Double-blind study of the addition of high-protein soya milk *v.* cows' milk to the diet of patients with severe hypercholesterolaemia and resistance to or intolerance of statins

C. R. Sirtori[1]*, F. Pazzucconi[1], L. Colombo[2], P. Battistin[1], A. Bondioli[1] and K. Descheemaeker[3]

[1]*Center E. Grossi Paoletti, Institute of Pharmacological Sciences, University of Milan, Italy*
[2]*S. Paolo Hospital, Milan, Italy*
[3]*Alpro N.V., Wevelgem, Belgium*

Total substitution of soyabean protein for animal protein in the diet has been repeatedly shown to lower plasma cholesterol levels in hypercholesterolaemic individuals. A new, highly palatable, high-protein soya drink may allow replacement of a significant percentage of animal protein in the diet. The soya drink was given, within a crossover design *v.* a cows' milk preparation of similar composition and taste, to twenty-one severely hypercholesterolaemic patients (mean baseline plasma cholesterol 8·74 mmol/l) with a history of resistance to or intolerance of statin treatment. Each dietary supplement was given for 4 weeks, with a 4-week interval between treatments, Plasma lipid levels were monitored every 2 weeks during each dietary sequence. The concomitant dietary treatment, which had been followed for a long time by all patients, was carefully monitored throughout the study. The soya supplementation reduced plasma total cholesterol level by 6·5 %, when given first, and by 7·4 % when given after cows' milk. When given first, cows' milk resulted in a small, non-significant reduction of plasma cholesterol level (−3·9 %), and when given after soya, it changed total plasma cholesterol to a minimal extent (−1·6 %). Changes in total and LDL-cholesterol levels after 2 and 4 weeks of soya *v.* cows' milk treatment were, thus, respectively −6·1, −7·0 and −6·2, −7·8 % (both *P* < 0·05). These first data from a double-blind study confirm a significant cholesterol-lowering effect of soyabean protein, even when only partly replacing animal protein in the diet, in individuals with extreme plasma cholesterol elevations.

Soyabean protein: Cows' milk: Hypercholesterolaemia

Total replacement of animal protein in the diet with vegetable protein, mainly derived from soyabeans, has been repeatedly shown to reduce total and LDL-cholesterol levels in hypercholesterolaemic individuals (Sirtori *et al.* 1977, 1979).

A recent meta-analysis of thirty-eight clinical trials has confirmed the beneficial effects of a diet high in soyabean protein, particularly in individuals with elevated serum total and LDL-cholesterol levels (Anderson *et al.* 1995). Several studies have indicated that a high daily intake of dietary soyabean protein can provide optimal results in these individuals (Sirtori *et al.* 1979, 1995; Carroll, 1991; Anderson *et al.* 1995). Studies in human subjects and appropriate animal models have, finally, documented that the mechanism does not appear to be linked to intestinal sterol loss (Calvert *et al.* 1981; Fumagalli *et al.* 1982; Anderson *et al.* 1984), but rather to the direct activation of LDL-receptors in liver cells (Lovati *et al.* 1987).

This mechanism of action of soyabean protein would thus appear to be different from that of cholesterol-lowering hydroxymethylglutaryl CoA reductase (*EC* 1.1.1.34) inhibitors (statins) that, instead, activate LDL-receptors as a consequence of inhibited sterol biosynthesis (Sirtori, 1993). A significant synergism was recently reported in rabbits between the hypocholesterolaemic effect of soyabean protein and that of a statin, with remarkable differences from casein-fed animals (Giroux *et al.* 1997).

In view of the frequent occurrence of severely hypercholesterolaemic patients who do not achieve satisfactory control of cholesterolaemia by statin treatment (Simons *et al.* 1992; Pazzucconi *et al.* 1995; Rubinstein & Weintraub, 1995), it appeared of interest to evaluate the effect of soyabean protein intake in such subjects. In a previous open study, total replacement of animal protein with a textured soya product over a 1-month period had resulted in a 15 % LDL-cholesterol reduction in patients with a primary resistance to statins (Sirtori *et al.* 1999). The availability of a high-protein milk analogue made with either

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* **Corresponding author:** Professor Cesare Sirtori, fax +39 02 29 404 961, email cesare.sirtori@unimi.it

casein or soyabean protein provided the opportunity for a controlled double-blind investigation in a series of severely hypercholesterolaemic patients, some of whom had shown either an insufficient response or a long-term tolerance to the cholesterol reduction induced by statins (Pazzucconi *et al.* 1995; Rubinstein *et al.* 1995), or significant side-effects.

