Effect of sample storage on the measurement of lipoprotein[a], apolipoproteins B and A-IV, total and high density lipoprotein cholesterol and triglycerides

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Abstract This study investigated the influence of long-term storage, for periods up to 24 months, and multiple freezing and thawing on the measured values of lipoprotein[a] (Lp[a]), apolipoproteins B and A-IV, total and high density lipoprotein (HDL) cholesterol and triglycerides using plasma samples stored at −80°C, −20°C, and 4°C. Samples stored at −80°C or −20°C showed significant changes in Lp[a] after 24 months, with a mean decrease of 7% and 13%, respectively ($P < 0.01$). The major part of the decrease occurred during the first freezing and thawing. In contrast, apolipoproteins B and A-IV decreased continuously over time ($P < 0.05$). The increase in plasma concentrations of total and HDL cholesterol and triglycerides was small but significant because of its uniformity. Multiple freezing and thawing influenced only the measured values of Lp[a] and apolipoprotein B. Comparison of samples stored at −80°C and −20°C showed no difference in any of the parameters at any time with the exception of Lp[a] after 18 and 24 months ($P < 0.05$). After a storage period of 24 months, immunoblotting with detection of apo[a] was possible from samples under each storage condition. ApoB and apoA-IV were detectable only in samples stored at −20°C or −80°C. These data, when compared to recent studies, suggest a critical role of the assay methodology in the reproducibility of measured Lp[a] and apolipoprotein plasma concentrations. We therefore recommend the examination of each system for measurement of long-term stored plasma samples.—**Kronenberg, F., E-M. Lobentanz, P. König, G. Utermann, and H. Dieplinger.** Effect of sample storage on the measurement of lipoprotein[a], apolipoproteins B and A-IV, total and high density lipoprotein cholesterol and triglycerides. *J. Lipid Res.* 1994. **35:** 1318–1328.

Supplementary key words sample handling • storage temperature • apolipoproteins • lipids

Routine clinical analysis of lipoproteins is usually performed with fresh plasma samples. Long-term prospective or retrospective epidemiological studies with multiple withdrawals of blood in single subjects, however, require adequate plasma sample storage. Such samples are individually frozen and stored and later analyzed all at the same time to eliminate any source of error from interassay variability. This necessitates a certain stability in the reactivity of various lipoproteins and apolipoproteins during storage in order to ensure reliable results.

The number of aliquots made from one plasma sample is also of importance. Too few aliquots will require analysis from samples that must be repeatedly thawed and refrozen.

The aim of this study was, therefore, to investigate the stability of measurements of lipoprotein[a], apolipoprotein A-IV (apoA-IV), apolipoprotein B (apoB), total cholesterol, HDL cholesterol and triglycerides kept at different storage temperatures for up to 24 months (long-term storage study) and the influence of repeated freezing-thawing cycles and of filling status of storage tubes (freezing and thawing study).

Lp[a] consists of an LDL-like particle and apolipoprotein[a] (apo[a]), which is linked to apoB-100 by a disulfide bond (1, 2). Several studies have shown that Lp[a] is an independent genetically determined risk factor for premature coronary heart disease and stroke (for a review see ref. 3). ApoA-IV is secreted by intestinal epithelial cells and is found mainly in the lipoprotein-free fraction of plasma (4). It might play an important role in reverse cholesterol transport by activating lecithin:cholesterol acyltransferase (LCAT) (5) and promotion of cholesterol efflux from adipose tissue cells (6). In recent years Lp[a] and reverse cholesterol transport have gained increasing importance in arteriosclerosis research.

Abbreviations: HDL, high density lipoproteins; PBS, phosphate-buffered saline; CV, coefficient of variation.

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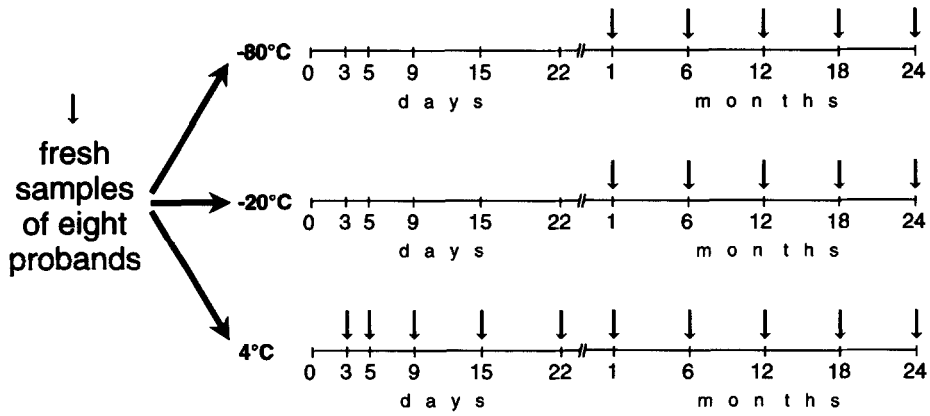

Fig. 1. Schematical illustration of the long-term storage study design. Vertical arrows mark the times of measurement. For explanation see text.

Apolipoprotein concentrations have been measured by various immunochemical techniques including radial immunodiffusion (RID), electroimmunoassay (EIA), radioimmunoassay (RIA), enzyme- as well as fluorescence-linked immunoassay (ELISA, FIA), immunonephelometric and turbidometric assays. Poly- and monoclonal antibodies have been used. Plasma lipids have been measured with commercially available kits for many years.

Only few and conflicting data are available on the influence of long-term storage (7, 8) and multiple freezing and thawing (9) on the plasma concentrations of Lp[a]. Craig et al. (7) showed a significant decrease of 46% in Lp[a] over a period of 6 months. However, the Helsinki heart study (8) reported no significant changes during an observation period of 8.5 years. Sgoutas and Tuten (9) were able to demonstrate with an ELISA kit significantly lower Lp[a] measurements after one freezing-thawing cycle from −20°C. The decrease was significant only after three freezing-thawing cycles from −70°C (9).

