The odds ratio for osteoporosis in patients with the *s* allele was 5.68 (95% confidence interval, 1.46–22). Thus, the *s* allele was present in 27.5% of the women with primary osteoporosis, but only in 6.2% of healthy controls. This overrepresentation of the *s* allele in patients with primary osteoporosis is consistent with previous reports showing an increased risk of fractures in post-menopausal heterozygote women, suggesting that the *COLIA1* Sp1 polymorphism has a potential value as a predictor of osteoporosis.

In conclusion, the introduction of an *Fnu*4HI restriction site allows us to perform *S* and *s* genotyping of the *COL1A1* Sp1 polymorphism by PCR-restriction fragment length polymorphism and polyacrylamide minigel electrophoresis. This method is faster, easier, provides better fragment resolution, and is more cost-effective than the method described previously by Grant et al. *(7)*. An additional advantage is that the second *Fnu*4HI restriction site can serve as an internal control for PCR cutting. Moreover, this study suggests that in premenopausal women with primary osteoporosis, there is a high prevalence of the *s* allele and the *Ss* genotype, reinforcing the suggestion that *COLIA1* genotype predisposes to osteoporosis.

References

1. Kanis JA, Melton LJ III, Christiansen C, Johnston CC, Khaltaev N. The diagnosis of osteoporosis. J Bone Miner Res 1994;9:1137–41.
2. Gueguen R, Jouanny P, Guillemin F, Kuntz C, Pourel J, Siest G. Segregation analysis and variance components analysis of bone mineral density in healthy families. J Bone Miner Res 1995;12:2017–22.
3. Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, et al. Prediction of bone density from vitamin D receptor alleles. Nature 1994;367:284–7.
4. Cooper GS, Umbach DM. Are vitamin D receptor polymorphism associated with bone mineral density ? A meta-analysis. J Bone Miner Res 1996;11:1841–9.
5. Kobayashi S, Inoue S, Hosoi T, Ouchi Y, Shiraki M, Orimo H. Association of bone mineral density with polymorphism of the estrogen receptor gene. J Bone Miner Res 1996;11:306–11.
6. Byers PH, Steiner RD. Osteogenesis imperfecta. Annu Rev Med 1992;43:269–82.
7. Grant SFA, Reid DM, Blake G, Herd R, Fogelman I, Ralston SH. Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen type I α1 gene. Nat Genet 1996;14:203–5.
8. Keen RW, Woodford-Richens KL, Grant SFA, Lanchbuey JS, Ralston SH, Spector TD. Type I collagen gene polymorphism is associated with osteoporosis and fracture. J Bone Miner Res 1997;12:S489.
9. Uitterlinden AG, Burger H, Huang Q, Yue F, McGuigan FEA, Grant SFA, et al. Relation of alleles of the collagen type Iα1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women. N Engl J Med 1998;338:1016–21.
10. Langdahl BL, Ralston SH, Grant SFA, Eriksen EF. An Sp1 binding site polymorphism in the *COLIA1* gene predicts osteoporotic fractures in both men and women. J Bone Miner Res 1998;13:1384–9.
11. Garnero P, Borel O, Grant SFA, Ralston SH, Delmas PD. Collagen Iα1 Sp1 polymorphism, bone mass and bone turnover in healthy French premenopausal women: the OFELY study. J Bone Miner Res 1998;13:813–7.
12. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:12–5.

Glycohemoglobin Results in Samples with Hemoglobin C or S Trait: A Comparison of Four Test Systems,

William L. Roberts,[1] Janelle M. Chiasera,[2] and Kory M. Ward-Cook[2]* ([1] The University of Utah Department of Pathology, Salt Lake City, UT 84112, and [2] The Ohio State University School of Allied Medical Professions, Columbus, OH 43210; * address correspondence to this author at: Board of Registry, American Society of Clinical Pathologists, 2100 Harrison St., Chicago, IL 60612; fax 312-738-5808, e-mail koryw@ascp.org)

A wide variety of commercial methods are available to measure glycohemoglobin (gHb). These methods measure various species of gHb, e.g., total gHb, hemoglobin A_1 (Hb A_1), or Hb A_{1c}. Some methods are based on charge differences between glycated and nonglycated hemoglobins (e.g., cation-exchange chromatography, electrophoresis, and isoelectric focusing), whereas boronate affinity methods depend upon the binding of the sugar groups on the hemoglobin molecule *(1)*. Immunoassay of Hb A_{1c} depends on the presence of an epitope that includes glucose and N-terminal amino acids of the β chain of hemoglobin. These methods usually measure Hb A_{1c} as a percentage of total hemoglobin. Some immunoassay methods also measure Hb S_{1c} or Hb C_{1c}, whereas others do not *(2)*. The National Glycohemoglobin Standardization Program (NGSP) was established to certify the various commercial methods so that they can be related to the candidate reference method used in the Diabetes Control and Complications Trial (DCCT) *(3, 4)*.

