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Survival and cardiovascular pathology of heterozygous Watanabe heritable hyperlipidaemic rabbits treated with pravastatin and probucol on a low-cholesterol (0.03%)-enriched diet

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Abstract This study was aimed at determining the effects of a combined pravastatin and probucol regimen on survival and vascular pathology of heterozygous Watanabe heritable hyperlipidaemic (WHHL) rabbits fed a low-cholesterol (0.03%)-enriched diet. Pravastatin monotherapy preceded the combined treatment. In animals receiving pravastatin and the enriched diet (verum group; $n = 6$), mean total serum cholesterol levels were consistently lowered at a dosage of 5 mg/kg pravastatin and with the combined treatment. Survival was increased (median 45 vs 25 months), while coronary atherosclerosis was less obstructive and altered to a more fibrous type than in controls ($n = 8$). The extent of aortic lesions, as determined by the relative plaque volume, was not related to survival in either group. However, aortic plaque types in verum group animals revealed less severe stages with a different composition and architecture, with a lower relative content of macrophage-derived foam cells and necrosis and a higher relative content of extracellular matrix. There was also a thicker fibrous cap than in control animals of similar age. Our data reveal a beneficial effect on survival of heterozygous WHHL rabbits when lipid-lowering and antioxidative treatment are combined. This appears to be due both to reduced coronary atherosclerosis and to a different, more stable type of atherosclerotic disease in this animal model.

Key words Atherosclerosis · Pathology · WHHL rabbits · Pravastatin · Probucol · Morphometric analysis · Survival

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Introduction

Watanabe heritable hyperlipidaemic (WHHL) rabbits develop spontaneous atherosclerosis as the result of a defect in the LDL receptor that causes familial hypercholesterolaemia. This strain of rabbits is a well-accepted animal model for human atherosclerosis [2, 6, 15]. Cholesterol-fed heterozygous WHHL rabbits develop atherosclerotic lesions similar to those seen in homozygous rabbits and humans [3]. The combined effects of a genetic disorder and diet may represent the more relevant model for the pathogenesis of atherosclerosis for the majority of humans [2, 3].

The HMG-CoA reductase inhibitor pravastatin has been shown to lower cholesterol serum levels, and it also reduces the extent of atherosclerotic disease in WHHL rabbits [34]. Recently, human studies have shown a significant reduction in cardiovascular morbidity and mortality with pravastatin, as well as an inhibitory effect on progression of coronary atherosclerosis [13, 22, 23, 27]. Probucol, a lipid-lowering and potently antioxidative drug, also exhibits antiatherosclerotic properties in homozygous WHHL rabbits, an effect that is thought to be caused mainly by its antioxidative potency [4, 7, 16, 33].

The present study was undertaken to determine the long-term effects of combined therapy with pravastatin and probucol on heterozygous WHHL rabbits fed a low-cholesterol (0.03%)-enriched diet. Total serum cholesterol levels, survival, aortic and coronary atherosclerotic disease and organ pathology were examined.

Methods

Randomly selected 8-month-old heterozygous WHHL rabbits ($n = 17$) from several litters bred at the animal facilities of the Medical Hospital of the University of Hamburg, were fed a low-cholesterol-enriched diet (0.03% w/w, Altromin no. 3/463, Altromin, Lage, Germany) for a period of 6 months. Three animals (2 females, 1 male) were then sacrificed as baseline controls, 8 animals (3 females, 5 males) continued the low-cholesterol-enriched diet as controls (control group) and 6 animals (3 females, 3 males) re-

Table 1 Mean total cholesterol levels (TC) [*sd* standard diet, *lc* low cholesterol, *prav* pravastatin (mg/kg body weight), *prob* 1g probucol, *p* no. of values used to calculate mean TC, *n* no. of animals per group, values represent medians (mg/dl)]

