

Stability of selected serum proteins after long-term storage in the Janus Serum Bank

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Abstract

Background: Human serum from biobanks is frequently used in prospective epidemiological studies. Long-term storage may modify its composition. A better understanding of the stability of the serum components may improve the interpretation of future studies.

Methods: The concentrations of selected proteins; immunoglobulins, carrier proteins and enzymes in samples stored at -25°C for 25 years and 2 years were compared with 1-month-old samples. For each length of storage time, 130 specimens were randomly selected from apparently healthy male blood donors aged 40–49 years. We examined the distribution of values, compared dispersion and localization of central tendency, and established reference intervals for each component.

Results: The study demonstrated non-significant or numerically small group differences in the concentrations of albumin, aspartate amino transferase, cystatin C, immunoglobulin E, immunoglobulin G, and sex hormone binding globulin. Mean values between fresh and 25-year-old samples suggested larger differences during storage for alanine amino transferase (-73.4%), creatinine kinase (-96.1%), insulin C-peptide (-98.7%), ferritin (-18.5%) and transferrin ($+8.2\%$).

Conclusions: The findings showed that long-term storage can introduce a considerable bias for vulnerable components.

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Keywords: biobank; biomarker; blood-based epidemiology; serum-proteins; stability; temperature.

Introduction

Changes during storage of biological samples may introduce bias in prospective epidemiological studies.

In cancer studies, the follow-up time can be long and storage of specimens over several decades may be necessary. A variety of proteins have been investigated in epidemiological studies. Development of new immunometric and mass-spectrometric methods makes it possible to identify disease markers, comprising newly synthesized proteins as well as post-translationally modified proteins (1). Amino acid residues at the active site may be oxidized rendering an enzyme inactive. Changes in the tertiary structure might also destroy its biological function and lead to aggregation and precipitation (2). Non-homogeneous deterioration can decrease the possibility of identifying differences between cases and controls in epidemiological studies (3).

The Janus Serum Bank was established in 1973 and comprises samples from subjects that have participated in the National Health examination program (90%) and blood donors (10%). The biobank is owned by the Norwegian Cancer Registry and is reserved for epidemiological cancer studies. After more than 35 years in existence, the biobank has accumulated more than 40,000 cancer cases for study. Gas chromatography-mass spectrometry and two-dimensional protein electrophoresis has been used previously to evaluate the stability of frozen sera in the Janus Serum Bank. The primary structure of the proteins studied was stable for up to 10 years (4, 5). However, proteins in low concentration could not be assessed by these methods. In this study, we assess the stability of eight serum proteins and three enzymes.

Materials and methods

Subjects

Serum samples were obtained from the blood donor sub-cohort in the Janus Serum Bank. Samples were selected from 390 men aged 40–49 years at blood draw, distributed in equally sized groups according to length of storage; 1 month, 2 years and 25 years.

Males were chosen as they have less intra- and inter-individual biological variation linked to sex hormones. The study group was homogeneous with respect to ethnicity; until 2004, donation of blood in Norway was restricted to people born in Northwest Europe, North America, Australia and New Zealand, resulting in a predominantly Caucasian cohort.

Sample handling

The sampling routine was fairly constant throughout the 25-year study period. Samples were collected between 8 am and 6 pm. The donors were not required to be fasting; therefore the influence of food intake could vary. Blood was drawn with the donor in a supine position and the collection tubes contained no additives or separating gel. The samples

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were allowed to clot at room temperature and stored over night at 4°C for 14–20 h, 18–26 h and 24–28 h for the 1-month-old, 2-year-old and 25-year-old samples, respectively. Subsequently, the samples were centrifuged and serum separated. The delay between separation and freezing was up to 30 min at room temperature. After centrifugation the serum was transferred to a 5 mL polypropylene tube. Centrifugation was performed in a Kubota KS-5200 C at 1300 *g* for 10 min, with a swing-out rotor RS-4. Samples were stored in covered paper boxes at –25°C in a commercial freezer warehouse. Direct exposure to light occurred only during initial sample handling, retrieval, thawing and aliquoting of samples. However, small leakage of light through the walls of the paper boxes cannot be excluded. The samples went through one thaw-freeze cycle for preparation of aliquots for the present study.

