*Biochimica et Biophysica Acta*, 1181 (1993) 279–286



BBADIS 61266

# Effect of pravastatin, a HMG CoA reductase inhibitor, on blood lipids and aortic lipidosis in cholesterol-fed White Carneau pigeons [1]

Peter Hadjiisky [a], Dominique Hermier [c], Jacqueline Truffert [b], Jean-Luc De Gennes [b] and Yves Grosgogeat [a]

[a] *Research Center on Cardiovasular Diseases,* [b] *Department of Endocrinology and Metabolism, Medical Faculty Pitié-Salpêtrière, Paris (France) and* [c] *INRA, Nouzilly, Monnaie (France)*

(Received 3 August 1992)
(Revised manuscript received 20 January 1993)

Key words: Cholesterol lowering; Lipoprotein lowering; Primary prevention of aortic lipidosis; Pravastatin; (White Carneau pigeon)

The effect of pravastatin, an inhibitor of HMG CoA reductase, on blood lipids and aortic lipidosis was studied in young cholesterol-fed White Carneau pigeons. The birds were fed with normal ('N group', $n = 20$) or atherogenic diet (grains + 0.4% cholesterol + 4% lard) alone ('C group', $n = 20$) and in association with pravastatin ('P group', $n = 20$). Plasma lipids and aortic intima lipidosis were studied after 3–5 and 8–12 months of the diet. Compared to the N group, pigeons from C group exhibited hypercholesterolemia (TC = 1000 mg/dl) and hyperlipoproteinemia of which level was independent of the duration of the diet. Total VLDL (VLDL + LDL) – cholesterol and apolipoprotein-B levels rose significantly 15, 8 and 4 times, respectively, whereas HDL were increased two times ($P < 0.01$) in females only. Macroscopically visible intima lipidosis areas covered 40% and 80% of aortic surface after 3–5 and 8–12 months of the diet. In P group, the increase in plasma lipid values was significantly lower than in WC from C group: −40% for total cholesterol (600 mg/dl) ($P < 0.01$), −71% for VLDL ($P < 0.001$), −53% for (VLDL + LDL) −cholesterol ($P < 0.01$) and −54% for apo-B ($P < 0.05$). HDL remained as high as in C group. Consequently TC/HDL-C ratio was improved and atherogenic risk of cholesterol was reduced by 41% ($P < 0.05$). Intima lipidosis areas were lowered by 35% ($P < 0.01$). We conclude that pravastatin treatment involves (1) a decrease in hypercholesterolemia and hyperlipoproteinemia and (2) a lowering in extensiveness and severity of macroscopically visible aortic lipidosis in cholesterol-fed White Carneau pigeon.

## Introduction

The 3-hydroxy-3-methylglutaryl-Co enzyme A reductase (HMG CoA reductase, E.C. 1.1.1.34) transforming CoA in mevalonic acid, is the most important rate-limiting enzyme in cholesterol biosynthesis. Excessive amounts of blood cholesterol inhibit this enzyme with consequently lowered endogenous cholesterol production [1]. Potent competitive inhibition of HMG CoA reductase is exerted by some metabolites isolated from fungi and recently reviewed, such as mevastatin (compactin), monacolin K, lovastatin (mevinolin) and simvastatin [2,3]. Pravastatin is one of the more recent member of this family and is produced by microbial transformation of mevastatin in active 6-hydroxy open acid and hydrophilic analog. Works in progress indicate its favorable tolerance and tissue selectivity for liver and intestine [4].

Recent papers reported the beneficial effect of HMG CoA reductase inhibitors (statins), including pravastatin, on human hypercholesterolemia [2,5–8]. These drugs act through inhibition of cholesterol biosynthesis resulting in (1) the increase in the number of LDL-receptors and enhanced removal of plasma LDL-cholesterol by liver cells [9]; (2) the inhibition of VLDL hepatic synthesis and secretion [10,11]. In animals, the response to HMG CoA reductase inhibitors varies from species to species. The inhibitors are ineffective at altering plasma cholesterol in small rodents (rats, mice, hamsters), but they are very effective in

Correspondence to: P. Hadjiisky, C.R.M.C.V., 105 Boulevard de l'Hôpital, 75013 Paris, France.

[1] Partial data were presented in abstract form in the Eur. Heart J., 1990, 11, 188.

Abbreviations: CE, cholesteryl esters; FC, free cholesterol; PL, phospholipid; PR, protein; TC, total cholesterol; TG, triacylglycerol; UA, uric acid; Gly, free glycerol; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; Apo-B, apolipoproteins.

altering of HMG CoA reductase activity [3]. Conversely, they exert potent cholesterol lowering effect in rabbit, dog, miniature pig and monkey [4,5,12].

The interaction between statins and atherosclerotic lesions is not well known. Relevant information is derived from experimental studies only in cholesterol-fed or genetically hyperlipemic rabbits [4,13–19]. Anti-atherogenic effect was reported in rabbits receiving a high-cholesterol diet and HMG CoA reductase inhibitor concomitantly [13,14,16]. Even in genetically hyperlipidemic rabbits (WHHL and St-Thomas strains) which develop spontaneous atherosclerosis, pravastatin [4,8,19,20], simvastatin [15] and lovastatin [17] administrated over some months have been found effective in reducing atherosclerotic aortic surface coronary stenosis and xantoma volume. In man with coronary artery disease, intensive lipid-lowering therapy with lovastatin involves angiographically demonstrable improvement of obstructive lesions [21].

