Fig. 1. Dose–response relationships of plasma holoTC and serum cobalamin with the metabolic markers plasma MMA and plasma tHcy, according to OC use.

The curves were obtained by additive gaussian generalized models. The models include age, serum creatinine, and smoking. The *solid lines* are the estimated dose–response curves, and the *shaded areas* are the 95% CIs. The *P* values indicate the significance of the smooth terms.

in OC users (Fig. 1; also see Table 2 in the online Data Supplement).

In HRT users, we found no significant differences in serum cobalamin or in plasma holoTC, MMA, and tHcy compared with controls. To our knowledge, there are currently no published data on the effects of HRT on plasma holoTC, and studies on serum cobalamin have not established a clear pattern *(4, 5)*. We observed that HRT use decreased the risk of having plasma MMA in the highest quartile but was not associated with significant changes in plasma MMA (Table 1). In another study investigating postmenopausal women >60 years, HRT users had significantly lower plasma MMA than nonusers *(5)*. Oral HRT lowered *(20)* or did not affect plasma tHcy *(4)*. Our findings indicate at least that use of HRT has no negative effect on cobalamin status.

In conclusion, both serum cobalamin and holoTC are lower by 25% in younger women taking OCs, but this is not associated with significantly higher concentrations of the metabolic markers of impaired cobalamin status, plasma MMA and plasma tHcy. This may suggest redistribution rather than depletion of intracellular cobalamin. Such hormonal effects may weaken the diagnostic utility of total cobalamin and holoTC. Further studies are warranted to decide whether OC users with marginal cobalamin status are prone to develop cobalamin deficiency. HRT use had no noticeable effect on circulating cobalamin, holoTC, or the metabolic markers.

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Effects of Preanalytical Factors on the Molecular Size of Cell-Free DNA in Blood, *K.C. Allen Chan,[1] Sze-Wan Yeung,[2] Wing-Bong Lui,[1] Timothy H. Rainer,[2] and Y.M. Dennis Lo[1*]* ([1] Department of Chemical Pathology and [2] Accident and Emergency Medicine Academic Unit, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region, China; * address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region, China; fax 852-2194-6171, e-mail loym@cuhk.edu.hk)

Recently, plasma DNA analysis has been increasingly explored for different clinical diagnostic purposes. Previous studies have shown that the concentration of plasma

DNA is increased in patients with neoplastic diseases *(1)*, pregnancy-related complications *(2, 3)*, trauma *(4)*, and certain autoimmune diseases *(5)*. In addition to quantitative analysis, qualitative changes in plasma DNA have also been investigated in different physiologic and pathologic conditions. In this regard, our group has shown that fetal DNA is shorter than the maternal counterpart in the plasma of pregnant women *(6)*, and this has allowed the enrichment of fetal DNA from maternal plasma by size fractionation *(7)*. It has also been shown that plasma DNA fragments are longer in patients suffering from a variety of neoplastic diseases *(8)*. In addition, circulating Epstein–Barr virus DNA molecules in patients with nasopharyngeal carcinoma have been found to consist mainly of short DNA fragments <180 bp in size *(9)*. Because the samples collected from patients and healthy controls in these studies were usually collected at different time points and under different conditions, it is important to ensure that the observed variations in the sizes of plasma DNA are the result of biological changes rather than artifacts attributable to differences in sample handling. Therefore, in this study, we investigated the effects of several preanalytical factors, including clotting, delayed separation of blood cells from plasma, freezing–thawing, and storage, on the integrity of circulating cell-free DNA.

We recruited 27 healthy volunteers for modules 1 to 4 of the study. Venous blood (30 mL) was collected from each volunteer into eight tubes containing EDTA and one plain tube. The samples serving as references were processed immediately after collection. Plasma was separated from the blood cells by centrifugation at 1600*g* for 10 min, and the plasma was then microcentrifuged at 16 000*g* for 10 min to ensure the complete removal of cells. DNA was extracted from the plasma samples with use of the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's recommendations and was stored at −80 °C until analysis.

Module 1. To evaluate the effect of clotting, blood collected in the plain tubes was allowed to stand at room temperature for 30 min to allow clotting. Serum was then separated from the blood cells by centrifugation and microcentrifugation as described above. DNA was extracted from the serum samples immediately and stored at −80 °C until analysis.

Module 2. To evaluate the effect of delayed separation of plasma from blood cells, we stored the blood samples in the EDTA tubes at either 4 °C or room temperature for 6 or 24 h after collection. Plasma was then isolated from the blood samples, and DNA was extracted as described above.