Instrumentation

The samples were analyzed for the following 11 components: alanine amino transferase (ALT), aspartate amino transferase (AST), albumin, creatinine kinase (CK), cystatin C, immunoglobulin E (IgE), immunoglobulin G (IgG), insulin C-peptide, ferritin, sex hormone binding globulin (SHBG) and transferrin. All methods, except for cystatin C, IgE and SHBG, were accredited according to Norwegian standard of general requirement for testing and calibration laboratories competence (NS-EN ISO/IEC 17025). The enzymes were analyzed by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) standardized methods. The analytical methods and corresponding coefficients of variation for each component are shown in Table 1. The group of samples that had been stored for 2 and 25 years were analyzed in the same batch. The samples that had been stored for 1 month were analyzed at a later time, but the same

methods and instruments were used. For IgE, all samples were analyzed in one batch.

Ethics

The study was approved by the Regional Committees for Research Ethics in Norway.

Statistics

To obtain normally distributed data for parametric tests, transformations (e.g., log-transformation and square root-transformation) were used where necessary. Visual inspection of z-score plots was used to check the distributions. Albumin was normally distributed. ALT, AST, C-peptide, CK, cystatin C, ferritin, IgE, IgG and transferrin required log-transformation, and SHBG was square root-transformed. Tukey's fence with a factor=2 (6) was used to eliminate outliers in t-test, F-test, and analysis of variance (ANOVAs) statistics. ANOVAs were used to test for differences in the mean values of the components in the three groups. A subsequent post-hoc Student Newman-Keuls test identified pair-wise group differences in cases where the ANOVA null hypothesis was rejected. Possible differences in dispersion (i.e., variances) with respect to the 1-month-old group were examined by use of F-tests. Reference limits with 90% confidence intervals were established for each component by means of bootstrap methods and rank-based non-parametric percentile estimates (RefVal) (7).

Parameters of central tendency (mean) and dispersion (SD) were used to evaluate stability. The numerical value of the difference in the level (e.g., distribution means or retransformed means) was compared with consensus acceptance criteria for bias of biochemical components in blood (8). For the components not represented in Ricós' (8) table,

Table 1 Analytical method and coefficient of variation (CV) and precision (I) in percent for the components.

Component	Analytical method	CV ^a	I ^b
ALT	Modular P (Roche) automatic analyzer, IFCC standardized enzymatic assay, UV detection	<4.0% at 81.0 and 173.0 U/L	12.2
Albumin	Modular P (Roche) automatic analyzer, colorimetric assay	<2.4% at 37.1 and 40.3 g/L	1.6
AST	Modular P (Roche) automatic analyzer, IFCC standardized enzymatic assay, UV detection	<3.0% at 122.0 and 272.0 U/L	6.0
CK	Modular P (Roche) automatic analyzer, IFCC standardized enzymatic assay, UV detection	<2.8% at 106 and 768 U/L	11.4
Cystatin C	Modular P (Roche) automatic analyzer, immunoturbidimetric assay	5% at 0.85 mg/L	NA
Ferritin	Modular E 170 (Roche), electrochemiluminescence immunoassay	10.0% at 6.1 µg/L and <7.0% at 77.8 and 446.1 µg/L	3.0
C-peptide	Auto DELFIA analyzer, solid phase time-resolved fluoroimmunoassay, two-monoclonal antibody	7.6%, 5.3% and 7.8% at 0.46, 2.06 and 3.70 nmol/L, respectively	NA
IgE total	Fluoroenzymeimmunoassay polyclonal antibody	9.3%, 5.7% and 9.5% at 13, 75 and 640 kU/L, respectively	NA
IgG	Modular P (Roche) automatic analyzer, immunoturbidimetric assay, polyclonal antibody	<4% at 9.1 and 14.0 g/L	2.3
SHBG	Modular E 170 (Roche), electrochemiluminescence immunoassay	5.0% and 5.3% at 39.1 and 73.0 nmol/L, respectively	6.1
Transferrin	Modular P (Roche) automatic analyzer, immunoturbidimetric assay	<4.0% at 1.57 and 3.19 g/L	1.5

^aCV, data from the laboratory at Rikshospitalet and Radiumhospitalet; ^bI, precision data published by Ricós; NA, not available.

we used an updated version from 2008 (9). We chose a significance level of 5%.

