Cholesterol lowering effects of soy protein, and how denatured protein may increase the risk for cardiovascular disease.

by

Lars Henrik Høie, M.D.

Domus & Medicus
Pilestredet Park 7
N-0176 Oslo, Norway

June 2010

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1078

ISBN 978-82-8264-053-4

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

Produced in co-operation with Unipub.
The thesis is produced by Unipub merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

This thesis is dedicated to:

Lisbet, Celine & Nicola

Else & Kåre

Acknowledgements

I am very grateful for the important feedback and comments to this thesis from:
Professor Dag Bruusgaard at the University of Oslo
Professor Anders Goksøyr at the University of Bergen
Nicole Armbruester for searching literature.

To the medical doctors who have made their important contributions to the clinical research presented in this material at:
Aarhus and Gentofte University Hospitals (Denmark)
Helsinki University Hospital (Finland)
Karolinska Institutet (Stockholm, Sweden)
Potsdam & Humbolt University Hospitals (Berlin, Germany).

To the scientists involved in the protein based product developments at:
Prinsen bv (Holland)
Campden & Chorleywood (England)
Norwegian University of Life Sciences (Norway)
TetraPak (Lund, Sweden).

Contents

4 Summary

The Food and Drug Administration (FDA) in the U.S. approved a health claim for soy protein in October 1999, i.e. that the intake of 25 g soy protein per day, or 6,25 g soy protein per serving, may prevent coronary artery disease by lowering cholesterol. The Joint Health Claims Initiative (JHCI) approved a similar health claim in the U.K. in 2002.

We have in this thesis confirmed that soy protein significantly lowers LDL, total cholesterol and other risk factors for cardiovascular disease (CVD), and that adding soy phospholipids and soy fiber doubles these lipid lowering properties. Surprisingly, we have documented that processing of soy protein using ultra heat treatment (UHT) not only ruins the cholesterol lowering effect of soy protein, but significantly increases blood lipids, with an approximately 20% increase in both LDL and total cholesterol after 8 weeks intake of 25 g soy protein per day. The same increases in serum lipids were found in the UHT treated milk protein based placebo beverage. UHT treated proteins are thereby potentially atherogenic, and may increase the risk for CVD.

By showing that denatured proteins may increase the risk for CVD, we have found a parallel with regards to fat processing resulting in trans fatty acids, which have been documented to increase the risk of CVD.

Our research has shown a potential increased CVD risk using high temperature treated protein beverages, such as UHT treated soy milk, milk & flavoured milk protein drinks, and soy protein based beverages - products which are used regularely by large consumer groups. Our results may thereby have important implications for consumers, legislators and for the food and beverage industries. These new and unexpected results were supported by a study where we developed a new non-denatured soy protein, which doubled serum lipid reductions compared to a conventional soy protein. The most likely explanation for our findings is that a) high temperature denatures proteins, and that b) bioactive peptides from digestion are altered, thereby becoming atherogenic.

Legislators including the FDA and JCHI may eventually revise their health claims for soy protein, and make these claims more specific, based on clinical documentation from manufacturers of soy protein regarding to lipid reductions. Ethanol is e.a. used by some of the soy producers in the processing of soy protein, and since alcohol denatures protein, ethanol processed soy proteins may also turn out to be atherogenic. If detrimental to health, possible health warnings may be considered by health authorities if UHT has been used in the processing of protein.

The studies in this thesis have been conducted in cooperation with medical doctors and other scientists at university hospitals in Aarhus, Copenhagen, Helsinki, Stockholm and Berlin, while the protein based product developments have been conducted in Holland, England, Norway and Sweden.

5 Introduction

According to the WHO, an estimated 17.5 million people died from cardiovascular disease (CVD) in 2005 worldwide, representing 30% of all deaths globally. Of these deaths, 7.6 million were due to heart attacks and 5.7 million were due to stroke.

Hypercholesterolemia, elevation of cholesterol, particularly low-density lipoprotein (LDL) levels in the blood, is known to increase the risk of CVD (1), which include coronary heart disease (heart attacks), cerebrovascular disease (stroke), peripheral artery disease and heart failure.

Increased lipid levels can lead to a build-up of fat deposits in the arterial walls. The blood vessels become narrower and less flexible, with a reduction of blood flow to the heart and brain. Blood vessels may thereby be blocked by blood clots, causing an insufficient supply of blood to the heart or the brain, which leads to heart attack or stroke (1).

Often, the underlying blood vessel disorders show no symptoms. A heart attack or stroke may be the first warning signs of the disease. According to EUROPSIRE, the prevalence of elevated total cholesterol (≥ 194 mg/dL,5.01 mmol/L) in patients with established coronary heart disease is 58.5% (2).

Since a 10% reduction in total cholesterol lowers the risk of cardiovascular events by up to 50% for older subjects with mild-to-moderate hypercholesterolemia, cholesterol lowering therapies are important (3, 4). LDL (low-density lipoprotein) cholesterol is regarded as the major atherogenic lipoprotein and has been identified by the NCEP (National Cholesterol Education Program) to be the primary target for cholesterol-lowering therapy (5).

Aside from genetic factors, which are regarded as the primary cause of hypercholesterolemia, food patterns also contribute to its prevalence. Dietary behavior has changed considerably in recent years. It has been demonstrated, that the average consumption of starch and complex carbohydrates has decreased, whereas the intake of fat, *trans* fatty acids and saturated fat, as well as simple carbohydrates has increased (6). Increased consumption of snacks and fast food, typically containing a high percentage of fat, has been observed in recent years. A sedentary lifestyle also contributes to a greater prevalence of elevated blood cholesterol levels.

The economic implications of hypercholesterolemia, increasing the risk of CVD, are important. In Europe, CVD is estimated to cost the EU 192 billion € per year (7). Compared to this the 2008 EU budget allocation to health care is 129.1 billion €. 57% of expenses related to CVD in the EU are due to direct health care cost, 21% to productivity loss and 22% to informal care for people with CVD.

Treatment of elevated plasma cholesterol concentrations includes dietary intervention, exercise programs, and drug treatment.

Dietary changes are the most important non-pharmacological measures to reduce serum lipid levels. Changed dietary patterns have an influence on modifying blood cholesterol le-

vels, which has been documented in many clinical investigations. Reducing the intake of saturated fat and trans fatty acids are regarded the most successful dietary approach.

Several studies have shown the beneficial effects of cholesterol-lowering foods, which are becoming increasingly important in the treatment of hypercholesterolemia and the reduction of CVD risk (8, 9). The intake of dietary supplements may be an alternative in individuals with moderately increased lipid values, which cannot be normalized by lifestyle modifications such as dietary changes and exercise. Soy-based supplements play a role in this context. Soy protein has been shown to reduce the risk of heart disease by lowering serum cholesterol levels. The U.S. Food and Drug Administration (FDA) approved the following health claim in 1999:"Diets low in saturated fat and cholesterol that include 25 g of soy protein per day may reduce the risk of heart disease."

A meta-analysis of 38 clinical trials concluded that the consumption of soy protein significantly decreases concentrations of total cholesterol, LDL cholesterol and triglycerides in the blood (11). Soy-based products have documented significant lipid reductions based on a composition of soy components such as isolated soy protein with high-standardized levels of isoflavones, cotyledon soy fiber and soy phospholipids (12, 13).

The mechanisms of the lipid-lowering effects of soy protein are still unclear, although there are possible explanations that need further investigation. Nevertheless, the use of soy-based diets has shown good efficacy and tolerability. A study investigating the willingness of people to consume soy foods for lowering cholesterol revealed that most of the participants were willing to consume soy as part of lifestyle modification to prevent CVD (14).

Many different soy products are currently on the market, and their cholesterol lowering properties may differ. The aim of this thesis is to examine the efficacy and safety of soy protein based products with regards to lowering lipid levels in the blood. Special focus is given to exploring the impact of processing on the cholesterol lowering properties of soy protein

6 Hypercholesterolemia

6.1 Definition of hypercholesterolemia

Hypercholesterolemia is defined as elevated levels of cholesterol. Cholesterol is a lipid which, together with cholesterol esters, phospholipids, and triglycerides, is transported in the blood as part of larger molecules called lipoproteins. They can be assigned to different categories and the five major families of lipoproteins are low-density lipoproteins (LDL), high-density lipoproteins (HDL), very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), and chylomicrons (15).

LDL cholesterol normally makes up 60-70% of total serum cholesterol and contains a single apolipoprotein, apo B-100 (apo B) that surrounds the fatty acids, keeping them soluble in the aqueous environment. In general, LDL transports cholesterol and triglycerides from the liver to peripheral tissues and regulates cholesterol synthesis at these sites. LDL cholesterol is well known to increase the risk of coronary heart disease (CHD) because it can be retained in arteries by arterial proteoglycans starting the formation of arterial plaques (16). Increased levels are associated with artherosclerosis, and thereby heart attack, stroke, and peripheral vascular disease.

In contrast, HDL cholesterol is inversely correlated to the risk of CVD. HDL normally carries around 20-30% of total serum cholesterol. Apo A-I and apo A-II are the major apolipoproteins of HDL (17).

Another lipoprotein subclass is VLDL, which is rich in triglycerides. They account for 10-15% of total serum cholesterol and are assembled in the liver from cholesterol and apolipoprotein. In the bloodstream it is converted to LDL, therefore it is a precursor of LDL. The intermediate lipoproteins IDL, reside between VLDL and LDL, but are included in LDL measurements in clinical practice (15, 18).

The fifth class of blood lipoproteins is chylomicrons, which are triglyceride-rich lipoproteins formed in the intestine from dietary fat and which appear in the blood after a fat-containing meal (18).

Table 1 presents different types of hypercholesterolemia classified according to Fredrickson (19), based on the pattern of lipoproteins on electrophoresis or ultracentrifugation. This classification system has been adopted by the WHO.

Hypercholesterolemia does not lead to specific symptoms and is usually discovered during routine medical tests or examinations for atherosclerotic cardiovascular disease. Sometimes xanthomas, deposits of cholesterol, can be found in individuals with hereditary forms of the disorder or in people with high levels of cholesterol. Deposits are especially found around the eyes or along the achilles tendon (20).

Table 1: Fredrickson Classification of Lipid Disorders (19)

Type	Associated clinical disorders	Labs description
Type I	decreased lipoprotein lipase (LPL) or altered ApoC2	elevated chylomicrons
Type IIa	LDL receptor deficiency	elevated LDL only
Type IIb	decreased LDL receptor and increased ApoB	elevated LDL and VLDL and triglycerides
Type III	defect ApoE synthesis	increased IDL
Type IV	increased VLDL production and decreased elimination	increased VLDL
Type V	increased VLDL production and decreased lipoprotein lipase	increased VLDL and chylomicrons

It is well documented that lipid disorders are associated with an increased risk of coronary heart disease, stroke and peripheral arterial disease. This is because excess cholesterol contributes to atherosclerosis, which eventually leads to CVD. Since LDL cholesterol is regarded as a high risk factor for CVD, LDL has been labeled “bad” cholesterol (16). In contrast, increased levels of HDL cholesterol are commonly assumed to reduce the risk of CVD and is therefore regarded as “good” cholesterol (21, 22).

6.2 Aetiology of hypercholesterolemia

There are both primary (genetic) and secondary causes of elevated cholesterol levels in the blood. Primary causes are single or multiple genetic mutations that result in an over production or defective clearance of triglycerides and LDL cholesterol, or in an underproduction or excessive clearance of HDL. Primary lipid disorders are common in patients who develop physical signs of hypercholesterolemia such as xanthomas, onset of premature atherosclerotic disease (age<60), a family history of atherosclerosis, or elevated serum cholesterol >240 mg/dL (>6.20 mmol/L) (23). In children, primary disorders are the most common cause (23).

In contrast, secondary causes contribute to most cases of hypercholesterolemia in adults.

The most important secondary cause in developed countries is a sedentary lifestyle with excessive dietary intake of saturated fat, cholesterol, and trans fatty acids (23).

Other secondary causes are diabetes mellitus and risk factors in the metabolic syndrome, nephrotic syndrome, hypothyroidism, anorexia nervosa, Zieve’s syndrome, excessive alco-

hol intake and drugs such as thiazides, β-blockers, retinoids, active antiretroviral agents and glucocorticoids (23).

Among these factors, diabetes is of special concern, because diabetics tend to have an atherogenic combination of increased levels of triglycerides, elevated small, dense LDL fractions and low HDL. One explanation may be that this combination of lipoproteins is caused by obesity and/or poor control of diabetes, which increases circulating fatty acids, leading to an increased hepatic VLDL production. Triglyceride-rich VLDL transfers triglycerides and cholesterol to LDL and HDL cholesterol, initiating the formation of triglyceride-rich, small dense LDL cholesterol and clearance of triglyceride-rich HDL cholesterol. Diabetic related hypercholesterolemia is often associated with increased caloric intake and physical inactivity. A high prevalence of hypercholesterolemia in insulin-dependent diabetes mellitus has been documented in several clinical studies (24, 25).

6.3 Treatment of hypercholesterolemia

In most western and industrialized countries, increased lipid levels are widespread. Data based on the Third National Health and Nutrition Examination Survey (NHANES III) showed that approximately 28% of the US population, or more than 50 million adults over 20 years of age suffer from hypercholesterolemia which warrants treatment (5). With respect to specific subpopulations, it was found that approximately 29% of caucasians, 24% of blacks, and 18% of hispanics were qualified for cholesterol-lowering treatment. The prevalence varies slightly between women and men: 30% of men and 26% of women need lipid-lowering treatment according to the National Cholesterol Education Program Adult Treatment Panel II (NCEP ATP II) criteria (26).

Another survey in Argentina revealed that one third of the population age 20 or older is affected by hyperlipidemia and hypertension (27).

Treatment of hypercholesterolemia depends on several factors such as lipid levels, individual heart risk factors and general health.

A desirable total cholesterol level is usually less than 200 mg/dL (5.17 mmol/L) (4). A level of 200 to 239 mg/dL (5.17-6.18 mmol/L) is borderline high, while a value greater than equal to 240 mg/dl (6.20 mmol/L) is considered to be high. Tables 2 and 3 summarize the classifications of lipids in blood.

The most important factor in treating lipid disorders is to reduce LDL cholesterol levels. Initially, it is reasonable to attempt to lower cholesterol levels by reducing dietary cholesterol intake.

Table 2: ATP III Classification of total cholesterol and LDL cholesterol (5)

Total Cholesterol in mg/dL (mmol/L)		**LDL Cholesterol in mg/dL (mmol/L)**	
		< 100 (< 2.58)	Optimal
< 200 (< 5.17)	Desirable	100-129 (2.58-3.33)	Near optimal/above optimal
200-239 (5.17-6.18)	Borderline High	130-159 (3.36-4.11)	Borderline High
≥ 240 (≥ 6.20)	High	160-189 (4.13-4.88)	High
		≥ 190 (≥ 4.91)	Very High

Table 3: ATP III Classification of serum triglycerides and HDL cholesterol (5)

Triglyceride Category in mg/dL (mmol/L)		**HDL Cholesterol in mg/dL (mmol/L)**	
< 150 (< 1.70)	Normal		
150-199 (1.70-2.25)	Borderline High	< 40 (< 1.03)	Low
200-499 (2.26-5.64)	High	≥ 60 (≥ 1.55)	High
≥ 500 (≥ 5.65)	Very High		

The American Heart Association (AHA) and other standard guidelines recommend the following measures (28):

- A diet that lowers caloric intake and reduces total fat and cholesterol intake
- Limiting daily fat intake to no more than 30% of total calories
- Cholesterol intake should be less than 300 mg daily
- Carbohydrate intake should total 55% to 60% of total daily calories
- Avoiding foods high in sugar
- Fiber intake from foods, not supplements, should total 25 to 30 grams daily
- Use of mono-saturated oils such as olive or canola oil
- Intake of five or more servings of fruit and vegetables daily
- Additional intake of antioxidants, vitamin C, beta-carotene and vitamin E in recommended amounts

A meta-analysis of large international nutrition studies revealed a mean decrease of cholesterol of only 2% with this dietary approach (29). Therefore, other lifestyle changes such as regular exercise (30), smoking cessation (31, 32, 33) and reduction of excessive alcohol intake should preferably also be implemented. Interestingly, soy protein is not mentioned, in spite of the approved health claim by the FDA.

It is important to identify and treat any potential underlying medical problems, such as hyperthyroidism and diabetes, which may cause or contribute to hypercholesterolemia.

If optimal lipid levels cannot be achieved by a dietary approach, the administration of lipid-lowering drugs should be considered. Such drugs are statins, bile acid sequestrants, fibrates and niacin (vitamin B3). Each category of medication has its own mode of action, cost and efficacy.

