Research article

Evaluation of the Effects of Freeze-Thaw Cycles on the Stability of Diabetes-Related Metabolic Biomarkers in Plasma Samples

Rebekka Gerwig[1*], Frederikke Høgh[1], Joachim Størling[2], Katja Kemp Jacobsen[3]

Corelab, Steno Diabetes Center Copenhagen, Herlev, Denmark[1].
Translational Type 1 Diabetes Research, Steno Diabetes Center Copenhagen, Herlev, Denmark[2].
Department of Technology, Faculty of Health and Technology, University College Copenhagen, Copenhagen, Denmark[3].

Background: **Repeatedly freezing and thawing of samples can affect the stability of biomarkers in plasma samples. There is a lack of studies reporting how these preanalytical factors affect the stability of diabetes-related metabolic biomarkers. This study investigated the effects of repeated freeze/thaw cycles (FTC) on the analysis of insulin, c-peptide, glucagon, total glucagon like peptide-1 (GLP-1), total glucose-dependent insulinotropic polypeptide (GIP), leptin and polypeptide YY (PYY).**

Material and Methods: **Plasma was prepared from blood samples collected from 10 healthy individuals. Each plasma sample was divided into 3 aliquots. An aliquot from each sample was analyzed immediately after preparation. The remaining aliquots were exposed to 3 and 5 repeated FTC. Samples were measured using a MESO Quickplex SQ 120 from Meso Scale Diagnostics LLC. (MSD) and the U-Plex Diabetes Combi 1 (hu) panel kit from MSD.**

Results: **The concentrations of GIP, GLP-1, insulin and PYY were statistical significantly affected by repeated FTC. After 5 FTC, the concentration of GIP was increased by 44 %, GLP-1 by 35 % and PYY by 22 %. There were no significant changes in the concentrations of glucagon, c-peptide and leptin after repeated FTC.**

Conclusions: **GIP, GLP-1 and PYY were significantly affected by repeated FTC. The concentration of these markers increased by 22-44 % with repeated FTC. Hence, repeated FTC can cause significant changes in the concentrations of the biomarkers. Our results suggest that caution should be exercised when comparing results of biomarkers between plasma samples that have been subject to FTC.**

Key words: Preanalytical, laboratory test, plasma, freezing and thawing.

Introduction

In relation to diabetes research, large-scale epidemiological studies and research projects are being carried out, collecting large amounts of human biological material. For logical reasons, all this material cannot be analyzed at the time of collection. The Clinical and Laboratory Standards Institute (CLSI) has developed guidelines for the proper handling of biological specimens and recommends that plasma specimens are frozen and thawed only once.[1] Despite this recommendation, the same

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*Corresponding author: Rebekka Gerwig, Borgmester Ib Juuls vej 83, Herlev, Denmark.
Email: rebekka.hanne.gerwig@regionh.dk

samples are often used repeatedly up to several times to recreate measurements and/or to determine additional results of multiple analytes.

Over the past decades, the expansion of biobanks has been excessive, which has led to decentralization of the biobanks due to limitations in storage capacity. A disadvantage of this is the fact that there may not be similar guidelines for how to collect and store the biobank samples across the different sectors of the biobanks. This heterogeneity between biobanks makes it difficult to properly compare various biospecimens among samples stored in different biobanks.

Forty-five percent of biobanks in Denmark do not have a Standard operating procedure (SOP) for sample handling. Based on a questionnaire sent to biobanks in Denmark, it has been found that only half of the participants thought that quality control of blood samples in biobanks would alter data quality and test results.[2] This indicates that the knowledge about how preanalytical factors such as FTC affect different biomarkers in the blood samples, is limited.

