

# Isolation and partial characterisation of a new strain of Ebola virus

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## Summary

We have isolated a new strain of Ebola virus from a non-fatal human case infected during the autopsy of a wild chimpanzee in the Côte-d'Ivoire. The wild troop to which this animal belonged has been decimated by outbreaks of haemorrhagic syndromes. This is the first time that a human infection has been connected to naturally-infected monkeys in Africa. Data from the long-term survey of this troop of chimpanzees could answer questions about the natural reservoir of the Ebola virus.

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## Introduction

Ebola virus was identified in 1976<sup>1</sup> when two outbreaks occurred simultaneously in Southern Sudan<sup>2</sup> and Northern Zaire.<sup>3</sup> The number of cases was 284 in Southern Sudan with 53% mortality and 318 in northern Zaire with 88% mortality. Another fatal case was reported one year later from the same zone of Zaire<sup>4</sup> and a small epidemic was recognised in 1979 in the same town of Sudan.<sup>5</sup> Serological tests suggested that the two strains, Zaire and Sudan, were different, and studies in mice and monkeys also confirmed differences in pathogenicity.<sup>6</sup> Numerous serological surveys have been reported from African populations, mainly using the indirect immunofluorescence assay (IFA), and the prevalence of anti-Ebola antibodies has ranged from 1 to 30%.<sup>7</sup> However, no clinical case has been confirmed for 15 years in Africa. Another strain of Ebola was isolated in 1989 during an outbreak of infection in cynomolgus monkeys (*Macaca fascicularis*) in quarantine in Reston, VA, USA.<sup>8</sup> The monkeys originated from the Philippines. 4 animal handlers were infected in this US facility. All developed antibodies without any clinical signs. Four animal handlers were also found seropositive in the originating Philippine facility, but none had a history of haemorrhagic disease. This Reston strain of Ebola appears to be highly pathogenic for some monkey species but not for man.

We report the isolation of a new strain of Ebola virus from a human case and its connection with an increased mortality among a troop of wild chimpanzees (*Pan troglodytes*) of Côte-d'Ivoire. Details of the epidemiological investigations and clinicopathological findings will be reported separately.

## Subjects and methods

Chimpanzees were studied for 15 years by ethologists<sup>9</sup> in the Tai National Park in western Côte-d'Ivoire. The troop, which numbered 80 animals in 1987, now numbers 33. Two abrupt episodes of mortality were noted in November 1992 (8 deaths) and again in November 1994 (12 deaths). Several dead chimpanzees were found with obvious signs of haemorrhages, but decomposition was too advanced to collect useful biological samples. One freshly dead chimpanzee was discovered on 16 November, 1994 and autopsied in the field. In an attempt to find the cause of death, formalin-fixed tissues from this animal were sent to France with blood samples from the two older males and one female from the troop and sera from members of the ethological team. A 34-year-old female who autopsied the chimpanzee developed a dengue-like syndrome on 24 November. She was hospitalised in Abidjan on the 26th for acute fever resistant to anti-malaria treatment and presented with acute diarrhoea and pruritic rash during the next few days. She was evacuated five days later to Switzerland where she recovered without sequelae.

## Results

Serological tests on these sera were performed against the antigens for the main African haemorrhagic fever viruses. These were IgG and IgM ELISA tests for Congo-Crimean haemorrhagic fever, Rift Valley fever, hantaviruses, yellow fever, Chikungunya, Dengue, and by immunofluorescence for Lassa, Ebola, and Marburg viruses. All the tests were negative. The serum from the patient was taken on 27 November during the febrile phase of her illness. The serum had been kept alternately at ambient temperature and at 4°C for 14 days. Despite these less than optimal conditions, we attempted viral isolation by inoculation of Vero E6 (monkey kidney) and AP61 (*Aedes pseudoscutellaris* mosquito) cells. No obvious cytopathic effect was noted after 12 days so blind passage was done. In the Vero E6 subculture some cells became refractile and detached from the monolayer after 5 days. Immunofluorescence assay was done on these cells with the immune ascitic fluids available in the lab. Anti-RVF, CCHF, Lassa, Hantaan for the haemorrhagic fever viruses and different alpha, toga, and bunyaviruses for the arboviruses were negative. However the haematoxylin-eosin colouration showed large eosinophilic cytoplasmic inclusions in numerous cells.