The present study evaluates the effect of pravastatin on White Carneau pigeon. This atherosclerosis-prone and spontaneously hypercholesterolemic model is particularly suitable for studying atherogenesis and for estimating the prevention or regression of atherosclerosis [22,23]. Atherosclerotic plaques in pigeon, morphologically and chemically similar to human lesions, occur predictably near the orifice of the celiac axis in all 6-year-old birds [22,24]. Cholesterol feeding, involving severe hypercholesterolemia, markedly accelerates the development of atherosclerosis and lesions may occur in juvenile birds [25,26]. Moreover, the changes in plasma lipoproteins and in their clearance from circulation have been studied extensively in this model [23].

Our study demonstrates that in the White Carneau pigeon, pravastatin is efficient, at least partly, in preventing lipid and lipoprotein profile alterations and development of aortic lipidosis induced by a cholesterol-rich diet.

## Materials and Methods

*Animals and diets*

Sixty White Carneau pigeons were provided by the Pigeon Selection Center in Bechanne, Saint-Etienne-du-Bois, France. They were 3 months old upon their arrival and had not yet developed spontaneous atherosclerosis [25]. The pigeons were housed in Courcelle-type standard lofts, with a small wire-enclosed space which allows limited exercise. Each loft holds 20–30 birds. Constituted pairs of pigeons were housed in two compartment nesting boxes in natural conditions of light and temperature. At 4 months of age, pigeons were randomly divided into three experimental groups of 10 pairs of birds: (1) the control group (N group) was fed the usual pigeon diet including 40–50% corn, 18–22% wheat, 18–22% green peas, 3% sunflower and 3% tare, 3% minerals and vitamins. The daily consumption was approximatively 50–60 g per pigeon providing 13–15% protein and 320 Kcal/100 g. During the reproduction period, the protein concentration was increased to 20%; (2) the cholesterol-fed group (C group) received an atherogenic diet consisting in the usual pigeon grain diet to which 0.4% cholesterol (w/w) and 4% lard (w/w) were added. Practically, after being pre-heated, the grains were coated with homogenized cholesterol and lard mixture under gentle agitation [25,26]. The daily supplies were weighed, vacuum-packed in plastic bags and stored at −20°C. Under those conditions, the daily consumptions per pigeon were the following: grains 35–45 g, cholesterol 200 mg, lard 2 g; (3) the pravastatin group (P group) was given the same atherogenic diet, associated to pravastatin (courtesy or Bristol-Myers Squibb Laboratories, France) dissolved in drinking water. The 'water-pravastatin mixture' was prepared each morning. Water consumption was monitored in order to insure a drug intake of 40 mg/kg/day (15–20 mg per pigeon).

*Experimentation duration.* In the pigeon, a cholesterol-rich diet is known to induce severe hypercholesterolemia that reaches a plateau after 2–3 months [23]. On the other hand, the degree of induced aortic lipidosis largely depends upon the duration of the atherogenic diet [24–26]. For these reasons, two subgroups were randomly constituted in each experimental group and received the diets for either 3–5 or 8–12 months.

All procedure carried out on the animals conformed to institutional and statutory guidelines.

*Blood samples analysis*

At the end of the experimental feeding, birds were fasted 18 h, anesthetized with pentobarbital (1 ml/kg) and promptly beheaded. Blood was collected in tubes containing EDTA, and plasma was separated by low speed centrifugation. Birds were sacrificed per three on the same day, one from each experimental group.

The following components were determined in plasma: triacylglycerol (TG), phospholipids (PL) and total cholesterol (TC) by enzymatic methods using the kits provided by Boehringer, Mannheim [28–30]; high density lipoprotein-cholesterol (HDL-C) after precipitation of apo-B-containing lipoproteins by phosphotungstic acid and magnesium chloride [31], using the kit provided by Bio-Merieux (Charbonnières-les-Bains, France); free glycerol (Gly) and uric acid (UA) [32] using the respective kits from Biotrol (Paris, France) and Bio-Merieux. Apolipoprotein-B (apo-B) levels were determined by immuno-nephelometric analysis [33], using polyclonal antibodies (Behring, Marburg, Germany) and a nephelometric analyser. Agarose gel electrophoresis was performed on whole plasma essentially as described by Noble [34]. Very low density lipopro-

TABLE I

*Plasma lipid profile in male (M) and female (F) pigeons from 'normal' (N group), 'cholesterol-fed' (C group) and 'cholesterol-fed and pravastatin-treated' (P group) pigeons*

| | | N group (mg/dl) | C group (mg/dl) | C/N ratio | P group (mg/dl) | P/N ratio | P/C ratio |
|---|---|---|---|---|---|---|---|
| TC | M | 305 ± 40 | 1423 ±180 | 4.7 *** | 719 ±119 | 2.3 ** | 0.5 ** |
| | F | 269 ± 20 | 780 ±180 | 2.9 ** | 543 ± 48 | 2.0 ** | 0.7 ** |
| HDL-C | M | 183 ± 40 | 202 ± 20 | 1.1 | 257 ± 30 | 1.4 | 1.3 |
| | F | 116 ± 20 | 255 ± 20 | 2.2 | 209 ± 30 | 1.8 * | 0.8 |
| VLDL+ | M | 122 ± 20 | 1221 ±260 | 10.0 *** | 462 ±110 | 3.8 ** | 0.3 ** |
| LDL-C | F | 153 ± 20 | 525 ±175 | 3.4 * | 334 ± 40 | 2.2 * | 0.6 * |
| Apo-B | M | 18 ± 5 | 241 ± 30 | 13.4 *** | 69 ± 20 | 3.8 ** | 0.3 ** |
| | F | 50 ± 30 | 89 ± 40 | 1.8 | 75 ± 20 | 1.5 | 0.8 |
| TG | M | 78 ± 20 | 147 ± 60 | 1.9 | 59 ± 16 | 0.7 | 0.4 |
| | F | 142 ± 70 | 139 ± 20 | 1.0 | 203 ± 95 | 1.4 * | 1.5 |
| PL | M | 424 ± 80 | 564 ±110 | 1.3 | 631 ± 60 | 1.5 | 1.1 |
| | F | 582 ±160 | 742 ±100 | 1.3 | 650 ±120 | 1.1 | 0.9 |
| GLY | M | 45 ± 7 | 74 ± 10 | 1.6 | 67 ± 7 | 1.5 | 0.9 |
| | F | 80 ± 20 | 54 ± 10 | 0.7 | 58 ± 9 | 0.8 | 1.1 |
| UA | M | 7.7± 1.4 | 9.9± 0.8 | 1.3 | 8.6 ± 1.0 | 1.1 | 0.9 |
| | F | 9.3± 1.2 | 9.8± 0.8 | 1.1 | 10.01± 0.8 | 1.1 | 1.0 |
| Number | | M = 6<br>F = 7 | M = 8<br>F = 10 | | M = 10<br>F = 8 | | |