Chow Conditions	Controls				Treatment Conditions	Cases				*P*
	Female	(*n*)	Male	(*n*)		Female	(*n*)	Male	(*n*)	
sd	73	(3)	56	(5)	sd	74	(3)	68	(3)	2
lc	374	(3)	219	(5)	lc	390	(3)	230	(3)	2
lc	323	(3)	179	(5)	lc, 1 mg prav	425	(3)	146	(3)	2
lc	303	(3)	230	(3)	lc, 10 mg prav	375	(3)	127	(3)	2
lc	416	(2)	282	(3)	lc, 30 mg prav	409	(3)	504	(3)	2
lc	693	(1)	540	(3)	lc, 5 mg prav	432	(2)	294	(3)	2
lc	491	(1)	236	(3)	lc, 5 mg prav+prob	228	(2)	123	(3)	4

ceived the low-cholesterol-enriched diet plus pravastatin for 11 months (verum group). In order to define the optimal cholesterol-lowering dose, pravastatin was given successively at a daily dose per kilogram of body weight of: 1 mg for 11 weeks, 10 mg for 4 weeks, 30 mg for 5 weeks and then 5 mg. After these 11 months, the verum group received the low-cholesterol-enriched diet plus 5 mg/kg pravastatin and 1 g probucol, while the control group continued on the low-cholesterol-enriched diet. Pravastatin supplementation of the diet was adjusted to body weights, which were determined periodically during the experiment. The amount of food supplied was 100 g/day; leftover food was weighed, and no significant differences in food intake were found between the animals. Total serum cholesterol levels were determined periodically throughout the experiment using a commercial kit (Boehringer Mannheim, Mannheim, Germany). Mean total serum cholesterol levels for each animal were calculated over total life time (mTC, deduced from the area under the curve for each animal over the total life time) and under the different treatment and chow conditions. The experimental procedure had been approved by an ethical committee.

After spontaneous death, a complete autopsy was performed and the aorta and internal organs were asservated in 4% neutral buffered formalin. The luminal surface of the aorta was photographed. The following specimens were embedded in paraffin: aorta (30 cross sections), heart (5 cross sections), liver (3 specimens), lungs, kidneys, spleen, skin, skeletal muscle, adrenals and gonads (1 specimen of each).

The extent of aortic atherosclerosis was determined macroscopically by relative aortic plaque area (RPA; precentage of total aortic surface, computer-assisted planimetry (Kontron Images, Eching, Germany) on photographs, magnified by a factor of 2.5) and histologically by mean aortic plaque thickness (mPT, [mm], computer-assisted measurement of the aortic plaque thickness at intervals of 1 mm at every cross-sectional level, perpendicular to the medial lamellae). Relative plaque volume (RPV, [%]) represents the ratio of total aortic plaque volume to the aortic lumen and was calculated by the following formula: $RPV = 4\pi \times RPA \times mPT \times L/F$, where L is the aortic length, and F, the total aortic surface.

The plaque composition (content of macrophage-derived foam cells, necrotic material, smooth muscle cells and extracellular matrix as a percentage of cross-sectional plaque area) was analysed semiquantitatively, using a six-step grading system (≤5%, ≤20%, ≤40%, ≤60%, ≤80%, >80%), and independently by three morphologists. The major cellular plaque constituents were analysed using immunohistochemical methods (i.e. monoclonal antibodies HHF 35 [Enzo Diagnostics, New York] for smooth muscle cells and RAM 11 [Dako, Hamburg, Germany] for macrophage-derived foam cells, diluted according to the manufacturers' recommendations), using the ABC technique. The major noncellular components (necrotic material and extracellular matrix) were analysed by histological methods, (staining with haematoxylin and eosin and a combined stain of Goldner's variant of Masson's trichrome, Van Gieson's elastica and the Kossa technique for calcification), as described previously [4]. The plaque type, a synopsis for plaque composition and architecture, was determined according to the classification of the AHA [30] and Stary [29]. Coronary atherosclerosis was determined using the following criteria: 1, diffuse or adaptive intimal thickening, 2–5, degree of stenosis <20%, 20–50%, 50–95%, >95%, according to the most severe lesion. Organ pathology was performed according to standard pathological criteria. Inter-observer agreement reached nearly 100%.