Recently, the effects of Hb AC and Hb AS on one gHb immunoassay method were assessed *(5)*. Samples containing Hb C trait showed modestly higher results than the HPLC method used. Although hemoglobin variants are relatively rare in Caucasians of northern European descent, the African-American population has an 8% prevalence of Hb AS and a 3% prevalence of Hb AC *(6, 7)*. In this study, we describe the influence of Hb AS and Hb AC on four DCCT-traceable gHb methods.

A total of 129 samples were collected in evacuated tubes containing EDTA as an anticoagulant. Samples with hemoglobin variants were identified by comparison of retention times on the Diamat system (Bio-Rad Clinical Laboratories) to known retention times for Hb S and Hb C. Of the 129 samples collected, 47 were homozygous for Hb A, 39 were heterozygous for Hb C, and 43 were heterozygous for Hb S. Samples were shipped on dry ice and stored at −70 °C until analysis.

Four commercial systems were used to measure gHb according to the manufacturers' instructions: the Diamat, the DCA 2000 (Bayer Diagnostics), the Unimate performed on a Cobas Mira L (Roche Diagnostics Systems), and the A1c 2.2 Plus (Tosoh Medics). Results for the Diamat and DCA 2000 methods using these samples have been published *(5)*. The purpose of this study was to investigate the effects of variant hemoglobins on the A1c 2.2 Plus and Unimate methods.

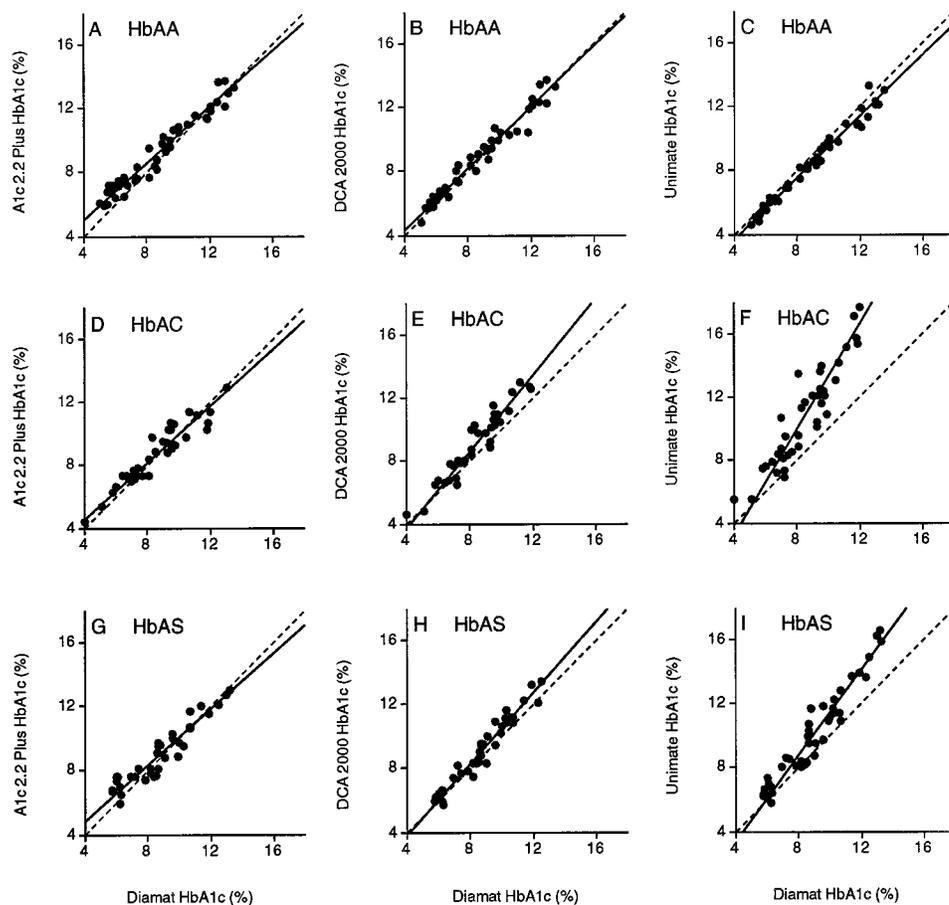Data analysis including Deming regression was per-

Fig. 1. Comparison of Hb A_{1c} results obtained by the Diamat, A1c 2.2 Plus, DCA 2000, and Unimate methods.