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

teins (VLDL) were separated from plasma by ultracentrifugation at 1.006 g/ml for a period of 24 h in a 50.3 Beckman rotor [35]. The following components were quantified according to procedures described elsewhere [36]: proteins by Lowry's method in VLDL and triglycerides, free (FC) and total cholesterol and phospholipids by enzymatic methods, using Bio-Merieux kits. Moreover, in some cases, cholesterol distribution among the plasma lipoprotein profile from male White Carneau was estimated in successive fractions after ultracentrifugation in a density gradient [36]. In plasma, low density lipoprotein-cholesterol (LDL-C) is usually calculated from Friedewald's formula: LDL-C = TC − (HDL-C + (TG)/5. Actually, in the present study, cholesterol from 'cholesterol' and 'pravastatin' groups non-transported by HDL was carried by VLDL and their remnants rather than by LDL. For this reason, 'non-HDL-C' was referred to as (VLDL + LDL) − C instead of LDL-C and the coefficient corresponding to TG/5 was omitted. In VLDL, cholesteryl esters (CE)

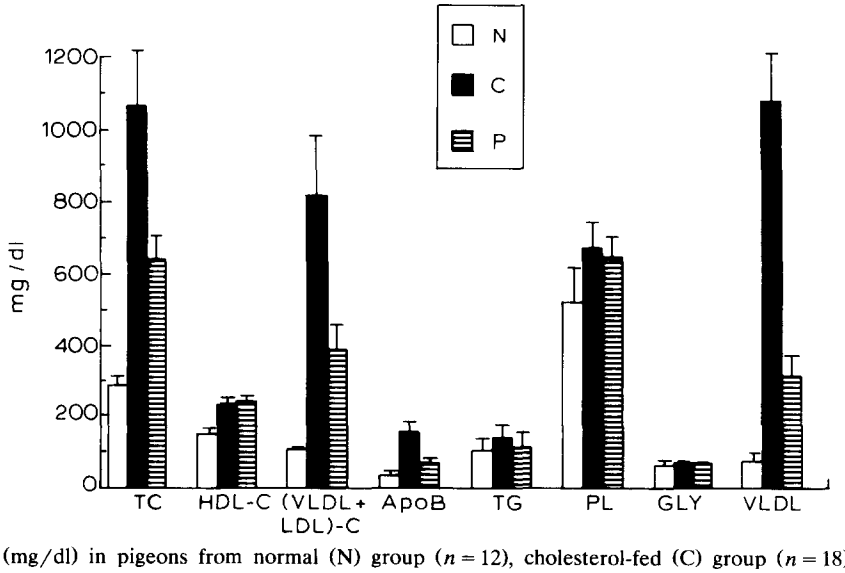


Fig. 1. Blood lipid values (mg/dl) in pigeons from normal (N) group ($n = 12$), cholesterol-fed (C) group ($n = 18$) and cholesterol-fed and pravastatin-treated (P) group ($n = 18$).

were calculated from the following formula: CE = (TC − FC) × 1.67. Total VLDL concentration was the sum of the various lipid and protein components, from which these components were expressed as percentages. Cholesterol-induced atherogenic risk was calculated from Castelli's formula (TC/HDL-C).

As concerns plasma lipid levels, parameters tended to plateau after 3 months of diet. For this reason, results from short-term and long-term subgroups did not differ significantly and were presented and discussed together.

*Determination of fatty streaks in area percent*

The aorta was rapidly removed, opened and examined. Precoeliac aortic area (preferential site of atherosclerotic lesions in White Carneau) was excised for further histological and histochemical studies. The remaining thoracic and superior abdominal aortas were fixed in 4% neutralized formaldehyde and stained in saturated Oil Red 0 solution for one hour. As a result, the intimal surface looked like a 'map' with more or less positive (red) and more or less prominent areas. Considering these two features, a 4-grade rating scale for lipidosis lesions was established: grade 1, poorly delineated and flat reddish areas; grade 2, sharply outlined and flat red areas; grade 3, sharply outlined and slightly elevated dark red areas; grade 4, very prominent dark red 'plaques'. In order to quantify these lesions, aortas were photographed and slides were projected (×13) on a grid with cross-points spaced 0.4 mm apart. Lipidosis areas were quantified by so-called 'points method'. At the magnification used, a cross-point corresponded to 0.16 $mm^2$.