One of the initiatives to solve this issue, has been establishing the International Organization for Standardization (ISO) standard for biobanks, ISO/DIS 20387. The Danish National Biobank is following the guidelines in this document but the biobank is not yet accredited.[3] The ISO 20387 standard neither mentions the need for temperature monitoring nor contains guidelines for keeping track of how many times samples are thawed and frozen.[4] Studies have shown that repeatedly freezing and thawing of samples affect the stability of various markers in plasma and serum samples.[5,6] To date, however, the stability of the diabetes-related metabolic factors insulin, c-peptide, glucagon, GIP, GLP-1, leptin and PYY has only been investigated in a few studies and with other methods than the MSD.[7–10] These biomarkers play a major role in the prognosis, diagnosis, treatment and research of diabetes.[7,10–14] Insulin for instance, is a hormone produced by beta cells in the pancreas and promotes the absorption of glucose from the blood into the liver, fat and skeletal muscle cells.[8,9] C-peptide is a polypeptide that can be measured to distinguish between T1 and T2 diabetes.[8,15] Glucagon has the opposite effect of insulin and activates the secretion of glucose from the liver cells into the blood.[13,16]

The seven biomarkers included in this study are easily degraded in the body and are therefore very unstable. Hence, this study hypothesized that the stability of the biomarkers will be further affected by FTC.[7,17–20] The aim of this study was to investigate how repeatedly freezing and thawing of plasma samples affect the stability of insulin, c-peptide, glucagon, GLP-1, GIP, leptin and PYY. This information is very relevant for diabetes research both at Steno Diabetes Center Copenhagen (SDCC) and worldwide.

Methods and materials

Setup/specimen collection

In this study venous blood samples were collected from ten healthy employees at SDCC, nine females and one male. All test subjects volunteered and gave spoken informed consent. The ten test subjects ate a meal consisting of bread, yogurt, cheese, jam and butter 30 min before sample collection. The calorie content of the meal was 729 kcal. The program used to design the meal and its composition was LifeSum from LifeSum AB. Inspiration for the composition and calorie content of the meal was taken from previous studies.[21,22] All test subjects were fasting for a minimum 12 hours before participating in the project, due to the fact, that the biomarkers investigated are secreted in response to food intake. The participants were permitted to drink water. Non-diabetic test subjects were recruited to ensure that the response to the food intake was normal, as well as the concentrations of the seven biomarkers were not in the low area of the reference values.

Blood samples were collected in BD™ P800 Blood Collection system containing DPP4 inhibitor, from Becton Dickinson A/S by

venipuncture. The samples were placed on ice immediately after collection, and stored for a maximum of 30 minutes, before centrifugation. All samples were centrifuged at 1000 x g for 10 minutes at 5° C as recommended by MSD. Plasma was extracted and aliquoted into three Eppendorf tubes per sample. One aliquot was analyzed immediately. The second aliquot was frozen at -80° C for at least 12 hours and thawed 3 times and the third aliquot was frozen at -80° C for at least 12 hours and thawed 5 times. All samples were thawed at room temperature for 15 minutes followed by 3 hours on ice (0°C).

MESO Quickplex SQ 120

The samples were analyzed in duplicates from the same aliquot on the Meso Quickplex SQ 120 from MSD. This method uses the sandwich enzyme-linked immunosorbent assay and electro chemiluminescence principle for detection of analytes. In this project, the U-Plex Diabetes Combo (hu) panel (MSD) measuring the total levels of insulin, c-peptide, glucagon, GLP-1, GIP, leptin and PYY was used to analyze the samples. Prior to analyzing, the samples were centrifuged at 2000 x g for 3 minutes by recommendations from MSD.

For each plate analyzed, a standard curve using two different calibrators was included. The calibrators containing known concentrations of analytes were included in the MSD kit.

Data handling and statistics

Bland-Altman plots were used to compare the baseline measurements with the 3 and 5 freeze/thaw measurements for each variable. This was used to evaluate systematic errors and to see if the differences between two measurements increased or decreased with changes in marker concentrations.