No data are available on the influence of sample storage on the plasma concentrations of apoA-IV. Conflicting results are published for apoB (10–13), total cholesterol (14–16), HDL cholesterol (17–25), and triglycerides (14–16).

MATERIALS AND METHODS

Long-term storage

The study design is schematically shown in **Fig. 1.** EDTA blood was collected from eight healthy subjects after an overnight fasting period and stored on ice for 20 min until centrifugation. After low-speed centrifugation for 15 min at 1,500 *g* at 4°C, plasma was separated and divided into multiple 500-μl aliquots in microtubes (Ratiolab, Dreieich, Germany) that were stored at different temperatures (−80°C, −20°C, and 4°C). For baseline values Lp[a], apoA-IV, apoB, total cholesterol, HDL cholesterol, and triglycerides were measured from fresh plasma within 8 h after blood collection. Samples stored at 4°C were analyzed on day 3, 5, 9, 15, 22, and after 1, 4, 6, 12, 18, and 24 months. After 1, 6, 12, 18, and 24 months, one aliquot of samples stored at −20°C and −80°C was thawed at room temperature and analyzed. The samples were shaken immediately before dilution and then kept in an ice bath during all dilution procedures. Each sample was diluted and analyzed in triplicate. To minimize variability of methodology, all three samples from storage conditions at −80°C, −20°C, and 4°C from each volunteer were measured on the same microtitre plate and by the same person during the entire study.

Additionally, two plasma samples with known concentrations of different lipoproteins and apolipoproteins were estimated in triplicate on each microtitre plate to serve as a quality control.

Freezing and thawing

The design of the study is illustrated in **Fig. 2.** EDTA blood was collected from six healthy subjects after an

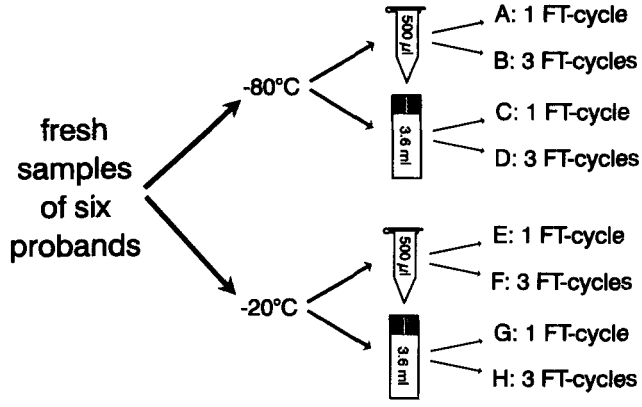

Fig. 2. Schematical illustration of the freezing and thawing study design. For explanation see text; FT-cycle, freezing-thawing cycle.

overnight fasting period and stored in an ice bath until centrifugation. After low-speed centrifugation for 15 min at 1,500 *g* at 4°C, plasma was separated and divided into portions of 500 μl, which were placed in 500 μl microtubes (Treff, Degersheim, Switzerland) as well as in 3.6-ml tubes (Nunc CryoTubes, Roskilde, Denmark). From each test person, half of the 500-μl tubes and half of the 3.6-ml tubes were stored at −20°C, the other half at −80°C. One aliquot in a 500-μl tube was stored in an ice bath. After 1 h one aliquot from each plasma specimen under different storage and tube conditions was thawed at room temperature, the closing cap was opened for 30 sec and reclosed, and the plasma was again stored at −20°C or −80°C. One hour later the same aliquots were again thawed and frozen. This procedure was repeated 1 h later. At this time, additional aliquots from each plasma specimen kept under different storage and tube conditions were thawed for the first time. In summary, we had eight different samples from each individual in addition to fresh plasma (Fig. 2): (A) 500-μl tube stored at −80°C, one freezing and thawing cycle; (B) 500-μl tube, −80°C, three cycles; (C) 3.6-ml tube, −80°C, one cycle; (D) 3.6-ml tube, −80°C, three cycles. (E)-(H) used the same kinds of tubes and freezing-thawing cycles as in (A)-(D), but storage was at −20°C. Five hours after blood collection, all freezing-thawing cycles were completed and analysis of the different samples began. During the analysis procedure all samples were stored in an ice bath. All nine different sample conditions from each volunteer were analyzed on the same microtitre plate. All samples were diluted and estimated in triplicate by the same person.

Laboratory procedures

Lp[a] quantification was performed as described elsewhere (26) with a double-antibody ELISA using an affinity-purified polyclonal apo[a] antibody for coating and the horseradish peroxidase-conjugated monoclonal 1A2 (Boehringer Mannheim, Germany) for detection. This anti-apo[a] antibody does not crossreact with plasminogen (27). The assay was performed in detail as follows. Microtitre plates (MaxiSorp F96, Nunc-Immuno Plate, Roskilde, Denmark) were coated with 5 μg/ml anti-apo[a], incubated for 3 h at 37°C, and then stored overnight at 4°C. Plates were washed with washing buffer (PBS + 0.5 ml Tween 20/l) and blocked with a 0.1% solution of casein in PBS for 30 min at 37°C. After washing, plasma samples and standards were incubated for 2 h at 37°C. Plasma samples were diluted with casein solution to such an extent that the readings for the diluted samples could be expected in the linear range of the calibration curve. For further analysis at the different time points, each sample was diluted in the same manner as during the first analysis on day 0. A commercially available Lp[a]-positive plasma from Immuno (Vienna, Austria) in a dilution series from 1:500–1:8,000 served as standard. All dilutions were made with a Hamilton diluter (Microlab 1000, Bonaduz, Switzerland). After further washing steps, the plate was incubated with the conjugated 1A2 antibody for 2 h at 37°C. The last washing procedure was followed by incubation with the substrate *o*-phenylene diamine for 25 min at room temperature and in darkness. The reaction was stopped with 2 M sulfuric acid. The absorbance was read with a Microplate Reader from Bio-Rad (Modell 3550, Richmond, CA) at a wavelength of 490 nm and was directly proportional to the concentration of Lp[a]. Lp[a] concentrations were expressed as total Lp[a] lipoprotein mass.

