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Endogenous and diet-induced hypercholesterolemia in nonhuman primates: effects of age, adiposity, and diabetes on lipoprotein profiles

Rania Shamekh[a,b], *Ellen H. Linden*[a,b], *Jennifer D. Newcomb*[a,b], *Xenia T. Tigno*[c], *Kai-Lin Catherine Jen*[d], *Michael A. Pellizzon*[e], *Barbara C. Hansen*[a,b,*]

[a] *Obesity, Diabetes and Aging Research Center and Department of Internal Medicine, Tampa, FL 33612, USA*
[b] *Obesity, Diabetes and Aging Research Center and Department of Pediatrics, Tampa, FL 33612, USA*
[c] *Department of Molecular Pharmacology and Physiology, College of Medicine, University of South Florida, Tampa, FL 33612, USA*
[d] *Department of Nutrition and Food Sciences, Wayne State University, Detroit, MI 48202, USA*
[e] *Research Diets, Inc, New Brunswick, NJ 08901, USA*

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ABSTRACT

Nonhuman primates (NHPs) share with humans many features of lipid metabolism and often develop all features of the metabolic syndrome, including hypertriglyceridemia and low high-density lipoprotein cholesterol, and have been used in many studies of potential therapeutics during the preclinical phase. Here we identify for the first time in middle-aged and older rhesus the natural occurrence of hypercholesterolemia, and this hypercholesterolemia develops despite maintenance on a low-cholesterol diet. The aims of this study were to (*a*) define normal and hypercholesterolemia in rhesus monkeys, (*b*) determine the factors associated with the development of hypercholesterolemia, (*c*) compare the lipoprotein profiles in adult rhesus monkeys fed a low-fat/low-cholesterol diet (LFLC) with the profiles of human subjects, and (*d*) determine the effect of a 16-week high-fat/high-cholesterol (HFHC) diet feeding on total cholesterol and lipoprotein profiles in middle-aged and older monkeys. In our colony, maintained on a constant diet with negligible cholesterol, the mean total cholesterol level in healthy nondiabetic monkeys was 3.7 ± 0.02 mmol/L, with hypercholesterolemia identified as the 95th percentile of the normal cholesterol distribution (≥5.2 mmol/L). Severe hypercholesterolemia developed in the HFHC-fed group; however, despite the high-fat diet composition, unexpectedly, no weight gain occurred in these NHPs. The diet-induced hypercholesterolemia differed significantly in lipoprotein pattern from that of the spontaneous hypercholesterolemia. In summary, despite ingesting only a LFLC, NHPs frequently develop hypercholesterolemia, reflecting lipoprotein patterns similar to human subjects; and this lipid profile of spontaneous hypercholesterolemia differs significantly from the hypercholesterolemia induced by an HFHC diet.

Dr Pellizzon is an employee of Research Diets, Inc, New, Brunswick, NJ 08901.

* *Corresponding author*. St Petersburg, FL 33701, USA. Tel.: +1 703 589 7112; fax: +1 727 767 7443.
E-mail address: bchansen@aol.com (B.C. Hansen).

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1. Introduction

Nonhuman primates (NHPs) are phylogenetically similar to humans, are known to take a similar omnivorous diet, and are similar in lipid metabolism and lipid profiles. All humans and NHPs are susceptible to the development of diabetes and dyslipidemia in middle age. As in human subjects, dyslipidemia is frequently associated with the progression from obesity to metabolic syndrome [1-3] and is further exacerbated by the development of type 2 diabetes mellitus (T2DM) [4-6]. This form of middle-aged–onset dyslipidemia develops even while ingesting a low-fat/low-cholesterol (LFLC) diet and is associated with increased serum triglycerides, reduced high-density lipoprotein cholesterol (HDL-C), and increased small dense low-density lipoprotein (LDL) particles, without alterations in LDL or total cholesterol [7]. The other major form of dyslipidemia in humans, leading to significantly increased risk of cardiovascular disease, is hypercholesterolemia. Although diet-induced hypercholesterolemia has been previously induced experimentally, particularly in young and young adult primates (age <15 years) [8-12], naturally occurring hypercholesterolemia while ingesting an LFLC diet in middle-aged (10-20 years) and aged (>20 years) monkeys has not been previously reported. A small group of monkeys was previously identified based on evidence of an LDL receptor deficiency [13-15]. Here we report naturally occurring (non–diet-induced) hypercholesterolemia, and this lipoproteinemia resembles familial (primary) hypercholesterolemia in humans.

The aims of the present study were to determine the incidence, characteristics, and predictors of spontaneous hypercholesterolemia in rhesus monkeys (*Macaca mulatta*) fed only an LFLC diet for life, together with comparison of the naturally occurring lipoprotein patterns to those of humans. We further examined the lipoprotein profiles in relation to age, obesity, metabolic syndrome, and T2DM. In addition, we determined whether a spontaneous increase in cholesterol levels affected the lipid responses to a high-fat/high-cholesterol (HFHC) diet.

We hypothesized that the HFHC diet induction of hypercholesterolemia would be strongly affected by age, body weight, and preexisting degree of hypercholesterolemia, with increased dietary cholesterol responsiveness expected in these middle-aged subjects. We also hypothesized that older, heavier monkeys, provided ad libitum intake of an HFHC diet, would be more susceptible to weight gain and would rapidly increase their adiposity.

2. Materials and methods

2.1. Primate colony and care

This study involved a primate colony consisting of 217 rhesus monkeys (*M mulatta*), 73% males, ranging in age from 3 to 40 years and in weight from 4 to 27 kg. All were born in breeding colonies in the United States and were of known age and history. Monkeys were housed in standard primate caging according to the *Guide for the Care and Use of Laboratory Animals* [16] and provided environmental enrichment (treats, toys, music, videos, visible neighbors, human contact). The environmental conditions were identical for all monkeys. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

2.2. Diet composition

The monkeys were fed, ad libitum, an LFLC monkey grain-based chow diet (Table 1A, B) (Lab Diet 5038, PMI Nutrition International, Richmond, IN) throughout their lives. The macronutrient composition listed in the table was provided by the specification sheet [17].

2.3. Blood samples and assays

All blood samples were obtained following an overnight (16-hour) fast, under sedation with ketamine. The total serum cholesterol was determined a minimum of twice a year for all monkeys beginning in mature adulthood (about age 8) censored for those still living. Other metabolic parameters obtained included the following: fasting plasma glucose (FPG) levels by the glucose oxidase method, fasting plasma insulin by radioimmunoassay (RIA method of Millipore, St Charles, MO), blood chemistry analysis by either Antech Diagnostic (Tampa, FL) or the Piccolo chemistry panel (Abaxis, Union City, CA), and the lipid profiles analyzed by the Vertical Auto Profile method (VAP; Atherotech, Birmingham, AL) and by the Abaxis Piccolo analyzer. Intravenous glucose tolerance tests were performed with a glucose infusion of 0.25 mg/kg to measure the glucose disappearance rate (Kglucose) calculated between 5 and 20 minutes (Kgluc 5-20) [18]. The multiple Kglucose analysis was used to test for the reproducibility of the intravenous glucose tolerance test disappearance rate determination.

2.4. Methods to establish normal cholesterol level and to define hypercholesterolemia

Among our colony of 217 adult rhesus monkeys, 123 monkeys were defined as normal and healthy (with no apparent illness, no diabetes, and blood chemistry enzyme levels all within the reference range). The remaining 94 primates in the colony either had T2DM or were prediabetic adult monkeys with impaired glucose tolerance (Kglucose <2.0%). The normal adult rhesus group was used to establish the average normal level (mean ± SEM), the range, and the distribution of total cholesterol (mean and median), with hypercholesterolemia identified as at or greater than the 95th percentile concentration. Based on this distribution, a group with the most normal blood cholesterol levels (median, ~3.6 mmol/L and less; n = 66) and a group with the highest cholesterol concentrations (>the 95th percentile of normal [>5.2 mmol/L], n = 22) were selected, and detailed lipoprotein fraction distributions in the 2 subgroups (of 217 monkeys) were determined using the VAPs.