Differences between the baseline test result and the test result at each FTC were examined by a 2-sided paired Wilcoxon test. Statistical significance was considered as a p-value <0,05. Data was converted into relative values (%). This was completed using the mean value for all measurements at 0 FTC as the baseline value. Then, the mean value from the relevant FTC (analysis value) was divided with the baseline value times 100 %.

$$\text{Relative value } \% = \frac{\text{Analysis result}}{\text{Baseline value}} \times 100\%$$

To assess whether the stability of the biomarkers was affected beyond the analytical variation, the results were illustrated using the mean relative value from every freeze/thaw cycle with a 95 % confidence interval (95% CI) where an analytical uncertainty of 20 % was included. The calculation was completed using the following formula:

$$95\%\ \text{CI} = \frac{Mean\ of\ relative\ analysis\ value\ \pm 1{,}96 \times \sqrt{2} \times CV\%ana}{\sqrt{\text{n}}}$$

When illustrating the results, a 5 % and 10 % bias line were included to assess the clinical significance of the results.

Results

The Bland-Altman plots (figure 1) illustrate that for most of the measured factors, several measurements were higher at 3 and/or 5 FTC as compared to the baseline (0 FTC). This was particularly evident for insulin, GIP, GLP-1, PYY and glucagon. For GIP, the higher measured concentrations were especially pronounced after 5 FTC, where nine out of ten measurements showed increased concentrations. For c-peptide and leptin there were no consistent differences between the measured values between baseline and 3/5 FTC.

The higher measured concentrations of insulin, GIP, GLP-1 and PYY after 3 and/or 5 FTC as compared to baseline were confirmed statistically significant by the Wilcoxon test. P-values showed significant differences between 0 and 3 FTC for GLP-1 (*p=0,005*), insulin (*p=0,048*) and PYY (*p=0,002*).

The test further showed significant differences between 0 and 5 FTC for GIP (*p= 0,013*), GLP-1 (p=0,048) and PYY(*p=0,019*). For GIP and GLP-1, none of the data points at 5 FTC or the total 95 % CI were within the 10 % bias

