

# Impairment of Endothelium-dependent Dilation Is an Early Event in Children with Familial Hypercholesterolemia and Is Related to the Lipoprotein(a) Level

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## Abstract

Familial hypercholesterolemia is associated with premature atherosclerosis. Since endothelial dysfunction is an early event in atherogenesis, we used a noninvasive method to assess endothelial function in the systemic arteries of 30 children aged 7–17 yr (median 11) with familial hypercholesterolemia (2 homozygotes, 28 heterozygotes, total cholesterol 240–696 mg/dl) and 30 healthy age- and sex-matched controls. Using high resolution ultrasound, the diameter of the superficial femoral artery was measured at rest, in response to reactive hyperemia (with increased flow causing endothelium-dependent dilation), and after sublingual glyceryltrinitrate (causing endothelium-independent vasodilation).

Flow-mediated dilation was present in the controls ( $7.5 \pm 0.7\%$ ) but was impaired or absent in the hypercholesterolemic children ( $1.2 \pm 0.4\%$ ,  $P < 0.0001$ ). Total cholesterol was inversely correlated with flow-mediated dilation ( $r = -0.61$ ,  $P < 0.0001$ ). In the hypercholesterolemic children, flow-mediated dilation was inversely related to the lipoprotein(a) level ( $r = -0.61$ ,  $P = 0.027$ ) but not to other lipid fractions. Glyceryltrinitrate-induced dilation was present in all subjects but was lower in the hypercholesterolemia group ( $10.0 \pm 0.6\%$  vs  $12.4 \pm 0.8\%$ ,  $P = 0.023$ ).

Thus, impaired endothelium-dependent dilation is present in children with familial hypercholesterolemia as young as 7 yr of age and the degree of impairment is related to the lipoprotein(a) level. (*J. Clin. Invest.* 1994. 93:50–55.) Key words: atherosclerosis • atherogenesis • ultrasound • cholesterol • flow

## Introduction

Although the clinical complications of atherosclerosis occur in adult life, the process of atherogenesis begins in childhood (1, 2). Hypercholesterolemia is an important risk factor for the early structural changes of atherosclerosis (3) and for cardiovascular morbidity and mortality in later life (4). In autopsy stud-

ies of young adults, a significant positive correlation between LDL cholesterol and the extent of atherosclerosis in the aorta and coronary circulation has been documented (3). Furthermore, a recent long-term prospective study has shown a strong association between the cholesterol level in young adult life and the later risk of cardiovascular disease (5). In particular, if levels of LDL cholesterol and lipoprotein(a) [Lp(a)]<sup>1</sup> are elevated, the risk of coronary disease is markedly increased (6).

Endothelial dysfunction is an important early event in atherogenesis (7, 8). Both spatial and temporal correlations between endothelial dysfunction and coronary atherosclerosis have been shown in animal models and in humans (9–11). Endothelial injury has been induced experimentally by hypercholesterolemia, particularly the LDL component (12). In clinical studies using invasive techniques, hypercholesterolemic adults have been shown to have impaired endothelium-dependent responses to a variety of pharmacologic stimuli, including infusions of acetylcholine (13, 14).

We have tested whether endothelial dysfunction can be demonstrated at a much earlier stage of the disease process, in the systemic arteries of young children with familial hypercholesterolemia. In addition, we have examined the relationship between disturbed arterial physiology and lipid subfractions at a stage when intervention might have the greatest benefit.

## Methods

**Subjects.** 30 children (17 girls, 13 boys) aged 7–17 yr (median 11) with familial hypercholesterolemia (FH) were studied. All were asymptomatic, normotensive, nondiabetic, and nonsmokers. FH was defined as total plasma cholesterol  $> 268$  mg/dl or LDL cholesterol  $> 196$  mg/dl with tendon xanthomas in the patient or in a relative (15). 28 of these children were heterozygotes and 2 were homozygotes. All 30 were prescribed lipid-lowering diet. Drug therapy was prescribed at the discretion of each patient's physician, and 12 were receiving lipid-lowering drugs (7 cholestyramine, 5 3-hydroxy-3-methylglutaryl CoA reductase inhibitors) for 1–7 yr. 30 healthy children (17 girls, 13 boys, aged 5–15, median 11 yr) served as controls. All were normotensive, nonsmokers, and had no family history of premature vascular disease. Each subject and/or their parents gave informed consent to the study, which was approved by the local committee on ethical practice.