2.5. Methods used for short-term experimental induction of hypercholesterolemia using feeding of an HFHC diet

Twenty-two normoglycemic middle-aged and older rhesus monkeys, ranging in age from 9 to 25 years and in body weight

Table 1

A. Percentage of calories by nutrient of the 2 diets: LFLC compared with HFHC

	LFLC diet (Lab Diet 5038) [a] (control) (kcal %)	HFHC diet (Research Diets) [b] (kcal %)
Protein	18.2	20
Carbohydrate	68.7	35
Fat	13.1	45

B. The percentage by weight provided by the 2 diets

	LFLC (% by weight)	HFHC (% by weight)
Protein	15.7 [c]	23.2 [d]
Carbohydrate	59.3	40.7
Starch	42.4	11.5
Sucrose	2.24	29.2
Fat	5 [e]	23.2 [f]
Cholesterol	0.0075 [g]	0.25 [h]
Saturated fatty acids	1.54	20.5
Lauric acid	ND	9.6
Myristic acid	ND	3.6
Caprylic	ND	1.56
Capric	ND	1.2
Caproic	ND	0.12
Palmitic	ND	2.06
Stearic	ND	2.26
Polyunsaturated fatty acids	1.04	1.7
Linoleic acid	1.66	1.5
α-Linolenic acid	0	0.22
Arachidonic acid	<0.01	0
Monounsaturated fatty acids	1.68	0.8
Palmitoleic acid	[a]	0
Oleic acid	[a]	0.85

C. Complete ingredient composition of HFHC

Ingredients	g
Casein	200
l-Cystine	3
Maltodextrin 10	100
Sucrose	245.6
Cellulose	50
Hydrogenated coconut oil	177.5
Soybean oil	25
Mineral mix (S40001)	50
Vitamin mix (V40001)	10
Choline bitartrate	2
l-Ascorbic acid, phosphate	3
Cholesterol	2.2
Banana flavor	5
FD&C red dye #40	0.05
Total	873.35

ND indicates not determined.
[a] Lab Diet 5038, Lab Diet, PMI Nutrition International, Richmond, IN.
[b] RD# D07110401 by Research Diets, New Brunswick, NJ.
[c] Protein contributed by soybean meal, dried whey, and other sources.
[d] Protein contributed by casein and L-cystine; sulfur containing amino acid.
[e] Coconut oil and soybean oil.
[f] Porcine animal fat.
[g] Cholesterol mainly from porcine animal fat; 23 mg cholesterol per 1000 kcal.
[h] 0.25% cholesterol = 542 mg cholesterol per 1000 kcal; monkeys of average body weight (16.5 kg) ingest ~840 kcal/d.

from 9 to 17 kg, were divided into 2 groups well matched for age, weight, sex, glucose tolerance, and fasting plasma insulin. At baseline, both groups of monkeys were ad libitum fed the LFLC grain-based primate diet (composition shown in Table 1), after which one group (n = 11) was assigned to continue on this diet, whereas the other group (n = 11) was transitioned onto a purified-ingredient HFHC diet containing 45% of kcal as fat (mostly hydrogenated coconut oil) and 0.25% cholesterol by weight (Table 1A-C). The hydrogenated coconut oil and cholesterol combination has been found to promote hypercholesterolemia in rhesus monkeys [9,10]. In addition, diets containing 45% to 60% kcal fat with coconut oil can promote obesity in both rats [19] and mice [20]; but the effects on inducing obesity in middle-aged, potentially obesity-susceptible monkeys were unknown. Although there are differences in background ingredients used in the LFLC and HFHC diets (ie, LFLC = grain-based ingredients, HFHC = purified ingredients) that go beyond the fat and cholesterol level, an HFHC diet based on purified ingredients allows one to properly balance levels of essential nutrients (ie, protein, vitamins, minerals). Note that formulation of a high-fat diet is possible by adding fat to a grain-based diet such as Purina 5038; but such a modification dilutes essential nutrients (and perhaps nonessential, influential compounds), making it compositionally different in every way than the original grain-based chow, and as such could cause nutrient deficiencies. In addition, purified-ingredient high-fat diets are successful in promoting diet-induced obesity and insulin resistance in rodents; so we used a similar strategy for attempting to promote diet-induced obesity in our monkeys, all of which had been historically fed a grain-based chow diet. Following a baseline period of the LFLC diet for 8 weeks, the experimental diet was provided for 16 weeks followed by return to the baseline LFLC diet, with further study for an additional 17 weeks. All food intakes were recorded daily throughout the study and converted to kilocalories of intake per day and kilocalories of intake per kilogram of body weight per day. Body weights were recorded biweekly. Fasting plasma glucose, fasting plasma insulin, blood chemistry, and serum lipids were analyzed during the baseline LFLC diet and after 16 weeks of the respective diets.

2.6. *Statistical analysis*

Results are presented as means ± SD or as means ± SEM, as specified. Correlation analyses used the NCSS statistical program (Kaysville, UT) and included all data for all monkeys in the colony, with all used to determine relationships between age, weight, and cholesterol levels. Paired *t* tests were performed to determine the significance of the differences between the responses to the LFLC diet and the HFHC diet cholesterol values. Student *t* tests (unpaired) were used for comparisons between the groups (HFHC diet and LFLC diet). Multiple regression analysis was also performed to determine the lipid fractions associated with changing lipid profiles in spontaneous hypercholesterolemia vs HFHC-diet–induced hypercholesterolemia. A P value <.05 was considered significant.

3. Results

3.1. *Defining normal cholesterol levels and diagnosing hypercholesterolemia in adult middle-aged rhesus monkeys fed an LFLC diet*

The mean total cholesterol concentration of healthy, nondiabetic, middle-aged and older adult rhesus monkeys (1582 determinations for 123 monkeys) was 3.7 ± 0.02 mmol/L (mean ± SEM) (143 ± 0.9 mg/dL) with a median of 3.6 mmol/L (140 mg/dL). In addition, to ensure that all monkeys contributed equally to the mean, the average total cholesterol value for each monkey was calculated; and the mean of means was determined to be 3.8 ± 0.07 mmol/L (mean ± SEM). To further analyze the cholesterol levels and fractions appropriate for diagnosis of spontaneous hypercholesterolemia in rhesus monkeys maintained on an LFLC diet, total cholesterol levels (1582 determinations in 123 normal adult monkeys), LDL cholesterol (LDL-C) (753 determinations for the same 123 monkeys), and HDL-C (787 determinations for the 123 monkeys) were ranked for each fraction; and the median, 5th, 15th, 85th, and 95th percentiles were determined using the approach of several human clinical trials for examining hyperlipidemia [21-23] (Fig. 1). In humans, mean cholesterol and hypercholesterolemia have been identified by the Centers of Disease Control and Prevention for persons in the United States (2006); however, these means are based on humans with a much higher average cholesterol intake than was ingested by the monkeys in the present study. Thus, more accurate comparisons would be either between humans and monkeys both on low-cholesterol diets or between the 2 when both are consuming a similar higher-cholesterol–containing diet (on average) [21]. In rhesus monkeys consuming a very low cholesterol diet (as is typical for the commonly used primate chows), mild hypercholesterolemia was identified as the 85th percentile for total cholesterol (≥4.7 mmol/L); and significant hypercholesterolemia was designated as the 95th percentile for total cholesterol concentration in the healthy nondiabetic group (5.2 mmol/L) (Fig. 1A). The related normal average LDL-C level in middle-aged healthy rhesus monkey was identified as 1.6 ± 0.02 mmol/L(mean ± SEM), with a median of 1.6 mmol/L. The 85th percentile indicated some elevation in the LDL-C concentration to be at least 2.2 mmol/L, and the 95th percentile established a very high LDL-C to be at least 2.8 mmol/L. The mean HDL-C level in the same normal adult rhesus group was 1.6 ± 0.02 mmol/L (mean ± SEM), with a median of 1.6 mmol/L. A reduced HDL-C level (one of the criteria for diagnosis of the metabolic syndrome) was determined to be less than or equal to 1.2 mmol/L (the 15th percentile), and a low HDL-C was determined to be less than or equal to 1.0 mmol/L (fifth percentile).