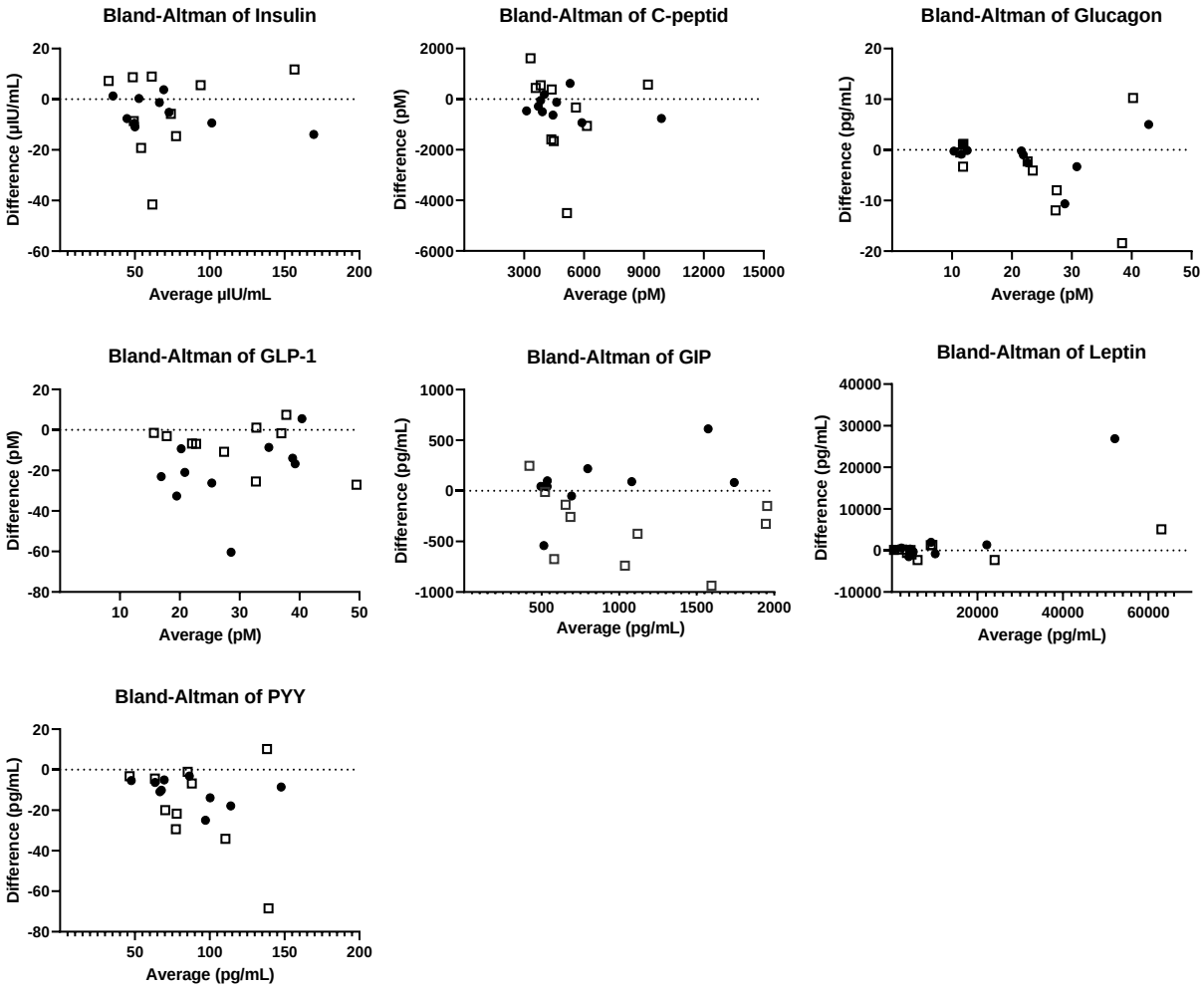

Figure 1. Bland-Altman plot for each biomarker, comparing 0 and 3, and 0 and 5 FTC's. Black circles marks `baseline – 3 FTC's and clear squares marks `baseline – 5 FTC's. The Y-axis shows the difference between the two paired measurements (0-3 or 0-5) and the x-axis represents the average of these measurements.

line, confirming that FTC affect the measured values beyond simple analytical variation. For GLP-1, the 95 % CI was
not within the 5 % bias line after 3 FTC, and for PYY the 95 % CI was not within the 5 % bias line after 5 FTC (figure 2).

Discussion

In this study, the effect of repeated FTC on the measurement of 7 metabolic plasma markers was evaluated using the MSD multiplexing technology. It was found that the measured concentrations of insulin, GIP, GLP-1 and PYY significantly increased as a result of FTC. For glucagon (p=0,23 + 0,16), c-peptide (p=0,08 + 0,62) and leptin (p=0,32 + 0,92) the results showed a tendency in increase in concentration due to multiple FTC, but this did not reach statistical significance.

When evaluating the performance of analysis in clinical practice, the observed bias should be compared to the desirable allowed bias.[23]

Data from Westgard shows that the desirable specification for inaccuracy (B%) for serum c-peptide is 7,1% and for serum insulin it is 15,5%. For the remaining analytes included in this study, B% was not available from