The most powerful lipid-lowering drugs are statins, which reduce the production of cholesterol in the liver. They work by inhibiting hydroxymethylglutaryl CoA reductase, which functions as a key enzyme in cholesterol synthesis (34). Using statins can reduce LDL cholesterol concentrations by as much as 30 to 50% (35), and is widely used.

Bile acid sequestrants, including colesevelam, colestipol, and cholestyramine, bind bile acids in the intestine and thereby reduce dietary cholesterol absorption. Bile acid sequestrants are mainly prescribed for the treatment of mild to moderate LDL cholesterol elevation, and are normally used with statins or with niacin (36, 37).

Niacin is a B-vitamin which lowers levels of both VLDL and LDL cholesterol and raises HDL cholesterol level. Fibrates, another category of lipid-lowering drugs, favorably alter lipid metabolism, resulting in lower triglyceride concentrations and higher HDL cholesterol (38).

All lipid-lowering drugs can be regarded as being effective, but their use may be accompanied by adverse events, such as muscle weakness, bloating, nausea, cramping and liver damage (39, 40, 41, 42).

Alternatives to such medications are dietary interventions such as restriction of the intake of saturated fatty acids (43, 44, 45) and *trans* fatty acids (46, 47), and increased intake of dietary fiber and protein-rich legumes, preferably soybeans (48, 49). Plant-sterols are also an effective dietary adjuvant (50, 51). In contrast to statins, sterols act by blocking the absorption of cholesterol in the intestine (52, 53). It has been shown, that the combination of sterols with other dietary components reduce elevated blood lipid levels, thereby providing an alternative treatment to medication (54).

The National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) guidelines are the most common reference when deciding who should be treated for hyperlipidemia (5).

Table 4, gives an overview of risk categories with corresponding LDL levels and medical treatment and goals.

Table 4: National Cholesterol Education Program Adult Treatment Panel III Guidelines for Treatment of Hyperlipidemia, CAD = coronary artery disease (5)

Risk Category	Begin Lifestyle Changes If:	Consider Drug Therapy If:	LDL Goal
High: CAD or CAD equivalents 10-yr risk > 20%	LDL ≥ 100 mg/dL (≥ 2.58 mmol/L)	LDL ≥ 100 mg/dL (≥ 2.58 mmol/L) (drug optional if < 100 mg/dL (< 2.58 mmol/L))	< 100 mg/dL (< 2.58 mmol/L); < 70 mg/dL (< 1.81 mmol/L) optional
Moderate high: ≥ 2 risk factors with 10-yr risk 10 to 20%	LDL ≥ 130 mg/dL (≥ 3.36 mmol/L)	LDL ≥ 130 mg/dL (≥ 3.36 mmol/L)	< 130 mg/dL (< 3.36 mmol/L); < 100 mg/dL (< 2.58 mmol/L) optional
Moderate: ≥ 2 risk factors with 10-yr risk < 10%	LDL ≥ 130 mg/dL (≥ 3.36 mmol/L)	LDL ≥ 160 mg/dL (≥ 4.13 mmol/L)	< 130 mg/dL (< 3.36 mmol/L); < 100 mg/dL (< 2.58 mmol/L); optional
Lower: 0-1 risk factor	LDL ≥ 160 mg/dL (≥ 4.13 mmol/L)	LDL ≥ 190 mg/dL (≥ 4.91 mmol/L) (drug optional if 160-189 mg/dL (4.13-4.88 mmol/L))	< 160 mg/dL (< 4.13 mmol/L)

Recent studies show that awareness and acceptance of the guidelines is high among practitioners, but implementation can be improved (56).

Another important issue is the compliance of patients during treatment of hyperlipidemia. Patients who need lipid-lowering therapy are likely to need it long-term, in many cases for life. However, many patients do not adhere to the recommended lifestyle modification or prescribed medication regimen (57). Hypercholesterolemia often does not have any symptoms and is often discovered during routine examinations (58, 59). Hence, people who need lipid-lowering therapy must be detected, e.g. by screening programs.

Several screening guidelines have been developed, which differ mainly in their recommendations concerning age to start screening, the best time interval between testing, and the age at which screening may stop.

The United States Preventive Task Force recommends the following:

- Lipid screening should start at age 35 in men and at age 45 in women.
- Screening is recommended for men aged 20 to 35 and for women aged 20 to 40 in patients with diabetes, a family history of cardiovascular disease before the age of 50 in male relatives or age 60 in female relatives, a family history of hyperlipidemia, or multiple coronary heart disease risk factors, such as smoking or hypertension.
- Screening should include total cholesterol and HDL cholesterol levels, and can be measured on non-fasting and fasting samples.
- The optimal time interval between screenings is uncertain; reasonable options in-

clude every five years, with a shorter interval for those with high-normal lipid levels and longer intervals for low-risk individuals with low or normal levels.

- The age to stop screening has not been established.
- Screening may be appropriate in older people who have never been screened, but repeated screening is less important in older people because lipid levels are less likely to increase after the age of 65.
- Treatment decisions should take into account the overall risk of heart disease, rather than lipid levels alone.

7 Soy products

Soybeans (*Glycine max*) are native to Asia, but today this plant is cultivated and consumed world wide. Its nutritional value is good with a high protein content of 40%, including all essential amino acids, and an excellent fat profile, as well as a high content of isoflavones which are plant estrogens (on average 1.2 mg per gram protein), prebiotic and satiety promoting fibers (23%), minerals (particulary calcium and magnesium), vitamins (particularly B1, B2, E and folic acid) (60, 61), and other bioactive components (saponins, trypsin inhibitors).

In recent years, the use of soy-based diets has become very popular for improving lipid profiles. The beneficial effects and good tolerability of soy-based products are well known (48, 62-64). The lipoprotein lowering effect of soy protein is of particular importance, and has been demonstrated in several clinical studies (65-72). The superiority of soy protein regarding the capability to reduce blood cholesterol levels compared to animal derived proteins has also been documented (73, 74).

The lipid lowering effect of soy is assumed to reduce the risk of CHD (75). A meta-analysis, including 38 clinical trials, has shown that supplementation with soy-based preparations led to average reductions of total and LDL cholesterols of 9.3% and 12.9 %, respectively (11).

This is supported by the US Food and Drug Administration (FDA) which in 1999 approved health claims for products providing a daily dosage of soy protein of at least 25 g, or 6.25 g/meal (76), and by the Joint Health Claims Initiative (JHCI) which approved similar health claims in the UK in 2002.

Furthermore, soy has also been shown to increase insulin sensitivity (72) and to prevent the development of diabetes (77).

Using soy-based diets is thereby a promising approach to treat elevated blood lipid levels, gaining acceptance in the general population. This is supported by a study investigating the willingness of people to consume soy foods to lower cholesterol. The findings revealed that most of the participants were willing to consume soy preparations as part of their lifestyle modification to prevent CVD (14).

8 Lipid lowering effect of soy

The active components in the soybean have not yet been fully elucidated and the mechanism of the lipid-lowering activity of soy protein remains unclear. It is likely that soy protein, peptides and isoflavones may all affect lipid metabolism and gene expression (78).

There are also other soy ingredients such as cotyledon fibers (79, 80) and phospholipids (81-84), which reduce serum lipid concentrations. Soy preparations vary not only in their content of soy ingredients, they also contain soy protein of varying structure and solubility, which may influence efficacy.

Potential mechanisms of action include the influence of soy on the endocrine system via the intestinal absorption of bile acids and dietary cholesterol, and on the hepatic metabolism of cholesterol and/or lipoproteins (85). A specific endocrine influence of soy protein on the insulin/glucagon ratio is suggested by Anderson (86).

It is assumed that the cholesterol lowering effect, especially the reduction of LDL, is caused by soy peptides, most probably from the 7S α-fraction of the globulins (87-89), which activate hepatic LDL receptors, and appear to increase the mRNA expression of LDL receptors in circulating human monocytes (90). According to Sirtori (1998), soy products provide a large amount of protein with high-quality amino acids, which seem to directly up-regulate LDL receptors by 50% or more (91). Additionally, Kohno documented a significant reduction of serum triacylglycerol levels and visceral fat due to dietary intake of β-conglycinin, which is a component of soy protein isolate (92).

Isoflavones are compounds that have structures similar to estrogen, with a weaker affinity to estrogen receptors. Therefore, it has been postulated that these compounds are partly responsible for the lipid lowering effects of soy protein (93). The two major soy isoflavones are genistein and daidzein (94). It is well known that mammalian estrogen can modulate blood lipid profile, by promoting reduction of LDL-cholesterol and increasing HDL-cholesterol.

It is not likely that isoflavones alone will decrease blood lipid levels, however they may influence the ability of soy protein to reduce serum lipid concentrations. It is likely that soy protein and isoflavones exert synergistic effects. Studies with the single compound could not show the same lipid-lowering effect as when both substances were administered together (95, 96).

In soy protein there are also other bioactive components such as saponins, trypsin inhibitors and bioactive peptides which may contribute to the lipid-lowering efficacy of soy, but more research is needed in this area.

9 Objectives of the thesis

The primary objective of the studies presented in this thesis was to investigate the cholesterol lowering effects of different soy based dietary supplements in patients with hypercholesterolemia (Articles 2-5), and in type 2 diabetic subjects (Article 1) with focus on the reduction of cardiovascular risk markers. Furthermore, two studies have documented how processing influences the lipid lowering effects of soy proteins in humans (Articles 5 & 6).

10 Materials and Methods

The published articles in this thesis represents 10 year of research projects studying the cholesterol lowering properties of soy protein, and of soy protein combined with soy fibre and soy phospholipids. Milk protein was used as control, since milk protein is known to have no significant lipid lowering properties, and has frequently been used as control in other studies.

When a soy protein based product proven to lower LDL and total cholesterol was made into a ready to drink product, which was intended to be a commercially available cholesterol lowering beverage, it was UHT treated to sterilize the product for longer shelf life. During this UHT process both the soy protein based drink and the milk protein based control beverage significantly increased lipid levels.

To study whether it was the denaturation of the protein structure which was responsible for the negative effects on blood lipids, we developed a new non-denatured soy protein. Substantial time, resources and efforts were used to make this new soy protein out of 1,2 tones of unprocessed soy flakes, and all processing of the new isolated soy protein had to be made in smaller batches of 30-40 kg.

In article 1, 25 type 2 diabetic patients from a hospital outpatient clinic were included, which had a controlled, double-blind crossover design. For each patient, the diets were isocaloric and had similar macronutrient composition up to the start of active and control treatment, respectively. The patients were randomly allocated to a 6-week active treatment or placebo. The soy supplement provided a daily dosage of 50 g isolated soy protein with a high isoflavone content (total isoflavones >165 mg) and 20 g soy cotyledon fiber. The control contained a daily dosage of 50 g casein and 20 g cellulose. Patients were instructed to mix half of their daily supplement in 250 ml water before breakfast and half before the evening meal and to consume as a beverage with the meal. The treatments were separated by a 3-week wash-out period.

Before each study period and during the last week of the 6-week treatment periods, participants had to weigh and record their food intake for 2 working days and one weekend day. The dietary records were validated by a registered dietitian to estimate the energy intake and

composition. Blood samples were taken after overnight fasting before the beginning of the study and on the last day of the two intervention periods. The main parameters determined in the blood tests were cholesterol (total, LDL, HDL), triglycerides, Apo B100, LDL/HDL-ratio, and homocystein. Additionally, clinical auscultatory and 24-hour ambulatory blood pressure, as well as 24-hour urine samples were collected. Five patients did not complete the study.

In article 2, 143 hypercholesterolemic patients selected from a hospital patient database took part in a randomised, controlled, double-blind, parallel-group, single-centre study. The hypercholesterolemic patients received yoghurt containing a soy composition based on isolated soy protein with standardized levels of isoflavones, cotyledon soy fibers and soy phospholipids, or a placebo yoghurt based on milk protein and cellulose fiber, over a period of 8 weeks. All patients were asked to consume one cup two times per day, both morning and afternoon, giving a daily dosage of 41.4 g soy protein in the active group and 24.4 g of milk protein in the control group. Prior to treatment, the patients followed an open, run-in dietary phase of 8 weeks, wherein all participants consumed a cholesterol-lowering diet in order to reduce the variability of lipid levels. During the study, all patients were seen in the clinic on weeks 0, 2, 4, 6 and 8 for compliance control and blood sampling for lipid measurements. The participants then had a final follow-up assessment after another 4 weeks without treatment. All patients were included in the efficacy analysis.

The study described in article 3 was performed as a prospective single cohort study including 53 hypercholesterolemic patients, of which four dropped out. The participants received a statin monotherapy for 6 weeks, followed by a combination of statin therapy and a soy preparation for 6 weeks and finally statin monotherapy alone for 6 weeks. During treatment with the soy preparation, two sachets containing 45 g of the soy product were to be consumed daily, corresponding to an intake of 30 g soy protein. There were six study visits where blood samples were drawn, vital signs measured, medication compliance recorded and adverse events monitored. Plasma lipid concentrations were measured for cholesterol (total, LDL, HDL) and triglycerides.

Article 4 concerns a randomized, controlled trial, which was performed to investigate the lipid lowering effect of two soy-based protein supplements in 117 hypercholesterolemic patients from an outpatient hospital clinic. The participants received their nutritional supplements after a run-in period of 2 weeks, after which baseline values were determined. The hypercholesterolemic patients were assigned to one of the following 3 study groups: active treatment 1, 77.5 g supplement containing 25 g soy protein; active treatment 2, 75.5 g supplement containing 15 g soy protein and 10 g milk protein; control, 76.5 g supplement containing 25 g milk protein. The participants received the preparation in two sachets which were dissolved in cold water and which were taken with their morning and evening meals. Additional assessments to the baseline examination were made 4, 6, and 8 weeks after base-

line and involved an evaluation of the general clinical condition of each patient, and where blood samples were analyzed to determine serum levels of total cholesterol, LDL and HDL cholesterol, triglycerides, apolipoprotein B, and lipoprotein (a).

The main aim of article 5 was to test an expected cholesterol lowering effects of an ultra heat treated (UHT) soy protein based drink. Eighty patients with hypercholesterolemia from an outpatient hospital clinic were enrolled in this randomized, controlled, double-blind trial. A four-armed study design was chosen, where the participants were assigned to one of the following four groups: Active treatment 1 (receiving 1l chocolate-flavored milk containing 24.4 g soy protein and 30.4 g milk protein daily) or control 1 (receiving 1l chocolate-flavored milk containing 43.3 g milk protein daily), and active treatment 2 (receiving 0.5l chocolate-flavored milk containing 12.2 g soy protein and 15.2 g milk protein daily) or control 2 (receiving 0.5l chocolate-flavored milk containing 21.7 g milk protein daily). The soy protein product and placebo preparation were premixed with low-fat (0.2%) chocolate-flavored milk and ultra heated (142°C for 4-6 sec) to sterilize the products for long term shelf life, and were distributed to the participants. Assessments took place at baseline and after 2 and 4 weeks. This involved clinical evaluation and blood sampling to determine serum concentrations of total, LDL and HDL cholesterol.

In article 6, the cholesterol lowering effect of two different soy proteins was investigated in 120 hypercholesterolemic patients from an outpatient hospital clinic in a randomized, double-blind, controlled trial. The patients received their study preparations, containing a total of 25 g protein, twice daily. Each dosage was stirred into 250 ml cold water and was taken with morning and evening meals. The active test preparation contained an isolated soy protein with the protein in its native non-denatured structural form, whereas the reference preparation contained a commercially available (conventionally) isolated soy protein which are heat treated. Furthermore, a preparation from milk protein, was given to a third group of patients in this study. Clinical assessments were performed four times throughout the study period. At each visit, the patient's general medical condition was evaluated and blood samples were taken.

Outcome measurements were total cholesterol, LDL and HDL cholesterol. Safety parameters such as hemoglobin, glucose and alanine aminotransferase to control liver function were determined in the initial and final evaluations.

Due to differences of the design in the various studies, different statistical tests were used.

Statistical analysis in article 1 was conducted using the Mann-Whitney test to compare the height and age for the groups using active and control treatments at the time of randomization, and Fisher's exact test to compare the gender distribution between these groups. Analysis of differences within and between treatments were performed using Wilcoxon's matched-pair signed rank test, applied regarding weight, waist, waist-hip ratio, 24-hour

blood pressure, fasting values of blood sample measurements and urine tests, whereas measures of glucose and insulin were evaluated using an analysis of variance with repeated measurements. Wilcoxon's matched-pair test and Fisher's exact test were also used in article 3.

A primary analysis was undertaken in article 2 on a modified intention-to-treat population, defined as those patients who received the study treatment for at least two weeks. To assess the validity of the results, a secondary analysis was performed, where missing values in the intention-to-treat population were substituted according to the last-observation-carried-forward method. Furthermore, the per-protocol population, consisting of only subjects who completed the study was analyzed.

