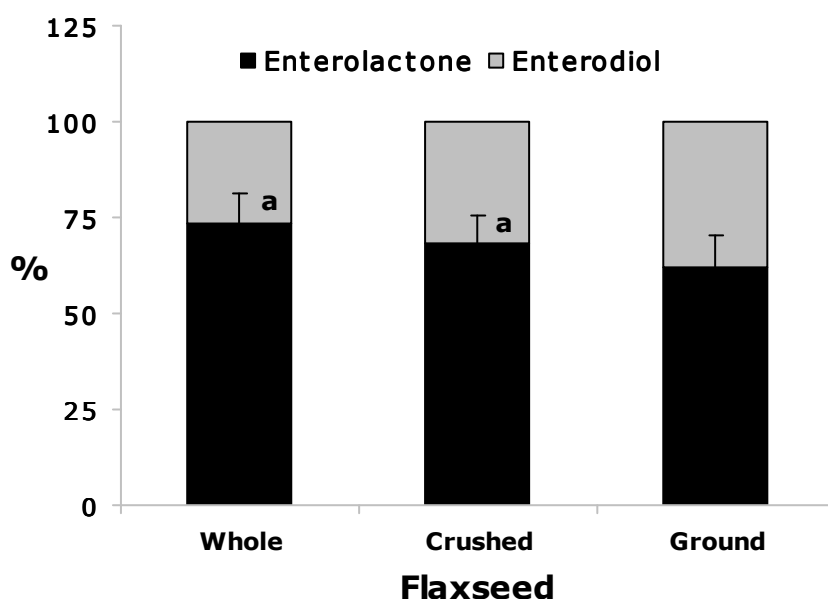
Figure 4.2 Contribution of enterolactone and enterodiol (%) to the total concentration of enterolignans in plasma of human subjects per supplement period. Values are means ± SEM, n=12. [a] Different from ground flaxseed (*P*≤ 0.05).

concentration of enterodiol (61 nmol/L) and of enterolactone (74 nmol/L) at the end of the first run-in/wash-out period. We excluded this value because at the end of the other run-in/wash-out periods, the concentrations of both enterolignans in this subject were much lower (enterodiol: 0.0 and 0.4 nmol/L; enterolactone 3.5 and 13.8 nmol/L). The overall mean baseline concentration of enterodiol in plasma was 1.9 ± 0.5 nmol/L and of enterolactone 9.5 ± 1.1 nmol/L (Table 4.1).

In most subjects (8 of 12), plasma concentrations of enterolactone were higher than plasma concentrations of enterodiol (the percentage of enterolactone ranged from 71 to 98%). In 3 subjects, the contribution of enterolactone (range 43 to 49%) was similar to that of enterodiol. In 1 subject, enterolactone contributed only 12% to the total concentration of enterolignans. The contribution of enterolactone to the total concentrations of enterolignans in plasma varied among the supplement periods (**Figure 4.2**). The percentages of enterolactone during the whole flaxseed period (73%) and crushed flaxseed period (68%) were both significantly higher than the percentage during the ground flaxseed period (62%). The percentages of enterolactone did not differ significantly between the whole and crushed flaxseed periods. The order of treatment had no effect on enterolactone percentages in plasma.

After consumption of flaxseed, the within-subject variation of plasma enterodiol concentrations ranged from 23 to 31% and that of enterolactone from 35 to 48% (**Table 4.2**). The between-subject variation of plasma enterodiol concentrations ranged from 82 to 98% and that of enterolactone from 45 to 81%. After the run-in/wash-out period, when plasma concentrations of enterolignans were low, the within- and between subject variations were slightly higher (range 49

Table 4.2 Within- and between-subject variations of enterodiol and enterolactone plasma concentrations after consumption of whole, crushed, and ground flaxseed (0.3 g/kg body weight per day) for 10 days[1].

	Within-subject variation (%)	Between-subject variation (%)	ICC (95% CI)
Enterodiol			
Whole flaxseed	31	82	0.87 (0.71-0.96)
Crushed flaxseed	27	98	0.93 (0.83-0.98)
Ground Flaxseed	23	98	0.95 (0.87-0.98)
Baseline[2]	65	104	0.72 (0.44-0.90)
Enterolactone			
Whole flaxseed	35	81	0.84 (0.65-0.95)
Crushed flaxseed	48	59	0.61 (0.28-0.85)
Ground Flaxseed	39	45	0.57 (0.23-0.83)
Baseline[2]	49	55	0.56 (0.22-0.83)

[1] Within- and between-subject CV are expressed as percentages, n = 12. The variations are based on 3 plasma samples taken on 1 day at the end of each supplement period.

[2] Baseline values are based on the run-in/wash-out-period before the ground flaxseed period.

to 104%). After consumption of flaxseed, the within-day ICCs of a single measurement were quite high for both enterolignans (Table 2). At low concentrations of enterodiol and enterolactone (baseline period) the ICCs of a single measurement were a little lower When we studied the within-day variation in more detail, we found that the concentration of enterolactone decreased significantly during the day in all 3 supplementation groups (**Figure 4.3**). The concentration of enterodiol decreased slightly during the day only after supplementation with crushed flaxseed.

DISCUSSION

In a randomized crossover study, we showed for the first time that crushing and milling of flaxseed improved the bioavailability of lignans 2- to 4-fold compared to whole flaxseed. This increased bioavailability is likely due to the improved accessibility of the colon bacteria to crushed and ground flaxseed.

In our study, the total plasma enterolignan concentration in subjects consuming 15-25 g ground flaxseed for 10 days (270 nmol/L) was higher than in Canadian subjects consuming 25 g ground flaxseed for 8 successive days (~85 nmol/L) [3]. A simple explanation for this difference is the lignan content of the flaxseed supplements, which was >2 times higher in our study (9.91 µmol/g) than in the Canadian study (2.97 µmol/g).

Steady-state plateau concentrations will be reached when the supplemental interval (10-14 h in this study) is smaller than 4 times the elimination half-life (4.4 h for enterodiol, 12.6 h for enterolactone) [30], which was true in our study. The elimination half-life of 12.6 h predicts that

steady state will be reached after 2 days (4 x 12.6 h); thus the 10 days of supplementation were sufficient. Nevertheless, fluctuations in enterolignan plasma concentrations are to be expected because of the noncontinuous dosing. To improve accuracy, 3 blood samples were taken on 1 day to measure the enterolignan concentrations. In spite of the diet restrictions and the standardized intake of flax supplements, there were large variations within (24-38%) and between subjects (46-98%). The within-subject variation was similar to that (31%) in 6 postmenopausal women consuming an experimental diet with 142 μg lignans/day [31]. Higher within-subject variations were observed over periods of weeks (67%) and months (68%) among 20 Finnish men and women consuming their normal diet (median concentration of enterolactone was 9.1 nmol/L) [32]. The ratio of within-subject and between-subject variation in our study was quite low, resulting in high ICCs for enterodiol (0.86-0.95) and enterolactone (0.57-0.84). Even at low concentrations, the ICC was relatively high (0.56-0.72; Table 4.2). Our results indicate that a single measurement will accurately reflect an individual's mean short-term concentration. Note, however, that this result was obtained in a well-controlled trial with standardized consumption of flaxseed. Moderately high ICCs of plasma enterolactone measurements have been observed over a period of weeks (0.79) and months (0.77) in men and women consuming their normal diet [32]. As expected, lower ICCs of serum measurements (enterolactone: 0.55; enterodiol 0.37) were observed over a 2-years period [33]. Our results and those of others indicate that plasma enterolactone and enterodiol are relatively good biomarkers. Although there was a relatively high within-subject variation, distinction between individuals is feasible as indicated by the relatively high ICC.

When we studied the within-subject variation in more detail, we observed that the daily variation of enterolactone was higher than that of enterodiol (Figure 4.3). This was surprising because the elimination half-life enterolactone, obtained in a single dose experiment, was longer than that of enterodiol [5], which would predict less variation [30]. It is possible that absorption and elimination are affected differently by enterodiol and enterolactone during chronic supplementation, leading to changes in elimination. Alternatively, the daily meal pattern may have influenced the availability of enterolignans. We observed that enterolactone concentrations in the morning were higher than late in the afternoon (Figure 4.3). In the morning, subjects consumed flaxseed supplements with breakfast (light meal), and in the evening with dinner (heavy meal). When flaxseed supplements are consumed with a heavy meal and before sleeping, the residence time in the colon will be longer, and conversion to enterolactone, the final metabolite, might increase. Enterolignans appear in plasma 8-10 h after consumption. Thus, increased conversion during the night might explain the high concentrations of enterolactone in the morning.

Quantitatively, enterolactone was the major metabolite of the plant lignans in flaxseed. The percentage of enterolactone differed slightly among the supplement periods. When subjects consumed ground flax, the percentage of enterolactone was lower than after consumption of

A

B
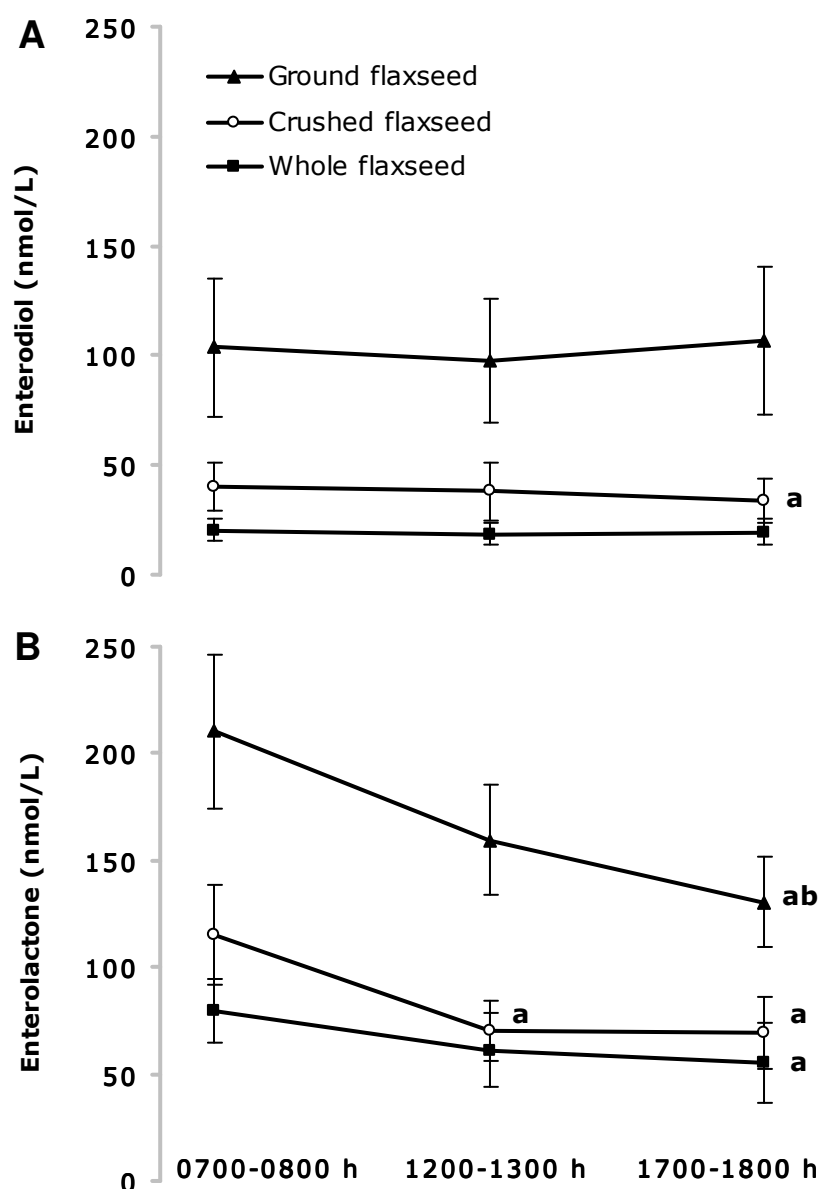
Figure 4.3 Plasma concentrations of enterodiol (A) and enterolactone (B) in human subjects at various times during the day after the supplement period. Values are means ± SEM, n = 12.
[a] Different from first sample (0700-0800 h); [b] different from second sample (1200-1300 h).

whole or crushed flax (Figure 4.2). Secoisolariciresinol, the major lignan in flaxseed, is first converted to enterodiol and thereafter to enterolactone by intestinal bacteria [34]. The capacity of the bacteria to convert enterodiol to enterolactone might be limiting when the highly accessible secoisolariciresinol diglucoside of ground flaxseed is given. This is supported by 2 studies [3, 35] in which the investigators observed decreasing percentages of enterolactone in urine with increasing consumption of ground flaxseed.

Overall enterolignans concentrations at the end of the 3 wash-out periods did not increase during this study. In addition, there was no effect of treatment order. This suggests that the bacteria in our 9-week study were not stimulated and that we measured only the effect of milling and

crushing of flax on lignan bioavailability. Although we found no evidence for it, stimulation of the colon bacteria due to chronic supplementation with flaxseed was suggested to occur in studies with longer exposures. In rats, Nicolle et al [36] observed that 2 weeks after supplementation with flaxseed, urinary enterolactone concentrations were still increasing. Similarly, in humans, plasma enterolactone concentrations further increased after 2 months supplementation with flaxseed [3, 11].

Throughout this study, the subjects consumed a diet poor in lignans. Although no dietary information was recorded, the low baselines suggest that compliance to the diet was good. However, in one subject, enterolignan concentrations were high (range 77 to 191 nmol/L) at the end of the first run-in/wash-out period. This was probably due to dietary noncompliance although the subject did not report this. In the data-analysis, we excluded this value because at the end of the other run-in/wash-out periods, the concentrations of both enterolignans were much lower in this subject and comparable to the baseline concentrations of other participants. We expected that this high enterolignan concentration would not affect the next sampling point because the length of the run-in/wash-out period was 11 days. When we excluded all outcomes of this subject, the relative bioavailability changed < 3%.

In conclusion, this study shows that bacteria in the colon are able to convert lignans from whole flaxseed to enterolignans, but crushing and milling of flaxseed substantially enhances the bioavailability of lignans from flax. The availability of lignans from food products, such as breads, will improve when whole seeds are replaced by crushed or ground seeds. The food matrix may thus be an important determinant of the bioavailability of lignans. Therefore, when assessing the dietary exposure to enterolignans, it is important to determine the bioavailability of lignans from other food sources, especially seeds. Enterolactone was the major metabolite in our study. Yet, the percentage of enterolignans was quite variable among individuals. Even within individuals the percentage of enterolignans varied slightly; the percentage of enterolactone was lower when subjects consumed ground flaxseed instead of whole flaxseed. Plasma enterolactone and enterodiol are expected to be suitable biomarkers because distinction between individuals is feasible as indicated by relatively high ICCs.

ACKNOWLEDGEMENTS

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ABSTRACT

Enterolignans are phytoestrogenic compounds derived from the conversion of dietary lignans by the intestinal micro flora that may be protective against cardiovascular diseases and cancer. In order to evaluate the use of enterolignans as biomarkers of dietary lignan intake, we have studied the relationship between plasma and dietary lignans. We determined the dietary intake of four lignans (secoisolariciresinol, matairesinol, pinoresinol and lariciresinol) using the EPIC food frequency questionnaire, and plasma enterodiol and enterolactone concentration using LC-MS/MS. The population consisted of 637 men and women, aged 19-75 years, participating in a case-control study on colorectal adenomas. Participants did not use antibiotics in the preceding calendar year. We found a modest association between lignan intake and plasma enterodiol (r_s = 0.09, P = 0.03) and enterolactone (r_s = 0.18, P < 0.001). The correlation of total lignan intake with plasma enterolignans was slightly stronger than that of only secoisolariciresinol plus matairesinol. The plasma concentrations of both enterodiol and enterolactone were associated with intake of dietary fiber and vegetable protein, but not with intake of other macronutrients. The relation between lignan intake and plasma enterodiol was modulated by age and previous use of antibiotics, whereas for enterolactone it was modulated by weight, current smoking and frequency of defecation. However, even when we included these non-dietary factors in the regression models the explained variance in plasma enterodiol and enterolactone remained low (2 and 13% respectively). Accounting for the bioavailability of lignans and including additional enterolignan precursors may improve the validity of enterodiol and enterolactone as biomarkers for dietary lignan intake.

Keywords: lignans, phytoestrogens, intake, biomarker, human

Relation between enterolignans in plasma and dietary intake of lignans

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Chapter 5

INTRODUCTION

Lignans are biphenolic compounds in plant foods, which belong to the group of phytoestrogens. Wholegrain products, vegetables, fruits, nuts, seeds, and beverages such as tea, coffee, and wine are dietary sources of lignans [1, 2]. Plant lignans can be converted by intestinal bacteria into so-called enterolignans: enterodiol and enterolactone. For about two decades only secoisolariciresinol (SECO) and matairesinol (MAT) were known to be precursors of enterolignans, but recently it was shown that also pinoresinol (PINO) and lariciresinol (LARI) are efficiently converted into enterolignans [3].

Enterolignans possess several biological activities, by which they may reduce the risk of cancer and cardiovascular diseases. Enterolignans have weak estrogen-like activity [4], may inhibit enzymes such as aromatase and 5α-reductase, and stimulate the production of sex hormone binding globulin (SHBG) [5]. In addition, plant lignans, and to a lesser extent also enterolignans have antioxidant activity [6]. In epidemiological studies some evidence for protection of lignans against hormone-related cancers and cardiovascular diseases was found, but results were not consistent [7]. Enterolignan concentrations in biological fluids have been used as a biomarker for lignan intake in several of these studies.

In order to assess the relative validity of these exposure measures, a few studies have examined the correlation between plasma and dietary lignans. So far, these studies have only found weak to moderate associations (Spearman r = 0.08-0.19) [8-10], possibly, because they only included two of the dietary lignan precursors (SECO plus MAT), and one of the metabolites (enterolactone). Horn-Ross et al [11] have used urinary enterodiol plus enterolactone as a biomarker for lignan intake, and found a similar correlation with intake of SECO and MAT (Spearman r = 0.17). In one study additional dietary lignans have been included, but lignan contents were only available for milk, bread and cereal products, and it did not improve the correlation with serum enterolactone compared to inclusion of only SECO plus MAT [12].

We have recently developed and validated methods to measure four dietary lignans in foods, and two enterolignans in plasma [13, 14]. In order to evaluate the use of enterolignans as biomarkers of dietary lignan intake, we have studied the relationship between plasma and dietary lignans in the POLIEP-study, a case-control study on colorectal adenomas. We included both plasma lignans enterodiol and enterolactone and four dietary lignans (LARI, PINO, SECO and MAT). In addition we identified other determinants of plasma lignans, which may modulate the relation between plasma and dietary lignans.

MATERIALS AND METHODS

Population

Subjects included in the current study were men and women participating in the POLIEP-study, a case-control study, designed to investigate gene-environment interactions and the risk of

Table 5.1 Characteristics of the study population according to tertiles of total plasma enterolignans (n = 637)

	I (n = 212)	II (n = 213)	III (n = 212)	P value[1]
Plasma enterolignans (nmol/L) [2]				
Total	5.0 (3.2-7.1)	15.5 (12.3-19.8)	40.6 (30.2-58.6)	
Enterodiol	0.7 (0.4-1.2)	1.4 (0.8-2.7)	2.8 (1.7-5.1)	
Enterolactone	3.8 (1.5-5.7)	13.8 (10.1-17.5)	37.3 (27.3-55.6)	
Lignan intake (µg/d) [2]				
Total	937 (740-1159)	1006 (798-1260)	1072 (873-1319)	<0.001
Lariciresinol	445 (348-542)	481 (377-577)	500 (406-625)	<0.001
Pinoresinol	303 (235-432)	334 (243-450)	377 (285-476)	<0.001
Secoisolariciresinol	181 (139-222)	187 (150-211)	189 (154-232)	0.17
Matairesinol	6 (3-11)	8 (4-11)	9 (5-13)	<0.001
General				
Age (y) [3]	54 ± 13	57 ± 12	57 ± 12	0.01
Female (%)	54	51	63	0.04
Weight (kg) [3]	79 ± 16	77 ± 15	73 ± 13	<0.001
Smoking (% current)	30	27	16	0.02
Physical activity (% low)	32	38	26	0.14
Bowel				
Polyps (total cases, %)	56	49	51	0.36
Indication for colonoscopy (%)				0.15
Complaints	65	62	62	
Screening	26	33	32	
Other/unknown	10	5	7	
Frequency of defecation (%, #/wk)				<0.001
>7	53	44	26	
5-7	37	43	54	
1-5	9	11	17	
<1	1	2	3	
Diet change because of bowel complaints (%)	24	30	31	0.22
Medication				
Antibiotics (% ever) [4]	78	77	74	0.57
Regular NSAID use (% ≥ 12 times/y)	26	36	30	0.30
Oral contraceptive use (% current) [5]	13	12	9	0.79

[1] normal distributed variables (ANOVA); skewed variables (Kruskal-Wallis); categorical variables (χ^2 test).

[2] Values are median (25th percentile, 75th percentile).

[3] Values are mean ± SD.

[4] Note that persons who used antibiotics in the previous calendar year have been excluded.

[5] In women only; n = 355.

colorectal adenomas. Participants were recruited among patients undergoing colonoscopy in ten clinics in the Netherlands between June 1997 and October 2002. The study design has previously been described in more detail [15, 16]. Eligible subjects were Dutch speaking, of European origin, aged 18-75 years at the time of colonoscopy, had no hereditary colorectal cancer syndromes (i.e., familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer), no chronic inflammatory bowel disease, no history of colorectal cancer, no (partial) bowel resection, or serious disabling morbidity. In the POLIEP-study cases were defined as those with at least one histologically confirmed colorectal adenoma ever in their life. In controls diagnosis of any type of adenomas was negative at the colonoscopy, and there was no history of any type of adenomas (based on medical record).

Plasma was available for 1385 of a total of 1477 subjects. Before a colonoscopy, subjects are fasted, and they receive medication to clean the colon. Because enterolignans are produced by intestinal bacteria, plasma enterolignan values are expected to be lower than normal at the time of colonoscopy. Therefore we excluded 350 subjects, whose blood samples were drawn on the same day a colonoscopy was performed. Antibiotic use can decrease enterolignan concentrations for 3-12 months [17]. We excluded an additional 397 subjects because they had taken antibiotics in the calendar year of blood sampling, or because data on antibiotic use were missing. One subject was excluded because information on body weight was missing, which made it impossible to calculate the lignan dose. After these exclusions, data of 637 subjects remained in the analyses, 331 cases with adenomatous polyps, and 306 endoscopy controls who never had polyps.

Data collection

Participants were requested to complete self-administered questionnaires on diet, medical history, and lifestyle according to habits in the year preceding their last colonoscopy. Habitual physical activity was estimated using a short questionnaire [18].

Dietary assessment. Dietary intake was assessed with the validated Dutch EPIC Food Frequency Questionnaire [19, 20]. This questionnaire enables estimation of the average daily consumption of 178 food items in the preceding year. Consumption data were converted into energy and nutrient data using the Dutch Food Composition Table [21]. Lignan intake was estimated using a recently developed database [2] including contents of LARI, PINO, SECO, and MAT of commonly consumed Dutch plant foods.

Collection of plasma samples. Non-fasting venous blood samples were taken into vacuum tubes containing EDTA. Samples were taken on average 4 months after colonoscopy. Samples were transported to our laboratory at Wageningen University in a foam fridge at 4°C. Within 48 h they were centrifuged at 1187g for 10 min at 4°C and then kept at -80°C until analysis.

Assay of plasma values

Plasma enterodiol and enterolactone were determined using a validated isotope-dilution LC-MS/MS method [14]. In brief, $^{13}C_3$ labeled enterodiol and enterolactone were added to the samples, and samples were enzymatically hydrolyzed in order to release aglycones from glucuronide and sulfate conjugates. Samples were extracted twice with diethyl ether, dissolved in 40% methanol/water, ether evaporated, filtered, and injected into the LC-MS/MS system. The samples were analyzed in 20 runs over a 12-week period. The between assay coefficient of variation was 14% for enterodiol and 10% for enterolactone. The limit of detection was 0.15 nmol/L for enterodiol and 0.55 nmol/L for enterolactone. When a plasma value was below the detection limit we assigned to that sample a value of 0.5 times the detection limit to enable log-transformation of skewed data.

Statistical analysis

Participants were classified according to tertiles of total plasma enterolignans. To test for differences in demographic and lifestyle characteristics between tertiles, we used analysis of variance for normally distributed variables, Kruskal-Wallis test for skewed variables and the chi-square test for categorical variables.

Spearman's rank order correlations were calculated for absolute lignan intake, as well as lignan dose (lignan intake per kg body weight) with plasma enterolignan concentrations. We have used linear regression to evaluate whether the correlations between lignan intake and plasma enterolignans differed between total, incident or prevalent cases and controls. To identify dietary and non-dietary determinants of plasma enterolignans, several regression models were used. Because the distribution of dietary and plasma lignans was skewed, log-transformed data were used. First, total lignan intake, age, sex, weight, smoking, physical activity, use of antibiotics (ever), polyps (ever), indication for colonoscopy and frequency of defecation were examined as potential determinants of plasma enterolignans by fitting univariate models. Secondly we constructed a 'full model' that included all these non-dietary variables. Finally, to identify dietary determinants of plasma enterolignan concentrations, we used multivariate models including the non-dietary determinants that were significant ($P < 0.05$) in the 'full model' for enterodiol or enterolactone, and energy intake. Dietary lignans, fiber, major energy providing nutrients, and major food sources of lignans were considered as potential dietary determinants of plasma enterolignan concentrations. We used one SD change in intake of potential dietary determinants to estimate the associated change in plasma enterolignan concentrations. Statistical analyses were performed using SAS software (version 9.1, SAS institute, Inc, Cary, NC).