Module 3. To evaluate the effect of freezing and thawing of plasma samples, we stored the plasma samples at −80 °C for 24 h and then allowed them to thaw at room temperature for 30 min. For evaluation of the effect of one freeze–thaw cycle, after the samples had thawed, we extracted the DNA and stored it at −80 °C until analysis. For evaluation of the effect of three freeze–thaw cycles, we repeated the freeze-thaw cycle two more times before DNA extraction.

Module 4. To evaluate the effect of storage of plasma samples, we separated plasma from the blood cells immediately after blood collection and stored it at −80 °C for 2 weeks before DNA extraction.

Module 5. To evaluate the effect of freezing and thawing of extracted DNA, we took 10 mL of venous blood from each of nine healthy volunteers, extracted the DNA from the plasma, and aliquoted it into three portions. One portion was stored at −80 °C until analysis. The other portions underwent either one or three additional freeze–thaw cycles as described above.

The plasma/serum DNA concentration in each sample was determined by three real-time quantitative PCR assays targeting the *leptin* gene. The amplicon sizes of the three real-time PCR assays were 105, 201, and 356 bp, respectively. The sequences of the primers and the probe and the set-up of the reactions have been described previously *(6)*.

For modules 1 to 4, samples from the same individual but subjected to different preanalytical conditions were analyzed in the same batch to avoid interanalysis variation. For module 4, all samples were analyzed in the same batch.

The concentrations measured in the 201- or 356-bp assays were compared with those measured by the 105-bp assay and expressed as the 201/105 and 356/105 ratios. An increase in these ratios indicates an increase in the observed size of plasma DNA, whereas a decrease in these ratios indicates the reverse.

The plasma/serum DNA concentrations for different handling protocols were compared by the Friedman test, followed by post hoc comparison with the control group with the Dunn method if the *P* value for the Friedman analysis was <0.05. The analyses were performed with SigmaStat Ver. 3.0 software.

The plasma/serum DNA concentrations measured by the 105-bp assay are shown in Fig. 1A. The median DNA concentrations for the serum samples and the fresh plasma samples were 975 and 600 copies/mL, respectively. The difference between these concentrations was statistically significant ($P < 0.05$). The increased serum DNA concentration suggests that additional DNA may be released from leukocytes into the serum during clotting. The plasma DNA concentrations were also significantly increased when the whole-blood samples were stored for 24 h at room temperature or at 4 °C. The median concentrations for the two groups were 1000 and 750 copies/mL, respectively. On the other hand, delayed separation of blood cells for up to 6 h, freezing and thawing of plasma samples up to three times, and storage of plasma at −80 °C for 2 weeks did not significantly affect the plasma DNA concentrations.

The 201/105 ratios of plasma/serum DNA for different preanalytical conditions are shown in Fig. 1B. The principle of the determination of the size of plasma DNA has been described previously *(9)*. The observation that assays with longer amplicons would give lower concentra-