Univariate comparisons of the differences between corresponding values versus baseline were accomplished by means of analysis of variance in article 4. In the case of significant group differences, comparison of pairs were made using Student's t test. Multivariate analysis of variance for repeated measurements was performed when no significant differences between the treatment groups and control were observed. Finally, a new multivariate nonparametric analysis of covariance for repeated measures was performed.

The data in article 5 were evaluated according to the full-analysis procedure. In addition to variance analysis and the F-test, the t-test for coupled observations was applied.
In article 6, analysis of variance and the F-test were used to test differences between mean changes that were observed in all 3 study groups. If differences occurred, the Student's t-test was used for paired group comparisons. All other data have been evaluated using descriptive methods, and have been performed by Realize & Analyse AG, Berlin, Germany.

All studies were approved by national ethics committees (Article 1: ethics committee of Aarhus County, Denmark; Article 2: ethics committee of the Joensuu Health Centre, Joensuu, Finland; Article 3: ethics committee in Copenhagen, Denmark; article 4-6: ethics committee of Humboldt University Medical School (Charité) in Berlin, Germany).

11 Results

11.1 Summaries of the six articles

Article 1

"Beneficial effects of a soy-based dietary supplement on lipid levels and cardiovascular risk markers in type 2 diabetic subjects"

K Hermansen, M Søndergaard, LH Høie, M Carstensen, B Brock

The objective of this controlled, randomized, double-blind crossover study was to investigate the effects of a dietary supplement containing soy protein, and a high-fixed level of isoflavones and cotyledon soy fiber on cardiovascular risk markers, blood glucose and insulin levels in type 2 diabetic patients. Twelve subjects were randomly allocated to a 6-week treatment with the active preparation and 8 patients to a 6-week treatment with placebo.

Subjects were instructed to mix half of their daily supplement in 250 ml water before breakfast and half before the evening meal and to consume them as a beverage with a meal.

Both treatments were separated by a 3-week wash-out period. The percentage mean treatment difference between soy protein and control showed significantly lower mean values after soy protein treatment for LDL cholesterol (10 ± 15%, $P < 0.05$), LDL/HDL ratio (12 ± 18%, $P < 0.05$), apolipoprotein (apo) B100 (30 ± 38%, $P < 0.01$), triglycerides (22 ± 10%, $P < 0.05$) and homocystein (14 ± 21%, $P < 0.01$). Total cholesterol was reduced in the soy protein group, however not significantly (8 ± 15%, $P < 0.08$) from the control group. No changes could be found in HDL cholesterol, apo B100/apo A1 ratio, plasminogen activator inhibitor1, factor VIIc, von Willebrand factor, fibrinogen, lipoprotein(a), glucose, HbA_{1c}, or 24-hour blood pressure when using soy protein. In conclusion, the active soy based dietary supplementation led to beneficial effects regarding reduction of cardiovascular risk markers in type 2 diabetic subjects.

Published in Diabetes Care 2001; 24 (2): 228-233

Article 2

"Isolated soy protein with standardized levels of isoflavones, cotyledon soy fibers and soy phospholipids improves plasma lipids in hypercholesterolemia: a double-blind, placebo-controlled trial of a yoghurt formulation"

P Puska, V Korpelainen, LH Høie, E Skovlund, KT Smerud

In this randomized, double-blind, controlled study, 143 subjects with hypercholesterolemia were enrolled and randomly assigned to the soy group (n=69) or the control group (n=74).

Over a period of 8 weeks, patients in the soy group received yoghurt containing a soy composition, based on isolated soy protein with standardized levels of isoflavones, cotyledon soy fibers and soy phospholipids. All patients were asked to take one cup twice a day, in the morning and afternoon. Prior to treatment, an open, run-in dietary phase of 8 weeks was followed, wherein all subjects consumed a cholesterol-lowering diet in order to reduce the variability of their lipid levels.

Efficacy parameters were determined five times during the intervention period and 4 weeks later. Significant differences in lipid-lowering effect was found in favor of the active soy intervention group compared with the control group with respect to total cholesterol (estimated mean difference 0.40 mmol/l; $P<0.001$), LDL-cholesterol (0.39 mmol/l; $P<0.001$), non-HDL cholesterol (0.40 mmol/l; $P<0.001$) and total/HDL-cholesterol ratio (0.23; $P=0.005$). There was no difference regarding effects on HDL-cholesterol, triacylglycerols or homocystein. It was found that the lipid-lowering effect occurred within the first two weeks and was not caused by weight loss. Gastrointestinal symptoms caused a significantly higher drop-out rate (fourteen v. three subjects) in the soy group. However, the safety profile for active soy was similar to the placebo group.

Published in British Journal of Nutrition 2004; 91: 393-401

Article 3

"Treatment with Abacor®, a soy-based dietary supplement, further reduces plasma concentrations of total and low-density lipoprotein cholesterol in statin-treated hypercholesterolaemic patients"

P Clausen, J Lindhardsen, LH Høie, S Stender

This study was performed to test whether a soy-based dietary supplement reduces cholesterol concentrations when administered to statin treated hypercholesterolemic patients. A total of 53 subjects with a plasma LDL-cholesterol concentration of >3.0mmol/l at baseline despite statin treatment were enrolled in this open label single center cohort study with six study visits. The participants received statin monotherapy for 6 weeks, statin and active treatment for 6 weeks and finally statin monotherapy for 6 weeks. During treatment with the active supplement two sachets of the soy product containing each 45 g were consumed daily, corresponding to an intake of 30 g soy protein per day. In total, 49 patients completed the study. It was found that plasma concentration of total cholesterol and LDL cholesterol were

significantly reduced at visit 5 (6 weeks of combination treatment) compared to the cholesterol mean values both after 6 weeks of statin monotherapy at visit 3 and of visit 6: 5.5 ± 0.1 vs. 5.9 ± 0.1mmol/l (P<0.0004) and 3.3 ± 0.1 vs. 3.6 ± 0.1mmol/l (P<0.0006). It was concluded that the soy-based dietary supplement further significantly reduces total cholesterol, mainly by reducing LDL cholesterol when given in combination with statins in hypercholersterolemic patients.

Published in Innovative Food Science and Emerging Technologies 2004; 5: 377-383

Article 4

"Lipid-lowering effect of 2 dosages of a soy protein supplement in hypercholesterolemia"

LH Høie, HJ Graubaum, A Harde, J Gruenwald, KD Wernecke

An 8-week randomized, controlled trial was performed to investigate the lipid lowering effect of a soy-based protein supplement in 117 patients. The participants were allocated to three different groups, one receiving 15 g/d soy protein, the second 25 g/d soy protein and the third control group with 25 g/d milk protein. The general clinical condition of each participant was determined at baseline, and after 4, 6 and 8 weeks. In the soy groups, LDL cholesterol levels decreased significantly by 1.1% and 5.9% respectively, whereas levels increased by 3.6% in the control group. Similar changes could be found for total serum cholesterol and apolipoprotein B. HDL cholesterol, triglycerides, homocysteine, folic acid, and vitamin B12 levels did not change significantly compared with baseline values in any study group. This study demonstrated that soy protein supplementation reduced serum cholesterol levels, and that 25 g soy protein was more effective compared to an intake of 15 g/d.

Published in Advances in Therapy 2005; 22 (2): 175-186

Article 5

"Ultra heat treatment destroys cholesterol-lowering effect of soy protein"

LH Høie, Å Sjoholm, M Guldstrand, HJF Zunft, W Lueder, HJ Graubaum, J Gruenwald

To investigate the dosage-dependent response on serum cholesterol after consuming an ultra-heat-treated soy protein preparation, a randomized, controlled, double-blind trial was conducted. A total of eighty subjects with hypercholesterolemia were assigned to one of the following four groups receiving 12.5 g or 25 g soy protein (active treatment) or casein ("pla-

cebo") daily over a period of 4 weeks. At baseline and after 2 and 4 weeks blood samples were taken to determine serum concentrations of total, LDL and HDL cholesterol.

It was found that LDL cholesterol concentrations were significantly increased compared to baseline in all study groups. The magnitude of this increase (17-21%) was similar in all active and control study groups, whether the UHT treated proteins beverages were based on soy or milk protein. The soy protein supplements previously shown to significantly reduce serum cholesterol, significantly increased cholesterol levels after ultra heat treatment in this study.

Published in International Journal of Food Sciences and Nutrition 2006; 57 (7/8): 512-519

Article 6

"Cholesterol-lowering effects of a new isolated soy protein with high levels of non-denatured protein in hypercholesterolemic patients"

LH Høie, M Guldstrand, Å Sjoholm, HJ Graubaum, J Gruenwald, HJF Zunft, W Lueder

The aim of this prospective, randomized, double-blind, controlled trial was to compare the effects of a new isolated soy protein in which the protein structure was kept in its native, non-denatured form (verum 1) versus a conventional isolated soy protein (verum 2) and milk protein (control) on plasma cholesterol levels. Over a period of 8 weeks, 120 patients were given verum 1, verum 2 or control containing a daily dosage of 25 g protein. Compared to control, total cholesterol levels were significantly reduced by 10.7% in the verum 1 group ($P<0.001$), and by 5.8% in the verum group 2 ($P=0.008$). The difference between the two verum groups lowering total cholesterol was significant ($P=0.008$). LDL cholesterol levels were significantly reduced with the non-denatured isolated soy protein in the verum 1 group, by 9.4% ($P=0.002$), and by 4.9% in the verum 2 group ($P=0.107$). The difference between the verum groups in lowering LDL cholesterol was significant ($P=0.05$). The lipid-lowering effects of the new isolated soy protein with its protein and peptide structures intact was approximately twice that of a conventional isolated soy protein.

Published in Advances in Therapy 2007; 24 (2): 439-447

11.2 Summary of the results.

Recently, we have published a paper (not included in the thesis) which summarizes the lipid lowering effects in our soy protein research, and how denatured protein causes an altered biological response (97). The enclosed figure summarizes the cholesterol lowering effects presented in the various clinical studies we have published.

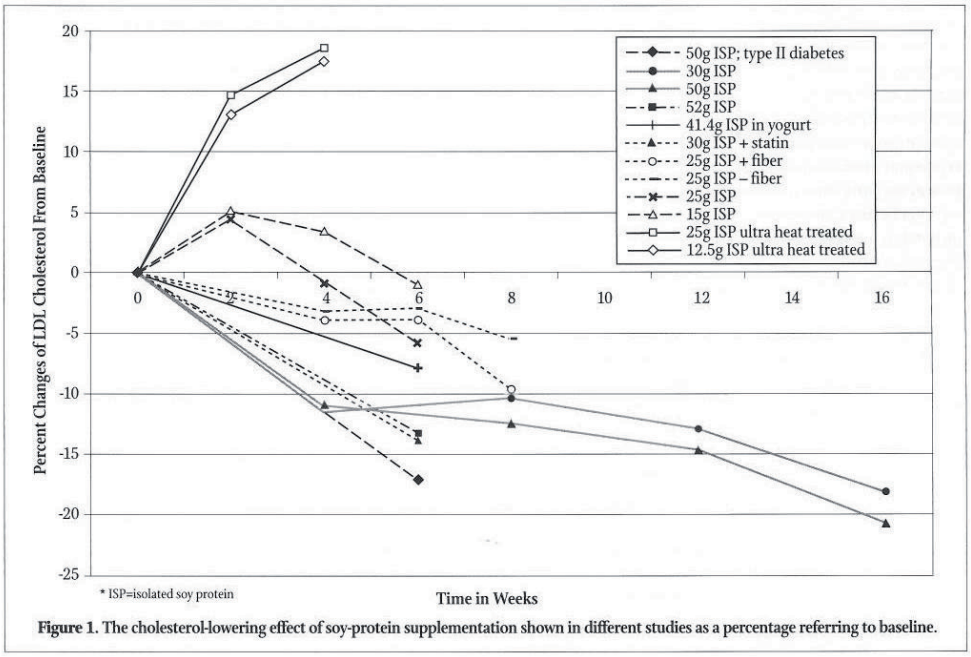

Figure 1. The cholesterol-lowering effect of soy-protein supplementation shown in different studies as a percentage referring to baseline.

Ultra heat treatment not only ruins the lipid lowering properties of soy protein, but significantly increased cholesterol levels, thereby being potentially atherogenic. The same was found for the milk protein based control drink which was UHT treated. Hence, for the first time to our knowledge a clear parallel with regards to the processing of proteins has been found, compared to the processing of polyunsaturated fatty acids into trans fatty acids, where also an increased risk of CVD has been documented.

12 Discussion

12.1 Design

Studies described in articles 1, 2, 4, 5 and 6 in this thesis, have a randomized, double-blind, controlled design in type 2 diabetic subjects (article 1) and in hypercholesterolemic patients (articles 2, 4, 5, 6). Furthermore, study 1 was performed as a crossover study. Article 3 presents an open label single center cohort study.

12.2 Weaknesses, strengths, arguments

In article 1, only 25 patients were included into the study, which is a small number for such an investigation. However, the cross over design strengthens the results. Otherwise the study designs and population sizes used in the clinical trials of this thesis were adequate. Five of the six studies were performed using a randomized, double-blind, controlled study design, which is regarded as the best standard for clinical trials. All studies were approved by ethics committees. Only the study reported in article 3 was conducted as an open label, single-center, cohort study without a control group, which can be regarded as a weakness for this study. However, the design of the study with an initial 6-week period of unchanged statin medication after dietary instructions should reduce a potential "cholesterol-lowering effect" by the participants' awareness of and possible lifestyle changes due to the study participation itself.

Dietary studies are normally influenced by several factors, such as seasonal or dietary adaptation and inaccurate self-reporting. In most of the studies the patients got dietary advice from a registered dietitian. Nevertheless, it cannot be ruled out that various individual dietary behaviors of the subjects had an impact on the results of the studies presented. Another approach was dietary food control by keeping a food diary. Unfortunately, this was considered to be inappropriate in the study reported in article 2. The study was intended to diminish any effect on normal life. Keeping a food diary was assumed to introduce an adverse effect on normal life of these subjects compared to frequent visits to the study nurse. A weakness in this study can also be seen from the fact that the two products compared, were not completely identical in fat content as there were manufacturing obstacles to maintain blinding in terms of appearance, smell and taste if the fat content was kept identical. It was assumed that this difference in fat content could explain some of the differences, which needs careful consideration in future studies. It is also questionable as to whether the control was actually an inactive substance, since the control group also showed a smaller reduction in lipids.

12.3 Patients

Article 1 investigates the effects of a soy-based dietary supplement on lipid levels and cardiovascular risk markers in type 2 diabetic subjects with serum lipid abnormalities. In all other studies, patients with hypercholesterolemia were studied. Subjects were selected from the patient database of different hospital outpatient clinics.

12.4 Efficacy

It has been documented that soy protein lowers elevated blood lipids in hypercholesterolemic subjects (11).

Dietary studies are influenced by a number of factors (98). Seasonal or dietary adaptation, as well as inaccuracy of self-reporting of dietary data may complicate the analyses. Furthermore, study design also varies considerably, with a treatment period of 5 to 24 weeks (67, 68, 90, 99) and soy protein intake of 17 to 125 g daily (72). Furthemore, various processing techniques may influence results. Subsequently, published results obtained from clinical studies in recent years have been inconsistent (63, 67, 68, 69, 71).

The study described in article 1 shows that the intake of a soy-based preparation significantly reduced LDL cholesterol levels, LDL/HDL ratio, apolipoprotein and triglycerides in diabetic patients. HDL cholesterol, however, remained unchanged. These findings are in accordance with the results in a meta-analysis with nondiabetic subjects performed by Anderson (11). Reduction of blood glucose and insulin levels were modest, which also have been found in other studies in diabetic patients (100, 101). The results document beneficial effects of an intervention with a soy-based preparation in reducing cardiovascular risk markers in type 2 diabetic subjects. The risk for coronary heart disease (CHD) is two to three times higher among adults with type 2 diabetes than in the general population (102, 103), and with a four to six times greater cardiovascular mortality (104). Serum lipid abnormalities in type 2 diabetes are normally characterized by decreased HDL cholesterol and hypertriglyceridemia, whereas total cholesterol and LDL cholesterol levels are similar to those in non-diabetic subjects.A rapid onset of the cholesterol-lowering effect was documented in article 2. Approximately half the lipid-lowering effect after 8 weeks treatment was observed after the first week.

It was demonstrated in article 3 that statin treated patients with moderate hypercholesterolemia significantly further reduced LDL- and total cholesterol, when given a soy-based preparation. This is surprising since the cholesterol-lowering effect of soy shown in previous studies has been found to positively correlate with baseline cholesterol concentration (11). The cholesterol-lowering effect of soy is also associated with gender. The results of article 3 showed that the lipid lowering effect was more pronounced in men, whereas no

significant effect was seen in women. This indicates that soy given to hypercholesterolemic patients treated with statins may be beneficial to men, but not to women.