A comparison of total cholesterol levels, LDL-C, and HDL-C in rhesus monkeys and humans [22,24-27], albeit on different diet types, is shown in Table 2.

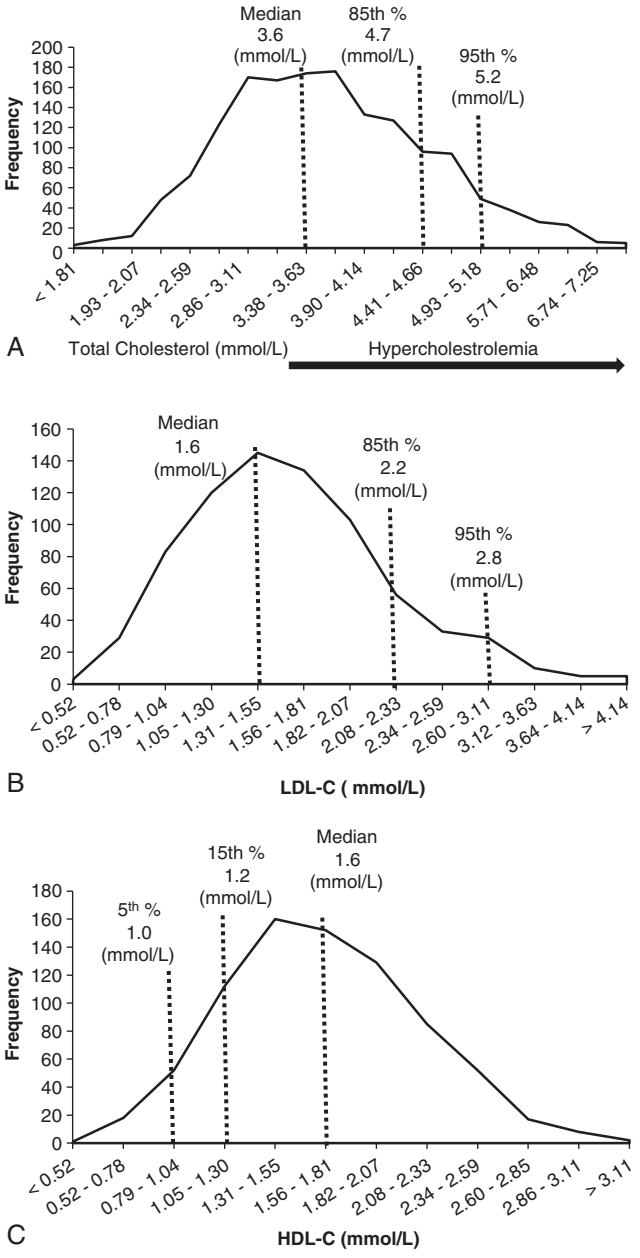

Fig. 1 – Frequency distribution of total cholesterol in normal, healthy, adult rhesus monkeys (ages 3-40 years, n = 123) (excluding any prediabetic or diabetic monkeys and monkeys with evidence of illness, eg, elevated enzymes) showing (A) the median and elevated (85th and 95th percentiles) total cholesterol levels (mean concentration ± SEM: 3.7 ± 0.02), (B) the median and elevated LDL-C levels (1.6 ± 0.02), and (C) the median and decreased (5th and 15th percentiles) HDL-C levels (1.6 ± 0.02).

3.2. *Effect of age and body weight on total cholesterol levels, LDL-C, and HDL-C in all adult monkeys (normal and DM/prediabetic, n = 217)*

The relationships between cholesterol levels and age (range, 3-40 years) and between cholesterol levels and body weight (range, 4-27 kg) in this colony of 217 monkeys were analyzed using more than 2000 determinations and are shown in Fig. 2. Total cholesterol tended toward a slight increase with age (r = 0.03, P = .07, not significant [NS]), and LDL-C increased slightly but significantly with age (r = 0.09, P = .01). HDL-C declined significantly with age (r = –0.27, P < .01). Neither total cholesterol nor LDL-C was related to body weight (Fig. 2);

Table 2 – Cholesterol levels among NHPs at least 3 years old and humans at least 20 years old consuming significantly different diets

	NHPs consuming a low-fat (5%) diet containing 0.07% by weight cholesterol [a]	Humans consuming an average of 510 mg cholesterol per day on a 30% fat diet [b]
Total cholesterol (mmol/L)	3.7	5.18
LDL-C (mmol/L)	1.63	2.59
HDL-C (mmol/L)	1.63	1.55

[a] Includes means using only healthy nondiabetic monkeys on a negligible cholesterol diet.
[b] The human group is a large population with a range of dietary cholesterol and without exclusion of diabetes or other conditions [22,25-27].

however, HDL-C declined significantly with increasing weight ($r = –0.15$, $P < .01$) (Fig. 2). Note that the evaluations of these associations included all monkeys in the colony, without regard to diabetic status.

In addition, triglycerides were significantly and positively associated with total cholesterol ($r = 0.4$, $P < .01$) and LDL-C ($r = 0.3$, $P < .01$) and negatively associated with HDL-C ($r = –0.5$, $P < .01$) (Fig. 3).

3.3. *Lipoprotein subclasses in non–diet-induced hypercholesterolemia*

The distribution of lipoprotein fractions in normal vs hyperlipoproteinemic monkeys was determined by VAPs. The group of 66 monkeys with total cholesterol levels at or less than the median of the nondiabetic group (≤3.6 mmol/L) was compared with the 22 monkeys with total cholesterol levels greater than the 95th percentile (≥5.2 mmol/L, severe hypercholesterolemia).

In the normal healthy group with the average or less total cholesterol levels, both the LDL-C ($r = 0.8$, $P < .01$) and the HDL-C ($r = 0.5$, $P < .01$) (but not the very low-density lipoprotein cholesterol [VLDL-C]) were significantly and positively correlated to the total cholesterol levels. In the most hypercholesterolemic monkeys (≥5.2 mmol/L, 95th percentile), the total cholesterol and LDL-C remained highly correlated ($r = 0.74$, $P < .01$), as was the total cholesterol to VLDL-C ($r = 0.6$, $P < .01$); but unlike in the normal group, in the hypercholesterolemic group, the HDL-C was not related to total cholesterol (Fig. 4) ($r = –0.36$, P = NS). There was no association between the spontaneous hypercholesterolemia and triglyceride concentration (data not shown).

To further determine the differential contribution of the various cholesterol subfractions to the total cholesterol levels in these 2 groups (normal and hypercholesterolemic), multiple regression analyses were performed to examine the relationships between total cholesterol and 7 fractions (HDL2, HDL3, LDL1, LDL2, LDL3, LDL4, and VLDL) within each group. In the monkeys with the most normal cholesterol levels (≤3.6 mmol/L), total cholesterol was significantly correlated with high-density lipoproteins HDL2 ($b = 1.2$, $P < .001$) and HDL3 ($b = 0.9$, $P < .001$); with low-density lipoproteins LDL1 ($b = 1.6$, $P < .001$), LDL2 ($b = 1.1$, $P < .001$), LDL3 ($b = 1.0$, $P < .001$), and LDL4 ($b = 1.3$, $P < .001$); and with VLDL ($b = 1.5$, $P < .01$), suggesting that all of the lipoprotein subclasses were predictors of and contributors to the total cholesterol level across the total cholesterol range from the median to the lowest. Rhesus monkeys with normal cholesterol levels showed the human-like LDL-C pattern A/B subclass (a mix of pattern A and pattern B).