Apolipoprotein B plasma concentrations were analyzed as previously described (28). The laboratory procedure was similar as that for the Lp[a] ELISA. An affinity-purified rabbit polyclonal antibody against apoB was used for coating (4 μg/ml) and the same antibody, labeled with horseradish peroxidase, for detection. All plasma samples were diluted 1:25,000 in casein solution. A calibrated standard (Calibration Serum Apolipoprotein, Boehringer Mannheim, Germany) in a dilution series from 1:7,500–1:120,000 served as secondary standard.

Apolipoprotein A-IV plasma concentrations were determined with a recently described ELISA (29). Briefly, affinity-purified polyclonal rabbit antiserum to human apoA-IV was used for coating microtitre plates (10 μg/ml) and for preparing a horseradish peroxidase-conjugated antibody. Plasma with known apoA-IV content served as calibration standard. The ELISA was performed as described above. All samples were diluted 1:2,500 (including preincubation for 1 h at room temperature in a 1:50 dilution of 4 M urea). The calibration standard was diluted in a series from 1:500–1:8,000.

Total cholesterol, HDL cholesterol and triglycerides were determined using commercially available kits from Boehringer Mannheim. Measurements were made on microtitre plates as previously described (30) with some modifications. For cholesterol measurement, 4 μl of the samples and four calibration standards (Preciset Cholesterol Calibrator, Boehringer Mannheim) were diluted by the diluter with Monotest cholesterol reagent (CHOD-PAP-method) 1:50 directly into the wells with a final volume of 200 μl. Calibration standards were used in final concentrations of 8, 4, 2, and 1 mg/dl. For HDL cholesterol measurement, 250 μl HDL cholesterol precipitation reagent (phosphotungstic acid/$MgCl_2$) was added to 100 μl plasma to precipitate the apoB-containing lipoproteins. After 10 min at room temperature the precipitated lipoproteins were removed by centrifuging at 10,000 rpm for 10 min. Of the clear supernatant solution, 50 μl was diluted 1:4 with Monotest cholesterol reagent solution directly into the wells of microtitre plates to a final volume of 200 μl. This ratio was constant for all samples. The standard series used for calibration was the same as for total cholesterol. For triglyceride measurement, 8 μl of the

plasma samples was diluted with triglyceride GPO-PAP reagent 1:25 in the same manner as cholesterol. A gravimetrically prepared glycerol served as standard (30). Precinorm L from Boehringer Mannheim was used as quality control for measuring total cholesterol, HDL cholesterol and triglycerides. After 15 min incubation time at room temperature the absorbance was read immediately at a wavelength of 490 nm with the ELISA reader.

The same lots of calibration standards were used for each assay type throughout the study.

Intra- and interassay coefficients of variation (CV) for the quantification assays used are shown in **Table 1.** The intraassay CV was calculated from the mean and the standard deviation of the measured values of two different samples in 20 replicates with the formula:

$$\frac{SD \times 100}{mean} = CV\ (\%)$$

The interassay CV was calculated with the same formula using the values of two different samples included in 16 runs over 2 months. The coefficients of variation remained constant over the 2-year period of the study.

At the end of the study the stability of samples stored at 4°C, −20°C, and −80°C was further investigated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. SDS-PAGE was performed under reducing conditions as previously described (31). A 6.6% or 15% polyacrylamide gel was used for apo[a] and apoB, or apoA-IV, respectively. The same antibodies used for detection in the ELISA procedures were used for immunoblotting.

Statistical analysis

As plasma concentrations of apolipoproteins A-IV and B, total and HDL cholesterol were normally distributed, the paired *t*-test for comparison of values between different storage conditions was used. For Lp[a] and triglycerides the nonparametric paired Wilcoxon test was applied. The statistical significance of changes over time was calculated by analysis of variance for repeated measurements. This statistical procedure avoids comparison of data between two points and examines the trends as a function of time because this analysis uses all the data rather than those for only two points. Therefore this procedure averages the effect of run-to-run variation. In a first run all data from every time point from day zero to the end of the study were included in this analysis of variance. To exclude the effect of the first freezing, in a second run all data from the end of the first month to the end of the study were included. Because of the skewness of Lp[a] and triglycerides the values of these two parameters were natural-log-transformed prior to analyzing variance. In the long-term storage study the variance for repeated measurements was analyzed between fresh plasma samples and samples stored for 1, 6, 12, 18, and 24 months at −80°C and −20°C. In a second step the same analysis was performed for the values for 1 to 24 months. The correlation coefficients (r) between fresh plasma samples and samples stored for 24 months at different temperatures as well as correlation coefficients (r) between different storage temperatures after 24 months were calculated. The percentage of change was calculated with the formula:

$$\frac{[\text{stored plasma}] \times 100}{[\text{fresh plasma}]} - 100 = \%\ \text{of change}$$

In the freezing–thawing study the statistical significance of change over freezing–thawing cycles was evaluated using the analysis of variance for repeated measurements.

TABLE 1. Intra- and interassay coefficients of variability (%) (CV)

Fraction	Intraassay CV	Interassay CV
Lp[a]	1.94	4.95
ApoB	4.44	9.72
ApoA-IV	4.52	6.56
Cholesterol	2.05	2.67
HDL cholesterol	2.37	3.83
Triglycerides	2.18	3.21

RESULTS

Long-term storage

Table 2 shows the mean plasma concentrations of various apolipoproteins and lipoproteins at different times and storage conditions. **Table 3** describes the same parameters for a storage time up to 6 months at 4°C. **Figure 3** A–F illustrates the percentage of change of these parameters at various times. Only data from the first 6 months are graphically illustrated for storage at 4°C. **Table 4** shows the correlation coefficients (r) of these parameters between fresh plasma and plasma stored for 24 months at different storage temperatures as well as correlation coefficients between different storage temperatures after 24 months.

