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CONTROLS FOR MONITORING THE DETERIORATION OF STORED BLOOD SAMPLES IN THE JAPAN MULTI-INSTITUTIONAL COLLABORATIVE COHORT STUDY (J-MICC STUDY)

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ABSTRACT

Cohort studies commonly store blood samples to measure the associations of biomarkers with disease risks for a long time after the study subjects are enrolled. To obtain valid measurements of the stored samples, monitoring their degree of deterioration is essential. The first stage of the Japan Multi-Institutional Collaborative Cohort (J-MICC) Study launched in 2005 included a project to validate the quality of stored blood samples. This project will compare the measurements of representative molecules over different storage periods (1, 4, and 8 years after sampling, and when a nested case-control study is conducted), different storage temperatures (–80 and –150°C), and different separation conditions (temperature and time) before storage. For these purposes, 28 ml of peripheral blood from 10 people was sampled four times annually, using two tubes for serum and two EDTA-Na tubes for plasma. These samples were treated using the process adopted for the J-MICC study protocol, and stored in tubes containing 300 µl of serum or plasma labeled with two-dimensional bar codes. The sampling was started in 2006, and some of the specimens will be stored until the end of the J-MICC Study in 2035. The resulting findings will produce valuable information on the stability of the molecules, not only for the J-MICC Study, but also for other cohort studies.

Key Words: Cohort study, Blood samples, Biomarkers, Long-term storage, Stability

INTRODUCTION

Several cohort studies worldwide are storing blood specimens for research purposes.[1-3] These studies hope to produce useful information on disease risks based on biomarkers.[4] Among Asian countries, Singapore, Taiwan, Malaysia, and Korea have started new cohort studies, while in Japan, productive cohort studies using blood samples are also under way. For example, the Japan Collaborative Cohort Study (JACC Study) with 130,000 participants, which started in 1988,[5,6] the Japan Public Health Center-based Cohort Study (JPHC Study) with 140,000 participants,[7] and

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the Life Span Study with 93,000 atomic bomb survivors and 20,000 controls are all ongoing.[8)]

In epidemiologic studies, the conditions used to separate and freeze serum and plasma vary depending on where the participants were enrolled. Ideally, the serum and plasma should be separated as soon as possible, stored in liquid nitrogen, and not thawed until time for measurements. However, blood specimens handling often fails to comply with this rule, especially in large studies. Accordingly, several studies have reported differences in the measurement results for different conditions of separation (time and temperature), freezing (temperature and period), and times of freezing and thawing.

No substantial changes (less than 4%) were found between plasma measurements made immediately and 7 days after blood was drawn for protein, albumin, apolipoproteins A1 and B, high-density lipoprotein, total cholesterol (T-C), and triglyceride for whole blood stored at 21°C. Under the same conditions, alanine transaminase (ALT or GPT), aspartate transaminase (AST or GOT), creatinine, and γ-glutamyl transferase (GGT) increased by more than 10%, while creatine kinase (CK) decreased by more than 10%. When whole blood was stored at 4°C, differences in the measurements of ALT, GGT, and CK were less than 4%.[9)]

Similar experiments have examined fat-soluble vitamins, indicating that α-carotene, β-carotene, lutein, lycopene, retinol, and α-tocopherol changed by less than 8%, and cryptoxanthin and γ-tocopherol by less than 11% for up to 7 days.[10)] Interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) were reported to be less stable; whole blood left unseparated for 4 h at room temperature caused a 14.3% reduction in IL-6 and 9.6% elevation in TNF-α. Freezing and thawing affected TNF-α more than IL-6, and leptin was relatively stable in comparison with the two cytokines. Plasma stored with lithium heparin and sodium citrate yielded lower averages for the three cytokines than serum or EDTA-added plasma.[11)]

Since the effects of separation and storage conditions vary for different analytes, studies on these conditions would be preferable for the analytes actually used in cohort studies. This article describes a project of the Japan Multi-Institutional Collaborative Cohort (J-MICC) that will examine the differences in measurements according to the separation conditions, freezing temperature, and freezing periods for several components of serum and plasma.

MATERIALS AND METHODS

The Japan Multi-Institutional Collaborative Cohort (J-MICC) Study

The Japan Multi-Institutional Collaborative Cohort (J-MICC) Study was launched in 2005, supported by a grant for Scientific Research on Special Priority Areas of Cancer from the Japanese Ministry of Education, Culture, Sports, Science and Technology.[12)] Although its main purpose is to detect and confirm gene-environment interactions of lifestyle-related diseases (mainly cancer) through the cohort analyses, it also includes cross-sectional analyses of lifestyle factors, biomarkers, and genotypes, as well as confirmation/screening of new biomarkers usable for the early diagnosis of cancer. The endpoints are cancer diagnosis and death. The participants diagnosed with cancer will be identified through population-based cancer registries, hospital cancer registries, questionnaires mailed and those administered during repeat visits, death certificates, health insurance data, and second-survey questionnaires.