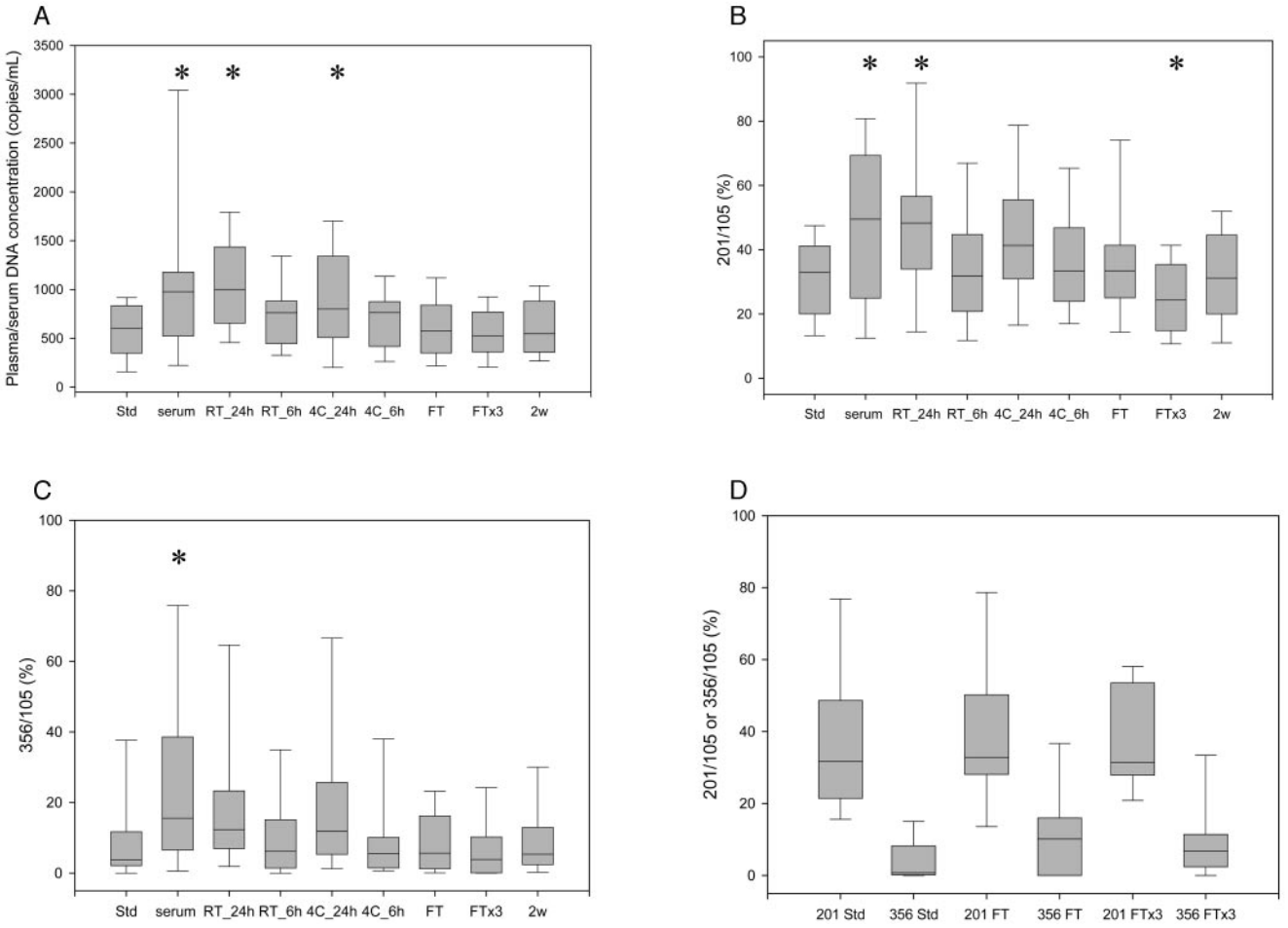

Fig. 1. Effect of various preanalytical conditions on plasma/serum DNA.

(*A*), plasma/serum DNA concentrations determined by the 105-bp assay. DNA was extracted from 800 μL of plasma/serum sample and eluted with 50 μL of water, and 5 μL of the eluted DNA solution was used as the template of each PCR reaction. Thus, the plasma DNA concentration was calculated by multiplying the DNA copy number per PCR by a factor of 12.5. For example, 1 copy/reaction is equivalent to 12.5 copies/mL of plasma/serum DNA. (*B*), 201/105 ratios of plasma/serum DNA. (*C*), 356/105 ratio of plasma/serum DNA. The *line* in the *middle* of each *box* represents the 50th percentile, and the *upper* and *lower limits* of each *box* represent the 75th and 25th percentiles, respectively. The *upper* and *lower error bars* represent the 90th and 10th percentiles, respectively. *Std*, plasma samples processed immediately after collection; *serum*, serum samples; *RT_24h*, plasma harvested after storage of whole blood at room temperature for 24 h; *RT_6h*, plasma harvested after storage of whole blood at room temperature for 6 h; *4C_24h*, plasma harvested after storage of whole blood at 4 °C for 24 h; *4C_6h*, plasma harvested after storage of whole blood at 4 °C for 6 h; *FT*, plasma samples subjected to one cycle of freezing and thawing; *FTx3*, plasma samples subjected to three cycles of freezing and thawing; *2w*, plasma samples stored at −80 °C for 2 weeks. *, significant difference compared with the standard group. (*D*), effects of repeated freezing and thawing on extracted DNA. *201*, fractional concentrations obtained by dividing the concentration of the 201-bp assay by that of the 105-bp assay; *356*, fractional concentrations obtained by dividing the concentration of the 356-bp assay by that of the 105-bp assay. There were no significant differences in the 201-bp or the 356-bp fractional concentrations among the three groups.

tions is unlikely to be attributable to the discrepancy in PCR efficiencies *(9)*. The median 201/105 ratios for the serum samples and the immediately handled plasma samples were 50% and 33%, respectively. The difference between these median ratios was statistically significant ($P < 0.05$). The median 201/105 ratios of samples stored for 24 h at room temperature and at 4 °C were 48% and 41%, respectively. The 201/105 ratio for samples stored at room temperature was significantly increased. A possible explanation for the increase in DNA size in serum and plasma samples processed after 24 h would be the release of high-molecular-weight DNA from leukocytes during clotting and prolonged storage. On the other hand, when the plasma samples were frozen and thawed three times, the 201/105 ratio was significantly decreased to 24%. This decrease in the 201/105 ratio indicates that the plasma DNA may become fragmented during repeated freezing and thawing of plasma. In contrast, delayed separation of plasma from blood cells for up to 6 h, freezing and thawing of plasma samples once, and storage of plasma at −80 °C for 2 weeks did not significantly affect the fractional concentration.