Results were compared using Deming regression analysis (———); an ideal comparison with slope = 1.00 and intercept = 0.0 is indicated by (- - - - - - -). Results for samples containing only Hb A are shown in *panels A* (n = 47; slope = 0.88 ± 0.03; intercept = 1.46 ± 0.26; r = 0.975; $S_{y|x}$ = 0.51), *B* (n = 45; slope = 0.96 ± 0.03; intercept = 0.51 ± 0.25; r = 0.982; $S_{y|x}$ = 0.46), and *C* (n = 47; slope = 0.96 ± 0.02; intercept = −0.12 ± 0.18; r = 0.990; $S_{y|x}$ = 0.35). Results for samples containing Hb C trait are shown in *panels D* (n = 36; slope = 0.90 ± 0.02; intercept = 0.95 ± 0.43; r = 0.949; $S_{y|x}$ = 0.61), *E* (n = 36; slope = 1.21 ± 0.06; intercept = −1.07 ± 0.51; r = 0.957; $S_{y|x}$ = 0.67), and *F* (n = 39; slope = 1.66 ± 0.10; intercept = −3.30 ± 0.89; r = 0.925; $S_{y|x}$ = 1.26). Results for samples containing Hb S trait are shown in *panels G* (n = 37; slope = 0.87 ± 0.05; intercept = 1.34 ± 0.43; r = 0.946; $S_{y|x}$ = 0.63), *H* (n = 40; slope = 1.11 ± 0.04; intercept = −0.61 ± 0.35; r = 0.975; $S_{y|x}$ = 0.49), and *I* (n = 43; slope = 1.34 ± 0.05; intercept = −1.89 ± 0.50; r = 0.965; $S_{y|x}$ = 0.79).

formed using EP Evaluator release 3 software (David G. Rhoads).

The mean percentage of the Hb S in samples with Hb AS was 38% (range, 25–41%), and the mean percentage of Hb C in samples with Hb AC was 38% (range, 29–41%). Samples from patients homozygous for Hb A served as controls for detecting calibration differences between methods. The Diamat was chosen as the comparison method, and Deming regression analysis was performed (Fig. 1).

The Diamat and A1c 2.2 Plus HPLC methods showed similar agreements for all three sample types (Fig. 1, A, D, and G). The DCA 2000 immunoassay and the Diamat agreed well for samples containing only Hb A (Fig. 1B), but for samples containing Hb S, and especially Hb C, slopes of the regression line were >1 (Fig. 1, E and H). The Unimate immunoassay method agreed with the Diamat on samples containing only Hb A (Fig. 1C), but the results were higher than the Diamat results for samples with Hb S (Fig. 1I) and even higher for samples with Hb C (Fig. 1F).

We calculated the mean difference attributable to the presence of a variant hemoglobin from Diamat results for each method at Diamat Hb A_{1c} values of 6% and 9% (Table 1). The results obtained with the A1c 2.2 Plus method were modestly lower than the results obtained with the Diamat for samples with Hb C trait at Hb A_{1c} concentrations of both 6% and 9% and minimally lower for samples with Hb S trait. The results obtained with the DCA 2000 method were higher than the results obtained with the Diamat at 9% Hb A_{1c} for both Hb AS and Hb AC. The results obtained with the Unimate were higher than

Table 1. Average differences from Diamat results for samples with Hb C or Hb S traits.[a]

Diamat	A1c 2.2 Plus		DCA 2000		Unimate	
	AC	AS	AC	AS	AC	AS
6.0	−0.38	−0.17	−0.06	−0.20	+1.03	+0.50
9.0	−0.32	+0.20	+0.69	+0.25	+3.13	+1.63

[a] Deming regression analysis was performed using the Diamat as the reference method. The average difference of each of the other three methods at Diamat medical decision levels of 6% and 9% was calculated for each hemoglobin trait. To correct for intermethod calibration differences, the mean difference for homozygous Hb A samples was subtracted from that calculated for samples containing the Hb C or Hb S trait.

the results obtained with the Diamat at 6% and 9% Hb A_{1c} for both Hb AS and Hb AC samples.

