

## Long Term Stability of Parameters of Lipid Metabolism in Frozen Human Serum: Triglycerides, Free Fatty Acids, Total-, HDL- and LDL-cholesterol, Apolipoprotein-A1 and B

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### Abstract

**Background:** In large epidemiological studies it is important to test the stability of biomarkers as a function of both temperature and duration of storage. In this study the stability of seven lipid parameters have been tested in human serum samples after storage at three different temperatures up to 1 year.

**Methods:** Serum samples of 16 human individuals were used in this study. The concentration of all parameters have been determined at T=0 and at several time points up to 1 year after storage at -20°C, -70°C and -196°C.

**Results:** Most of the lipid biomarkers, cholesterol, triglycerides HDL- and LDL cholesterol, apolipoprotein-A1 and -B are stable on long-term storage for one year at the three temperatures tested. The levels of both HDL- and LDL cholesterol showed a small decrease for samples stored at -20°C only. The free fatty acids, however, are not stable and showed a decrease to about 80% of the starting value. The rank order between the samples, however, remained very good.

**Conclusions:** This study shows that free fatty acids are not stable in human serum samples probably due to thawing and freezing effects, although the rank order and correlation between the samples from different time points remained the same. HDL- and LDL-cholesterol showed a very small deviation on storage at -20°C. The other lipid parameters remained perfectly stable in this study. No differences were observed between storage at -70°C or -196°C. Therefore it is advised to store serum samples at -70°C for longer periods.

**Keywords:** Storage temperature; Cholesterol; Triglycerides; Free Fatty Acids; HDL-cholesterol; LDL-cholesterol apolipoprotein-A1; Apolipoprotein-B

### Introduction

In epidemiological research the stability of the serum or plasma parameters during long-term storage is of great importance. Storage is one part of the whole process of blood withdrawal, blood processing to serum or plasma and storage of these end products. It is also important that sample suitability and the effect of any delay between blood withdrawal and processing on the measurement of analytes are assessed. The time between blood withdrawal and processing (centrifugation) must be kept as short as possible or at least constant for all samples. Parameters of lipid metabolism are often determined to assess the nutritional status or to establish the risk of chronic diseases, mainly cardiovascular-related diseases [1-4] but also others, such as cancer [5,6], diabetes [7,8] and cognitive diseases [9,10]. Biomarkers of the lipid metabolism are triglycerides, free fatty acids, total cholesterol, HDL-cholesterol, LDL-cholesterol and the apolipoproteins A1 and B.

In the present study the stability and performance of these biomarkers have been tested in serum of human volunteers during storage up to 12 months at three different temperatures.

### Materials and Methods

For the stability study of triglycerides, free fatty acids, total cholesterol, HDL- and LDL-cholesterol, human serum samples of 16 healthy human volunteers (blood donors) were used. Samples were obtained from the Central Blood Laboratory of the Red Cross in Amsterdam with written permission of the volunteers. At the day of blood withdrawal 2x8 mL blood was collected in serum tubes (Vacuette serum clot activation tubes (Greiner)). The serum samples were prepared within two hours, divided in aliquots and stored at different temperatures.

For the stability study of apolipoprotein-A1 and -B, human serum samples of 16 healthy human volunteers were used, with approval of the Medical Ethical Committee TNO under no 98/36. At the day of blood withdrawal 2x8 mL blood was collected in serum tubes (Vacuette serum clot activation tubes (Greiner)). The serum samples were prepared within two hours, divided in aliquots and stored at different temperatures.

The initial concentrations for the parameters at T=0 have been determined within 4 hours. Measurements for cholesterol, triglycerides, HDL- and LDL-cholesterol were performed in samples stored at -20°C at time points 0, 0.14, 0.5, 1, 2, 6 and 12 months. For samples stored at -70°C at time points 0, 0.5, 2, 6 and 12 months and for samples stored at -196°C at time points 0, 1, 2, 6 and 12 months.

Measurements for apolipoproteins A1 and B were performed in samples stored at  $-20^{\circ}\text{C}$  at time points 0, 0.14, 1, 2, 3, 6, 9 and 12 months. For samples stored at  $-70^{\circ}\text{C}$  at time points 0, 0.5, 1, 2, 3, 6, 9 and 12 months and for samples stored at  $-196^{\circ}\text{C}$  at time points 0, 0.5, 1, 2, 3, 6, 9 and 12 months (Table 1).

