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## Effect of Repeated Freeze-Thaw on Serum Biomarkers Associated with Eye Disease

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**Background:** Serum biomarkers are associated with eye diseases, which results in the need for cryopreservation of serum samples. However, the effect on serum biomarker levels of repeatedly freezing and thawing remains poorly understood. The aim of this study was to evaluate the effects of repeated freeze-thaw on the serum levels of the protein, complement C3c (C3c), the micromolecule, uric acid (UA), and the enzyme, angiotensin-converting enzyme (ACE).

**Material/Methods:** Serum samples were obtained from 50 patients who attended an ophthalmic outpatient department. Following baseline measurements, the serum samples from each subject were divided into aliquots and stored at  $-80^{\circ}\text{C}$  for further analysis, following between one to six freeze-thaw cycles. The serum levels of C3c, UA, and ACE were measured immediately after the stored serum samples were thawed.

**Results:** The serum level of C3c was significantly changed after the first freeze-thaw cycle ( $p < 0.05$ ), and a significant alteration in serum ACE levels occurred after the third freeze-thaw cycle ( $p < 0.05$ ). The serum UA level remained unchanged after all freeze-thaw cycles. Repeated freeze-thaw cycles significantly increased the serum levels of C3c and decreased the serum levels of ACE. The serum levels of C3c, UA, and ACE, respectively were significantly correlated ( $p < 0.001$ ), while the correlation coefficient for C3c and UA were improved when compared with ACE.

**Conclusions:** Repeated freeze-thaw can have variable effects on the serum levels of biomarkers, C3c, UA and ACE, which supports the need for quality control of cryopreserved serum for biomarker evaluation.

**MeSH Keywords:** **Biological Markers • Biological Specimen Banks • Serum • Specimen Handling**

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## Background

Until recently, ocular diseases were regarded as focal diseases, and few serum biomarkers were used for laboratory diagnosis and for the study of eye diseases. However, there is increasing published evidence from laboratory studies supporting the use of serum biomarkers, including complement C3, angiotensin-converting enzyme (ACE), uric acid (UA), bilirubin, and gamma-glutamyl transferase (GGT), which have been shown to be significantly associated with eye diseases such as age-related macular degeneration (AMD) [1–6], diabetic retinopathy (DR) [7–11], hypertensive retinopathy [12] and glaucoma [13]. Previous studies from our laboratory have also shown that serum biomarkers that are significantly associated with glaucoma include complement C3 [14], UA [15], and D-dimer [16]. These published studies on the role of serum biomarkers in eye diseases might provide a future development in clinical diagnosis and patient monitoring, and in understanding eye diseases, with the future implementation of prospective controlled clinical studies.

Because prospective clinical studies require the use of numerous cryopreserved serum samples, obtained from laboratory biobanks, it is important to ensure that accurate results may be obtained from stored samples. The pre-analytical phase is the primary source of analytical errors [17], which includes physiological factor, sample collection methods, and endogenous interference factors [18]. During the design process of clinical serum biomarker studies, physiological patient factors, including age, sex, and lifestyle, and endogenous interference factors, including drug treatments and the presence of circulating antibodies, are usually recorded and may be controlled. However, factors that affect sample collection and storage are not always considered or controlled and include blood collection tubes and anticoagulants used, centrifugation speed and time, storage temperature and duration, and for frozen serum storage, freeze-thaw cycles.

In particular, cryopreserved serum samples from the same biobank might undergo various sample handling procedures that result in repeated freeze-thawing. Although previously published studies have shown that storage temperature, storage time, freeze-thaw cycles, and other factors could affect the serum or plasma levels of several biomarkers [19–22], there is still limited guidance for the optimal selection of cryopreservation of these samples for clinical monitoring and clinical studies. Repeated freeze-thaw is the almost inevitable for cryopreserved samples, as multiple testing or re-analysis leads to repeated freeze-thaw. The relatively limited storage volume of biobanks also limits using multiple smaller aliquots of serum samples to reduce the effect of the freeze-thaw process. However, the increased sensitivity of detection methods now requires much smaller sample volumes to complete biomarker analysis, which

means that if serum samples were frozen as small aliquots, these small individual samples might be thawed for analysis without thawing and refreezing the whole serum sample.