We requested samples of the patient's late serum from the clinic and two samples from 16 December 94 and 4 January 95 gave bright fluorescence on large cytoplasmic inclusions by IFA. The inclusions were thought to be viral

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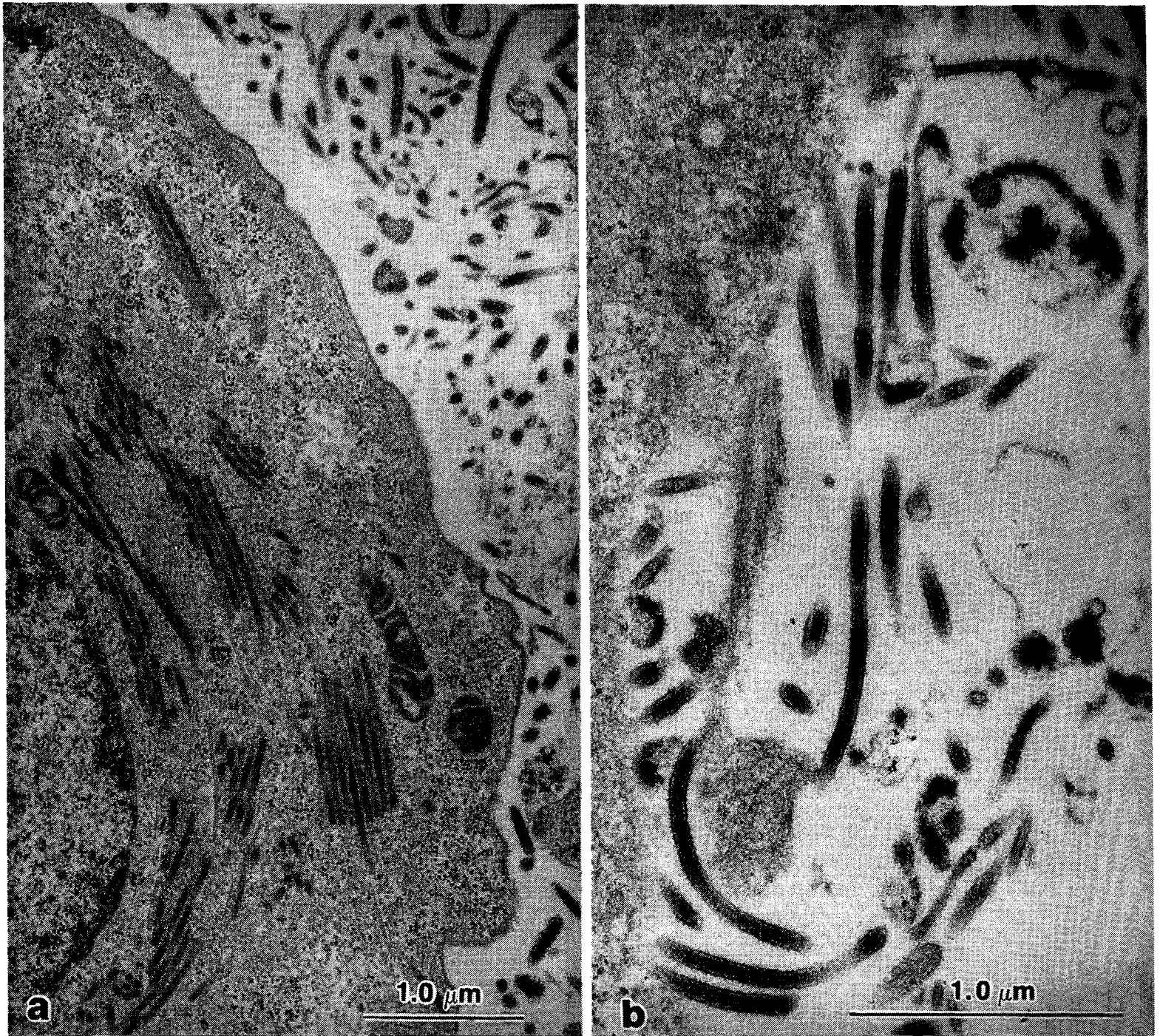


Figure 1: Electron micrograph of a thin section through Vero E6 cells inoculated with the serum of the human patient. Particle with typical filovirus morphology (b) and prominent inclusions formed by nucleocapsids in the cytoplasm (a).

antigens recognised by the patient's antibodies. Electron microscopy performed on the Vero cells from the same subculture revealed the characteristic morphology of a filovirus (figure 1). The late sera of the patients gave a doubtful result on the only Ebola reagent that we had: polyvalent slides prepared with Vero cells infected by the filoviruses Marburg and Ebola Reston, and the arenavirus Lassa.