Parameter	Average $\pm$ SD	Range	Median
Triglycerides	1.68 $\pm$ 0.27	1.2-2.15	1.65
Free fatty acids	0.53 $\pm$ 0.12	0.38-0.89	0.48
Total cholesterol	5.29 $\pm$ 0.44	4.45-6.1	5.27
HDL-cholesterol	1.32 $\pm$ 0.19	1.06-1.63	1.3
LDL-cholesterol	3.11 $\pm$ 0.28	2.68-3.70	3.15
Apolipoprotein-A	1.68 $\pm$ 0.29	1.27-2.53	1.68
Apolipoprotein-B	1.32 $\pm$ 0.31	0.70-1.86	1.39

**Table 1:** Initial parameters of oxidative stress for the 16 volunteers

### Measurements of parameters

The apolipoprotein A1 and B were measured with an auto-analyzer (Cobas Fara, Roche Diagnostics, Almere, The Netherlands) with dedicated kits from Roche Diagnostics. All other parameters have been determined in serum with an auto analyzer (Hitachi 912, Roche Diagnostics) with the use of dedicated kits supplied by Roche Diagnostics. The measurements of FFA were performed with the NEFA kit (Non-Esterified Fatty Acids) from Wako Chemicals, Neuss, Germany. This kit measures all free fatty acids which a carbon chain length from C-6 to C-24 with a recovery between 90 and 103%.

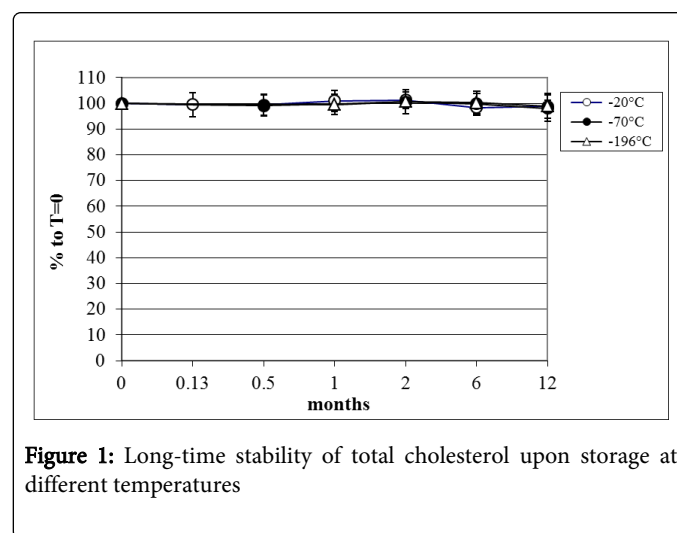
The measurements were performed as a single measurement, except on day 0 (duplicate measurements). Each day, a number of quality control samples were included to exclude the potential effect of long-term assay drift as much as possible. The inter assay coefficients of variation were determined by daily measurements in duplicate using manufactured quality control samples (Precinorm L obtained from Roche Diagnostics for cholesterol, triglycerides and HDL- and LDL-cholesterol; AHSL-1 serum obtained from Randox for FFA; Apolipoprotein T control obtained from Roche Diagnostics for the apolipoproteins). The inter assay CVs were 5.6% for cholesterol at the level of 4.39 mmol/L, 7.5% for triglycerides at the level of 1.40 mmol/L, 1.8% for HDL-cholesterol at the level of 1.20 mmol/L, 6.5% for LDL-cholesterol at the level of 2.43 mmol/L, 8.5% for free fatty acids at the level of 1.28 mmol/L, 1.7% for apolipoprotein-A1 at the level of 1.67 g/L and 2.6% for apolipoprotein-B at the level of 1.14 g/L.

For long-term stability samples have been stored for 12 months at  $-20^{\circ}\text{C}$ ,  $-70^{\circ}\text{C}$  or  $-196^{\circ}\text{C}$ . Samples stored at  $-20^{\circ}\text{C}$  were kept in a freezer room with a daily temperature check. Samples stored at  $-70^{\circ}\text{C}$  were kept in a refrigerator equipped with a temperature recorder and a sound alarm. Samples stored at  $-196^{\circ}\text{C}$  were kept in liquid nitrogen in a container equipped with an automatic filling device and a sound alarm.

At all time points the statistical difference (95 % confidence interval) of the values from the initial value at  $T=0$  were determined with a t-test for two-samples assuming equal variances.