Therefore, in order to better direct the selection of cryopreserved serum samples from biobanks for future prospective studies involved in ophthalmology, the aim of this study was to investigate the effect of repeated freeze-thaw on the levels of three types of serum biomarkers related to eye diseases, the protein, C3c, the micromolecule, UA, and the enzyme, ACE. This study was designed to evaluate the effects of repeated freeze-thaw cycles on the serum concentration of these three biomarkers with strict control of other factors, which were the same for blood collection tubes and anticoagulants, centrifugation speed and time, and serum storage temperature.

## Material and Methods

### Patients

A total of 50 outpatients (26 men and 24 women) between 19–75 years old (mean age, 41 years) were enrolled in this study and were randomly selected from our hospital on the same day. Patients with hyperlipidemia, hyperbilirubinemia, or other severe diseases, including hemolytic diseases, which could interfere the detection of biochemical indices, were excluded. The serum samples collected from each patient were for the laboratory testing ordered by the physician. No additional blood was taken from the subjects. All patients were informed of the study and signed an informed consent to participate in the study. The study design and procedures were in accordance with the guidelines of the Helsinki Declaration on human experimentation, and the study was approved by the Ethics Committee of the Eye and ENT Hospital, Shanghai Medical College, Fudan University, Shanghai, China.

### Blood collection and processing

Blood samples were obtained in the morning after the subjects had fasted for eight hours. Peripheral blood samples from each subject were collected into two 5 mL vacuum tubes (Zhi Yuan Development of Medical Science, Co., Ltd., Wuhan, China) with a coagulant, by standard venepuncture. The sample tubes were left in upright position for 30 min at room temperature for complete clot formation. All samples were then centrifuged at 2,593 xg for 10 minutes. Serum samples were checked visually for hemolysis or lipemia to avoid possible interference with the analysis [23,24]. Then, the serum of each subject was pooled into a plain 5.0 ml snaplock tube (Axygen, INC., CA, USA), and a total of 2.8ml of serum was aliquoted into seven 0.6 ml snaplock tubes (Axygen, INC., CA, USA) (0.4ml per tube) for each subject.

**Table 1.** Serum levels of biomarkers under each freeze-thaw cycle.

Biomarker	n	T0	T1	T2	T3	T4	T5	T6
C3c (mg/dL)	50	99.381±	107.452±	110.149±	116.637±	122.341±	130.409±	127.972±
		15.801	17.401	17.801	19.600	21.122	22.605	23.080
p			<b>0.002*</b>	<b>&lt;0.001*</b>	<b>&lt;0.001*</b>	<b>&lt;0.001*</b>	<b>&lt;0.001*</b>	<b>&lt;0.001*</b>
UA (mol/L)	50	0.286±	0.300±	0.302±	0.315±	0.317±	0.319±	0.318±
		0.082	0.084	0.085	0.089	0.089	0.089	0.090
p			0.415	0.354	0.095	0.071	0.057	0.069
ACE (U/L)	50	41.960±	41.660±	42.380±	36.320±	33.340±	34.120±	34.520±
		14.975	15.271	15.662	15.537	16.219	15.526	14.011
p			0.921	0.891	<b>0.048*</b>	<b>0.002*</b>	<b>0.012*</b>	<b>0.012*</b>

All concentrations are written as mean ±SD. The Kolmogorov-Smirnov test was used for normality. Paired sample t-test and Wilcoxon matched pairs signed rank tests were used to compare the values obtained from each freeze-thaw cycle (T1–T6) and the baseline values (T0). C3c – complement component 3c; UA – uric acid; ACE – angiotensin-converting enzyme. \* p<0.05 for the significant difference of serum biomarkers level between the experimental measurement (T1–T6) and the baseline measurement (T0).