RESULTS

As expected, participants in the highest tertile for total plasma enterolignans had higher intakes of all four dietary lignans than participants with lower plasma enterolignan concentrations, although the difference was not significant for SECO (**Table 5.1**). Participants in the highest tertile of total plasma enterolignans were on average older, more likely to be female, of lower weight, less likely to smoke, and more likely to have a low frequency of defecation than participants with lower plasma enterolignan concentrations.

The mean (95% CI) plasma concentrations in the total population were (geometric means) 1.4 (1.3-1.6) nmol/L for enterodiol and 11.3 (10.2-12.5) nmol/L for enterolactone (**Table 5.2**). Six percent (n = 39) of the samples was below the detection limit of 0.15 nmol/L for enterodiol and 1% (n = 6) was below the detection limit for enterolactone of 0.55 nmol/L. The mean (95% CI) total lignan intake was (geometric mean) 989 (963-1016) µg/d.

The correlation of total lignan intake with plasma enterodiol (r_s = 0.09) was weaker than that with plasma enterolactone (r_s = 0.18) (Table 5.2). When lignan intake was expressed per kg body weight (lignan dose), the correlations with plasma enterolignans were stronger than those for absolute lignan intake. Most of the correlations of plasma enterodiol and enterolactone with the individual lignans were slightly weaker than those with total lignan intake, and the correlations of intake of SECO and MAT with plasma enterodiol were not statistically significant. The correlation

Table 5.2 Spearman correlation coefficients between lignan intake and plasma lignan concentration in 637 men and women in the POLIEP-study.

		Plasma lignans (nmol/L) [1]	
		Enterodiol 1.4 (1.3-1.6)	Enterolactone 11.3 (10.2-12.5)
Lignan intake (µg/d) [1]		r_s	r_s
Lariciresinol	463 (451-475)	0.09 [a]	0.18 [c]
Pinoresinol	328 (317-340)	0.10 [a]	0.18 [c]
Secoisolariciresinol	180 (176-185)	0.02	0.10 [b]
Matairesinol	6 (6-7)	0.07	0.21 [c]
Total	989 (963-1016)	0.09 [a]	0.18 [c]
Lignan dose (µg/kg/d) [1]			
Lariciresinol	6.2 (6.0-6.4)	0.12 [b]	0.24 [c]
Pinoresinol	4.4 (4.2-4.6)	0.13 [c]	0.23 [c]
Secoisolariciresinol	2.4 (2.3-2.5)	0.06	0.18 [c]
Matairesinol	0.1 (0.1-0.1)	0.09 [a]	0.23 [c]
Total	13.2 (12.8-13.6)	0.12 [b]	0.24 [c]

[1] Geometric mean (95% CI).

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

Table 5.3a Determinants of plasma enterodiol in 637 men and women in the POLIEP-study.

	Univariate model			Full model [1]	
	β	*P* value	Adj-R²	β	*P* value
Total plant lignans [2]	0.112	0.03	0.6	0.094	0.95
Age (y)	0.011	0.008	1.0	0.012	0.007
Sex (female vs. male)	0.080	0.44	0.1	0.012	0.93
Weight (kg)	-0.007	0.03	0.6	-0.006	0.11
Smoking (ex vs. never)	0.063	0.59	0.3	0.014	0.91
Smoking (current vs. never)	-0.200	0.12	0.3	-0.144	0.30
Physical activity score	-0.007	0.89	0.2	-0.031	0.54
Antibiotics (ever vs. never) [3]	0.237	0.05	0.5	0.275	0.03
Polyps (ever vs. never)	-0.092	0.36	0.0	-0.177	0.14
Indication for colonoscopy			0.3		
Complaints vs. other/unknown	-0.061	0.76		-0.124	0.55
Screening vs. other/unknown	-0.011	0.96		-0.011	0.96
Frequency of defecation (#/wk)			0.2		
5-7 vs.>7	0.108	0.33		0.118	0.31
1-5 vs. >7	0.097	0.56		0.099	0.58
<1 vs. >7	0.315	0.39		0.303	0.41
Diet change because of bowel complaints	0.122	0.28	0.0	0.018	0.88

[1] Adjusted for all other variables in the table; adjusted-R² = 2.1.

[2] per SD increment (= 345 µg/d).

[3] Note that persons who used antibiotics in the previous calendar year have been excluded.

between total lignan intake and plasma enterolignans did not differ between total, incident or prevalent cases and controls (*P* for interaction all ≥ 0.41, results not shown).

In the univariate models, total lignan intake, age, weight and ever use of antibiotics were significantly associated with plasma enterodiol (**Table 5.3a**). In the 'full model' only age and ever use of antibiotics remained statistically significant. For the 'full model' the total explained variance was 2.1%. Total lignan intake, age, weight, current smoking and frequency of defecation were significantly associated with plasma enterolactone in both the univariate and the 'full model' (**Table 5.3b**). The total explained variance of enterolactone concentrations was 12.7%.

We then further evaluated the dietary determinants of enterodiol and enterolactone after adjustment for age, weight, energy intake, use of antibiotics, current smoking and frequency of defecation. Plasma enterolactone was significantly associated with all four individual dietary lignans, whereas plasma enterodiol was only significantly associated with intake of LARI and PINO (**Table 5.4**). The increase in plasma enterodiol and enterolactone associated with one SD increase in intake of fiber or one SD increase in intake of vegetable protein was larger than that

Table 5.3b. Determinants of plasma enterolactone in 637 men and women in the POLIEP-study.

	Univariate model			Full model [1]	
	β	*P* value	Adj-R²	β	*P* value
Total plant lignans [2]	0.201	<0.001	2.2	0.179	<0.001
Age (y)	0.013	0.002	1.3	0.011	0.01
Sex (female vs. male)	0.140	0.18	0.1	0.158	0.19
Weight (kg)	-0.015	<0.001	2.7	-0.013	<0.001
Smoking (ex vs. never)	0.074	0.53	2.2	0.029	0.81
Smoking (current vs. never)	-0.438	<0.001		-0.348	0.009
Physical activity score	0.053	0.29	0.0	0.009	0.86
Antibiotics (ever vs. never) [3]	-0.170	0.17	0.1	-0.119	0.33
Polyps (ever vs. never)	-0.033	0.75	0.1	-0.030	0.79
Indication for colonoscopy			0.2		
Complaints vs. other/unknown	0.204	0.32		0.208	0.30
Screening vs. other/unknown	0.364	0.10		0.345	0.10
Frequency of defecation (#/wk)			4.9		
5-7 vs.>7	0.533	<0.001		0.540	<0.001
1-5 vs. >7	0.626	<0.001		0.656	<0.001
<1 vs. >7	1.293	0.001		1.389	<0.001
Diet change because of bowel complaints	0.216	0.07	0.4	0.187	0.10

[1] Adjusted for all other variables in the table; adjusted-R² = 12.7.

[2] per SD increment (= 345 µg/d).

[3] Note that persons who used antibiotics in the previous calendar year have been excluded.

associated with one SD increase in total lignan intake. None of the other nutrients was significantly associated with plasma enterolignan concentrations. Wholegrain wheat bread was the only lignan-containing food for which the consumption was significantly associated with plasma enterodiol. For enterolactone also consumption of fruits, nuts and seeds, and wine were significantly associated with its plasma concentrations. Beer consumption was significantly inversely associated with plasma enterolactone.

DISCUSSION

In the present study we found a modest positive association between plasma enterolignans and dietary lignan intake. The Spearman correlation coefficient of plasma enterodiol was 0.09 ($P <$ 0.001), and of enterolactone 0.18 ($P <$ 0.001). As expected, the correlations improved (enterodiol r_s = 0.12; enterolactone r_s = 0.24) when lignan dose was used instead of lignan intake.

An important strength of our study is that we used a newly developed database [2] that includes four major plant lignans, SECO, MAT, PINO and LARI, whereas other studies included only SECO plus MAT. Furthermore, in contrast to other databases, all values in this database were obtained

using one analytical method (LC-MS/MS) [13] with an identical sample preparation for all food items. Lignan intake in the year preceding colonoscopy was estimated based on the habitual consumption of 178 food items in the Dutch EPIC-FFQ, which had a satisfactory reproducibility and relative validity compared to 12 monthly 24-h recalls for the main food groups contributing to lignan intake [19]. In addition, we have included both enterodiol and enterolactone, to assess the internal exposure (plasma concentrations), whereas most of the previous studies only included enterolactone.

A drawback of our study may be that the population exists of persons who underwent colonoscopy. Both cases and controls often had bowel complaints and/or a positive (family) history of abdominal polyps or cancer. Thus, results for this population may have differed from those for a 'healthy' population. 28% of the study participants indicated that they changed their diet because of their bowel complaints, but their plasma enterolignan levels were not significantly different from those of participants who did not change their diet. In addition, plasma enterolignan concentrations did not differ between persons who underwent a colonoscopy indicated by complaints, screening or other/unknown reasons. And finally, the correlation between total lignan intake and plasma enterolignans did not differ between cases and controls. So, to the extent that we could verify this in our study, we found no evidence that the relation between plasma enterolignan levels and dietary lignan in our population differs from that in a healthy population, and we have combined cases and controls in the analyses.

Vegetables, black tea, wholegrain bread, fruits, and wine were the most important lignan sources. Although vegetables and black tea contributed both more then 20% to the total lignan intake, they were not associated with plasma enterolignan concentrations. Consumption of wholegrain bread, fruits, nuts and seeds, and wine contributed less to the total lignan intake, but all were significantly associated with plasma enterolignan concentrations. This indicates that there are differences in the bioavailability from different foods, or in the reliability of the intake measurements. Indeed, the relative validity of the EPIC-FFQ compared to 12 monthly 24-hour recalls was relatively low for vegetables, and higher for bread, fruits and nuts and seeds [19].

Although the correlation of lignan intake with the intake of dietary fiber is relatively strong (r_s = 0.6, P < 0.001; results not shown) and the variation in intake is similar, the increase in plasma enterolignans associated with one SD intake in fiber was larger than that associated with one SD increase in total lignan intake. Perhaps fiber-rich foods also contain other enterolignan precursors such as syringaresinol and sesamin that we did not take into account, but that may also be converted to enterolignans [3, 22]. This is supported by the fact that the association between intake of dietary fiber and enterolactone was only slightly attenuated from β = 0.296 to β = 0.215 nmol/L/SD fiber intake (P = 0.007; results not shown), i.e., it remained clearly positive, when lignan intake was also included in the multivariate model.

Plasma enterodiol concentrations were positively associated with total lignan intake, age and

antibiotic use. We have excluded participants who used antibiotics in the calendar year preceding the colonoscopy. Therefore it is remarkable that we found a significant positive association between plasma enterodiol and antibiotic use. Apparently, antibiotic use can have a long-term effect on plasma enterolignans, and it does not affect plasma enterodiol and enterolactone concentrations in a similar way. A possible explanation for this finding is that the bacteria involved in the production of enterodiol and enterolactone from dietary precursors are not the same [23, 24]. So far, one bacterial strain capable of catalyzing the oxidation of enterodiol to enterolactone has been identified [25]. Destruction of this strain by antibiotics may increase the relative concentration of enterodiol, due to reduced conversion to enterolactone.

In agreement with previous studies we found that plasma enterolactone concentrations were associated with age, and inversely associated with weight, frequency of defecation and current smoking. Kilkinnen et al [26] found that serum enterolactone concentrations were higher in men and women with constipation. They also found that serum enterolactone concentrations were associated with age, and inversely associated with smoking and obesity in women, but not in men. Horner et al. [27] have reported that plasma enterolactone concentrations were associated with age, and inversely associated with body mass index.

The correlation between total dietary lignan intake and plasma enterolactone (r_s = 0.18, P < 0.001), was similar to correlations found previously for lignan density (SECO plus MAT/energy intake) and serum enterolactone concentrations (Spearman r = 0.18-0.19 [8, 12]), and for the correlation between intake of SECO plus MAT and urinary enterodiol plus enterolactone (spearman r = 0.17-0.25; [11]. However, it was stronger than the correlation between SECO plus MAT estimated using 12 monthly 24h-recalls and plasma enterolactone reported by Bhakta et al. [10] for South Asian and native British women in the UK, (r_s = 0.10, NS; and 0.08, NS).

In our study, associations between lignan intake and plasma enterolignans were stronger when we included four dietary lignans, than when we included only SECO plus MAT. This indicates that inclusion of four dietary lignans better reflects the exposure to enterolignans than only the inclusion of SECO plus MAT. In the study of Hedelin et al. [12] seven additional dietary lignan were included, but the correlation between lignan intake and serum enterolactone was not stronger than when only SECO and MAT were included. However, data on these additional lignans were only available for milk, bread and cereal products. It should also be noted that with the inclusion of four dietary lignans and two lignan metabolites, both the dietary lignan intake and plasma enterolignan concentrations may still be underestimated, because more enterolignan precursors [3, 22], as well as lignan metabolites [28] have been identified. In addition, the estimation the dietary lignan exposure could be improved by taking into account seasonal and variety variation in lignan content, and differences in bioavailability of lignans from different foods [29]. Food processing may affect both the lignan content and bioavailability from foods, e.g. crushing or milling substantially improved the bioavailability of lignans from flaxseed [30].

Table 5.4. Dietary determinants of plasma enterolignans in 637 men and women in the POLIEP-study [1]

	mean ± SD	Enterodiol 1.4 (1.3-1.6) [2] β	P value	Enterolactone 11.3(10.2-12.5) [2] β	P value
Dietary lignans					
LARI (µg/d)	488 ± 157	0.133	0.02	0.230	<0.001
PINO (µg/d)	362 ± 153	0.118	0.03	0.166	0.002
SECO (µg/d)	191 ± 64	0.052	0.35	0.176	0.001
MAT (µg/d)	9 ± 7	0.082	0.14	0.228	<0.001
SECO+MAT (µg/d)	200 ± 68	0.057	0.30	0.185	<0.001
Total (µg/d)	1045 ± 345	0.125	0.03	0.218	<0.001
Nutrients					
Energy (kJ/d) [3]	8354 ± 2437	-0.076	0.16	-0.014	0.79
Fiber (g/d)	23 ± 7	0.149	0.03	0.296	<0.001
Carbohydrates (g/d)	223 ± 69	-0.045	0.68	0.085	0.43
Vegetable protein (g/d)	27 ± 9	0.178	0.03	0.357	<0.001
Animal protein (g/d)	49 ± 17	-0.033	0.65	-0.120	0.10
Monounsaturated fat (g/d)	30 ± 12	0.033	0.77	-0.028	0.80
Polyunsaturated fat (g/d)	15 ± 6	0.121	0.11	0.106	0.15
Saturated fat (g/d)	33 ± 12	-0.014	0.89	-0.152	0.14
Alcohol (g/d)	13 ± 17	-0.019	0.74	-0.043	0.43
Foods					
Vegetables (g/d)	114 ± 49	-0.005	0.92	0.043	0.40
Fruits (g/d)	190 ± 57	0.015	0.78	0.131	0.01
Wholegrain wheat bread (g/d)	56 ± 60	0.140	0.007	0.167	0.001
Other bread (g/d)	77 ± 70	-0.001	0.11	-0.002	0.05
Nuts and Seeds (g/d)	8 ± 16	0.071	0.18	0.163	0.002
Black tea (g/d)	219 ± 224	0.077	0.14	0.077	0.13
Coffee (g/d)	392 ± 293	-0.025	0.64	0.001	0.99
Wine (g/d)	45 ± 80	0.016	0.75	0.126	0.01
Beer (g/d)	88 ± 248	-0.003	0.96	-0.115	0.03

[1] Adjusted for age, weight, energy intake, use of antibiotics, current smoking, and frequency of defecation (4 categories: <1, 1-5, 5-7 or >7 times/week); β given per SD

[2] Geometric mean (95% CI).

[3] Adjusted for the same variables as above, except energy intake.

We assumed that the plasma concentrations of enterodiol and enterolactone reflect steady state concentrations because enterolignans are eliminated slowly, their precursors are present in many foods and beverages, and they are eaten several times a day [31]. Thus, one plasma sample will probably reflect exposure for a longer period. Indeed, the reliability coefficient estimated from

three yearly serum samples was moderately high for enterolactone (0.55; 95% CI: 0.41-0.69), but lower for enterodiol (0.37; 95% CI: 0.21-0.53) [32].

Several researchers have pointed out the large intra- and inter-individual variation in plasma enterolignan concentrations due to differences in the composition of the colonic micro flora [33, 34]. Knowledge on the bacterial strains responsible for the conversion of dietary lignans to enterolignans is emerging [23, 24], but so far it is not possible to take into account the micro flora composition as a factor modulating the relation between plasma and dietary lignans. We could adjust for habitual diet composition, sex, age, previous use of antibiotics, smoking, presence of polyps, and frequency of defecation, which all may affect the composition of the micro flora. However, when we included these non-dietary factors, in the multivariate adjusted models, the explained variance in plasma enterodiol and enterolactone concentrations remained low (2% and 13% respectively).

In summary, we found a modest positive association between dietary lignan intake and plasma enterodiol and enterolactone. When four dietary lignans were included the associations between dietary lignan intake and plasma enterolignan concentrations were stronger than when only SECO plus MAT were included. However, even when we also included age, weight, energy intake, previous use of antibiotics, current smoking and frequency of defecation in the regression models the explained variance in plasma enterodiol and enterolactone remained low. Accounting for the bioavailability of lignans and including additional enterolignan precursors, may improve the validity of enterodiol and enterolactone as biomarkers of dietary lignan intake.

ACKNOWLEDGEMENTS

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PART B

Enterolignans and their relation with diseases

ABSTRACT

Lignans are biphenolic compounds that occur in foods of plant origin such as whole grains, seeds, fruits and vegetables, and beverages, such as coffee and tea. Plant lignans are converted by intestinal bacteria into the enterolignans, enterodiol and enterolactone. Enterolignans possess several biological activities, whereby they may influence carcinogenesis. We studied the associations between plasma enterolignans and the risk of colorectal adenomas in a Dutch case-control study. Colorectal adenomas are considered to be precursors of colorectal cancer. Cases (n = 532) with at least one histologically confirmed colorectal adenoma and controls (n = 503) with no history of any type of adenoma were included. Plasma enterodiol and enterolactone concentrations were measured by liquid chromatography with tandem mass spectrometry. Associations were stronger for incident than for prevalent cases. When only incident cases (n = 262) were included, high compared to low plasma concentrations of enterodiol were associated with a reduction in colorectal adenoma risk after adjustment for confounding variables. Enterodiol odds ratios (95% confidence intervals) were 1.00, 0.69 (0.42-1.13), 0.60 (0.37-0.99), and 0.53 (0.32-0.88) with a significant trend (P = 0.01) through the quartiles. Although enterolactone plasma concentrations were 10-fold higher, enterolactone's reduction in risk was not statistically significant (P for trend = 0.09). Use of oral antibiotic therapy could decrease plasma concentrations of enterolactone. Exclusion of antibiotic users resulted in similar odds ratios for both enterolignans, but the association for enterolactone became somewhat stronger (P = 0.05 vs. P = 0.09). We observed a substantial reduction in colorectal adenoma risk among subjects with high plasma concentrations of enterolignans, in particular, enterodiol. These findings could be important in the prevention of colorectal adenomas.

Keywords: lignans; enterolactone; enterodiol; colorectal adenoma; case-control studies

Plasma enterolignans are associated with lower colorectal adenoma risk

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Chapter 6

INTRODUCTION

Lignans are biphenolic compounds that occur in foods of plant origin such as whole grains, seeds, especially flaxseed, fruits and vegetables, and beverages such as coffee and tea [1]. Following consumption, plant lignans are converted by intestinal bacteria into the enterolignans, enterodiol and enterolactone [2-4].

No epidemiological studies on the relation between lignan exposure and risk of colon cancer have been published. Associations between other cancers and urinary or plasma enterolignans are inconsistent. Inverse associations for breast cancer were reported in 4 case-control studies, and 1 prospective study [5-9], whereas no associations were found in 5 prospective studies [10-14]. No associations were observed between plasma enterolactone and prostate cancer in 3 nested case-control studies [15-17]. In contrast, Hernandez et al. [18] reported a positive association between plasma enterodiol concentrations and premalignant lesions of the cervix.

Enterolignans possess several biological activities *in vitro*, such as antioxidant activity [19, 20], reduction of cell proliferation of colonic mucosal cells [21], stimulation of sex hormone-binding globulin synthesis [22] and inhibition of aromatase enzyme [23], whereby they may influence carcinogenesis. Animal experiments regarding lignans and colon cancer are limited and inconsistent [24]. The two most studied sources of plant lignans are flaxseed and rye bran. Rye bran decreased colonic tumors in carcinogen treated-rats [25]. Furthermore, the number of aberrant crypt foci was significantly reduced in the distal colon in rats fed with 2.5% or 5% flaxseed or defatted flaxseed [26]. However, other studies showed that neither diets with 0.5% defatted flaxseed [27], 5% flaxseed, or 30% rye bran [28], nor the pure plant lignans secoisolariciresinol diglucoside and matairesinol [29], were able to decrease intestinal adenoma formation in APC-min mice. Hence, evidence for a protective effect of lignans on colon carcinogenesis is still contradictory.

We studied the association between plasma enterolignans and colorectal adenomas in a case-control study. Colorectal adenomas are generally regarded as precursors of colorectal cancer.

MATERIALS AND METHODS

Study population

A retrospective case-control study (the POLIEP study) was conducted in the Netherlands between June 1997 and October 2002. The study, which was designed to investigate gene-environment interactions and the risk of colorectal adenomas, has been described in detail elsewhere [30, 31]. In brief, participants were recruited among patients undergoing endoscopies, later referred to as the index endoscopy, in 10 outpatient clinics in the Netherlands. We defined cases as those with at least one histologically confirmed colorectal adenoma ever in their life. In controls, diagnosis of any type of adenomas was negative at the index endoscopy, and they had no history of any type of adenomas (based on medical records). Ninety percent of the cases and 87% of the controls

underwent a full colonoscopy (i.e., full colonoscopy or sigmoidoscopy combined with X-ray). Other subjects did not have full visualization of the colon, i.e., they had a sigmoidoscopy without X-ray or a colonoscopy in which the cecum was not reached. Response rates varied from 35% to 91% in different outpatient clinics, overall response was 54%.

Eligible subjects were Dutch-speaking persons of European origin and aged between 18 and 75 years at the time of the index endoscopy. They did not suffer from inflammatory bowel diseases and did not have a history of colorectal cancer, (partial) bowel resection, or serious disabling morbidity. Furthermore, they had no hereditary colorectal cancer syndromes (i.e., familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer).

Of 1477 eligible participants, we excluded 92 subjects because plasma samples were not available. We additionally excluded 350 subjects whose blood was drawn on the same day as their index endoscopy or of whom the date of blood sampling was missing. As patients are not allowed to eat solid foods for 24 h prior to endoscopy and they receive medication to clean the colon, intestinal bacteria are (partially) washed away and plasma enterolignan concentrations no longer reflect long-term exposure to enterolignans in these patients. Data analyses included 1035 participants: 532 cases and 503 controls. The medical ethical committees of all participating hospitals and of Wageningen University approved the study protocol and all participants have provided written informed consent.

Data collection

Cases and controls were asked to complete self-administered questionnaires on diet, medical history, and lifestyle, relating to their habits in the year preceding their index endoscopy. Dietary intake was assessed with a standardized and validated semiquantitative food frequency questionnaire that was originally developed for the Dutch cohort of the European Prospective Investigation into Cancer and Nutrition [32, 33]. Information on demographic and lifestyle factors, like smoking habits, physical activity level [34], and family history, was obtained from a self-administered questionnaire.

Collection of plasma samples

For cases and controls, nonfasting venous blood samples were taken into vacuum tubes containing EDTA. Samples were taken, on average, 4 months after admission to hospital for endoscopy. Samples were transported to our laboratory at Wageningen University in a foam fridge containing cooling materials at 4°C. Within 48 h, samples were centrifuged at 1187 x g for 10 minutes at 4°C, and then kept at -80°C until analysis.

Assay of plasma samples

The concentrations of enterodiol and enterolactone in plasma were measured by liquid

chromatography with tandem mass spectrometry using triply ^{13}C-labeled isotopes [35]. The samples were analyzed in 20 runs over a 12-week period. The between-run coefficient of variation of the quality control plasma samples was 10% for enterolactone and 14% for enterodiol. The limit of detection was 0.55 nmol/L for enterolactone and 0.15 nmol/L for enterodiol. Seven percent of the subjects had concentrations below the detection limit of enterodiol. Only 3% had concentrations below the detection limit of enterolactone. Lab technicians were blinded to the status of the subjects.

Data analysis

To assess the association between plasma enterolignans and colorectal adenomas, we used logistic regression models. First, we calculated odds ratios (ORs) and 95% confidence intervals per quartile of enterolignan concentrations in plasma. Quartiles of plasma enterodiol and plasma enterolactone were based on the distribution among controls. The quartile with the lowest enterolignan concentration (Q1) was used as a reference. A multivariate logistic regression model was used to account for the effect of several potential confounding factors, i.e., age, sex, body mass index, physical activity, smoking, alcohol intake, regular use of nonsteroidal anti-inflammatory drugs (≥12 times a year), family history of colorectal cancer, indication for endoscopy, use of oral contraceptives, and use of hormone replacement therapy (FULL model). Variables were dropped from the model when they did not change the ORs by > 10% (backwards modeling). "Time interval between endoscopy and blood sampling", and "outpatient clinic" were included as covariates, but because they did not change the ORs by > 10%, they were not included in the FULL model. Because age and sex distributions differed between cases and controls, and are related to development of colorectal adenomas, these variables remained in the model at all times.