TABLE II

*VLDL concentration and chemical composition*

Results are means ± S.E.M. of the number of pigeons given in parenthesis.

| | "N group" (6) | "C group" (12) | "P group" (10) |
|---|---|---|---|
| Concentration (mg/dl) | 72 ± 21 | 1070 ± 130 *** | 410 ± 82 °°° |
| Composition (%) | 5.4 ± 0.5 | 11.9 ± 0.7 *** | 9.6 ± 1.0 ° |
| Free cholesterol | 17.4 ± 2.5 | 45.9 ± 3.1 ** | 38.0 ± 0.4 ° |
| Triglyceride | 37.7 ± 2.7 | 10.4 ± 2.6 ** | 15.7 ± 3.6 ° |
| Phospholipid | 17.0 ± 0.5 | 21.3 ± 0.8 | 21.2 ± 0.7 |
| Protein | 23.7 ± 2.4 | 10.4 ± 0.7 *** | 14.9 ± 1.9 °° |

Effect of cholesterol vs control: ** $P < 0.01$; *** $P < 0.001$;
Effect of pravastatin vs Cholesterol: ° $P < 0.05$; °° $P < 0.01$; °°° $P < 0.001$.

*Statistical analysis*

Quantitative results were expressed as means ± S.E.M. Means between groups were compared using unpaired Student's *t*-test. The significance threshold chosen was $P < 0.05$.

## Results

*Plasma parameters*

Dietary-induced modifications of plasma lipid parameters were reported in Table I and Fig. 1. They corresponded to dramatic alterations of the lipoprotein profile, as described by VLDL composition and cholesterol distribution (Table II and Fig. 2).

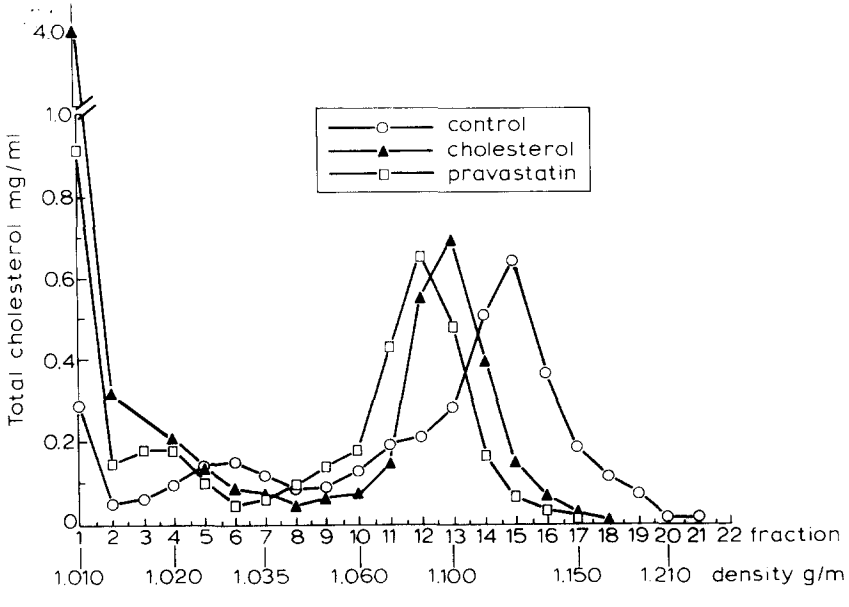


Fig. 2. Quantitative distribution of total cholesterol after separation of lipoproteins from male White Carneau plasma by density gradient ultracentrifugation: fraction 1 corresponded to VLDL, fractions 2–3 to IDL, fractions 4–8 to LDL and fractions 9–20 to HDL.

All control pigeons, aged 7–16 months at the end of the experimental period, exhibited a mild hypercholesterolemia when compared to human cholesterol levels (305 mg/dl in males and 269 mg/dl in females) (Table I). Individual variations were important, especially among females in which they were probably related to vitellogenesis (3 laying females were even withdrawn from the study). In males birds, 60% cholesterol was transported by HDL, whereas this percentage was only 43% in females. The remaining plasma cholesterol was thus transported primarily by LDL, VLDL having only a minor role. Actually, when it could be determined, total VLDL concentration in these fasted birds was found to be only 70 mg/dl (Table II). This low VLDL level also indicated that apo-B and triacylglycerol that were in greater amounts in males than in females, were surprisingly high, especially in females (Table I). Besides, the composition of VLDL in controls was normal, whatever the sex: triglyceride prevailed (38%), while cholesterol (free and esterified) accounted for only 23% of the particle weight (Table II) and corresponded to a plasma concentration of 20 mg/dl only.

The pigeons fed the atherogenic diet quickly developed a marked hypercholesterolemia that plateaued after 3–4 months of diet. The response in males was systematically more abrupt and significant than in females (1420 mg/dl versus 780 mg/dl) (Table I). In both sexes, hypercholesterolemia was associated with a dramatic increase in VLDL level (1070 mg/dl compared to 70 mg/dl in control) (Table II). Besides, VLDL composition was dramatically modified, with cholesterol (58%) prevailing over triacylglycerol (10%). The distribution of cholesterol along the density gradient prepared from plasma indicated that, in cholesterol-fed birds, cholesterol was mainly transported by VLDL and their remnants (Fig. 2). After electrophoresis of agarose gel, these cholesterol-rich VLDL appeared as a band clearly distinct from the one corresponding to LDL from control pigeons (Fig. 3).

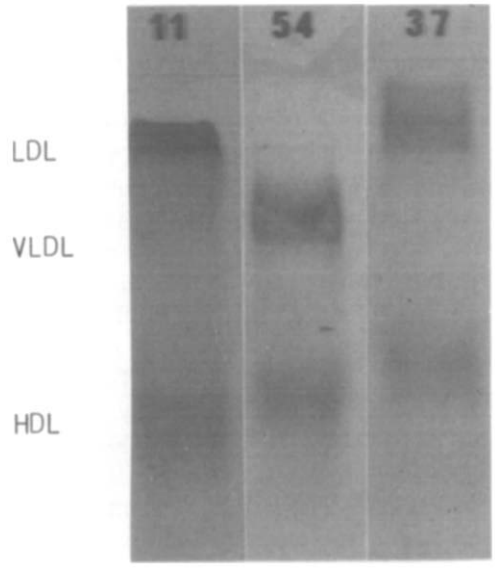


Fig. 3. Agarose gel electrophoretic patterns of plasma lipoproteins from normal-fed (11) cholesterol-fed (54) and cholesterol-fed and pravastatin-treated pigeons (37).