Six aliquot of each subject were frozen at –80°C immediately for analysis, and the remaining one aliquot was used for the required clinical test for each patient and for the baseline measurement (T0). After 24 hours, all frozen serum in the tubes was thawed at room temperature (about 25°C) for approximately 1.5 hours until completely thawed. One aliquot from each subject was then mixed thoroughly by inverting the sample 15 times and centrifuged at low speed before analysis (T1). Other aliquots were refrozen at –80°C immediately for later analysis. This freeze-thaw cycle was repeated once every day for the next six consecutive days to undertake the freeze-thaw processing. The results of testing for each freeze-thaw cycle were marked as T1, T2, T3, T4, T5, and T6, respectively.

### Biochemical analysis

The levels of complement component C3c (C3c), uric acid (UA) and angiotensin-converting enzyme (ACE) in serum were measured using the Roche Cobas c702 biochemical automatic analyzer. The concentration of C3c was measured by immunoturbidimetry with the Roche reagent (Roche Diagnostics GmbH, Mannheim, Germany), and UA was also measured by enzymatic colorimetry with Roche reagent (Roche Diagnostics GmbH, Mannheim, Germany). The level of ACE was measured by a continuous monitoring assay with an open-channel reagent (Kangte Bio-Tech, Co., Ltd., Zhejiang, China). As stated by the manufacturer, the inter-assay and intra-assay variability was <10% for all assays. Quality control of the biochemical automatic analyzer was performed each day before sample detection with two levels of control materials. Internal laboratory controls were analyzed daily over the three-year study period, with typical monthly coefficients of variation (CVs) between 2–4% and no significant changes in the values.

### Statistical analysis

The distribution of the variables was determined by Kolmogorov-Smirnov normality test. The significant difference between serum biomarkers levels from any two detections was determined by the paired samples t-test, or Wilcoxon matched pairs signed rank test. The linear regression model was used to determine the trends of serum biomarker levels following the effect of repeated freeze-thaw cycles, which used the serum biomarkers level as a dependent variable and the number of freeze-thaw cycles as an independent variable. An estimate was considered to be significant if the multiple correlation coefficient (R) was >0.2 and the p-value was <0.05 [17]. The correlation of serum biomarker levels between the various detections (T0, T1–T6) was determined by the Pearson correlation test or the Spearman correlation test. A P-value <0.05 was considered to be statistically significant. All statistical analysis was performed by using the SPSS licensed statistical package for Windows, Version 24.0 (SPSS Inc., Chicago, IL, USA).