After performing analyses for all subjects, cases were grouped into incident and prevalent cases. Incident cases (n = 262) were defined as those with a histologically confirmed colorectal adenoma at the index endoscopy, but no history of any type of polyps. Prevalent cases (n = 254) were defined as those with or without an adenoma at the index endoscopy, but with a history of at least one histologically confirmed colorectal adenoma. When data on prevalence of former polyps were missing, cases (n = 16) were excluded from these analyses. Because use of oral antibiotic therapy could decrease urinary and serum concentrations of enterolactone for 3 to 12 months [36, 37], additional analyses were done with incident cases excluding subjects using antibiotic therapy within the same calendar year as the blood sampling.

Finally, we calculated ORs and 95% confidence intervals for enterodiol and enterolactone using concentrations on a continuous scale. As increments, we used the difference between the median concentrations of the lowest and the highest quartile. For enterodiol this increment was

Table 6.1. Characteristics of the study population

	Cases (n=532)	Controls (n=503)	*P*
General			
Age (y) [1]	59.6 ± 9.6	52.8 ± 13.6	<0.01
Female (%)	47	62	<0.01
Family history of colorectal cancer (% yes)	24	21	0.30
Indication for endoscopy (%)			
Complaints	44	80	<0.01
Screening	47	10	
Other/ unknown	9	10	
Lifestyle characteristics			
Body mass index (kg/m^2) [1]	26.2 ± 3.8	25.5 ± 4.3	<0.01
Smoking, ever (%)	66	56	<0.01
Alcohol consumption (g/d) [2]	8.8 (0.9; 23.8)	4.0 (0.3; 14.1)	<0.01
Physical activity (% low)	39	33	0.11
Medication			
Regular nonsteroidal anti-inflammatory drug use (≥12 time/y; % yes)	27	32	0.12
Antibiotics within the same calendar year of blood sampling (%)	21	25	0.12
Oral contraceptive use (% ever) [3]	68	77	0.01
Hormone replacement therapy (% yes) [3]	20	15	0.16
Plasma concentrations of enterolignans			
Enterodiol (nmol/L) [2]	1.3 (0.6-3.1)	1.5 (0.7-3.1)	0.38
Enterolactone (nmol/L) [2]	11.2 (4.4-25.4)	11.6 (4.6-26.3)	0.99
Total enterolignans (nmol/L) [2]	13.5 (6.2-29.4)	14.4 (6.0-28.7)	0.84

[1] Values are means ± SD.

[2] Values are medians (25th percentile- 75th percentile).

[3] Among women only (n = 250 cases and n = 312 controls).

4.9 nmol/L, and for enterolactone it was 39.1 nmol/L. In these analyses, concentrations were log-transformed because the distributions were skewed. Before log-transformation, we assigned a standard value (detection limit divided by two) to values of enterodiol and enterolactone that were below the detection limit. Tests for linear trend, representing potential dose-response effects, were done by fitting of a continuous variable. If *P* ≤ 0.05 (two-sided), the effect was considered significant. All statistical analyses were performed using SAS statistical analysis package (version 9.1; SAS Institute, Inc., Cary, NC).

Table 6.2 Adjusted ORs and 95% confidence intervals for plasma enterodiol and enterolactone concentrations in relation to colorectal adenomas.

	Q1	Q2	Q3	Q4	*P* for trend [1]	continuous model [1]
Enterodiol (nmol/L); cut off (median)	**<0.7 (0.4)**	**0.7-1.5 (1.0)**	**1.5-3.1 (2.1)**	**>3.1 (5.3)**		**Δ 4.9**
total cases/ controls (n)	149/126	142/126	107/126	134/125		532/503
BASIC: age, sex	1.0	0.90 (0.63-1.29)	0.69 (0.47-1.00)	0.83 (0.58-1.19)	0.20	0.90 (0.77-1.06)
FULL [2]	1.0	0.85 (0.53-1.36)	0.56 (0.35-0.91)	0.67 (0.42-1.07)	0.10	0.84 (0.68-1.03)
FINAL: age, sex, indication for endoscopy	1.0	0.88 (0.60-1.29)	0.61 (0.41-0.91)	0.75 (0.51-1.11)	0.09	0.86 (0.73-1.03)
incident cases/ controls (n)	81/126	68/126	58/126	55/125		262/503
BASIC: age, sex	1.0	0.80 (0.53-1.23)	0.67 (0.43-1.03)	0.64 (0.41-0.99)	0.03	0.81 (0.66-0.98)
FULL [2]	1.0	0.65 (0.39-1.11)	0.62 (0.37-1.05)	0.48 (0.28-0.84)	0.01	0.75 (0.60-0.94)
FINAL: age, sex, antibiotic use	1.0	0.69 (0.42-1.13)	0.60 (0.37-0.99)	0.53 (0.32-0.88)	0.01	0.75 (0.60-0.94)
prevalent cases/ controls (n)	65/126	71/126	47/126	71/125		254/503
BASIC: age, sex	1.0	1.04 (0.67-1.61)	0.68 (0.42-1.10)	1.02 (0.66-1.59)	0.90	0.99 (0.81-1.20)
FULL [2]	1.0	1.33 (0.62-2.89)	0.47 (0.21-1.04)	1.10 (0.52-2.33)	0.81	1.04 (0.75-1.43)
FINAL: age, sex , family history of colorectal cancer, indication for endoscopy	1.0	1.22 (0.63-2.39)	0.57 (0.28-1.13)	1.10 (0.57-2.14)	0.83	1.03 (0.77-1.37)
Enterolactone (nmol/L); cut off (median)	**<4.6 (1.6)**	**4.6-11.6 (7.8)**	**11.6-26.3 (17.3)**	**>26.3 (40.6)**		**Δ 39.1**
total cases/ controls (n)	137/126	132/126	133/125	130/126		532/503
BASIC: age, sex	1.0	0.89 (0.62-1.28)	0.89 (0.62-1.28)	0.79 (0.55-1.14)	0.46	0.88 (0.62-1.24)
FULL [2]	1.0	0.83 (0.52-1.33)	0.86 (0.53-1.38)	0.75 (0.46-1.22)	0.37	0.81 (0.51-1.29)
FINAL: age, sex, indication for endoscopy	1.0	0.81 (0.55-1.20)	0.74 (0.50-1.10)	0.66 (0.44-0.98)	0.12	0.75 (0.52-1.08)
incident cases/ controls (n)	78/126	70/126	54/125	60/126		262/503
BASIC: age, sex	1.0	0.87 (0.57-1.32)	0.66 (0.43-1.03)	0.67 (0.43-1.04)	0.11	0.72 (0.48-1.08)
FULL [2]	1.0	0.74 (0.44-1.25)	0.69 (0.40-1.19)	0.63 (0.36-1.10)	0.15	0.67 (0.40-1.15)
FINAL: age, sex, antibiotic use	1.0	0.72 (0.44-1.18)	0.61 (0.37-1.02)	0.63 (0.38-1.06)	0.09	0.64 (0.40-1.04)
prevalent cases/ controls (n)	55/126	57/126	77/125	65/126		254/503
BASIC: age, sex	1.0	0.95 (0.59-1.52)	1.30 (0.83-2.04)	0.95 (0.60-1.51)	0.68	1.09 (0.72-1.66)
FULL [2]	1.0	0.91 (0.41-2.01)	1.39 (0.64-3.06)	0.98 (0.45-2.13)	0.79	1.10 (0.53-2.29)
FINAL: age, sex , family history of colorectal cancer, indication for endoscopy	1.0	0.76 (0.38-1.53)	1.09 (0.56-2.13)	0.72 (0.36-1.41)	0.71	0.89 (0.48-1.65)

[1] Log-transformed continuous variables were used in this model. The increments are based on the difference of the median concentrations in quartile 1 and quartile 4. The increment of enterodiol is 4.9 nmol/L; the increment of enterolactone is 39.1 nmol/L. Tests for linear trend were done by fitting of a continuous variable.

[2] FULL model: adjusted for age, sex, alcohol, nonsteroidal anti-inflammatory drugs, body mass index, physical activity, smoking, family history of colorectal cancer, antibiotic use, and indication for endoscopy.

RESULTS

Compared to controls, cases were more likely to be male, to be older, and have a slightly higher body mass index (**Table 6.1**). Furthermore, cases consumed more alcohol, and were more likely to smoke. The use of oral contraceptives, for women only, was higher among controls than among cases. The major indications for endoscopy among cases were complaints (44%), including bowel complaints, rectal bleeding and defecation problems, and screening (47%). For controls these numbers were 80% for complaints and 10% for screening. Plasma enterodiol and enterolactone concentrations did not differ between cases and controls. Indication for endoscopy was different for prevalent and incident cases. Prevalent cases underwent endoscopy because of screening (83%) and incident cases underwent endoscopy because of complaints (76%, data not shown).

For total cases and controls, the adjusted OR estimates for the continuous model (FINAL model) showed no significant associations (**Table 6.2**). Only in the highest quartile of plasma enterolactone was the OR estimate significant. When analyses were done for incident cases only, the OR estimates showed that higher concentrations of enterodiol and enterolactone were associated with a reduction in risk of colorectal adenomas in a dose-dependent manner. This was particularly clear for enterodiol; the adjusted OR in the highest quartile was 0.53 (95% confidence intervals, 0.32-0.88; P = 0.01). In prevalent cases, no association was observed between either of the enterolignans and colorectal adenomas. ORs did not change substantially when we excluded subjects who did not have full visualization of the colon in incident and prevalent cases (data not shown).

Use of antibiotics, as expected, reduced enterolactone concentration in plasma substantially, but not the enterodiol concentration. The median concentration of enterolactone was 6.9 nmol/L in subjects using antibiotic therapy, whereas the concentration in the other subjects was 13.6 nmol/L. The concentration of enterodiol was 1.5 nmol/L in subjects using antibiotic therapy, and 1.2 nmol/L in the other subjects.

Therefore, in an additional analysis, we included only incident cases and controls who did not use antibiotics within the same calendar year as the blood was drawn. In this analysis 161 cases and 306 controls were included. For both enterolignans the age- and sex-adjusted ORs were similar to the FINAL model of incident cases, in which we adjusted for age, sex, and use of antibiotic therapy. Enterodiol ORs in the quartiles (from lowest to highest) were 1.00, 0.62 (0.36-1.07), 0.51 (0.29-0.89), and 0.48 (0.27-0.85). The OR on a continuous scale was 0.74 (0.57-0.96) with a significant trend (P = 0.02). Enterolactone ORs in the quartiles were 1.00, 0.75 (0.43-1.31), 0.64 (0.36-1.14), and 0.60 (0.34-1.06). The OR on a continuous scale was 0.58 (0.33-0.99) with a significant trend (P = 0.05) as well (data not shown).

DISCUSSION

Our study shows that increased plasma concentrations of enterodiol and enterolactone are associated with a considerable reduction in colorectal adenoma risk in a dose-dependent manner. This inverse association was observed in incident cases only, and not in prevalent cases. No other epidemiological studies on the relation between lignan exposure and risk of colorectal adenomas or colorectal cancer have been published before. However, consumption of lignan-containing products, such as cereals, nuts and grains, fruits, vegetables, and tea has been associated with lower risks of colorectal cancer, reviewed in [38].

In our study, inverse associations between enterolignan concentrations and colorectal adenomas were observed in incident cases, whereas no associations were observed in prevalent cases. This might be due to the time interval between the exposure assessment and the diagnosis of the first colorectal adenomas, which was different for incident and prevalent cases. For both incident and prevalent cases the exposure to plasma enterolignans was measured shortly after the index endoscopy, whereas the time of first diagnosis of colorectal adenomas was earlier in prevalent than in incident cases. Prevalent cases were diagnosed prior to the index endoscopy (they have a history of colorectal adenomas); incident cases were first diagnosed at the index endoscopy itself. When the time interval becomes longer the association between plasma enterolignans and colorectal adenomas might be weakened. Several factors, such as diet, disease status, and medication might have influenced the enterolignan plasma concentrations. The presence of adenomas usually does not provide any symptoms, reducing the possibility that people may have changed their dietary habits or that the tumor may influence plasma levels. However, due to perceived risks and/or to intestinal complaints prevalent cases might have changed their diet (information bias). This might have changed the intake of plant lignans and as a result their enterolignan plasma concentrations. Therefore, the inverse association between plasma enterolignans, which we observed in incident cases, might have diluted in prevalent cases. Other factors that might have influenced plasma concentrations of enterolignans, such as use of antibiotics, and time between endoscopy and blood sampling, were similar in incident and prevalent cases. Family history of colorectal cancer was equally distributed among prevalent and incident cases. Thus, for prevalent cases the blood sample may reflect an improper exposure time window to study the relation between the formation of first colorectal adenomas and plasma enterolignans.

An advantage of our study is that we used plasma enterolignans, rather than dietary recall or records to measure the exposure to lignans. The concentration of enterolignans in plasma is not dependent on memory, and takes into account metabolism by the colonic flora and bioavailability as well. Enterolignans will accumulate in plasma when consumed 2-3 times a day [39]. Thus, steady state plasma concentrations of enterodiol and enterolactone are likely to be achieved because plant lignans are present in many foods and beverages. Therefore, plasma enterolignans are

expected to be suitable biomarkers of lignan exposure over a period up to 2-years [40] and may be used to evaluate the effects of lignans. Because blood samples were collected after the index endoscopy, misclassification of exposure due to changes in diet or lifestyle might be a concern (information bias). However, as we do not expect incident cases and controls to be aware of risk factors for colorectal adenomas, there is no reason to believe that this misclassification is differential. Furthermore, both controls and incidents cases underwent endoscopy primarily for complaints (controls 80% and incident cases 76%). Hence, if they would change their diet because of these complaints in this short period, this would have been similar in controls and incident cases.

Another important issue in case-controls studies concerns selection bias. The response rate was rather low and varied widely by clinic. However, selection procedures were identical for incident cases and controls, reducing the possibility for differential selection bias. Unfortunately, we do not have data on plasma concentrations of patients not participating in the study to further evaluate the possible selection bias due to nonresponse.

In this study use of antibiotics within the same calendar year decreased plasma enterolactone and not enterodiol concentrations. Our results suggest that enterolactone concentrations are more affected by antibiotic use than enterodiol concentrations. When these antibiotics users were excluded from the analysis, the inverse association between plasma enterolactone and risk of colorectal adenomas became significant, although the ORs changed only slightly.

Enterodiol was similarly associated with colorectal adenomas as enterolactone in this study, although concentrations of enterodiol were 5- to 10-fold lower. This suggests that in the human body enterodiol might be more active than enterolactone. Enterodiol had a higher antioxidant capacity than enterolactone *in vitro* [19, 20]. On the other hand, enterolactone was two times more effective than enterolactone to inhibit growth of colon tumor cells [21]. Therefore, more work is needed to sort out whether real biological differences exist.

In summary, our study shows for the first time that both plasma enterolignans are associated with a lower risk of first colorectal adenomas. Although colorectal adenomas are considered to be precursors of colorectal cancer, only about 5% of colorectal adenomas are estimated to become malignant, which takes 5 to 10 years [41]. To further investigate the role of enterolignans on the development of colorectal cancer more prospective studies or recurrence trials are needed.

ACKNOWLEDGEMENTS

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ABSTRACT

Plant lignans are present in foods such as whole grains, seeds and nuts, fruits and vegetables, and beverages. They are converted by intestinal bacteria into the enterolignans, enterodiol and enterolactone. Enterolignans possess several biological activities whereby they may influence carcinogenesis. We investigated the association between plasma enterodiol and enterolactone on colorectal cancer risk in a prospective study.

Among more than 35,000 participants, 160 colorectal cancer cases were diagnosed after 7.5 years of follow-up. Cohort members frequency matched to the cases on age, sex, and study center, were selected as controls (n = 387). In the source population median plasma concentrations were 0.9 nmol L for enterodiol and 8.8 nmol L for enterolactone. No statistically significant associations between plasma enterodiol and enterolactone and risk of colorectal cancer were detected. The odds ratio for the highest versus the lowest quartile of enterodiol was 1.11 (95% confidence interval (CI), 0.56-2.20; P for trend = 0.75), and that of enterolactone 1.70 (95% CI, 0.88-2.37; P = 0.15) after adjustment for known dietary and lifestyle risk factors for colorectal cancer. The relation between plasma enterolactone and colorectal cancer risk was modified by sex (P = 0.06), and by body mass index (P <0.01); among women and subjects with high body mass index, we observed increased risks. The association between plasma enterodiol and colorectal cancer risk was modified by smoking status (P < 0.01; among current smokers an increased risk).

In conclusion, the findings of this nested case-control study do not support the hypothesis that high plasma enterodiol or enterolactone concentrations are associated with reduced risk of colorectal cancer. Further studies are needed to examine the risk associations in subgroup analysis according to sex, body mass index, and smoking status.

Keywords: lignans, enterolactone, enterodiol, colorectal cancer, prospective studies

Plasma enterolignan concentrations are not associated with colorectal cancer risk in a nested case-control study

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Chapter 7

INTRODUCTION

Dietary patterns high in fruits and vegetables, whole grains, nuts and seeds, and low in red and processed meats are associated with lower risk of colorectal cancer in humans [1, 2]. Because lignans occur in many of these foods of plant origin [3], the aforementioned beneficial effect might be partly related to lignans. Plant lignans are biphenolic compounds that are converted by intestinal bacteria to enterolignans, enterodiol and enterolactone [4-6]. First, plant lignans are converted to enterodiol and subsequently to enterolactone. Matairesinol, one of the plant lignans, can be converted directly to enterolactone [5].

Only *in vitro* or animal-based studies investigated the role of lignans in colorectal carcinogenesis. In a study with four colon cancer cell lines (LS174T, CaCo-2, HCT-15, and T-84), enterodiol and enterolactone, each at 100 µmol L concentrations, demonstrated estrogen-independent growth inhibitory effects [7]. At a concentration of 40 µmol L enterodiol and enterolactone induced cell cycle arrest of colon cancer cells (SW480) [8]. Furthermore, enterodiol and enterolactone possess several biological activities *in vitro*, potentially influencing carcinogenic processes, such as antioxidant activity [9, 10], stimulation of sex hormone-binding globulin synthesis [11] and inhibition of aromatase enzyme [12]. Animal experiments studying the effect of lignans in colon carcinogenesis are limited and inconsistent [13]. The two most studied sources of plant lignans are flaxseed and rye bran. Rye bran decreased the number of colonic tumors in carcinogen-treated rats [14]. Furthermore, the number of aberrant crypt foci was significantly reduced in the distal colon in rats fed 2.5% or 5% flaxseed or 2.5% or 5% defatted flaxseed [15]. However, other studies showed that neither diets with 0.5% defatted flaxseed [16], 5% flaxseed, 30% rye bran [17], nor the pure plant lignans, secoisolariciresinol diglucoside and matairesinol [18] were able to decrease intestinal adenoma formation in APC-min mice. Hence, evidence for a protective effect of lignans on colon carcinogenesis is still contradictory. To date, no epidemiological studies have examined the associations between enterolignans and colorectal cancer. However, we recently observed an inverse association between plasma concentrations and colorectal adenomas risk in a case-control study [19]. Colorectal adenomas are generally regarded as precursors of colorectal cancer, although only about 5% of colorectal adenomas are estimated to become malignant, which may take 5-10 years [20]. Therefore, we investigated the role of enterolignans in a prospective study, in which subjects developed colorectal cancer, and we explored effect modification by tumor site and gender. Previous studies have suggested differences in etiology between the sub sites of the colorectal tract [21]. The colon and rectum arise from different embryonic tissue and serve different functions [22]. The duration of exposure to and the composition of bowel content differ between colon and rectum. Moreover, molecular aspects of tumor genesis also seem to differ between sub sites of the large bowel [23, 24]. Additionally, women are less likely than men to develop colon cancer [25], and postmenopausal hormone replacement therapy has been shown to reduce even further colon cancer risk for women by up to 25% [26]. Furthermore, in the present study we evaluated

effect modification by body mass index and smoking status for explorative reasons.

METHODS

Study population and baseline data-collection

We conducted a nested case-control study within the Monitoring Project on Cardiovascular Disease Risk Factors (PPHV, Dutch acronym), a large survey of cardiovascular risk factors in the Netherlands [27]. This survey was carried out between 1987 and 1991 among more than 35,000 men and women, aged 20-59 years. They were examined at the Municipal Health Services in three Dutch municipalities: Amsterdam, Doetinchem and Maastricht. All participants gave written informed consent and approval was obtained from the medical ethics committees of Leiden University and the Netherlands Organization for Applied Scientific Research.

Baseline examinations included anthropometric measurements, blood sampling and self-administered questionnaires. Trained technicians, who were instructed by the same physician, carried out the measurements. Height and weight were measured, with the participants wearing indoor clothing and no shoes. The questionnaire provided information about demographic variables, current medication, cigarette smoking, alcohol consumption, and physical activity. Dietary habits were estimated using a semiquantitative food frequency method that was evaluated for reproducibility and tested against an extensive dietary history [28].

Follow-up for cancer incidence

Follow-up for cancer incidence was based on computerized record linkage to the national and regional cancer registries between 1987 and mid 2003, providing the diagnosis, tumor site classification and morphology (according ICD-O-2 classifications).

Nested case-control design

We used a nested case-control design, which represents a cost-efficient alternative to a traditional full-cohort analysis. According to this approach all incident colorectal carcinomas (n=161) were identified; of these 101 were colon carcinomas and 60 were rectosigmoid junction or rectum carcinomas. In addition, a random sample of controls (n=165) was selected from the full cohort at baseline, frequency matched on age (5 year intervals), sex and study center. One case was excluded because no control with the same matching criteria as this case was selected. We simultaneously selected controls (n=222) at random from this cohort for a nested case-control analysis on myocardial infarction. In this subset controls were excluded when they reported a history of myocardial infarction or heart surgery at baseline or if follow-up data on myocardial infarction were missing. In this subset plasma enterolignan concentrations were analyzed as well. We combined the two control groups for our data-analysis. Only controls with the same matching factors as the cases were selected. In total 160 cases and 387 controls were

used for data-analysis. When data analysis was restricted to the case-control subset excluding the myocardial infarction controls, risk estimates did not change substantially (data not shown).

Laboratory analyses

The concentrations of enterodiol and enterolactone in plasma were measured by liquid chromatography with tandem mass spectrometry using triply ^{13}C-labeled isotopes [29]. The samples were analyzed in 22 runs over an 8-week period. The within-run coefficient of variation ranged from 3-6% and the between-run coefficient of variation ranged from 10-14% for both enterolignans. The limit of detection was 0.55 nmol L for enterolactone and 0.15 nmol L for enterodiol. Lab technicians were blinded to the status of the subjects. Cases and controls were randomly distributed over the runs.

Data analysis

Concentrations of enterodiol and enterolactone were log-transformed prior to the analyses to improve normality. For continuous study characteristics PROC GLM (SAS statistical software, version 9.1, SAS institute, Inc., Cary, NC) was used to compare means and to calculate a *P* for trend between quartiles of enterolignan concentrations in the control group (n=387) accounting for the matching factors, age, sex, and study center. For categorical variables quartile differences were evaluated based on the Cochran-Mantel-Haenszel statistics. The relationships of plasma enterodiol and enterolactone concentrations with colorectal cancer risk were analyzed by conditional logistic regression using odds ratios (OR) and 95% confidence intervals (95% CI). The ORs were examined by quartiles of enterodiol and enterolactone concentrations with cutoff points based on the distribution among controls. For all the models, linear trends were tested using median concentrations of the quartiles. Risk estimates were computed in a crude model (adjusted for matching variables, which are controlled for automatically by design). Additionally, two different sets of covariates were used: adjusted model 1 included lifestyle factors: body mass index (kg m^2), status of smoking (never, ex, current), duration intensity of smoking (pack year), alcohol intake (g d), physical activity at work (low versus high), physical activity at leisure time (low versus high), education level (low, medium, and high), and aspirin use (yes versus no); adjusted model 2 additionally included dietary intake of energy, calcium, fiber, meat, fish, fruits, vegetables, tea, wine, and whole grain bread. Fruits and vegetables, tea, wine, and whole grain bread are sources of plant lignans. We included these sources of plant lignans in order to evaluate whether the observed effect for enterolignans could be attributed to other components. Furthermore, analyses were conducted using plasma enterolignan concentrations continuously with the interquartile ranges (1.50 nmol L for enterodiol and 14.61 nmol L for enterolactone) as increments, and a *P* for trend based on the continuous variables.

In order to explore potential modification of the effects of enterolignans by cancer site (colon and

rectosigmoid plus rectum), sex, body mass index (<25 versus ≥25 kg m²), and smoking status (never, ex, current) we performed stratified analyses in a reduced model. For the reduced model we selected lifestyle and dietary covariates, which significantly contributed to the reduced model (P <0.05) based on the log likelihood ratio test using the SCORE option in the software package. The reduced model was adjusted for matching factors and included the covariates body mass index, alcohol intake, current smoking status, and physical activity at leisure time. In the subgroup analyses for women, menopausal status and oral contraceptive use were considered as additional confounders. To test for statistical interaction with sex, body mass index, and smoking status we obtained the P value for their product terms with enterolignans. In order to assess the effect of undiagnosed tumors already present at baseline, analyses were also performed excluding cases diagnosed in the first 2 years of follow-up.