The subjects are individuals aged 35–69 years who enrolled in response to study announcements in specific areas, inhabitants attending health checkup examinations conducted by local governments, visitors at health checkup centers, and patients at a cancer hospital. The number of subjects was set at 100,000 throughout Japan. The enrollment period started in October 2005 and extends to March 2010. The second survey is scheduled for 5 years after enrollment, with

participants followed until 2025.

The J-MICC Central Office is at the Nagoya University Graduate School of Medicine. Ten participating research groups (Cohort Study Executing Groups) will send baseline data and blood samples (buffy coat, serum, and plasma) anonymized with an identification number (J-MICC ID) to the central office. The data from the second survey and follow-up will be linked using the J-MICC ID. This study is expected to produce many new findings on lifestyle and genetic traits associated with lifestyle-related diseases, including cancer, in Japan. The protocol of the J-MICC Study was approved on July 20, 2005 by the Ethics Review Committee of Nagoya University School of Medicine (Approval number 253).

Samples stored in J-MICC Study

The J-MICC Study collects blood samples in a 7-ml vacuum tube for serum and a 7-ml EDTA-2Na-containing vacuum tube for plasma and buffy coat. For plasma separation, two centrifugations are required to eliminate white blood cells more completely. Usually, 3 ml of serum, 3 ml of plasma, and 0.8 ml of buffy coat are obtained. The blood samples to be sent to the central office consist of one storage tube containing 300 µl of buffy coat, another four containing 300 µl of serum, and a final four containing 300 µl of plasma. The remainder will be used for research by each cohort study group. There is a 10-digit two-dimensional bar code on the bottom of each storage tube.

Aims of control sample storage

The project aims to compare the measurements of representative molecules according to storage-period duration (1, 4, and 8 years after sampling and when a nested case-control study is conducted using the stored J-MICC Study samples: aim 1) storage temperature (–80 and –150°C; aim 2) and different separation conditions (temperature and hours until storage: aim 3).

The procedure used to store control samples

In the protocol, fasting blood was drawn from 10 apparently healthy volunteers (5 men and 5 women) aged 20 or over in the morning at Nagoya University Daiko Medical Center, Nagoya, Japan. The process of serum/plasma separation and storage was the same as that used in the J-MICC Study. The study protocol requires that blood be processed within 24 h of being drawn, and that it be stored quickly using storage tubes containing 300 µl at –80°C. The separation was conducted in a room at a temperature of 23 to 25°C.

RESULTS

To obtain the required information, we drew fasting blood from 10 volunteers (5 men and 5 women) four times in 1 year, twice for serum and twice for plasma, as shown in Table 1. The volunteers were J-MICC Study staff and employees at the Daiko Medical Center aged 25–51 years. Table 2 shows the separation and storage conditions used for each sample. The samples were identified using five parameters: Y-D-P-B-S, where Y is the year at sampling, D is the order of sampling within the year, P is the participant, B is the sequence in which the four blood tubes were drawn from 1 to 4, and S is the sequence number for the storage tubes from 1 to 8. For example, 2006-1-1-1-1 indicates the first storage tube separated from the first blood tube drawn from participant 1 during the first blood drawing in 2006.

The measurement items in Table 2 are identified as Fixed and Unfixed; the former are specified in Table 3, and the latter indicate molecules to be measured during a nested case-control

Table 1 Blood drawing schedule for control samples to evaluate the effects of temperature before separation (Tm before Sp), time to freezing (Time to Fr) in hours (h), and storage temperature (St Tm)

Blood drawn	Sample	*n*	Tm before Sp (°C)	Time to Fr (h)	St Tm (°C)	Aim
First	Serum	10	Room Tm	4	–80/–150	1 & 2
Second	Plasma	10	Room Tm	4	–80/–150	1 & 2
Third	Serum	10	Room Tm/4	1, 2, 4, or 8	–80	3
Fourth	Plasma	10	Room Tm/4	1, 2, 4, or 8	–80	3

Four tubes of blood were sampled from each person, and the blood in each tube was aliquoted into eight storage tubes (300 µl per tube), resulting in 32 storage tubes per person. The aims were to compare the measurements of representative molecules according to storage period (1, 4, and 8 years after sampling, and when a nested case-control study is conducted using the stored J-MICC Study samples: aim 1) storage temperature (–80 and –150°C; aim 2) and different separation conditions (temperature and hours until storage: aim 3).