## Results

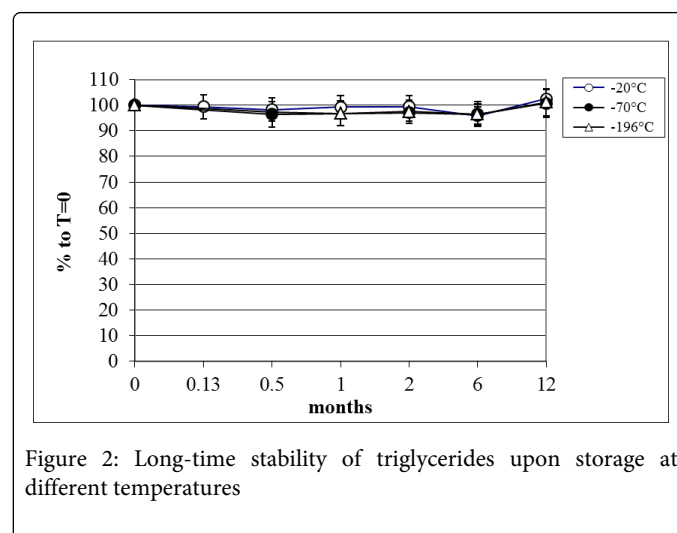
For total cholesterol the long-term stability is good as is shown in Figure 1. At all time points up to one year no statistically significant change compared with the initial value was observed at all temperatures. Also no statistically significant change was observed between the mean concentrations in the samples stored at different temperatures.



**Figure 1:** Long-time stability of total cholesterol upon storage at different temperatures

### Triglycerides

For triglycerides the long-term stability is good as is shown in Figure 2. At all time points up to 1 year no statistical significant change compared with the initial value was observed at all temperatures. Also no statistically significant change was observed between the mean concentrations of total cholesterol in the samples stored at different temperatures.



**Figure 2:** Long-time stability of triglycerides upon storage at different temperatures

### Free fatty acids

For free fatty acids (FFA) the long-term stability was declined during storage as is shown in Figure 3. Both the short- and long-term stability shows a temperature dependency. On storage at  $-20^{\circ}\text{C}$  a decline to 80% is observed after 3 days. Storage at  $-70^{\circ}\text{C}$  and  $-196^{\circ}\text{C}$

shows also a similar decrease in the mean value of FFA to about 80% of its original value after 15 days. Between 15 days and 1 year of storage no further decrease was observed at all temperatures. The control sample remains constant during the whole period, so the decrease can probably ascribed to a freeze-thaw effect. At the time point of 12 months the rank order, compared with the measurements at T=0, remained the same for all three temperatures. Correlation coefficients were 0.937, 0.941 and 0.932 for the samples stored at at -20°C, -70°C and -196°C, respectively. Also the rank orders between the samples stored at -20°C, and -70°C and between samples stored at -20°C and -196°C for 12 months was very good with correlation coefficients of 0.993 and 0.998, respectively.

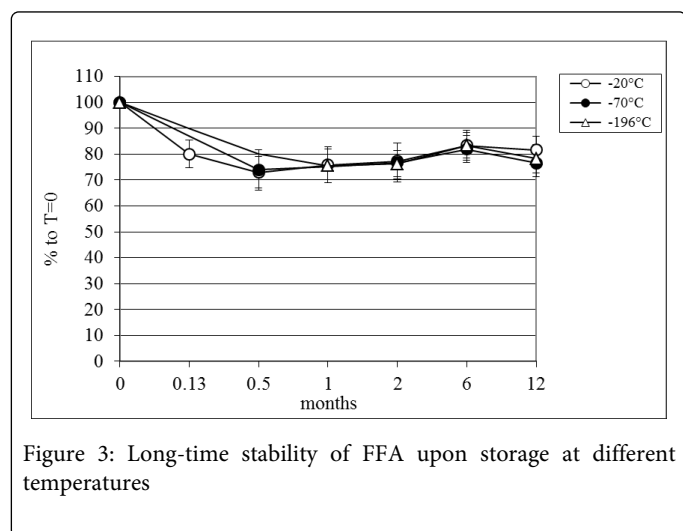


Figure 3: Long-time stability of FFA upon storage at different temperatures

### HDL-cholesterol

For HDL-cholesterol the long-term stability is good as is shown in Figure 4. At all time points up to 2 months no statistical significant change compared with the initial value was observed at all temperatures. At the time points of 6 and 12 months the samples stored at -20°C showed a small, but statistically significant decrease ( $p < 0.00001$ ) compared with the values at -70°C and -196°C. At the time point of 12 months the rank order remained the same for the measurements at -20°C and -196°C with a correlation coefficient of 0.942. These data have been presented before as part of a study on the stability of paraoxonase [11].