Comparison between samples stored at −80°C and −20°C showed no difference in any of the parameters at any time with the exception of Lp[a] after 18 and 24 months ($P < 0.05$) (Table 2). After 24 months, however, the correlation coefficients between samples stored at −20°C or −80°C were highly significant for all measured parameters (Table 4).

We always found a highly significant correlation be-

TABLE 2. Influence of long-term storage at different temperatures on Lp[a], apolipoproteins and lipids (mean ± SD)

Fraction	Fresh Samples	Storage Condition	1 mo.	6 mos.	12 mos.	18 mos.	24 mos.	P, fresh to 24 mos.[m]	P, 1 to 24 mos.[m]
		°C							
Lp[a]	23.99 ± 23.28	−80	21.46 ± 19.29	21.82 ± 19.72	21.31 ± 19.10	22.46 ± 20.48[d]	22.86 ± 21.36[d]	<0.01	n.s.
		−20	21.17 ± 19.04	20.53 ± 17.79	20.74 ± 18.09	20.16 ± 17.79[d]	21.01 ± 18.78[d]	<0.001	n.s.
		4	22.79 ± 23.39	23.28 ± 23.28	24.48 ± 22.72	28.51 ± 28.70	25.98 ± 26.11	—	—
ApoB	94.4 ± 29.4	−80	94.8 ± 22.9	94.8 ± 23.9[h]	91.9 ± 25.0[h]	91.1 ± 24.1	87.8 ± 24.9	<0.05	<0.01
		−20	94.9 ± 23.1	93.7 ± 22.2[g]	94.2 ± 24.1[h]	85.7 ± 22.5	89.6 ± 29.6	<0.05	<0.05
		4	97.5 ± 25.1	81.4 ± 22.0[d,b]	55.8 ± 7.9[e,b]	85.7 ± 44.6	67.4 ± 25.7	—	—
ApoA-IV	11.56 ± 3.90	−80	11.53 ± 3.37	11.72 ± 3.88[g]	11.04 ± 3.46[k]	10.64 ± 2.13[k]	10.22 ± 2.27[k]	<0.05	<0.05
		−20	11.47 ± 3.39	11.72 ± 3.54[h]	11.32 ± 3.34[k]	10.73 ± 2.28[k]	10.45 ± 2.50[k]	<0.05	<0.05
		4	11.97 ± 3.12	9.05 ± 3.56[e,a]	3.20 ± 1.44[f,c]	1.33 ± 0.91[f,c]	0.99 ± 0.72[f,c]	—	—
Cholesterol	162.5 ± 24.9	−80	162.6 ± 22.4	166.5 ± 23.1	168.3 ± 24.9	163.0 ± 21.8	165.8 ± 24.1	<0.01	<0.01
		−20	163.1 ± 22.8	166.9 ± 22.3	166.1 ± 23.8	163.1 ± 23.6	165.2 ± 23.6	n.s.	n.s.
		4	160.1 ± 23.6	172.8 ± 30.0	184.0 ± 34.4	222.0 ± 86.4	207.5 ± 59.9	—	—
HDL chol.	54.09 ± 14.58	−80	56.03 ± 15.66[h]	54.84 ± 14.52	55.49 ± 14.70[h]	56.44 ± 15.70	56.08 ± 15.26	<0.01	n.s.
		−20	56.00 ± 15.57[h]	55.32 ± 15.27	55.60 ± 15.34[h]	55.59 ± 15.32	55.34 ± 14.05	n.s.	n.s.
		4	51.23 ± 13.63[b,c]	48.61 ± 16.13	43.01 ± 13.15[b,c]	104.19 ± 89.14	118.92 ± 101.04	—	—
TG	96.6 ± 123.3	−80	99.4 ± 126.9	97.5 ± 119.7	100.2 ± 130.7	98.2 ± 122.2	99.3 ± 121.4[g]	<0.05	n.s.
		−20	99.1 ± 126.3	101.0 ± 121.3	100.8 ± 129.9	97.1 ± 120.5	99.1 ± 123.5[g]	<0.01	<0.01
		4	99.3 ± 125.7	101.2 ± 124.2	96.8 ± 119.2	123.1 ± 124.9	118.1 ± 125.1[d,a]	—	—

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$, for comparison with values stored at −80°C.
[d] $P < 0.05$, [e] $P < 0.01$, [f] $P < 0.001$, for comparison with values stored at −20°C.
[g] $P < 0.05$, [h] $P < 0.01$, [k] $P < 0.001$, for comparison with values stored at 4°C.
[m] No statistical evaluation was done for samples stored at 4°C because measured values after 6 months were too scattered for comparison; n.s., not significant.

tween fresh plasma samples and samples stored at −80°C or −20°C for a period of 24 months (Table 4). Triglycerides and Lp[a] showed the highest correlation with fresh plasma samples.

Lp[a] stored at −80°C or −20°C showed significant changes after 24 months with a mean decrease of 7% and 13%, respectively (Table 2 and Fig. 3A). The major part of these changes occurred during the first month. Storage at 4°C produced a continuous decrease, which became significant after 15 days, and in the course of time the recovery of measured values became very scattered (Table 3 and Fig. 3A).

Samples stored at −80°C for 24 months showed mean changes for apoB, apoA-IV, total cholesterol, HDL cholesterol and triglycerides of −5.4% ($P < 0.05$), −9.1% ($P < 0.05$), +2.2% ($P < 0.01$), +3.7% ($P < 0.01$), and +6.8% ($P < 0.05$), respectively. Samples stored at −20°C showed very similar results: −4.8% ($P < 0.05$), −9.1% ($P < 0.05$), +1.8% (n.s.), +3.1% (n.s.) and +4.8% ($P < 0.01$), respectively (Fig. 3 B–F).

Figure 4 shows the immunoblots with detection of apo[a], apoB, and apoA-IV for one sample at each storage condition after 24 months. Apo[a] was well detectable from samples of all three storage conditions. ApoB and apoA-IV were only detected in samples stored at −20°C or −80°C.