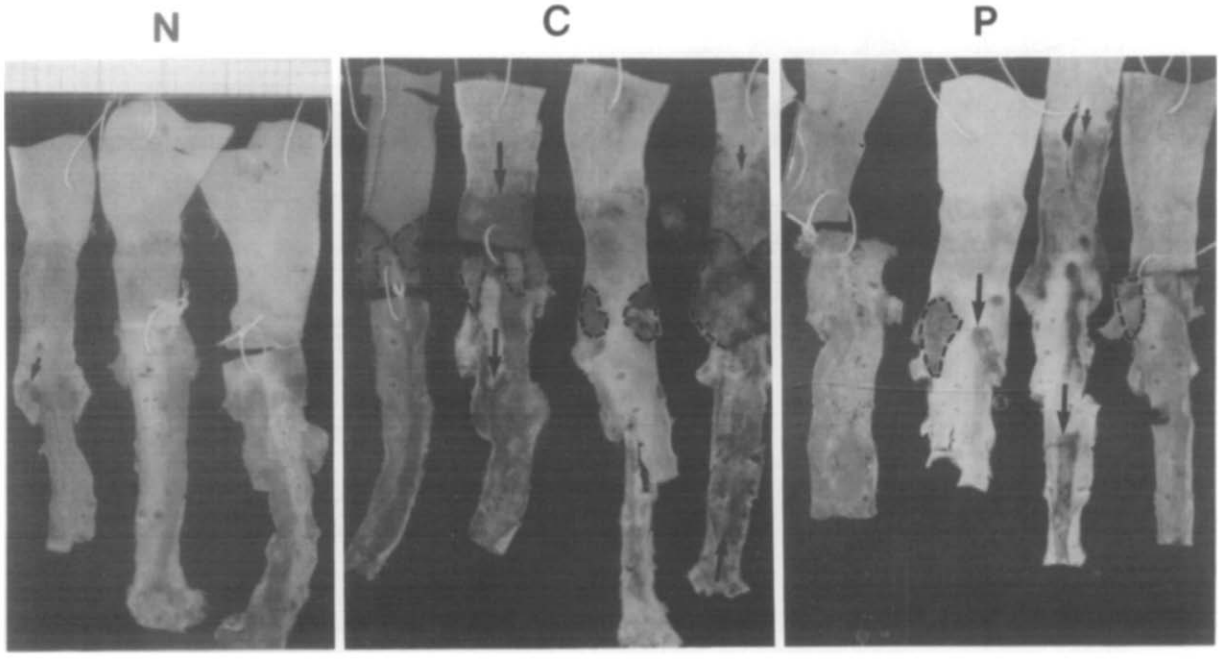


Fig. 4. Intima lipidosis in Oil-Red-stained opened aortas from normal-fed (N), cholesterol-fed (C) and cholesterol-fed and pravastatin-treated pigeons (P): N = normal aspect; C = severe and extent intima lipidosis with prominent grade 3, and grade 4 areas; P = less developed intimal lipidosis. Short arrow = grade 2 lesion; long arrow = grade 3 lesion; grade 4 lesion (atherosclerotic plaques) are surrounded.

Surprisingly, LDL-cholesterol was greatly reduced, whereas the peak corresponding to HDL-cholesterol exhibited a shift towards the lower density range, but was not quantitatively modified in these male pigeons.

The prevalence of cholesterol-rich VLDL was even more striking in males and was paralleled by an increase in plasma levels of apo-B and acylglycerol, whereas these two components were not significantly modified in cholesterol-fed females compared to controls (Table I). On the contrary, HDL-cholesterol increased in females only. However, in both sexes, apo-B-containing lipoproteins (VLDL, IDL and LDL) predominated over HDL. Consequently, cholesterol-induced atherogenic risk (CIAR) increased from 1.9 to 4.6.

In pigeons given pravastatin concomitantly with the atherogenic diet, the effect of dietary cholesterol was significantly blurred, since hypercholesterolemia was reduced to 40% ($P < 0.01$) when compared to untreated birds (Fig. 1). This preventive effect had different impacts on the various lipoprotein classes. It was dramatic on VLDL. This VLDL-lowering effect was mostly quantitative ($-71\%$, $P < 0.001$), since VLDL particles remained rich in cholesterol (38%) and poor in triacylglycerol (16%) (Table II). Similarly, apo-B and triacylglycerol concentration were also reduced, $-54\%$, ($P < 0.05$) and $-20\%$ (ns) respectively (Table I). The analysis of the density gradient profile showed that pravastatin tended to reequilibrate the transport of cholesterol in favour of LDL (Fig. 2). Similarly, lipoprotein profile on agarose gel tended to be normalized, with a $\beta$-migrating band typical of LDL (Fig. 3). On the other hand, HDL-cholesterol did not appear to be modified by the treatment (Table I).

*Aortic lipidosis*

The term 'lipidosis' refers to either flat or prominent intimal lesions observed after lipid staining of opened aorta (Fig. 4).

In Control pigeons, the aortas showed only topographically very limited lipid lesions that covered 13% of their surface (Table III). Grade 1 lipidosis areas predominated in the upper portion of the thoracic aorta. At the abdominal level, a few grade 2 lipid areas were observed. Aortas were virtually free of grade 3 and 4 lesions.