RESULTS

Characteristics of the controls are presented in **Table 7.1.** Body mass index decreased with increasing concentrations of plasma enterolignans. Highest percentages of current smokers were observed in the lowest quartiles of plasma enterodiol and enterolactone. Alcohol intake increased with quartiles of plasma enterolactone. Education level was higher with increasing enterolactone plasma concentrations (P = 0.01), but not with enterodiol. Physical activity at work and leisure time, and use of aspirin were not related to both plasma enterolignans. Additionally, the consumption of important dietary sources of plant lignans, such as fruits, vegetables, whole grain products, tea, and wine, increased with increasing concentrations of plasma enterolignans. The consumption of meat and of fish, were not related to plasma enterolignans. Fiber and calcium intake increased with increasing concentrations of plasma enterolignans. In women menopausal status and oral contraceptive use did not differ between quartiles (Table 7.1).

No statistically significant associations between plasma enterodiol and enterolactone and risk of colorectal cancer were present in the highest versus lowest quartiles or in the continuous model using the interquartile ranges (1.50 nmol L for enterodiol and 14,61 nmol L for enterolactone) as increments (**Table 7.2**). In the model adjusted for lifestyle factors (adjusted 1) the OR (95%CI) for continuous enterodiol concentrations was 1.03 (0.96-1.11, P = 0.46), and for enterolactone the OR was 1.46 (0.86-2.48, P = 0.16). In the model adjusted for lifestyle and dietary factors (adjusted 2), the OR for enterodiol was 1.03 (0.95-1.11, P = 0.47), and for enterolactone 1.56 (0.89-2.72, P = 0.12). The adjusted models 1 and 2 did not alter the crude risk estimates more than 10% (Table 7.2). In the third quartile of enterodiol concentrations ORs were significantly higher than the lowest quartile both in the crude model as well as in the adjusted models. However, the ORs over the quartiles were not linear as indicated by the P for trend (Table 7.2).

In the reduced model the continuous risk estimates (95%CI) were 1.02 (0.95-1.10) for enterodiol, and 1.41 (0.84-2.35) for enterolactone (**Table 7.3**). When stratifying for cancer site no consistent

Table 7.1 Characteristics of controls[1]

Concentration (nmol L); Cut off (median)	Enterodiol					Enterolactone				
	Q1 <0.45 (0.26)	Q2 0.45-0.89 (0.66)	Q3 0.90-1.95 (1.24)	Q4 ≥1.96 (3.00)	P value	Q1 <4.11 (2.26)	Q2 4.11-8.79 (6.63)	Q3 8.80-18.74 (13.08)	Q4 ≥18.75 (29.32)	P value
Lifestyle factors										
Body mass index, (kg m²)	26.2 ± 0.4	25.0 ± 0.4	25.8 ± 0.4	24.5 ± 0.4	0.01	26.6 ± 0.4	25.4 ± 0.4	24.7 ± 0.4	24.7 ± 0.4	<0.01
Alcohol (g d)	11.5 ± 1.4	13.2 ± 1.4	11.3 ± 1.4	13.9 ± 1.4	0.44	9.9 ± 1.4	13.2 ± 1.4	12.7 ± 1.4	14.1 ± 1.4	0.05
Smoking (%)					<0.01					0.03
never	18	25	34	27		23	24	27	30	
ex	29	27	26	42		28	29	28	39	
current	53	48	39	32		49	47	45	31	
Smoking (pack years)	16.3 ± 1.6	15.7 ± 1.5	12.1 ± 1.6	13.2 ± 1.6	0.07	14.8 ± 1.6	15.8 ± 1.6	14.1 ± 1.6	12.6 ± 1.6	0.23
Physical activity work (% high)	34	30	27	33	0.74	29	34	32	30	0.98
Physical activity leisure time (% high)	45	53	53	48	0.63	47	50	48	53	0.48
Education (%)					0.34					0.01
low	74	72	68	71		79	73	71	62	
medium	15	15	14	13		11	15	11	20	
high	11	13	18	16		10	12	18	18	
Use of aspirin (% yes)	27	30	18	28	0.70	29	22	24	27	0.86
Dietary factors										
Energy (kJ d)	6496±215	7285±211	7325±212	7375±214	<0.01	6757±215	7244±212	7195±214	7278±214	0.12
Fiber (g d)	14.7 ± 0.6	17.4 ± 0.6	18.0 ± 0.6	18.4 ± 0.6	<0.01	15.4 ± 0.6	17.0 ± 0.6	17.7 ± 0.6	18.5 ± 0.6	<0.01
Calcium (mg d)	850 ± 43	944 ± 42	1004 ± 43	978 ± 43	0.03	863 ± 43	965 ± 42	959 ± 43	988 ± 43	0.06
Meat (g d)	86 ± 4	87 ± 4	84 ± 4	83 ± 4	0.43	86 ± 4	88 ± 4	84 ± 4	82 ± 4	0.25
Fish (g d)	11.5 ± 1.3	10.7 ± 1.3	12.5 ± 1.3	12.4 ± 1.3	0.42	10.9 ± 1.3	12.4 ± 1.3	11.0 ± 1.3	12.7 ± 1.3	0.51
Fruits (g d)	88 ± 7	92 ± 7	104 ± 7	112 ± 7	<0.01	89 ± 7	97 ± 7	100 ± 7	111 ± 7	0.03
Vegetables (g d)	122 ± 6	127 ± 6	140 ± 6	131 ± 6	0.18	122 ± 6	134 ± 6	128 ± 6	135 ± 6	0.28

Table 7.1 continued

Concentration (nmol L); Cut off (median)	Enterodiol					Enterolactone				
	Q1 <0.45 (0.26)	Q2 0.45-0.89 (0.66)	Q3 0.90-1.95 (1.24)	Q4 ≥1.96 (3.00)	P value	Q1 <4.11 (2.26)	Q2 4.11-8.79 (6.63)	Q3 8.80-18.74 (13.08)	Q4 ≥18.75 (29.32)	P value
Dietary factors (continued)										
Tea (g d)	184 ± 34	275 ± 33	247 ± 33	353 ± 34	<0.01	233 ± 34	242 ± 34	280 ± 34	301 ± 34	0.11
Wine (g d)	7.6 ± 5.5	20.9 ± 5.4	22.7 ± 5.4	28.4 ± 5.4	<0.01	7.6 ± 5.4	13.6 ± 5.3	29.8 ± 5.4	28.6 ± 5.3	<0.01
Whole grain bread (g d)	45.6 ± 9.3	78.1 ± 9.2	67.7 ± 9.2	85.7 ± 9.3	0.01	46.3 ± 9.2	57.4 ± 9.1	81.8 ± 9.2	91.4 ± 9.2	<0.01
Risk factors in women only										
Contraceptive use (% ever)	57	70	64	73	0.27	51	74	63	75	0.10
Postmenopausal women (%)	57	43	41	59	0.82	60	42	46	53	0.69

[1] Controls (n=387) randomly selected from source population; adjusted for matching factors, age, sex and study center; continuous variables are expressed as means ± SEM with P for trend; categorical variables are expressed as percentages with P value for differences estimated with the Cochran-Mantel-Haenszel statistics.

Table 7.2. Multivariate analysis of all colorectal cancers and log-transformed continuous variables for plasma enterolignans

	Q1	Q2	Q3	Q4	P for trend [1]	Continuous model [2]	P value
Enterodiol [3]							
cases/controls (n)	26/97	39/97	59/97	36/96		160/387	
Crude [4]	1.00	1.24 (0.69-2.23)	1.90 (1.07-3.37)	0.99 (0.54-1.84)	0.53	1.01 (0.95-1.09)	0.69
Adjusted 1 [5]	1.00	1.37 (0.73-2.56)	2.06 (1.12-3.79)	1.07 (0.55-2.06)	0.59	1.03 (0.96-1.11)	0.46
Adjusted 2 [6]	1.00	1.29 (0.68-2.45)	2.01 (1.09-3.71)	1.11 (0.56-2.20)	0.75	1.03 (0.95-1.11)	0.47
Enterolactone [3]							
cases/controls (n)	26/96	36/98	51/96	47/97		160/387	
Crude [4]	1.00	1.16 (0.64-2.13)	1.78 (1.00-3.16)	1.47 (0.82-2.64)	0.23	1.42 (0.88-2.31)	0.15
Adjusted 1 [5]	1.00	1.15 (0.61-2.18)	1.68 (0.91-3.10)	1.51 (0.81-2.84)	0.22	1.46 (0.86-2.48)	0.16
Adjusted 2 [6]	1.00	1.23 (0.65-2.34)	1.80 (0.97-3.34)	1.70 (0.88-3.27)	0.15	1.56 (0.89-2.72)	0.12

[1] P for trend are based on median concentrations in quartiles.

[2] Log-transformed continuous concentrations of enterolignans are used in this model; the increments are based on the interquartile ranges (1.50 nmol L for enterodiol and 14.61 nmol L for enterolactone).

[3] Quartiles cut off-points are described in Table 7.1.

[4] Crude model: adjusted for matching factors by design

[5] Adjusted model 1: Additionally adjusted for lifestyle factors: body mass index, alcohol intake, smoking status (never, ex, current), duration smoking, physical activity at leisure time and work, aspirin use, and education level.

[6] Adjusted model 2: Additionally adjusted for lifestyle factors (adjusted 1) and dietary factors: energy, fiber, calcium, meat, fish, fruits, vegetables, tea, wine, and whole grain bread.

associations were observed between enterolignan concentrations and cancer risk (Table 7.3). When stratifying according to sex, there was an increased risk for women with increasing concentrations of enterolactone (OR with 95% CI: 2.63 (1.09-6.33)); this was not observed for enterodiol concentrations, nor in men. For enterolactone the ORs among men and women were almost significantly different (*P* for interaction = 0.06). In women menopausal status and oral contraceptive were not considered confounders because they did not significantly change the risk estimates (data not shown). When analyzing the data for women by menopausal status, an increased risk was observed for enterolactone in postmenopausal women (n = 33; OR: 5.12, 95%CI: 1.25-21.0), whereas no association was observed in premenopausal women (n = 38; OR: 1.37, 95%CI: 0.34-5.56). However, *P* for interaction was not significant (0.29). This was not observed for enterodiol. When stratifying for body mass index we observed an increased risk for subjects with a high body mass index ($\geq$ 25 kg m^2) with increasing concentrations of enterolactone (OR with 95% CI: 2.44 (1.17-5.08)). For enterodiol the OR was 1.09 (0.99-1.20) in subjects with high body mass index; *P* for interaction was <0.01 for enterolactone, and 0.16 for enterodiol. In never smokers, plasma enterodiol was inversely associated with colorectal cancer risk (*P* = 0.05); in current smokers the relation between enterodiol and cancer risk was positively associated (*P* = 0.02); in former smokers no association was observed (*P* for interaction with smoking status <0.01). These estimates were not consistent with plasma enterolactone concentrations, where no associations with colorectal cancer risk were observed (Table 7.3).

The mean time period from blood collection to diagnosis was 7.5 years, with a range from 5 months to 15 years. The association between enterodiol and enterolactone and colorectal cancer was not substantially altered when subjects diagnosed within two years of follow-up (n = 14) were excluded (data not shown). Nor were any consistent associations observed between plasma enterodiol and enterolactone concentrations and colorectal cancer risk according to years of follow-up: ORs (95%CI) in the strata 0-7.5 years follow-up, and 7.5-15 years follow-up were for enterodiol 1.02 (0.93-1.12) and 1.04 (0.94-1.15), respectively, and for enterolactone 1.61 (0.80-3.27) and 1.25 (0.65-2.40), respectively (data not shown in table).

DISCUSSION

This is the first report on the relation between plasma enterolignans and colorectal cancer risk, studied in a prospective design. The results of this case-control study nested within a cohort of more than 35,000 subjects do not support the hypothesis that high plasma enterodiol or enterolactone concentrations are associated with reduced risk of colorectal cancer. In our study plasma enterolignan concentrations were similar to concentrations in other epidemiological studies.

In epidemiological studies associations between other cancers and urinary or plasma enterolignans are inconsistent. Inverse associations for breast cancer were reported in four case-

control studies, and one prospective study [30-34], whereas no associations were found in five prospective studies [35-39]. No associations were observed between plasma enterolactone and prostate cancer in three nested case-control studies [40-42]. In contrast, Hernandez et al. [43] reported a positive association between plasma enterodiol concentrations and premalignant lesions of the cervix.

Recently, we reported a case-control study in which we observed an inverse association between plasma concentrations of enterolignans and colorectal adenomas risk [19]. In the present study we were not able to confirm the inverse association between plasma enterolignans and colorectal cancer risk. Colorectal adenomas are widely accepted as precursors of colorectal cancer in humans. However, only few adenomas develop into carcinomas. Enterolignans might decrease or delay the development of colorectal adenomas, but once adenomas are formed enterolignans may no longer influence the further development into cancer. Colorectal carcinogenesis has been demonstrated to involve the accumulation of genetic alterations, in which individual dietary factors, such as lignans, may play a small but not crucial role. Therefore, the possible protective role of enterolignans in the development of colorectal adenomas might no longer be observed when looking at the development of colorectal cancer, which involves multiple factors and takes several years or even decades. Another reason could be that we lack statistical power to see any association. With the size of this study, we would be able to demonstrate an OR of <0.47 (80% power; two-sided) in the highest versus the lowest quartile. For individual dietary factors, like enterolignans, this is a very large effect.

Although plasma enterolignan concentrations were not associated with a reduced risk of colorectal cancer in the entire study population, some subgroup analyses are noteworthy. High plasma concentrations of enterolactone were associated with an increased risk of colorectal cancer in women, especially in postmenopausal women. Although no underlying mechanism has been established yet, the interaction with sex, and menopausal status, suggest that an estrogen-related hormonal mechanism might be involved.

Evidence is accumulating for a possible protective effect of estrogens: at all ages, women are less likely than men to develop colon cancer [25], and postmenopausal hormone replacement therapy has been shown to reduce even further the colon cancer risk for women by up to 25% [26]. It has been suggested that enterolignan have anti-estrogenic effects: they bind to estrogen receptors [44, 45], but their efficacy is less, resulting in blocking of the effect of estradiol. This could become of crucial importance in postmenopausal women who have low endogenous levels of estradiol.

Furthermore, we observed an increased risk for subjects with high body mass index. Body weight and body mass index have been found to be positively related to risk of colon cancer in men, whereas weaker or no associations have been reported for women [46]. Because of the small numbers in our study we cannot further address the combined effects of body mass index, sex, and menopausal status.

Table 7.3. Subgroup analysis

	cases (n)	Enterodiol[1] (1.5 nmol L)	P for trend	Enterolactone[1] (14.6 nmol L)	P for trend
Reduced model	158	1.02 (0.95-1.10)	0.53	1.41 (0.84-2.35)	0.19
Cancer site					
Colon cancer	98	1.02 (0.94-1.11)	0.59	1.17 (0.64-2.13)	0.63
Rectal cancer	60	1.04 (0.93-1.17)	0.47	2.00 (0.90-4.42)	0.09
Sex					
Male	87	1.03 (0.94-1.13)	0.56	0.98 (0.52-1.85)	0.94
Female	71	1.02 (0.91-1.14)	0.76	2.63 (1.09-6.33)	0.03
P for interaction			*0.77*		*0.06*
Body mass index					
low (<25 kg m^2)	67	0.93 (0.82-1.05)	0.24	0.65 (0.28-1.50)	0.31
high (≥25 kg m^2)	90	1.09 (0.99-1.20)	0.07	2.44 (1.17-5.08)	0.02
P for interaction			*0.16*		*<0.01*
Smoking status					
Never	44	0.84 (0.70-1.00)	0.05	3.04 (0.99-9.35)	0.05
Ex	45	1.01 (0.90-1.14)	0.85	0.83 (0.32-2.13)	0.69
Current	51	1.19 (1.03-1.38)	0.02	1.33 (0.56-3.12)	0.52
P for interaction			*<0.01*		*0.55*

[1] Enterodiol and enterolactone are log-transformed; ORs are expressed for an increment of interquartile ranges (1.5 nmol L for enterodiol and 14.6 nmol L for enterolactone) and based on a reduced model (= additionally adjusted for body mass index, alcohol, current smoking status, and physical activity at leisure time).

An important issue in observational studies concerns bias. In our study, the most potential source of bias might originate from misclassification of exposure. The increased risk observed in women and in subjects with high body mass index could be the result of slower intestinal motility. Prevalence of constipation, which indicates slower intestinal motility, is higher in women than in men [47, 48]. Constipation appears to be positively associated with serum enterolactone and is an important independent determinant of enterolactone concentration [49]. Constipation may lead to more complete metabolism and absorption of lignans. Thus higher enterolactone concentrations might be associated with slower intestinal motility. This will result in prolonged exposure to toxins in the colon, thus increasing the risk of colorectal cancers [50, 51]. Unfortunately, we have no data available on defecation patterns or constipation in this study.

In this study, the association between plasma enterodiol and colorectal cancer risk was modified by smoking status. We observed among current smokers an increased risk, and among never smokers an inverse risk. Although we did not observe similar modification with plasma enterolactone, it is interesting to note that this modification is consistent with observations on intake of fruits and vegetables and smoking status in a large European cohort study (EPIC) (personal communication Bueno-de-Mesquita, RIVM, The Netherlands). Fruits and vegetables are important sources of plant lignans, and plasma enterolignans might be markers of fruit and vegetable intake.

An important strength of our study is its prospective design. Plasma samples were obtained up to 15 years prior to the diagnosis of colorectal cancer, making the presence of cancer at the time of blood donation unlikely. Furthermore, our study design ensures identical collection and handling of blood samples from case and control subjects. The main limitation of our study is the lack of information on antibiotic use in the year prior to the blood sampling. Use of antibiotics reduces plasma enterolactone concentrations, and this effect is sustained for several months [52]. Nevertheless, we have no reason to assume that use of antibiotics prior to the study differed between cases and controls. Finally, information on family history of colorectal cancer was not available. Although some hereditary forms of colorectal cancer exist such as familial adenomatous polyposis and hereditary nonpolyposis colorectal cancer, they constitute no more than 10% of total colorectal cancer occurrence, most colorectal cancer cases occur sporadically via polyp-cancer sequence [53].

In summary, the findings of this nested case-control study do not support the hypothesis that high plasma enterodiol or high plasma enterolactone concentrations are associated with reduced risk of colorectal cancer. Further studies are needed to examine the risk associations in subgroup analysis according to sex, body mass index, and smoking status. Additionally, studies are needed to identify determinants of plasma enterolignans in order to evaluate their use as biomarkers of exposure.

ACKNOWLEDGEMENTS

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ABSTRACT

Plant lignans, present in foods such as whole grains, seeds and nuts, fruits and vegetables, and beverages, have been shown to protect against coronary heart disease in large observational studies. Plant lignans are converted by intestinal bacteria into enterodiol and enterolactone. In a prospective study (1987-1998) the association between plasma enterodiol and enterolactone and nonfatal myocardial infarction risk was investigated.

During follow-up of 15,107 participants, 237 incident nonfatal myocardial infarction cases were diagnosed. Controls (n = 283) were frequency matched to the cases on age, sex, and study center.

No statistically significant associations between plasma enterodiol and enterolactone and risk of nonfatal myocardial infarction were detected. The odds ratio for the highest versus the lowest quartile of enterodiol was 1.21 (95% confidence interval (CI): 0.70, 2.12; P for trend = 0.74), and that of enterolactone 1.51 (95% CI: 0.87, 2.61; P for trend = 0.12) after adjustment for known dietary risk factors for coronary heart disease. No effect modification was observed for sex, menopausal status, or smoking status.

In conclusion, the results of this prospective study do not support the hypothesis that high plasma enterodiol or enterolactone concentrations are associated with a reduced risk of nonfatal myocardial infarction.

Keywords: *enterolactone; enterodiol; plasma; myocardial infarction; cardiovascular diseases; prospective studies*

Plasma enterolignans are not associated with nonfatal myocardial infarction risk

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Chapter 8

INTRODUCTION

Diets rich in whole grain and fruit and vegetable fiber have been shown to protect against coronary heart disease in large observational studies [1, 2]. The nutrients and substances responsible for these protective effects have been extensively explored. Recently, lignans have received attention.

Lignans are biphenolic compounds present in foods such as whole grains, seeds and nuts, fruits and vegetables, and beverages [3]. They are converted by intestinal bacteria into the enterolignans, enterodiol and enterolactone. Among others, enterolignans have demonstrated antioxidant capacity at 10-100 μmol/L in vitro [4, 5]. Furthermore, enterolactone has the capacity to increase hepatic low-density lipoprotein (LDL) receptor activity (at 50 μmol/L), and thus may lower circulating LDL cholesterol [6]. These effects provide potential mechanisms for the postulated preventive influence on the development of coronary heart diseases.

Several animal studies support this hypothesis. In rabbits fed a high-cholesterol diet, the isolated plant lignan secoisolariciresinol diglucoside reduced hypercholesterolemic atherosclerosis by 73% and this effect was associated with a decrease in total and LDL cholesterol by 33 and 55%, respectively [7]. In a supplementary study with a lignan complex isolated from flaxseed, the reduction of hypercholesterolemic atherosclerosis was accompanied with marked decreases in oxidative stress as well [8]. In humans this cholesterol lowering capacity of lignans could not be confirmed [9]. However, in contrast to the animal studies, the women were not hyperlipidemic and did not follow a high-cholesterol diet.

Up to now, epidemiological evidence for a protective role of lignans on cardiovascular diseases is limited and inconsistent. One Finnish prospective study reported that high plasma concentrations of enterolactone were associated with a lower risk of acute coronary events [10], and cardiovascular disease-related death in men [11]. However, another Finnish prospective study among male smokers found no association between serum enterolactone and nonfatal myocardial infarction or coronary death [12]. In a Dutch prospective study using dietary intake of plant lignans as exposure measure, lignan intake was also not associated with coronary heart disease or cerebrovascular events in women [13]. This latter study included only the plant lignans secoisolariciresinol and matairesinol, which contribute only 25% to the total intake of plant lignans if lariciresinol and pinoresinol, two other important precursors of enterolignans, would be taken into account as well [14]. Although risk estimates were not significant in the latter two studies [12, 13], the estimates were below unity. The Finnish studies measured only enterolactone concentrations in serum, and not enterodiol, whereas the Dutch prospective study limited their exposure measure to dietary intake of two plant lignans. Furthermore, the analyses were restricted to men (Finnish studies) or to middle-aged and elderly women (Dutch study).

To further evaluate the association between lignans and the risk of coronary heart disease, we conducted a prospective study among men and women aged 20 to 59 years and used both

plasma enterodiol and enterolactone concentrations as exposure measures. To be able to compare our results with others, we also carried out a stratified data analysis according to sex, menopausal status, and smoking status.

MATERIALS AND METHODS

Study population

We conducted a nested case-control study within the Monitoring Project on Cardiovascular Disease Risk Factors, a large survey of cardiovascular risk factors in the Netherlands [15]. This survey was carried out between 1987 and 1991 among 35,488 men and women, aged 20-59 years. They were examined at the Municipal Health Services in three Dutch municipalities: Amsterdam (n = 11,488), Doetinchem (n = 11,350) and Maastricht (n = 12,650). All participants gave written informed consent and approval was obtained from the medical ethics committees of Leiden University and the Netherlands Organization for Applied Scientific Research.

Data collection

Baseline examinations included anthropometric measurements, blood sampling and a self-administered questionnaire. Trained technicians, who were instructed by the same physician, carried out the measurements. Height and weight were measured, with the participants wearing indoor clothing and no shoes. Nonfasting blood samples were obtained in EDTA-coated vacuum tubes.

The questionnaire provided information about demographic variables, (parental) history of cardiovascular diseases, hypertension, hypercholesterolemia, and other diseases, current medication, cigarette smoking, alcohol consumption, and physical activity. Dietary intake was assessed using a short (70-item) self-administered semi-quantitative food-frequency questionnaire that was evaluated for reproducibility and compared to an extensive dietary history [16]. Nutrient intake was calculated using the computerized version of the Netherlands food table [17].

Morbidity follow-up

A morbidity follow-up was carried out in two out of three regions, i.e. Doetinchem and Maastricht. A random sample (69%) of the participants from Doetinchem received a follow-up questionnaire 6 and/or 11 years after baseline examinations. All living participants from Maastricht still residing in the Netherlands (91%) received a follow-up questionnaire in 1998 (7-11 years after the baseline examination). The response to the follow-up questionnaire was 80% in Doetinchem (n = 6247) and 77% in Maastricht (n = 8860).

The follow-up questionnaires provided, among others, information on history of myocardial infarction, coronary artery bypass grafts, and percutaneous transluminal coronary angioplasty.

Subjects who reported a myocardial infarction in the follow-up questionnaire, but no myocardial infarction or heart surgery at baseline were considered to have incident nonfatal myocardial infarction.

In the follow-up questionnaire used in Maastricht, additional questions were asked regarding the prevalence of myocardial infarction. First, nonfatal myocardial infarction had to be confirmed by a general practitioner. Furthermore, the questionnaire in Maastricht inquired about hospital treatment as a result of the infarction. In Maastricht n = 133 cases out of n = 171 cases reported hospital treatment because of their infarction. To provide us with insight into the reliability of our results, these cases were used in a subgroup analysis, as they may constitute a more reliable case group.

The mean (standard deviation) time to first myocardial event in cases was 4.5 (2.5) years.

Nested case-control design

We used a nested case-control design to examine the association between plasma enterolignan concentrations and the risk of nonfatal myocardial infarction. According to this approach all incident nonfatal myocardial infarction (n = 245) were identified. In addition, a random sample of individuals who reported no history of myocardial infarction or heart surgery at baseline and no myocardial infarction at follow-up (n = 245) was selected as a control group, frequency matched on age (5 year intervals), sex and study center. Plasma sample were not available for n = 8 cases and n = 14 controls. One case was excluded because no control with the same matching criteria as this case was available. Simultaneously, we selected controls for another nested case-control analysis on colorectal cancer from this cohort. For these controls plasma enterolignan concentrations were available as well. Therefore, we were able to add another 52 individuals with the same matching factors and exclusion criteria as the myocardial infarction cases to our control group. In total 236 cases and 283 controls were used for data-analysis.