TABLE 3. Influence of long-term storage at 4°C on the plasma concentrations of Lp[a], apolipoproteins and lipids (mean ± SD)

Storage	Lp[a]	ApoB	ApoA-IV	Cholesterol	HDL Chol.	Triglycerides
Fresh plasma	23.99 ± 23.28	94.4 ± 29.4	11.56 ± 3.90	162.5 ± 24.9	54.09 ± 14.58	96.6 ± 123.3
3 days	23.37 ± 22.89	95.6 ± 23.8	11.41 ± 4.07	158.7 ± 24.0[b]	52.53 ± 14.85[a]	98.3 ± 123.8[a]
5 days	23.48 ± 23.78	98.3 ± 30.6	11.45 ± 3.91	163.0 ± 24.0[b]	53.00 ± 14.81[b]	97.6 ± 124.9[b]
9 days	22.80 ± 21.64	103.7 ± 27.9	11.20 ± 2.75	157.9 ± 23.5[c]	51.04 ± 14.24[c]	98.5 ± 124.7[b]
15 days	21.53 ± 21.47[c]	96.9 ± 28.7	11.15 ± 2.67	165.7 ± 23.1[c]	52.53 ± 14.58[c]	98.5 ± 125.6[b]
22 days	22.06 ± 22.20[c]	97.8 ± 22.4	11.58 ± 3.32	158.6 ± 24.5[c]	51.14 ± 14.13[c]	100.8 ± 124.2[c]
1 month	22.79 ± 23.39[c]	97.5 ± 25.1	11.97 ± 3.12	160.1 ± 23.6[c]	51.23 ± 13.63[c]	99.3 ± 125.7[c]
4 months	21.32 ± 20.84[c]	89.2 ± 22.5[b]	9.61 ± 3.58[b]	173.1 ± 27.0[c]	47.49 ± 14.03[c]	104.0 ± 129.6[c]
6 months	23.28 ± 23.28[b]	81.4 ± 22.0[c]	9.05 ± 3.56[c]	172.8 ± 30.0[c]	48.61 ± 16.13[b]	101.2 ± 124.2[c]

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$, for comparison with values of fresh plasma considering all previous measured values (analysis of variance for repeated measurements).

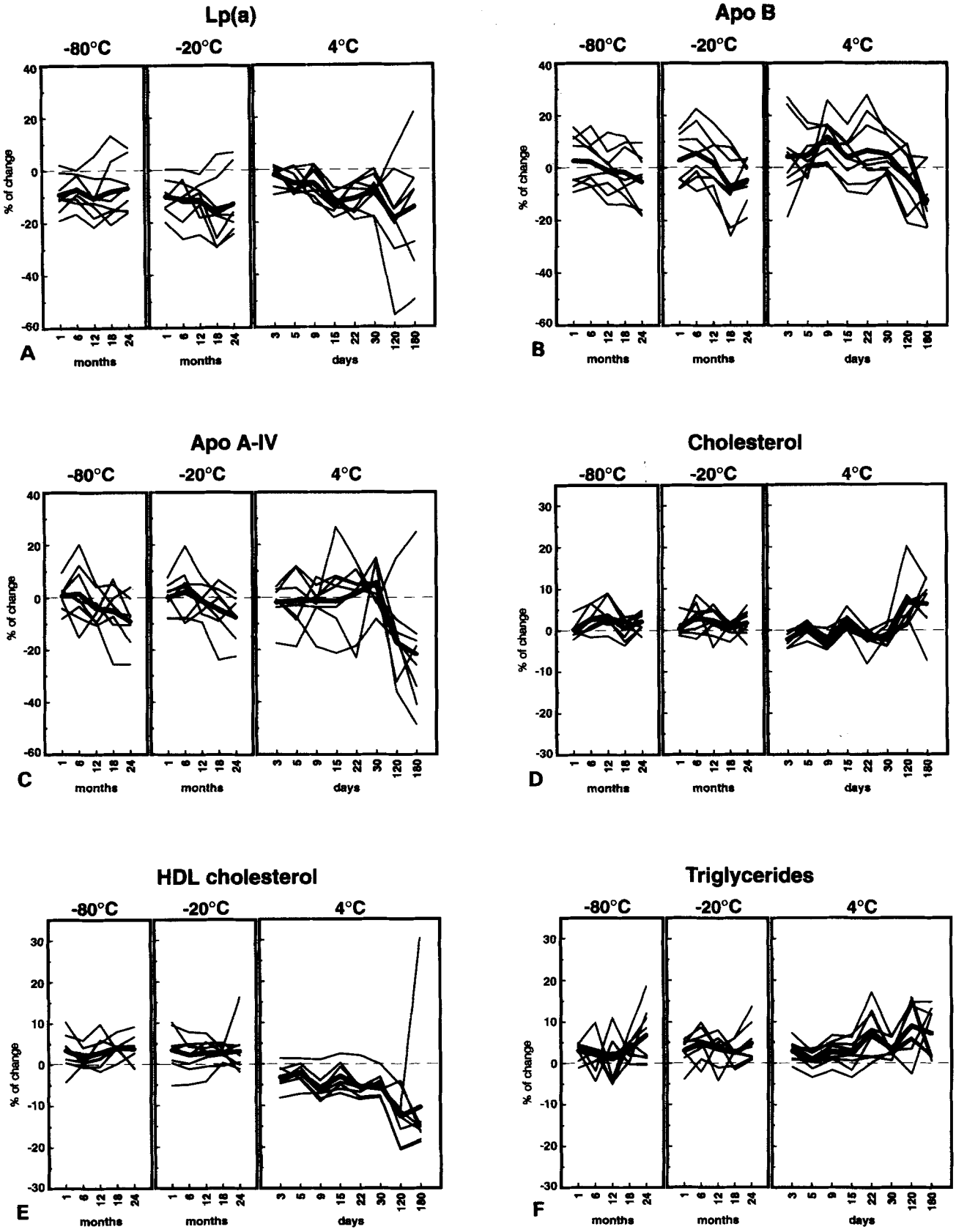

Fig. 3. Percentage of change for each sample over time at different storage conditions. The bold line is the calculated mean value of all eight samples.