In White Carneau pigeons fed a high cholesterol diet for 3 to 5 months, lipidosis covered 40% of the intimal surface with development of severe grade 3 lesions. The most severe lesions were located in the inferior aorta. In the subgroup receiving the atherogenic diet for 8–12 months, lipidosis was more extensive (78%). Responsiveness variability was blunted and each individual lipidosis index was higher than 50%. Lesions were also more severe. Grade 2, 3 and 4 lesions covered 60% of the aortic surface. Grade 4 lesions, seen in 3 birds out of the 'preceliac lesional areas', looked like atherosclerotic plaques on microscopical contral examinations.

In the pravastatin group, the development of fatty

TABLE III

*Intimal lipidosis-surface morphometry (I) in normal (N group), cholesterol-fed (C group) and cholesterol-fed and pravastatin-treated (P group) pigeons after 3–5 and 8–12 months of diet*

Data are expressed as means ± S.E.M. (rounded off) of oil-red 0 stained surfaces (in $mm^2$) obtained by point methods. $S_1 \ldots_4 \times 100/TS$, when $S_1 -_4$ = surface taken up by a given grade of lipidosis. TS = total intimal surface. LI (lipidosis index) = % of total Oil Red positive surface $[(S_1 \pm S_2 \pm S_3 \pm S_4) \times 100/TS]$.

| | TS ($mm^2$) | Grade 1 LI | | Grade 2 | | Grade 3 | | Grade 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $S_1$ ($mm^2$) | % (a) | $S_2$ ($mm^2$) | % (a) | $S_3$ ($mm^2$) | % (a) | $S_4$ ($mm^2$) | % | |
| N group ($n = 11$) | 211 ± 11 | 26 ± 5 | 12 ± 2 | 2 ± 0.7 | 1 ± 0.3 | 0.4 ± 0.3 | 0.2 ± 0.2 | 0 | 0 | 13 ± 2 |
| C group | | | | | | | | | | |
| 3–5 mo ($n = 11$) | 226 ± 7 | 53 ± 7 | 23 ± 3 | 21 ± 9 | 9 ± 4 | 17 ± 10 | 8 ± 5 | 0 | 0 | 40 ± 5 |
| 8–12 mo ($n = 7$) | 202 ± 8 | 47 ± 10 | 22 ± 4 | 58 ± 19 | 28 ± 9 | 45 ± 17 | 22 ± 8 | 12 ± 7 | 6 ± 4 | 78 ± 6 |
| Total ($n = 18$) | 217 ± 6 | 50 ± 6 | 23 ± 2 | 36 ± 5 | 17 ± 3 | 28 ± 1 | 55 ± 6 | 5 ± 3 | 2 ± 1 | 55 ± 6 |
| P group | | | | | | | | | | |
| 3–5 mo ($n = 12$) | 230 ± 11 | 56 ± 10 | 24 ± 4 | 10 ± 3 | 4 ± 1 | 1 ± 0.8 | 0.6 ± 0.4 | 1 ± 1 | 0.5 ± 0.5 | 29 ± 5 |
| 8–12 mo ($n = 6$) | 191 ± 9 | 48 ± 9 | 25 ± 5 | 36 ± 14 | 21 ± 9 | 8 ± 3 | 4 ± 2 | 2 ± 2 | 1 ± 1 | 51 ± 7 |
| Total ($n = 18$) | 217 ± 9 | 53 ± 7 | 24 ± 3 | 19 ± 6 | 10 ± 3 | 3 ± 1 | 2 ± 0.7 | 2 ± 1 | 0.7 ± 0.5 | 37 ± 4 |
| C/N ratio | 1.02 | 1.9 *** | | 18.5 *** | | 6.5 *** | | 2 | | 4.0 *** |
| P/N ratio | 1.02 | 2.0 *** | | 9.5 *** | | 10 *** | | 0.7 | | 2.6 *** |
| P/C ratio | 1 | 1.04 | | 0.51 * | | 0.15 ** | | 0.35 | | 0.66 *** |

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

streaks was substantially delayed. In White Carneau pigeons on 'short-term diet', the total lipid-containing surface was less extended than in the cholesterol group (29% versus 40%, $P < 0.05$). The most significant ($P > 0.001$) protective effect of pravastatin was exerted on grade 2 and 3 lesions: their surface were found to be 2 and 13 times smaller, respectively, than in the 'short-term cholesterol group'.

In pigeons on 'long-term diet', the effect of pravastatin was more marked. The intimal surface taken up by lipids was less extended than the corresponding cholesterol subgroup (50% versus 80%). This protective effect was mostly exerted on the development of grade 3 lesions. They covered less than 4% of the intimal surface in the pravastatin group versus 22% in cholesterol group ($P < 0.001$). The impact on the extension of grade 4 lesions (plaques) has been more difficult to evaluate as the preceliac aortic segment was removed for further histological and histochemical analyses. Comparisons were carried out in pigeons with 'plaques' located out of the preceliac lesional area: grade 4 lesions observed in two pravastatin-treated birds covered each about 6% of intimal surface in each bird versus 8%, 9% and 25% measured in three pigeons from the cholesterol group.