Results

The mean age was 44.6, 45.4 and 42.1 years for samples from the 1-month, 2-year and 25-year-old groups, respectively. The latter group had a lower mean age. Therefore, we explored the effect of age-adjustment. For most components the effect was negligible, but correction for age was retained for albumin and SHBG. Visible hemolysis, defined as more than 0.2 g/L hemoglobin in serum (10), was not reported for any sample. Table 2 shows the medians and inter-quartile ranges for all groups (raw data with outliers included).

Table 3 shows the results of ANOVA and variance ratio tests. AST, cystatin C, IgE, IgG and SHBG did not show statistically significant differences at the group level. In contrast, the rest of the components including ALT, albumin, CK, ferritin, insulin C-peptide and transferrin did show differences. Generally there was an increase in dispersion with increasing storage time, except for albumin, for 2-year/1-month samples ratio. Details are given for each component in Table 3.

Relative percent differences in mean values between the groups are shown in Table 4. In addition, the recommended bias criteria based on estimates of total biological variation are also given. The recommended maximal bias for deviance from the reference limits is $0.25 (CV_w^2 + CV_b^2)^{1/2}$, where CV_w represents the within-subject variation and CV_b represents between-subject or between-group variation. Relevant coefficients of total biological variation have been published by Ricós (8). Some of the components showed substantial differences in mean values when 25-year-old samples were compared to fresh samples; ALT (−73.4%), CK (−96.2%), ferritin (−18.5%), insulin C-peptide (−98.7%) and, although less pronounced, transferrin (+8.2%). Albumin and SHBG demonstrated a relative difference in mean below or close to the bias criteria after 25 years of storage.

Table 5 gives the calculated, recommended (11) or currently used reference limits for the components.

Results from the 1-month group may be used to evaluate the effect of the procedure on the initial sample handling. Results from 2-year and 25-year groups may be used to evaluate the influence of storage.

Discussion

In this study we report long-term stability for a broad panel of serum proteins. We found that immunoglobulins are quite robust, and carrier proteins slightly less stable. The stability pattern for polypeptides was heterogeneous and there were large differences in stability for the enzymes studied.

The three-dimensional structure of proteins is critical for a molecule's properties and function, and the folding is dependent on the primary structure. Hydrophobic interaction is probably the major contributor to stability of the folded state, but other interactions, such as hydrogen bonds, van der Waals' and electrostatic interactions also have a stabilizing effect (12). Post-translational modifications like phosphorylation and glycosylation influence the stability of proteins, the activity of enzymes, and interaction with other molecules (13). Antibodies, enzymes and carrier proteins are examples of globular proteins consisting of functional domains and flexible parts that are accessible to proteases.

Pre-analytical sample handling issues such as prolonged clot time at 4°C for 24 h has shown negligible or no effect on SHBG, CK, albumin, AST and ALT (14–19). However, Boyanton and Zhang (20, 21) found a significant increase in albumin of 2.3% and 8.5%, respectively. The lack of specific information on individual pre-analytical sample-handling is a weakness of our study. To estimate the potential contribution from differences in clot time, an experiment was performed with four simultaneously collected blood samples from ten healthy individuals. The samples were allowed to clot for an hour at room temperature, and then separated immediately (0 h), or after 14 h, 22 h and 28 h following storage at 4°C. The rate of change was examined by using regression analysis ($Y = a + bt$), where Y was the concentration ratio with reference to the 0 h sample. For C-peptide, SHBG and ferritin the change was negligible (slope smaller than

Table 2 Median and inter-quartile range for the components.