TABLE 4. Correlation coefficients (r) between fresh plasma and samples stored for 24 months at different storage temperatures as well as correlation coefficients (r) between samples stored at different temperatures for a period of 24 months

	Correlation between Fresh Plasma and Samples Stored for 24 mos. at			Correlation between Different Storage Temperatures after 24 mos.		
	−80°C	−20°C	4°C	−80°C vs. −20°C	−80°C vs. 4°C	−20°C vs. 4°C
Lp[a]	0.992[b]	0.976[b]	0.820[a]	0.996[b]	0.882[a]	0.918[b]
ApoB	0.936[b]	0.959[b]	0.045	0.987[b]	0.054	−0.017
ApoA-IV	0.984[b]	0.980[b]	0.552	0.964[b]	0.525	0.524
Cholesterol	0.990[b]	0.980[b]	0.417	0.953[b]	0.316	0.520
HDL chol.	0.990[b]	0.992[b]	0.590	0.993[b]	0.554	0.580
TG	1.000[b]	1.000[b]	0.982[b]	1.000[b]	0.980[b]	0.981[b]

[a] $P < 0.01$; [b] $P < 0.001$.

Freezing and thawing

Multiple freezing and thawing of plasma stored in tubes filled either without air space (A, B, E, F) or with more than 3 ml air space (C, D, G, H) had no influence on the measured values of apoA-IV, total and HDL cholesterol or triglycerides (**Table 5** and **Fig. 5**). Samples stored in 3.6-ml tubes at −80°C showed significantly lower values for Lp[a] after one and three freezing–thawing cycles in comparison to fresh plasma samples. The decrease in 3.6-ml samples stored at −20°C and 500-μl samples stored at −80°C was of borderline significance.

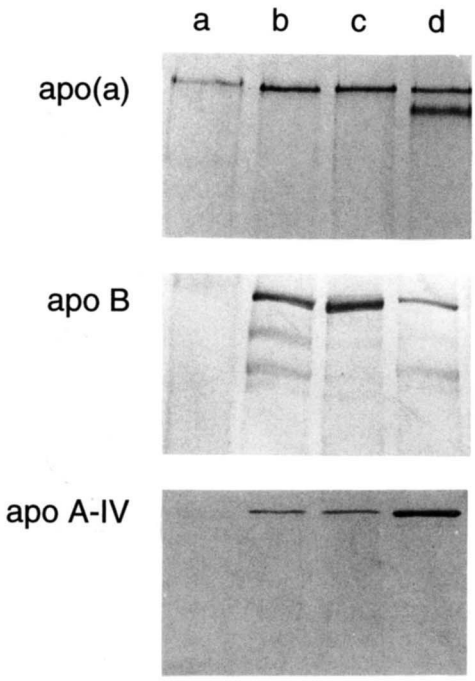

Fig. 4. Immunoblot analysis of apo[a], apoB and apoA-IV from one sample stored for 24 months at 4°C (lane a), −20°C (lane b) and −80°C (lane c) and one standard sample (lane d). The apo[a] type was determined as S3 and is in agreement with repeated phenotyping of fresh samples from the same donor.

ApoB recovery was very scattered, and because of these irregular changes we found the only significant alterations for 3.6-ml tubes stored at −80°C.

DISCUSSION

Controversy prevails on the influence of storage conditions on lipoprotein assays. One of our major findings is that there is no significant difference in the measurement stability of most studied lipids and apolipoproteins between samples stored at −80°C or −20°C for a period up to 24 months (Table 2). Only Lp[a] values were significantly lower in samples stored at −20°C than at −80°C with a mean difference of 6%. This significant difference was first observed after 18 months of storage. For Lp[a] Craig et al. (7) did not find a difference between the two storage temperatures over a period of 6 months, which agrees with our data. But they reported a highly significant continuous decrease of 46% for Lp[a] within 6 months, when measured by RID. After 6 months we found a mean decrease of 7% in samples stored at −80°C and 11% at −20°C. After 24 months we measured 7% and 13% lower values, respectively. This decrease was significant at both times, but clearly lower in comparison to Craig et al. (7), whose investigations were done with sera. Nothing is known about the influence of coagulation factors on the stability of Lp[a]. Most of the decrease in our study occurred during the first month (9% and 10%, respectively) and not during long-term storage (Fig. 3). From the end of the first month to the end of the second year we found no significant change in measured Lp[a] plasma concentrations. Considering the results of multiple freezing and thawing (Table 5), we suppose that the decrease in measured values is caused by the first freezing and thawing. It therefore appears that long-term storage has only little influence on the stability of Lp[a] or, to put it more precisely, on the stability of the measured values. Our ELISA system consists of an affinity-purified poly-

TABLE 5. Influence of freezing-thawing and filling status of tubes on Lp[a], apolipoproteins and lipids at different storage temperatures