Table 2 Sample number (Year - Date - Participant number - Blood collection tube number - Storage tube number) and preparation/storage conditions for serum: temperature before separation (Tm before Sp), time to freezing (Time to Fr), storage temperature (Tm of St), time to measurement (Time to Ms), items measured (It Ms), and study aim

Sample number	Tm before Sp (°C)	Time to Fr (h)	Tm of St (°C)	Time to Ms (years)	It Ms	Aim
Y-1-i-1-(1,2)	Room Tm	4	–80	1	Fixed	1
Y-1-i-1-(3,4)	Room Tm	4	–80	4	Fixed	1
Y-1-i-1-(5,6)	Room Tm	4	–80	8	Fixed	1
Y-1-i-1-(7,8)	Room Tm	4	–80	At analysis	Fixed	1
Y-1-i-2-(1,2)	Room Tm	4	–80	1	Fixed	1
Y-1-i-2-(3,4)	Room Tm	4	–80	4	Fixed	1
Y-1-i-2-(5,6)	Room Tm	4	–80	8	Fixed	1
Y-1-i-2-(7,8)	Room Tm	4	–80	At analysis	Fixed	1
Y-1-i-3-l	Room Tm	4	–80	At analysis	Unfixed	2
Y-1-i-3-m	Room Tm	4	–150	At analysis	Unfixed	2
Y-1-i-4-l	Room Tm	4	–80	At analysis	Unfixed	2
Y-1-i-4-m	Room Tm	4	–150	At analysis	Unfixed	2
Y-3-j-1-n	Room Tm	1	–80	At analysis	Unfixed	3
Y-3-j-2-n	Room Tm	2	–80	At analysis	Unfixed	3
Y-3-j-3-n	Room Tm	4	–80	At analysis	Unfixed	3
Y-3-j-4-n	Room Tm	8	–80	At analysis	Unfixed	3
Y-3-k-1-n	4	1	–80	At analysis	Unfixed	3
Y-3-k-2-n	4	2	–80	At analysis	Unfixed	3
Y-3-k-3-n	4	4	–80	At analysis	Unfixed	3
Y-3-k-4-n	4	8	–80	At analysis	Unfixed	3

Blood was scheduled to be drawn four times per year, with dates 1 and 3 for serum collection and dates 2 and 4 for plasma collection. Participant number is given as i = 1–10 (10 people), j = 1–5 (5 people), and k = 6–10 (5 people). Storage tube number is given as l = 1–4, m = 5–8, and n = 1–8. "At analysis" means that the samples will be measured at analysis in a nested case-control study. The measurement items will not be fixed until the analysis. The aims were to compare the measurements of representative molecules according to storage period (1, 4, and 8 years after sampling and when a nested case-control study is conducted using the stored J-MICC Study samples: aim 1) storage temperature (–80 and –150°C), aim 2), and aim 3) different separation conditions (temperature and hours until storage).

Table 3 Fixed measurement items for control samples in the Japan Multi-Institutional Collaborative Cohort (J-MICC) Study

Sample number	First measurement	Sample	Items to be measured
2006-1-i-o-(1,2)	September 20, 2007	Serum	T-C, HDL-C, LDL-C, TG, AMY
2006-2-i-o-(1,2)	September 20, 2007	Plasma	T-C, HDL-C, LDL-C, TG, AMY
2007-1-i-o-(1,2)	To be measured in 2008	Serum	CPK, AST, ALT, LDH, ALP
2007-2-i-o-(1,2)	To be measured in 2008	Plasma	Renin activity, Pancreas PL
2008-1-i-o-(1,2)	To be measured in 2009	Serum	Not determined
2008-2-i-o-(1,2)	To be measured in 2009	Plasma	Not determined

All samples were 600 μl. Blood collection tube number is expressed as o = 1–4. T-C: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglyceride; CPK: creatinine phosphokinase; AST: aspartate transaminase; ALT: alanine transaminase; LDH: lactase dehydrogenase; ALP: alkaline phosphatase; AMY: amylase; and PL: phospholipase.

study in the J-MICC Study. The fixed measurement items in Table 3 consisted of protein, enzymatic activity, or lipid. The items of total cholesterol (T-C), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG) and amylase (AMY) were measured in control plasma and serum samples on September 20, 2007. The items were measured as follows: a UV-End method using cholesterol dehydrogenase for T-C [13]; homogenous enzymatic methods using commercial Cholestest N-HDL and Cholestest LDL kits (Daiichi Pure Chemicals, Tokyo, Japan) for HDL-C and LDL-C, respectively; an enzymatic method using glycerol 3-phosphotransferase and glycero-3-phosphate:O_2-oxidoreductase for T-G; an enzymatic method with 2-chloro-4-nitrophenyl-4-galactopyranosylmaltoside as a substrate using a commercial CicaLiquid-N AMY kit (Kanto Chemical, Tokyo, Japan) for AMY. [14] All assays were performed at the SRL Laboratory in Tokyo, Japan.