## Results

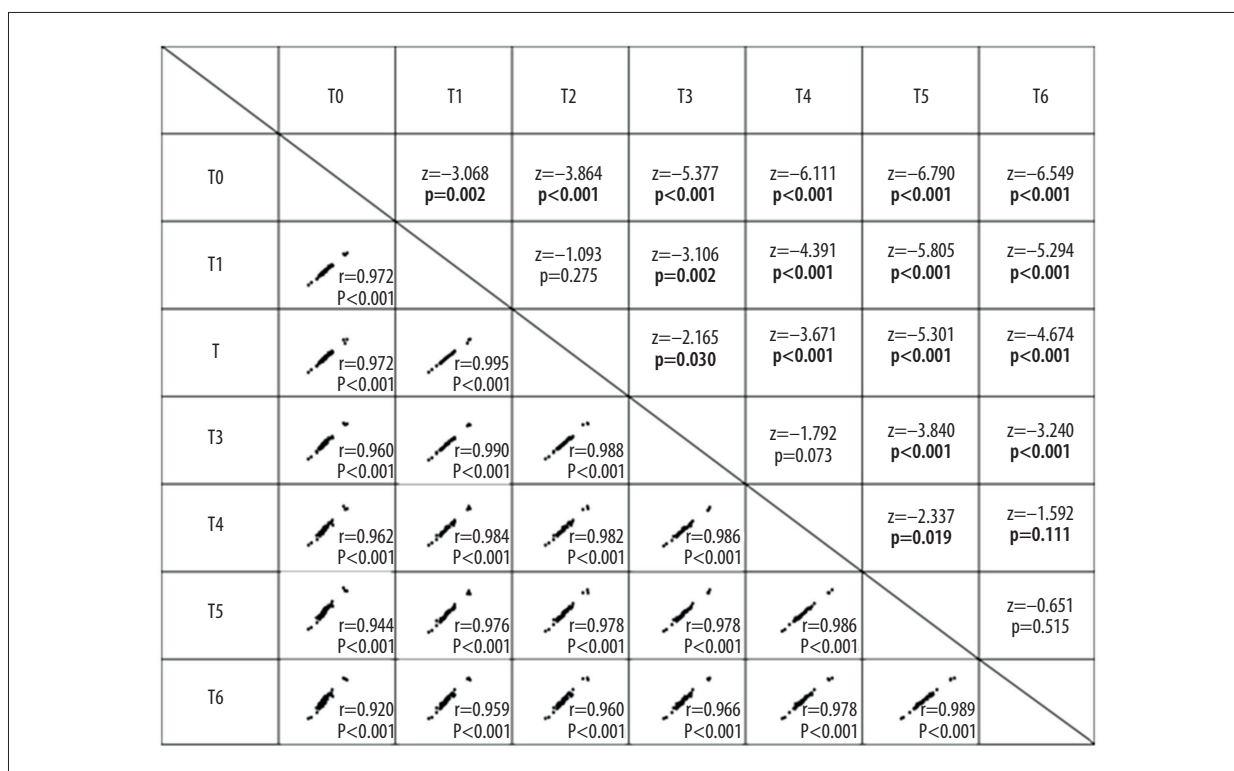
### Stability of serum biomarkers tolerated six freeze-thaw cycles

As shown in Table 1, the serum concentration of complement C3c was vulnerable to the effect of repeated freeze-thaw, which significantly changed after the first freeze-thaw cycle (p<0.05). The serum angiotensin-converting enzyme (ACE) level was relatively stable for the first and second freeze-thaw cycles and significantly altered following the third freeze-thaw cycle (p<0.05). The serum level of uric acid (UA) was stable and remained unchanged during all six freeze-thaw cycles.

**Table 2.** Linear regression analysis serum levels of biomarkers under the effect of freeze-thaw cycles.

Condition		C3c	UA	ACE
Repeated freeze-thaw cycles	$\beta$	5.139	0.005	-1.659
	R	<b>0.462</b>	<b>0.123</b>	<b>0.212</b>
	p	<b>&lt;0.001</b>	<b>0.022</b>	<b>&lt;0.001</b>

Linear regression was used to analyze the following:  $\beta$  – regression coefficient; R – multiple correlation coefficient; C3c – complement component 3c; UA – uric acid; ACE – angiotensin-converting enzyme.  $R>0.2$  and  $p<0.05$  are considered as significant for the linear relation between the number of freeze-thaw cycles and the level of serum biomarkers.