Certification of gHb methods has aided in the reduction of differences between methods, as demonstrated in a recent College of American Pathologists gHb survey (8). All four methods in this study have been certified as traceable to the DCCT by the NGSP. Both the DCA 2000 and Unimate methods yielded data consistent with certification by the NGSP when Hb A samples were analyzed. When samples containing Hb C trait were analyzed with the DCA 2000 method, the mean result was substantially higher than that obtained with the Diamat at 9% Hb A_{1c}. When samples containing Hb C and Hb S traits were analyzed with the Unimate method, the mean result was substantially higher than that obtained with the Diamat at 9% Hb A_{1c}. When the A1c 2.2 Plus method was designated the reference method, the DCA 2000 results again were substantially higher for samples containing Hb C, as were the Unimate results for samples containing both Hb C and Hb S (data not shown).

These apparent overestimations of Hb A_{1c} by the Unimate method in particular would appear to be clinically significant. It is noteworthy that both immunoassays showed significant differences from the Diamat HPLC method only at Hb A_{1c} values above the upper limits of the reference interval. Previous reports that indicated that the DCA 2000 method is not affected by hemoglobin variants used only samples from nondiabetic patients (2, 9). These observations confirm the necessity of the NGSP guidelines requiring bias estimates at 6% and 9% Hb A_{1c} (3). The package insert for the Unimate method indicates that specimens containing Hb C and Hb S variants may yield higher than expected Hb A_{1c} results, but the magnitude of this problem is not quantified.

There are several possible explanations for the higher values seen with the immunoassay methods compared with the HPLC methods for samples containing Hb C trait. One explanation is that the HPLC methods are underestimating the true Hb A_{1c} value. Both HPLC methods express Hb A_{1c} as a percentage of total Hb A, as previously recommended (10), whereas the immunoassay methods express results as a percentage of total hemoglobin. Inspection of a Diamat chromatogram reveals the presence of a peak eluting after Hb A but before Hb C that probably is Hb C_{1c}. This peak is adequately resolved from Hb A when the Diamat is operated using an extended program.

Another possible explanation is that the immunoassay methods overestimate the true Hb A_{1c} concentration. The monoclonal antibody used in the DCA 2000 and Unimate assays is identical (11). Hb S and Hb C differ from Hb A by a single amino acid substitution at the sixth position of the β chain (Glu to Val or Glu to Lys, respectively). These amino acid substitutions are very close to the glycated amino terminus that is recognized by the monoclonal antibody in the immunoassays. It is possible that the glycated variant hemoglobins Hb C_{1c} and Hb S_{1c} have a higher affinity than Hb A_{1c} for the mouse monoclonal antibody-latex particles used in the assay. The difference

between the DCA 2000 and Unimate immunoassays might arise from the proteolytic digestion that occurs with the Unimate assay. Pepsin, the protease used in the Unimate method, can cleave the β chain of Hb A, Hb C, and Hb S between amino acids 3 and 4, 7 and 8, 11 and 12, and 14 and 15 (12). The sequence of the tripeptide generated is not altered; however, the sequences of the peptides containing 7, 11, and 14 amino acids are altered at the sixth position in Hb C and Hb S. We hypothesize that these peptides and intact Hb C_{1c} have an increased affinity for the anti-Hb A_{1c} antibody. The difference between the DCA 2000 and Unimate assays thus may be attributable to different steric effects at the antigen recognition site produced by differences in the latex particles to which the common monoclonal antibody is attached.

The magnitude of differences between methods observed in this study was dependent on the type of hemoglobin present, the percentage of Hb A_{1c} in the sample, and the method used. The presence of a hemoglobin variant such as Hb S or Hb C may adversely impact the accuracy of a gHb result. Physicians and laboratorians should be able to refer to the manufacturer's technical literature to judge whether the presence of a common hemoglobin variant has a clinically significant effect on the test result. More information needs to be collected on the effect of various hemoglobin variants on specific Hb A_{1c} test systems. One advantage of an HPLC method for gHb analysis is that the analyst may be alerted to the presence of a variant hemoglobin in many cases, whereas no such warning is generated by immunoassay methods.