Compared with monkeys with normal cholesterol levels, in monkeys with severe spontaneous hypercholesterolemia (³5.2 mmol/L, 95th percentile), total cholesterol was similarly significantly correlated with high-density lipoprotein HDL3 ($b = 1.2$, $P < .001$); with low-density lipoproteins LDL1 ($b = 1.5$, $P < .001$), LDL2 ($b = 0.9$, $P < .001$), LDL3 ($b = 0.8$, $P < .001$), and LDL4 ($b = 0.6$, $P < .001$); and with VLDL ($b = 1.1$, $P < .01$). There was, however, no correlation of total cholesterol to HDL2 ($b = 0.4$, P = NS). Thus, all of the lipoprotein subclasses were predictors of the total cholesterol level except for HDL2 (Fig. 4). In addition, all lipoprotein subclasses in monkeys with spontaneous hypercholesterolemia (Fig. 5A) showed proportional increases in relation to the total cholesterol, preserving the pattern A/B subclass of the LDL-C.

3.4. *Effects of an HFHC diet*

The effects of an HFHC diet were compared with the standard LFLC diet in 2 groups of monkeys, matched as shown in Table 3, with each group having a range of endogenous cholesterol levels.

We specifically determined the ability of the HFHC diet (Table 1A, B) to produce weight gain in middle-aged and older rhesus monkeys and to induce hypercholesterolemia in monkeys with a wide range of starting cholesterol levels, and examined the various lipid fractions for similarities and differences between the spontaneous hypercholesterolemia in response to an LFLC diet and the profile following an HFHC diet.

3.5. *Serum cholesterol and lipoprotein fractions during the high-cholesterol diet feeding*

Compared with their own baseline total plasma cholesterol level (4.9 ± 0.34 mmol/L), monkeys fed with the HFHC diet for 16 weeks showed the expected significant increase in total plasma cholesterol (12.1 ± 1.23 mmol/L) ($P < .001$); and the LFLC-diet–fed monkeys showed no change in cholesterol levels or lipid fractions during the experimental period. In the HFHC-diet–fed group, the LDL-C was significantly increased from a baseline concentration of 2.4 ± 0.27 to 8.9 ± 1.25 mmol/L on the HFHC diet ($P < .001$) in parallel with the change in total cholesterol ($r = 0.98$, $P < .01$). The VLDL-C increased significantly from 0.3 ± 0.02 mmol/L at baseline to 0.8 ± 0.18 mmol/L ($P < .05$). The HDL-C was not affected by the HFHC diet (baseline, 2.2 ± 0.23 mmol/L vs end of HFHC diet, 2.4 ± 0.31 mmol/L; NS). In addition, serum triglyceride baseline levels (0.7 ± 0.09 mmol/L) tended to increase in the HFHC-fed monkeys (1.0 ± 0.15 mmol/L) (Fig. 6), although this change did not reach significance ($P = .06$). Neither the HFHC diet nor the LFLC diet affected FPG, glucose tolerance, fasting plasma insulin, or blood pressure during this 16-week period.

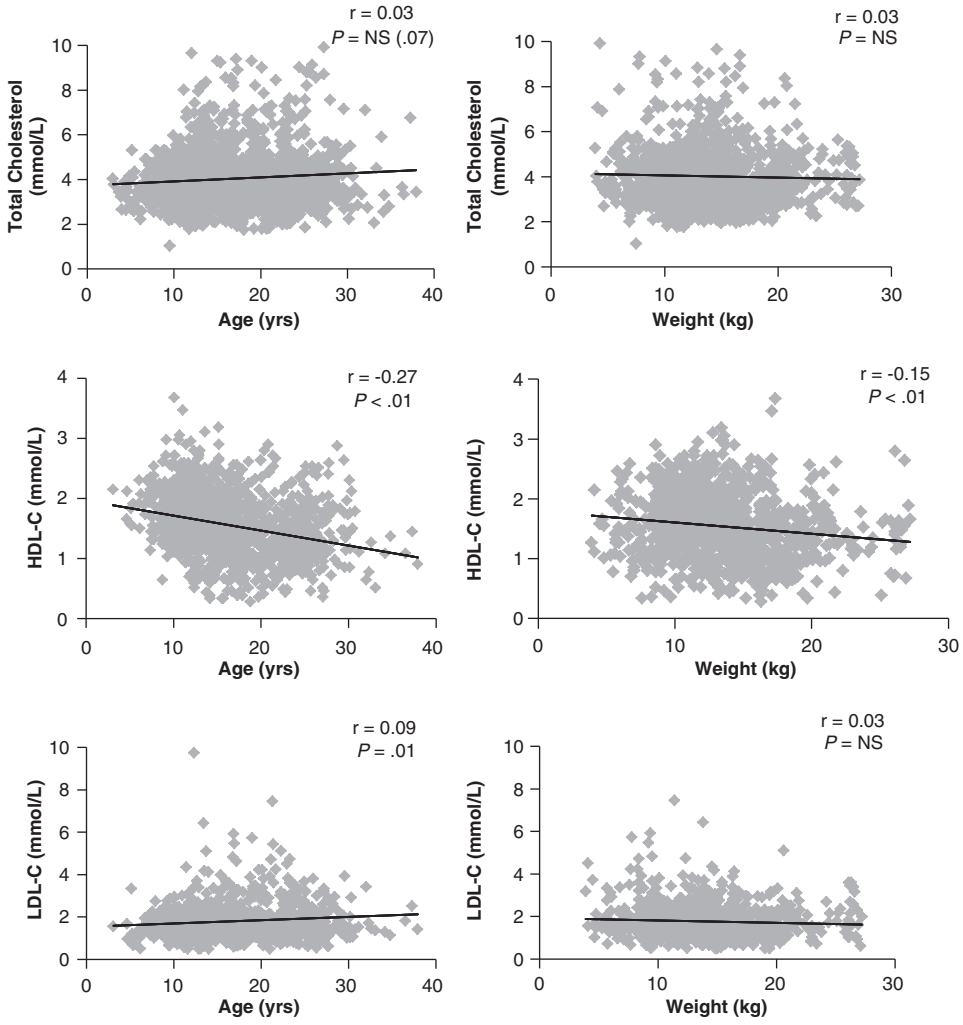

Fig. 2 – Effect of age and body weight on total cholesterol, LDL-C, and HDL-C levels in all adult monkeys (no exclusions). HDL-C showed significant negative association with age ($r = -0.27$, $P < .01$) and weight ($r = -0.15$, $P < .01$). Both LDL-C and total cholesterol tended to increase with age ($P < .01$ and $P < .07$, respectively). Neither total cholesterol nor LDL-C was related to body weight.

In response to the HFHC diet, some monkeys were more sensitive to the dietary cholesterol and showed greater increases in serum cholesterol (by >13 mmol/L) compared with others who showed only a relatively modest increase in serum cholesterol (~2.5-mmol/L increase). Baseline cholesterol level played no role in this wide range of hypercholesterolemia in response to the HFHC diet.

Over the age range of 9 to 25 years, age played no role in the degree of hypercholesterolemia induced by the high-cholesterol diet.

3.6. *Lipoprotein subclasses in diet-induced hypercholesterolemia*

To determine the relative contribution of various lipid fractions to the total cholesterol levels in diet-induced hypercholesterolemia, multiple regression analysis was performed between total cholesterol and 7 lipoprotein fractions (HDL2, HDL3, LDL1, LDL2, LDL3, LDL4, and VLDL). Under this diet-induced hypercholesterolemia, the total cholesterol was most highly related to LDL1 ($b = 1.3$, $P < .05$), LDL2 ($b = 1.2$, $P < .05$), LDL3 ($b = 1.3$, $P < .05$), and VLDL ($b = 2.1$, $P < .05$) (Fig. 4). Multiple regression analysis showed no relationship between total cholesterol and the LDL4 and HDL fractions. In addition, the HFHC diet induced an exclusive significant increase in LDL-1, LDL-2, and LDL-3 compared with their baseline levels, leading to a shift in the total LDL-C subclass from pattern A/B to pattern A (Figs. 5B and 6). Fig. 5C provides a combination of the data in Fig. 5A and B, better illustrating the highly unique lipoprotein profile induced by the HFHC diet compared with both the normal profiles of the healthy monkeys and the profiles of those with spontaneous hypercholesterolemia, as well as the baseline profile of the HFHC-diet monkeys before the diet experiment.