Laboratory analyses

Plasma total and high-density lipoprotein (HDL) cholesterol were determined enzymatically using a Boehringer test-kit within three weeks after storage [18]. HDL cholesterol was determined after precipitation of apoB containing lipoproteins with magnesium phosphotungstate [19].

Plasma concentrations of enterodiol and enterolactone were measured by liquid chromatography with tandem mass spectrometry using triply ^{13}C-labeled isotopes [20] in samples that were stored at −20 °C for 10-15 years. The samples were analyzed in 22 runs over an 8-week period. The within-run coefficient of variation ranged from 3-6% and the between-run coefficient of variation ranged from 10-14% for both enterolignans. The limit of detection was 0.55 nmol/L for enterolactone, and 0.15 nmol/L for enterodiol. Cases and controls were randomly distributed over the runs, whereas lab technicians were blinded to the status of the subjects.

Data analysis

For continuous study characteristics PROC GLM (SAS statistical software, version 9.1, SAS institute, Inc., Cary, NC) was used to compare means and to calculate a *P* for trend between quartiles of enterolignan concentrations in the control group (n = 283). For categorical variables quartile differences were evaluated based on the Cochran-Mantel-Haenszel statistics.

First, the relationships of possible confounders and of plasma enterodiol and enterolactone concentrations with nonfatal myocardial infarction risk were analyzed univariately by conditional logistic regression that automatically controls for the matching factors, age, sex, and study center, using odds ratios (OR) and 95% confidence intervals (95% CI). The ORs were examined by quartiles of enterodiol and enterolactone concentrations with cutoff points based on the distribution among controls. Concentrations of enterodiol and enterolactone were log-transformed prior to the analyses to improve normality. Linear trends were tested using median concentrations of the quartiles. Furthermore, analyses were conducted using plasma enterolignan concentrations continuously with the interquartile ranges (1.3 nmol/L for enterodiol and 13.7 nmol/L for enterolactone) and 10 nmol/L as increments, and a *P* for trend based on the continuous variables. With the size of this study, we would be able to demonstrate an OR of <0.49 (80% power; two-sided) in the highest versus the lowest quartile.

Potential confounding factors include: body mass index (kg/m^2), status of smoking (never, ex, current), duration/ intensity of smoking (pack year), alcohol intake (g/d), diabetes (yes versus no), systolic blood pressure (mmHg), diastolic blood pressure (mmHg), hypertension (yes versus no; according to World Health Organization guidelines: systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg and/or use of blood pressure lowering medication), total cholesterol (mmol/L), HDL cholesterol (mmol/L), ratio of total and HDL cholesterol, aspirin use (yes versus no), physical activity at work (low versus high), physical activity at leisure time (low versus high), and education level (low, medium, and high).

Multivariate conditional logistic regression models were used to account for the effect of confounders. Covariates were included in the multivariate model, when they changed the ORs by more than 10% when added to a univariate model that contained plasma enterodiol or enterolactone (model A). Body mass index, current smoking status (versus ex smokers plus never smokers), systolic blood pressure, total and HDL cholesterol concentration, and the ratio total/HDL cholesterol changed the ORs in the univariate analysis and were included in the multivariate model. Additionally, a reduced model (model B) was formed, in which only covariates from model A were selected that significantly contributed to the model (*P* < 0.05) based on the log likelihood ratio test using the SCORE option in SAS (SAS statistical software, version 9.1, SAS institute, Cary, NC). Model B included the covariates current smoking status, systolic blood pressure, and the ratio of total/ HDL cholesterol. In the subgroup analyses for women, menopausal status was also tested as confounder.

Table 8.1 Baseline characteristics of controls (n = 283) adjusted for matching factors, age, sex, and study center by quartiles of plasma enterolignan concentrations

	Enterodiol					Enterolactone				
	Q1	Q2	Q3	Q4	P value	Q1	Q2	Q3	Q4	P value
Cut off (nmol/L)	<0.4	0.4-0.8	0.8-1.7	>1.7		<3.8	3.8-7.9	7.9-17.5	>17.5	
(median)	(0.2)	(0.6)	(1.1)	(3.0)		(2.0)	(6.0)	(11.4)	(26.9)	
Lifestyle factors [1]										
Body mass index (kg/m²)	26.3 (0.4)	25.1 (0.4)	26.0 (0.4)	24.4 (0.4)	0.01	27.0 (0.4)	24.9 (0.4)	25.1 (0.4)	24.7 (0.4)	< 0.01
Alcohol (g/d)	12.1 (1.7)	12.8 (1.7)	11.9 (1.7)	13.4 (1.6)	0.67	10.5 (1.6)	13.2 (1.7)	12.6 (1.7)	13.9 (1.6)	0.19
Smoking (%)					< 0.01					0.10
never	16	28	35	28		22	27	31	27	
ex	31	32	26	45		32	31	31	41	
current	53	40	38	27		46	43	38	32	
Pack years of smoking	16.3 (1.7)	14.2 (1.7)	10.0 (1.7)	13.6 (1.6)	0.12	16.0 (1.7)	14.9 (1.7)	11.3 (1.7)	12.1 (1.7)	0.04
History of diabetes	1	0	0	3	0.39	1	0	0	3	0.29
Blood pressure (mmHg)										
Systolic	124 (2)	120 (2)	119 (2)	123 (2)	0.74	125 (2)	121 (2)	120 (2)	121 (2)	0.09
Diastolic	80 (1)	78 (1)	77 (1)	78 (1)	0.16	81 (1)	77 (1)	76 (1)	78 (1)	0.08
Hypertension (% yes)	31	15	17	24	0.46	32	19	16	21	0.12
Cholesterol (mmol/L)										
Total	5.86 (0.12)	5.61 (0.12)	5.84 (0.12)	5.95 (0.11)	0.34	6.00 (0.12)	5.81 (0.12)	5.60 (0.12)	5.85 (0.11)	0.22
High-density lipoprotein	1.18 (0.04)	1.24 (0.04)	1.19 (0.04)	1.26 (0.04)	0.22	1.15 (0.04)	1.25 (0.04)	1.22 (0.04)	1.25 (0.04)	0.07
Physical activity work (% high)	36	41	21	33	0.28	33	30	42	26	0.75
Physical activity leisure time (% high)	43	57	51	57	0.19	47	50	50	60	0.16
Education (%)					0.54					0.12
low	72	73	57	75		75	69	72	61	
medium	18	13	19	12		12	20	9	21	
high	9	15	24	13		13	11	19	18	
Postmenopausal women (%)	48	46	28	49	0.28	53	49	38	36	0.17

Table 8.1 (continued)

	Enterodiol					Enterolactone				
	Q1	Q2	Q3	Q4	*P* value	Q1	Q2	Q3	Q4	*P* value
Cut off (nmol/L)	<0.4	0.4-0.8	0.8-1.7	>1.7		<3.8	3.8-7.9	7.9-17.5	>17.5	
(median)	(0.2)	(0.6)	(1.1)	(3.0)		(2.0)	(6.0)	(11.4)	(26.9)	
Dietary factors [1]										
Total energy (kJ/d)	6574 (266)	7637 (267)	7436 (270)	7549 (264)	0.02	6925 (269)	7253 (269)	7612 (273)	7409 (268)	0.14
Fiber (g/d)	15.0 (0.8)	18.7 (0.8)	18.3 (0.8)	19.5 (0.8)	< 0.01	16.1 (0.8)	16.9 (0.8)	19.2 (0.8)	19.3 (0.8)	< 0.01
Fruits (g/d)	89 (8)	104 (8)	105 (8)	109 (8)	0.10	92 (8)	98 (8)	111 (8)	107 (8)	0.10
Vegetables (g/d)	123 (7)	133 (8)	131 (8)	139 (7)	0.19	125 (7)	134 (7)	133 (8)	134 (7)	0.44
Whole grain wheat bread (g/d)	43 (11)	86 (11)	63 (11)	97 (11)	< 0.01	52 (11)	51 (11)	79 (11)	107 (11)	< 0.01
Tea (g/d)	185 (40)	210 (40)	257 (40)	335 (40)	< 0.01	231 (40)	191 (40)	284 (41)	282 (40)	0.17
Coffee (g/d)	623 (47)	686 (47)	604 (47)	515 (47)	0.06	631 (47)	626 (47)	601 (48)	570 (47)	0.33
Wine (g/d)	4.5 (5.1)	10.8 (5.1)	23.2 (5.2)	30.2 (5.0)	< .01	4.8 (5.1)	12.7 (5.1)	19.2 (5.1)	32.0 (5.1)	< 0.01
Fish (g/d)	11 (1)	9 (1)	10 (1)	12 (1)	0.52	10 (1)	10 (1)	11 (1)	11 (1)	0.31
Meat (g/d)	87 (4)	95 (4)	83 (4)	84 (4)	0.29	89 (4)	88 (4)	92 (4)	80 (4)	0.23

[1] Continuous variables: values are means (standard error), *P* for trend; Categorical variables: values are percentages, *P* value with the Cochran-Mantel-Haenszel statistics

In order to explore the potential effect modifiers sex, menopausal status, and smoking status, we performed stratified analyses with model B. To test significant interaction, we calculated a *P*-value for the product terms. Additionally, we analyzed the subset of cases (n = 133) who reported hospital treatment as a result of their infarction (see *morbidity follow-up*).

RESULTS

Baseline characteristics of the controls are presented in **Table 8.1.** Regarding the main risk factors of coronary heart disease, only smoking status and body mass index were related to enterodiol or enterolactone concentrations in plasma. The mean body mass index was highest in the lowest quartile of plasma enterodiol and enterolactone. Highest percentages of current smokers were observed in the lowest quartiles of plasma enterodiol and enterolactone. Other lifestyle factors point in the direction of a healthy lifestyle in subjects with higher enterolignan concentrations: lower blood pressure, lower percentage of hypertension, higher HDL cholesterol concentrations, increasing physical activity during leisure time, and higher educational level, but differences were not statistically significant. Consumption of important dietary sources of plant lignans, such as whole grain products, tea, and wine, were positively associated with concentrations of plasma enterolignans. Furthermore, the intake of fiber was positively associated with concentrations of plasma enterolignans.

As expected, known risk factors significantly increased coronary heart disease risk when tested in a univariate model. ORs were as follows: body mass index (kg/m²; OR: 1.08; 95% CI: 1.03, 1.14), current smoking (yes versus no; OR: 2.22; 95% CI:1.55, 3.17), history of diabetes (yes versus no; OR: 3.62; 95% CI: 0.97, 13.5), systolic blood pressure (mmHg; OR: 1.03; 95% CI: 1.01, 1.04)), diastolic blood pressure (mmHg; OR: 1.03; 95% CI: 1.01, 1.05), hypertension (yes versus no; OR: 1.96; 95% CI: 1.31, 2.91), and total cholesterol concentrations (mmol/L; OR: 1.78; 95% CI: 1.47, 2.16). HDL cholesterol (mmol/L; OR: 0.10; 95% CI: 0.05, 0.19) and physical activity at leisure time (high versus low; OR: 0.64; 95% CI: 0.45, 0.91) significantly lowered the univariate risk.

No statistically significant associations between plasma enterodiol and enterolactone and risk of myocardial infarction were present, whether analyzed by the highest versus lowest quartiles or using the interquartile ranges (1.3 nmol/L for enterodiol and 13.7 nmol/L for enterolactone) or 10 nmol/L as increments (**Table 8.2**). In the crude model using the interquartile ranges as increments, the OR (95% CI) was 0.99 (0.96, 1.03; *P* = 0.70) for enterodiol concentrations and 0.98 (0.68, 1.42; *P* = 0.91) for enterolactone. The effect estimates in the adjusted models (model A and B) were similar to each other (Table 2). In model B, adjusted for current smoking, systolic blood pressure, and total/ HDL cholesterol ratio, the OR for enterodiol was 1.03 (0.99, 1.07; *P* = 0.22), and for enterolactone 1.40 (0.92, 2.13; *P* = 0.12). Comparable results for both enterodiol and enterolactone were obtained when computing ORs based on a 10 nmol/L increment.

Table 8.2 Adjusted odds ratios and 95% confidence intervals for plasma enterodiol and enterolactone concentrations in relation to nonfatal myocardial infarctions using conditional logistic regression models

	Q1	Q2	Q3	Q4	P for trend	Continuous models [1]		P value
Enterodiol (nmol/L)	<0.4	0.4-0.8	0.8-1.7	>1.7		Δ 1.3	Δ 10	
cases/ controls (n)	60/71	54/71	70/70	52/71		236/283		
Crude	1.0	0.90 (0.55, 1.47)	1.15 (0.71, 1.88)	0.85 (0.52, 1.40)	0.51	0.99 (0.96, 1.03)	0.94 (0.66, 1.32)	0.70
Adjusted model A [2]	1.0	1.21 (0.69, 2.13)	1.66 (0.95, 2.91)	1.18 (0.67, 2.07)	0.86	1.03 (0.98, 1.07)	1.27 (0.86, 1.88)	0.24
Adjusted model B [3]	1.0	1.18 (0.67, 2.07)	1.62 (0.93, 2.82)	1.21 (0.70, 2.12)	0.74	1.03 (0.99, 1.07)	1.28 (0.87, 1.88)	0.22
Enterolactone (nmol/L)	<3.8	3.8-7.9	7.9-17.5	>17.5		Δ 13.7	Δ 10	
cases/ controls (n)	66/71	57/71	49/70	64/71		236/283		
Crude	1.0	0.88 (0.54, 1.42)	0.75 (0.45, 1.22)	0.98 (0.61, 1.59)	0.87	0.98 (0.68, 1.42)	0.98 (0.71, 1.36)	0.91
Adjusted model A [2]	1.0	1.07 (0.62, 1.85)	1.16 (0.66, 2.06)	1.49 (0.85, 2.61)	0.14	1.39 (0.90, 2.12)	1.33 (0.92, 1.94)	0.13
Adjusted model B [3]	1.0	1.07 (0.62, 1.85)	1.17 (0.67, 2.07)	1.51 (0.87, 2.61)	0.12	1.40 (0.92, 2.13)	1.34 (0.93, 1.94)	0.12

[1] In these continuous models enterolignans concentrations are log-transformed. The increments are based on the interquartile ranges of enterodiol (1.3 nmol/L) and enterolactone (13.7 nmol/L), and on a 10 nmol/L change in plasma concentrations.

[2] Adjusted model A: current smoking, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, ratio total/ HDL cholesterol.

[3] Adjusted model B: current smoking, systolic blood pressure, ratio total/ HDL cholesterol.

Table 8.3 Subgroup analysis with adjusted odds ratios and 95% confidence intervals for plasma enterodiol and enterolactone concentrations in relation to nonfatal myocardial infarctions

	cases (n)	Enterodiol (1.3 nmol/L)	*P* for trend	Enterolactone (13.7 nmol/L)	*P* for trend
Adjusted model B [1]	236	1.03 (0.99, 1.07)	0.22	1.40 (0.92, 2.13)	0.12
Sex					
Male	169	1.02 (0.97, 1.07)	0.44	1.60 (0.98, 2.60)	0.06
Female	64	1.07 (0.97, 1.17)	0.16	0.94 (0.39, 2.28)	0.90
P for interaction			*0.94*		*0.54*
Menopausal status					
premenopausal	34	0.99 (0.86, 1.15)	0.94	0.16 (0.02, 1.03)	0.05
postmenopausal	30	1.17 (1.00, 1.36)	0.05	2.27 (0.57, 8.95)	0.24
P for interaction			*0.80*		*0.29*
Smoking status					
never	34	1.02 (0.92, 1.12)	0.75	0.86 (0.34, 2.29)	0.80
ex	57	1.01 (0.94, 1.08)	0.82	1.89 (0.83, 4.30)	0.13
current	134	1.05 (0.99, 1.12)	0.09	1.51 (0.82, 2.75)	0.18
P for interaction			*0.51*		*0.24*
Maastricht cohort	170	1.03 (0.99, 1.08)	0.16	1.37 (0.84, 2.23)	0.21
Maastricht + hospital treatment	133	1.03 (0.99, 1.09)	0.16	1.41 (0.83, 2.40)	0.21

[1]Adjusted model B: current smoking, systolic blood pressure, ratio total/ HDL cholesterol

When stratifying according to sex, we observed a borderline significant increased risk for men with increasing concentrations of enterolactone (OR: 1.60; 95% CI: 0.98, 2.60, *P* for trend 0.06). This was not observed for enterodiol concentrations, nor in women (**Table 8.3**). In women menopausal status was not considered a confounder as it did not significantly alter the risk estimates (data not shown). When analyzing the data for women by menopausal status, we observed an increased risk in postmenopausal women (OR: 1.17; 95% CI: 1.00, 1.36) for enterodiol. For enterolactone a borderline significant decreased risk was observed (OR: 0.16; 95% CI: 0.02, 1.03) in premenopausal women, whereas no significant association was observed in postmenopausal women (OR: 2.27; 95% CI: 0.57, 8.95). When stratifying for smoking status no consistent associations were observed between enterolignan concentrations and myocardial infarction risk. *P* for interaction was not significant in all stratified analysis (Table 8.3).

The association between enterodiol and enterolactone and nonfatal myocardial infarction was not substantially altered when analysis was restricted to cases who reported hospital treatment as a result of their infarction (Table 8.3), or when data analysis was restricted to the case-control subset excluding the colorectal cancer controls (data not shown).

DISCUSSION

The results of this nested case-control study with subjects aged 20 to 59 years at baseline do not support the hypothesis that high plasma enterodiol or enterolactone concentrations are associated with reduced risk of nonfatal myocardial infarction after adjustment for potential confounders.

An important strength of our study is its prospective design. Plasma samples were obtained up to 15 years prior to the diagnosis of myocardial infarction. Furthermore, our study design ensures identical collection and handling of blood samples from case and control subjects. Finally, plasma enterodiol and enterolactone were used as markers of long-term exposure. In comparison with the assessment of plant lignan intake, plasma enterolignans have the advantage that they integrate nutritional exposure from various sources, including unknown sources, that they take into account the bioavailability from different food matrices, and that they are not based on memory. However, our study has also some limitations. First, nonfatal myocardial infarction was identified through self-reported questionnaires. In the Maastricht part of the cohort, the questionnaire provided also information on hospital treatment as a result of their infarction. This is more unlikely to be filled in incorrectly. The results did not alter when analysis was restricted to this sub cohort. Furthermore, our study was relatively small (n = 237 cases). However, it was large enough to detect an effect similar in size to that reported before in Finland [10, 11], but in general such large effects are unlikely in case of dietary components. Another limitation of our study might be the lack of information on antibiotic use in the year prior to blood sampling. Use of antibiotics reduces plasma enterolactone concentrations, and this effect is sustained for several months [21]. Nevertheless, we have no reason to assume that use of antibiotics prior to the study differed between cases and controls.

Up to now, only one observational study showed inverse associations between serum enterolactone and acute myocardial risk [10] and cardiovascular mortality [11] in men (aged 42 to 60 years at baseline). In two other prospective studies [12, 13], one using serum enterolactone concentrations and one using dietary intake of plant lignans as exposure measure, no associations were observed between lignans and cardiovascular diseases. In the Finnish prospective study that showed inverse associations [10, 11], the risk of acute myocardial infarction was significantly lower in the highest versus lowest quartile, with an OR of 0.35 (95% CI: 0.14, 0.88). The risk of coronary heart disease related death (OR: 0.44; 95% CI: 0.20, 0.96) and risk of total cardiovascular disease related death (OR: 0.55; 95% CI: 0.29, 1.01) was decreased as well.

In the other Finnish study [12] among male smokers aged 50 to 69 years at baseline the OR of highest versus the lowest quintile of serum enterolactone for nonfatal myocardial infarction was 0.67 (95% CI: 0.37, 1.23). In Dutch middle-aged and elderly women (49 to 70 years of age at baseline; around 90% postmenopausal women) the highest versus the lowest quartile of plant lignan intake the ORs were 0.92 (95% CI: 0.65, 1.29) for coronary heart disease and 0.89 (95% CI: 0.66, 1.19) for total cardiovascular diseases [13].

In contrast to our study, all these previous studies [10-13] reported ORs below unity. The explanation for the different results with the present study is not clear, but may be at least partly due to the differences in study populations. Our population was relatively young (20 to 59 years of age at baseline) and included both men and women. However, when we stratified according to sex, we observed a borderline significant increased risk in men, which is opposite to the results found by Vanharanta et al [10, 11]. Furthermore, underlying dietary patterns, including sources of lignans, might differ between The Netherlands and Finland. In Finland whole grain products, especially rye bread, and fruits, like berries, are main sources of lignans [22], whereas in The Netherlands main sources are beverages, like tea and coffee, and vegetables [14]. So, confounding by other dietary compounds, present in these products, or factors related to these diets, may have played a role. In the Dutch prospective study by van der Schouw et al [13], not all known major precursors of enterolignans were measured; lariciresinol and pinoresinol were not taken into account. Furthermore, they used a method which included 7 category scores, instead of exact lignan contents of foods for calculation of the lignan intake. These scores were bases on the highest reported value found in the Lature. For some products, the content of secoisolariciresinol and matairesinol was derived from the amount of enterolignans produced after in vitro fermentation. This leads to an overestimation of the amount of secoisolariciresinol and matairesinol if the product also contains other enterolignans precursors, and may also have led to misclassification of participants and invalid ORs [14].

To a large extent the support for a potential protective effect of lignans on cardiovascular diseases is based on human intervention trials using flaxseed as source of lignans. Several authors reported a decrease in serum total cholesterol and LDL cholesterol concentrations after supplementation with 38 to 50 g flaxseed for several weeks in normal [23] and hyperlipidemic subjects [24, 25]. Besides lignans, flaxseed contains also high concentrations of α-linolenic acid, which might have caused the beneficial effect. The health benefits of isolated plant lignans have been examined in animal models [7, 8]. Those studies showed a reduction in total cholesterol and LDL cholesterol. As far as we know, the only study using a lignan complex isolated from flaxseed in humans, did not show any effect on plasma lipid concentrations or antioxidant capacity [9]. Note however, that these subjects were healthy postmenopausal women; the effect of lignans might be different in hyperlipidemic subjects with a different cardiovascular disease risk profile.

We did not observe any cross-sectional association between plasma lignan concentrations and total or HDL-cholesterol. In another cross-sectional study the intake of plant lignans was not associated with total and LDL cholesterol [26], but associated with lower systolic and diastolic blood pressure and a lower prevalence of hypertension [27], which is also a known risk factor of coronary heart disease. In our study, high concentrations of plasma enterodiol and enterolactone were not associated with lower systolic or diastolic blood pressure.

Several mechanisms may contribute to the proposed protective effect of enterolignans on coronary heart disease. Due to the biphenolic structure of enterolignans, they could act as antioxidants and through this contribute to cardiovascular health. Enterolactone has demonstrated some potential to act as an antioxidant in concentrations of 10 to 100 μmol/L [5]. However, these concentrations are high in comparison with concentrations detected in plasma (95% enterolactone concentrations in our study are <50 nmol/L). Moreover, the tendency to circulate in conjugated form, leaving none or only one free hydroxyl to act as an antioxidant [28], suggests that enterolactone is unlikely to contribute to the antioxidant defense.

In summary, the findings of this nested case-control study do not support the hypothesis that high plasma enterodiol or high plasma enterolactone concentrations are associated with reduced risk of nonfatal myocardial infarction.

ACKNOWLEDGEMENTS

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General discussion

Chapter 9

As described in *Chapter 1*, the main objectives of this thesis were a) to determine the bioavailability of major plant lignans in humans and b) to quantify the associations between plasma enterolignans and colorectal adenomas, colorectal cancer, and coronary heart disease risk in observational studies.

Sufficient bioavailability from the diet is a prerequisite for its potentially protective role in cardiovascular diseases and most forms of cancer. In this last chapter we give a brief overview of the main findings and critically discuss the strengths and weaknesses of the studies described in this thesis. Furthermore, we give recommendations for future research and summarize important conclusions.

MAIN FINDINGS

The findings of the studies described in this thesis are schematically summarized in **Figure 9.1**. First, we obtained a simple, rapid, and sensitive method for simultaneous quantification of both enterolignans applicable for the analysis of large numbers of samples (*Chapter 2*). To evaluate the role of lignans on the development of chronic diseases it is essential to understand to what extent they are available in the human body. Plant lignans are transported to the colon where they are converted into the enterolignans, enterodiol and enterolactone, by intestinal bacteria. In a single dose study with a purified plant lignan, described in *Chapter 3*, we showed that enterodiol and enterolactone absorption started only 8-10 h after consumption, which confirms the predominant role of microorganisms in the colon, and that they were eliminated slowly. A substantial part (~40%) of the enterolignans was excreted in urine, and thus at least 40% has been available in the blood circulation. The measured mean residence times and elimination half-lives indicate that enterolignans will accumulate in plasma when consumed 2-3 times a day, and will reach steady state concentrations. As lignans are present in many foods this is very likely to happen. In a randomized crossover study, described in *Chapter* 4, we found that the relative bioavailability of lignans from flaxseed can be improved by milling or crushing. Furthermore, we found that the intake of lignan-rich foods, defecation frequency, and body mass index were major determinants of enterodiol and enterolactone plasma concentrations (*Chapter 5)*. The correlations between plasma enterolactone concentrations and the intake of individual plant lignans, secoisolariciresinol, matairesinol, pinoresinol, and lariciresinol, ranged from 0.10 for secoisolariciresinol to 0.21 for matairesinol, and that for enterodiol from 0.02 for secoisolariciresinol to 0.10 for pinoresinol. The correlation between total plasma enterolignan concentrations and total intake of plant lignans was 0.18.