No data relating mean blood glucose and Hb A_{1c} in patients with Hb C or S trait have been published to our knowledge. The red cell survival in patients with Hb S trait is comparable to that seen in patients who are homozygous for Hb A (13). We presume the same is true for Hb C trait, but no published study could be found. We hypothesize that Hb A_{1c} results measured by the Diamat or A1c 2.2 Plus methods in patients with Hb C or Hb S trait correspond to the same mean blood glucose values as they would in patients homozygous for Hb A.

References

1. Goldstein DE, Little RR, Weidmeyer HM, England JD, McKenzie EM. Glycated hemoglobin: methodologies and clinical applications. Clin Chem 1986;32:B64–70.
2. Weykamp CW, Penders TJ, Muskiet FAJ, van der Slik W. Influence of hemoglobin variants and derivatives on glycohemoglobin determinations, as investigated by 102 laboratories using 16 methods. Clin Chem 1993;39:1717–23.
3. National Glycohemoglobin Standardization Program. Background and rationale. http://www.missouri.edu/~diabetes/ngsp/1.html.
4. Goldstein DE, Little RR. Monitoring glycemia in diabetes. Short-term assessment. Endocrinol Metab Clin N Am 1997;26:475–86.
5. Roberts WL, McCraw M, Cook CB. Effects of sickle cell trait and hemoglobin c trait on determinations of Hb A_{1c} by an immunoassay method. Diabetes Care 1998;21:983–6.
6. Schneider RG, Hightower B, Hosty TS, Ryder H, Tomlin G, Atkins R, et al. Abnormal hemoglobins in a quarter million people. Blood 1976;48:629–37.
7. Meyer LM, Adams JG III, Steinberg MH, Miller IE, Stokes N. Screening for sickle cell trait: the Veterans Administration National Sickle Cell Program. Am J Hematol 1987;24:429–32.
8. College of American Pathologists. Glycohemoglobin survey set GH2-A. Northfield, IL: CAP, 1998:2–8.
9. Weykamp CW, Martina WV, van der Dijs FPL, Penders TJ, van der Slik W,

Muskiet FAJ. Hemoglobin S and C: reference values for glycohemoglobin in heterozygous, double-heterozygous and homozygous subjects, as established by 13 methods. Clin Chim Acta 1994;231:161–71.

10. Martina WV, Martijn EG, van der Molen M, Schermer JG, Muskiet FAJ. β-N-Terminal glycohemoglobin in subjects with common hemoglobinopathies: relation with fructosamine and mean erythrocyte age. Clin Chem 1993;39:2259–65.

11. Holownia P, Bishop E, Newman DJ, John GW, Price CP. Adaptation of latex-enhanced assay for percent glycohemoglobin to a Dade Dimension® analyzer. Clin Chem 1997;43:76–84.

12. Konigsberg W, Goldstein J, Hill RJ. The structure of human hemoglobin VII: the digestion of the β chain of human hemoglobin with pepsin. J Biol Chem 1963;238:2028–33.

13. Barbedo MMR, McCurdy PR. Red cell life span in sickle cell trait. Acta Haematol 1974;51:339–43.

Simple Precipitation-based Method for the Screening of Type III Hyperlipoproteinemia

Matthias Nauck,[1] Lioba Glatt,[1] Winfried März,[1] Giso Feussner,[2] and Heinrich Wieland[1] ([1] University Hospital of Freiburg, Department of Clinical Chemistry, Hugstetter Strasse 55, 79106 Freiburg i. Br., Germany, and [2] University Hospital of Heidelberg, Department of Endocrinology, D-69115 Heidelberg, Germany; * author for correspondence: fax 49-761-270 3444, e-mail manauck@mzl200.ukl.uni-freiburg.de)