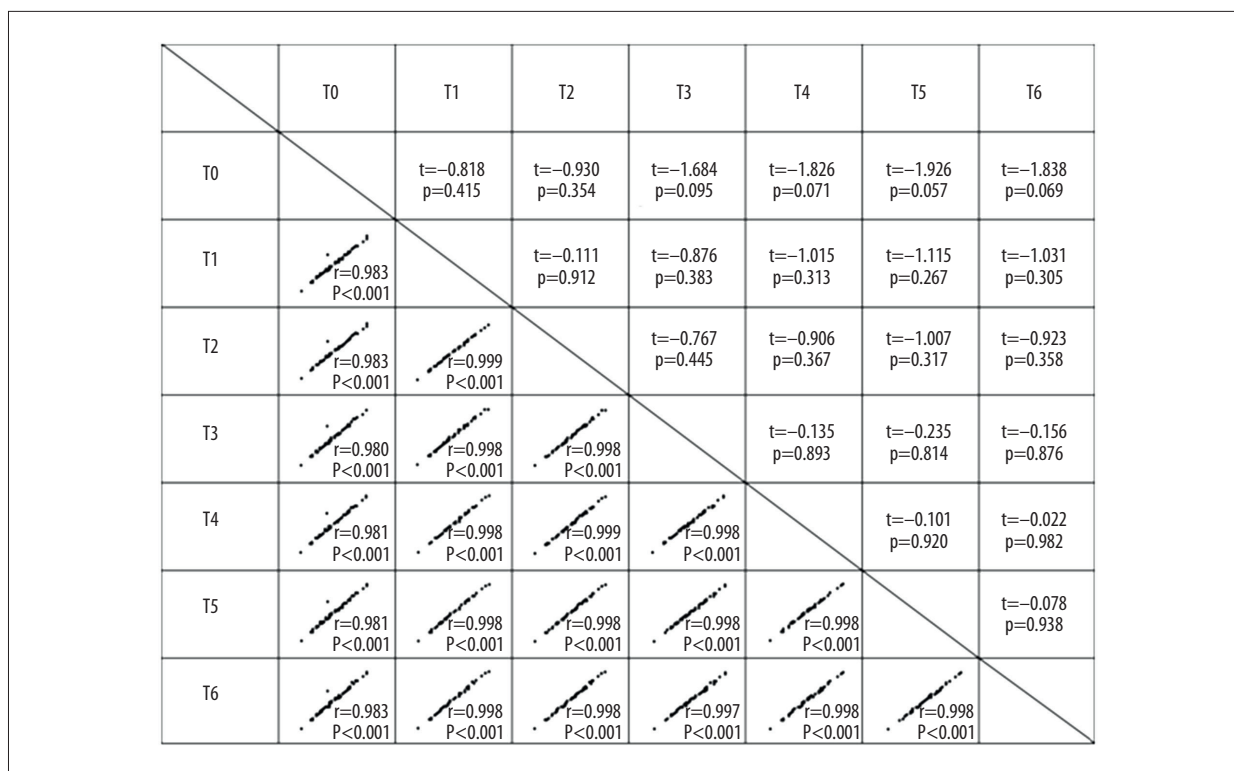
**Figure 1.** The difference and correlation of serum C3c level between any two detections that underwent six freeze-thaw cycles. The upper area shows the difference of serum C3c level between any two detections. Wilcoxon matched-pairs signed-ranks test was used to compare the values.  $P<0.05$  for the significant difference of serum C3c level among freeze-thaw cycles. The lower area shows the correlation of serum C3c level between any two detections. The Spearman correlation test was used to analyze the correlation.  $P<0.05$  for the significant correlation of serum C3c level between freeze-thaw cycles.

**Comparison of levels of serum biomarkers that underwent six freeze-thaw cycles**

The linear regression analysis showed that the serum levels of C3c and ACE were significantly correlated with the number of freeze-thaw cycles (Table 2). The C3c concentration in serum increased as the number of freeze-thaw cycles ( $R=0.462$ ,  $p<0.001$ ), while the UA serum concentrations slightly increased with the number of freeze-thaw cycles. The activity of serum ACE decreased significantly with the number of freeze-thaw cycles ( $R=0.212$ ,  $p<0.001$ ).

**The difference in serum biomarkers level between any two freeze-thaw cycles**

Analysis of serum C3c, UA, and ACE levels between the various freeze-thaw cycles (Figures 1–3) showed that the serum level of C3c was significantly different ( $p<0.05$ ) in most of the comparisons between two detections between all six freeze-thaw cycles, except the difference of values between T1 and T2, T3 and T4, T6 and T4, and T5. However, there was no significant difference in serum UA level between any two detections between six freeze-thaw cycles. The serum level of ACE



**Figure 2.** The difference and correlation of the serum micromolecule uric acid (UA) level between any two detections that underwent six freeze-thaw cycles. The upper area shows the difference of serum UA level between any two detections. Paired samples t-test was used to compare the values.  $P < 0.05$  for the significant difference of serum UA level among freeze-thaw cycles. The lower area shows the correlation of serum UA level between any two detections. The Pearson correlation test was used to analyze the correlation.  $P < 0.05$  for the significant correlation of serum UA level between freeze-thaw cycles.