### LDL-cholesterol

For LDL-cholesterol the long-term stability is good as is shown in Figure 5. At all time points up to 2 months no statistical significant change compared with the initial value was observed at all temperatures. At the time points of 6 and 12 months the samples stored at -20°C showed a small, but statistically significant decrease ( $p < 0.0001$ ) compared with the values at -70°C and -196°C. At the time point of 12 months the rank order remained the same for the measurements at -20°C and -196°C with a correlation coefficient of 0.925.

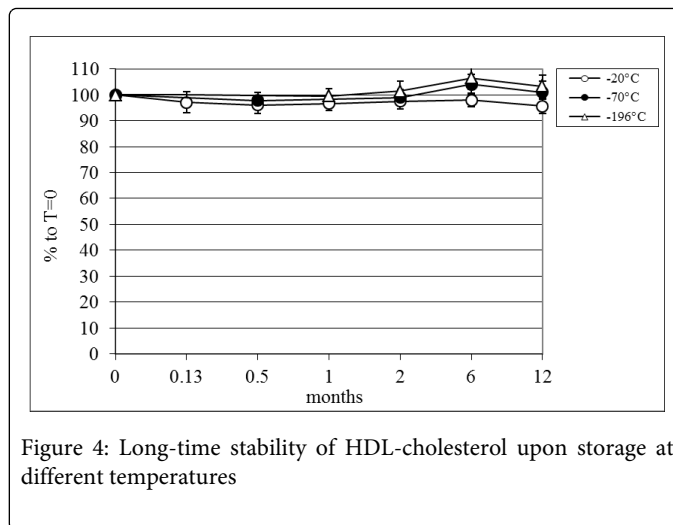


Figure 4: Long-time stability of HDL-cholesterol upon storage at different temperatures

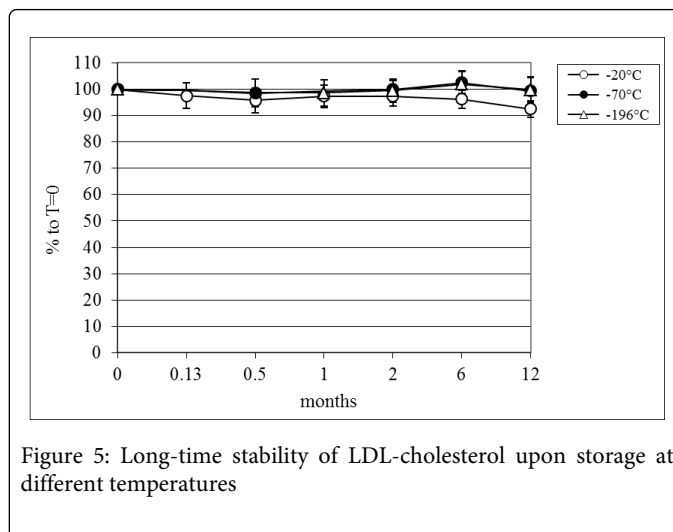


Figure 5: Long-time stability of LDL-cholesterol upon storage at different temperatures

### Apolipoprotein-A

For apolipoprotein-A the long-term stability is good as is shown in Figure 6. At all time points up to 1 year no statistical significant change compared with the initial value was observed at all temperatures. The control sample also showed a gradual decrease to about 97% of the average value at T=0.

### Apolipoprotein-B

For apolipoprotein-B the long-term stability is good as is shown in Figure 7. At all time points up to 1 year almost no statistical significant change compared with the initial value was observed at all temperatures. Only at the time points of month 6 and 12 some small deviations were observed, probably due to instability of the measurements. At time point of 12 months the average values of all temperatures were similar to that at T=0.

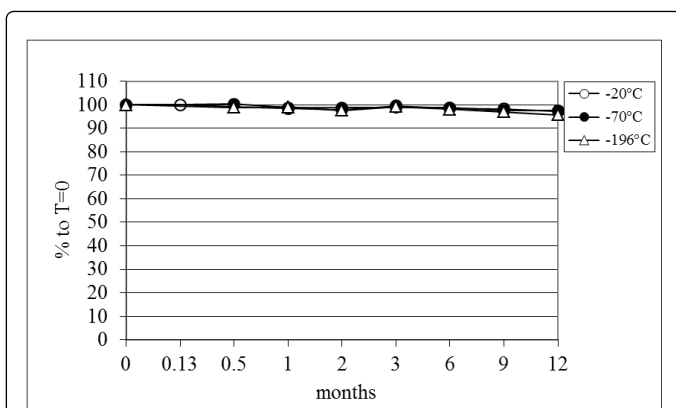


Figure 6: Long-time stability of apolipoprotein-A upon storage at different temperatures