In two observational studies we evaluated the role of lignans on the development of chronic diseases. In a case-control study, plasma enterodiol and enterolactone were inversely associated with first colorectal adenoma risk (odds ratio (OR) in highest quartile of plasma enterodiol: 0.53, *P*
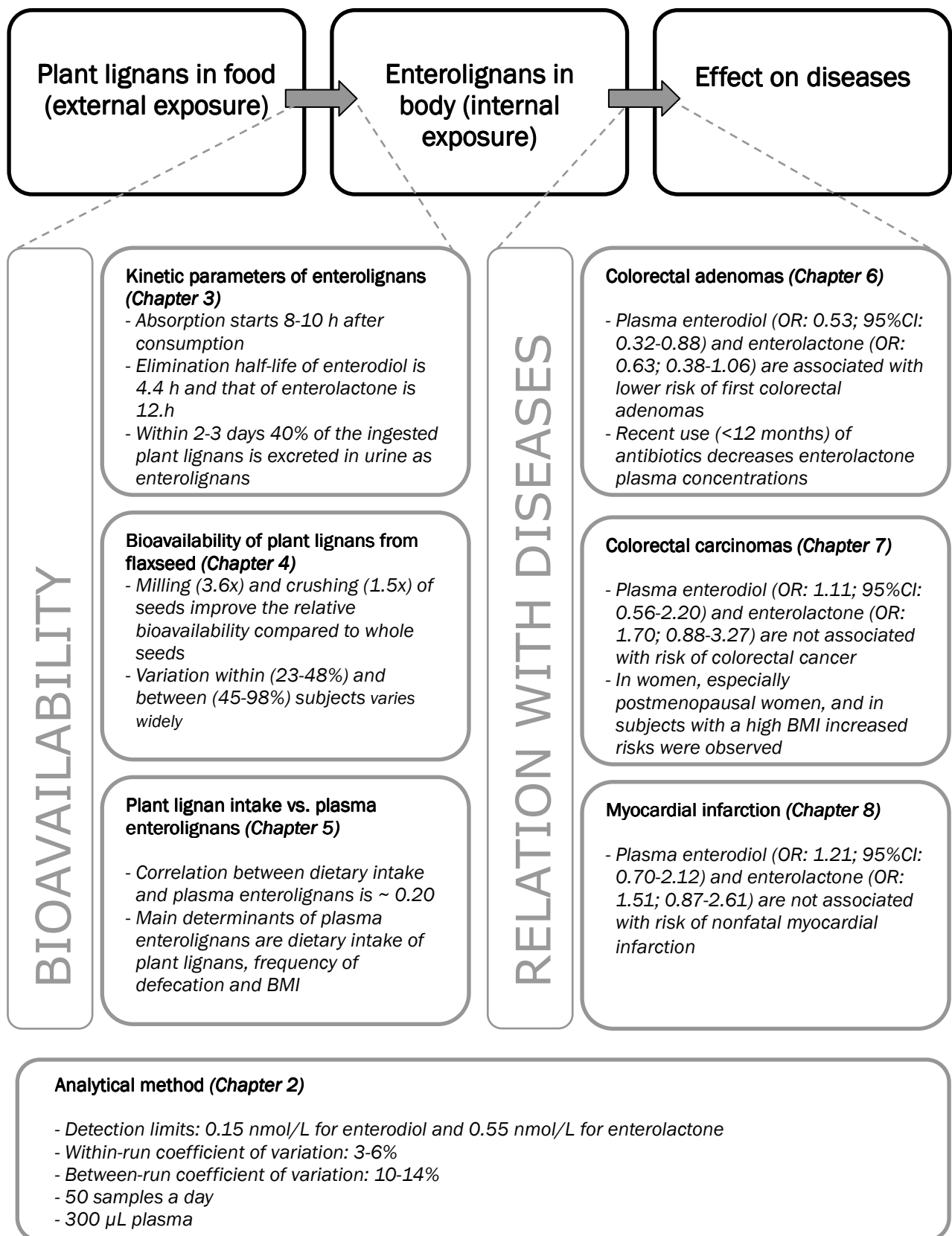
Plant lignans in food (external exposure) → Enterolignans in body (internal exposure) → Effect on diseases

BIOAVAILABILITY

Kinetic parameters of enterolignans *(Chapter 3)*
- *Absorption starts 8-10 h after consumption*
- *Elimination half-life of enterodiol is 4.4 h and that of enterolactone is 12.h*
- *Within 2-3 days 40% of the ingested plant lignans is excreted in urine as enterolignans*

Bioavailability of plant lignans from flaxseed *(Chapter 4)*
- *Milling (3.6x) and crushing (1.5x) of seeds improve the relative bioavailability compared to whole seeds*
- *Variation within (23-48%) and between (45-98%) subjects varies widely*

Plant lignan intake vs. plasma enterolignans *(Chapter 5)*
- *Correlation between dietary intake and plasma enterolignans is ~ 0.20*
- *Main determinants of plasma enterolignans are dietary intake of plant lignans, frequency of defecation and BMI*

RELATION WITH DISEASES

Colorectal adenomas *(Chapter 6)*
- *Plasma enterodiol (OR: 0.53; 95%CI: 0.32-0.88) and enterolactone (OR: 0.63; 0.38-1.06) are associated with lower risk of first colorectal adenomas*
- *Recent use (<12 months) of antibiotics decreases enterolactone plasma concentrations*

Colorectal carcinomas *(Chapter 7)*
- *Plasma enterodiol (OR: 1.11; 95%CI: 0.56-2.20) and enterolactone (OR: 1.70; 0.88-3.27) are not associated with risk of colorectal cancer*
- *In women, especially postmenopausal women, and in subjects with a high BMI increased risks were observed*

Myocardial infarction *(Chapter 8)*
- *Plasma enterodiol (OR: 1.21; 95%CI: 0.70-2.12) and enterolactone (OR: 1.51; 0.87-2.61) are not associated with risk of nonfatal myocardial infarction*

Analytical method *(Chapter 2)*
- *Detection limits: 0.15 nmol/L for enterodiol and 0.55 nmol/L for enterolactone*
- *Within-run coefficient of variation: 3-6%*
- *Between-run coefficient of variation: 10-14%*
- *50 samples a day*
- *300 μL plasma*

Figure 9.1 Schematic overview of main findings

for trend = 0.01, and that for enterolactone: 0.63, *P* for trend = 0.09) after adjustment for age, sex, and antibiotic use (*Chapter 6*). In a prospective study, no statistically significant associations between plasma enterodiol and enterolactone and risk of colorectal cancer were present (OR in highest quartile of plasma enterodiol: 1.11, *P* for trend = 0.75, and that for enterolactone: 1.70, *P* for trend = 0.15) after adjustment for known dietary and lifestyle risk factors for colorectal cancer (*Chapter 7*). However, we observed increased risks in women (enterolactone: OR 2.63; *P* for trend = 0.03*)*, especially postmenopausal women, in subjects with a high body mass index (enterolactone: OR 2.44; *P* for trend = 0.02), and in current smokers (enterodiol: OR 1.19; *P* for trend = 0.02). In the same prospective study, plasma enterodiol and enterolactone were not associated with risk of nonfatal myocardial infarction (OR in highest quartile of plasma enterodiol: 1.21, *P* for trend = 0.74, and that for enterolactone: 1.51, *P* for trend = 0.12) after adjustment for known risk factors for myocardial infarction (*Chapter 8)*.

It can be concluded that a substantial part of the plant lignans in the diet becomes available in the blood circulation and can play a role in the prevention of chronic diseases. However, our results show that increased plasma concentrations are only associated with lower risk of colorectal adenomas, and not with a risk reduction of colorectal carcinomas or nonfatal myocardial infarction. Possibly, in some groups (women and subjects with high body mass index) the risk might even increase.

METHODOLOGICAL CONSIDERATIONS

In this paragraph we discuss the strengths and weaknesses of the single and multiple dose studies and observational studies described in this thesis. We address the study designs, study populations, and exposure and outcome measurements that we used to determine the bioavailability of enterolignans and to quantify their associations with chronic diseases.

SINGLE AND MULTIPLE DOSE STUDIES IN HUMANS

Study population

In our single and multiple dose studies, subjects, mostly students, were ~25 years old and generally healthy (self-reported). None of them had diarrhea or had used antibiotics or other medication in the past three months. As illustrated in **Table 9.1**, the habitual plasma enterolignan concentrations measured in these subjects were relatively high (5 nmol/L for enterodiol and 27 nmol/L for enterolactone) compared to concentrations measured in subjects in our epidemiological studies (~1 nmol/L for enterodiol and ~10 nmol/L for enterolactone). As the same analytical method and procedures were used for the quantification of enterolignans in plasma, analytical bias cannot be the explanation for these differences. Time of storage of plasma samples, which is longer in our prospective studies, in which samples are collected at baseline, might have reduced plasma concentrations of enterolignans. However, this is not likely

Table 9.1 Concentrations plasma[1]

	n	Enterodiol nmol/L	Enterolactone nmol/L
Ch. 3. Single dose study with SDG[2]	12		
Baseline[3]		3.4 ± 3.9	7.2 ± 3.6
Habitual[4]		5.1 ± 5.1	27.5 ± 27.1
Maximum after SDG consumption		73.2 ± 39.8	55.9 ± 30.2
Ch. 4. Multiple dose study with flaxseeds[5]	12		
Baseline[3]		1.9 ± 0.5	9.5 ± 1.1
Whole flaxseeds		20 ± 5	65 ± 16
Crushed flaxseeds		37 ± 11	85 ± 17
Ground flaxseeds		103 ± 31	167 ± 25
Ch. 6. Case-control study, colorectal adenomas			
Cases	532	1.3 (0.6; 3.1)	11.2 (4.4; 25.4)
Controls	503	1.5 (0.7; 3.1)	11.6 (4.6; 26.3)
Ch. 7. Nested case-control study, colorectal carcinomas			
Cases	160	1.1 (0.6; 1.9)	11.6 (5.6; 20.5)
Controls	387	0.9 (0.5; 2.0)	8.8 (4.2; 18.8)
Ch. 8. Nested case-control study, myocardial infarction			
Cases	237	0.8 (0.4; 1.5)	7.5 (3.3; 19.1)
Controls	283	0.8 (0.4; 1.7)	7.9 (3.8; 17.5)

[1] Concentrations are means ± SD (*Chapters 3 and 4*) or median (25th; 75th percentile) (*Chapters 6-8*).

[2] SDG = secoisolariciresinol diglucoside; Single dose = 1.31 µmol SDG/kg bodyweight.

[3] Baseline concentrations were measured after a diet poor in lignans: 7 days for single dose study and 11 days for multiple dose study (see *Chapters 3 and 4* for dietary guidelines).

[4] Habitual concentrations were measured before the study.

[5] Multiple dose = 0.3 g flaxseeds (~3 µmol plant lignans)/ kg bodyweight per day for 10 days.

the case as stability of plasma enterolactone concentrations over a 2-year period is relatively high [1]. In this study duration of storage had no effect on the plasma concentrations. More likely, these young volunteers have a higher degree of health consciousness and are expected to eat more food to fulfill energy requirements and consume more fiber-rich products, like whole grain products, fruits and vegetables, which have high lignan concentrations and in addition stimulate the activity of the microflora in the colon, resulting in higher plasma concentrations of enterolignans. These observations are in line with others who have observed similar relatively high enterolactone concentrations in human intervention studies (30-40 nmol/L) [2-4] and low concentrations in population based studies (8-20 nmol/L) [5-11].

Our bioavailability data are based on studies with healthy volunteers. In subjects, who have a less active microflora due to, for example, less consumption of fibers, we expect that plasma

enterolignan concentrations, and thus the bioavailability, will be lower. Currently, there is little information on the influence of age on the bioavailability of lignans [12]. As absorption and metabolic processes might slow down with aging, this may change the kinetic parameters of enterolignans. We speculate that elimination half-lives of enterolignans will increase and as a consequence, concentrations of enterolignans will fluctuate less. This will result in more stable biomarkers in elderly as compared to younger subjects.

Study design

We investigated the extent of absorption and excretion of enterolignans in a single dose study. To calculate pharmacokinetic parameters it is essential to have a sufficient number of specimen sampling points. A specific sampling schedule was designed based on a pilot study, which covered the increase in plasma and urine concentrations of enterolignans and their return to baseline. Collection of plasma and urine for 5 days appeared to be adequate to calculate pharmacokinetic parameters of enterodiol and enterolactone. So far, published studies investigating the absorption and excretion of enterolignans collected plasma and urine only for the first 24 h. As a consequence, they were not able to properly calculate pharmacokinetic parameters, such as elimination half-life and mean residence time. The elimination half-life is necessary to predict when steady state concentrations (3.5 x elimination half-life) will be reached[13]. Treatment, run-in, and wash-out periods of at least 7 days, which we used in our multiple dose study, were more than sufficient to reach steady state concentrations.

The relative bioavailability of enterolignans from flaxseeds was studied in a multiple dose randomized crossover study. An advantage of this design is that factors that could contribute to the variation between subjects, like the activity and composition of the microflora and frequency of chewing, are corrected for automatically because each treatment is compared within the same subject. This is important because plasma enterolignan concentrations vary widely between different people. However, even within subjects variations are observed. To increase precision further, flaxseed supplements were given twice a day to reduce daily fluctuations in plasma concentrations, and at the final day of each treatment period 3 plasma samples were taken during the day to calculate mean concentrations per subject.

Randomization, blinding, and compliance

In the single dose study, randomization, blinding, and compliance to the treatment were no issues because each individual received the same treatment and consumption of the single dose was monitored. In the multiple dose study, the order of treatments was randomly allocated to the subjects so that each order group was represented twice. It was not possible to blind the subjects to the type of treatment because the specific treatments were recognizable (whole, crushed, or ground flaxseeds). However, we believe that this could have had only a slight impact on the results

of the study, as we do not expect that the recognizability of the treatments would have influenced the activity of the microflora in the colon or the enterolignan concentrations in plasma, and as a consequence the relative bioavailability. Furthermore, statistical analysis showed that there was no effect of order of treatment. Identical baseline concentrations at the end of each washout period showed that the bacteria were not stimulated during the study. For both the single and multiple dose studies, the laboratory personnel were blinded to the treatments of the participants.

The compliance in the multiple dose study was monitored by means of pre-printed diaries in which subjects wrote down the time of consumption of the supplied products and by collection of used packages twice a week. Although we cannot rule out that subjects did not consume the entire dosages, they could have emptied their packages before handing them in, the high enterolignan concentrations at the end of the treatment periods supported that the compliance was good.

Background diet

To measure the bioavailability of a dietary compound it would be ideal when baseline concentrations are reduced to zero and interference from other dietary sources of the compound could be avoided. However, this is not feasible when lignans are involved. Because lignans are present in almost all food products of plant origin it is impossible to follow a lignan free diet for several days. Instead, subjects had to follow a diet poor in lignans during our single and multiple dose studies. Furthermore, we used relatively high doses of purified plant lignan or flaxseed (80-240 µmol/day) compared to the habitual intake of lignans in the Dutch population (1-6 µmol/day) [14]. Although no dietary information was recorded, the low baseline concentrations (Table 9.1) suggest that compliance with the diet was good.

EPIDEMIOLOGICAL STUDIES

Study population and design

We applied two frequently used study designs, a retrospective case-control and a prospective nested case-control study design. In the case-control study both controls and colorectal adenoma cases were recruited among patients undergoing endoscopies. Because blood samples were collected approximately 4 months after the index endoscopy, misclassification of exposure due to changes in diet or lifestyle might be a concern (information bias). The presence of adenomas usually does not provide any symptoms, reducing the possibility that people may have changed their dietary habits. Furthermore, in our case-control study both controls and incident cases underwent endoscopy primarily for intestinal complaints. So, if they would change their diet because of these complaints in this short period, this would have preceded the occurrence of the endoscopy and blood collection and would have been similar in controls and incident cases. A

limitation of a retrospective case-control design is that we cannot rule out that enterolignan plasma concentrations might be influenced by the presence of adenomas. Thus, the observed associations might not be causally related.

In the nested case-control study, in which plasma samples were taken at baseline prior to the diagnosis of colorectal cancer and myocardial infarction, information bias is not regarded a problem. Moreover, we evaluated the potential impact of systematic errors by excluding colorectal cancer cases diagnosed in the first 2 years of follow-up. The results were not substantially altered when excluding these cases.

Exposure assessment

In our epidemiological studies we used plasma enterodiol and enterolactone as markers of long-term internal exposure. In comparison with the assessment of plant lignan intake, plasma enterolignans have the advantage that they integrate nutritional exposure from various sources, including unknown sources, they take into account the bioavailability from different food matrices, and they are not based on memory. Furthermore, they take into account the activity and composition of the colonic microflora, which affect enterolignan concentrations, as described above. However, use of plasma enterolignans as measure of internal exposure has some limitations as well. Because the measured biomarker on a single day is rarely the same as the underlying true exposure, misclassification of the exposure might occur and severely bias the observed associations towards the null. In our epidemiological studies, however, we expect steady state plasma enterodiol and enterolactone to be suitable long-term measures of internal exposure. Steady state plasma concentrations of enterodiol and enterolactone are achieved because enterolignans are eliminated slowly, and their precursors are present in many foods and beverages and eaten several times a day. However, recent antibiotic use or a combination of fasting and laxative medication can have such a large impact on the microflora and the absorption of enterolignans in the colon that these treatments will decrease steady state concentrations and no longer reflect long-term exposure. In populations in which people recently used antibiotics, this should be taken into account when using plasma enterolignans as measure of long-term exposure. In our case-control study we adjusted for recent antibiotic use. This was not possible in our prospective study because no information on antibiotic use was available. Future cases may have higher prevalence of antibiotic use. This phenomenon might induce a spurious inverse association in the early years of follow-up, but there was no evidence in our data to support this idea.

In epidemiological studies biomarkers are used to make a distinction between individuals. Although plasma enterolignan concentrations vary widely within subjects, variation between subjects is even larger. The intra class correlation coefficient (ICC) represents the proportion of variance in the measure explained by the between-subject variation. High ICC values (close to 1)

represent excellent reproducibility, and distinction between individuals is feasible. The ICCs in our multiple dose study were quite high (enterodiol: 0.86-0.95; enterolactone: 0.57-0.84). This is in line with the moderately high ICCs of plasma enterolactone measurements that have been observed over a period of weeks (0.79) and months (0.77) in men and women consuming their normal diet [15]. As expected, lower ICCs of serum measurements (enterolactone: 0.55; enterodiol 0.37) were observed over a 2-y period [1]. Thus, our results and those of others indicate that plasma enterolactone and enterodiol are relatively good biomarkers of internal exposure; one plasma sample will reflect exposure for a longer period when the underlying dietary pattern is relatively stable and recent antibiotic use is corrected for.

Confounding

In our observational studies we adjusted for several factors that are related to both the exposure and the outcome measure and influence the association of interest (confounding). In the prospective study the confounding factors age, sex and study center were controlled by matching of cases and controls. Other potential confounding factors in this study and in the case-control study were adjusted for when the odds ratio changed by more than 10% if the variable was added to the model. For colorectal adenomas an important confounding factor, besides age and sex, was antibiotic use. Although we adjusted for these factors, residual confounding might have occurred as a result of inadequate control for the confounding factors. In our case-control study information on antibiotic was limited; we only had data on the calendar year of the last antibiotic therapy. To reduce residual confounding data on frequency and duration of antibiotic use, and number of months between the last therapy and blood sampling would have been more useful. In our prospective study no information was available on use of antibiotics. However, we expect that antibiotics were used less frequently in this study population than in the case-control study, in which the majority participants had intestinal complaints.

As the odds ratios remained approximately stable after adjusting for most possible confounding factors in both observational studies, we conclude that confounding is not a major issue in our observational studies.

INTERPRETATION OF FINDINGS

In this paragraph we will compare our findings to others, address the similarities and discrepancies, and discuss them. We will evaluate the analytical method, the bioavailability of lignans, the use of plasma enterolignans as marker of dietary or internal exposure, and the associations between enterolignans and chronic diseases.

Analytical method

Plasma enterolignans are frequently used as markers of internal exposure. A commonly used

analytical method in epidemiological research is time-resolved fluorimmunoassay. A major drawback of this method is that enterodiol, one of the two enterolignans, cannot be measured. We developed a liquid chromatography-tandem mass spectrometry method using triply ^{13}C-labeled isotopes for the simultaneous quantification of enterodiol and enterolactone. Because the reproducibility and sensitivity of our method is comparable to the time-resolved fluorimmunoassay, our method is an excellent more adequate alternative to measure plasma enterolignans in epidemiological studies.

Bioavailability of lignans

Bioavailability can be defined as the fraction of an ingested compound that can be used for metabolic processes and storage in the body. The bioavailability is a crucial factor influencing the biological activity in the human body. Thus far, information on the fate of enterolignans in the human body is very limited. We are the first who determined pharmacokinetic parameters of enterodiol and enterolactone, and gathered data on absorption, metabolism, distribution and excretion. Similar studies have been performed regarding other phytoestrogens, such as isoflavones and their metabolites, e.g., daidzein and genistein [12].

After conversion of plant lignans to enterolignans in the human colon, enterodiol and enterolactone are absorbed and will reach the blood circulation and target tissues. As metabolism is extensive, enterodiol and enterolactone might be more important for potential health effects than the parent compounds. To date, in vitro and in vivo studies have focused on the bioactivity of enterolignans. Therefore, we concentrated on the internal dose of enterolignans, and not on that of plant lignans. In our single and multiple dose studies we used secoisolariciresinol diglucoside and flaxseed, which contains a high amount of plant lignans (>98% secoisolariciresinol). The bioavailability from other plant lignans has not been studied. The extent of conversion by the microorganisms in the colon has been shown to be specific for the different plant lignans in vitro [16]. Secoisolariciresinol was converted to enterolignans for 72%, matairesinol for 62%, pinoresinol for 55%, and lariciresinol for 101% after incubation with human fecal flora for 24 h; other plant lignans had conversion rates <15%. Thus, the impact on bioavailability of enterolignans from other plant lignans remains to be studied.

The central role of microorganisms on the bioavailability is also illustrated by the effect of antibiotic therapy on enterolignan concentrations. We observed a substantial reduction of plasma enterolactone concentrations in subjects using antibiotic therapy in the year prior to blood sampling. Interesting to note is that plasma enterodiol concentrations were not reduced in our study. This suggests that enterolactone concentrations are more affected by antibiotic use than enterodiol concentrations. This makes sense, because for most plant lignans, except matairesinol, enterodiol is the microbial precursor of enterolactone. In addition, we observed that defecation frequency is also negatively related to plasma concentration of enterolignans. Less

frequent defecation will lead to extended residence in the colon and consequently to more complete metabolism and absorption of lignans and influence the bioavailability.

In conclusion, we determined the extent of absorption and excretion of enterolignans after consumption of secoisolariciresinol diglucoside. Bioavailability of enterolignans from other plant lignans has not been studied. Furthermore, our studies supported the role of microorganisms on the bioavailability. Recent knowledge regarding the specific microorganisms that are responsible for the conversion of plant lignans to enterolignans [17, 18] should be further examined in human studies.

Plasma enterolignans as biomarker of dietary intake

In *Chapter 5* we evaluated the use of plasma enterolignans as indicators of dietary intake. The correlation between dietary intake and plasma enterolignans was rather low (0.2) compared to other biomarkers of dietary intake. For some markers in plasma, e.g. carotenoids and vitamin E, correlations with questionnaire measurements have been reported to be low as well (0.1- 0.4), whereas the correlation can be as high as 0.6 or 0.8 for vitamin C or linoleic acid [19]. Although this low correlation may partially be due to the fact that the food frequency questionnaire was not developed specifically for assessment of lignan intake (e.g., it lacked adequate information on the consumption of multigrain breads and seeds), the low correlations we found are not surprising, taking into account that most biomarkers mentioned above are the authentic compounds present in foods, whereas, enterolignans are metabolites of plant lignans, and undergo conversion to variable extents. Besides the intake of lignans-rich foods, major independent determinants of enterodiol and enterolactone plasma concentrations were defecation frequency, and body mass index. The role of microorganisms is also illustrated by the effect of antibiotic therapy on enterolignan concentrations. We observed a 2-fold reduction of plasma enterolactone concentrations in subjects using antibiotic therapy in the year prior to blood sampling. Thus, the use of plasma enterolignans as marker of dietary intake (external exposure) has some restrictions, both regarding its potential for the level of intake and the ranking of individuals.

Plasma enterolignans as biomarker of internal exposure

In the epidemiological studies described in this thesis, plasma concentrations were used as markers of internal exposure. The potential errors resulting from this choice may depend on the disease endpoint of interest. To have an effect on most chronic diseases, absorption of enterolignans is a prerequisite, and plasma concentrations are regarded suitable biomarkers of internal exposure. However, when studying the association between lignans and colorectal adenomas or carcinomas, absorption of lignans might not be essential since both the plant lignans and enterolignans might play a role in carcinogenesis at the luminal colon side, rather than via the systemic blood flow (or both). When lignans act at the luminal colon side, it is

questionable whether plasma enterolignans would be a good marker in that case. However, plasma concentrations of enterodiol and enterolactone are positively related to the intake of lignan-rich foods [20-22]. This suggests that the amount of plant lignans and enterolignans in the colon, which is the intermediate site, could be reflected by enterolignans plasma concentrations as well. Besides, the use of plasma concentrations as marker of internal exposure is preferred in human studies because local exposure measures, such as tissue concentrations, would impose a considerable burden to the volunteers involved.