Specific reagents provided by the Centers for Disease

Strains	Immunofluorescence assay				IgG ELISA				IgM capture ELISA			
	CI	Z	S	R	CI	Z	S	R	CI	Z	S	R
Days post onset												
3	0	0	0	0	0	0	0	0	1250	0	0	0
7	0	0	0	0	0	0	0	0	15 000	0	0	0
23	2048	512	64	64	6000	1600	<100	200	10 000	0	0	0
40	4096	128	<64	<64	10 000	1600	<100	200	3000	0	0	0
78	2048	64	<64	<64	5500	800	<100	200	200	0	0	0

Antigens tested: Slide (Immuno Fluorescence Assay) and borate triton extract (ELISA) of Vero E6 cells infected by Ebola strains Côte d'Ivoire (CI), Zaire (Z), Sudan (S) and Reston (R).

Table 1: Kinetics of antibody titres of the patient against four different Ebola antigens

Control verified characterisation of an Ebola strain. Polyclonal antibodies from rabbit immunised with Ebola Zaire, and a pool of monoclonal antibodies reactive with the three known Ebola strains gave positive results by immunofluorescence assay (IFA). We prepared an ELISA antigen by a borate/triton X-100 extraction of the membrane proteins of the infected cells. The IgG titres of the patient's sera by IFA and ELISA showed large antigenic differences between the new strain and the three known Ebola viruses. The most reactive of the other antigens appeared to be Zaire (table 1). However, the

Strains	Centers for Disease Control monoclonal antibodies							
	1	2	6	8	9	10	16	20
Zaire	+++	-	++	++	+	-	++	++
Sudan	++	-	+++	+++	-	+	-	+
Reston	++	++	++	-	-	+	++	++
Côte d'Ivoire	+++	-	++	+	-	-	++	+

The monoclonals were tested diluted 1/100 on slides prepared with VeroE6 cells infected by the different Ebola strains Côte d'Ivoire (CI), Zaire (Z), Sudan (S) and Reston (R).

Table 2: Reactivity of the different Ebola strains against 8 monoclonal antibodies

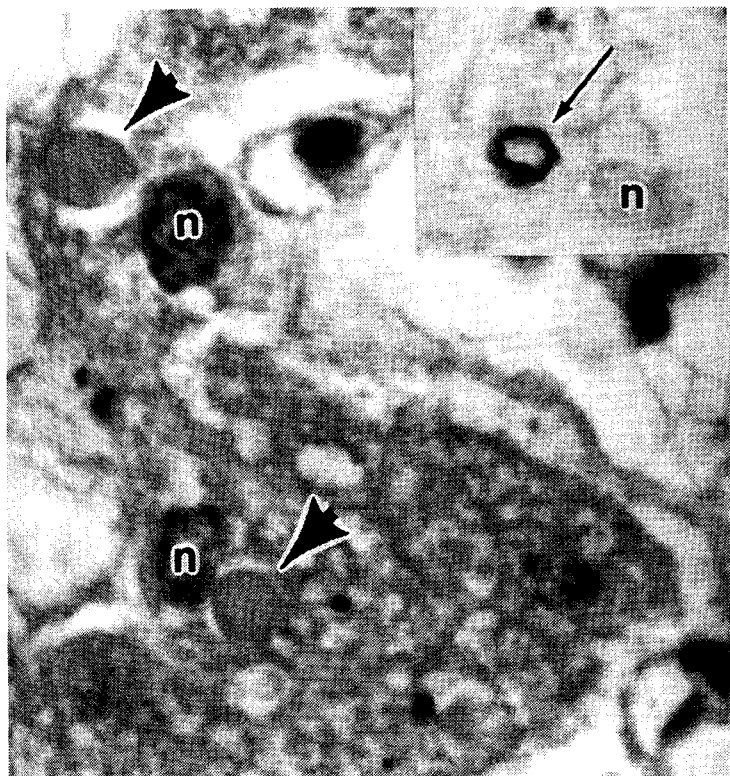


Figure 2: Liver tissue of a wild dead chimpanzee (Côte-d'Ivoire) formalin fixed embedded section stained with haematoxylin-eosin showing two large eosinophilic intracytoplasmic inclusions (arrow heads) in hepatocytes. Insert: Ebola specific immuno-peroxydase staining showing reactivity at the periphery of such intracytoplasmic inclusion (thin arrow). n=nuclei ( $\times 1250$ ).