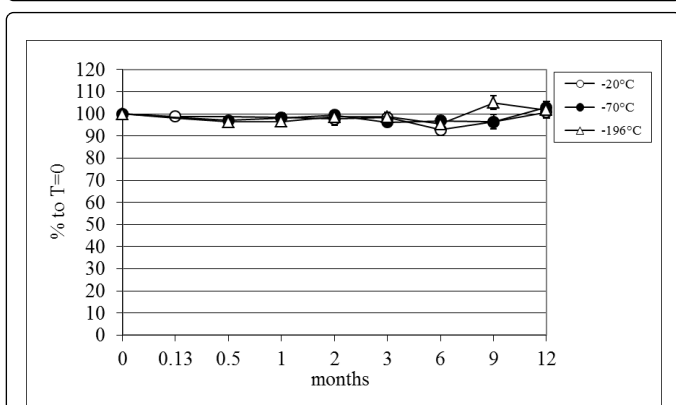


Figure 7: Long-time stability of apolipoprotein-B upon storage at different temperatures

## Discussion

Most of the lipid biomarkers tested here are stable on long-term storage for one year at the three temperatures tested, -20°C, -70°C and -196°C. This conclusion is valid for total cholesterol, triglycerides and apolipoprotein-A1 and -B.

For the free fatty acids, however, there is a sharp decrease to about 80% of the starting value at the first time point of 4, 15 or 30 days of storage. The level of 80% remains constant until one year of storage, for the three temperatures tested. No statistically significant differences were observed between the mean values of the samples stored at the three temperatures. This observation may indicate that the main problem for the analysis of fatty acids is the process of freezing/thawing. Whether the availability of some fatty acids is decreased after freezing or the assay performance is affected cannot be concluded from this study. The kit insert of the NEFA assay from Wako, based on a sequence of enzymatic reactions, does not mention this possible problem of freezing of samples.

The levels of both HDL- and LDL cholesterol in samples stored after 6 and 12 months showed a small but statistically significant decrease for samples stored at -20°C only. The rank order between the samples stored at -20°C compared with those stored at -70°C or

-196°C during 12 months remained very good. Also the rank order between the samples measured before freezing and after 12 months of storage at the three different temperatures was still very good (correlation coefficients were 0.932-0.941).

In literature, a few short-term studies were reported [12-14] and some long-term studies on the long-term stability of lipid parameters on storage. Hodson et al. [15] reported a study in which the fatty acid compositions of plasma lipid constituents remain constant for 4 years at -80°C. Hron and Menahan [16] described a study in which free fatty acids showed an increase to 200% of the initial value after storage for 300 days at -15°C. Ito et al. [17] reported that in a single pooled serum sample, total cholesterol and triglycerides decreased in concentration to 90% after 6 years of storage at -80°C. In a paper of Elliott and Peakman [18] another study was referred in which all lipid parameters showed no decrease after storage for 6 years at -80°C and -180°C. On storage at -20°C and -40°C some parameters showed a lower stability. Experimental details of this study were not given. The only well-described study is from Kronenberg et al. [19] in which they studied the stability of all lipid parameters in EDTA-plasma of 8 probands. For apolipoprotein-A1 and -B, a small but statistically significant decrease of 6-10% was observed after storage for 24 months at both -20°C and -80°C. For total and HDL-cholesterol an increase was observed of 2-4% at both -20°C and -80°C, which was ascribed to the freezing process.

The limitations of the present paper are that all serum samples originate from healthy volunteers. No serum samples from sick participants were included. Furthermore the representation as percentage relative to T=0 may mask the underlying actual concentration data, but the results from all participants showed the same behavior with respect to the stability across the whole concentration range.

In conclusion, this study shows that some care should be taken concerning the temperature of storage of serum samples intended for future measurements of free fatty acids and to a lower extent of HDL- and LDL-cholesterol. The problem for the stability of the free fatty acids can probably be assigned to freezing process. The rank order between the samples of t=0 and 12 months, however, remained the same. For HDL- and LDL-cholesterol. A storage temperature of -20°C is not to be preferred although the rank order between the samples stored at different temperatures remained the same. For epidemiological studies storage conditions of -20°C for longer periods are acceptable, if all samples have been treated in the same way and stored under the same conditions. For all biomarkers there was no difference between storage for 12 months at -70°C or -196°C, therefore expensive facilities for storage at -196°C in liquid nitrogen is needless and can be avoided.