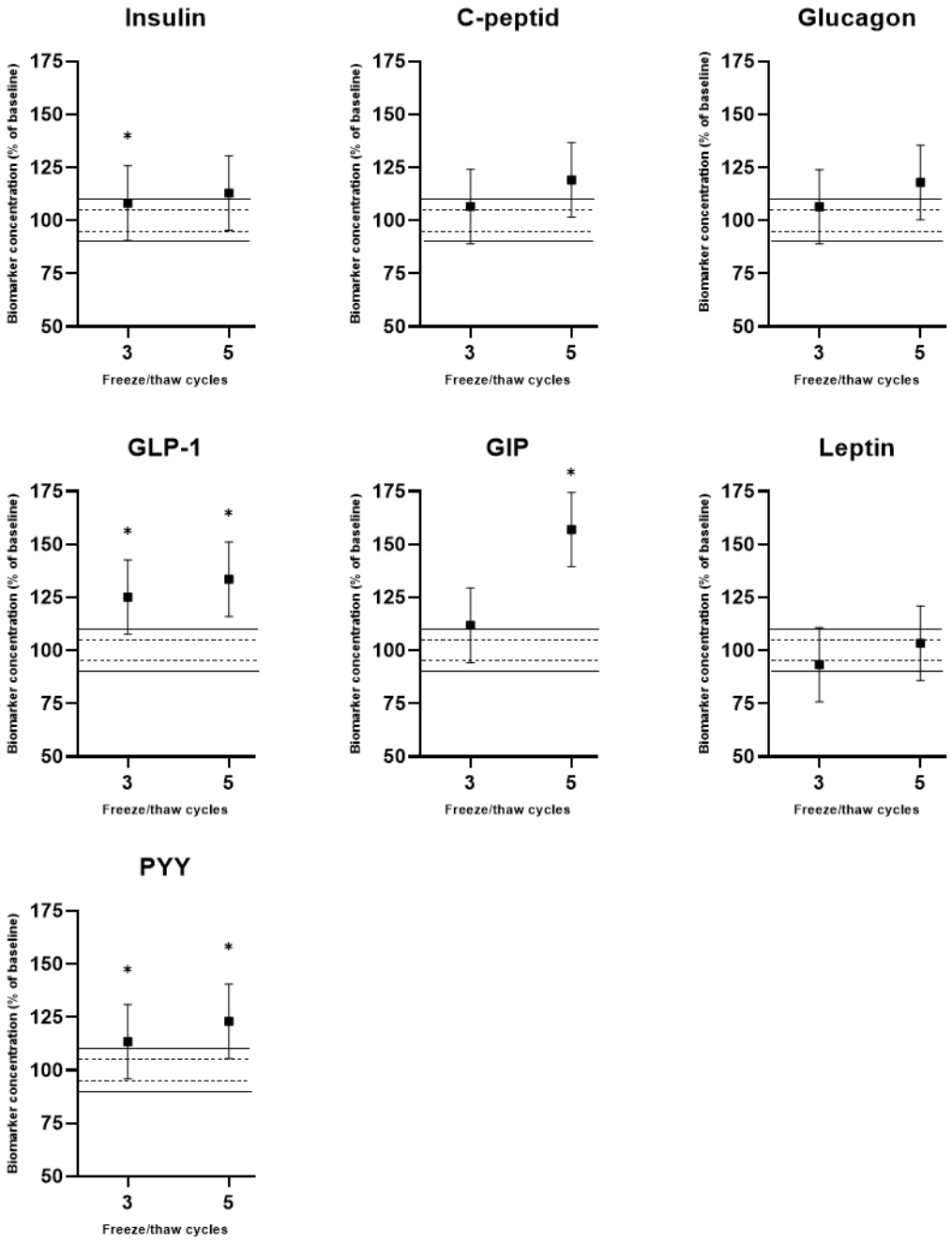

Figure 2. The change in concentration illustrated as means with 95%CI of the 7 biomarkers after 3 and 5 FTC's. The y-axis represents the relative values with 100 % being the baseline value. The dotted line shows 5 % bias limit. The black line shows 10 % bias limit.

95% CI was calculated as $\frac{Mean\ of\ relative\ analysis\ value \pm 1{,}96 \times \sqrt{2} \times CV\%ana}{\sqrt{n}}$

* = p<0,05.

Westgard. Further, the biological variation of the analytes were not available and we were not able to calculate the B% using the formula from Westgard.[23] At SDCC biochemical validated analyses has a maximum inaccuracy/bias between 5-10%. With all this in mind, 5 % and 10 % bias was used when illustrating the results.