Component	Median (inter quartile range, 25%–75%)		
	1-month-old n = 130	2-year-old n = 130	25-year-old n = 130
ALT	18 (13–22)	10 (7–14)	4 (3–7)
Albumin	44.5 (42.8–45.8)	42.6 (41.3–43.9)	45.0 (43.3–47.1)
AST	28 (25–32)	28 (25–32)	28 (24–33)
CK	115.0 (78.0–154.0)	64.0 (49.0–86.5)	5.0 (1.0–10.0)
Cystatin C	0.77 (0.72–0.82)	0.77 (0.70–0.82)	0.74 (0.68–0.81)
Ferritin	52.5 (40.6–52.5)	51.0 (38.8–70.0)	46.3 (28.1–74.0)
IgE	29.0 (11.4–70.3)	36.8 (14.1–71.6)	32.7 (11.8–75.1)
IgG	9.47 (8.46–10.64)	9.64 (8.43–10.9)	9.5 (8.1–10.7)
C-peptide	1.38 (1.0–1.98)	1.3 (0.9–1.8)	0.02 (0.01–0.03)
SHBG	30.7 (24.0–41.4)	31.7 (24.5–39.7)	35.6 (29.4–47.2)
Transferrin	2.6 (2.44–2.89)	2.56 (2.34–2.73)	2.84 (2.59–3.1)

Table 3 Analysis of variance (ANOVA) and variance ratio of the mean between the groups.

Component	ANOVA for group differences, F-value [m, n] ^a (p-value)	Post-hoc Student Newman-Keuls test p-value			Variance ratio test F-value ^b [k, l] (p-value)	
		Comparison of storage group			Comparison of storage group	
		0–2 years	2–25 years	0–25 years	2/0	25/0
ALT	276.8 [385, 2] (<0.001)	<0.001	<0.001	<0.001	1.73 [128, 129] (0.001)	1.60 [128, 129] (0.004)
Albumin	37.7 [387, 2] (<0.001)	<0.001	<0.001	<0.001	0.61 [129, 129] (0.002)	1.32 [129, 129] (0.06)
AST	0.364 [380, 2] (0.70)	>0.5	>0.5	>0.5	1.26 [126, 127] (0.09)	2.0 [126, 128] (<0.001)
CK	630.4 [382, 2] (<0.001)	<0.001	<0.001	<0.001	1.12 [127, 127] (0.26)	6.95 [127, 129] (<0.001)
Cystatin C	2.74 [384, 2] (0.07)	>0.5	0.09	0.06	1.08 [128, 129] (0.3)	1.28 [128, 128] (0.08)
Ferritin	4.3 [383, 2] (0.015)	0.07	0.27	0.01	0.79 [128, 127] (0.09)	2.10 [128, 129] (<0.001)
IgE	0.32 [386, 2] (0.77)	0.46	>0.5	>0.5	1.01 [129, 129] (0.47)	1.27 [129, 129] (0.25)
IgG	0.26 [385, 2] (0.84)	>0.5	>0.5	>0.5	0.78 [129, 128] (0.05)	0.87 [129, 129] (0.21)
C-peptide	2555 [386, 2] (<0.001)	<0.001	<0.001	<0.001	1.35 [129, 129] (0.045)	2.03 [129, 129] (<0.001)
SHBG	7.7 [386, 2] (<0.001)	0.47	<0.001	0.009	0.79 [128, 129] (0.09)	1.21 [128, 129] (0.14)
Transferrin	26.6 [384, 2] (<0.001)	0.03	<0.001	<0.001	1.27 [128, 128] (0.09)	1.47 [128, 129] (0.014)

^aDegrees of freedom (m, n) m=total number of samples – number of groups, n=number of groups – 1; ^bdegrees of freedom (k, l) k=numbers of samples in first group – 1, l=numbers of samples in second group – 1.