3.7. *Body weight and caloric intake with high- and low-fat/cholesterol diets*

No differences were observed in body weight, total calorie intake, and calorie intake per kilogram of body weight in

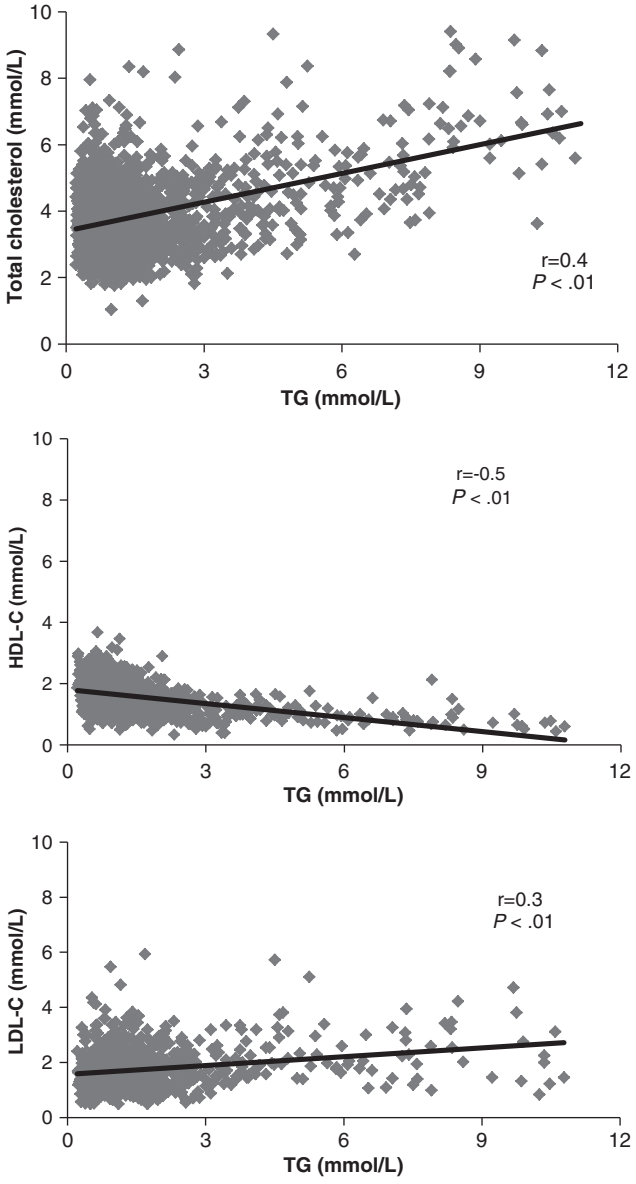

Fig. 3 – Correlation analysis of triglycerides and cholesterol in all adult monkeys. Triglycerides showed significant positive association with total cholesterol ($r = 0.4$, $P < .01$) and LDL-C ($r = 0.3$, $P < .01$) and a significant negative association with HDL-C ($r = -0.5$, $P < .01$). TG indicates triglycerides.

monkeys fed the HFHC diet for 16 weeks compared with their own baseline or compared with the matched group of monkeys fed the LFLC diet. Thus, unexpectedly, these middle-aged monkeys failed to show weight gain over a 16-week period on a high-fat diet (baseline body weight, 12.1 ± 0.8 kg; 16-week body weight. 11.8 ± 0.8 kg) (mean ± SEM).

4. Discussion

Adult rhesus monkeys represent a valuable animal model for studies of obesity, metabolic syndrome, and T2DM [1-4,6,28]. We have previously shown the extraordinary similarity of the dyslipidemia of monkeys and humans that have developed the metabolic syndrome, a very common precursor to overt T2DM in both species [4]. That form of dyslipidemia includes elevated triglyceride levels, low HDL-C, and little or no elevation of LDL-C; and a number of drugs have been shown to effectively improve this dyslipidemia in humans and in [28-30,57-62] monkeys . In the present study, we have sought and characterized a second form of naturally occurring dyslipidemia in rhesus monkeys, one which is again similar between humans and monkeys: hyperlipoproteinemia and, more specifically, hypercholesterolemia. One of the goals of the current study was to determine the total cholesterol level above which a diagnosis of hypercholesterolemia should be established for adult rhesus monkeys consuming a low-cholesterol typical-chow diet. The diagnosis of hypercholesterolemia in rhesus monkeys using methods comparable to those used in humans is very useful in identifying naturally occurring hypercholesterolemia against which therapeutic interventions and pharmaceutical agents targeting hypercholesterolemia should be tested. In the present study of our total colony, all maintained for a lifetime on a low-fat (13% of kcal) and low-cholesterol (0.07% by wt) diet and excluding prediabetic and diabetic monkeys, the mean (±SD) total cholesterol level of adult, nondiabetic rhesus monkeys was 3.7 ± 0.91 (SEM = 0.02) mmol/L, with a median of 3.6 mmol/L, similar to a previous report of Smuncy et al [31] in another group of rhesus (~3.7 ± 0.95 mmol/L [mean ± SD]; n =288; age range, 6-36 years). Young cynomolgus monkeys (*M fascicularis*, n = 106) weighing less than or equal to 5.5 kg were also reported to have similar normal total cholesterol levels (3.7 ± 0.73 mmol/L [mean ± SD]) [32]. In adult humans, the mean total cholesterol has been reported to be 5.2 ± 0.01 mmol/L (200 ± 0.5 mg/dL) [21,22]; however, this level is associated with a high-fat diet (30%) and a cholesterol intake averaging 510 mg/d, shown in Table 3. If hypercholesterolemia is defined in NHPs, using the same approach as has been used to define it in humans (≥the 85th percentile or ≥the 95th percentile [21-23], adjusting proportionately for the ~1.4 mmol/L (55 mg/dL) lower basal normal cholesterol level in NHPs receiving the very low cholesterol diet), then the diagnosis of hypercholesterolemia in rhesus monkeys on a low-cholesterol diet should be made when the total cholesterol level is at least 4.7 mmol/L (180 mg/dL). This level is likely to be comparable to the diagnosis of hypercholesterolemia at a level of at least 6.2 mmol/L (≥240 mg/dL) in humans with their much higher cholesterol-containing diet [22,25-27]. Hypercholesterolemia has been estimated by the Centers for Disease Control and Prevention for US adults (age range, 20-74 years) to affect 16% of the population (≥6.2 mmol/L or 240 mg/dL) [21], and this is consistent with the approach of defining hypercholesterolemia as greater than or equal to the 85th percentile. Alternatively, the 95th percentile may be used; and in the case of rhesus monkeys with a low-cholesterol diet, this concentration would be greater than or equal to 5.2 mmol/L. The American Heart Association recommends that total blood cholesterol be maintained at less than 5.2 mmol/L (200 mg/dL) (the current population average) [21], that LDL-C concentration be less than 2.6 mmol/L (100 mg/dL), and that HDL-C be at least 1.6 mmol/L (60 mg/dL) [22,25-27]. An increase in total cholesterol to greater than 6.2 mmol/L (240 mg/dL) together with LDL-C levels greater than 4.1 mmol/L (160 mg/dL)