The indication of a possible gender difference in response to soy may reflect the importance of the isoflavone content. Isoflavones are natural plant estrogens from soy which weakly bind to estrogen receptors and may thereby initiate a cholesterol-lowering effect similar to that of estradiol found in other studies (105). However, the number of women participating in the study presented in article 3 was small and a possible gender difference with regards to the plasma cholesterol lowering effect of soy needs further investigation.

Results in article 4 demonstrate that the lipid-lowering effect is influenced by the amount of soy protein intake daily. The reduction of lipids resulting from a daily intake of 25 g soy protein was documented to be larger than that of a daily intake of 15 g.

LDL cholesterol, which is a major risk factor for cardiovascular disease, was significantly reduced in all of the studies of this thesis, except when the soy protein was UHT-treated (article 5), when LDL and total cholesterol significantly increased.

There are different alternatives of processing soy-beans, and most of the manufacturing steps are accompanied by heat treatments (106), which alter the protein structure (107). An overview of the literature on the effects of temperature on food proteins and its implications on functional properties is given by Kilara and Sharkasi (108).

The conventional ISP achieved only moderate lipid lowering effects in a previous clinical study (70), and in a proteomic investigation it was shown to be highly fragmented in its protein constituents (109).

In conventional processing of proteins the structure may be altered, which may influence biological effects (110). Soy protein previously shown to reduce serum cholesterol had no such lipid-lowering effect after ultra-high heat treatment, when both total and LDL-cholesterol levels very surprisingly significantly increased, as documented in article 5. In a follow-up study using the same soy protein without the preceding ultra heat treatment, total cholesterol and LDL cholesterol were significantly reduced during a 4-week treatment period (article 5).

It is likely that the significant increase in blood lipid levels found after ultra heating of the soy protein is due to heat-induced denaturating of the protein which changes the constituents of the soy protein responsible for the serum cholesterol reduction, including soy peptides from 7S globulins.

It took considerable time and effort to develop the new isolated soy protein (ISP) for the study presented in article 6, and the new ISP could only be manufactured in approximately 30 kg batches from 1.2 tonnes of defatted soybean flakes. Microbiological growth was controlled in the new ISP by freeze drying down to a very low moisture content of approximately 5%. In the hypercholesterolemic patients participating in this study the new ISP, in which the major part of the protein was in its non-denatured form with its native protein and pep-

tide structures intact, was compared with a commercially available ISP. The reduction of total and LDL cholesterol was significantly more pronounced with the new ISP, which was twice as effective in lowering lipids when directly compared to the commercially available ISP.

Wright and Boulter (111) detected a thermal transition ascribed to 11S globulin (glycinin), and it was found that the 7S form of glycinin is especially sensitive to heating, with denaturation occuring at a lower temperature (about 60°C) than the 11S globular form (112, 113).

Recent investigations by Amigo-Benavent (114) show that the quality, antigenicity and antioxidant activity of soy protein can be affected by the intensity of thermal processing. An immunoblotting assay revealed modifications in the antigenic response of soy foods, suggesting that the processing had altered the structure of soy allergens.

Coward (115) suggests that chemical modification of isoflavones in soyfoods during cooking and processing may influence the bioavailability and pharmacokinetic properties of soy. Of importance is the composition of the isoflavone glucoside conjugates which can alter rates of absorption and metabolism. β-glucosides can be malonylated, acetylated or unesterified in the 6´´-OH position.

It has been demonstrated that treatment of soy flour or soy protein concentrate with temperatures of up to 190°C does not change the total isoflavone content (115, 116). However, the portion of the 6´´-O-malonyl derivates decreases due to decarboxylation, which may have an important effect on bioavailability.

When soy protein denatures, the globular structure opens, resulting in long-chain proteins, which can form insoluble aggregates (107, 117). Apparently, a higher degree of aggregation reduces the amount of bioactive soy peptides from digestion that can be absorbed through the intestines.

More research is needed to investigate the impact of processing of soy protein, especially with regards to the impact of UHT in increasing lipids, which again may increase the risk for cardiovascular disease.

Increased cardiovascular risk is also associated with trans fatty acids (TFA). Most trans fats consumed today are industrially created by partially hydrogenating unsaturated fats at high temperatures, making them more saturated and transforming cis-double bonds to trans-double bonds. Consumption of trans fats is known to increase the risk of coronary heart disease by raising the levels of LDL cholesterol and lowering HDL levels (118, 119, 120). Also an increase of plasma triacylglycerol levels (121) and a relationship between TFA and systemic inflammation, endothelial dysfunction (122, 123) and increased risk of type 2 diabetes has been observed (124).

In addition, TFA derived from industrially produced sources have specific HDL cholesterol-lowering properties (125). Another study revealed that replacement of dietary unsatu-

rated fatty acids by TFA not only decreased HDL levels in healthy men and women, but also impaired flow-mediated vasodilatation of the brachial artery, which is a risk marker for coronary heart disease (126, 127).

Current dietary recommendations to keep TFA intake as low as possible and the growing awareness of those recommendations by consumers and food regulatory agencies have been major driving forces for the edible oil industry and food manufacturers to develop alternative fats and oils with nutritionally improved fatty acid compositions (128, 129, 130).

In 2006 the U.S. Food and Drug Administration (FDA) issued a final rule that requires the declaration of the amount of TFA present in foods, including dietary supplements, on the nutrition label. For the purpose of nutrition labeling, TFA are defined as the sum of all unsaturated fatty acids that contain one or more isolated (i.e. non-conjugated) double bonds in a *trans* configuration. It is assumed, that this approach will lead to the prevention of 600 to 1200 cases of coronary heart disease (CHD) and 240-480 deaths each year, saving US$ 900 mill. to US$ 1.8bn in yearly medical costs (128).

In Denmark, legislation virtually eliminated the intake of industrially produced trans fatty acids (IP-TFA), by banning any food with an IP-TFA content > 2% of total fat within a few years (129).

Oilseeds with modified fatty acid composition, (e.g. enhancement of the content of long-chain omega-3 fatty acids or conjugated linoleic acid) have been developed to increase the delivery of these fatty acids directly into the food supply or indirectly as use for feed ingredients for livestock. New processing technologies have been developed to create dietary fats and oils with specific physiologic functions related to risk factors for CVD (130).

Changing the structure of protein may alter its biological effects, as we have documented in Article 5. New processing technologies have been developed to create dietary proteins with specific physiologic functions. Investigations have been performed, e.g. by Kwok (131), who carried out optimization of the thermal processes for soy milk to obtain a minimum degradation of sensory and nutritional qualities. However, no one has to our knowledge previousely documented that UHT treatment of proteins may alter the biological effects in humans by significantly increasing cholesterol levels, thereby possibly increasing the risk for CVD.

12.5 Safety

Consumer safety has the highest priority also when it comes to dietary interventions. Suitable parameters to assess safety of a product are drop-out rates and questionnaires documenting side effects and well-being.

Drop-out rates were very low in articles 1 and 3-5 of this thesis. In most cases, the consumption of the soy-based preparations was very well tolerated, with slight temporary gastrointestinal discomfort being the most common side effect.

In article 2, a considerable drop-out-rate was reported. Fourteen subjects (20.3%) from the active soy group, and three (4.1%) from the control group dropped out of the study during the intervention period. The high fiber intake may partly explain gastrointestinal symptoms reported by these subjects. A lower dosage taken for a longer term may be as effective, but this has to be confirmed in further dose-response studies.

A higher number of drop-outs was also reported in article 6. Of the 120 patients included, 88 completed the study, the other 32 (26.7%) withdrew prematurely, mainly because they did not like the taste of the study preparation. Since there was a high drop-out-rate in the verum groups taking soy (12 and 8) as well as in the placebo group (12), it is likely that the intolerance was not caused by the soy ingredients.

Safety was assessed by measurements of a number of risk factors and biochemical parameters, which were determined at the initial and final evaluation. In article 2 for example, hematology and biochemistry blood tests were taken (alanine aminotransferase, aspartate aminotransferase, creatinine, glucose and uric acid), as well as a physical examination including blood pressure and heart rate. No significant changes in any of the hematological or biochemical laboratory variables were found. The same results were reported in the other articles.

13 Conclusions

This thesis comprises the description and results of six clinical studies investigating the cholesterol lowering effects of soy products and soy protein based diets. In article 1, the effects of a soy-based dietary supplement was investigated on lipid levels and cardiovascular risk markers in type 2 diabetic subjects with serum lipid abnormalities. In the other studies, patients with hypercholesterolemia were examined. The studies were performed at university hospitals in the Nordic countries and Germany, with a study population ranging from 25 to 143 patients. The study period, which was often accompanied by wash-out periods before starting a particular treatment or between different treatments, varied from 4 to 8 weeks.

The results of the study described in article 1, investigating the effects of a soy-based dietary supplement on lipid levels and cardiovascular markers in type 2 diabetic subjects, demonstrated that consumption of the soy-based formulation reduced cholesterol and other risk factors for CVD, which was also seen in individuals with near-normal lipid values. The lipid-lowering effects was observed in article 2 after 1-2 weeks of intervention, and was not caused by weight loss. This rapid onset of effect may be of particular benefit to those who need motivation during the early phases of treatment, and who are not able to comply with

lifestyle modification programs. Article 3 showed that treatment with a soy-based dietary supplement significantly further reduced plasma concentrations of total and LDL cholesterol in statin treated hypercholesterolaemic patients. Therefore, a soy-based diet represents an attractive alternative to increasing the dosage of statins. The results of study 4 also demonstrated that soy protein supplementation reduced serum cholesterol levels, and that 25 g of soy protein was more effective than 15 g per day.

Article 5 describes the cholesterol-lowering effects of soy proteins after ultra heat treatment. A soy protein supplement, previously shown to significantly reduce serum cholesterol, significantly increased LDL and total cholesterol after UHT treatment, which is likely caused by changes in the protein structure. This was supported by the results in article 6, where the cholesterol-lowering effect of a new isolated soy protein, with high levels of non-denaturated protein, was compared to a conventional isolated soy protein in hypercholesterolemic patients. The lipid-lowering effects of the new isolated soy protein, with its protein and peptide structures intact, was approximately twice as large when compared to the conventional isolated soy protein. All soy products used in the clinical trials were found to be well tolerated and safe, causing only minor side effects.

These results may have important implications when it comes to the health effects and safety of proteins based foods and ready to drink products in the way the food and beverage companies process proteins. All UHT drinks, which have been ultra heated for long shelf life, whether containing soy protein, milk protein or other proteins, may be atherogenic and increase the risk for cardiovascular disease. The finding that a non-denaturated soy protein was twice as effective in lowering lipid levels compared to a conventional soy protein which is heat treated with some denaturation, further document these results.

Conventional soy proteins will have been heat treated during their processing by most producers, whereby some of the cholesterol lowering properties is lost. Some of the major soy producing companies use ethanol in their processing, which denatures protein, and as such ethanol processing may also be atherogenic.

There is limited literature with regards to processing/heating of protein based foods and drinks and changes in their biological and functional properties. More research is needed to document potential side-effects, efficacy and safety with regards to the processing of dietary proteins, and the implications for human health.

Competing interests

The author is a co-founder and shareholder of Nutri Pharma ASA, which has sponsored the studies presented in this thesis.

14 References

1. WHO. Reducing risks, promoting healthy life. The World Health Report 2002, Geneva.

2. EUROASPIRE I and II Group. European Action on Secondary Prevention by Intervention to Reduce Events: Clinical reality of coronary prevention guidelines: a comparison of EUROASPIRE I and II in nine countries. Lancet 2001; 357: 995-1001.

3. Baigent C, Keech A, Kearney PM, et al. Cholesterol Treatment Trialists' (CTT)Collaborators. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet 2005; 366: 1267-1278.

4. Law MR, Wald NJ, Thompson SG. By how much and how quickly does reduction in serum cholesterol concentration lower risk of ischaemic heart disease? BMJ 1994; 308: 367-372.

5. National Cholesterol Education Program (NECP). Third report of the expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). Circulation 2002; 106: 3143-3421.

6. WHO. Healthy nutrition. Preventing nutrition-related diseases in Europe. WHO Regional publications. European Series 1988; 24: 127.

7. European Heart Network. European cardiovascular disease statistic. 2008 edition. http://www.ehnheart.org. 18.03.2008.

8. Jenkins DJ, Kendall CW, Faulkner DA, Nguyen T, Kemp T, Marchie A, Wong JM, *et al.* Assessment of the longer-term effects of a dietary portfolio of cholesterol-lowering foods in hypercholesterolemia. Am J Clin Nutr 2006; 83 (3): 582-591.

9. Farriol M, Jorda M, Delgado G. Past and current trends in soy supplementation: a bibliographic study. Nutr Hosp 2006; 21 (4): 448-451.

10. Christensen R, Kristensen PK, Bartels EM, Bliddal H, Astrup A. Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomised trials. Lancet 2007; 370: 1706-13.

11. Anderson JW, Johnstone BM, Cook-Newell ME. Meta-analysis of the effects of soy protein intake on serum lipids. N Engl J Med 1995; 333: 276-282.

12. Anderson JW, Høie LH. Weight Loss and Lipid Changes with Low-Energy Diets: Comparator Study of Milk-Based versus Soy-Based Liquid Meal Replacement Interventions. Journal of the American College of Nutrition 2005; 24 (3): 210-216.

13. Høie LH, Morgenstern ECA, Gruenwald J, Graubaum HJ, Busch R, Lueder W, Zunft HJF. A double blind-blind placebo-controlled clinical trial compares the cholesterol-lowering effects of two different soy protein preparations in hypercholesterolemic subjects. European Journal of Nutrition 2005; 44: 65-71.

14. Schryver T, Smith C. Participant's willingness to consume soy foods for lowering cholesterol and receive counseling on cardiovascular disease by nutrition professionals. Public Health Nutr 2006; 9 (7): 866-874.

15. Hegele RA. Plasma lipoproteins: genetic influences and clinical implications. Nat Rev Genet 2009; 10 (2): 109-121.

16. Mensink RP, Aro A, Den Hond E, German JB, Griffin BA, ter Meer HU, Mutanen M, Pannemans D, Stahl W. PASSCLAIM – Diet-related cardiovascular disease. Eur J Nutr 2003; Suppl. 1: I6-I27.

17. Brewer HB Jr, Rader DJ. HDL: structure, function and metabolism. Prog Lipid Res 1991; 30 (2-3): 139-144.

18. Vance DE, Vance JE. Biochemistry of lipids, lipoproteins and membranes (New Comprehensive Biochemistry). Elsevier Science, 5th edition, 2008.

19. Fredrickson DS, Lees RS. A system for phenotyping hyperlipidemia. Circulation 1965; 31: 321-327.

20. James W, Berger T, Elston D. Andrew's diseases of the skin: Clinical dermatology. 10th dition, Saunders Elsevier. 2006.

21. Harper CR, Jacobson TA. New perspectives on the management of low levels of high-density lipoprotein cholesterol. Arch Intern Med 1999; 159: 1049-1057.

22. Jacobs DR, Mebane IL, Bangdiwala SI, et al. High density lipoprotein cholesterol as a predictor of cardiovascular disease mortality in men and women: the follow-up study of the Lipid Research Clinics Prevalence Study. Am J Epidemiol 1990; 131: 32-47.

23. Merck. Merck Manuals Online Medical Library. http://www.merck.com/mmpe/print/sec12/ch159/ch159b.html. Last update September 2008.

24. Thomas MC, Nestel PJ. Management of dyslipidaemia in patients with type 2 diabetes in Australian primary care. MJA 2007: 186 (3): 128-130.

25. Winocour PH, Durrington PN, Ishola M, Hillier VF, Anderson DC. The prevalence of hyperlipidaemia and related clinical features in insulin-dependent diabetes mellitus. QJM 1989; 70 (3): 265-276.

26. National Cholesterol Education Program (NECP). Second report of the expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel II). Circulation 1994; 89: 1333-1445.

27. De Sereday MS, Gonzalez C, Giorgini D, De Loredo L, Braguinsky J, Cobenas C, Libman C, Tesone C. Prevalence of diabetes, obesity, hypertension and hyperlipidemia in the central area of Argentina. Diabetes Metab 2004; 30 (4): 335-339.

28. Lichtenstein AH, Appel LJ, Brands M, et al. Diet and lifestyle recommendations revision 2006. Circulation 2006; 114: 82-96.

29. Ramsay IE, Yeo WW, Jackson PR. Dietary reduction of serum cholesterol concentration: time to think again. Brit Med J 1991; 303: 953-957.

30. Ring-Dimitriou S, Von Duvillard SP, Paulweber B, Stadlmann M, Lemura LM, Peak K, Mueller E. Nine months aerobic fitness induced changes on blood lipids and lipoproteins in untrained subjects versus controls. Eur J Appl Physiol. 2007; 99 (3): 291-9.