Familial type III hyperlipoproteinemia (HLP) is characterized by the accumulation of cholesterol-rich remnants (β-VLDL). The differential diagnosis of type III HLP is clinically important because patients with type III HLP develop premature coronary artery disease (CAD) and peripheral atherosclerosis and because type III HLP responds well to dietary treatment and fibric acid derivatives *(1, 2)*. Pathogenetically, type III HLP is related to dysfunctional isoforms of apolipoprotein (apo) E. At the *APOE* gene locus, three common alleles exist, designated $\epsilon2$, $\epsilon3$, and $\epsilon4$ *(3–5)*. apoE2 is defective in its binding to lipoprotein receptors *(6, 7)*. Because of the impaired catabolism of chylomicron and VLDL remnants, individuals homozygous for apoE2 reveal detectable amounts of β-VLDL in their plasma. β-VLDLs are atypical lipoproteins with a density <1.006 kg/L and β-mobility on agarose gel electrophoresis. Compared with normal VLDL, β-VLDLs are cholesterol-enriched; compared with normal LDL, they are relatively enriched in triglycerides. More than 90% of patients with type III HLP are homozygous for apoE2, but only ~1 in 20 individuals carrying the E2/2 phenotype finally develops type III HLP *(8)*. Those homozygous carriers of apoE2 having small amounts of β-VLDL in their plasma not sufficient to produce overt hyperlipidemia have been classified as suffering from normolipidemic dysbetalipoproteinemia. The term type III HLP, in contrast, is applied to hyperlipemic individuals only.

Clinical characteristics such as palmar, tendon, and/or tubero-eruptive xanthomas do not occur in all individuals with type III HLP. To establish biochemically the diagnosis of type III HLP, the following criteria have been applied in this study: (*a*) presence of increased cholesterol and triglycerides at 2500 mg/L or more, (*b*) an increased ratio of VLDL-cholesterol (VLDL-C) to VLDL-triglycerides (VLDL-TG; >0.4), and (*c*) an increased ratio of VLDL-C to total triglycerides (>0.3) *(9–11)*.

We studied in total 1317 sera from women and men, ages 20–65 years. Among the participants with apoE phenotypes other than E2/2 (n = 1288) were 468 CAD patients recruited at the University Hospital, Freiburg or at the Benedikt-Kreutz-Klinik, Bad Krozingen, and 820 clinically healthy individuals, recruited at the Rheintalklinik, Bad Krozingen or employees of the BASF, Ludwigshafen. Twenty-nine apoE2 homozygotes were studied, including 21 samples obtained at the University Hospital of Heidelberg. In 8 of the 12 individuals with type III HLP, signs of atherosclerosis were present (66%). Two of the 17 apoE2 homozygotes without manifest type III HLP showed CAD (12%). Informed consent was obtained from each individual participating in this study; all procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Blood was drawn after an overnight fast and allowed to clot at room temperature; serum was obtained by centrifugation at 1500*g* for 15 min. All analyses were performed within 2 days after blood collection at the University Hospital of Freiburg. Cholesterol and triglycerides, without blanking for glycerol, were determined enzymatically (Boehringer Mannheim) using a Hitachi type 747 (total cholesterol and triglycerides) or a Wako 30R analyzer (supernates of precipitation reactions), respectively. The total CVs for the methods were <2%.

LDL-like lipoproteins were isolated using 100 μL of serum and 1000 μL of dextran sulfate (DS)/MgCl$_2$ precipitation reagent (Quantolip®; Immuno GmbH) and incubated for at least 10 min. The resulting precipitate was separated by a 5-min centrifugation, and the cholesterol and triglyceride content of the soluble lipoproteins (mainly VLDL and HDL) was measured in the supernate. Cholesterol and triglycerides associated with the precipitated lipoproteins (LDL-C$_{DS}$ and LDL-TG$_{DS}$, respectively) were calculated as total cholesterol or triglycerides minus supernate cholesterol or triglycerides, respectively.

A combined ultracentrifugation (UC) and precipitation assay was used as the comparison method *(12, 13)*. In this method, the recoveries of the bottom fraction (LDL plus HDL) was between 97% and 102%, whereas the recoveries of the top fraction (VLDL) was between 75% and 103%. All lipid measurements of one sample—lipids and lipoprotein fractions—were performed in the same analytical runs. The between-day CVs for LDL-C$_{DS}$, LDL-TG$_{DS}$, LDL-C$_{UC}$, and HDL-C$_{UC}$ were below 3% and 4%, respectively.

apo E phenotyping was performed by isoelectric focusing on agarose and immunofixation *(14, 15)*. Regression analyses were performed using the method of Passing and Bablok *(16)*.

We first wished to compare the results for LDL-C obtained with the DS precipitation method with those obtained with UC. In this comparison, we excluded 45 samples in which a recently developed algorithm indicated that the DS precipitation was incomplete because of