**Lipid analysis.** Total plasma cholesterol was measured in all 60 subjects (FH group and controls) using the cholesterol C-system high performance cholesterol oxidase 4-aminophenazone method (Boehringer Mannheim Diagnostica GmbH, Mannheim, Germany). Measurement of plasma lipids and lipoproteins in the 28 children with heterozygous FH was performed on EDTA plasma after a 14-h overnight fast. Plasma triglyceride was measured using the glyceryl phosphate oxidase 4-aminophenazone high performance enzymatic colori-

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1. **Abbreviations used in this paper:** FH, familial hypercholesterolemia; FMD, flow-mediated dilation; GTN, glyceryltrinitrate; Lp(a), lipoprotein (a); SFA, superficial femoral artery.

metric test (Boehringer Mannheim Diagnostica GmbH). HDL cholesterol was measured after precipitation of apoprotein B containing lipoproteins and LDL cholesterol was calculated according to the Friedwald formula (16). Lp(a) was measured using an ELISA method (Immuno GmbH, Sevenoaks, United Kingdom).

**Study design.** Arterial, endothelial, and smooth muscle function was studied noninvasively, as described previously (17). High resolution ultrasound was used to measure changes in arterial diameter in response to reactive hyperemia (with increased flow producing an endothelium-dependent stimulus to vasodilation) and to glyceryltrinitrate (GTN, an endothelium-independent vasodilator) (128XP/10 with a 7.0 MHz linear array transducer; Acuson, Mountain View, California).

The subject rested in the supine position for 10 min before the first scan and remained supine throughout the study. The superficial femoral artery (SFA) was scanned in longitudinal section and the center of the vessel was identified when the clearest images of the anterior and posterior walls of the artery were obtained. The transmit zone was set to the level of the anterior vessel wall. Depth and gain settings were optimized to identify the lumen to vessel wall interface. Images were magnified with the resolution box function leading to a television line width of  $\sim 0.065$  mm. Machine settings were kept constant during each study.

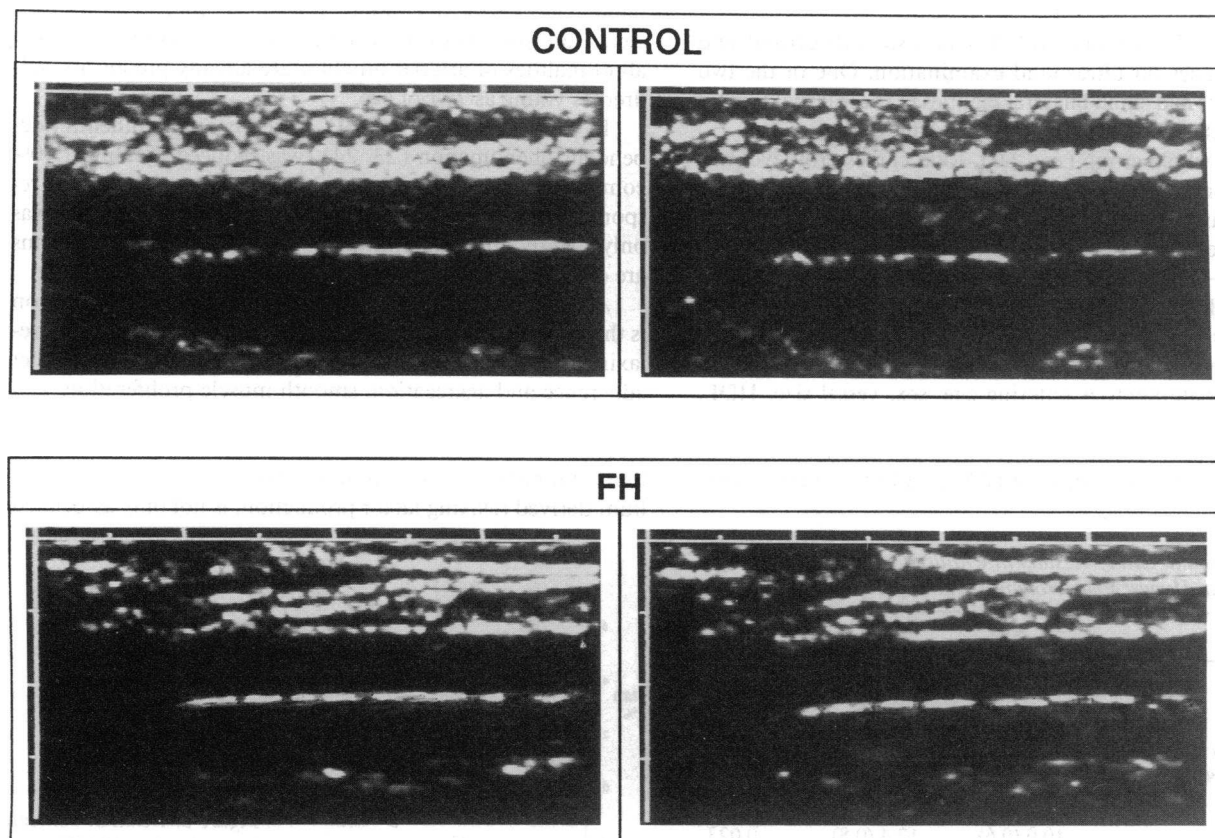
Flow increase was induced by inflation of a blood pressure tourniquet placed around the thigh, distal to the target artery, to  $\sim 250$  mmHg. The cuff was released after 4–5 min and the artery was scanned continuously for 30 s before and 90 s after cuff deflation. 10 min later, a second resting scan was recorded. GTN (400  $\mu$ g spray) was then administered sublingually and the artery was scanned 3–4 min later.