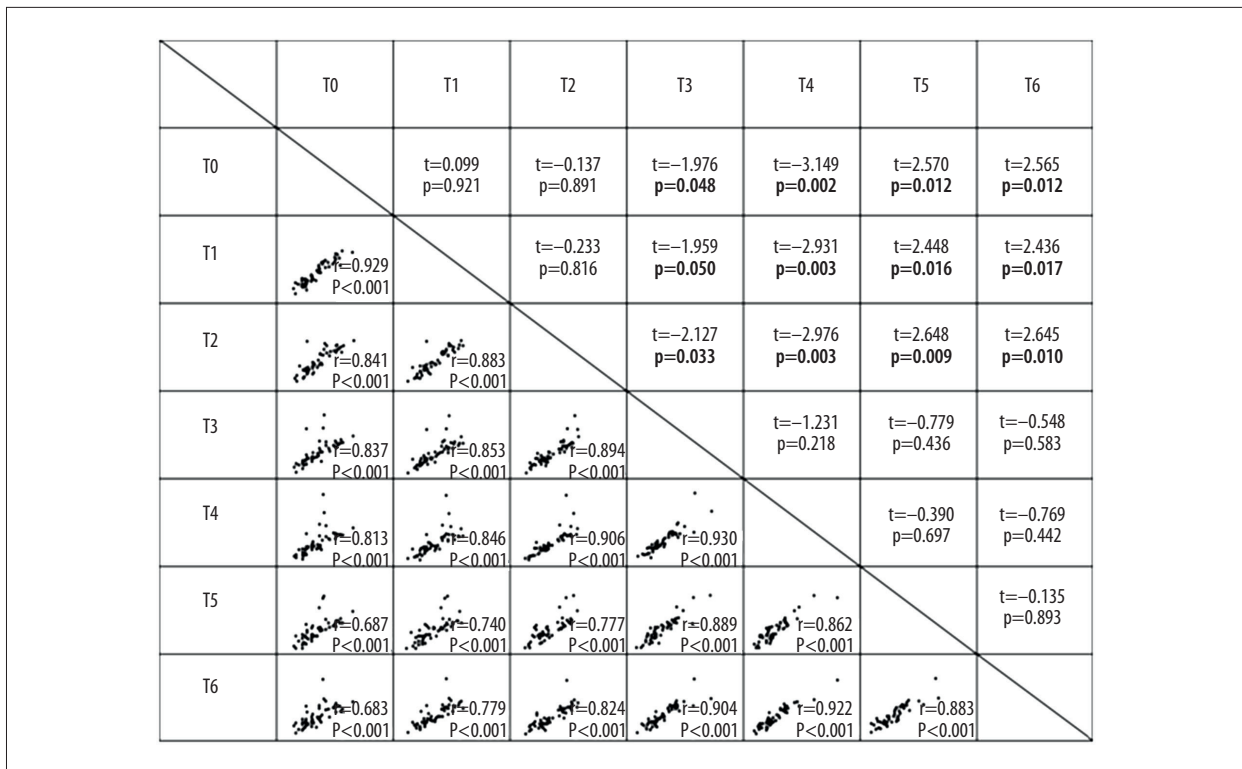
did not alter significantly ( $p > 0.05$ ) at the first two freeze-thaw cycles. Since the third freeze-thaw cycle, there was a significant difference ( $p < 0.05$ ) in serum ACE level between any two detections, although there was no significant difference between T3, T4, T5, and T6. Therefore, the serum level of C3c and ACE might be more sensitive to the effect of repeated freeze-thaw than UA.

### The correlation of serum biomarker levels between any two detections

Analysis of the correlation of serum C3c, UA, and ACE levels between various detections (Figures 1–3), showed significant correlations ( $p < 0.001$ ) of serum levels of C3c, UA and ACE between any two detections during six freeze-thaw cycles and the correlation between the serum C3c and UA level (all  $r > 0.920$ ) were stronger than for serum ACE (all  $r > 0.680$ ). For serum C3c, the more the serum samples were freeze-thawed, the worse the correlation coefficient was found.