0.0003 h⁻¹). For ALT, albumin, AST, CK and transferrin, there was significant increase in the values corresponding to 4.6%, 0.6%, 2.6%, 0.7% and 0.6%, respectively, for 9 h difference in average clot time between samples collected in 2004 and 1979. The

numerical values are small compared to the observed changes given in Table 4. Ideally, one would like to analyze samples from the same individuals, collected at the same time, stored for different time intervals, and analyzed simultaneously with the same instru-

Table 4 Percent difference of mean levels and biological variation.

Component	Percent difference of mean ^a			Recommended maximum bias (B) in percent B ^b
	0–2 years	2–25 years	0–25 years	
ALT	–41.1 ^c	–54.8 ^c	–73.4 ^c	12.0
Albumin	–5.5 ^c	+3.8 ^c	–1.9 ^c	1.3
AST	–1.2	–2.0	+2.0	5.4
CK	–41.1 ^c	–93.5 ^c	–96.1 ^c	11.5
Cystatin C	–0.8	–2.4	–3.3	3.4
Ferritin	–12.1	–7.3	–18.5 ^c	5.0
IgE	+12.3	–7.1	+4.3	NA
IgG	+1.4	–0.4	+1.0	4.3
C-peptide	–8.4 ^c	–98.6 ^c	–98.7 ^c	4.1
SHBG	–1.7	+8.91 ^c	+7.1 ^c	11.1
Transferrin	–3.4 ^c	+12.1 ^c	+8.2 ^c	1.3

^aAll values were retransformed after ANOVA except for albumin; ^bB values from Ricós et al. (8) and for Cys C, C-peptide and transferrin from Westgard (9); ^cSignificant changes, for p-value <0.05 ; NA, not available.

Table 5 Bootstrap generated and recommended reference values.

Component, Unit	Lower level (CI) ^a Upper level (CI) ^a			
	1 month	2 years	25 years	Recommended ^b or in-house ^c reference limits
ALT, U/L	9 (8–10) 39 (32–45)	4 (3–5) 29 (23–35)	3 (3–3) 13 (11–15)	10 ^b 78
Albumin, g/L	39.4 (38.8–40.1) 49.2 (48.0–50.4)	39.2 (38.4–39.6) 46.2 (45.6–46.8)	39.5 (38.8–40.2) 50.7 (48.6–52.8)	37.7 ^b 45.8
AST, U/L	19 (15–23) 44 (39–50)	19 (18–21) 51 (40–64)	17 (13–21) 55 (50–61)	14 ^b 41
CK, U/L	47 (40–54) 273 (240–306)	24 (14–33) 214 (142–287)	1 (1–1) 43 (22–65)	52 ^b 366
Cystatin C, mg/L	0.61 (0.56–0.67) 0.99 (0.89–1.08)	0.59 (0.55–0.63) 0.94 (0.90–0.98)	0.56 (0.50–0.62) 0.98 (0.91–1.05)	0.53 ^c 1.01
Ferritin, µg/L	24.9 (20.1–29.7) 205.7 (176.1–235.4)	20.1 (17.8–22.4) 161.5 (99.4–223.5)	9.3 (4.2–14.5) 186.3 (133.2–239.5)	19 ^b 310
IgE, kU/L	2.7 (0.7–4.7) 464.5 (356.7–572.4)	3.1 (0.6–5.6) 487.4 (265.2–709.6)	2.2 (0.6–3.4) 581.1 (378.2–783.9)	< 120 ^c
IgG, g/L	6.6 (6.3–6.9) 13.4 (13.1–13.8)	6.58 (6.20–6.96) 14.7 (12.6–16.8)	6.7 (6.4–7.0) 14.5 (13.0–16.1)	6.1 ^c 14.9
C-peptide, nmol/L	0.55 (0.47–0.64) 3.50 (3.00–4.00)	0.41 (0.36–0.46) 3.43 (3.09–3.77)	0.009 (0.009–0.009) 0.062 (0.053–0.070)	0.3 ^c 2.4
SHBG, nmol/L	15 (13–17) 64 (60–68)	14 (12–15) 55 (50–59)	14 (11–18) 69 (65–72)	15 ^c 85
Transferrin, g/L	2.0 (1.8–2.2) 3.3 (3.2–3.5)	1.9 (1.7–2.1) 3.3 (3.1–3.6)	2.2 (2.1–2.3) 4.0 (3.6–4.4)	2.2 ^c 3.9