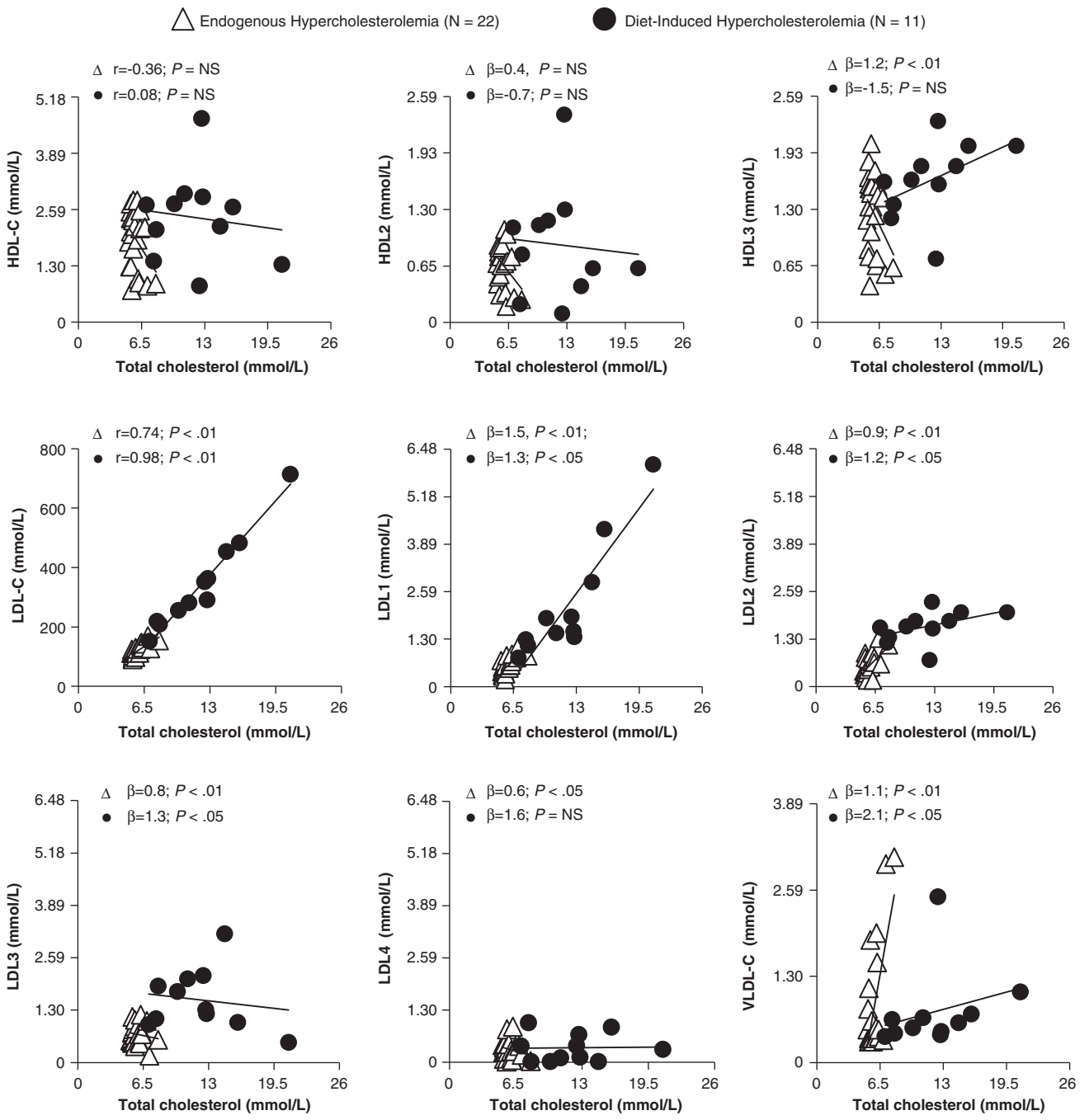

Fig. 4 – Correlation analysis of total cholesterol and lipoprotein subclasses in endogenous hypercholesterolemia compared with diet-induced hypercholesterolemia in rhesus monkeys. With endogenous hypercholesterolemia, total cholesterol was significantly correlated with LDL-C (r = 0.7, P < .01), LDL1 subclass (b = 1.5, P < .01), LDL2 subclass (b = 0.9, P < .01), LDL3 (b = 0.8, P < .01), and LDL4 (b = 0.6, P < .05). There was also a positive correlation between total cholesterol and VLDL-C (b = 1.1, P < .01) and between total cholesterol and the HDL3 subclass (b = 1.2, P < .01) particle. There was no relationship between total cholesterol and HDL-C or the HDL2 subclass. Diet-induced hypercholesterolemia showed a significant positive correlation between total cholesterol and LDL-C (r = 0.9, P < .01), LDL1 subclass (b = 1.3, P < .05), LDL2 subclass (b = 1.2, P < .05), LDL3 subclass (b = 1.3, P < .05), and VLDL-C (b = 2.1, P < .05), but not with HDL-C or the HDL-C subclasses.

and HDL-C less than 1.0 mmol/L (40 mg/dL) in males and less than 1.3 mmol/L (50 mg/dL) in females constitutes a lipid profile that is identified as a major risk factor for heart disease. In addition, the American Heart Association recommends that triglyceride levels be maintained at less than 3.9 mmol/L (150 mg/dL). We have identified the respective levels for rhesus monkeys as total blood cholesterol less than 3.7 mmol (143 mg/dL), LDL-C less than 1.6 (63 mg/dL), HDL-C at least 1.6 mmol/L (63 mg/dL), and triglycerides levels less than 1.1 mmol/L (100 mg/dL).

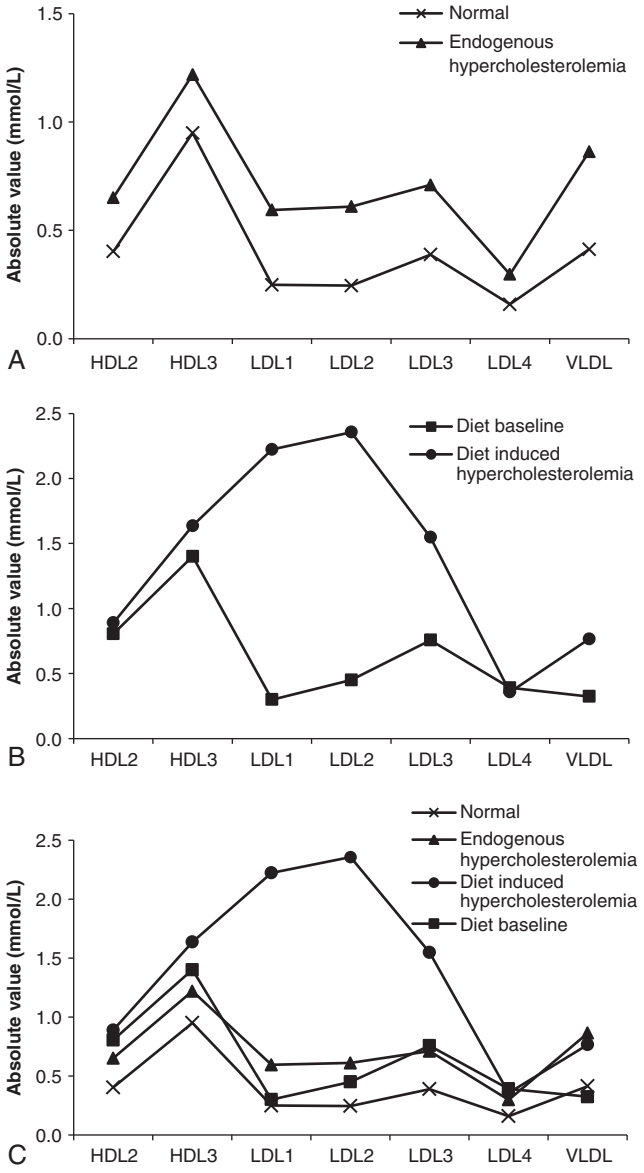

Fig. 5 – Distribution of lipoprotein subclasses in endogenous hypercholesterolemia compared with diet-induced hypercholesterolemia in rhesus monkeys. A, Endogenous hypercholesterolemic monkeys showed consistent elevation in all lipoprotein fractions with greatest proportionate increases in the LDL1, LDL2, and VLDL fractions compared with normal monkeys. B, By contrast, the diet-induced hypercholesterolemic monkeys showed highly significant increases ($P < .01$) in LDL1, LDL2, and LDL3 compared with their own baseline cholesterol levels. C, The combined data panels (A) and (B) illustrate the extraordinary difference between the profiles of diet-induced hypercholesterolemia and the profiles of normal animals, hypercholesterolemic animals, and the preexperimental baselines for these diet-induced monkeys.