31. McBride PE. The health consequences of smoking: cardiovascular diseases. Med Clin North Am 1992; 76: 333-353.

32. Jonas MA, Oates JA, Ockene JK, Hennekens CH. Statement on smoking and cardiovascular disease for health care professionals. Circulation 1992; 86: 1664-1669.

33. U.S. Department of Health and Human Services. The health benefits of smoking cessation. A Report of the Surgeon General. U.S. Department of Health and Human Services, Public Health Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health. DHHS pub. No. (CDC) 90-8416, Washington, D.C.: U.S. Department of Health and Human Services, 1990. 627 pages.

34. Sviridov D, Nestel P, Watts G. Statins and metabolism of high density lipoprotein. Cardiovasc Hematol Agents Med Chem 2007; 5 (3): 215-221.

35. Jones P, Kafonek S, Laurora I, Hunninghake D. Comparative dose efficacy study of atorvastatin versus simvastatin, pravastatin, lovastatin, and fluvastin in patients with hypercholesterolemia (the CURVES study. Am J Cardiol 1998; 81 (5): 582-587.

36. Aldridge MA, Ito MK. Colesevelam hydrochloride: a novel bile acid-binding resin. Ann Pharmacother 2001; 35: 898-907.

37. Wong NN. Colesevelam: a new bile acid sequestrant. Heart Dis 2001; 3: 63-70.

38. Otvos JD, Collins D, Freedman DS. Low-density lipoprotein and high-density lipoprotein particle subclasses predict coronary events and are favorably changed by gemfibrozil therapy in the veterans affairs high-density lipoprotein intervention trial. Circulation 2006; 113: 1556-63.

39. Shepherd J, Cobbe SM, Ford I, et al. Prävention der KHK durch Pravastatin bei Männern mit Hypercholesterinämie. Münch Med Wschr 1996; 138:21-28.

40. Frishman WH, Horn J. Statin-drug interactions: not a class effect. Cardiol Rev 2008; 16 (4): 205-212.

41. Paragh G, Fülöp P. Impact of lipid lowering therapy on liver function. Orv Hetil 2009; 150 (26): 1205-1012.

42. Filippatos TD, Mikhailidis DP. Lipid-lowering drugs acting at the level of the gastrointestinal tract. Curr Pharm Des 2009; 15 (5): 490-516.

43. Ginsberg HN, Kris-Etherton P, Dennis B, et al. Effects of reducing dietary saturated fatty acids on plasma lipids and lipoproteins in healthy subjects: the Delta Study. Protocol 1. Arterioscler Thromb Vasc Biol 1998; 18: 441-449.

44. Mueller-Bohn T. Atheroskleroseprophylaxe-Bericht zum Fortbildungskongress in Malente. DAZ 1996; 136: 2453-58.

45. Mensink RP, Katan MB. Effects of dietary fatty acids on serum lipids and lipoproteins: a meta-analysis of 27 trials. Arterioscler Thromb 1992; 12: 911-919.

46. Aro A, Jauhiainen M, Partanen R, et al. Stearic acid, trans-fatty acids, and dairy fat: effects on serum and lipoprotein lipids, apolipoproteins, lipoptrotein(a), and lipid transfer proteins in healthy subjects. Am J Clin Nutr 1997; 65: 1419-1426.

47. Judd JT, Clevidence BA, Muesing RA, et al. Dietary trans fatty acids: effects on plasma lipids and lipoproteins of healthy men and women. Am J Clin Nutr 1994; 59: 861-868.

48. Reynolds K, Chin A, Lees KA, Nguyen A, Bujnowski D, He J. A meta-analysis of the effect of soy protein supplementation on serum lipids. Am J Cardiol 2006; 98 (5): 633-640.

49. Zdrenghea D, Pop D, Colcear D. Effect of dietary isoflavones on plasma cholesterol. Rom J Intern Med 2006; 44: 353-363.

50. Plana N, Nicolle C, Ferre R, Camps J, et al. Plant sterol-enriched fermented milk enhances the attainment of LDL-cholesterol goal in hypercholesterolemic subjects. Eur J Nutr 2008; 47: 32-39.

51. Rudkowska I, Jones PJH. Functional foods for the prevention and treatment of cardiocascular diseases: cholesterol and beyond. Expert Rev Cardiovasc Ther 2007; 5 (3): 477-490.

52. Ostlund RE Jr., Racette SB, Stenson WF. Inhibition of cholesterol absorption by phytosterol-replete wheat germ compared with phytosterol-depleted wheat germ. American Journal of Clinical Nutrition 2003; 77 (6): 1385-1389.

53. Brufau G, Canela MA, Rafecas M. Phytosterols: physiologic and metabolic aspects related to cholesterol-lowering properties. Nutr Res 2008; 28 (4): 217-225.

54. Patch CS, Tapsell LC, Williams PG, Gordon M. Plant sterols as dietary adjuvants in the reduction of cardiovascular risk: theory and evidence. Vasc Health Risk Manag 2006; 2 (2): 157-162.

55. Grundy SM, Cleeman JI, Bairey Merz CN, Brewer Jr. HB, Clark LT, Hunninghake DB, Pasternak RC, Smith Jr. SC, Stone NJ. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III Guidelines. Circulation 2004; 110: 227-239.

56. Hobbs FD, Erhardt L. Acceptance of guideline recommendations and perceived implementation of coronary heart disease prevention among primary care physicians in five European countries: the reassessing European attitudes about cardiovascular treatment (REACT) survey. Fam Pract 2002; 19: 596-604.

57. LaRosa JH, LaRosa JC. Enhancing drug compliance in lipid-lowering treatment. Arch Fam Med 2000; 9 (10): 1169-1175.

58. Lopez AD, Mathers CD, Ezzati M, et al. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet 2006; 367: 1747-1757.

59. Manuel DG, Lim J, Tanuseputro P, Anderson GM, et al. Revisting Rose: strategies for reducing coronary heart disease. BMJ 2006; 332: 659-662.

60. Perkins EG. Composition of soybeans and soybean products. In: Pracitcal Handbook of soybean processing and utilization. Erickson DR (ed.). AOCS Press. 1997: 9-28.

61. KeShun L. Chemistry, Technology and Utilization. Chapman & Hall. 1997.

62. Ristic Medic D, Ristic V, Arsic A, Postic M, Ristic G, Blazenic Mladenovic V, Tepsic J. Effects of soybean D-LeciVita product on serum lipids and fatty acid composition in type 2 diabetic patients with hyperlipidemia. Nutr Metab Cardiovasc Dis 2006; 16 (6): 395-404.

63. Puska P, Korpelainen V, Høie LH, Skovlund E, Lahti T, Smerud KT. Soy in hypercholesterolaemia: a double-blind, placebo-controlled trial. EJCN 2002; 56: 352-357.

64. Friedman M, Brandon DL. Nutritional and health benefits of soy proteins. J Agric Food Chem 2001; 49 (3): 1069-1086.

65. Maesta N, Nahas EA, Nahas-Neto J, Orsatti FL, Fernandes CE, Traiman P, Burini RC. Effects of soy protein and resistance exercise on body composition and blood lipids in postmenopausal women. Maturitas 2007; 56 (4): 350-358.

66. Torres N, Torre-Villalvazo I, Tovar AR. Regulation of lipid metabolism by soy protein and its implication in diseases mediated by lipid disorders. J Nutr Biochem 2006; 17 (6): 365-373.

67. Tonstad S, Smerud KT, Hoie L. A comparison of the effects of 2 doses of soy protein or casein on serum lipids, serum lipoproteins, and plasma total homocysteine in hypercholesterolemic subjects. Am J Clin Nutr 2002; 76: 78-84.

68. Merz-Demlow BE, Duncan AM, Wangen KE, et al. Soy isoflavones improve plasma lipids in normocholesterolemic, premenopausal women. Am J Clin Nutr 2000; 71: 1462-1469.

69. Teixeira SR, Potter SM, Weigel R, et al. Effects of feeding 4 levels of soy protein for 3 and 6wk on blood lipids and apolipoproteins in moderately hypercholesterolemic men. Am J Clin Nutr 2000; 71: 1077-1084.

70. Crouse JR, Morgan T, Terry JG, et al. A randomized trial comparing the effect of casein with that of soy protein containing varying amounts of isoflavones on plasma concentrations of lipids and lipoproteins. Arch Intern Med 1999; 159: 2070-2076.

71. Washburn S, Burke GL, Morgan T, et al. Effects of soy protein supplementation on serum lipoproteins, blood pressure, and menopausal symptoms in perimenopausal women. Menopause 1999; 6: 7-13.

72. Andersen P, Seljeflot I, Abdelnoor M, Arnesen H, Dale PO, Løvik A, Birkeland K. Increased insulin sensitivity and fibrinolytic capacity after dietary intervention in obese women with polycystic ovary syndrome. Metabolism 1995; 44 (5): 611-613.

73. Bricarello LP, Kasinski N, Bertolami MC, Faludi A, Pinto LA, Relvas WG, Izar MC, Ihara SS, Tufik S, Fonseca FA. Comparison between the effects of soy milk and nonfat cow milk on lipid profile and lipid peroxidation in patients with primary hypercholesterolemia. Nutrition 2004; 20 (2): 200-204.

74. Desroches S, Mauger JF, Ausman LM, Lichtenstein AH, Lamarche B. Soy protein favorably affects LDL size independently of isoflavones in hypercholesterolemic men and women. J Nutr. 2004; 134 (3): 574-579.

75. Hermansen K, Dinesen B, *et al.* Effects of soy and other natural products on LDL:HDL ratio and other lipid parameters: A literature review. Advances in Therapy 2003; 20 (1): 50-78.

76. Food and Drug Administration. Food labelling health claims; soy protein and coronary heart disease. Fed Regist 1999; 64: 57699-57733.

77. Yang G, Shu X-O, Jin F, Elasy T, Li H-L, Li Q, Huang F, Gao Y-T, Zheng W. Soyfood consumption and risk of glycosuria: a cross-sectional study within the Shanghai Women's Health Study. Eur J Clin 2004; 58: 615-620.

78. Anderson JW. Diet first, then medication for hypercholesterolemia. JAMA 2003; 290: 531-533.

79. Lo GS, Goldberg AP, Lim A, et al. Soy fiber improves lipid and carbohydrate metabolism in primary hyperlipidemic subjects. Atherosclerosis 1986; 62: 239-248.

80. Brown L, Rosner B, Willet WW, Sacks FM. Cholesterol-lowering effects of dietary fiber: a meta-analysis. Am J Clin Nutr 1999; 69: 30-42.

81. Kirsten R, Heintz K, Nelson K, Hesse K, Schneider E, Oremek G, Nemeth N. Polyenylphosphatidylcholine improves the lipoprotein profile in diabetic patients. Int J Clin Pharmacol Ther 1994; 32: 53-56.

82. Dewailley P, Moulin S, Rouget JP, et al. Effects of polyenylphosphatidyl choline on lipoproteins in patients with hypercholesterolemia. Med Welt 1985; 36: 367-369.

83. Ovesen L, Ebbesen K, Olesen E. The effects of oral soybean phospholipids on serum cholesterol, plasma triglyceride, and serum high-density lipopoprotein cholesterol concentrations in hyperlipidemia. J Parenter Enteral Nutr 1985; 9: 716-719.

84. Sirtori CR, Zucchi-Dentone C, Sirtori M, et al. Cholesterol-lowering and HDL-raising properties of lecithinated soy proteins in type II hyperlipidemic patients. Ann Nutr Metab 1985; 29: 348-357.

85. Potter SM. Soy Protein and Cardiovascular Disease: The impact of bioactive components in soy. Nutr Reviews 1998; 56 (8): 231-235.

86. Anderson JW, Smith BM, Washnock CS. Cardivascular and renal benefits of dry bean and soybean intake. Am J Clin Nutr 1999; 70 (Suppl.): 464S-474S.

87. Lovati MR, Manzoni C, Gianazza E, Arnoldi A, Kurowska E, Carroll KK, Sirtori CR. Soy protein peptides regulate cholesterol homeostasis in Hep G2 cells. J Nutr 2000; 130: 2543-2549.

88. Manzoni C, Lovati MR, Gianazza E, et al. Soybean protein products as regulators of liver low-density lipoprotein receptors. II. Alpha prime rich commercial concentrate and alpha prime deficient mutant differently affect low-density lipoprotein receptor activity. J Agr Food Chem 1998; 46: 2481-2484.

89. Lovati MR, Manzoni C, Corsini A, et al. Low density lipoprotein receptor activity is modulated by soybean globulins in cell culture. J Nutr 1992; 122: 1971-1978.

90. Baum JA, Teng H, Erdman JW Jr, *et al.* Long-term intake of soy protein improves blood lipid profiles and increases mononuclear cell low-density-lipoprotein receptor messenger RNA in hypercholesterolemic, postmenopausal women. Am J Clin Nutr 1998; 68: 545-551.

91. Sirtori CR, Lovati MR, Manzoni C, Gianazza E, Bondioli A, Staels B, Auwerx J. Reductions of serum cholesterol by soy proteins: clinical experience and potential molecular mechanisms. Nutr Metab Cardiovas Dis 1998; 8: 334-340.

92. Kohno M, Hirotsuka M, Kito M, Matsuzawa Y. Decrease in serum triacylglycerol and visceral fat mediated by dietary soybean b-conglycinin. J Atheroscler Thromb 2006; 13: 247-255.

93. Tham DM, Gardner CD, Haskell WL. Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. J Clin Endocrinol Metab 1998; 83: 2223-2235.

94. Kaufman PB, Duke JA, Briefmann H, Boik J, Hoyt Je. A comparative survey of leguminous plants as sources of the isoflavones, genistein and diadzein: implications for human nutrition and health. J Altern Complement Med 1997: 3 (1): 7-12.

95. Clair RS, Anthony M. Soy, isoflavones and atherosclerosis. Handb Exp Pharmacol 2005; 170: 301-323.

96. Gardner CD, Newell KA, Cherin R, Haskell WL. The effect of soy protein with or without isoflavones relative to milk protein on plasma lipids in hypercholesterolemic postmenopausal women. Am J Clin Nutr 2001; 73 (4): 728-735.

97. Grünwald J, Høie LH, Stier H. Cardiovascular Benefits of Soy Supplements. IMCJ

Aug/Sept 2009, vol 8, No. 4: 30-40.

98. Sandström B. Quality criteria in human experimental nutrition research. Eur J Clin Nutr 1995; 49: 315-322.

99. Wong WW, Smith EOB, Stuff JE, et al. Cholesterol-lowering effect of soy protein in normocholesterolemic and hypercholesterolemic men. Am J Clin Nutr 1998;68 (suppl): 1358S-1389S.

100. Tsai AC, Vinik AI, Lasichak L, Lo GS. Effects of soy polysaccharide on postprandial plasma glucose, insulin, glucagons, pancreatic polypeptide, somatostatin, and triglyceride in obese diabetic patients. Am J Clin Nutr 1987; 45: 596-601.

101. Mahalko JR, Sandstead HH, Johnson LK, Inman LF, Milne DB, Warner RC, Haunz EA. Effect of consuming fiber from corn bran, soy hulls, or apple powder on glucose tolerance and plasma lipids in type II diabetes. Am J Clin Nutr 1984; 39: 25-34.

102. Kannel WB, D'Agostino RB, Wilson PW, Belanger AJ, Gagnon DR. Diabetes, fibrinogen, and risk of cardiovascular disease: the Framingham experience. Am Heart 1990; 120: 672-676.

103. Laakso M, Ronnemaa T, Lehto S, Puuka P, Kallio V, Pyorala K. Does NIDDM increase the risk of coronary heart disease similarly in both low- and high-risk populations? Diabetologia 1995; 38: 487-493

104. Kannel WB, McGee DL. Diabetes and cardiovascular disease: the Framingham Study. JAMA 1979; 241: 2035-2038.

105. Knight DC, Eden JA. A review of the clinical effects of phytoestrogens. Obstetrics & Gynecology 1996; 87: 897-904.

106. Lusas EW, Riaz MN. Soy protein products: Processing nd use. J Nutr. 1995; 125: 573S-580S.

107. Petruccelli S, Anon MC. Thermal aggregation of soy protein isolates. J Agric Food Chem 1995; 43: 3035-3041.

108. Kilara A, Sharkasi TY. Effects of temperature on food proteins and its implications on functional properties. Crit Rev Food Sci Nutr 1986; 23 (4): 323-395.

109. Gianazza E, Eberini I, Arnoldi A, et al. A proteomic investigation of isolated soy proteins with variable effects in experimental and clinical studies. J Nutr 2003; 133: 9-14.