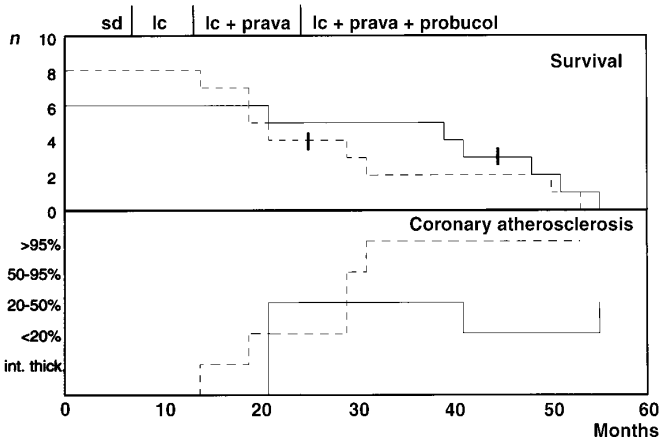
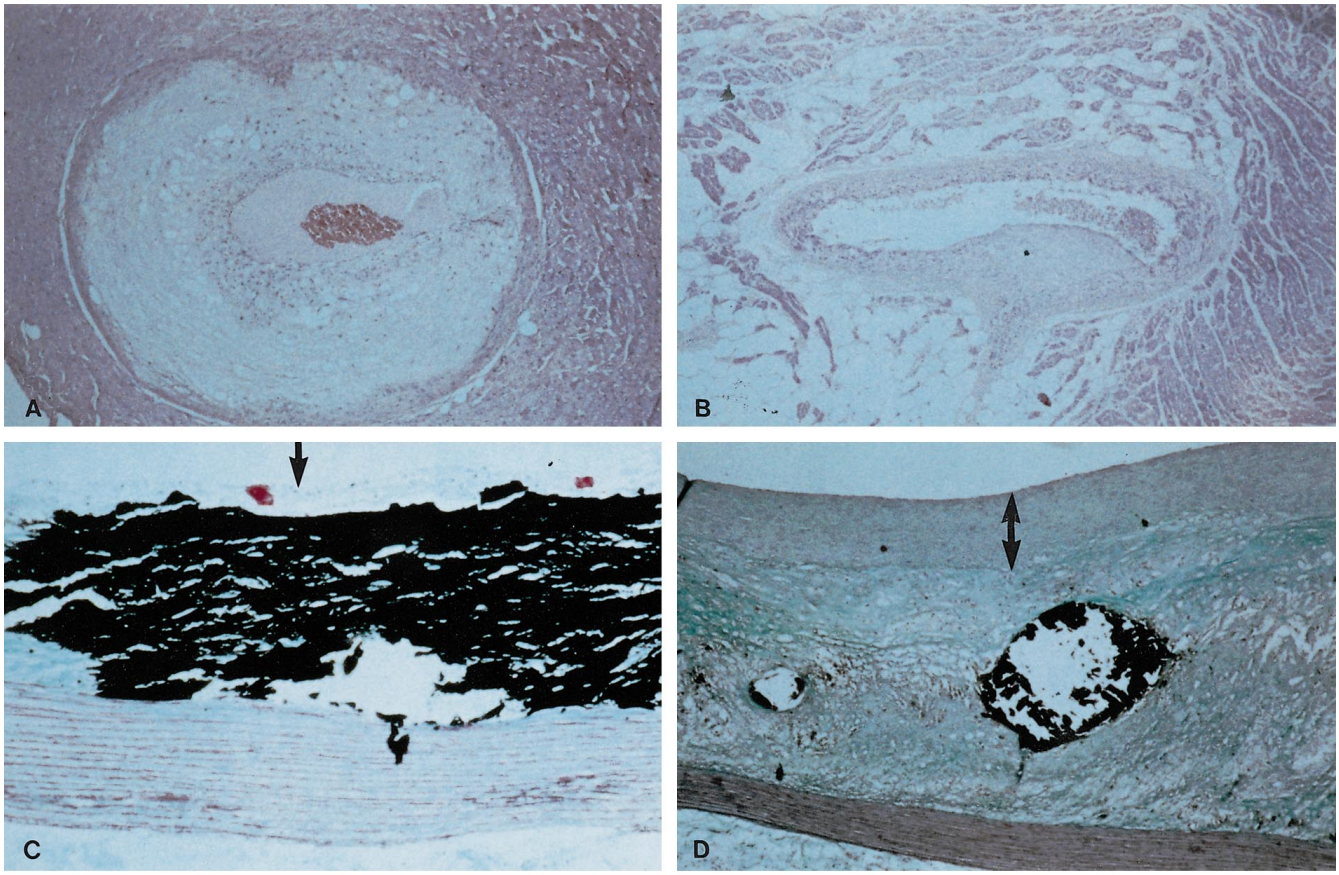


Fig. 1 Survival and coronary atherosclerosis of verum (*continuous line*) and control animals (*dotted line*) shown by Kaplan–Meier curves. *Above* dietary and treatment periods are shown, standard diet (*sd*), low cholesterol-enriched diet (*lc*), pravastatin monotherapy (*prava*) and combined pravastatin and probucol therapy (*prava + probucol*). *Grey bars* median survival, *int. thick.* = intimal thickening

Data are presented as median values. Statistical analyses were carried out by the Mann–Whitney U-test. Owing to the low numbers of animals at similar ages the U-test cannot be used for morphometric analyses.