IgM antibodies from the patient reacted only with the strain isolated from the patient in an ELISA immunocapture assay. This difference was confirmed by the use of monoclonal antibodies (table 2). Among a panel of 8, none of the specific anti-Reston and anti-Zaire and anti-Sudan monoclonal antibodies reacted with the new virus. However, a monoclonal antibody known to react with all Ebola strains confirmed that this virus belonged to the Ebola serogroup. We tested its pathogenicity for suckling mice by intracerebral inoculation and no death was noted.

A second serum sample drawn from the patient while in the Swiss clinic on 1 December, 1994 and kept at 4°C was received by us on 10 February, 1995. The same virus was quickly isolated in Vero E6 cells from this serum. Antigen capture ELISA previously reported by Ksiazek et al<sup>10</sup> detected a titre of 512 for the first serum and 8192 for the second. Despite the poor storage and transport conditions plus two cycles of freezing and thawing of the sera, the titre was still 1000 pfu/mL.

We notified the Swiss clinicians and the Ivorian health authorities by 6th February. We received 20 sera from people in contact either with the patients (colleagues, health personnel, friends) or with the chimpanzee samples (lab technicians). None had developed antibodies to this virus.

## Discussion

The available epidemiological data suggest an Ebola epizootic among the chimpanzees as the cause of deaths and the contact with infectious blood or tissues during the necropsy as the cause of the human infection. To investigate this hypothesis further, slides prepared from different organs of the dead chimpanzee were examined after haematoxylin-eosin-saffron fixation or after virus specific immunohistochemistry with a pool of cross-reactive monoclonal antibodies.<sup>8</sup> The spleen showed

extensive areas of fibrinoid necrosis of the red pulp. Necrosis was strongly marked around the lymphoid follicles with pyknosis and karyorrhexis of lymphoid cells. The liver lesions consisted of numerous foci of necrosis randomly distributed in the lobules. Isolated degenerative hepatocytes and multinucleated hepatic cells were seen throughout the parenchyma. A small number of single large amorphous acidophilic inclusions was seen in the cytoplasm of hepatocytes, near the necrotic foci (figure 2). The results are close to those reported from the only 5 human cases autopsied during the 1976 Zaire and Sudan outbreaks<sup>11</sup> and to data obtained by experimental inoculation of monkeys with Ebola.<sup>12</sup> The Ebola-specific immunohistochemical staining of the liver showed small aggregates mainly in the hepatocytes near to the portal ducts and sometimes in the Kupfer cells, but also showed a high immunoreactivity at the periphery of the intracytoplasmic inclusions (figure 2, insert). Pieces of liver and spleen kept in formalin were not in good enough shape to permit electron microscopy.

The natural reservoir for Ebola viruses has not been identified and Johnson's hypothesis—that Ebola is a plant virus has not yet been rebutted.<sup>11</sup> Because of the high mortality seen in this episode, apes are unlikely to be the reservoir. However, the data available from the long term survey of this troop of chimpanzees by Boesch et al and the fact that the Taï forest is protected from human activities will allow us to design studies able to answer the questions; what is the natural reservoir of this virus and does it infect the human population in the region?

Our preliminary data demonstrates that this new strain is serologically related to, but distinct from, the deadly Ebola Zaire. The new strain is lethal for chimpanzees and we may suppose for humans: the patient developed a severe syndrome similar to that described among the Ebola cases who survived.<sup>12,13</sup> Our work answers the recent question about the risk of emergence of Ebola and related viruses—non-diagnosed sporadic cases of Filovirus infections certainly occur among poachers and populations feeding on monkeys.<sup>7</sup> Although risk of infection after exposure to infected tissue may be high, the transmissibility from human patients seems to be low outside haemorrhagic episodes. Progress in applying good nursing practices such as disposable injection devices due to the AIDS epidemic may have circumvented a second Yambuku episode in the Côte-d'Ivoire.

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We also thank all the members of the Special Pathogens branch of the CDC for providing us reagents to confirm the novelty of this strain, particularly Dr Tony Sanchez who prepared the monoclonal antibodies.