110. Friedman M. Nutritional concequences of food processing. Forum Nutr. 2003; 56: 350-352.

111. Wright DJ, Boulter D. Differential scanning calorimetric study of meals and constituents of some food grain legumes. J Sci Food Agric 1980; 31: 1231.

112. Mills EN, Marigheto NA, Wellner N, et al. Thermally induced structural changes in glycinin, the 11S globulin of soya bean (Glycine max.) – an in situ spectroscopic

study. Biochim Biophys Acta 2003; 1648: (1-2): 105-114.

113. Lakemond CM, De Jongh HH, Hessing M, et al. Heat denaturation of soy glycinin: influence of pH and ionic strength on molecular structure. J Agric Food Chem 2000; 48 (6): 1991-1995.

114. Amigo-Benavent M, Silván JM, Moreno FJ, et al. Protein quality, antigenicity, and antioxidant activity of soy-based foodstuffs. J Agric Food Chem; 2008; 56 (15): 6498-6505.

115. Coward L, Smith M, Kirk M, Barnes S. Chemical modification of isoflavonesin soy-foods during cooking and processing. Am J Clin Nutr 1998; 68 (Suppl.): 1486S-1491S.

116. Mahungu SM, Diaz-Mercado S, Li J, Schwenk M, Singletary K, Faller J. Stability of isoflavones during extrusion processing of corn/soy mixture. J Agric Food Chem 1999; 47: 279-284.

117. Arrese EL, Sorgentini DA, Wagner JR, Anon MC. Electrophoretic, solubility, and functional properties of commercial soy protein isolates. J Agric Food Chem 1991; 39: 1029-1032.

118. Asp NG. Fatty acids in focus – the good and the bad ones. Scandinavian Journal of Food and Nutrition 2006; 50 (4): 150.

119. Wolfram G. Dietary fatty acids and coronary heart disease. Eur J Med Res 2003; 8 (8): 321-324.

120. Lichtenstein AH, Erkkilä AT, Lamarche B, et al. Influence of hydrogenated fat and butter on CVD risk factors: remnant-like particles, glucose and insulin, blood pressure and C-reactive protein. Atherosclerosis 2003; 171 (1): 97-107.

121. Katan MB, Zock PL, Mensink RP. Trans fatty acids and their effects on lipoproteins in humans. Annu Rev Nutr 1995; 15: 473-493.

122. Mozzafarian D, Katan MB, Ascherio A, et al. Trans fatty acids and cardiovascular disease. N Engl J Med 2006; 354: 1601-1613.

123. Lopez-Garcia E, Schulze MB, Meigs JB, et al. Consumption of trans fatty acids is related to plasma biomarkers of inflammation and endothelial dysfunction. J Nutr 2005; 135: 562-566.

124. Hu FB, Manson JE, Stampfer MJ, et al. Diet, lifestyle, and the risk of type 2 diabetes mellitus in women. N Engl J Med 2001; 345: 790-797.

125. Chardigny JM, Destaillats F, Malpuech-Brugère C, et al. Do trans fatty acids from industrially produced sources and from natural sources have the same effect on cardiovascular disease risk factors in healthy subjects? Results on the trans Fatty Acids Collaboration (TRANSFACT) study). Am J Clin Nutr 2008; 87 (3): 515-516.

126. De Roos NM, Bots ML, Katan MB. Replacement of dietary saturated fatty acids by

trans fatty acids lowers serum HDL cholesterol and impairs endothelial function in healthy men and women. Arterioscler Thromb VAsc Biol 2001; 21: 1233-1237.

127. De Roos NM, Shouten EG, Katan MB. Trans fatty acids, HDL-cholesterol, and cardiovascular disease. Effects of dietary changes on vascular reactivity. Eur J Med Res 2003; 8 (8): 355-357.

128. Moss J. Labeling of trans fatty acid content in food, regulations and limits – the FDA view. Atheroscler Suppl. 2006; 7 (2): 57-59.

129. Stender S, Dyerberg J, Astrup A. Consumer protection through a legislative ban on industrially produced trans fatty acids in foods in Danmark. Scan J Food Nutr 2006; 50 (4): 155-160.

130. Flickinger BD, Huth PJ. Dietary fats and oils: technologies for improving cardiovascular health. Curr Atheroscler Rep 2004; 6 (6): 468-476.

131. Kwok KC, Liang HH, Niranjan K. Optimizing conditions for thermal processes of soy milk. J Agric Food Chem 2002; 50 (17): 4834-4838.

15 Published Articles

1. Hermansen K, Søndergaard M, Høie LH, Carstensen M, Brock B. Benefitial effects of a soy-based dietary supplement on lipid levels and cardiovascular risk markers in type 2 diabetic subjects. Diabetes Care 2001; 24 (2): 228-233.

2. Puska P, Korpelainen V, Høie LH, Skovlund E, Smerud KT. Isolated soy protein with standardized levels of isoflavones, cotyledon soy fibers and soy phospholipids improves plasma lipids in hypercholesterolemia: a double-blind, placebo-controlled trial of a yoghurt formulation. British Journal of Nutrition 2004; 91: 393-401.

3. Clausen P, Lindhardsen J, Høie LH, Stender S. Treatment with Abacor, a soy based dietary supplement, further reduces plasma concentrations of total and low-density lipoprotein cholesterol in statin-treated hypercholesterolaemic patients. Innovative Food Science and Emerging Technologies 2004; 5: 377-383.

4. Høie LH, Graubaum HJ, Harde A, Gruenwald J, Wernecke KD. Lipid-lowering effect of 2 dosages of a soy supplement in hypercholesterolemia. Advances in Therapy 2005; 22 (2): 175-186.

5. Høie LH, Sjøholm Å, Guldstrand M, Zunft HJF, Lueder W, Graubaum HJ, Gruenwald J. Ultra heat treatment destroys cholesterol-lowering effect of soy protein. International Journal of Food Sciences and Nutrition 2006; 57 (7/8): 512-519.

6. Høie LH, Guldstrand M, Sjøholm Å, Graubaum HJ, Gruenwald J, Zunft HJF, Lueder W. Cholesterol-lowering effects of a new isolated soy protein with high levels of non-denatured protein in hypercholesterolemic patients. Advances in Therapy 2007; 24 (2): 439-447.

Article 1

Beneficial effects of a soy-based dietary supplement on lipid levels and cardiovascular risk markers in type 2 diabetic subjects

K Hermansen, M Søndergaard, LH Høie, M Carstensen, B Brock

Published in Diabetes Care 2001; 24 (2): 228-233

Article 2

Isolated soy protein with standardized levels of isoflavones, cotyledon soy fibers and soy phospholipids improves plasma lipids in hypercholesterolemia: a double-blind, placebo-controlled trial of a yoghurt formulation

P Puska, V Korpelainen, LH Høie, E Skovland, KT Smerund

Published in British Journal of Nutrition 2004; 91: 393-401

British Journal of Nutrition (2004), **91**, 393–401
DOI: 10.1079/BJN20031074

Isolated soya protein with standardised levels of isoflavones, cotyledon soya fibres and soya phospholipids improves plasma lipids in hypercholesterolaemia: a double-blind, placebo-controlled trial of a yoghurt formulation

Pekka Puska[1]*, Vesa Korpelainen[2], Lars H. Høie[3], Eva Skovlund[4] and Knut T. Smerud[5]

[1]*National Public Health Institute, Mannerheimintie 166, FIN-00300 Helsinki, Finland*
[2]*North Karelia Project, Joensuu, Finland*
[3]*Nutri Pharma A/S, Copenhagen, Denmark*
[4]*Section of Medical Statistics, University of Oslo, Oslo, Norway*
[5]*Smerud Medical Research International AS, Oslo, Norway*

(Received 17 April 2003 – Revised 23 October 2003 – Accepted 12 November 2003)

The objective was to study whether a yoghurt containing isolated soya protein with standardised levels of isoflavones, cotyledon soya fibres and soya phospholipids is more effective in lowering total and LDL-cholesterol than a placebo. One hundred and forty-three subjects were randomised to the soya group (n 69) or to the placebo (n 74). The mean baseline levels were 7·6 and 5·1 mmol/l for total and LDL-cholesterol, respectively. Fasting serum lipoproteins were assessed five times during the 8-week intervention period, and 4 weeks thereafter. The results were analysed by a mixed model for unbalanced repeated measurements. During the intervention, there were highly significant differences in lipid-lowering effect in favour of the active soya intervention group compared with the control group. The significant differences were for total cholesterol (estimated mean difference 0·40 mmol/l; $P<0·001$), LDL-cholesterol (0·39 mmol/l; $P<0·001$), non-HDL-cholesterol (0·40 mmol/l; $P<0·001$) and for the total:HDL-cholesterol ratio (0·23; $P=0·005$). There was no difference in the effects on HDL-cholesterol, triacylglycerols or homocysteine. The lipid-lowering effect occurred within 1–2 weeks of intervention, and was not due to weight loss. The safety profile for active soya was similar to the placebo group, except for gastrointestinal symptoms, which caused a significantly higher dropout rate (fourteen v. three subjects) among the subjects taking active soya.

Soya protein: Fibre: Isoflavones: Phospholipids: Hypercholesterolaemia

It is well established through epidemiological research that hypercholesterolaemia is among the major causes for CHD and that changes in diet may influence this and other risk factors for such disease (Pietinen *et al.* 1996; Grundy, 2000; Stamler *et al.* 2000; Vartiainen *et al.* 1994). Several large, prospective intervention studies using statins have further demonstrated that reducing the cholesterol level also reduces the risk of cardiovascular morbidity and mortality (The Scandinavian Simvastatin Survival Study Group, 1994; Shepherd *et al.* 1995; The Long Term Intervention with Pravastatin in Ischemic Disease (LIPID) Study Group, 1998; Heart Protection Study Collaborative Group, 2002).

A public health approach to primary prevention of CHD is to call for lifestyle changes; for example, a reduced intake of cholesterol and saturated fat, exercising, weight control and smoking cessation (Anonymous, 2002). There is still some debate whether or when drug intervention is indicated for primary prevention, partly because of cost considerations and partly because of concerns over the safety of lipid-lowering drugs. Reports from the UK indicate that patient and primary-care compliance with public recommendations are not followed entirely (Anonymous, 1998; Monkman, 2000; Primatesta & Poulter, 2000). As with other patient groups, hypercholesterolaemic patients also may be incapable of modifying their lifestyle significantly, they may be unable to comply with statin treatment (Avorn *et al.* 1998), or they may simply not want intervention with drugs. Various dietary supplements and functional foods have thus recently been introduced as alternatives to pharmaceuticals. In our view, before being acknowledged in clinical practice, such products should have their efficacy and tolerability documented by well-designed, prospective, randomised, double-blind and rigorously monitored clinical trials.

The lipid-lowering efficacy of soya protein is supported by the fact that the US Food and Drug Administration (1999) has approved a health claim for products giving

* **Corresponding author:** Professor Pekka Puska, fax +358 9 474 48408, email pekka.puska@ktl.fi

a daily dose of soya protein of at least 25 g. A pertaining problem with clinical studies of soya proteins has been that the variability of products has been huge, as each soya-based product may have different soya components, either isolated or not. In particular, using alcohol extractions for isolating the soya protein will also remove isoflavones, components that due to their oestrogenic or anti-oestrogenic effects very well may affect serum lipid levels. A recent meta-analysis (Anderson *et al.* 1995) reviewed several controlled studies and concluded that the dietary substitution of soya protein for animal protein might decrease the risk of cardiovascular disease by decreasing serum cholesterol, LDL-cholesterol and triacylglycerol concentrations, and by slightly increasing serum HDL-cholesterol levels.

The active components of soya have not yet been fully elucidated. It has been suggested (Anderson, 2003) that soya protein, peptides and isoflavones may work together to produce effects on lipid metabolism and gene expression. Several potential mechanisms of action have been suggested including an influence on the endocrine system, on the intestinal absorption of bile acids and dietary cholesterol, or on the hepatic metabolism of cholesterol and/or lipoproteins (Potter, 1998) and a more specific endocrine influence of soya protein on the insulin:glucagon ratios (Anderson *et al.* 1999). Soya peptides, most probably from the 7S α-fraction of the globulins (Lovati *et al.* 2000), are supposed to activate hepatic LDL receptors, and they appear to increase the mRNA expression of LDL receptors in circulating human monocytes (Baum *et al.* 1998).

Isoflavones have structures similar to mammalian oestrogens and they are capable of binding to oestrogen receptors. Depending on the hormonal status of the individual they seem to exhibit either oestrogenic or anti-oestrogenic effects. Therefore it has been postulated that premenopausally isoflavones may act anti-oestrogenic, whereas they could act post-menopausally as oestrogen receptor agonists. It is known that mammalian oestrogens can modulate the blood lipid profile, promoting decreases in LDL-cholesterol and increases in HDL-cholesterol. Oestradiol also shows potent cardioprotective effects on blood vessels (Potter, 1998). Isoflavones alone will not decrease blood lipid levels as described, but they influence the ability of soya protein preparations to reduce serum lipid concentrations.

Anderson *et al.* (1999) proposed several other mechanisms by which soya fibres could influence fasting and postprandial serum lipid protein concentrations. Due to the alteration of gastric emptying and intestinal transit time, dietary fibres could alter the kinetics of lipid absorption. Dietary fibre may decrease lipid absorption by modifying pancreatic secretion, the variation of intestinal motility, or by changing transport barriers such as the unstirred layer. Dietary fibre may affect the secretion rates of insulin or other pancreatic or intestinal hormones, which will change lipid and lipoprotein synthesis and secretion rates. Another suggested mechanism is that the soluble fibre of soya beans is fermented in the colon and generates SCFA such as acetate, butyrate or propionate, which inhibit hepatic lipid synthesis (Nishina & Freedland, 1990).

Phospholipids from soya beans may protect against atherosclerosis development (Navder *et al.* 2000). The most obvious action of unsaturated lecithin is the protection of LDL against oxidation and peroxidation. Oxidised LDL are involved in the development of atherosclerotic lesions. Peroxidation of LDL-lipids could damage apo B-100, the surface protein of LDL and VLDL. This may increase atherogenicity, because normal catabolism through the regulated LDL receptor pathway is blocked. This results in an enhanced cholesterol uptake via an unregulated scavenger pathway, which leads to the cholesterol overloading of macrophages and the development of foam cells, a key component of atherosclerotic plaques. One major mechanism of phospholipids or soyabean lecithin is the activation of reverse cholesterol transport. This includes the activation of lecithin-cholesterol-acetyltransferase, increased cholesterol uptake by HDL and leads to an increased biliary excretion of cholesterol (Rosseneu *et al.* 1979; Ishida *et al.* 1988; Hsia *et al.* 1996; Polichetti *et al.* 1996, 2000; LeBlanc *et al.* 1998).

Abacor® is a new product based on isolated soya protein and cotyledon soya fibres containing standardised levels of isoflavones as well as soya phospholipids. Initial studies in subjects with mild-to-moderate hypercholesterolaemia have demonstrated that this product is efficient in lowering cholesterol (Puska *et al.* 2002; Tonstad *et al.* 2002). These early results were obtained by administering the soya product in a powder formulation to be mixed with water before intake. The aim of the present study was to demonstrate similar lipid-lowering effects with this active soya product when administered in a yoghurt formulation.

Methods

Subjects

Subjects were screened from the patient database of the Joensuu Health Centre area in North Karelia, Finland. Eligible subjects had to meet selection criteria including: written informed consent; total serum cholesterol concentration of 6·7–9·9 mmol/l; serum triacylglycerols of $<4{\cdot}5$ mmol/l; age 18–75 years for men and 45–70 years for post-menopausal women (at least 6 months with no vaginal bleeding after menopause). Subjects were to have no significant signs of cardiovascular, renal, hepatic, endocrine or gastrointestinal disease; no familial hypercholesterolaemia; no type I or II diabetes mellitus treated with insulin; no past or concomitant use of statins, *n*-3-fatty acids or other lipid-lowering drugs (including any cholesterol-lowering functional foods) during the last 8 weeks before randomisation. They were to have had no hormone replacement therapy within the past 6 months; have no drug or alcohol dependency; no eating disorder; and no plans to lose weight during the study.

Study design and laboratory analyses

Subjects with hypercholesterolaemia were informed about the study and invited to participate. After having given their informed consent to participate in the study, the subjects were followed for 8 weeks in an open, run-in dietary phase. During this time all subjects were asked to consume a cholesterol-lowering diet in order to reduce the variability of their baseline lipid values as recommended

by The Swedish Medical Product Agency and the Norwegian Medicines Control Agency (1996). Their guidance is similar to the American Heart Association step I cholesterol-lowering diet (Anonymous, 2002) which recommends that the energy intake from fat should not exceed 30 % of the total energy. After a baseline medical assessment, subjects who met the selection criteria were randomised to one of the two study groups. They were advised to take one yoghurt serving twice daily for the next 8 weeks as part of their normal morning and evening meal. It was attempted to keep the energy intake as constant as possible by advising the subjects to reduce their morning and evening meal with approximately the same amount of energy as they received through the study products. All subjects were seen in the clinic at week 0, 2, 4, 6 and 8 for compliance control (deemed by counting returned and unused containers) and blood sampling for lipid measurements. The subjects then had a final follow-up assessment after another 4 weeks without administration of any study treatment.