^aCI, 90% confidence interval; ^breference limits from Nordic reference interval projects (NORIP), men 40–49 years (10);

^cin-house reference value for men 40–49 years (Rikshospitalet, University Hospital, Department of Medical Biochemistry).

ments. As this is not feasible we chose to study serum samples drawn from similar groups of individuals of the same gender, age, and category (blood donors) at different points back in time. This, however, assumes that the populations studied are unchanged. We evaluated parameters at the group level, describing the serum values by the mean, median, variance, and reference limits. The number of subjects in each group was large (130); exceeding the recommended number of 120 for reference range studies (22, 23). The 2-year-old and 25-year-old samples were analyzed simultaneously. The 1-month-old group was analyzed somewhat later but with the same instrumentation and with long-term CVs that indicated stable analytical performance.

The assay used to determine proteins may be affected by interference from lipemia, cross-reacting substances, and other exogenous interferences due to pre-analytical conditions and matrix reaction. This may lead to falsely increased or decreased analyte concentration (24). Immunoglobulins, cystatin C and transferrin have been determined by immunoassays using polyclonal antibodies directed against a number of epitopes on the molecule. This increases the possibility that partly degraded components are also being measured. The immunoturbidimetric assay for determination of total IgG used a polyclonal antibody which recognized all four subclasses of IgG. The immunoassays for ferritin, SHBG and insulin C-peptide were of a sandwich type with two monoclonal antibodies and could be more readily influenced by degradation. There are at least 24 isoforms of ferritin,

and no epitope mapping is presently available for ferritin and SHBG according to Roche Diagnostics product support. For C-peptide, the actual epitopes for antibody binding are not known.

Determination of activity by an enzymatic assay requires an intact site for the substrate. However, loss of activity might be caused by changes in conformation of the enzymes as well as degradation such as hydrolysis of peptide bonds or removal of functional groups. Serine and threonine are common residues at the enzyme active site and may be exposed to oxidation resulting in lower enzyme activity (25). Modification of amino acids, even those far from the active site, may reduce activity.

Immunoglobulins

One important finding in this study was a non-significant difference in concentrations with respect to storage time for both IgE and IgG. The material in the Janus Serum Bank is dedicated to epidemiological cancer studies, and IgG antibodies against virus and bacteria have been tested in a number of studies. A study of breast cancer and Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) on Janus sera reported no evidence of decay in concentrations of specific IgG antibodies over time (Ann Richardson, personal communication, 7/8-2008). Concentrations of serum IgE are involved in allergy and asthma, and have also been associated with lung, breast, rectum, prostate and colorectal cancer (26–29). Ovary et al. (30) observed that IgE was stable for at least 1 year at

–20°C and for 4 years at –70°C. Henderson et al. (31) reported that it was possible to obtain reliable IgE measurements in sera stored for more than 30 years at –20°C. The calculated upper reference limit for IgE for all groups exceeded the value given by the kit supplier based on a presumably non-allergic population (32). This latter result is difficult to explain as we were not aware of a high prevalence of allergy in our study group.

Transport and storage proteins

The concentration of transport proteins may vary according to the concentration of components they carry. Both ferritin and transferrin correspond to iron concentrations in blood. In an earlier publication, we reported normal concentrations of total iron in the samples included in this study (33). Ferritin (474 kDa) consists of 24 identical peptide subunits (apo-ferritin) connected via flexible components (34), and may thus be vulnerable to a moderately degradation. The slightly higher concentration of transferrin (8.2%) in the 25-year group may be partly attributed to sublimation (ice to vapor) estimated at 4.1% for the oldest samples (25 years) included in this study (33); the small additional increase is unexplained.