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## Baseline serum cholesterol and treatment effect in the Scandinavian Simvastatin Survival Study (4S)

Scandinavian Simvastatin Survival Study Group\*

We examined the relation between the risk of major coronary events (coronary death and non-fatal myocardial infarction) and baseline cholesterol levels in patients with coronary heart disease, randomised to placebo or simvastatin therapy in the Scandinavian Simvastatin Survival Study (4S). The relative risk reduction in the simvastatin group was 35% (95% CI 15-50) in the lowest quartile of baseline low-density-lipoprotein cholesterol and 36% (19-49) in the highest. Simvastatin significantly reduced the risk of major coronary events in all quartiles of baseline total, high-density-lipoprotein, and low-density-lipoprotein cholesterol, by a similar amount in each quartile.

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The Scandinavian Simvastatin Survival Study (4S) showed that in patients with previous myocardial infarction or stable angina pectoris, simvastatin reduced the risk of death by 30% (95% CI 15-24,  $p=0.0003$ ), due to a 42% reduction (27-54) in the risk of death from coronary heart disease, and reduced the risk of major coronary events (coronary death and non-fatal myocardial infarction) by 34% (25-41,  $p<0.00001$ ).<sup>1</sup> Over the median 5.4 years of follow-up, simvastatin produced mean changes in total cholesterol, low-density-lipoprotein (LDL) cholesterol, and high-density-lipoprotein (HDL) cholesterol of -25%, -35%, and +8%, respectively. 622 (28%) of the 2223 patients randomised to placebo and 431 (19%) of the 2221 randomised to simvastatin had one or more major coronary events.<sup>2</sup> In patients with previous infarction the extent of myocardial damage has greater predictive value than lipid concentrations. In patients with angina only and in those without severe myocardial damage (such as those studied in 4S), lipid levels may be more relevant to prognosis.<sup>3-7</sup> We have now examined the relation between baseline lipids in 4S and the risk reductions produced by simvastatin, which is an important issue in the decision to treat individual patients with coronary heart disease.

The study methods have been reported.<sup>1,8</sup> 4444 men and women aged 35-70 years with coronary heart disease, and serum

total cholesterol 5.5-8.0 mmol/L and triglyceride 2.5 mmol/L or less on a lipid-lowering diet, were randomly allocated to placebo or treatment with simvastatin 20-40 mg daily. Lipids were analysed at the central laboratory of the study in serum obtained 2 weeks before and at randomisation. The primary endpoint was total mortality; although there were 438 deaths, the secondary endpoint (major coronary events), is more appropriate for subgroup analyses because it is not diluted by non-coronary events, and because more than 1000 patients had one or more such events, which provides greater statistical power. Patients were ranked into quartiles according to the mean of the two values. (Quartile size varies slightly because of tied ranks.) The number and percentage of patients with one or more major coronary events were calculated for each quartile. The percent reduction in LDL cholesterol at 12 months after randomisation was calculated for each quartile of baseline LDL. Relative risk and 95% CIs in each quartile were obtained by Cox's regression analysis, with treatment group the only factor in the model; adding baseline covariates related to major coronary events had no material effect. Differences between quartiles were assessed by a test for homogeneity. In addition, interactions between baseline lipids and both major coronary events and total mortality were assessed with treatment, baseline lipid value, and treatment by baseline-lipid-value included in the regression model.

The percentage of patients with major coronary events tended to be higher with increasing quartile of total and LDL cholesterol, as well as LDL/HDL cholesterol ratio, and with decreasing quartile of HDL cholesterol (table). The reductions in relative risk were similar in each quartile and did not differ significantly between quartiles for any of the variables examined ( $p>0.25$  for each of the twenty-four pairwise comparisons). There was also no significant treatment by baseline-value interaction for any lipid ( $p>0.4$  in each case).

Thus there was no evidence that the reductions in relative risk produced by simvastatin were dependent on the baseline lipid concentration; however, the absolute risk reductions tended to be less in quartiles in which fewer placebo patients had a major coronary event. Furthermore, the upper bound of the 95% CI of the relative risk was below unity (indicating a statistically significant benefit of simvastatin) in every quartile separately, including those with the lowest total and LDL cholesterol and the highest HDL cholesterol. Essentially the same pattern of reduction in total mortality was observed in each quartile, but there were too few deaths in individual quartiles for adequate statistical power. Again, there was no significant treatment by baseline-value interaction for any lipid ( $p>0.3$  in each case). The mean percent reductions of LDL cholesterol in the simvastatin group at 12 months' follow-up compared with the level in the placebo group were similar in each quartile of baseline LDL cholesterol (range 32-37%).

Since patients in the lowest quartile of baseline LDL cholesterol experienced substantial benefit on simvastatin, it can be argued that the appearance of coronary heart disease shows that the LDL-cholesterol concentration,

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