For insulin, glucagon, c-peptide, and leptin it is unclear whether the changes in concentrations of the measured biomarkers are due to repeated freezing and thawing of the samples or analytical errors, since the 95 % CI are only partially within the bias lines. If the maximum allowed analytical error (CV% ana) was lower, the range of the 95 % CI would

decrease and might alter the reliability of the results. However, the 95 % CI of GLP-1 and GIP were not within the 10 or 5 % bias line after 5 FTC and the 95 % CI was not within the 5 % bias line after 3 FTC for GLP-1 and after 5 FTC for PYY. This indicates that FTC affects the concentration of these 3 biomarkers beyond analytical variation. A limitation in this study is the sample size of only ten. If the sample size were bigger, the 95 % CI might also have been smaller and thereby the statistical power of the results more robust.

The results show that GLP-1 concentrations in plasma are significantly affected by 3 (p=0,005) and 5 (p=0,048) FTC and GIP is significantly affected after 5 FTC (p=0,013). For both of these biomarkers the concentration increased by approximately 35% following FTC. Insulin was significantly affected after 3 FTC (p=0,048) and PYY after 3 (p=0,0002) and 5 (p=0,019) FTC. A study measured twenty-seven cytokines in plasma after 6 FTC and found that IL-1B concentration decreased and CCL5 concentrations increased, both changes being statistically significant.[24] Another study by Chen et. al used LC-MS method and $^{12}C/^{13}C$ – dansyl-chloride labeling of several metabolites when evaluating the effects of FTC. The results from this study showed a great difference in changing patterns as the FTC increased for each metabolite. Some concentrations decreased and some increased.[25] A possible mechanism being responsible for the increase in concentration of the metabolites could be the degradation of plasma proteins due to the number of FTC. Hoshiyama et. al found that GIP binds to albumin, IgG and transferrin, all plasma proteins found in blood.[26] When the blood samples are subjected to repeated FTC these proteins could degrade, and thereby release more GIP, which will result in an increase in concentration of the biomarker in the sample. Today many studies are investigating the importance of GIP and GLP-1 in diabetes and how these biomarkers can be used in the diagnostic phase and treatment of diabetes. If the samples used in a study are frozen 5 or more times, the results could be misinterpreted and would not reflect the true concentration of GLP-1 in the sample. This would cause poor reproducibility and development of a treatment based on wrong results. Overall, the quality of the samples can be altered by repeated FTC and this can greatly affect the validity and reliability of research studies.

As shown both in this and in many other studies, different biomarkers are affected differently by preanalytical variables.[24,27] This is especially problematic when samples are collected and stored in population-based biobanks, where the samples are used for multiple different purposes and studies, investigating a wide range of (patho) physiological conditions and diseases. The evolution of multiplex analyses like the one from MSD used in this study, also increases the need for knowledge about how the different preanalytical variables affect different biomarkers.

Ensuring that samples are not affected at all by any preanalytical variables is very difficult. It should be standard procedure to track and document every variable the sample is subject to. This can accomplished by using the Standard Pre-analytical Code (SPREC) system. This system makes it easy to document how samples have been handled from collection. It includes preanalytical values such as type of sample, primary container, delay of centrifugation and long term storage conditions.[28] Implementing this system in every biobank, will make it easier for scientists to select the right samples for their studies. However, the SPREC system fails to document the of number of freeze/thaw cycles. Scientists should also improve the documentation of the condition and quality of the samples used for their research and be very transparent in this area.

Conclusion

The results in this study underline that repeated FTC of plasma samples can affect the measured concentrations of protein markers including the total concentrations of PYY and

the incretin hormones GIP and GLP-1. The results show that repeatedly freezing and thawing of samples can cause a statistically significant difference in the results, but not necessarily have a clinical significance. The study emphasizes the need for precaution when repeatedly thawing and freezing samples meant for measuring PYY, GIP and GLP-1. Future studies should include a larger sample size, and from both non-diabetic and diabetic patients in order to cover a larger range in measurements.

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