Phantom studies performed in our laboratory have confirmed that changes in diameters of 0.1–0.2 mm can be detected accurately with

this method (18). Furthermore, we (17) and others (19, 20) have found a low coefficient of variation for measurements of arterial diameter (interobserver error) and a high correlation between consecutive control measurements within a study.

**Data analysis.** Scans were recorded on super-VHS tapes and arterial diameters were measured directly from the tape by two independent observers “blinded” to the scan sequence and the identity of the subject. Vessel diameters were measured from the anterior to the posterior interface between media and adventitia (the “m-line”) at a fixed distance from an anatomical marker, usually the flow divider between the superficial and profunda branches of the common femoral artery. The mean diameter was calculated from four cardiac cycles incident with the R-wave on the electrocardiogram (ECG). For the hyperemia scan, vessel diameter was measured 45 s after cuff release (Fig. 1). Diameter changes were derived as percent change relative to the first baseline scan (100%). Baseline blood flow (measured during the first baseline scan) was estimated by multiplying angle-corrected, pulsed Doppler recordings of the flow-velocity integral by  $\pi$  and the square of the radius of the artery. Reactive hyperemia was calculated as the maximum flow measured during the first 15 s after cuff deflation divided by the baseline flow.

**Statistics.** Descriptive data are expressed as mean  $\pm$  SEM. The FH and control groups were compared using two sample *t* tests. For the whole group, the relationships between the dependent variables flow-mediated dilation (FMD) and GTN-induced dilation and sex, vessel size, and total cholesterol level were explored by both univariate and multivariate regression analysis. For the 28 children with heterozygous FH, the relationship between FMD and GTN-induced dilation and age, sex, vessel size, cholesterol, triglycerides, HDL, LDL, apolipoproteins A and B, and Lp(a) levels was explored by univariate analysis and multiple regression models. The distribution of Lp(a) levels was nor-



**Figure 1.** Scans from the superficial femoral artery of a control child (top panels) and from a child with FH (lower panels), showing the artery at rest (left) and after reactive hyperemia (right). In the control subject, the artery dilates  $\sim 10\%$ , and in the child with FH there is no increase in arterial diameter during increased flow.

mal in this group except for three subjects who had very high levels (> 50 mg/dl); therefore, the analyses were repeated excluding these three children. Statistical significance was inferred at a *P* value of < 0.05.