To achieve aim 1, the same samples from one person will be measured 1 year later [Y-1-i-1-(1,2) and Y-1-i-2-(1,2) for serum and Y-2-i-1-(1,2) and Y-2-i-2-(1,2) for plasma], 4 years later [Y-1-i-1-(3,4) and Y-1-i-2-(3,4) for serum and Y-2-i-1-(3,4) and Y-2-i-2-(3,4) for plasma], 8 years later [Y-1-i-1-(5,6) and Y-1-i-2-(5,6) for serum and Y-2-i-1-(5,6) and Y-2-i-2-(5,6) for plasma], and at the time of a nested case-control study [Y-1-i-1-(7,8) and Y-1-i-2-(7,8) for serum and Y-2-i-1-(7,8) and Y-2-i-2-(7,8) for plasma]. All measurements have been done in duplicate to evaluate the reproducibility of the measurement methods.

In accordance with our protocol, T-C, HDL-C, LDL-C, TG, and AMY were measured for a 600 μl mixture from two tubes (2006-D-i-B-1 and 2006-D-i-B-2, where D = 1 for serum and D = 2 for plasma, and B = 1, 2 for duplicated measurements for the same blood sample) for 20 serum samples and 20 plasma samples on September 20, 2007 (Table 3). The difference in the measurements between two corresponding samples of the same blood from one person was less than 5% of the average for any biomarker, with the maximums being 4.9% for AMY in serum and 3.9% for TG in plasma (Table 4).

To meet aim 2, which is to examine the differences in measurements between storage at –80 and –150°C, Y-1-i-(3,4)-l stored at –80°C and Y-1-i-(3,4)-m stored at –150°C will be used for serum, and the corresponding samples (D = 2) for plasma. To meet aim 3, samples D = 3 for serum and D = 4 for plasma will be used.

As of December 2007, blood has been collected seven times. The first control blood collection was planned for serum, but the schedule was changed to plasma because blood tubes for serum could not be prepared for 10 people. Table 5 shows the actual conditions for each control blood collection. It took more than 30 min to draw blood from the 10 volunteers, which resulted in a

Table 4 Analysis of fixed measurement items in control samples stored in 2006 for the J-MICC Study

		T-C	HDL-C	LDL-C	TG	AMY
Serum	Mean ± SD (mg/dl or units/L)	216.2±48.6	62.6±18.0	129.2±39.8	146.5±112.3	83.7±29.2
	Range (mg/dl or units/L)	147–303	41–102	85–204	61–362	52–155
	Range: difference percentages (%)	0.0–2.4	0.0–2.5	0.0–2.2	0.0–2.9	0.0–4.9
Plasma	Mean ± SD (mg/dl or units/L)	200.6±42.0	63.5±17.7	113.4±31.9	139.5±124.3	77.0±30.7
	Range (mg/dl or units/L)	152–272	38–93	77–169	60–454	45–154
	Range: difference percentages (%)	0.0–2.0	0.0–3.0	0.0–1.5	0.0–3.9	0.0–2.2

All items in serum and plasma were measured on September 20, 2007. Difference percentages (%) = difference value/mean. SD: standard deviation. T-C: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglyceride; and AMY: amylase.

Table 5 Control samples stored in 2006–2007 for the J-MICC Study: time taken to draw (Time for BD) and time between when blood was drawn from the last participant and freezing (Time to Fr)

Year	Sample	Date drawn	Participants (*n*)	Time for BD (min)	Time to Fr
2006 First	Serum	Oct. 26, 2006	10	34	5 h 41 min
2006 Second	Plasma	July 14, 2006[a)]	10	32	4 h 32 min
2006 Third	Serum	Feb. 21, 2007	10	42	1 h 13 min, 2 h 3 min, 4 h 3 min, 8 h 3 min
2006 Fourth	Plasma	Apr. 4, 2007	10	38	1 h 7 min, 2 h 2 min, 4 h 2 min, 8 h 0 min
2007 First	Serum	June 5, 2007	10	32	4 h 42 min
2007 Second	Plasma	July 26, 2007	10	38	5 h 1 min
2007 Third	Serum	Nov. 20, 2007	10	37	1 h 39 min, 2 h 34 min, 4 h 7 min, 8 h 24 min
2007 Fourth	Plasma	Not done			