## Discussion

This study was designed to evaluate the effect of repeated freeze-thaw on the serum levels of three serum biomarkers associated with eye diseases, the protein, complement C3c (C3c), the micromolecule, uric acid (UA), and the enzyme, angiotensin-converting enzyme (ACE). The serum C3c level significantly changed from the first freeze-thaw cycle and there were significant differences in most comparisons between two detections or analyses between all six freeze-thaw cycles. These study findings supported the possibility that the serum C3c level was quite sensitive to the effect of repeated freeze-thawing. The serum levels of ACE could not be maintained for more than two freeze-thaw cycles. However, the serum level of UA was unchanged during six freeze-thaw cycles. There was a significant correlation between serum C3c and UA levels between analyses, which might indicate that the relative clinical evaluation for individuals who had repeated freeze-thaw cycles might be unchanged. Therefore, for future clinical studies that use stored serum samples, it would be important to ensure the reliability of stored serum analysis data and to choose appropriate serum samples from biobanks depending on the aim of studies and the kinds of biomarkers



**Figure 3.** The differences and correlation of serum angiotensin-converting enzyme (ACE) levels between any two detections that underwent six freeze-thaw cycles. The upper area shows the difference of serum angiotensin-converting enzyme (ACE) level between any two detection methods. Paired sample t-test and Wilcoxon matched pair and signed rank tests were used to compare the values.  $P < 0.05$  represented a significant difference of serum ACE level between freeze-thaw cycles. The lower area shows the correlation of serum ACE level between any two detection methods. The Pearson correlation test and the Spearman correlation test were used to analyze the correlation.  $P < 0.05$  for the significant correlation of serum ACE level between freeze-thaw cycles.

involved in studies. For example, in epidemiologic studies in which relative risk or odds ratio might be the primary and key study factor [25], cryopreserved serum samples that have undergone different freeze-thaw cycles might or might not introduce significant effects on the validity of the results, but complete information for each cryopreserved sample stored in biobanks is recommended.

Complement C3 is the main component of the complement system involved in innate and acquired immunity, and its activation is central to all the three complement pathways [26]. C3c is a main proteolytic fragment of C3 [26] and is a relatively stable conversion product of C3 compared with the short half-life of other complement system components [27]. Therefore, the concentration of C3c in serum has been used to evaluate the activation of the complement system. Yang et al. showed that the level of C3a in serum had a trend to rise slightly with increasing cycles of freeze-thaw [28]. In the present study, it was also found that the serum C3c concentration increased with the increasing cycles of freeze-thaw. Since repeated freeze-thaw could result in severe damage or dissociation in

the complement protein [29], it is possible that the above phenomenon might be caused by the existence of continuous dissociation of the complement C3 or C3b fragment after repeated freeze-thaw cycles.

The UA micromolecule, is the end-product of metabolic turnover of purine nucleotides, involved in endothelial dysfunction, oxidative stress, vasoconstriction, and platelet aggregation, and is considered to be a marker of systemic inflammation [30]. Previously published studies had focused on the association between serum UA levels and the pathogenic mechanism of various diseases, with elevated serum UA being associated with the development of microcirculatory complications, such as diabetic retinopathy [31].

Therefore, further studies to explore the effects of repeated freeze-thaw on serum UA levels and managing the storage condition of serum samples might support the accuracy of results of related studies. A previously published study on the stability of serum UA reported that the serum levels were significantly altered after the second freeze-thaw cycle, and

its concentrations per freeze-thaw cycle showed an increasing trend over time [22]. However, no significant alteration in serum UA levels after six freeze-thaw cycles was observed in the present study, although the UA concentration showed a slight, but non-significant increase with repeated freeze-thawing. Also, considering the correlation coefficient of serum UA levels between various detections of repeated freeze-thaw cycles was relatively high and stable, the serum UA level from cryopreserved samples, it is possible that serum UA measurements could be utilized even if the samples had been previously thawed several times.