## Results

**Lipid data.** Total plasma cholesterol in the control subjects was  $164 \pm 27$  mg/dl (range 107–204). Total cholesterol was significantly increased in the FH group ( $312 \pm 16$  mg/dl,  $P < 0.0001$ ). In the FH children, HDL level was  $47 \pm 1.4$  mg/dl (range 31–61) and LDL level was  $224 \pm 11$  mg/dl (range 151–374). Triglyceride levels were normal ( $86 \pm 6$  mg/dl). Apolipoprotein A was  $130 \pm 4$  mg/dl (range 96–195). Apolipoprotein B was  $132 \pm 6$  mg/dl (range 70–215). Lp(a) was  $24.8 \pm 3.2$  mg/dl (median 18.5, interquartile range 13–31, range 7.2–70).

**Vascular studies.** Vessel size was similar in both groups (4.83 mm in control vs 4.79 mm in FH subjects,  $P = 0.807$ ). The mean resting blood flow in the SFA was  $> 269 \pm 24$  ml/min in controls and  $232 \pm 17$  ml/min in the FH group ( $P = 0.138$ ). The mean flow increase observed immediately after cuff deflation was  $414 \pm 19\%$  in the control and  $419 \pm 20\%$  in the FH children ( $P = 0.876$ ) (Table I).

In the arteries of the control children, FMD was  $7.5 \pm 0.7\%$  and GTN-induced dilation was  $12.4 \pm 0.8\%$  (Fig. 2). FMD was absent (< 2%) in 3 of these 30 children. In the FH subjects, FMD was significantly impaired ( $1.2 \pm 0.4\%$ , range  $-2.01$ – $5.8\%$ ,  $P < 0.0001$ , Table I). In 22 subjects (73%) FMD was absent. GTN-induced dilation was mildly attenuated in FH subjects compared with controls ( $10.0 \pm 0.6\%$ , range 4.0–16.5%,  $P = 0.023$ ) (Table I, Fig. 2).

29 of the 30 children with FH had smooth carotid and femoral arteries on ultrasound examination. One of the two homozygotes had small bilateral plaques in the internal carotid artery, which did not cause flow disturbance.

For the whole group ( $n = 60$ ), total cholesterol level was inversely correlated with FMD ( $r = -0.58$ ,  $P < 0.0001$ ) but not with GTN-induced dilation. A similar result was obtained on multivariate analysis (total cholesterol related inversely to FMD,  $r = -0.61$ ,  $P < 0.0001$ ). For the 28 heterozygous children with FH, the only significant correlate of FMD on univariate analysis was Lp(a) ( $r = -0.51$ ,  $P < 0.01$ ) (Fig. 3). This inverse correlation between FMD and Lp(a) was still observed on multivariate analysis entering age, sex, vessel size, HDL, LDL, triglycerides, and Lp(a) ( $r = -0.61$ ,  $P = 0.027$ ) (Table II). Similar results were obtained if total cholesterol was entered into this model instead of HDL and LDL. Furthermore,

Table I. Vascular Study Results in Children with Familial Hypercholesterolemia and Controls

	FH	Controls	<i>P</i>
Age (yr)	10.9 (0.46)	10.9 (0.45)	0.92
Vessel size*	4.79 (0.10)	4.83 (0.10)	0.807
Baseline flow (ml/min)*	232 (17)	269 (24)	0.138
Hyperemia (%)	419 (20)	414 (19)	0.876
FMD (%)	1.2 (0.4)	7.5 (0.7)	<0.0001
GTN (%)	10.0 (0.6)	12.4 (0.8)	0.023

Results are expressed as mean  $\pm$  SE. \* As measured during the first (resting) scan.

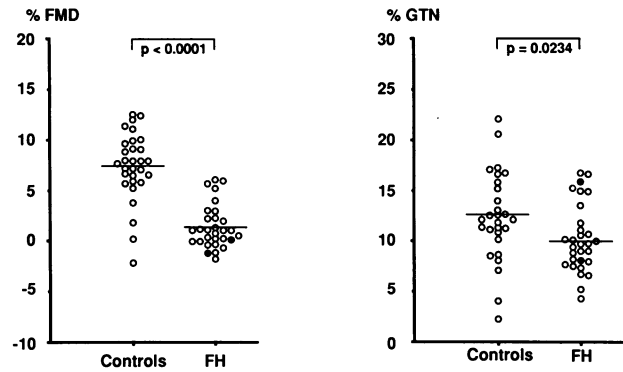


Figure 2. Flow-mediated and GTN-induced dilation in the controls and children with FH. Horizontal lines, group means; ● subjects with homozygous FH.

when these multivariate analyses were repeated excluding the three children with very high Lp(a) levels, almost identical results were obtained (Lp[a] inversely related to FMD,  $r = -0.55$ ,  $P = 0.029$ ).