Subjects and methods

Patients

Twenty-one patients with severe hypercholesterolaemia, thirteen of genetic origin (familial hypercholesterolaemia), eight of a sporadic nature, all with a total plasma cholesterol level ≥7·8 mmol/l and LDL-cholesterol ≥5·8 mmol/l in the absence of drug therapy, with normal or mildly elevated triacylglycerol levels (< 2 mmol/l), participated in the study. Their major biochemical and clinical characteristics are reported in Table 1.

Only one of the patients had symptomatic coronary artery disease, i.e. an effort-related angina not requiring surgical vascularization. Exclusion criteria included: type I or type II diabetes needing medication, alcoholism or alcoholic liver disease, and severe ailments such as cancer, degenerative mental disease, and heart or kidney failure.

Of the selected patients, six had shown an inadequate response to statin treatment (mainly simvastatin); two were non-responders and four had shown tolerance to the statin effect, expressed as an escape phenomenon after 6 months or longer of successful therapy (Pazzucconi *et al.* 1995; Rubinstein *et al.* 1995). Thirteen had shown some form of intolerance: five had abnormalities in laboratory tests of liver function (all were carriers of a previous hepatitis B infection); one had an episode of severe rhabdomyolysis; the remaining patients complained of various forms of subjective intolerance to one or more statins, mainly at the gastrointestinal level, with refusal to continue intake of these drugs.

The protocol was fully explained to the participants during group information sessions before the study. In these sessions, the patients were allowed to taste the chocolate-flavoured soya and cows' milk drinks. All signed a consent form for the study, approved by the Ethical Committee of the Center E. Grossi Paoletti.

Experimental protocol

The study was a randomized crossover trial. All patients, after quitting statin treatment (for at least 2 months), were maintained on their low-cholesterol, elevated polyunsaturated : saturated fatty acid diet (Sirtori *et al.* 1986), which had been followed by all for at least 2 years before the present study. With the low-lipid, high polyunsaturated : saturated fatty acid diet, and maintenance of body weight within normal limits, patients generally presented, off drug therapy, LDL-cholesterol levels fluctuating by no more than ± 5 %.

After this run-in period, at the end of which two blood samples (2 weeks apart) were collected in order to verify the stability of plasma lipoprotein levels, the patients received a slightly changed dietary schedule, with self-selected food items reducing the total protein content to approximately 10 % of total dietary energy, mainly from vegetables, allowing, however, two or three meals per week of lean meat (not more than 20 g protein), at the expense of the vegetables. Examples of this dietary schedule are given in Table 2, providing respectively a low (5852 kJ) and an average daily energy intake (8360 kJ). This type of dietary management allowed the introduction of the approximately 35 g/d of soyabean or cows' milk proteins. These were especially prepared in order to achieve an approximately doubled protein content (7 % *v.* 3·5 % in normal cows' milk) and were given as two daily portions, each of 250 ml fluid, in coded carton packages, delivered, similarly to commercial fruit juices, with an attached straw. Cartons were identical and the coding was not known either to the patients or to the investigators. The two products were virtually indistinguishable in taste. The study had a design similar to that of a double-blind drug study. Patients were kept on each product for 4 weeks, being examined after 2 and 4 weeks. At the 4-week visit, milk intake (either soya or cows') was interrupted and patients returned after 4 more weeks, following which they were given the alternate milk, always in double-blind conditions.

Fasting blood samples for the determination of plasma lipid and lipoprotein levels were obtained twice during the run-in period and at 2 and 4 weeks during each arm of the crossover trial. A final sample was drawn 4 weeks after the end of the second experimental period. Participants were weighed before each treatment began and at each clinic visit. Sitting blood pressure was also taken at each visit after a 5 min rest.