Fasting blood samples were taken, after 5 min supine rest, during each visit for measurement of the efficacy lipid variables. Total cholesterol, HDL-cholesterol and triacylglycerols were analysed for every visit, whereas homocysteine was measured at baseline and at the end of therapy. Blood tests for safety haematology and biochemistry (alanine aminotransferase, aspartate aminotransferase, creatinine, glucose and uric acid) as well as a brief physical examination including the control of blood pressure and heart rate were taken only at baseline and at the end of treatment. All lipids were analysed at the Department of Biochemistry at the National Public Health Institute, Helsinki, Finland. The concentrations of LDL-cholesterol were calculated according to the Friedewald *et al.* (1972) method. As soya products contain high levels of the isoflavones genistein and daidzein (Adlercreutz, 1998), plasma concentrations were measured by fluoroimmunoassay (Wang *et al.* 2000) after 8 weeks treatment as an objective measure of treatment compliance. Weight was measured at each visit, but there was no detailed diary recording in order to control energy intake. Tolerability was assessed by recording spontaneously reported adverse events, as well as by actively asking an open, non-leading question to the patient during every visit whether they had experienced any undesirable medical event since their last clinic visit.

Study product

The subjects were asked to take one cup per twice-a-day serving, both morning and afternoon, giving a daily dose of soya protein of 41·4 g for the Abacor® group. The active Abacor® yoghurt (the active soya treatment) was manufactured by Protein Technology International Inc., St Louis, MO, USA (soya protein and fibres) and Lucas Meyer, Hamburg, Germany (soya lecithin), whereas the placebo yoghurt was the commercially available product 'Lett Yoghurt Naturell', manufactured by Tine Sør, Kristiansand, Norway. The products were matched to each other in terms of taste, appearance, smell and flavour by adding cherry jam. The finished products were manufactured and quality controlled by the Agricultural University of Norway, Ås, Norway. The study products were presented in identical, unmarked (except for subject number) containers. A detailed comparison of the contents of each study treatment is presented in Table 1.

Statistical analysis

This was a randomised, placebo-controlled, double-blind, parallel-group, single-centre study. It was estimated that with approximately seventy patients in each group, the power to detect a 10 % difference in LDL-cholesterol between the two study groups would be at least 90 % with a 5 % significance level (two-sided). Neither the investigators nor the patients knew the computer-generated randomisation code, block size or the results of the blood lipid concentrations until after the statistical analysis. Statistical analyses were conducted before breaking the randomisation code.

The primary analysis was undertaken on a modified intention-to-treat population, defined as those subjects who received the study treatment for at least 2 weeks. Changes in serum lipids over time were analysed by a mixed model for unbalanced repeated measurements employing the SAS® 6.12 software (SAS Institute, Cary, NC, USA). Parameters were estimated by the method of restricted maximum likelihood, and an unstructured covariance matrix was applied. The model included time and treatment group as the main effects and in addition the interaction between treatment and time. When no statistically significant interaction was found, the interaction term was excluded from the model. The last measurement before the start of treatment was included as a covariate in the model. Total cholesterol, HDL-cholesterol, non-HDL-cholesterol, LDL-cholesterol, LDL:HDL ratio, total:HDL-cholesterol ratio and triacylglycerols were analysed separately. Analysis of covariance was used to estimate the effect of treatment on homocysteine. The active soya treatment and placebo were compared for a total of eight efficacy variables.

In order to assess the robustness of the results, we conducted secondary analyses. In the intention-to-treat population, missing values were substituted according to the last-observation-carried-forward method. In addition, the per-protocol population consisting of study completers only (i.e. fifty-three subjects in the active soya group and seventy in the placebo group) was analysed. Both secondary analyses demonstrated that the data obtained in our primary analysis were robust and consistent.

Table 1. Contents of the yoghurt products

	Active soya	Placebo
Energy (kJ)	1484	994
Protein (g)		
Isolated soya protein*	20·7	0
Milk protein	0	12·2
Total fibre (g)	4·5	0
Fat (g)	2·84	1·6
Soya lecithin (g)	1·76	0

* Standardised as 3·7 mg isoflavones/g soya protein.

Ethics and administration

The study protocol and informed consent form were approved by the ethics committee of the Joensuu Health Centre, Joensuu, Finland. An independent contract research organisation (Smerud Medical Research International AS, Oslo, Norway) was responsible for monitoring the study according to the International Conference on Harmonization guidelines on good clinical practice principles (European Agency for the Evaluation of Medicinal Products, 1996) and for overall quality assurance.

Results

A total of 146 subjects were admitted for the screening of selection criteria, and were subsequently randomised to receive the study products. Three subjects withdrew their consent either before or immediately after taking the first dose. Hence 143 subjects, sixty-nine in the active soya group and seventy-four in the placebo group, have been included in the analysis of efficacy. Fig. 1 shows the flow of participants through each stage of the study. The two groups were well matched at baseline (Table 2) for sex, age, body weight, BMI, blood pressure, heart rate as well as for cigarette smoking and alcohol use.

A significant proportion of patients dropped out from the study during the intervention period, as they did not tolerate the product. The difference between the study groups was statistically significant ($P=0{\cdot}03$) as fourteen subjects (20·3 %) from the active soya group, and three (4·1 %) from the placebo group dropped out, respectively. The reason for withdrawal was either 'refusal to take more study product' or 'adverse events'. The subjects complained about nausea, vomiting, stomach pain and a feeling of stomach swelling, but these symptoms were usually mild and transient. Furthermore, three of the subjects in the active soya group who refused to continue taking the study product did so after a contamination (mould growth) was found in their yoghurt cups. There were no significant changes in any of the haematological or biochemical laboratory variables.

Lipid variables

Table 3 shows the mean screening and baseline concentrations for the lipid efficacy variables. Our statistical model incorporated the results from all biweekly visits, and the mean changes from baseline as well as the estimated difference in reductions, are also presented. The difference between the active soya treatment and the placebo was statistically significant for total cholesterol-, LDL-cholesterol-, and non-HDL-cholesterol-lowering and for the total:HDL-cholesterol and LDL:HDL-cholesterol ratios, whereas no differences for HDL-cholesterol changes, or for triacylglycerols or homocysteine were found. The serum concentrations over time for total cholesterol and LDL-cholesterol are presented in Fig. 2. The reduction occurred rapidly, within 1–2 weeks, and continued up to 8 weeks. Upon withdrawal after 8 weeks of the study products, serum concentrations for both groups returned to their baseline values.

Isoflavone levels

After therapy, the genistein levels were 762·1 (SD 1001·9) nmol/l in the active soya group and 7·8 (SD 11·7) nmol/l in the placebo group. For daidzein, the after-therapy levels were 127·0 (SD 120·5) and 2·6 (SD 5·1) nmol/l, for the active soya and placebo groups, respectively. These results confirmed that genistein and daidzein were indeed good means to distinguish those patients being reasonably compliant with taking study products and those who were not.

Haemodynamics

There were no significant changes in blood pressure or heart rate (data not shown) for either group during the study. The active soya group had systolic blood pressure of 130·0 (SD 15·8) mmHg at screening, 129·6 (SD 15·8) mmHg after the 8-week intervention, and 129·9 (SD 14·2) mmHg at 4 weeks after the study. However the results in the placebo group were 132·4 (SD 17·1) mmHg at screening, 131·3 (SD 15·7) mmHg after 8 weeks intervention, and 132·1 (SD 19·1) mmHg at 4 weeks after the intervention. For diastolic blood pressure, the findings were 80·5 (SD 8·7) mmHg at screening, 80·6 (SD 8·2) mmHg after

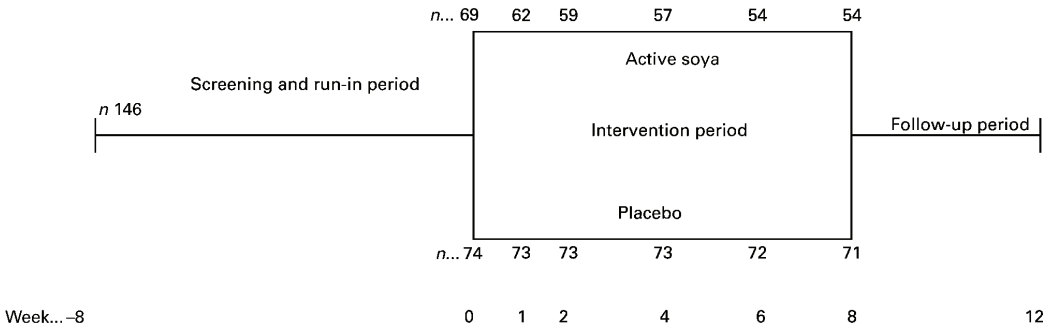

Fig. 1. Study flow-chart and number of subjects at each study stage.

Table 2. Subject characteristics at baseline
(Mean values and standard deviations)

	Active soya		Placebo	
	Mean	SD	Mean	SD
Patients (*n*)				
Total	69		74	
Males	33		34	
Females	36		40	
Age				
Range (years)	30–70		35–70	
Median (years)	58		58	
Weight (kg)				
Males	82·2	9·0	84·5	10·8
Females	72·7	15·3	68·7	12·8
BMI (kg/m^2)				
Males	26·6	2·9	27·1	3·7
Females	28·1	5·3	26·4	5·0
Systolic blood pressure (mmHg)	130·0	15·8	132·4	17·1
Diastolic blood pressure (mmHg)	80·5	8·7	81·1	9·4
Heart rate (beats/min)	70	10	69	10
Daily cigarette smokers (% of patients)	13		11	
Alcohol users (% of patients)	32		30	

the 8-week intervention, and 80·1 (SD 8·6) mmHg at 4 weeks after the study for the active soya group. The corresponding results in the placebo group were 81·1 (SD 9·4) mmHg at screening, 81·3 (SD 9·7) mmHg after the 8-week intervention, and 80·1 (SD 9·9) mmHg at 4 weeks after the intervention.

Body weight

Body weight was measured in every single clinic visit for all patients. The groups were comparable at baseline, and there were no significant changes in mean weight throughout the duration of the study. Females in the active soya group had a slight mean change from 72·7 (SD 15·3) to 73·7 (SD 15·8) kg compared with a similarly negligent change from 68·7 (SD 12·8) to 69·6 (SD 12·9) kg in the placebo group. For males, the mean change was in the opposite direction, from 82·2 (SD 9·0) to 79·9 (SD 17·8) kg in the active soya group and from 84·5 (SD 10·8) to 82·6 (SD 18·9) kg in the placebo group.

Discussion

The present 8-week diet intervention trial in subjects with hypercholesterolaemia demonstrated that a product based on isolated soya protein with standardised levels of isoflavones, cotyledon soya fibres and soya phospholipids had modest, but clinically significant, lipid-lowering effects compared with a placebo. The findings confirm that in a population with mild-to-moderately elevated plasma lipids, effects on cholesterol lowering can be obtained with a functional food.

Law *et al.* (1994) have shown that for mild-to-moderate hypercholesterolaemic subjects, a 10 % reduction in total cholesterol lowers the risk of cardiovascular events by up

Table 3. Serum concentrations of efficacy variables
(Mean values and standard deviations)

	Pre-run-in (screening)				Post run-in (baseline)				Mean change from baseline		Estimated difference in reduction		
	Active soya		Placebo		Active soya		Placebo				Active soya *v.* placebo		
Variable	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Active soya	Placebo		95 % CI	*P*
Total cholesterol (mmol/l)	7·60	0·62	7·62	0·63	7·53	0·79	7·59	0·77	− 0·42 (− 5·6 %)	− 0·02 (0·3 %)	0·40	(0·26, 0·55)	< 0·001
HDL-cholesterol (mmol/l)	1·70	0·50	1·74	0·46	1·67	0·53	1·72	0·45	0·05 (3·0 %)	0·06 (3·5 %)	− 0·01	(− 0·05, 0·03)	0·60
Non-HDL-cholesterol (mmol/l)					5·86	0·76	5·86	0·82	− 0·36 (− 6·1 %)	0·04 (0·7 %)	0·40	(0·25, 0·55)	< 0·001
LDL-cholesterol (mmol/l)	5·17	0·63	5·13	0·65	5·11	0·69	5·13	0·75	− 0·40 (− 7·8 %)	− 0·01 (− 0·2 %)	0·39	(0·24, 0·54)	< 0·001
LDL:HDL-cholesterol ratio					3·29	0·91	3·14	0·83	− 0·16 (− 4·9 %)	0·05 (1·6 %)	0·21	(0·10, 0·32)	0·003
Total:HDL-cholesterol ratio					4·81	1·21	4·62	0·98	− 0·12 (− 2·4 %)	0·11 (2·4 %)	0·23	(0·10, 0·36)	0·005
Triacylglycerols (mmol/l)	1·63	0·92	1·68	0·80	1·68	0·80	1·67	0·58	0·10 (6·0 %)	0·10 (6·0 %)	0	(− 0·15, 0·15)	1·00
Homocysteine (μmol/l)					10·41	2·74	11·16	5·50	2·44 (23·4 %)	1·86 (16·7 %)	− 0·58	(− 1·48, 0·32)	0·20

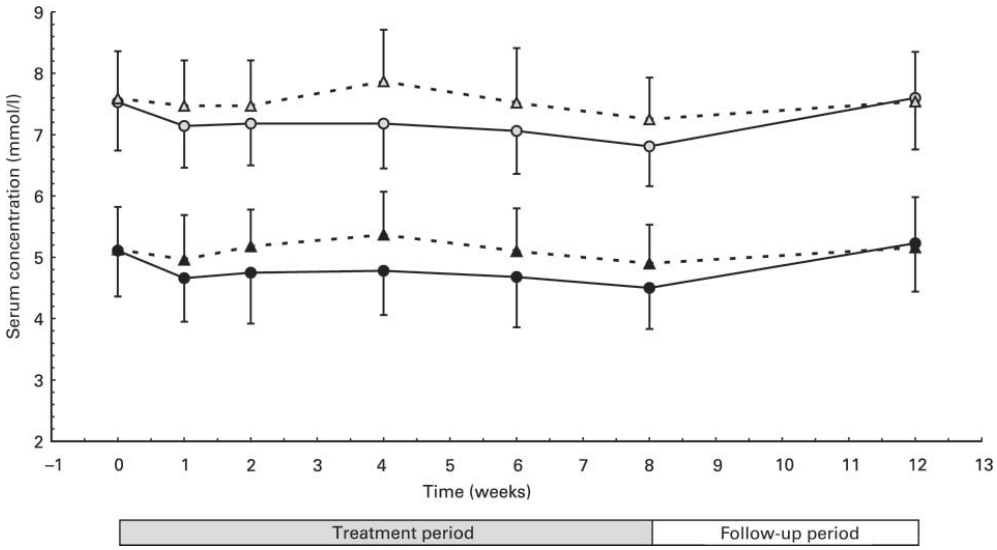

Fig. 2. Serum concentration *v.* time for total cholesterol in the active soya group (–○–), total cholesterol in the placebo group (- -Δ- -), LDL-cholesterol in the active soya group (–●–), and LDL-cholesterol in the placebo group (- -▲- -). Mean values are shown, with standard deviations represented by vertical bars.

to 50 % for a 40-year-old individual, and gradually less for older subjects. However, extrapolating risk reduction based on cholesterol alone is probably much too crude. With regard to the risk of CHD, it is not known whether otherwise healthy subjects would benefit from taking soya fibre for several years; neither has long-term safety been studied carefully.

Despite the test product being a dietary supplement by classification, we designed and conducted the study according to the guidelines for developing medicinal products. We employed both general principles (International Conference on Harmonization topics E6, E8, E9, and E10; European Agency for the Evaluation of Medicinal Products, 1996, 1997, 1998, 2000) and specific ones for the treatment of dyslipoproteinaemia (European Agency for the Evaluation of Medicinal Products, 2001). It is well known, and comprehensively reviewed by Sandström (1995), that dietary supplement studies include a number of confounding factors, since such intake most often includes other dietary components as well. Seasonal or dietary adaptation, as well as inaccuracy of self-reporting of dietary data, further complicate the analysis. Strict dietary food control could have controlled changes of individual food intake. The study intended to diminish any effect on normal life settings. The effect of keeping a food diary could have introduced an adverse effect on normal life of these subjects compared with only frequent visits to the study nurse, and was considered inappropriate.