## Discussion

Familial hypercholesterolemia is an autosomal, dominant lipoprotein disorder with a prevalence of  $\sim 1:500$  in its heterozygous form (21). Heterozygotes have a 100-fold increased risk of premature death from coronary heart disease (15). In homozygous FH, clinical coronary disease can occur in childhood. The metabolic consequences of the LDL-receptor deficiency are present from birth (22) and our results show that abnormalities of arterial function are already present in children as young as 7 yr of age.

In children with FH, FMD, which is an endothelium-dependent phenomenon (23, 24), was reduced by almost 90% compared with healthy controls. In contrast, the GTN response, which tests smooth muscle relaxation directly, was only slightly attenuated in the FH group. These observations are consistent with endothelial dysfunction.

A major functional consequence of endothelial dysfunction is the inability to produce and release endothelium-derived relaxing factor, a local vasodilator, which also inhibits platelet adherence and aggregation, smooth muscle proliferation, and endothelial cell-leukocyte interactions, all of which are important early events in atherogenesis (8). Recent evidence suggests that administration of L-arginine, the substrate for endothelium-derived relaxing factor production, is not only associated

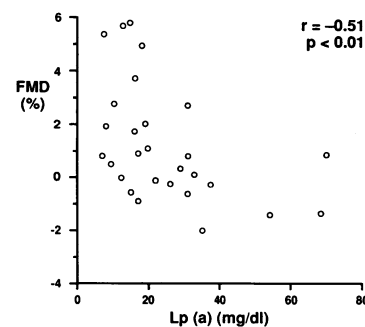


Figure 3. Relation between Lp(a) level and FMD in the superficial femoral arteries of the children with heterozygous FH ( $n = 28$ ).

*Table II. Multivariate Regression Analysis for Determinants of Flow-mediated Dilation in 28 Children with Heterozygous Familial Hypercholesterolemia*

Variable	Partial regression coefficient	P
Age	+0.41	0.19
Sex	-0.25	0.34
Vessel size	-0.37	0.18
LDL	-0.11	0.60
HDL	-0.08	0.68
Triglycerides	-0.08	0.74
Lp(a)	-0.61	0.027

with preserved endothelium-dependent responses but is also antiatherogenic (25). This implies that endothelial dysfunction is not merely a marker of early atherosclerosis but may be intimately involved in its pathogenesis.

Hypercholesterolemia, especially LDL cholesterol, is known to cause endothelial injury *in vitro*, with attenuation of the normal endothelium-dependent relaxation occurring long before the formation of atherosclerotic lesions (13, 26–29). *In vitro*, native LDL cholesterol appears to impair reversibly endothelium-dependent dilation minutes after exposure (30), and oxidized LDL cholesterol causes slowly developing and apparently irreversible inhibition of endothelium-dependent relaxation (31, 32). In experimental animals with diet-induced hypercholesterolemia, arteries show impaired vasodilation to acetylcholine in the absence of atheromatous lesions (30, 33). In clinical studies, selective loss of endothelium-dependent vasodilation to acetylcholine has been documented in hypercholesterolemic adults with angiographically normal coronary arteries (34) and has been found to be associated with the degree of elevation of serum cholesterol (35). Our findings show that the damaging interaction between hypercholesterolemia and the endothelium may begin even earlier, in the first decade of life.

We have investigated which lipid subfractions might be related to early vascular injury in these children. In our relatively small group, HDL and LDL levels were not correlated with impaired endothelial function. However, a significant relation between Lp(a) and FMD was observed, independent of the total cholesterol level. Lp(a) has recently been documented as a risk factor for premature atherosclerosis (36–38). Patients with FH suffering from coronary heart disease have higher Lp(a) levels than FH patients free of coronary artery disease (39, 40). The mechanism by which high levels of Lp(a) are related to atherosclerosis is unclear, but hypotheses include inhibition of plasminogen activity (41) and alteration of LDL-mediated delivery of cholesterol to the vessel wall (6). Our data represent the first evidence that elevated Lp(a) levels may be related to endothelial injury *in vivo*.