Hypercholesterolemia is primarily a disorder of middle and older age in humans, and this is also the case in NHPs. Although the incidence of hypercholesterolemia was higher in the older monkeys, among adult animals older than 8 years

Table 3 – Baseline characteristics of the 2 groups of monkeys, ad libitum fed either the LFLC or a specific HFHC diet

Sex	LFLC diet		HFHC diet	
	Male = 6	Female = 5	Male = 6	Female = 5
Age (y)	15 ± 2.2	11 ± 1.6	14 ± 1.3	14 ± 1.7
Weight (kg)	13 ± 1.1	11 ± 1.3	14 ± 1.1	10 ± 0.5
FPG (mmol/L)	3.7 ± 0.16	3.8 ± 0.14	3.9 ± 0.19	3.7 ± 0.1
Blood pressure (systole/diastole) (mm Hg)	126/70	126/61	126/65	116/60
Plasma insulin (pmol/L)	472.3 ± 158.3	620.9 ± 34.7	354.2 ± 68.1	465.3 ± 68.8

Data presented as mean ± SEM.

(fully mature; estimated human equivalent of 25 years old), age, per se, had no effect on the development of spontaneous hypercholesterolemia. We observed no differences between male and female monkeys in any lipid variables. Similar to humans [34], HDL-C in rhesus monkeys tended to decrease with age (as the frequency of metabolic syndrome and prediabetes increases). Interestingly, age has been significantly correlated with total cholesterol only in young monkeys [6,34] or young humans [35]. In the current study, body weight was associated with decreasing HDL-C and a tendency toward increasing LDL-C. Human studies have shown that LDL-C tended to increase with age only during the adulthood period until the age of 60 years in men and 70 years in women [36], whereas HDL-C tended to decrease with age [33,37]. The increase in LDL-C was due to decreased LDL-C catabolism and clearance by the LDL receptor and also due to hormonal changes associated with aging [36,37]. The decrease in HDL-C has been attributed to aging-associated hormonal changes in both men and women and to the increase in inflammatory cytokines[38,39], such as interleukin-6 and tumor necrosis factor–α, associated with aging [33,37]. Interestingly, in very old age (>60 in men and >70 in women) total cholesterol, LDL-C, and HDL-C all tended to decrease [36,37,40], which can partly be explained by the survival factor, as healthier subjects with low LDL-C had a better survival [36], and could also be due to aging-associated weight loss.

The increase in blood lipids can be either a primary disorder or a secondary disorder related to either diet or underlying disease [13,41]. A well-known cause of spontaneous hypercholesterolemia in humans is familial (monogenic) hypercholesterolemia [13-15,41]. Familial hypercholesterolemia is clinically associated with a mutation in the LDL-R gene or a less likely mutation in the apolipoprotein B gene [13,41]. The cause of spontaneous hypercholesterolemia in NHPs has yet to be determined, although several monkeys have been identified with a similar LDL-R mutation [14,15].

One of the goals of the current study was to determine the distribution of lipoprotein fractions in spontaneous hypercholesterolemia in comparison with the lipoprotein fraction concentrations in diet-induced hypercholesterolemia. In humans, as well as in monkeys [8,12], LDL-C is divided into 4 main subclasses, LDL1 to LDL4, according to size and

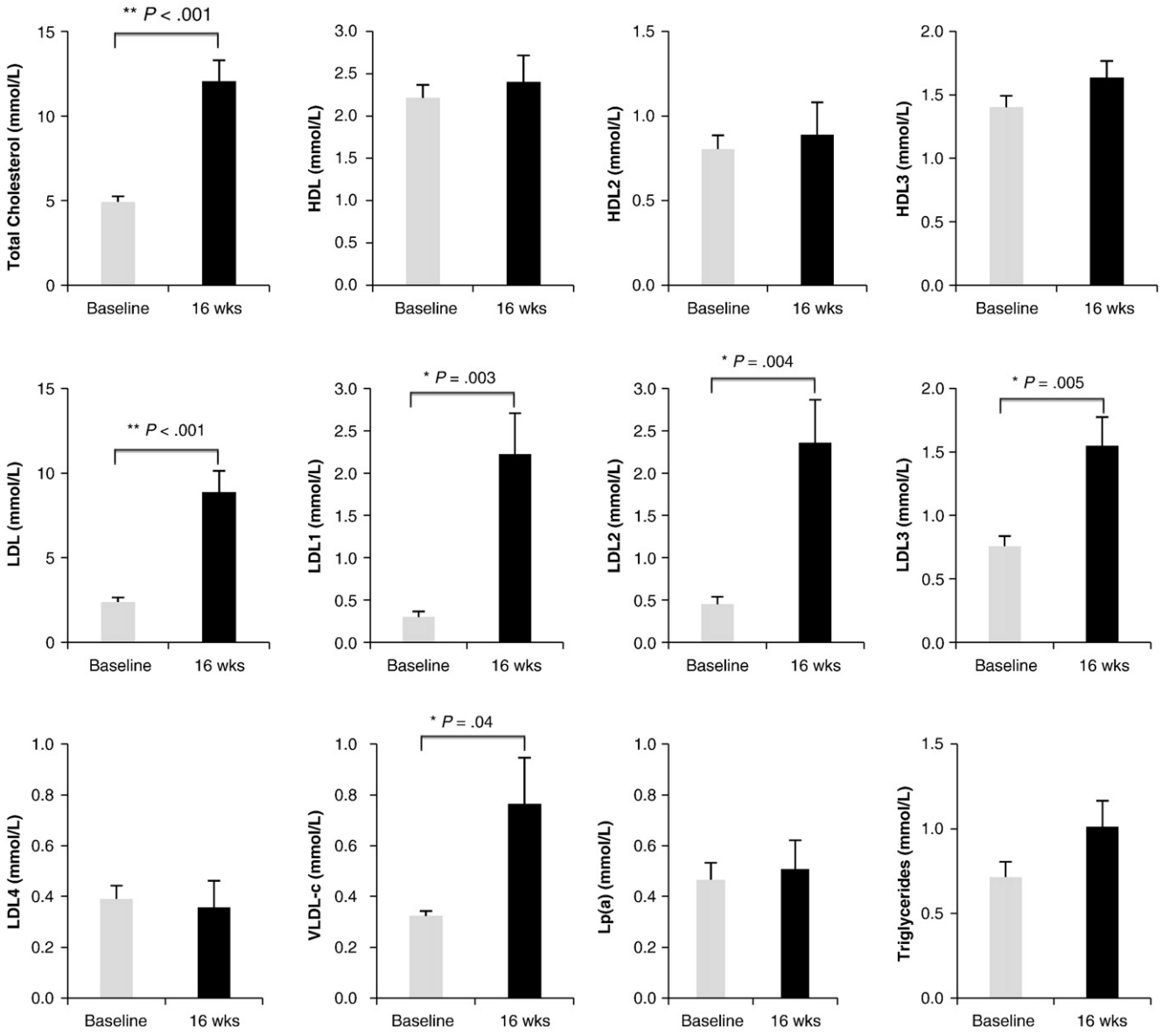

Fig. 6 – Change in cholesterol and lipoprotein fractions after 16 weeks of the HFHC diet in rhesus monkeys compared with their baseline LFLC diet values (n = 11 monkeys). After 16 weeks of an HFHC diet, there was a significant increase in the total cholesterol ($P < .001$), LDL-C ($P < .001$), and VLDL-C ($P < .04$) in monkeys compared with their own baseline levels. Further analysis of the LDL-C and HDL-C subclasses showed significant increases in LDL1, LDL2, and LDL3 subclasses ($P < .01$) in monkeys with diet-induced hypercholesterolemia.

cholesterol composition [42,43]. LDL1 is the largest particle, whereas LDL2 is a medium-sized particle; LDL1 and LDL2 together form pattern A. LDL3, a small-sized particle, and LDL4, the smallest-sized particle, together form the small dense particle pattern B. In addition, the small dense pattern B was found to be associated with increased levels of triglycerides and decreased levels of HDL-C, thus making small dense pattern B the most atherogenic LDL-C subclass [44].