As discussed before, high ICCs of enterolignan plasma concentrations suggest that distinction between individuals is feasible. Besides, the correlation between plasma concentrations and dietary intake is rather low, which suggest the importance of other determinants on enterolignan plasma concentrations than the dietary intake of plant lignans. Therefore, plasma enterolignan concentrations are preferred as biomarkers of exposure compared to the intake of dietary plant lignans.

In conclusion, plasma concentrations are regarded suitable for the measurement of the long-term internal exposure to enterolignans.

Associations of enterolignans with chronic diseases

The relation between plasma enterolignans and **colorectal adenomas and carcinomas** was evaluated in observational studies. We observed a substantial reduction in first colorectal adenoma risk among subjects with high plasma concentrations of enterolignans, in particular enterodiol. Colorectal adenomas are widely accepted as precursors of colorectal cancer in humans. However, only few adenomas (~5%) develop into carcinomas. We observed that high plasma enterodiol or enterolactone concentrations were not associated with reduced risk of colorectal cancer in a prospective study. Colorectal carcinogenesis takes several years or even decades and has been demonstrated to involve the accumulation of genetic alterations, in which individual dietary factors, such as lignans, may play a small but not crucial role. Enterolignans might decrease or delay the development of colorectal adenomas, but once adenomas are formed enterolignans may no longer influence the further development into cancer. To date, no other epidemiological studies on colorectal adenomas and carcinomas have been published.

Although plasma enterolignan concentrations were not associated with a reduced risk of colorectal cancer, we observed increased risks in women, especially in postmenopausal women. No specific underlying mechanism has been established yet, but the interaction with sex, and menopausal status, suggest that an estrogen-related hormonal mechanism might be involved. The interaction of enterolignans with hormonal mechanisms is also supported by epidemiological studies on hormone related cancers, such as breast cancer, in which inverse associations were observed (Table 1.1).

In conclusion, enterolignans might decrease or delay the development of colorectal adenomas,

but once adenomas are formed enterolignans may no longer influence the further development into cancer. The role of enterolignans on hormone related cancer mechanism remains to be studied.

The relation between lignans and **cardiovascular diseases** has been studied before. We observed no association between plasma enterodiol or enterolactone concentrations and risk of nonfatal myocardial infarction. This is similar to two prospective studies [23, 24], were the authors did not observe any associations between the intake of dietary plant lignans and coronary heart disease or cerebrovascular events, and between serum enterolignans and nonfatal myocardial infarction or coronary death. Only one prospective observational study showed inverse associations between serum enterolactone and acute myocardial risk [25] and cardiovascular mortality [26]. Based on all these epidemiological studies it is hard to draw firm conclusions. The populations studied were quite different in age and sex distributions. Furthermore, to determine the association between lignans and cardiovascular diseases different exposure measures and disease outcome measures were used and the follow-up time varied among these studies.

To a large extent, the support for a potential protective effect of lignans on cardiovascular diseases is based on in vitro and animal studies. Enterolignans have demonstrated antioxidant capacity in vitro [27, 28]. Furthermore, enterolactone has the capacity to increase hepatic low-density lipoprotein (LDL) receptor activity, and thus may lower circulating LDL cholesterol [29]. In rabbits fed a high-cholesterol diet, secoisolariciresinol diglucoside reduced hypercholesterolemic atherosclerosis and this effect was associated with a decrease in total and LDL cholesterol [30]. However, this cholesterol lowering capacity and antioxidant activity of lignans could not be confirmed in healthy postmenopausal women [31].

In conclusion, our data do not support a protective effect of enterolignans against myocardial infarction. To date, no plausible mechanisms are apparent that would support a role of enterolignans in cardiovascular diseases.

FUTURE RESEARCH

In the lignan project, we studied the bioavailability, i.e., the extent of absorption and excretion of enterolignans. Therefore, we were able, to some extent, to evaluate the use of plasma concentration as biomarker of exposure. Simultaneously, we accumulated more evidence on the relation between lignans and chronic diseases from well-designed observational studies. Although we contributed to a large extent to the understanding of the fate of lignans in humans, a number of questions remain to be answered. Up until now, research has been focused on the estrogenic activity of enterolactone and enterodiol. Recently, evidence is accumulating that also plant lignans as such are absorbed[32] and excreted in urine[33], be it to a lesser extent than the enterolignans. The biological impact of plant lignans as such is not known, as is the impact of enterolignans on

other pathways. Additional effects may occur as well as differences between various tissues and stages of disease pathophysiology. Furthermore, epidemiological evidence regarding the potential protective effects of lignans on chronic diseases is inconsistent and incomplete. While data on the role of lignans is still limited, integrated research at the level of the population, and the individual, and the cell would be suitable in future projects.

Bioavailability of plant lignans and enterolignans

Plant lignans are metabolized extensively in the colon. In this thesis we focused on the availability of enterolignans and their relation with chronic diseases. However, we do not know the fate of the plant lignans themselves and the concentrations that circulate in the body. Recently, Milder et al. [34] reported inverse associations between the intake of matairesinol, one of the plant lignans, and cardiovascular diseases, cancer, and all-cause mortality risk, but not with the other enterolignan precursors. This could be explained by a more efficient conversion of matairesinol to enterolactone, but might also indicate that plant lignans themselves can have effect on the development of chronic diseases. This needs to be further investigated. In vivo (human or animal) studies should focus on the fate of plant lignans and their metabolites, the target tissues, and factors that influence absorption and metabolism, and thus the bioavailability of lignans.

Relation between enterolignans and chronic diseases

The analytical method we developed is suitable to measure plasma enterodiol and enterolactone concentrations on a large scale. It would be useful to accumulate more evidence on the relation between enterolignans and chronic diseases from well designed prospective studies.

We observed an inverse association between plasma concentrations of enterolignans and first colorectal adenoma risk in a retrospective case-control study. This association has to be confirmed in a prospective study. We did not observe a beneficial association of enterolignans on colorectal carcinomas. It could be that enterolignans reduce the development of benign adenomas and not the adenomas that develop into malignant carcinomas. It would be interesting to study if the impact of enterolignans will be modified by type and/or size of the adenomas. Furthermore, it would be interesting to examine the role of enterolignans on the recurrence of adenomas.

Although plasma enterolignan concentrations were not associated with a reduced risk of colorectal cancer overall, the possible interaction with endogenous hormonal status needs to be addressed. Because enterolignans are able to bind to sex hormone-binding globulin [35-37], and they stimulate their synthesis [38], they could block or enhance the estrogenic effects of endogenous estrogens depending on their concentration. The interaction of enterolignans with endogenous estrogens and sex hormone-binding globulin are attractive to study in observational studies with high risk groups, such as women, especially postmenopausal women, in whom we observed

increased risks of colorectal carcinomas.

In these observational studies, detailed information on activity of the microflora (recent use of antibiotics, constipation, or defecation frequency) is important, to formulate eligibility criteria, and/or to address effect modification and/or to account for confounding.

Pathways and mechanisms

To a large extent, the support for a potential protective role of lignans on cardiovascular diseases is based on the cholesterol-lowering capacity and antioxidant activity observed in *in vitro* and animal studies. Up to now, these mechanisms could not be confirmed in humans [31, 39]. Note however, that the subjects in these studies were all healthy postmenopausal women; the effect of lignans might be different in hyperlipidemic subjects with a different cardiovascular disease risk profile. Because no clear underlying mechanism has been established yet, this needs to be addressed first.

The majority of in vitro studies used concentrations of enterolignans that are out of the physiological range. It is not clear whether apart from enterodiol and enterolactone other metabolites or their precursors also have a relevant biological effect. In vitro studies should study the role of these other compounds using relevant concentrations. In addition, challenge studies in humans or animals consuming high and low doses of lignans could be performed to identify possible target genes. From plasma samples collected in human studies or from tissues collected in animal experiments, RNA could be isolated and gene expression studied to find new biochemical pathways affected by lignans. Micro-array technology can be used to unravel mechanisms of biological effects of lignans. New biochemical pathways involved might be found since this technology allows us to study a large number of molecular effects at the same time.

PUBLIC HEALTH IMPLICATIONS

We observed a substantial reduction in colorectal adenoma risk among subjects with high plasma concentrations of enterolignans. However, because this is the first and only study that shows an inverse association between enterolignans and colorectal adenomas, and we were not able to confirm this association in colorectal carcinomas, it is too early to give dietary recommendations.

In addition, our data do not support a strong protective effect of lignans against myocardial infarction. At this point, there is lack of consistent evidence to offer recommendations regarding the consumption of lignan-rich foods.

CONCLUSIONS

Plant lignans are converted to enterolignans, and a substantial part of enterolignans enter the blood circulation, and are subsequently excreted in urine. Enterolignans will accumulate and reach steady state concentrations in plasma when consumed 2-3 times a day. As lignans are

present in many foods this is very likely to happen. The availability of lignans from food products, such as breads, will improve substantially when whole seeds are replaced by crushed or ground seeds. Besides the intake of plant lignans, use of antibiotic therapy, defecation frequency, and body mass index are independent determinants of plasma concentrations of enterolignans. In observational studies using biomarkers as long-term exposure measures it is necessary to take these determinants into account.

Our data suggest a protective effect of enterolignans against colorectal adenomas. However, the protective effect could not be confirmed for colorectal carcinomas. Furthermore, our data do not support a protective role of lignans against the development of nonfatal myocardial infarction. At this point, there is not enough evidence to give recommendations regarding the consumption of foods rich in lignans.

It would be very interesting to study if the effect of enterolignans would be modified by type and/or size of the adenomas. In addition, the possible interaction with endogenous hormonal status in the development of colorectal cancer requires further investigation. Up to now, there are still very few data available on the pathways and mechanisms in which enterolignans and plant lignans may play a role. Relevant concentrations should be used to study their role.

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Summary

Lignans are biphenolic compounds that occur in foods of plant origin. Some plant lignans can be converted into the enterolignans, enterodiol and enterolactone, through a series of reactions mediated by the bacterial flora in the colon. Enterolignans are present in a range of biological fluids. It has been shown in vitro that they decrease, among others, the proliferation of breast tumor cells, colon tumor cells, and vascular endothelial cells, and influence the activity of steroid metabolizing enzymes. Furthermore, they possess antioxidant, antigenotoxic, and anti-angiogenic activity. Because of these activities, they may affect the development of cancer and coronary heart disease. Indeed the exposure to lignans (dietary intake and plasma/ urinary concentrations) has been associated with several cancers and cardiovascular diseases (*Chapter 1*). However, these studies have yielded inconsistent results. Studies on the relation between lignans and breast cancer risk were contradictory. No associations were observed with prostate cancers. For cancers other than breast and prostate too few studies have been performed to draw conclusions. No studies on colorectal cancer risk are published. Strong inverse associations were found for plasma enterolactone concentrations and the risks of coronary heart diseases, although only 2 out of 4 studies showed a statistical significant protective association.

Sufficient absorption from the diet is a prerequisite for its potentially protective role in cancer (except perhaps for colon cancer) and cardiovascular disease. This thesis describes studies to gain further insight in the bioavailability of plant lignans and into the relationship between plasma enterolignans and colorectal adenomas, colorectal carcinomas and myocardial infarction.

In order to do this, we developed a simple, rapid, and sensitive method for simultaneous quantification of both enterodiol and enterolactone in plasma at low concentrations applicable for the analysis of large numbers of samples (*Chapter 2*).

Absorption, metabolism and excretion

In *Chapter 3* we studied to what extent plant lignans are converted to enterolignans, absorbed, and excreted in humans. We performed a single dose study in humans with a purified plant lignan, secoisolariciresinol diglucoside. Enterodiol and enterolactone absorption started 8-10 hours after consumption of secoisolariciresinol diglucoside, which confirms the predominant role of microorganisms in the colon, and they were eliminated slowly. A substantial part (~40%) of enterolignans was excreted in urine, and thus at least 40% had been available in the blood circulation. The systemic exposure to enterolactone was approximately two times the exposure to enterodiol. This might be explained by enterohepatic circulation of enterodiol and enterolactone. However, because enterodiol will be oxidized into enterolactone in the colon, predominantly enterolactone will be re-absorbed. The measured mean residence times and elimination half-lives indicate that enterolignans will accumulate in plasma when consumed 2-3 times a day, and will reach steady state. As lignans are present in many foods this is very likely to happen.

Influence of food matrix

In the single dose study described above, secoisolariciresinol diglucoside was dissolved in water and consumed just before breakfast. Therefore, the conversion and absorption of enterolignans could not have been influenced by a food matrix. However, the extent of conversion and absorption of enterolignans might be different in the human diet, were plant lignans are part of a food matrix.

Flaxseed is a small hard-coated seed and contains high amounts of plant lignans. Although flaxseed is a relatively minor dietary component, it is increasingly being incorporated into a variety of food products, such as bread, muesli bars and breakfast cereals, or used as a supplement. We questioned whether lignans in whole seeds are accessible to bacteria in the colon. In a randomized crossover study twelve healthy subjects supplemented their diet with whole, crushed, or ground flaxseed for 10 successive days (*Chapter 4*). At the end of these periods plasma was drawn and analyzed. We found that the mean relative bioavailability of enterolignans from whole compared to ground flaxseed was 28%, whereas that of crushed compared to ground flaxseed was 43%. The availability of lignans from food products, such as breads, will improve substantially when whole seeds are replaced by crushed or ground seeds.

Determinants of plasma enterolignan concentrations

Plasma enterolignan concentrations (internal exposure) are not solely the result of the amount of plant lignans consumed, but also influenced by factors that have an effect on the bioavailability, such as the food matrix, as described above. In our single and multiple dose studies large variations in plasma and urinary enterolignan concentrations were observed between individuals, although they received the same amount of lignans. The most likely explanation for this observed variation is the difference in colonic microflora. In the colon most plant lignans are first converted to enterodiol and subsequently to enterolactone. As far as we know, matairesinol is the only plant lignan that can be directly converted to enterolactone. Some individuals had such low concentrations of enterolactone, even after supplementation with high dosages of plant lignans, that we wondered whether they were able to convert enterodiol to enterolactone (*Chapters 3 and 4)*. The central role of microorganisms is also illustrated by the effect of antibiotic therapy on enterolignan concentrations. We observed a substantial reduction of plasma enterolactone concentrations in subjects using antibiotic therapy in the year prior to blood sampling (*Chapter 6*). Interestingly, plasma enterodiol concentrations were not reduced in these subjects. This suggests that microorganisms that are capable to convert enterodiol are more susceptible to antibiotic use. To identify important determinants of plasma enterolignan concentrations, we studied the association between plasma enterolignan concentrations and dietary and lifestyle factors in an observational study (*Chapter 5)*. Major independent determinants of enterodiol and enterolactone plasma concentrations were intake of lignan rich foods, such as, whole grain products, nuts and

seeds, defecation frequency, and body mass index. The correlations between plasma enterolactone concentration and the intake of individual plant lignans, secoisolariciresinol, matairesinol, pinoresinol, and lariciresinol, ranged from 0.10 for secoisolariciresinol to 0.21 for matairesinol, and that for enterodiol ranged from 0.02 for secoisolariciresinol to 0.10 for pinoresinol (*Chapter 5*). The correlation between plasma enterolactone concentration and total intake of plant lignans was 0.18. These correlations are rather low compared to other biomarkers of dietary intake. For some markers in plasma, e.g. carotenoids and vitamin E, correlations with questionnaire measurements have been reported to be rather low (0.1- 0.4), whereas the correlation can be as high as 0.6 or 0.8 for vitamin C or linoleic acid. The low correlations we found are not surprising, taking into account that most biomarkers are the authentic compounds present in foods, whereas, enterolignans are metabolites of plant lignans, and undergo conversion to variable extents. The composition and activity of the microflora are the major factors in this metabolic conversion of dietary lignans to plasma enterolignans, as discussed before. Thus, in studies using plasma enterolignans as a marker of long-term exposure, data relevant to the activity of the microflora, e.g., recent use of antibiotic therapy, constipation, or defecation frequency, is essential.

Biological variation

In spite of the diet restrictions and the standardized intake of flax supplements we did observe high variations of plasma enterolignans within (24-38%) and between-subjects (46-98%) in our multiple dose study (*Chapter 4*). When we studied the within-subject variation in more detail, we found that the concentration of enterolactone decreased significantly during the day, leading to a higher day to day variation than that of enterodiol. This was surprising because the elimination half-life enterolactone was longer than that of enterodiol (*Chapter 3*), which would predict less variation. Possibly, absorption and distribution of enterodiol and enterolactone are differently affected during chronic supplementation leading to changes in elimination. Alternatively, the daily meal pattern may have influenced the availability of enterolignans. When flaxseed supplements are consumed with a heavy meal and before sleeping, the residence time in the colon will be longer, and conversion to enterolactone, the final metabolite, might increase. Thus, increased conversion during night might explain high concentrations of enterolactone in the morning.

In epidemiological studies biomarkers are used to make a distinction between individuals. Although enterolignan concentrations vary widely within subjects, variation between subjects is if even larger.

The intra class correlation coefficient (ICC) represents the proportion of variance in the measure explained by the between-subject variation. High ICC values (close to 1) represent excellent reproducibility, and distinction between individuals is feasible. The ICCs in our multiple dose study

were quite high (enterodiol: 0.86-0.95; enterolactone: 0.57-0.84). Our results and those of others indicate that plasma enterolactone and enterodiol are relatively good biomarkers (*Chapter 4)*.

In conclusion, we expect plasma enterodiol and enterolactone to be suitable biomarkers. Steady state plasma concentrations of enterodiol and enterolactone are achieved because plant lignans are present in many foods and beverages and eaten several times a day. And thus, one plasma sample will reflect exposure for a longer period. However, antibiotic use or a combination of fasting and laxative medication can have such a large impact on the microflora and the absorption of enterolignans in the colon that these treatments will decrease steady state concentrations, as discussed, and no longer reflect long-term exposure. This should be taken into account when using plasma enterolignans as measure of exposure.

Enterolignans and colorectal adenomas and carcinomas

The relation between plasma enterolignans and colorectal adenomas was studied in a case-control study with men and women aged between 18 and 75 years (*Chapter 6)*. Plasma enterodiol and enterolactone were inversely associated with first colorectal adenoma risk (odds ratio in highest quartile of plasma enterodiol: 0.53, *P* for trend = 0.01, and that for enterolactone: 0.63, *P* for trend = 0.09) after adjustment for age, sex, and antibiotic use. Although enterolactone plasma concentrations were 10-fold higher, enterolactone's reduction in risk was not statistically significant. Although colorectal adenomas are considered to be precursors of colorectal cancer, only about 5% of colorectal adenomas are estimated to become malignant. To further investigate the role of enterolignans on the development of colorectal cancer, we studied the relation between plasma enterolignans and colorectal carcinomas in a prospective study (*Chapter 7)*. No statistically significant associations between plasma enterodiol and enterolactone and risk of colorectal cancer were present (odds ratio in highest quartile of plasma enterodiol: 1.11, *P* for trend = 0.75, and that for enterolactone: 1.70, *P* for trend = 0.15) after adjustment for known dietary and lifestyle risk factors for colorectal cancer. However, the association between plasma enterolactone and colorectal carcinoma risk was increased in women, especially postmenopausal women, and in subjects with a high body mass index. This could mean that estrogen-related mechanisms might play a role. In current smokers a positive association was observed between plasma enterodiol and colorectal carcinomas. However, this was not consistent with plasma enterolactone concentrations.

In conclusion, we observed a substantial reduction in colorectal adenoma risk among subjects with high plasma concentrations of enterolignans, in particular enterodiol. However, high plasma enterodiol or enterolactone concentrations were not associated with reduced risk of colorectal cancer in a prospective study. More prospective studies or recurrence trials are needed to evaluate the role of enterolignans on the development of colorectal adenomas and cancer.

Enterolignans and cardiovascular diseases

The relation between plasma enterolignans and nonfatal myocardial infarction was studied in a prospective study (*Chapter 8*). No statistically significant associations between plasma enterodiol and enterolactone and risk of myocardial infarction were present (odds ratio in highest quartile of plasma enterodiol: 1.21, *P* for trend = 0.74, and that for enterolactone: 1.51, *P* for trend = 0.12) after adjustment for known risk factors for myocardial infarction. Our results do not support the hypothesis that high plasma enterodiol or enterolactone concentrations are associated with reduced risk of nonfatal myocardial infarction.

Conclusions

The main findings from these studies are summarized and discussed in *Chapter 9.* Plant lignans are converted to enterolignans, and a substantial part of enterolignans enter the blood circulation, and are subsequently excreted in urine. Enterolignans will accumulate in plasma when consumed 2-3 times a day. As lignans are present in many foods this is very likely to happen. The availability of lignans from flaxseed-containing food products, such as breads, will improve substantially when whole seeds are replaced by crushed or ground seeds. Besides the intake of plant lignans, use of antibiotic therapy, defecation frequency, and body mass index are independent determinants of plasma concentrations of enterolignans. In observational studies using biomarkers as exposure measures it is necessary to take these determinants into account.

Our data suggest a protective role of enterolignans against colorectal adenomas. However, the inverse association could not be confirmed for colorectal carcinomas. Furthermore, our data do not support a protective role of lignans against the development of nonfatal myocardial infarction. Defecation frequency and use of antibiotic therapy, both predictors of plasma enterolignan concentrations, were not measured in the studies regarding colorectal carcinomas and myocardial infarction. Therefore, misclassification of exposure might have attenuated the true associations between plasma enterolignans and risk of these chronic diseases. At this point, there is not enough evidence to give recommendations regarding the consumption of foods rich in lignans.

Deze samenvatting is geschreven voor niet
wetenschappelijk publiek

Samenvatting

Inleiding

Lignanen zijn stoffen die voorkomen in planten. Sommige van deze plantaardige lignanen kunnen door bacteriën in de dikke darm worden omgezet in de enterolignanen enterodiol en enterolacton. Er zijn aanwijzingen dat enterolignanen een aantal in mens en dier voorkomende enzymsystemen kunnen beïnvloeden. Hierdoor zouden ze de ontwikkeling van kanker en hart- en vaatziekten kunnen beïnvloeden. In enkele epidemiologische onderzoeken is inderdaad een verband aangetoond tussen blootstelling aan lignanen (gemeten d.m.v. de inname van plantaardige lignanen of d.m.v. de concentraties van enterolignanen bloed of urine) en verscheidene soorten kanker en hart- en vaatziekten (*Hoofdstuk 1*), maar de resultaten zijn niet eenduidig. Resultaten uit onderzoeken naar de relatie tussen lignanen en borstkanker waren tegenstrijdig. In onderzoeken naar prostaatkanker werd geen verband aangetoond en voor andere kankers zijn er tot nog toe te weinig onderzoeken uitgevoerd om daaruit harde conclusies te trekken. Onderzoeken naar de relatie tussen lignanen en dikke darmkanker waren tot nu toe nog niet gedaan. Tussen plasmaconcentraties van enterolignanen en het risico op coronaire hartziekten zijn inverse verbanden gevonden (dus hoe hoger de concentratie hoe lager het risico op de ziekte). Deze beschermende verbanden werden in 2 van de 4 tot nu toe uitgevoerde onderzoeken gevonden.

Willen enterolignanen een rol spelen in het voorkomen van kanker en hart- en vaatziekten dan is het noodzakelijk dat de lignanen worden opgenomen uit de voeding (behalve misschien voor dikke darmkanker). In dit proefschrift worden onderzoeken beschreven die verder inzicht geven in de biobeschikbaarheid (opname en uitscheiding) van lignanen en in de relatie tussen enterolignanen en dikke darmpoliepen, dikke darmkanker en hartinfarct. Om dit te kunnen doen hebben we een simpele en snelle analysemethode ontwikkeld, die enterodiol en enterolacton in plasma kan meten in zeer lage concentraties. Deze methode is geschikt voor het analyseren grote aantallen bloedmonsters (*Hoofdstuk 2*). In de observationele onderzoeken beschreven in dit proefschrift zijn de enterolignaanconcentraties steeds bepaald in plasma.

Opname, metabolisme en uitscheiding

In *Hoofdstuk 3* hebben we gekeken naar de hoeveelheid enterolignanen die wordt opgenomen en uitgescheiden na het eten van plantaardige lignanen. Dit hebben we gedaan in een interventiestudie bij mensen die eenmalig een bepaalde hoeveelheid gezuiverde plantaardige lignanen (secoisolariciresinol diglucoside) hebben gegeten. De opname van enterodiol en enterolacton begon 8-10 uur na de inname van het plantaardige lignaan. Deze late opname bevestigd de rol van de bacteriën in de dikke darm. Een behoorlijk deel (~40%) werd vervolgens langzaam uitgescheiden via de urine en was dus ook beschikbaar in het bloed. De blootstelling in het lichaam was twee keer groter voor enterolacton dan voor enterodiol. Dit kan mogelijk verklaard worden door de heropname van de enterolignanen. Via de gal komen de lignanen

nogmaals in de dikke darm terecht en kunnen daar opnieuw worden opgenomen. In de dikke darm kan enterodiol in enterolacton worden omgezet. Hierdoor zou er meer enterolacton kunnen worden opgenomen.

De gemiddelde verblijftijd en de halfwaardetijd (dat is de tijd die het kost om 50% van de aanwezige lignanen in het lichaam af te breken of uit te scheiden) van enterolignanen geven aan dat wanneer men 2-3 keer per dag lignanen eet, enterolignaanconcentraties in het lichaam zullen toenemen en een stabiel niveau zullen bereiken. Omdat lignanen voorkomen in veel verschillende producten is dit zeer aannemelijk.