Fraction	Fresh Plasma	1 Cycle	3 Cycles	P^a
Lp[a]	35.09 ± 32.89			
500-μl tubes, −80°C		34.26 ± 32.12	33.09 ± 31.00	0.06
500-μl tubes, −20°C		34.31 ± 31.70	34.84 ± 32.29	n.s.
3.6-ml tubes, −80°C		33.84 ± 32.01	31.59 ± 30.60	0.008
3.6-ml tubes, −20°C		34.77 ± 32.30	33.66 ± 31.00	0.07
ApoB	78.0 ± 27.4			
500-μl tubes, −80°C		83.1 ± 17.6	80.1 ± 21.7	n.s.
500-μl tubes, −20°C		84.4 ± 29.3	90.1 ± 25.1	0.10
3.6-ml tubes, −80°C		84.4 ± 21.1	77.6 ± 24.0	0.04
3.6-ml tubes, −20°C		83.3 ± 19.9	87.1 ± 25.0	n.s.
ApoA-IV	14.87 ± 3.16			
500-μl tubes, −80°C		14.79 ± 3.27	15.36 ± 3.44	n.s.
500-μl tubes, −20°C		14.62 ± 3.29	15.44 ± 3.14	n.s.
3.6-ml tubes, −80°C		15.03 ± 3.33	14.87 ± 3.92	n.s.
3.6-ml tubes, −20°C		14.98 ± 3.86	14.69 ± 3.23	n.s.
Cholesterol	179.7 ± 33.6			
500-μl tubes, −80°C		179.1 ± 35.5	182.8 ± 33.9	n.s.
500-μl tubes, −20°C		180.2 ± 33.9	183.2 ± 32.7	n.s.
3.6-ml tubes, −80°C		178.7 ± 35.5	180.7 ± 33.9	n.s.
3.6-ml tubes, −20°C		180.8 ± 34.2	177.8 ± 30.0	n.s.
HDL chol.	45.64 ± 11.71			
500-μl tubes, −80°C		45.85 ± 11.17	46.69 ± 11.90	n.s.
500-μl tubes, −20°C		46.38 ± 11.51	47.28 ± 11.57	n.s.
3.6-ml tubes, −80°C		45.63 ± 11.47	45.65 ± 11.00	n.s.
3.6-ml tubes, −20°C		45.94 ± 11.42	45.86 ± 11.29	n.s.
TG	91.9 ± 50.5			
500-μl tubes, −80°C		93.3 ± 50.8	93.8 ± 50.0	n.s.
500-μl tubes, −20°C		93.0 ± 50.4	94.8 ± 50.1	n.s.
3.6-ml tubes, −80°C		92.8 ± 50.7	93.8 ± 50.8	n.s.
3.6-ml tubes, −20°C		93.3 ± 50.0	92.2 ± 50.6	n.s.

[a]The statistical significance of change over freezing-thawing cycles was evaluated using analysis of variance for repeated measurements; n.s., not significant.

clonal anti-apo[a] antibody for coating and a monoclonal 1A2 for detection. Obviously, this monoclonal 1A2 antibody is directed at an antigenic epitope of apo[a] that remains relatively intact during long-term storage. This epitope has been shown to reside on the highly repetitive kringle IV domain of apo[a] and encompasses the linear sequence PEYYPN (H. Dieplinger and G. Utermann, unpublished results). The Lp[a] measurement from plasma stored at 4°C was very stable for the first 9 days. Afterwards we found decreased values that were significant because of their uniformity: all eight samples showed lower concentrations than fresh samples. When mean values (Tables 2 and 3) and percentage of change during the first months (Fig. 3A) were analyzed, a mean decrease was detected that was lower than in frozen samples and not as scattered. Measuring Lp[a] within 1 month from samples stored at 4°C is therefore just as precise as from samples stored at −20°C or −80°C. Phenotyping of apo[a] from samples stored for 24 months at 4°C was still possible, provided samples had concentrations higher than 10 mg/dl. Phenotyping of samples stored at −20°C or −80°C for a period of 24 months was problem-free. It is important to note that comparisons were done with the same samples under various conditions of storage. Thus there was no change in the isoform type unless changes were a result of degradation of apo[a]. Degradation, however, has been excluded by immunoblotting which showed the same pattern in repeatedly analyzed fresh samples from the same donor.

Multiple freezing and thawing of plasma samples had an influence on the measured Lp[a] values (Table 5 and Fig. 5). In all storage conditions we found lower Lp[a] values that were significantly lower in 3.6-ml tubes stored at −80°C and borderline significantly lower in 3.6-ml tubes stored at −20°C and 500-μl tubes stored at −80°C. These findings are similar to those of Sgoutas and Tuten (9) who found a significant decrease in samples stored at −20°C after one freezing-thawing cycle and in samples stored at −80°C after three cycles. They used a commercially available ELISA system. The same samples measured with an immunoturbidometric assay (ITA) showed a significant decrease after three and four cycles, respectively. From our data for Lp[a] measurement, we recommend that storage conditions never be changed in an ongoing study and that fresh and frozen plasma samples never be compared.