In order to maintain optimal compliance with the prescribed regimen, patients were carefully monitored as to dietary intake. They kept daily food records, indicating the consumption of test diet foods and any food items that were not part of the therapeutic diet, along with the number of

Table 1. Characteristics of the patients participating in the study

(Mean values, standard deviations and ranges for twenty-one subjects (eight male, thirteen female)

	Mean	SD	Range
Age (years)	51·9	13·5	23–70
Height (m)	1·674	0·092	1·55–1·83
Initial weight (kg)	68·5	12·6	45–92
Final weight (kg)	66·7	11·9	46–87
Initial BMI (kg/m^2)	24·4	3·6	18·7–31·2
Total plasma cholesterol at baseline (mmol/l)	8·74	0·74	7·88–10·47
LDL-cholesterol at baseline (mmol/l)	6·39	0·95	5·16–8·26

Table 2. Examples of daily dietary schedules used during the study*

Meal	Component	Quantity
5852 kJ (1400 kcal) diet		
Breakfast	Tea or coffee	
	Bread	50 g
Lunch	Pasta or rice	50 g
	Maize oil	7 g
	Vegetables†	130 g
	Maize oil	7 g
	Bread	50 g
	Fruit‡	100 g
Dinner	Pasta or rice	50 g
	Maize oil	7 g
	Vegetables†	130 g
	Maize oil	7 g
	Bread	50 g
	Fruit‡	100 g

Diet provided (% energy): carbohydrate 56·7, fat 25·1 (P : S 2·75 for soya milk, 2·65 for cows' milk), protein 18·2.

Meal	Component	Quantity
8360 kJ (2000 kcal) diet		
Breakfast	Tea or coffee	
	Bread	75 g
Lunch	Pasta or rice	80 g
	Maize oil	10 g
	Vegetables†	200 g
	Maize oil	10 g
	Bread	75 g
	Fruit‡	150 g
Dinner	Pasta or rice	80 g
	Maize oil	10 g
	Vegetables†	200 g
	Maize oil	10 g
	Bread	75 g
	Fruit‡	150 g

Diet provided (% energy): carbohydrate 59·9, fat 24·3 (P : S 2·75 for soya milk, 2·65 for cows' milk), protein 15·8.

P : S, polyunsaturated fatty acid : saturated fatty acid.

* In both dietary schedules patients consumed, daily, two cartons (500 ml) of milk, either soya or cows', containing (g/l): protein 70, fat 22 (P : S 2·0 for soya milk, 0·4 for cows' milk, with a total difference between the two milks of < 5 g saturated fat/d), digestible sugars 15, soluble indigestible sugars (guar-gum based) 27·4. The isoflavone content of the soya drink consisted of (mg/l): total genistein 40·34, total daidzein 21·29, total glycetin 2·65, leading to a total daily consumption of about 32 mg isoflavones. The 500 ml milk, either soya or cows' provided 1124 kJ/d.

† Any fresh seasonal vegetable, raw or cooked, with the exception of potatoes and legumes. At two or three meals per week lean meat (30 g for the 5852 kJ diet, 50 g for the 8360 kJ diet) was included.

‡ Any fresh seasonal fruit.

soya or cows' milk cartons taken daily. Dietary records were collected during the last 3 d of each treatment period, and were analysed for nutrient intake by the Dietosystem for Windows Program (DIETOSYSTEM, Milan, Italy), specifically tailored for the Italian food items. Compared with the dietary schedules given in Table 2, patients did not generally deviate by more than 10–15 % in their 3 d records.

Laboratory methods

All patients underwent a complete laboratory evaluation, including urinalysis and haematology, at baseline and at the end of the controlled investigation. At all visits they underwent a complete lipid and lipoprotein evaluation including total cholesterol and triacylglycerol levels by enzymic methods (using a Cobas Mira Bio Reactor, Roche, Basel, Switzerland), HDL-cholesterol by selective precipitation with dextran–MgCl₂ (Warnick *et al.* 1982), followed by calculation of LDL-cholesterol levels by the Friedewald formula (Friedewald *et al.* 1972), as well as fasting glucose levels.