As an enzyme, ACE plays an important role in many physiological and metabolic events in the human body, especially in the renin angiotensin aldosterone system (RAAS) [32]. ACE regulates the systemic and renal circulation by converting angiotensin I into the vasoconstrictor molecule angiotensin II and is also involved in cardiovascular and renal homeostasis [6]. The activity of ACE in serum is not routinely detected, and the best methods of maintaining the quality of ACE data from cryopreserved serum samples are important. The activity of the enzyme, ACE is significantly influenced by storage temperature and other factors, and in this study, all the cryopreserved serum samples were frozen at  $-80^{\circ}\text{C}$  and thawed at room temperature, and the large changes in temperatures could have a great impact on the activity of the enzyme. The influence of temperature might explain the results showing that the activity of ACE declined with the increasing cycles of freeze-thaw. Also, the serum ACE levels between various detections following repeated freeze-thaw cycles were more significantly affected than C3c and UA. However, the reagent for detecting ACE was an open-channel reagent, which was used in the Roche Cobas c702 biochemical automated analyzer in this study. To some extent, the quality of reagent might affect the measurement of serum ACE levels, and result in the worse correlation of ACE among various detections than for C3c and UA.

To shed light on the true alterations in serum biomarker levels during disease, including eye disease, the possible sources of serum detection errors must be minimized [33,34]. Previous studies have reported that various factors might result in detection errors, such as storage duration, storage temperature, and freeze-thaw cycles. The present study was designed to focus on the effect of repeated freeze-thaw on biomarkers in serum samples and to observe the trends in changes of levels of serum biomarkers during several freeze-thaw cycles, and included controlling the storage duration of adjacent freeze-thaw cycles to 24 hours, and the storage temperature to  $-80^{\circ}\text{C}$ . Based on the results of this study, the pre-analytical phase was a major source of error, it might indicate the importance of biomarker selection for analysis in cryopreserved serum samples. For detection of micromolecule biomarkers, such as UA, the cryopreserved serum samples could be used

in various studies and the effect of freeze-thaw might have less effect on the results. However, for the detection of protein biomarkers, such as C3c, cryopreserved serum samples that undergo different freeze-thaw cycles should be used and interpreted very carefully, or avoided, in clinical studies. For the measurement of enzyme biomarkers, such as ACE, fresh serum samples or cryopreserved samples with no or few freeze-thaw cycles should be used.

This study had several limitations. Other pre-analytical factors were not simultaneously evaluated in this study, which could have affected the quality of the samples and influenced the study findings. However in our previously unpublished studies, an evaluation of the effect of repeated freeze-thaw on serum samples stored in different temperatures, including  $-20^{\circ}\text{C}$ ,  $-35^{\circ}\text{C}$ , and  $-80^{\circ}\text{C}$  was undertaken. The findings of the present study indicated that for serum samples stored by freezing at different temperatures, the effect of repeated freeze-thaw on serum concentration of the same biomarker was different. In future studies, it might be possible to explore the effects of various pre-analytical factors and their interaction on the findings of biomarkers in stored serum samples. Also, in this clinical study, only three serum biomarkers were analyzed, mainly for reasons of cost. Future studies are recommended to include a greater range of serum biomarkers, classified into different types, to determine the effects of freeze-thaw and to optimize serum storage processes.

## Conclusions

The effect of repeated freeze-thaw cycles on three serum biomarkers of eye disease, the protein, complement C3c (C3c), the micromolecule, uric acid (UA), and the enzyme, angiotensin-converting enzyme (ACE), was significant although repeated freeze-thaw is recognized to be inevitable for cryopreserved serum samples in biobanks. In this study, the effect of repeated freeze-thaw on three serum biomarkers was different, which supports the importance of complete information regarding cryopreserved serum samples before analysis, including the number of freeze-thaw cycles for biobank samples. Also, the findings of this study support the view that it is important to select appropriate cryopreserved samples from biobanks depending on the aim and design of the study.

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## Conflict of interest

None.

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