Invloed van de voedselmatrix

In het hierboven beschreven onderzoek werd zuiver secoisolariciresinol diglucoside opgelost in water gebruikt. Deelnemers aten dit vlak voor hun ontbijt. Hierdoor kon de omzetting en opname van enterolignanen nauwelijks worden verstoord door de voedselmatrix. Wanneer lignanen worden gegeten via de 'gewone' dagelijkse voeding kan de voedselmatrix de mate van omzetting en opname van enterolignanen wel beïnvloeden.

Lijnzaad is een voedingsmiddel rijk aan plantaardige lignanen en heeft een zeer harde buitenkant. Hoewel lijnzaad een relatief klein onderdeel van onze dagelijkse voeding vormt, wordt het steeds vaker verwerkt in producten zoals meergranenbrood, mueslirepen en ontbijtgranen. Hierdoor kan het een belangrijke bijdrage leveren aan de totale inname van plantaardige lignanen. We vroegen ons af of plantaardige lignanen uit lijnzaad wel kunnen worden omgezet in enterolignanen, omdat de bacteriën in het lichaam misschien geen toegang hebben tot de plantaardige lignanen door de harde buitenkant van het zaad. Daarom hebben we een interventiestudie uitgevoerd waarbij twaalf gezonde personen drie maal 10 dagen lang lijnzaad supplementen aten; de zaden werden heel, gebroken of gemalen gegeten (*Hoofdstuk 4*). Aan het einde van iedere 10 dagen werd er bloed afgenomen en dit werd geanalyseerd. In dit onderzoek vonden we dat de biobeschikbaarheid van enterolignanen uit hele zaden slecht 28% was ten opzichte van gemalen zaden. Wanneer de zaden gebroken waren was de biobeschikbaarheid 43% ten opzichte van gemalen zaden. De beschikbaarheid van lignanen in producten zoals brood zal dus aanzienlijk verbeteren wanneer hele zaden worden vervangen door gemalen of gebroken zaden.

Determinanten van enterolignanen in plasma

Enterolactonconcentraties in plasma worden niet alleen bepaald door de hoeveelheid lignanen die iemand eet, maar kunnen ook worden beïnvloed door factoren zoals de biobeschikbaarheid en de invloed van de voedselmatrix, zoals hierboven beschreven. In onze interventiestudies varieerden de enterolignaanconcentraties in bloed en urine enorm, ook al aten de deelnemers evenveel plantaardige lignanen. De variatie was niet alleen groot tussen personen, maar ook

binnen personen. Een logische verklaring voor deze schommelingen is het verschil in samenstelling van dikke darmbacteriën. In de dikke darm worden de meeste plantaardige lignanen eerst omgezet in enterodiol en vervolgens in enterolacton. Tot nu toe is matairesinol de enige bekende plantaardige lignaan die direct in enterolacton kan worden omgezet. Sommige deelnemers hadden zulke lage concentraties van enterolacton, zelfs na het eten van veel plantaardige lignanen, dat we ons afvroegen of ze wel in staat waren om enterolacton uit enterodiol te vormen (*Hoofdstuk 3 en 4*). De centrale rol van dikke darmbacteriën blijkt ook uit het effect van antibioticagebruik op de enterolactonconcentraties. In mensen die in het voorgaande jaar antibiotica gebruikt hadden waren de enterolactonconcentraties veel lager dan bij mensen die dat niet hadden gebruikt (*Hoofdstuk 6*). Interessant is dat de enterodiolconcentraties nauwelijks waren beïnvloed. Dit geeft aan dat de bacteriën die enterodiol omzetten in enterolacton waarschijnlijk gevoeliger zijn voor het gebruik van antibiotica, dan de bacteriën die plantaardige lignanen omzetten in enterodiol.

Om belangrijke determinanten van enterolignaanconcentraties in plasma te bepalen hebben we de relatie tussen plasmaconcentraties en voedingsgewoonten en levensstijlfactoren bekeken in een observationeel onderzoek (*Hoofdstuk 5*). Belangrijke determinanten van enterodiol- en enterolactonconcentraties waren de inname van lignaanrijke voedingsmiddelen, zoals volkoren producten, noten en zaden en andere factoren, zoals de frequentie van ontlasten en de Body Mass Index (= lichaamsgewicht (kg)/ lengte (m)^2). De correlaties tussen enterolacton en de inname van de afzonderlijke plantaardige lignanen, secoisolariciresinol, matairesinol, pinoresinol en lariciresinol, varieerde van 0.10 voor secoisolariciresinol tot 0.21 voor matairesinol; die voor enterodiol varieerde van 0.02 voor secoisolariciresinol tot 0.10 voor pinoresinol (*Hoofdstuk 5*). De correlatie tussen enterolacton en de totale inname van plantaardige lignanen was 0.18. Deze correlaties zijn redelijk laag vergeleken met andere biomarkers voor inname van voedingsstoffen. Voor sommige biomarkers zijn de correlaties met voedingsvragenlijsten ook laag (0.1-0.4), zoals voor carotenoïden en vitamine E, maar voor bijvoorbeeld vitamine C en linolzuur zijn de correlaties veel hoger (0.6-0.8). De lage correlaties die wij vonden zijn niet zo verwonderlijk als we bedenken dat de meeste voedingsstoffen onveranderd in het bloed terecht komen, terwijl enterolignanen omzettingsproducten zijn van plantaardige lignanen, gevormd door bacteriën in de dikke darm. De samenstelling en activiteit van de darmflora zijn belangrijke factoren voor de mate van omzetting van plantaardige lignanen in enterolignanen, zoals eerder beschreven.

Samenvattend kunnen we stellen dat gegevens die de activiteit van de darmflora kunnen beïnvloeden, zoals recent gebruik van antibiotica, verstopping of frequentie van ontlasten belangrijk zijn in onderzoeken waarin enterolignanen in plasma worden gebruikt als biomarkers voor de langetermijnblootstelling.

Biologische variatie

In ons lijnzaadonderzoek zagen we, ondanks de dieetvoorschriften en de gestandaardiseerde inname van lijnzaad, een grote variatie in enterolignaanconcentraties zowel binnen (24-28%) als tussen personen (46-98%) (*Hoofdstuk 4*). Wanneer we de variatie binnen personen in meer detail bestudeerden, zagen we dat de concentratie van enterolacton gedurende de dag omlaag ging. Hierdoor was de variatie voor enterolactonconcentraties groter dan die voor enterodiol. Dit hadden we niet verwacht, omdat de halfwaardetijd van enterodiol juist korter is dan die van enterolacton (*Hoofdstuk 3*). Een kortere halfwaardetijd impliceert een kleinere variatie. Mogelijk heeft een frequentere consumptie van lignanen invloed op de opname- en distributieprocessen in het lichaam, zodat omzetting en uitscheiding in het lichaam anders verlopen dan na een eenmalige consumptie. Ook zou het dagelijks eetpatroon van invloed kunnen zijn op de beschikbaarheid van enterolignanen. Als plantaardige lignanen worden gegeten tijdens het avondeten en voor het slapen gaan zou de verblijftijd in de dikke darm langer zijn en zo de omzetting naar enterolacton, het eindproduct, verhogen. Een verhoogde omzetting tijdens de nacht zou de hoge concentraties in de ochtend kunnen verklaren.

In epidemiologische onderzoeken worden biomarkers gebruikt om onderscheid tussen mensen te kunnen maken. Hoewel enterolactonconcentraties binnen een persoon enorm kunnen verschillen, is het verschil tussen personen groter. De 'intra class correlation coefficient' (ICC) is een maat voor de spreiding, die wordt verklaard door de variatie tussen personen. Hoge ICC waarden (dicht bij 1) geven een goede herhaalbaarheid aan en het is dan mogelijk om goed onderscheid te maken tussen individuen. De ICCs in de interventiestudie met lijnzaad waren redelijk hoog (enterodiol: 0.86-0.95; enterolacton: 0.57-0.84). Deze resultaten en die van anderen geven aan dat concentraties van enterolacton en enterodiol in plasma relatief goede biomarkers zijn (*Hoofdstuk 4*).

Samenvattend kunnen we stellen dat we verwachten dat enterodiol en enterolacton bruikbare biomarkers zijn. Omdat plantaardige lignanen in veel voedingsmiddelen voorkomen en dus regelmatig en verspreid over de dag gegeten worden kunnen enterolignaanconcentraties een stabiel niveau bereiken. Eén plasmamonster kan dus de blootstelling voor een langere periode weergeven. Maar wanneer mensen antibiotica of laxeermiddelen hebben gebruikt of hebben gevast dan kan dat een zo grote impact hebben op de darmflora dat enterolignanen niet meer gevormd worden en de plasmaconcentraties niet langer een maat zijn voor de langetermijnblootstelling. Dit moet dus worden meegenomen als enterolignanen in plasma worden gebruikt als maat voor blootstelling.

Enterolignanen en dikke darmpoliepen en dikke darmkanker

De relatie tussen enterolignanen en dikke darmpoliepen (een voorstadium van dikke darmkanker) werd bestudeerd in een patiënt-controle-onderzoek bij mannen en vrouwen tussen de 18 en 75

jaar (*Hoofdstuk 6*). Enterodiol en enterolacton waren negatief geassocieerd met het risico op dikke darmpoliepen (odds ratio in het hoogste kwartiel van enterodiol: 0.53, *P* voor trend = 0.01, en dat voor enterolacton: 0.63, *P* voor trend = 0.09) na correctie voor leeftijd, geslacht en antibioticagebruik. Hoewel de concentraties van enterolactone ongeveer 10 keer hoger waren dan die van enterodiol was de risicoverlaging van enterolacton niet statistisch significant. Over het algemeen worden dikke darmpoliepen beschouwd als een voorstadium van dikke darmkanker, maar slechts ~5% van de darmpoliepen worden uiteindelijk ook kwaadaardig. Om de rol van enterolignanen in de ontwikkeling van dikke darmkanker verder te bestuderen hebben we in een prospectief epidemiologisch onderzoek gekeken naar de relatie tussen enterolignanen en dikke darmkanker (*Hoofdstuk 7*). We vonden geen statistisch significante verband tussen enterodiol of enterolacton en het risico op dikke darmkanker (odds ratio in het hoogste kwartiel voor enterodiol 1.11, *P* voor trend = 0.75, en dat voor enterolacton: 1.70, *P* voor trend = 0.15) na correctie voor bekende risicofactoren voor dikke darmkanker. Wel vonden we een verhoogd risico op dikke darmkanker bij vrouwen, speciaal bij vrouwen na hun menopauze, en bij mensen met een hoge BMI. Dit kan betekenen dat oestrogeengerelateerde mechanismen een rol zou kunnen spelen. Bij de groep huidige rokers werd een positieve associatie gevonden tussen enterodiol en dikke darmkanker. Dit was echter niet consistent met enterolacton.

Samenvattend kunnen we stellen dat we een aanzienlijke verlaging van het risico op darmpoliepen bij mensen met hoge enterolignaanconcentraties zagen, zeker bij die van enterodiol. Maar zowel enterodiol als enterolacton waren niet geassocieerd met een verlaagd risico op dikke darmkanker. Om de rol van enterolignanen in de ontwikkeling van dikke darmkanker te evalueren zijn meer prospectieve onderzoeken en herhalingsstudies nodig.

Enterolignanen en hart- en vaatziekten

De relatie tussen enterolignanen en niet-fataal hartinfarct werd bestudeerd in een prospectief onderzoek (*Hoofdstuk 8*). We vonden geen statistisch significante verbanden tussen enterodiol of enterolacton en het risico op een hartinfarct (odds ratio in het hoogste kwartiel voor enterodiol 1.21, *P* voor trend = 0.74, en dat voor enterolacton: 1.51, *P* voor trend = 0.12) na correctie voor bekende risicofactoren voor een hartinfarct. Onze resultaten weerleggen de hypothese dat hoge concentraties van enterodiol of van enterolacton het risico op een niet-fataal hartinfarct verlagen.

Conclusies

De belangrijkst bevindingen uit deze onderzoeken zijn samengevat en besproken in *Hoofdstuk* 9. Plantaardige lignanen worden omgezet in enterolignanen en een aanzienlijk deel van deze enterolignanen komt in de bloedcirculatie terecht en wordt vervolgens uitgescheiden via de urine. De concentratie enterolignanen neemt toe en bereikt een stabiel niveau wanneer men meerdere keren per dag lignanen eet. Omdat lignanen in veel verschillende producten voorkomen is dit

zeker te verwachten. De beschikbaarheid van lignanen uit lijnzaadbevattende producten, zoals broden, zal verbeteren wanneer hele zaden worden vervangen door gebroken of gemalen zaden. Naast de inname van plantaardige lignanen, zijn het gebruik van antibiotica, de frequentie van ontlasten en de BMI onafhankelijke determinanten van enterolignaanconcentraties in plasma. In observationele studies is het belangrijk om rekening te houden met deze determinanten wanneer enterolignaanconcentraties worden gebruikt als maat voor de langetermijnblootstelling.

Onze gegevens suggereren een beschermende rol van enterolignanen bij de ontwikkeling van darmpoliepen. Deze inverse associatie konden we echter niet bevestigen bij dikke darmkanker. Verder ondersteunen onze gegevens geen beschermende rol van lignanen op de ontwikkeling van een niet-fataal hartinfarct. De frequentie van ontlasten en het gebruik van antibiotica, beide voorspellers van enterolignaanconcentraties in plasma, waren niet gemeten in de onderzoeken naar dikke darmkanker en hartinfarcten. Daarom zou misclassificatie van de blootstelling de associatie tussen enterolignanen en het risico op deze ziekten kunnen hebben afgezwakt. Tot nu toe is er geen voldoende bewijs om aanbevelingen te doen ten aanzien van de consumptie van voedingsmiddelen die rijk aan lignanen zijn.

Dankwoord

Hartelijk dank!

Het boekje dat u zojuist van voor tot achter hebt doorgelezen eindigt met een dankwoord. Dit boekje is één van de hoogtepunten van mijn promotieonderzoek. Maar meer waarde nog hecht ik aan de fijne contacten met mijn collegae. Ik wil mijn waardering voor jullie uitspreken en jullie ongelofelijk bedanken!

Allereerst wil ik mijn beide promotoren en copromotor bedanken. Pieter, ik vond je een fijne promotor. Je maakte altijd ruim de tijd voor afspraken en bereidde die ook goed voor. Onze gesprekken waren altijd vruchtbaar en nooit ging ik de deur uit zonder nieuwe inzichten. Ik wil je bedanken voor het sparren en meedenken tijdens de verschillende fasen van het project en met name voor de inbreng van je epidemiologische kennis. Bedankt! Frans, steevast 5 minuten te laat en 10 minuten eerder weg, maar je gaf onze vergaderingen altijd kleur. Altijd klaar met kritische vragen. Veel van die 'eenvoudige' vragen leken lastig te beantwoorden en hebben mij bewuster leren kijken naar mijn onderzoek. Ook aan het einde, bij het schrijven van de discussie, heb je mij boven mijn onderzoek uitgetild. Leuk uitzicht! Met jouw kijk voorkwam je dat ik teveel in details verzandde. Bedankt! Peter, jij bent degene geweest die het intensiefst betrokken was bij mijn onderzoek. Van jou heb ik heel erg veel geleerd, eigenlijk teveel om op te noemen. Toch een poging. Heldere doelstellingen en duidelijke methodes waren altijd de inzet. Van jou heb ik het vak van 'wetenschapper' geleerd. Je bent een goede leermeester. Ik kon altijd bij je binnenlopen voor korte of lange vragen. Je was altijd nieuwsgierig naar de resultaten en enthousiast om verder te gaan. Bedankt ook voor het vertrouwen dat je me gegeven hebt. Dat motiveert mij om verder te gaan in de wetenschap. Bedankt!

Ilja, in de lijst van officiële begeleiders kom je niet voor, maar de praktijk is duidelijk anders geweest. Je bent gedurende mijn volledige aanstelling mijn dagelijkse begeleider geweest. Zeer betrokken bij het onderzoek en ook bij het schrijven van de wetenschappelijke publicaties. Je leerde me logische discussies op te bouwen en heldere conclusies te trekken. Kwaliteit heb jij me geleerd. Bedankt! Michel, zonder jou was ik nu nog steeds bezig geweest. Ik kan me voorstellen dat je het op den duur ook echt zat was, die duizenden monsters (voor de frisse lezer: max. 40 monsters per dag, dus reken maar uit). Ik ben blij dat je me hebt willen helpen. Ook je weekenden en avonden zijn er bij tijd en wijle aan gesneuveld omdat de MS weer 'vrij' was. Bedankt! Ivon, gelukkig was jij er ook. We zijn een hele tijd samen opgetrokken, want we begonnen tegelijk aan hetzelfde project. Je was mijn steun en toeverlaat en ik waardeer je vriendschap enorm. Gedeelde smart is halve smart. En straks ben jij aan de beurt.

Ivon, Vincent, Ilja, Peter en Marjolein, met jullie heb ik heel wat van de wereld gezien. Een klooster in Duitsland, een prachtige zaal in Vichy, een stamkroeg in Helsinki en ontbijten in San Fancisco, onvergetelijke momenten. Dini, Betty en Michel, bedankt voor jullie hand- en spandiensten. Tijdens de interventiestudies hebben jullie met heel wat droogijs rondgereden. Ook zijn de noordpoolexpedities naar het RIVM om niet te vergeten. Bedankt! Ivon, Vincent, Linette, Esther, Wendy en Armand, mede AIO's, alle anderen van onze groep, door jullie waren de jaren op het RIKILT een aangename tijd en een fijne plek om naar toe te gaan. Het was altijd erg gezellig. Bedankt voor jullie vriendschap en collegialiteit!

Tom Vree, fijn dat je coauteur wilde zijn. In korte tijd heb jij mij wegwijs gemaakt in de wondere wereld van farmacokinetische parameters. Bedankt! Ook Patrick Mulder, bedankt voor je lessen over de MS, jou bijdrage kwam goed van pas. Ellen, mijn komst naar de Universiteit werd steeds uitgesteld en is uiteindelijk kort maar krachtig geweest. Jij hebt me geholpen en mij in korte tijd wegwijs gemaakt in de POLIEP studie. Bedankt! Ook Petra, Mariken en Maureen wil ik bedanken. Ik kon altijd bij jullie binnen lopen met definitie- en SAS problemen, ook al hadden jullie het zelf erg druk. Kristel, mijn kamergenootje voor zeer korte tijd, je hebt er voor gezorgd dat de uurtjes op de vakgroep erg gezellig waren. Bedankt! Verder wil ik bedanken: Lucy en Joke en alle andere bloedprikkers, die er voor zorgde dat mijn interventiestudies vlekkeloos verliepen. Lucy, bedankt voor het pionierswerk met de Venflon en de overnachtingen in ziekenhuis 'Gelderse Vallei'. Je moet er wat voor overhebben. Els bedankt voor je ondersteuning tijdens de supplementen-studies. Zonder proefpersonen geen onderzoek. Jullie hebben veel moeten doorstaan. Zelfs tijdens jullie slaap werden jullie verstoord voor de wetenschap. Jullie hebben je voorbeeldig gedragen. Bedankt!

Bas, je begeleidde mij tijdens mijn verblijf op het RIVM. Dank je voor het zorgvuldig doornemen van mijn manuscripten en je fijne samenwerking! Jolanda Boer en Hendriek Boshuizen bedankt voor jullie adviezen. Jan van der Laan, bedankt voor je voorbereidingen, zowel voor de toegang tot de bloedmonsters als voor de toegang tot de juiste directories. Bedankt!

Verder wil ik ook alle andere collegae op de vakgroep Humane Voeding en het RIVM bedanken. Ook al waren mijn aanwezige dagen op een paar handen te tellen, ik wil jullie bedanken voor de gezellige lunches en de koffiepauzes bij het kopieerapparaat. Bedankt! Brian en Martinet, bedankt voor de uurtjes in de auto. Ook wil ik alle ICT'ers op het RIKILT (Jan en Ruud) en op het RIVM (Hans) bedanken voor alle digitale oplossingen. Bedankt!.

Ivon en Anneke, fijn dat jullie mijn paranimfen willen zijn!

Natuurlijk wil ik ook mijn familie en mijn vrienden bedanken. Ik wil jullie bedanken omdat jullie er altijd voor mij zijn. En dat is al genoeg. Dank voor jullie luisterend oor, vriendschap en de nodige afleiding en nog veel meer.

Sjoerd, ook jou wil ik hier bedanken. Dank voor je liefde, je rust en je vertrouwen. Je staat altijd voor me klaar. Ik hoop binnenkort weer wat meer tijd te hebben voor jou en Loïs. Daar gaan we van genieten!

Bovenal wil ik mijn hemelse Vader bedanken voor de rust en liefde die Hij mij iedere dag geeft. Dag aan dag draag U mij. *"HEER, hoog als de hemel is uw liefde, tot in de wolken reikt uw trouw"*, psalm 36:6.

Anneleen

About the author

ABOUT THE AUTHOR

Anneleen Kuijsten was born on 2nd April 1975 in Wageningen, The Netherlands. After completing secondary school in 1993 (VWO, RSG Enkhuizen), she studied 'Human Nutrition' at Wageningen University. As part of that study she conducted one research project at the chair group of 'Human and Animal Physiology' and two projects at the division of 'Human Nutrition and Epidemiology'. In her first MSc project she studied the effect of arginine vasopressin on the stress-induced hormone surges in rats. For her second MSc thesis she went to Kenya to participate in a project that studied the effect of iron and malaria prophylaxis supplementation to control anemia in children aged 2-36 months. In her final MSc thesis she investigated the public health relevance of hepatitis B virus infection and peanut butter intake with respect to hepatocellular carcinoma in a case-control study (Sudan). She obtained her MSc degree in September 2000. In 2001 Wageningen University appointed her as a PhD-fellow to conduct research on the bioavailability of enterolignans and their relation to several chronic diseases, as is described in this thesis. This project was carried out at RIKILT - Institute of Food Safety in Wageningen, at the National Institute of Public Health and Environment (RIVM) in Bilthoven, and at the division of Human Nutrition, Wageningen University. During this period, she attended several courses and conferences.

PUBLICATIONS

- **Kuijsten A**, Arts ICW, Hollman PCH, van 't Veer P, Kampman E. Plasma enterolignans are associated with lower colorectal adenoma risk. *Cancer Epidemiology Biomarkers & Prevention* 2006; 15:1132-1136.
- **Kuijsten A**, Arts ICW, van 't Veer P, Hollman PCH. The relative bioavailability of enterolignans in humans is enhanced by milling and crushing of flaxseed. *Journal of Nutrition* 2005; 135:2812-2816.
- **Kuijsten A**, Buijsman MNCP, Arts ICW, Mulder PPJ, Hollman PCH. A validated method for the quantification of enterodiol and enterolactone in plasma using isotope dilution liquid chromatography with tandem mass spectrometry. *Journal of Chromatography B* 2005; 822:178-184.
- **Kuijsten A**, Arts ICW, Vree TB, Hollman PCH. Pharmacokinetics of enterolignans in healthy men and women consuming a single dose of secoisolariciresinol diglucoside. *Journal of Nutrition* 2005; 135:795-801.
- Omer RE, **Kuijsten A**, Kadaru AM, Kok FJ, Idris MO, El Khidir IM, van 't Veer P. Population-attributable risk of dietary aflatoxins and hepatitis B virus infection with respect to hepatocellular carcinoma. *Nutrition and Cancer* 2004; 48:15-21.
- Verhoef H, West CE, Nzyuko SM, de Vogel S, van der Valk R, Wanga MA, **Kuijsten A**, Veenemans J, Kok FJ. Intermittent administration of iron and sulfadoxine-pyrimethamine to control anaemia in Kenyan children: a randomised controlled trial. *The Lancet* 2002; 360:908-914.

TRAINING AND SUPERVISION PLAN

Discipline specific courses

- *Nutritional and lifestyle epidemiology [VLAG], Wageningen [NL], 2001*

- *HPLC-course 2 [Varian], Etten-Leur [NL], 2001*

- *Pharmacokinetics [LACDR], Oss [NL], 2001*

- *Epidemiology course [by K. Rothman; RIVM], Bilthoven [NL], 2002*

- Principles of epidemiologic data analysis [Erasmus Summer School], Rotterdam [NL], 2004

- Regression analysis [NIHES], Rotterdam [NL], 2004

National and international conferences

- Meetings NWO Nutrition, Arnhem [NL], 2001-2005

- Meetings NWO 'Verantwoorde Voeding', Utrecht [NL] 2002-2006

- Symposium 'Biomoleculair onderzoek voor gezond en veilig voedsel', Wageningen [NL], 2001

- International Conference '40-year results of the Seven Countries Study', Eefde [NL], 2002

- 3rd Thematic day 'Food safety', Wageningen [NL], 2002

- Congress 'Phytochemistry and Biology of Lignans', Bornheim-Walberberg, [DE], 2003

- 1st International Conference on Polyphenols and Health, Vichy [FR], 2003

- 12th International Conference on Polyphenols, Helsinki [FI], 2004

- 2nd International Conference on Polyphenols and Health, Davis [US], 2005

- European Congress of Epidemiology, Utrecht [NL], 2006

General courses

- PhD week [VLAG], Bilthoven [NL], 2001

- Scientific writing [CENTA], Wageningen [NL], 2003

- Talent classes [NWO], Bilthoven [NL], 2004

- Career perspectives [Wageningen Graduate Schools], Wageningen [NL], 2005

Optional courses and activities

- PhD Study Tour to Switzerland, Italy, and Germany, 2001

- Preparation PhD research proposal, Wageningen University [NL], 2001-2005

- Discussion groups 'Brainstorm' and 'Epidemiology' at the division of Human Nutrition, Wageningen University [NL], 2001-2005

COLOFON