As the present study was an early proof-of-concept one, we aimed to include subjects with the highest probability of showing an effect, and thus we included subjects with as high lipid levels as possible. However, the subjects were not so hypercholesterolaemic that intervention with a statin or another licensed lipid-lowering drug would be mandatory according to public guidelines at the time of planning the study. The meta-analysis by Anderson *et al.* (1995) showed that sex does not affect the response to soya protein, but whether isoflavones could have lesser effects in women with premenopausal oestrogen levels was not addressed in their paper. Consequently, we chose not to include this patient group in the present study.

The placebo was a standard low-fat yoghurt available on the consumer market. Cherry jam was added to this yoghurt in order to make it similar to the soya yoghurt. The two products were not completely identical in fat content as there were manufacturing obstacles to maintain blinding in terms of appearance, smell and taste if the fat content should be kept identical. Obviously, this difference in fat content may explain some of the differences, and needs careful considerations in future studies. Whether this choice was truly a placebo is also questionable, as the placebo group also showed a mean reduction in lipids, and regression to the mean would also be one possible explanatory factor.

For the primary efficacy variable (LDL-cholesterol), there was a highly significant difference between the active soya treatment and the placebo corresponding to a mean reduction from baseline of 0·42 mmol/l (corresponding to a 7·8 % difference in reduction) for the subjects taking active soya. Similar results, demonstrating a consistent beneficial effect, were also seen for total cholesterol and non-HDL-cholesterol, as well as for the LDL:HDL-cholesterol and total:HDL-cholesterol ratios. The present results suggest that the effect of soya protein and fibre are somewhat higher than seen in previously reported studies with the same duration of treatment (Anderson *et al.* 1995; Baum *et al.* 1998; Wong *et al.* 1998; Crouse *et al.* 1999; Merz-Demlow *et al.* 2000; Teixeira *et al.* 2000).

The rapid onset of the cholesterol-lowering effect was striking, even if this phenomenon is well known from other dietary intervention studies. About half of the effect after 8 weeks was seen already after the first week of intervention. These early lipid changes could be due to weight loss, but upon inspection we were not able to explain our findings by the weight data. This rapid onset of effect may prove beneficial to patients who need motivation in the early phases of treatment, and in particular for those patients who are not able to comply with lifestyle modification programmes.

In recent years, homocysteine has been introduced as a potential risk factor for atherosclerosis (Boushey *et al.* 1995; Gerhard & Duell, 1999; Wald *et al.* 2002). A previous study with Abacor® in a ready-to-mix beverage form (Tonstad *et al.* 2002) had demonstrated favourable effects with a daily dose of 30–50 g soya protein, but the present results were not able to confirm this effect in the yoghurt formulation.

It has been shown that related products based on soya are efficient in reducing weight and maintaining the weight loss over time (Rössner & Flaten, 1997; Ryttig *et al.* 1997). One could also have expected some weight-reduction benefit from participating in the present study as the subjects were asked to follow a standardised healthy diet scheme. We expected that subjects who are followed carefully with bi-weekly weight measurements in a medical clinic by health professionals would be likely to adhere truthfully to the dietary programme and in general lose weight because of greater focus on and awareness of a healthy lifestyle. The present results did not demonstrate any significant weight reduction even if a slight tendency in the male groups was noted, but 8 weeks may be too short to see any effects.

The yoghurt formulation used in the present study was not well tolerated. Microbiological (mould) contamination in some of the soya yoghurt cups from one shipment partly explained the dropout problems in the active soya group. However, even when correcting for this, the number of dropouts and complaints due to gastrointestinal symptoms were higher in the active soya group. The high intake of fibre may partly explain the gastrointestinal symptoms reported by some subjects. The usability of this prototype product is also limited in practice because of the relatively large volume of yoghurt, almost 300 ml twice daily. Whether the difference in yoghurt composition (energy or fatty acids or fermenting organism) influenced the results should be studied in later product development and clinical investigations. A lower dose taken for a longer term might be as or even more efficient but this remains to be confirmed in further dose–response investigations. Except for the gastrointestinal symptoms, both study groups showed a similar tolerability profile and the study did not indicate any particular safety concerns. However, the present study was not designed to address the safety issue specifically, and larger therapeutic use studies or post-marketing surveillance must be undertaken in order to shed more light on this aspect.

In conclusion, the present study supports a significant lipid-lowering effect of isolated soya proteins with standardised levels of isoflavones, cotyledon soya fibres and soya phospholipids. Some product development is recommended in order to avoid the gastrointestinal symptoms, and to reduce the volume to be ingested. Dependent on evidence from confirmatory and long-term trials, this soya yoghurt might then be a first intervention step in hypercholesterolaemia in addition to general dietary changes and possibly as an add-on to statin therapy. The rapid onset of effect may prove particularly beneficial to patients who need motivation in the early phases of intervention.

Acknowledgements

We would like to acknowledge the dedicated assistance by Pertti Puhakka (sub-investigator), Liisa Purmonen (study nurse), Timo Lahti, Merja Sariola and Irene Wassdal Fuglem (monitoring), Arne Husebø (data management), and Joerg Gruenwald, Eve Morgenstern and Bo Dinesen (comments on the manuscript). The study products were provided by The Agricultural University of Norway, Ås, Norway. Nutri Pharma ASA, Oslo, Norway supported the study financially.

References

Adlercreutz H (1998) Epidemiology of phytoestrogens. *Ballières Clin Endocrinol Metab* **12**, 605–623.

Anderson JW (2003) Diet first, then medication for hypercholesterolemia. *J Am Med Assoc* **290**, 531–533.

Anderson JW, Johnstone BM & Cook-Newell ME (1995) Meta-analysis of the effects of soy protein intake on serum lipids. *N Engl J Med* **333**, 276–282.

Anderson JW, Smith BM & Washnock CS (1999) Cardiovascular and renal benefits of dry bean and soybean intake. *Am J Clin Nutr* **70**, Suppl., 464S–474S.

Anonymous (1998) Joint British recommendations on prevention of coronary heart disease in clinical practice. British Cardiac Society, British Hyperlipidaemia Association, British Hypertension Society, endorsed by the British Diabetic Association. *Heart* **80**, Suppl. 2, 1S–29S.

Anonymous (2002) Third Report of the National Cholesterol Education Program (NCEP). Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III final report). *Circulation* **106**, 3143–3421.

Avorn J, Monette J, Lacour A, Bohn RL, Mogun H & LeLorier J (1998) Persistence of use of lipid-lowering medications: a cross-national study. *J Am Med Assoc* **279**, 1458–1462.

Baum JA, Teng H, Erdman JW Jr, *et al.* (1998) Long-term intake of soy protein improves blood lipid profiles and increases mononuclear cell low-density-lipoprotein receptor messenger RNA in hypercholesterolemic, postmenopausal women. *Am J Clin Nutr* **68**, 545–551.

Boushey CJ, Beresford SA, Omenn GS & Motulsky AG (1995) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *JAMA* **274**, 1049–1057.

Crouse JR III, Morgan T, Terry JG, Ellis J, Vitolins M & Burke GL (1999) A randomized trial comparing the effect of casein with that of soy protein containing varying amounts of isoflavones of plasma concentrations of lipids and lipoproteins. *Arch Intern Med* **159**, 2070–2076.

European Agency for the Evaluation of Medicinal Products (1996) *ICH Topic E6: Note for Guidance on Good Clinical*

Practice, CPMP/ICH/135/95. London: European Agency for the Evaluation of Medicinal Products.

European Agency for the Evaluation of Medicinal Products (1997) *ICH Topic E8: Note for Guidance on General Considerations for Clinical Trials*, CPMP/ICH/291/95. London: European Agency for the Evaluation of Medicinal Products.

European Agency for the Evaluation of Medicinal Products (1998) *ICH Topic E9: Note for Guidance on Statistical Principles for Clinical Trials*, CPMP/ICH/363/96. London: European Agency for the Evaluation of Medicinal Products.

European Agency for the Evaluation of Medicinal Products (2000) *ICH Topic E10: Note for Guidance on Choice of Control Groups in Clinical Trials*, CPMP/ICH/364/96. London: European Agency for the Evaluation of Medicinal Products.

European Agency for the Evaluation of Medicinal Products (2001) *Concept Paper on the Development of a Committee for Proprietary Medicinal Products (CPMP) Note for Guidance on the Evaluation of Medicinal Products for the Treatment of Dyslipoproteinaemia*, CPMP/EWP/512/01. London: European Agency for the Evaluation of Medicinal Products.

Friedewald WT, Levy RI & Fredrickson DS (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* **18**, 499–502.

Gerhard GT & Duell PB (1999) Homocysteine and atherosclerosis. *Curr Opin Lipidol* **10**, 417–428.

Grundy SM (2000) Early detection of high cholesterol levels in young adults. *J Am Med Assoc* **284**, 865–867.

Heart Protection Study Collaborative Group (2002) MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20 536 high-risk individuals: a randomised placebo-controlled trial. *Lancet* **360**, 7–22.

Hsia SL, He JL, Nie Y, Fong K & Milikowski C (1996) The hypocholesterolemic and antiatherogenic effects of topically applied phosphatidylcholine in rabbits with heritable hypercholesterolemia. *Artery* **22**, 1–23.

Ishida T, Koba K, Sugano M, Imaizumi K, Watanabe S & Minoshima RM (1988) Cholesterol levels and eicosanoid production in rats fed phosphatidylinositol or soybean lecithin. *J Nutr Sci Vitaminol* **34**, 237–244.

Law MR, Wald NJ & Thompson SG (1994) By how much and how quickly does reduction in serum cholesterol concentration lower risk of ischaemic heart disease? *BMJ* **308**, 367–372.

LeBlanc MJ, Gavino V, Perea A, Yousef IM, Lévy E & Tuchweber B (1998) The role of dietary choline in the beneficial effects of lecithin on the secretion of biliary lipids in rats. *Biochim Biophys Acta* **1393**, 223–234.

Lovati MR, Manzoni C, Gianazza E, Arnoldi A, Kurowska E, Carroll KK & Sirtori CR (2000) Soy protein peptides regulate cholesterol homeostasis in Hep G2 cells. *J Nutr* **130**, 2543–2549.

Merz-Demlow BE, Duncan AM, Wangen KE, Xu X, Carr TP, Phipps WR & Kurzer MS (2000) Soy isoflavones improve plasma lipids in normocholesterolemic, premenopausal women. *Am J Clin Nutr* **71**, 1462–1469.

Monkman D (2000) Treating dyslipidaemia in primary care (editorial comment). *BMJ* **321**, 1299–1300.

Navder KP, Baraona E & Lieber CS (2000) Dilinoleoylphosphatidylcholine protects human low density lipoproteins against oxidation. *Atherosclerosis* **152**, 89–95.

Nishina PM & Freedland RA (1990) The effects of dietary fiber feeding on cholesterol metabolism in rats. *J Nutr* **120**, 800–805.

Pietinen P, Vartiainen E, Seppänen R, Aro A & Puska P (1996) Changes in diet in Finland from 1972 to 1992: impact on coronary heart disease risk. *Prev Med* **25**, 243–250.

Polichetti E, Diaconescu N, de la Porte PL, Malli L, Portugal H, Pauli AM, Lafont H, Tuchweber B, Yousef I & Chanussot F (1996) Cholesterol-lowering effect of soyabean lecithin in normolipidaemic rats by stimulation of biliary lipid secretion. *Br J Nutr* **75**, 471–481.

Polichetti E, Janisson A, de la Porte PL, Portugal H, Léonardi J, Luna A, La Droitte P & Chanussot F (2000) Dietary polyenylphosphatidylcholine decreases cholesterolemia in hypercholesterolemic rabbits: role of the hepato-biliary axis. *Life Sci* **67**, 2563–2576.

Potter SM (1998) Soy proteins and cardiovascular disease: the impact of bioactive components in soy. *Nutr Rev* **56**, 231–235.

Primatesta P & Poulter NR (2000) Lipid concentrations and the use of lipid lowering drugs: evidence from a national cross sectional survey. *BMJ* **321**, 1322–1325.

Puska P, Korpelainen V, Høie LH, Skovlund E, Lahti T & Smerud K (2002) Soy in hypercholesterolemia: a double-blind, placebo-controlled trial. *Eur J Clin Nutr* **56**, 352–357.

Rosseneu M, Declercq B, Vandamme D, Vercaemst R, Soetewey F, Peeters H & Blaton V (1979) Influence of oral polyunsaturated and saturated phospholipid treatment on the lipid composition and fatty acid profile of chimpanzee lipoproteins. *Atherosclerosis* **32**, 141–153.

Rössner S & Flaten H (1997) VLCD versus LCD in long-term treatment of obesity. *Int J Obes Relat Metab Disord* **21**, 22–26.

Ryttig KR, Flaten H & Rössner S (1997) Long-term effects of a very low calorie diet (Nutrilett) in obesity treatment. A prospective, randomized, comparison between VLCD and a hypocaloric diet + behaviour modification and their combination. *Int J Obes Relat Metab Disord* **21**, 574–579.

Sandström B (1995) Quality criteria in human experimental nutrition research. *Eur J Clin Nutr* **49**, 315–322.

Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, McKillop JH & Packard CJ (1995) Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *N Engl J Med* **333**, 1301–1307.

Stamler J, Daviglus ML, Garside DB, Dyer AR, Greenland P & Neaton JD (2000) Relationship of baseline serum cholesterol levels in 3 large cohorts of younger men to long-term coronary, cardiovascular, and all-cause mortality and to longevity. *J Am Med Assoc* **284**, 311–318.

Teixeira SR, Potter SM, Weigel R, Hannum S, Erdman JW Jr & Hasler CM (2000) Effects of feeding 4 levels of soy protein for 3 and 6 weeks on blood lipids and apolipoproteins in moderately hypercholesterolemic men. *Am J Clin Nutr* **71**, 1077–1084.

The Long Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group (1998) Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. *N Engl J Med* **339**, 1349–1357.

The Scandinavian Simvastatin Survival Study Group (1994) Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet* **344**, 1383–1389.

The Swedish Medical Product Agency and the Norwegian Medicines Control Authority (1996) Workshop: *Treatment of Hyperlipidaemia. 1996–1992*. Stockholm: The Swedish Medical Product Agency and the Norwegian Medicines Control Authority.

Tonstad S, Smerud K & Høie L (2002) A comparison of the effects of 2 doses of soy protein or casein on serum lipids, serum lipoproteins, and plasma total homocysteine in hypercholesterolemic subjects. *Am J Clin Nutr* **76**, 78–84.

US Food and Drug Administration (1999) Food labeling: health claims; soy protein and coronary heart disease. Federal register:

October 26, 1999 (Volume 64, Number 206) Rules and regulations: 21 CFR part 101 (Docket no. 98P-0683): 57699–731. www.wais.access.gpo.gov

Vartiainen E, Puska P, Pekkanen J, Tuomilehto & Jousilahti P (1994) Changes in risk factors explain changes in mortality from ischaemic heart disease in Finland. *BMJ* **309**, 23–27.

Wald DS, Law M & Morris JK (2002) Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *BMJ* **325**, 1202–1206.

Wang GJ, Lapcik O, Hampl R, Uehara M, Al-Maharik N, Stumpf K, Mikola H, Wahala K & Adlercreutz H (2000) Time-resolved fluoroimmunoassay of plasma daidzein and genistein. *Steroids* **65**, 339–348.

Wong WW, O'Brian Smith E, Stuff JE, Hachey DL, Heird WC & Pownell HJ (1998) Cholesterol-lowering effect of soy protein in normocholesterolemic and hypercholesterolemic men. *Am J Clin Nutr* **68**, Suppl., 1385S–1389S.

Article 3

Treatment with Abacor®, a soy-based dietary supplement, further reduces plasma concentrations of total and low-density lipoprotein cholesterol in statin-treated hypercholesterolaemic patients

P Clausen, J Lindhardsen, LH Høie, S Stender

Published in Innovative Food Science and Emerging Technologies 2004; 5: 377-383

Article 4

Lipid-lowering effect of 2 dosages of a soy protein supplement in hypercholesterolemia

LH Høie, HJ Graubaum, A Harde, J Gruenwald, KD Wernecke
Published in Advances in Therapy 2005; 22 (2): 175-186

Article 5

Ultra heat treatment destroys cholesterol-lowering effect of a soy protein

LH Høie, Å Sjoholm, M Guldstrand, HJF Zunft, W Lueder, HJ Graubaum, J Gruenwald

Published in International Journal of Food Sciences and Nutrition 2006; 57 (7/8): 512-519

Article 6

Cholesterol-lowering effects of a new isolated soy protein with high levels of non-denaturated protein in hypercholesterolemic patients

LH Høie, M Guldstrand, Å Sjoholm, HJ Graubaum, J Gruenwald, HJF Zunft, W Lueder

Published in Advances in Therapy 2007; 24 (2): 439-447