[a)] On July 14, 2006, since vacuum tubes for serum separation could not be prepared for 10 people, plasma was sampled instead.

difference in the time until sample freezing. Although the time until freezing was scheduled to be predetermined (1, 2, 4, or 8 h), the handling of the serum/plasma for storage sometimes took longer than we expected, resulting in a longer time until freezing. The third-time samples were collected in 2007, with some tubes being stored in a refrigerator at 4°C within 5 min. These blood samples did not clot completely, with the result that it took longer to separate serum using centrifugation, which also caused a delay before freezing.

DISCUSSION

Although the quality control of stored blood samples is important, few papers report how the problem has been managed in various cohort studies. We in the J-MICC Study group have been discussing this since the early stage of the study, and have drawn up a protocol for control blood samples. The protocol focuses on three main aims, which may partly support the validity of the findings from biomarker analyses in the J-MICC Study.

In this protocol, 10 samples were to be drawn each time blood was sampled. There was no rigid statistical basis for this number. Since sampling was scheduled for four times a year, for at least 4 years from 2006 to 2009, the enrollment period of the J-MICC Study, 10 people (5 men and 5 women) seemed to be a practical maximum. Their ages ranged from the 20s to

the 50s. The measurements of five biomarkers covered the normal range for the population. The volunteers were not always the same because of scheduling problems. In total, about 20 people participated in the first seven blood samplings. Since the donated blood was completely anonymized, there was no advantage to volunteering.

The results derived from the control measurements seemed to be directly applicable to the J-MICC Study since the storage tubes and amounts of sample in the tubes were the same as those actually used in the J-MICC Study; and the procedures used for serum/plasma separation and storage are the same ones used by the cohort study executing group. Eight hours at room temperature before freezing constituted the worse-case condition in the J-MICC Study.

Using standard laboratory methods, 300 μl is the minimum volume needed to measure one component of serum or plasma. The five items selected, T-C, HDL-C, LDL-C, TG, and AMY, can be measured using an automated method that requires 600 μl. Therefore, for the measurements, we mixed serum/plasma from two storage tubes (Y-D-P-B-1 and Y-D-P-B-2) and sent them to the company doing the measurements. Two independent samples from one person gave quite similar measured values for the 5 items, ensuring that their reproducibility was high. The measurements made 1 year after the blood was drawn will be compared with those made 4 and 8 years after the blood was drawn, and with the results of a nested case-control study in the J-MICC Study.

In the J-MICC Study, the blood samples are expected to be used for proteomics analyses.[15-18] The time between when the blood is drawn and frozen is measured routinely for some of the samples collected in the J-MICC Study. All samples were separated and frozen on the day the blood was drawn. A pilot study of the proteomics analysis will be conducted using the control samples and samples from the J-MICC Study.

Some studies have examined marker stability after long-term storage.[19-23] Shih demonstrated that average decreases for serum T-C, HDL-C, and TG occurred for up to 7 years in storage at –70°C, although these changes were not statistically significant.[19] A cohort study in Japan reported that C-reactive protein in blood samples was higher 13.8 years after storage than before storage at –80°C.[20] The stability of antioxidant micronutrients, such as carotenoids and retinol, in plasma stored at –70°C for 4 years was also monitored.[23] The long-term storage effect in our samples will be estimated by monitoring the stability at intervals. The results obtained from assays of aliquots of the same serum or plasma sample will provide useful information for developing storage procedures.

Furthermore, at analysis we will compare the marker stability of samples stored at –80°C with those stored at –150°C. Ideally, the control sample items need to be measured both at baseline and the time of analysis. However, it is difficult to fix all of the measurement items at baseline in cohort studies with long study periods. Unfixed items in aim 2 stored at –80 and –150°C will be used to confirm the validity of storage at –80°C or, at least, whether the stability is the same as that for samples stored at –150°C.

In conclusion, we described a project used to store control blood samples to examine the differences in measurements among the different separation and storage conditions. Since quality control of stored blood samples is essential to validate the findings of biomarker analyses, it is necessary to store control blood samples, especially in cohort studies with a long follow-up period. The findings of this project will be used for the J-MICC Study, and possibly other cohort studies as well.

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