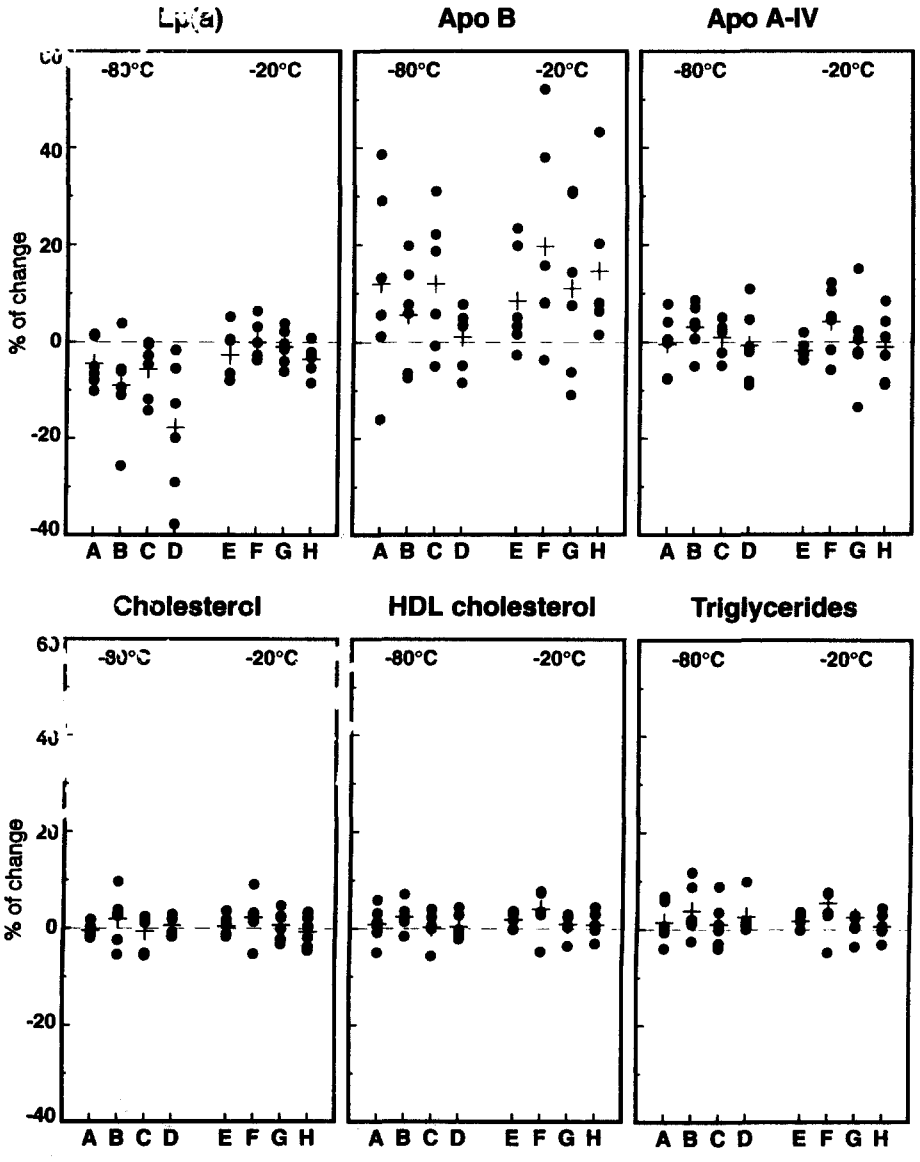

Fig. 5. Percentage of change in measured values from multiple freezing and thawing and filling status of tubes in six plasma samples in comparison to values measured for fresh plasma samples. (A) 500-μl tube stored at −80°C, one freezing and thawing cycle; (B) 500-μl tube, −80°C, three cycles; (C) 3.6-ml tube, −80°C, one cycle; (D) 3.6-ml tube, −80°C, three cycles; (E) to (H) same kind of tubes and freezing-thawing cycles as for (A) to (D), but storage at −20°C. The mean value of all six samples is graphically illustrated as "+."

During the first months we observed a small increase in measured apoB in samples stored at −80°C and −20°C, and thereafter a decrease of 5% after 24 months for both temperatures ($P < 0.05$, Table 2). In samples stored at 4°C we saw the same pattern with an increase during the first month and a decrease thereafter (Table 3). These findings are in agreement with La Belle et al. (10) who used a monoclonal ELISA system. Other groups found no change after 11 months (11) or a significant 6.8% decrease after 18 months when using an ELISA system (12), and a 6.5% decrease after 22 months (13) with radial immunodiffusion, with all samples stored at −70°C. We must bear in mind that the changes observed in our study are smaller than the interassay CV, which is nearly 10% for this ELISA because of the two steps needed to reach a final dilution of 1:25,000. This high interassay CV also explains the wide dispersion of changes in measured values.

The apoA-IV values measured showed a continuous decrease over time ($P < 0.05$, Table 2). The changes after

24 months were 9% for samples stored at −80°C and 7.5% for samples stored at −20°C. The values for samples stored at 4°C were very stable within the first month. From 6 months until the end of the study an abrupt drop of more than 90% was seen. After 24 months immunoblotting showed no detectable apoA-IV band in samples stored at 4°C, which suggests that apoA-IV had disintegrated and/or the epitopes were no longer detectable for our antibody against apoA-IV. In contrast to Lp[a], the decrease in apoA-IV was continuous and not caused by the first freezing and thawing. This was confirmed by the freezing–thawing study, where we observed no significant changes from multiple freezing and thawing.

Total and HDL cholesterol increased in samples stored at −80°C during the observation time (Table 3, Fig. 3, D and E). These small increases (+2.2% and +3.7%, respectively) are statistically significant because of their uniformity. However, these differences are in the range of the interassay variability and therefore meaningless. Other groups did not observe a difference in total cholesterol during shorter storage periods (14, 15) or showed an increase of nearly 15% during 2 weeks storage at −20°C (16). No data have been published on long-term storage exceeding 2 years. A large number of studies investigated HDL cholesterol stability, the results being controversial: increase (17, 18) as well as decrease (19–22) and no change (23) were reported. Bachorik et al. (24, 25) reported a decrease in samples stored at −20°C and −70°C, but the changes correlated with HDL cholesterol levels.

With a test kit from Boehringer, Pini et al. (16) observed a 10.4% increase in triglycerides after 2 weeks of storage at −20°C. Other studies did not confirm such changes (14, 15). We also found a small but significant increase of 6.8% for samples stored at −80°C and 4.8% for those stored at −20°C.

As illustrated in Fig. 3, D–F and Fig. 5, total cholesterol, HDL cholesterol, and triglycerides always showed very little scatter, and recovery was close to 100% in both studies, the long-term storage study and the freezing–thawing study. In these three parameters the influence of vaporization caused by a large air space in the tubes was only minor (Table 5).

When examining all these data, we have to consider the interassay CVs of the quantification systems used (Table 1). For all lipids and apolipoproteins the difference between fresh and frozen samples was always lower than the twofold interassay CV. Only the Lp[a] plasma concentrations from samples stored at −20°C for 24 months showed a greater decrease (−13%) than did the twofold interassay CV. In the case of Lp[a] we have to bear in mind that most of the decrease occurred during the first month and that the long-term storage had only little influence on the measured values. Therefore, we recommend not to change the storage conditions in subgroups of samples in an ongoing study and not to compare values from frozen stored samples with fresh plasma samples.

We are well aware about the problems of generalization of our conclusions from eight samples. Because of the very uniform trends in the samples from all eight probands we feel that, despite the small group size, we are able to generalize our results with care. On the other hand, it is safe to assume strong differences between the various assay types used when we compare our results with those of others (7).

In summary, we found only few significant differences between samples stored at −80°C and −20°C and smaller changes in long-term observations in all lipoproteins and apolipoproteins than did other investigators. Because of the different results in measuring Lp[a] and other apolipoproteins in comparison to other quantification systems, an investigation of the influence of long-term storage and repeated freezing and thawing of plasma samples appears necessary for each antibody and assay type used.

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