In the current study, multiple regression analysis of lipoprotein fractions in monkeys with a total cholesterol within the reference range (less than or equal to the median) showed that both HDL-C subclasses (HDL2 and HDL3) and LDL-C subclasses (LDL1 to LDL4) were all significantly correlated to the total cholesterol in normal healthy monkeys reflecting the pattern A/B subclass of the LDL-C. As monkeys developed spontaneous hypercholesterolemia, all cholesterol subclasses increased, with a proportionate contribution of each subclass to the total cholesterol preserving the pattern A/B subclass of the LDL-C.

Studies [45] have shown that an increase in small dense LDL-C particles, primarily LDL3 (<25 nm, pattern B), is strongly associated with coronary artery disease; and this is the predominant form of LDL-C in patients with hyperlipidemia [46,47]. However, others have concluded that, within LDL-C, both the large LDL particles and the small dense LDL particles contribute to the risk of coronary heart disease [48,49]. As we have reported, cardiac-related death is the predominate cause of natural deaths in LFLC-diet–maintained rhesus [50].

In the present study, we have shown that 16 weeks of ad libitum feeding of an HFHC diet containing saturated fat of coconut origin led to a significant increase in total cholesterol, LDL-C, and VLDL-C, with no significant change in HDL-C or triglycerides and no change in body weight. The diet containing hydrogenated coconut oil was chosen because this fat increases non–HDL-C cholesterol compared with other fats, an effect that has been attributed to the higher concentrations of 12 and 14 carbon saturated fatty acids that are more potent cholesterol raisers compared with the 16 and 18 carbon saturated fatty acids of the LFLC diet [10]. Moreover, the inclusion of 0.25% cholesterol (542 mg cholesterol per 1000 kcal) in this HFHC diet promoted a further increase in serum cholesterol.

Other studies of high-fat feeding in NHPs have reported similar results. Feeding of high fat (35% kcal of fat; a mix of beef tallow, soy oil, and butter) with and without 0.4 mg/kcal of cholesterol added to the diet [8], a diet high in saturated fat (31% kcal, as either corn oil or coconut oil) with or without cholesterol (0.3 mg/kcal) [9], a diet rich in saturated fat (palm oil) [12], or a diet with a high fat (37% fat from fish oil, safflower oil, and lard) and high cholesterol content (0.4 mg/kcal) [51] (comparable to the 0.5 mg/kcal in the current diet) all induced a remarkable increase in serum cholesterol in cynomolgus, rhesus, and African green monkeys. Interestingly, in the study of Baker et al [8] as well as in the present study, some rhesus monkeys showed a much greater response to the HFHC diet than others. The cause of this enhanced responsiveness to dietary cholesterol is unknown.

In the current study, multiple regression analysis of the lipoprotein fractions in diet-induced hypercholesterolemia showed a significant increase in the LDL1 and LDL2 (large buoyant) subclass. The LDL3 subclass was also significantly increased in response to the diet, and the LDL4 did not change in response to the HFHC diet. These findings indicate a shift from the pattern A/B subclass of LDL-C, which is the predominate pattern in normal hypercholesterolemia and spontaneous hypercholesterolemia, to the pattern A subclass of the LDL-C, which is considered a significant characteristic of the diet-induced hypercholesterolemia. In humans, the Kraus group [52,53] has reported similarly that those with pattern A on a high-fat diet exhibited pattern B on a low-fat diet.

When Baker and colleagues [8] analyzed the distribution of cholesterol fractions in response to a high-fat diet and a high-cholesterol diet in rhesus monkeys, they found that, during the high-fat feeding, HDL formed 50% of the total cholesterol, whereas the remainder was carried by LDL1, LDL2, VLDL, and intermediate-density lipoprotein. In contrast, it was found that the addition of 0.4 mg/kcal of cholesterol to provide a high-fat and high-cholesterol diet altered the lipoprotein fraction distribution. Monkeys with the highest response to the high-cholesterol diet showed increases in LDL1, intermediate-density lipoprotein, and VLDL, respectively, with no change in LDL2 [8]. In the current study, however, the HFHC diet produced significant increases in both LDL1 and LDL2. In the study of Baker et al [8], there was a remarkable decrease in HDL by 50% after 18 weeks of high-cholesterol feeding. This decrease in the HDL-C in the study of Baker et al was similar to the current study because, at baseline, HDL-C formed 45% of total cholesterol, whereas after 16 weeks of HFHC diet, HDL-C constituted only 20% of the total cholesterol. Note that although the percentage of HDL relative to total cholesterol declined, the absolute concentration increased.

In the current study, monkeys fed an HFHC diet for 16 weeks failed to gain weight and failed to increase or otherwise change their daily caloric intakes. A similar study using a group of African green monkeys fed a high-fat/high-cholesterol diet for 5 years showed that neither the fat content of the diet nor the fatty acid components of the had any effect on body weight [12]. The present results are similar to those reported by Howard and colleagues [54] on the effect of dietary fat content and body weight change in humans. They observed that a decrease in fat content of the diet can lead to decreased body weight only if it is associated with a decrease in total caloric intake. Similarly, a recent clinical trial studying the effect of various dietary macronutrients (proportion of fat, protein, and carbohydrate) on the degree of weight loss among 800 overweight and obese subjects between the ages of 30 and 70 years old [55] found no differences in body weight change related to diet composition. After 2 years of comparing the effect of 4 diets with different macronutrient proportions on body weight, they concluded that weight loss was achieved by reduced energy intake regardless of the macronutrient content or fat content of the diets [55]. Consequently, our finding that an HFHC diet did not induce weight gain is similar in that the total calorie intake remained unchanged regardless of the fat and cholesterol content and that no change in body weight was observed.

It is important to mention that the dietary compositions of the HFHC and LFLC diets varied significantly with respect to other ingredients aside from fat and cholesterol. Some of these differences could have contributed to the observed differences in lipoprotein profiles of monkeys on either of these 2 diets. For example, one source of protein in the LFLC diet was soybean meal, whereas the protein source in the HFHC diet was casein. Previous work in cynomolgus monkeys suggested that high-fat and high-cholesterol diets containing soy protein and isoflavones reduced LDL-C and increased HDL-C relative to those containing casein [56]. Therefore, although the main contributor to the increase in LDL-C by HFHC relative to LFLC was likely due to the difference in the cholesterol content between these diets, it is possible that the protein sources present in these diets also had some influence. Perhaps other factors contributed to differences in lipoprotein profiles, so future studies will also include monkeys fed a matching LFLC diet with the same background ingredients used in the HFHC diet.

In summary, consumption of a healthy LFLC diet post-weaning can, nevertheless, result in significant endogenously produced hypercholesterolemia in predisposed rhesus monkeys. Similarly, an HFHC diet can induce an exaggerated increase in total cholesterol and an altered lipoprotein profile. Interestingly, spontaneous hypercholesterolemia was a result of a proportional increase in all lipoprotein subclasses with a predominance of the pattern A/B subclass, whereas diet-induced hypercholesterolemia produced significant increases in the large buoyant LDL-C subclasses LDL1 and LDL2 and a predominance of the pattern A subclass. The dietary fat percentage and cholesterol content had no effect on body weight or total caloric intake. Both spontaneous hypercholesterolemia and diet-induced hypercholesterolemia are similar

to those reported in humans and make the NHP an excellent model for studying the efficacy and mechanisms of action of agents aimed at lowering cholesterol in patients with high cholesterol [57-62].

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