Results

Within 5 months of starting the low-cholesterol-enriched diet, mean total serum cholesterol levels increased 5-fold in females (73 to 382 mg/dl) and 4-fold in males (56 to 224 mg/dl). When verum group animals were compared with control animals, mean total serum cholesterol levels were found to be lower in males at doses of 1, 5, 10 mg/kg pravastatin (23%, 46%, 45%, respectively) and in females only at 5 mg/kg (54%), whereas at other doses of pravastatin the mean total serum cholesterol levels increased. During the combined treatment mean total serum cholesterol levels in verum animals were lower by about 50% in both sexes than in control animals (Table 1).



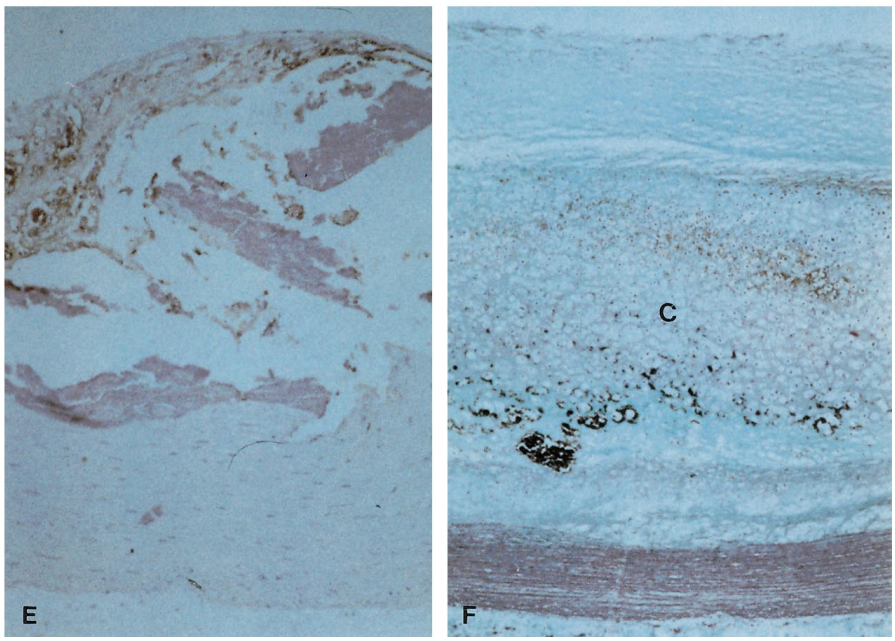


Fig. 2A, B Comparison of typical coronary atherosclerosis between a control (**A**, no. 6) and a verum animal (**B**, no. 8). Note the severely stenosing circular and macrophage-derived foam cell- as well as lipid- and necrosis-rich lesion in the control animal compared with the denser and more fibrous lesion in the verum animal, which is located at a branch site. **C, D** Typical aortic lesion of a control (**C**, no. 13) and a verum animal (**D**, no. 14). Notice the large lesion with a thick fibrous cap (*double arrow*) in the verum animal and the thin macrophage-rich cap (*arrow*) overlying a calcified necrotic core (*black*) in the control animal. **E** Immunohistochemical stain for macrophages (*brown*) infiltrating the fibrous cap of the plaque in the control animal. **F** Large aortic atherosclerotic lesion with cartilage (*c*) formation in a verum animal (no. 8). **C**, **D, F** combined stain, **E** immunohistochemical stain with RAM11

The longest lifespan was 55 months and did not much differ between the two groups; 2 verum animals died at slightly higher ages (51 and 55 months) than 2 control animals (50 and 53 months). Median survival was prolonged against that in the control group (45 vs 25 months, $P = 0.07$; Fig. 1). In the control animals coronary atherosclerosis worsened with age and was occlusive or nearly occlusive after 31 months without further changes in older animals (Fig. 1). In contrast, in verum animals the degree of stenosis never exceeded 50%. Coronary atherosclerosis showed histological differences between the two groups, being of a more dense and fibromuscular type in the verum animals (Fig. 2A, B). Myocardial infarctions were evident histologically in 2 con-

Table 2 Age, mean total serum cholesterol level over total life time (*mTC*) and results of morphometric analyses (*RPA* relative aortic plaque area, *mPT* mean aortic plaque thickness, *RPV* relative aortic plaque volume, *mfc* macrophage-derived foam cells, *nec* necrotic material, *smc* smooth muscle cells, *ecn* extracellular matrix, *c* control, *v* verum, *f* female, *m* male)