## Discussion

Applied atherogenic diet was very effective in pigeons which developed a severe hypercholesterolemia associated with a markedly modified lipoprotein contents and ratios. The VLDL which were in lesser amounts in normally-fed pigeons, showed up to 18-fold increases ($P < 0.001$) and became the principal cholesterol carrier (Table II). These VLDL could be identified as atherogenic $\beta$-VLDL on the basis of their high free and esterified cholesterol content and abnormal mobility on agarose gels [27]. Their origin is still controversial. In the cholesterol-fed rabbit, they have been shown to be similar to chylomicron remnants or secreted by the liver [37]. According to Barakat's hypothesis [27], VLDL in pigeons should be of portal and hepatic origin. In the present study, hepatic cholesterol synthesis is severely depressed, and cholesterol transported by $\beta$-VLDL is ultimately of dietary origin. However, since the pigeons were fasted overnight before slaughtering, it is therefore likely that $\beta$-VLDL represented the form under which dietary cholesterol was reassociated with lipoprotein particles in the liver and then secreted. Contrary to findings in mammals [38], no decrease in HDL levels have never been observed in cholesterol-fed pigeons [27]. In the present study, HDL levels even have a tendency to rise in females. Consequently, the increase of atherogenic risk of cholesterol was more striking in males than in females. The reason why the female pigeon seems to be more resistant to plasma changes induced by cholesterol-rich diet is not clear. Despite the fact that laying females were excluded from the present study, it is likely that, even in the non-laying females, the hormonal status interferes with the synthesis and uptake of lipoproteins in relation to the needs of triglyceride and cholesterol for vitellogenesis and steroïdogenesis [23,27].

Pravastatin tended to reduce hypercholesterolemia ($-40\%$) and to change plasma lipoprotein profile in both male and female White Carneau pigeons. Plasma cholesterol distribution among the lipoproteins was still aberrant but the levels of $\beta$-VLDL and their remnants (IDL) were significantly decreased as well as the concentration of apo-B. The HDL, predominant in control pigeons, increased in cholesterol-fed pigeon and remained as elevated after pravastatin treatment. As a result, the TC/HDL ratio was markedly improved and cholesterol-induced atherogenic risk was reduced by 41% ($P < 0.05$) in the pravastatin-treated group. The dramatic decrease in the plasma $\beta$-VLDL level without a change in its composition may be due to either a decrease in production or an increase in uptake. Actually, both hypotheses are likely, since pravastatin exerts its pharmacological effects in different targets. Despite the fact that both the cholesterol group and the pravastatin group received the same amount of dietary cholesterol, the intestinal production of cholesterol-rich lipoproteins may be impaired by pravastatin. It was recently reported that this drug may interact in vitro with cholesterol absorption, esterification and secretion by inhibiting the activity of enterocytic acyl-cholesterol acyltransferase (ACAT) [39,40]. Moreover, pravastatin may reinforce the effect of cholesterol in order to inhibit the synthesis and secretion of cholesterol by the liver [41]. Consequently, the output of both exogenous and endogenous cholesterol is severely depressed by the drug. In parallel, pravastatin may stimulate the clearance of $\beta$-VLDL-cholesterol by enhancing its uptake by hepatic receptors. This effect, demonstrated in mammals for LDL [42], may be of considerable importance in the pigeon since hypercholesterolemia fails to completely inhibit receptor-dependent clearance of $\beta$-VLDL in this species [43]. The persistance of elevated HDL might be of importance in cellular efflux of cholesterol, as shown in vivo and in vitro studies [44].

The treatment by pravastatin modulated significantly the extent and severity of cholesterol-induced aortic lipidosis. In the pravastatin group, the aortic surface taken up by lipidosis was 35% smaller than in pigeons in the cholesterol group. Grade 1 lesions containing only lipid-loaded monocytes-macrophages * were not affected by pravastatin treatment. This 'resistance' is probably due to persisting hypercholesterolemia. Indeed, although cholesterol and lipoprotein levels were improved in pravastatin-treated birds,

they remained 2–3 times higher (600 mg/dl) than those in control pigeons, that is to say, far beyond the threshold for plaque development [23]. Inversely, the protective effect of pravastatin was evident ($P < 0.001$) upon prominent (Grades 3 and 4) lipidosis areas containing large smooth muscle cell compounds. In those cases, the statin may act as anti-atherogenic agent through mechanisms independent from their lipid-lowering effect.

In conclusion, preventively applied pravastatin treatment involves (1) a decrease in induced hypercholesterolemia and hyperlipoproteinemia and (2) a lowering in extensiveness and severity of macroscopically visible aortic lipidosis in cholesterol-fed White Carneau pigeons.

## Acknowledgements

We are grateful to Remy Cheron, Mireille Peuchmaurd, Jean-Pierre Lagarde and Marie-Christine Federspiel for their excellent technical assistance, and to Veronique Villareal for expert preparation of manuscript. We acknowledge the financial support from 'Squibb Laboratories' France and Claude Bernard Association, Paris, France.