Statistical analyses

Data were analysed with a repeated measures two-factor ANOVA in a crossover design test, according to an 'intention to treat' protocol. In order to take into account the crossover design of the study, the procedure of Wallenstein & Fischer (1977) was followed. As statistical significance was only seen by the comparison of pre- *v.* post-intervention data by Student's *t* test within a soya-diet period (see later), a one-way ANOVA was performed on the data of all patients pooled into a single soya milk–cows' milk sequence, thus simulating a linear model study. Data from both groups when on soya milk were added at times 0, 2 and 4 weeks, data from the cows' milk periods were equally treated, thus obtaining a single group of twenty-one patients. Analysis according to the Bonferroni method was then applied in order to detect significant differences.

Results

All twenty-one patients completed the trial. Acceptability of the two products was excellent in seventeen patients, adequate in three and only one complained of significant side-effects. Side-effects occurred mainly during the cows' milk period, probably as a result of lactose intolerance (present in about 50 % of the adult Mediterranean population; Ladas *et al.* 1991), and led to a moderate weight loss during the cows' milk period in some of the patients, with an overall mean body weight reduction of 1–2 %. While this may have resulted in a fall in plasma cholesterol, according to the 'intention to treat' statistical analysis, all patients were considered in the final calculation. During soya milk and washout periods, however, weight was essentially constant. None of the weight changes reached statistical significance. Questioning of the patients at the end of the study as to the indication of which milk was soya and which was cows' resulted in correct responses by only twelve out of twenty-one, thus supporting the good comparability of the two products. No changes in major laboratory values (biochemical and haematological) were recorded at the end of either the first or the second period of study (results not shown).

Lipid and lipoprotein changes

During the soya milk treatment, total plasma cholesterol level decreased by 6·5 % in the group that had soya milk as the first treatment and by 7·4 % in the group treated with soya milk after the cows' milk supplementation. Cows' milk reduced plasma cholesterol levels clearly below baseline only when given first (−3·9 %); when given second the reduction was only −1·6 % (neither of the two statistically significant). Accordingly, LDL-cholesterol levels were reduced respectively by −5 % and −11 % (this latter *P* < 0·01 *v.* baseline by Student's *t* test) in the two different
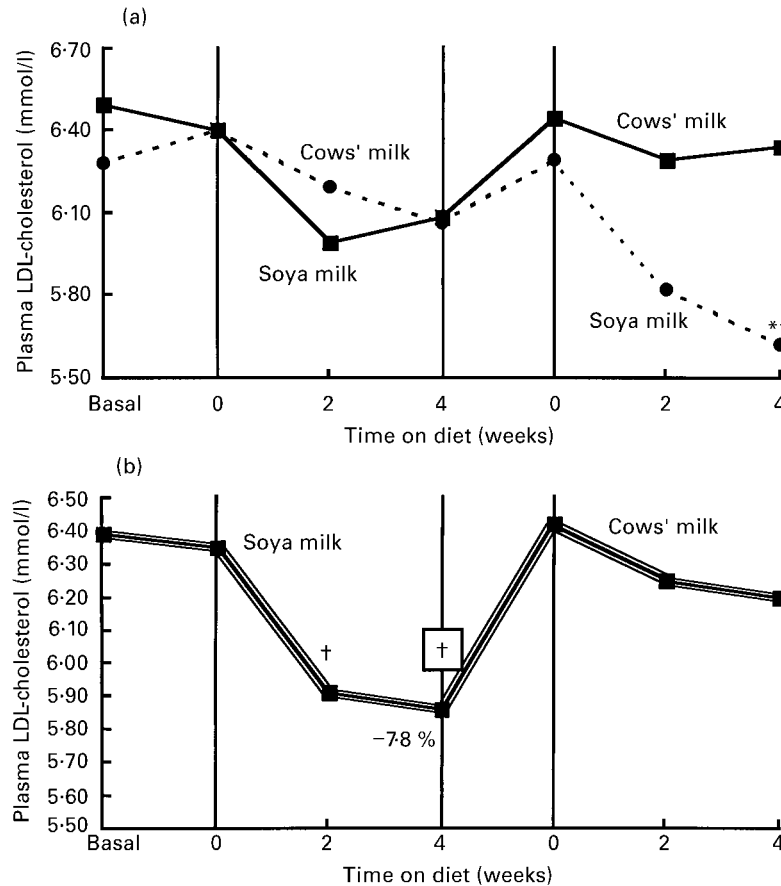
Fig. 1. Changes in plasma LDL-cholesterol concentrations of hypercholesterolaemic patients undergoing dietary intervention with high-protein soya or cows' milk drinks (500 ml/d). (a) Mean values in the two sequences for all twenty-one subjects. (●- - -●), Sequence cows' milk–soya milk; (■–■), sequence soya milk–cows' milk. (b) Data normalized into a single sequence cows' milk–soya milk, more clearly indicating the significant effect of soya milk *v.* cows' milk on LDL-cholesterol levels. Mean value was significantly different from baseline: **$P < 0.01$ (Student's *t* test). Mean values were significantly different from those for cows' milk: †$P < 0.05$ (one-way ANOVA after Bonferroni's correction).