The simple noninvasive test used in this study enables accurate and reproducible assessment of vascular responses in humans (17). Flow-mediated dilation of large systemic arteries depends on the ability of the healthy endothelium to release relaxing factors (23, 42). We have induced a condition of increased flow by distal circulatory arrest followed by reperfusion. This method has been shown to produce dilation of the proximal artery by increase in flow rather than by altering cardiovascular reflexes, changing arterial distending pressure, or via the release of ischemic metabolites (43). In our laboratory,

we have demonstrated that equivalent cuff occlusion of the contralateral limb has no effect on ipsilateral arterial diameter. In contrast, GTN produces vasodilation by acting directly on smooth muscle. GTN was an effective vasodilator in both controls and FH children, although there was a modest impairment of GTN response in the latter group. A similar trend has been observed previously in small groups of hypercholesterolemic adults with either angiographically normal coronary arteries (34) or minimal coronary irregularities (44) and also in the superficial femoral artery of patients with mild atherosclerosis (45). The vasodilator response observed after GTN administration is a function of both direct smooth muscle relaxation and the response to the hyperemia caused by GTN-induced dilation of the resistance vessels (46). Loss of this flow component to the GTN response may explain the mildly impaired response seen in the FH group. In the setting of hypercholesterolemia, superoxide anions have been shown to inactivate nitric oxide and this also might contribute to the reduced responses of our FH patients, both to increased flow and to an exogenously administered nitrovasodilator (47). In addition, a smooth muscle abnormality in the conduit arteries of these children cannot be excluded. It should be noted, however, that the dilation to GTN in the FH group was sufficiently great ( $10 \pm 0.6\%$ ) so as not to preclude endothelium-dependent dilation.

Impaired flow-mediated dilation was found in three children in the control group. As the process of atherosclerosis begins in childhood and adolescence (48), even in normocholesterolemic subjects, these children may indeed already have abnormal arteries. Serial evaluation during long-term follow-up will be required to investigate this possibility.

In this study we investigated vascular physiology in the superficial femoral artery of children. In previous work we have defined the relationship between vessel size and FMD in systemic arteries in humans and found that the diameter of the SFA in children is within the optimal range for resolution and response (17). We have not studied the coronary arteries as these are not accessible to external ultrasound. Atherosclerosis, however, is a diffuse process, and clinical and autopsy data suggest that the disease often follows a parallel course in the coronary and proximal femoral arteries (49, 50). Other investigators have studied proximal segments of the femoral arteries to monitor the effects of lipid-lowering therapy on the regression of atherosclerotic lesions (51). It is likely that impaired endothelium-dependent dilation in the SFA of our children with FH reflects abnormalities of other sites in the arterial tree such as the carotid and coronary arteries.

In established disease, cholesterol-lowering therapy can attenuate progression of human coronary and femoral atherosclerosis and can lead to regression of existing plaques (51–53). In early disease, cholesterol-lowering therapy might be beneficial if it was associated with an improvement in endothelial function. In a primate model of early atherosclerosis, cholesterol-induced endothelial dysfunction has been reversed by reversion to a low-fat diet (10), and similar findings have recently been reported in adult men (35), using cholesterol-lowering drug therapy. In humans, the reduction in LDL cholesterol produced by a low-fat, low-cholesterol diet is small (54) and, despite the addition of lipid-lowering drugs in some of our patients, none had cholesterol or LDL levels as low as those shown to be beneficial in atherosclerosis regression studies in adults. There is legitimate concern about the administration of

lifelong therapy with cholesterol-lowering agents (55) and many pediatricians therefore avoid their use (56). Our findings suggest that therapy may be especially important in children with FH who also have an elevated Lp(a) level, as this group may have a particularly high risk of atherosclerosis. Treatment is currently available to lower LDL cholesterol but not Lp(a) levels. Whether the early abnormalities in arterial physiology can be reversed with any intervention will require prospective investigation, which is facilitated by the availability of a noninvasive test for the study of endothelial and smooth muscle function.

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