## References

1. Laakso M, Lehto S, Penttilä I, Pyörälä K (1993) Lipids and lipoproteins predicting coronary heart disease mortality and morbidity in patients with non-insulin-dependent diabetes. *Circulation* 88: 1421-1430.
2. Shai I, Rimm EB, Hankinson SE, Curhan G, Manson JE, et al. (2004) Multivariate assessment of lipid parameters as predictors of coronary heart disease among postmenopausal women: potential implications for clinical guidelines. *Circulation* 110: 2824-2830.
3. Pischon T, Girman CJ, Sacks FM, Rifai N, Stampfer MJ, et al. (2005) Non-high-density lipoprotein cholesterol and apolipoprotein B in the prediction of coronary heart disease in men. *Circulation* 112: 3375-3383.

4. Walldius G, Jungner I (2006) The apoB/apoA-I ratio: a strong, new risk factor for cardiovascular disease and a target for lipid-lowering therapy--a review of the evidence. *J Intern Med* 259: 493-519.
5. Santos CR, Schulze A (2012) Lipid metabolism in cancer. *FEBS J* 279: 2610-2623.
6. Muntoni S, Atzori L, Mereu R, Satta G, Macis MD, et al. (2009) Serum lipoproteins and cancer. *Nutr Metab Cardiovasc Dis* 19: 218-225.
7. Jiang R, Schulze MB, Li T, Rifai N, Stampfer MJ, et al. (2004) Non-HDL cholesterol and apolipoprotein B predict cardiovascular disease events among men with type 2 diabetes. *Diabetes Care* 27: 1991-1997.
8. Bruno G, Merletti F, Biggeri A, Barger G, Prina-Cerai S, et al. (2006) Effect of age on the association of non-high-densitylipoprotein cholesterol and apolipoprotein B with cardiovascular mortality in a Mediterranean population with type 2 diabetes: the Casale Monferrato study. *Diabetologia* 49: 937-944.
9. Panza F, D'Introno A, Colacicco AM, Capurso C, Pichichero G, et al. (2006) Lipid metabolism in cognitive decline and dementia. *Brain Res Rev* 51: 275-292.
10. Morley JE, Banks WA (2010) Lipids and cognition. *J Alzheimers Dis* 20: 737-747.
11. Beekhof PK, Gorshunskaya M, Jansen EH (2012) Long term stability of paraoxonase-1 and high-density lipoprotein in human serum. *Lipids Health Dis* 11: 53.
12. Trichopoulos A, Kalaidzidou C, Kalandidi A (1976) The relationship between plasma non-esterified fatty acids concentration and conditions of storage. *Clin Chim Acta* 69: 355-356.
13. Jackson C, Best N, Elliott P (2008) UK Biobank Pilot Study: stability of haematological and clinical chemistry analytes. *Int J Epidemiol* 37 Suppl 1: i16-22.
14. Tanner M, Kent N, Smith B, Fletcher S, Lewer M. Stability of common biochemical analytes in serum gel tubes subjected to various storage temperatures and times pre-centrifugation. *Ann Clin Biochem* 45: 375-379.
15. Hodson L, Skeaff CM, Wallace AJ, Arribas GL (2002) Stability of plasma and erythrocyte fatty acid composition during cold storage. *Clin Chim Acta* 321: 63-67.
16. Hron WT, Menahan LA (1981) A sensitive method for the determination of free fatty acids in plasma. *J Lipid Res* 22: 377-381.
17. Ito Y, Nakachi K, Imai K, Hashimoto S, Watanabe Y, et al. (2005) Stability of Frozen Serum Levels of Insulin-like Growth Factor-I, Insulin-like Growth Factor-II, Insulin-like Growth Factor Binding Protein-3, Transforming Growth Factor $\beta$ , Soluble Fas, and Superoxide Dismutase Activity for the JACC Study. *J Epidemiol* 15: S67-73.
18. Elliott P, Peakman TC; UK Biobank (2008) The UK Biobank sample handling and storage protocol for the collection, processing and archiving of human blood and urine. *Int J Epidemiol* 37: 234-244.
19. Kronenberg F, Lobentanz EM, König P, Utermann G, Dieplinger H (1994) 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* 35: 1318-1328.