groups when receiving the soya milk supplementation. By analysing data with repeated measures two-way ANOVA, total and LDL-cholesterol changes occurring with the soya milk supplementation *v.* cows' milk did not reach statistical significance (Fig. 1(a)). However, when analysing pooled data, i.e. data from the two series of patients, into one single sequence (soya milk–cows' milk), one-way ANOVA indicated a statistically significant effect. The Bonferroni method revealed that after soya milk treatment total and LDL-cholesterol levels were significantly reduced by 6·1 and 7·0 % after 2 weeks and by 6·2 and 7 8·% after 4 weeks (all $P < 0.05$) *v.* cows' milk (Fig. 1(b)).

Triacylglycerol, HDL-cholesterol and fasting glucose levels did not show any important variations during the whole study. Only at the 2-week point was a rise of triacylglycerol level (+ 13·3 %) observed in the group receiving cows' milk supplementation as the second treatment period; this finding was not confirmed at the 4-week determination. Blood pressure changes were also minimal and not statistically significant (results not shown).

Discussion

Addition or substitution of soyabean proteins to the diet of hypercholesterolaemic individuals has consistently resulted in a significant plasma cholesterol reduction. As yet, however, there has been no controlled investigation of this phenomenon, taking into account all of the variables that might potentially be involved in the hypocholesterolaemic effect. Some authors have raised the point that differences in the dietary cholesterol intake might have affected results (Lopez-Miranda *et al.* 1994), although in all of our major studies we managed to provide an equivalent cholesterol intake in diets with and without soyabean proteins (Lovati *et al.* 1987; Sirtori *et al.* 1999). The cholesterol contents of the two supplements in this study were essentially identical, i.e. close to nil. Furthermore, it was questioned whether soya, because of the presence of complex, at times not fully characterized components, might affect plasma cholesterol levels by other mechanisms. 'Fibre-like' properties, due to the presence of saponins or dietary fibres, and endocrine or

'hormone-like' properties have been suggested, because of concomitant endocrine changes or the presence of isoflavones. The potential of soyabean protein to act by fibre-like mechanisms was clearly ruled out by earlier studies (Calvert *et al.* 1981; Fumagalli *et al.* 1982; Anderson *et al.* 1984) and more recent clinical experiments (Bakhit *et al.* 1994). In contrast to other fibre-rich vegetables, the cholesterol reduction with soyabean protein is not associated with an increased faecal excretion of neutral or acidic sterols and/or to changes in the plasma cholesterol turnover, but rather to the stimulation of the LDL-receptor activity in liver cells (Calvert *et al.* 1981; Fumagalli *et al.* 1982; Anderson *et al.* 1984; Lovati *et al.* 1987). A potential endocrine mechanism, i.e. an increased glucagon : insulin ratio, was indicated by our group many years ago (Noseda *et al.* 1982), and is supported by a recent clinical study in mildly hypercholesterolaemic patients (Frühbeck *et al.* 1997). This would, however, again result in increased liver LDL-receptor activity.