Animal no. sex/group	age (months)	mTC (mg/dl)	RPA (%)	mPT (mm)	RPV (%)	mfc (%)	nec (%)	sms (%)	ecm (%)	Plaque type[b] (AHA, Stary)
1 m/c	14	232	97	0.23	23	40	20	60	20	2
2 f/c	18	262	63	0.42	30	60	20	20	40	6
3 m/c	19	131	7	0.06	1	5	5	80	20	3
4 f/v	20	290	95	0.42	47	40	20	20	40	4
5 f/c	21	265	97	0.40	49	20	40	20	40	5
6 m/c	28	380	93	0.74	95	40	40	20	40	6
7 f/c	31	473	71	0.42	34	20	20	40	40	6
8 m/v	38	308	94	0.96	79	20	40	20	80	5
9 m/v	41	172	22	0.19	4	5	5	60	60	5
10 m/v	48	126	51	0.22	15	5	40	20	60	6
11 m/c	50	180	100	0.68	64	20	80	20	40	7
12 f/v	51	298	97	0.39	47	5	40	20	60	6
13 m/c	53	216	100	0.44	50	20	80	20	20	7
14 f/v	55	312	100	0.89	87	5	80	20	40	7

[a] Animals are in order of age
[b] Numbers in this column are aortic plaque types

trol animals (nos. 6 and 11). Verum animals showed fewer foci of myocardial fibrosis than controls. Intrapulmonary and intrarenal arterial lesions revealed fibromuscular intimal thickening increasing with age and showed no difference between the two groups in severity or type. All animals showed epithelial fatty changes in the liver, which were more severe in control animals. Portal mononuclear infiltrates of the liver were observed in both groups, with predominance in verum animals. No difference was detected in foci of interstitial nephritis, whereas renal interstitial fibrosis was less severe in verum animals. There were no histologically evident differences between the two groups in the other organs analysed.

In "baseline" animals, aortic and coronary lesions were not detected macroscopically or histologically. The extent of aortic lesions determined by RPA, mPT and RPV was not related to survival in either group. Furthermore, RPA, mPT and RPV were not found to be related to mTC when compared in animals of similar ages (Table 2). Two verum animals (nos. 8 and 14) with a high RPV had large fibrous plaques with a huge core consisting of variably calcified cartilage (Table 2, Fig. 2F). The control female with the highest mTC (no. 7) had a relatively small RPV (Table 2).

A lower percent content of macrophage-derived foam cells and necrosis and a higher percent content of extracellular matrix were found in verum animals than in controls, whereas the percent content of smooth muscle cells was the same in both groups (Table 2). Generally, with increasing age the percent of necrosis increased and that of macrophage-derived foam cells decreased; that of extracellular matrix showed a maximum in middle aged animals. There was no relation between the percent content of smooth muscle cells and ageing. The percent of smooth muscle cells was high in animals with a small RPV (nos. 3 and 9) and in animal no. 1, which died first at the age of 14 months (Table 2). Aortic plaques of verum animals, especially at more advanced ages, were more fibrous and had a thicker fibrous cap containing fewer macrophages (Fig. 2C–E). Analysis of aortic plaque types, determined according to the classification of the AHA and Stary, revealed more severe stages in the control group than in verum animals at similar ages (Table 2).

Discussion

The present study was aimed at determining long-term effects of a combined treatment with pravastatin and probucol on survival and cardiovascular disease, using low-cholesterol-fed (0.03% cholesterol, w/w) heterozygous WHHL rabbits (WHHL-Hh) as a model for human disease [2, 3]. Our data, obtained over a period of 4.5 years (55 months), show a beneficial effect of this regimen on survival, coronary atherosclerosis and the composition and architecture of aortic atherosclerotic disease.