## References

1 Brown, M.S. and Goldstein, J.L. (1986) Science 32, 34–47.
2 Alberts, A.M. (1988) In Hypercholesterolemia Clinical and Therapeutic Implications (Stokes, J., Mancini, M., eds). Atherosclerosis Reviews (Raven Press), New York, 18, 123–131.
3 Endo, A.(1988) In Atherosclerosis VIII (Crepaldi, G., Gotto, A.M., Manzato, E. and Baggio, G., eds), pp. 775–780. Elsevier, Amsterdam.
4 Tsujita, Y. and Watanabe, Y. (1989) Cardiovascular Drug Reviews (Raven Press), 7/2, 110–126.
5 Hoeg, J.M. and Brewer Jr., H.B. (1987) J. Am. Med. Assoc. 258, 3532–3536.
6 Hoeg, J.M. (1989) In Atherosclerosi VIII (Crepaldi, G., Gotto, A.M., Manzato, E. and Baggio, G., eds). pp. 785–89. Elsevier, Amsterdam.
7 Mol, M.J.T.M., Erkelens, D.W., Gevers Leuven, J.A., Shouten, J.A. and Stalenhoef, A.F.H. (1988) Atherosclerosis 69, 131–137.
8 Hagemenas, F.C., Pappu, A.S. and Illingworth, D.R. (1990) Eur. J. Clin. Invest. 20, 150–157.
9 Goldstein, J.L. and Brown, M.S. (1983) In The metabolic basis on inherited diseases (Stanbury et al. eds), pp. 672–712. McGraw-Hill, New York.
10 Watanabe, Y., Ito, T., Saeki, M., Kuroda, M., Tanzawa, K. et al. (1981) Atherosclerosis 38, 27–31.
11 Kasim, S.E., Le Boeuf, R.C., Khilnani, S., Tallapaka, L., Dayananda, D. and Jen, K.L.(1992) J. Lipid Res. 33, 1–7.
12 Kritchevsy, D., Tepper, S.A. and Klurfeld, D.M. (1981) Pharmacol. Res. Commun. 13, 921–926.
13 Fiedler, M., Thiery, J., Hartge, K.W., Genschel, M., Pfeiffer, M., Walli, A.K. and Seidel, D. (1988) 8th Intern. Symp. Atheroscl., Rome, Abstract Book (CIC Rome), p. 945.
14 Woolf, N. (1988) In Hyperlipidemia: clinical and therapeutic implications. (Stokes, Y. and Mancini, M. eds). Atherosclerosis Reviews, Vol. 18, pp. 25–47. Raven Press, New York.
15 Fukno, Y., Nagashima, M., Saitoh, A. and Kobayashi, Y. (1988) 8th Intern. Symp. Atheroscler., Rome, Abstract Book (CIC Rome), p. 276.
16 Kobayashi, M., Ishida, F., Takahashi, T., Taguchi, K., Watanabe, K., Ohmura, I. and Kamei, T. (1989) Atherosclerosis 83, 69–80.
17 La Ville, A.E., Seddon, A.M., Shaikh, M., Rowles, P.M., Woolf, N. and Lewis, B. (1989) Atherosclerosis 78, 205–210.
18 Shiomi, M., Ito, T.,Watanabe, Y., Tsujita, Y., Kuroda, M., Arai, M., Fukami, M., Fukushige, J. and Tamura, A. (1990) Atherosclerosis 83, 69–80.
19 Niendorf, A., Beisiegel, U., Harsch, M., Bräsen, J.H., and Niendorf, D.S. (1991) 9th Intern. Symp. Atherosclerosis, Rosemont, USA, Abstract book, p. 481.
20 Watanabe, Y., Ito, T., Shiomi, M., Tsujita, Y., Keiroda, M. et al. (1988) Biochim. Biophys. Acta 960, 294–302.
21 Brown, G., Albers, J.J., Fisher, L.D., Schaefer, S.M., Lin, J.T., Kaplan, C., Zhao, X.Q., Bisson, B.D., Fritzpatrick, V.F. and Dodge, H.T. (1990) N. Engl. J. Med. 323, 1289–98.
22 Prichard, R.W., Clarkson, T.B., Lofland, H.B., Goodman, H.O., Herndon, N. and Netsky, M.G. (1962) J. Am. Med. Assoc. 179, 49–52.
23 Saint-Clair, R.W. (1983) Fed. Proc. 42, 2480–2485.
24 Svindland, A.D., Gallagher, P.J. and Wallo, L. (1986) Acta Path. Microbiol. Immunol. Scand. Sect. A 94, 285–290.
25 Clarkson, T.B. and Lofland, H.B. (1967) Arch. Pathol. 84, 513–516.
26 Jerome, W.G. and Lewis, J.C. (1985) Am. Pathol. 119, 210–222.
27 Barakat, H.A. and Saint-Clair, R.W. (1985) J. Lipid Res., 26, 1252–1268.
28 Kessler, G. and Lederer, H. (1966) In Automation in analytical chemistry – Technicon Symposia 1965. (L.T. Skeggs Jr., ed.) pp. 341–344. Mediad. Inc., New York.
29 Takayama, M., Itoh, S., Nagasaki, T. and Tanimizu, I. (1977) Clin. Chim. Acta 19, 1350–1355.
30 Abell, L.L., Levy, B.B., Brodie, B.B. and Kendall, F.E. (1952) J. Biol. Chem. 195, 357–362.
31 Lopez-Virella, M.F.L., Stone, P., Ellis, S. and Coluell, J.A. (1977) Clin. Chem. 23, 882–884.
32 Barham, D. and Trinder P. (1972) Analyst 97, 142–145.
33 Wider, G.E., Kulnigg, E., Molinari, E., Hotschek, H. and Bayer, P.M. (1982) J. Clin. Chem. Clin. Biochem. 20, 1–8.
34 Noble, R.P. (1968) J. Lipid. Res. 9, 693–700.
35 Havel, T.J., Eder, H.A. and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345–1353.
36 Hermier, D., Forgez, P. and Chapman, M.J. (1985) Biochim. Biophys. Acta 836, 105–118.
37 Thompson, K.H. and Zilversmith, D.B. (1983) J.Nutr. 113, 2002.
38 Slater, H.R., Shepard, J. and Packard, C.J. (1982) Biochim. Biophys. Acta 713, 435–445.
39 Ishida, F., Lizuka, Y. and Kamei, T. (1989) Chem. Pharm. Bull (Tokyo) 37, 1635–1636.
40 Kempen, H.J.M., Vermeer, M., de Wit, E. and Havekes, L.M. (1991) Arteriosclerosis 11, 146–153.
41 Reagan, J.W., Miller, L.R. and Saint-Clair, R.W. (1990) J. Biol. Chem. 265, 9381–9391.
42 Grundy, S.M. (1988) N. Engl. J. Med. 319, 24–33.
43 Reagan, J.W. and Saint-Clair, R.W. (1991) Biochim. Biophys. Acta 1081, 202–210.
44 Johnson, W.J., Mahlberg, F.H., Rothblat, G.H. and Phillips, M.C. (1991) Biochim. Biophys. Acta 1085, 273–298.

* Data from our Histological and Morphometrical Analysis (paper in preparation).