We have recently reported that in most studies carried out in Italy from 1981 onwards, the soyabean protein preparations administered to patients were essentially free of isoflavones (Sirtori *et al.* 1997); this was not the case for the soya milk used in the present study, which indeed contained a small amount of isoflavones (about 32 mg/d). This amount is well below that indicated as potentially useful to exert vascular protective effects in a recent editorial (Finkel, 1998). Furthermore, in most studies examining the possible cholesterol-reducing effect of isoflavones, daily doses were well above 50 mg/d (Nestel *et al.* 1997; Hodgson *et al.* 1998; Potter, 1998). Isoflavones, because of their inhibitory action on tyrosine kinase (*EC* 2.7.1.112), a major regulator of LDL-receptor activity in liver cells (Grove *et al.* 1991), might possibly exert a detrimental effect on LDL levels. Data from primates, indicative of a direct cholesterol-lowering effect of ethanol-extractable components of soyabean protein (mainly isoflavones) (Anthony *et al.* 1996), were not confirmed by a more recent study in gerbils (Tovar-Palacios *et al.* 1998); furthermore, in a very recent study on apolipoprotein E knockout mice a soyabean protein isolate exerted impressive anti-atherosclerotic activity *v.* no effect of the ethanol-extractable components (Ni *et al.* 1998). Although the objective of the present study was to make a double-blind comparison of two similarly palatable dietary additions, not to contribute to the issue of the role of isoflavones in lipid lowering, the data obtained are indicative, if anything, of a somewhat lower hypolipidaemic effect than expected, particularly compared with the previous open study with a preparation without isoflavones (Sirtori *et al.* 1999). The patients selected in the present study had, however, more severe hypercholesterolaemia and the dietary design was different. This in fact, did not require total substitution of dietary proteins with the milk/s but only a predominant intake of vegetables as the major additional source of protein, thus not reaching the high percentage protein intake indicated as appropriate in previous studies (Sirtori *et al.* 1995). It had, moreover, a very low fat and high polyunsaturated : saturated fatty acid content, shown in previous comparative investigations to somewhat reduce the efficacy of the soyabean protein diet (Sirtori *et al.* 1979), thus clearly indicating that the dietary effect was over and above that exerted by the lipid changes in the diet.

In addition to the predictable differences between an open study and the present double-blind investigation, the intestinal effects of the reference product, cows' milk, detected in lactose intolerant patients, may have been responsible for a modest cholesterol reduction, thus erroneously reducing the comparative efficacy of soya milk. The results of the present study also confirm a recent investigation, with a similar protocol, carried out in subjects with borderline hypercholesterolaemia; in these, a statistically significant improvement of the LDL : HDL cholesterol ratio, more marked in subjects with a more significant elevation of baseline plasma cholesterol levels, was reported after soyabean protein and/ or oil supplementation (Kurowska *et al.* 1997).

There is growing interest in the use of soyabean proteins for the treatment of human disease. Indications range from cancer prevention, where a role for isoflavones is indicated (Cassidy *et al.* 1994), to the reduction of atherosclerotic disease, particularly in high-risk individuals, e.g. hypercholesterolaemic subjects (Anderson *et al.* 1995; Sirtori *et al.* 1999). Very recently the US Food and Drug Administration proposed allowing some soyabean products to carry a label indicating that they may reduce heart disease risk (Finkel, 1998).

The mechanisms of the cholesterol-reducing effect of soyabean protein are, as yet, incompletely clarified: novel data from primate studies indicate, however, that the addition of a soyabean protein regimen to a standard cholesterol-lowering treatment may result in astonishing improvements in the lipid and lipoprotein profile (Wilson *et al.* 1998). The present study, carried out in severely hypercholesterolaemic patients, who had shown inadequate response to or intolerance of the most commonly used drug treatment for hypercholesterolaemia, provides the first data from a double-blind study on the effects of the dietary addition of a readily available, well tolerated soyabean protein-based dietary product. In view of the recently reported additive effects of soyabean protein to the hypocholesterolaemic action of a statin in experimental animals (Giroux *et al.* 1997), it seems now of special interest to evaluate this dietary addition in patients where the response to the major lipid lowering drugs is inadequate.

Acknowledgement

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