Median survival was nearly twice as long in verum animals as in controls and was inversely related to the severity of coronary atherosclerosis, which never exceeded 50% stenosis in verum animals. Furthermore, histologically evident myocardial infarctions occurred only in the control group. In verum animals the extent of coronary atherosclerosis was lowered and the plaque type modulated, giving a more dense and fibromuscular appearance (Fig. 2A, B). Aortic plaques in verum animals revealed a different type of atherosclerosis rather than merely a reduced plaque mass: severe lesions occurred later; there was a lower relative content of macrophage-derived foam cells and necrosis; and lesions had a more fibrous appearance with a thicker fibrous cap (Fig. 2C–E). Two animals with a high plaque volume (nos. 8 and 14) were from the verum group, and histological analysis revealed large fibrous aortic plaques with a partly calcified chondromatous core (Fig. 2F). Recent publications suggest that cartilage and bone formation within atherosclerotic

plaques are formed by active mechanisms [9] thought to represent reparative processes [24]. Pravastatin has been shown not to inhibit smooth muscle cell proliferation [8], thus permitting better reparative activity.

A combined treatment with pravastatin and probucol has been shown to reduce cholesterol levels [26], apparently due mostly to pravastatin, as we have found while comparing the phases of 5 mg pravastatin and the combined treatment. Surprisingly, a consistent lipid-lowering effect of pravastatin monotherapy was seen at only 5 mg/kg for both males and females, although pravastatin monotherapy also produced a beneficial effect on survival (Fig. 1). Studies with pravastatin monotherapy have revealed a dose-dependent lowering effect on total cholesterol levels [25]. The absence of this effect in this study may have been caused by the natural history of cholesterol levels in WHHL-Hh rabbits [10], and the fact that cholesterol feeding preceded the treatment may also have attenuated the effect of the monotherapy. Increasing cholesterol levels after a change of dose of HMG CoA reductase inhibitors were observed in the males in this study and have also been reported by other authors as an escape phenomenon [35]. However, each dosage was given for several weeks and should have had some effect. Our data do not resolve this issue: both pravastatin monotherapy and the combination of pravastatin and probucol had beneficial effects on survival coinciding with reduced coronary atherosclerotic lesions.

Our observations are in agreement with recent data in humans: lowering serum cholesterol significantly reduces morbidity and mortality from coronary heart disease [13, 19, 22, 23, 27]. Differences in plaque types between verum and control animals might be due to lower serum cholesterol levels in the verum group. Similar effects of a lipid-lowering therapy with pravastatin on plaque composition and extent have recently been shown in WHHL rabbits [28]. However, the oldest control animals had lower mTC but even more complicated lesions than verum animals. Additional mechanisms must thus be involved in the genesis of this phenomenon. We suggest that the altered plaque type might be attribuable to the effects of probucol. One crucial point in atherogenesis is the oxidation status of the lipids involved [31, 33], and data from studies in animals show a dramatic reduction of planimetrically determined aortic atherosclerotic disease progression with probucol treatment [16] with cholesterol-matched conditions [7]. This effect is due, at least in part, to the antioxidative properties of probucol on lipoprotein particles [21], although other effects, including enhanced reverse cholesterol transport [12], may also play a part. Probucol has been shown to alter the composition of atherosclerotic lesions in WHHL rabbits mostly by reducing macrophage content and the necrotic material content [4, 20, 32]. Further, a different plaque architecture after probucol treatment has been described, suggesting more stable lesions [4]. The findings of the present study are in agreement with these observations and show an association with an increased longevity. Although pravastatin also bears antioxidative properties [17], probucol is a more powerful candidate for antioxidative effects than pravastatin.

The key event in sudden arterial occlusion and ischaemia is rupture of an atherosclerotic lesion with subsequent thrombo-haemorrhagic occlusion; this is not directly related to the size of the lesion [14]. Factors that determine the risk of plaque rupture are such morphological criteria as the presence and size of an atheromatous core and the presence, thickness and roughness of the fibrous cap, and weakening features of the latter, such as macrophage infiltration, collagen degradation and smooth muscle cell damage. All these changes can be caused by an aggressive oxidative environment [11]. This study shows that the composition of atherosclerotic lesions is changed by a combined treatment using pravastatin and probucol and that the plaque architecture is altered, leading to more fibrous plaques and thicker fibrous caps containing less macrophages. In patients with established cardiovascular disease, who are at higher risk of myocardial infarction [18], a radical lipid-lowering strategy is thought to be able to inhibit progression and to lead to a reduction in lipid content, thus influencing the morphology of atherosclerotic lesions [5, 19]. In humans, only one study has investigated the pathobiological effects of a combined lipid-lowering and antioxidative treatment using probucol and lovastatin: this showed an improvement of endothelium-dependent vasomotion [1].

Our study demonstrates the importance of a synergistic effect when cholesterol is lowered and lipoproteins are protected against oxidation, which seems to shift atherosclerosis towards a more stable plaque type and to result in increased survival.

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