Despite a statistically significant difference, albumin and SHBG demonstrated a relative difference of the mean less than or close to the recommended bias criteria after 25 years of storage. In contrast to our findings, Høstmark et al. (35) reported a significant increase in the albumin level, estimated to be 0.5% per year in the Janus samples after 22 years of storage. They suggested that the results could be attributed to unfolding of the protein during the analytical process used at that time causing false high values. The SHBG median shifted slightly upwards and the dispersion was significantly increased after 25 years of storage. The increase in dispersion of values was possibly an effect of inter-individual differences in loss of water from the vials. Some degradation of SHBG has been reported after more than 40 years of storage at –20°C (36), while Holl et al. (16) found a small increase with storage-time.

Polypeptides

C-peptide has been an important biomarker for cancer of the colon, pancreas, kidney, and aggressive prostate cancer (37). This polypeptide is formed when insulin pro-peptide is cleaved to become an active hormone. C-peptide (3 kDa, 31 aa) has a longer half-life than insulin, and is useful as an indirect measure of insulin secretion (38). A small polypeptide may be more accessible to attack from proteases, and Melles et al. (38) have suggested endopeptidase degradation of C-peptide in serum. We found almost complete loss of C-peptide following 25 years of storage, suggesting that analysis is useless after long-term storage. Cystatin C (13 kDa, 120 aa), a non-glycosylated single polypeptide chain and a potent inhibitor of cysteine proteases (39), is produced at a constant rate in all nucleated cells and is used primarily as a bio-

marker of glomerular filtration rate. However, Kos et al. (40) have shown a significant correlation between increased serum cystatin C and malignant progression in melanoma and colorectal cancer. Being a stable protein, cystatin C could be suitable for epidemiological studies based on biobank samples.

Enzymes

There was a considerable variation in the stabilities of individual enzymes. Both ALT and CK showed significant lower median values with storage-time. After 25 years of storage CK had almost complete loss of activity. CK is known to be relatively labile unless stored with various thiol compounds (41). Enzyme activity seems to decrease as a result of sulfhydryl oxidation or internal disulfide bond formation. Some loss of CK activity in serum samples within 24 h at –20°C have been reported (41–43). Jackson et al. (44) suggested the use of mass concentration rather than enzyme activity when measuring CK following long-term storage. ALT was unstable during long-term storage at –20°C. A mean loss of 46% of enzyme activity by day six has been reported (45). In contrast, AST was relatively stable. Previous studies of AST stability used different storage times and temperatures, and are not consistent (46–49). Jung et al. (50) found that ALT and AST were stable for 10 months at –196°C, while CK was unstable even under these conditions. The activity-based assays for CK, AST and ALT indicated that CK and ALT had considerably lower levels of activity after 2 years storage, while AST seemed to be more stable. One possible explanation is that ALT and CK contain two serine residues in the catalytic site that are susceptible to modification, whereas AST possesses neither serine nor threonine in the corresponding region (51). Difference in the enzyme's flexible regions or in the bonds between subunits may also impact on the stability of enzymes during long-term storage.

Stability of serum constituents is crucial for epidemiological projects based on biobank material. The quality of a biobank sample depends on pre-analytic sample handling, mechanisms of degradation during storage and the analytic methods used for analysis.

Conclusions

We found a great variation in stability between selected proteins. Immunoglobulins were relatively stable, while some enzymes seemed to be particularly fragile. Assays that rely on the three-dimensional structure may be vulnerable to protein modification such as oxidation. Use of polyclonal antibodies in immunoassay procedures may give a more reliable measurement, even if the proteins are partly degraded. The utility of assays that use monoclonal antibodies may be compromised if intact epitopes are not present. Catalytic site may be susceptible to modification during long-term storage and destroy enzymatic activity. Systematic search for serum proteins as biological indicators or biomarkers for cancer can be of limited

value unless the long-term stability and the quality of the assay are addressed. Careful matching with respect to storage time might therefore be essential for comparison of biobank material.

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