The 356/105 ratios for serum/plasma DNA are shown in Fig. 1C. The median 356/105 ratio for freshly prepared plasma samples was 12%, and that of the serum samples was 26%. The difference between these ratios was statistically significant ($P < 0.05$). Although the median 356/105 ratios for samples stored for 24 h at room temperature and

at 4 °C increased to 21% and 20%, respectively, the differences did not reach statistical significance ($P > 0.05$). There was also no significant difference in the 356/105 ratios for samples subjected to other preanalytical conditions.

Because we have shown that repeated freezing and thawing of plasma samples would affect the integrity of plasma DNA, it is logical to investigate whether freezing and thawing of extracted DNA would also lead to fragmentation of DNA. The 201/105 and 356/105 ratios for plasma DNA subjected to one and three cycles of additional freezing and thawing of extracted DNA are shown in Fig. 1D. There was no significant change in DNA concentration when the extracted DNA was frozen and thawed up to three times.

In this study, we have shown that clotting and delayed separation of plasma from blood cells for 24 h significantly increases the concentration and observed size of cell-free DNA in blood samples. Moreover, we have also shown that repeated freezing and thawing of plasma samples, but not extracted DNA, leads to fragmentation of DNA. Therefore, blood samples collected for investigation of the integrity of circulating DNA should be handled within 6 h after collection. The harvested plasma should be aliquoted into smaller portions to avoid repeated freezing and thawing of samples. Alternatively, DNA can be extracted from the plasma samples for storage because DNA appears to be more resistant to fragmentation when stored in DNA extraction solution than in plasma.

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Biochip for K-*ras* Mutation Screening in Ovarian Cancer, *Gerhild Fabjani,[1,2] Gernot Kriegshaeuser,[3] Andreas Schuetz,[4] Lothar Prix,[4] and Robert Zeillinger[1,3*]* ([1] Department of Obstetrics and Gynecology, Division of Gynecology, Medical University of Vienna, Vienna, Austria; [2] Ludwig Boltzmann Institute for Gynecology and Gynecologic Oncology, Vienna, Austria; [3] ViennaLab Labordiagnostika GmbH, Vienna, Austria; [4] Biofocus GmbH, Recklinghausen, Germany; * address correspondence to this author at: Department of Obstetrics and Gynecology, Division of Gynecology, Medical University of Vienna, Vienna, Austria, Waehringer Guertel 18-20, 1090 Vienna, Austria; fax 43-1-40400-7832, e-mail robert.zeillinger@meduniwien.ac.at)

Ovarian carcinoma is the fifth most common female cancer type and the most common cause of death from gynecologic malignancies in the Western world *(1)*. The three members of the *ras* gene family, H-*ras*, K-*ras*, and N-*ras*, are among the most common oncogenes associated with human neoplasms *(2)*. Mutations in the K-*ras* gene are frequently found in malignant neoplasms: 90% of adenocarcinomas of the pancreas; 50% of colon, 30% of lung, and 50% of thyroid tumors; and 30% of myeloid leukemia cases, respectively *(3)*. K-*ras*-activating mutations occur in codons 12 and 13 and seldom in codon 61, and lead to constitutive activation of the protein by increasing GDP/GTP exchange or decreasing GTPase activity of the protein, thus leading to increased cell proliferation.

K-*ras* mutation frequencies seem to be highly related to tumor histology. In general, K-*ras* mutations occur more frequently in mucinous tumors, including borderline malignancies, than in nonmucinous tumors such as serous carcinomas *(4–8)*. K-*ras* mutations are more common in borderline serous tumors than in serous carcinomas, suggesting distinct etiologies *(5, 9–11)*.

A biochip application for detection of the 10 most common mutations of K-*ras* codons 12 and 13 *(12)* combines mutant-enriched amplification with a highly specific hybridization protocol. The chip appears suitable for the detection of K-*ras* mutations in human feces *(12)*. An improved biochip platform, called GeneStiX (ViennaLab Labordiagnostika GmbH), is designed to meet the needs of molecular diagnostic applications. Up to 400 different DNA capture oligonucleotides can be immobilized on the tip of a special plastic stick contained in a cylindrical tube. This allows hybridization with low volumes in a closed system (tube) and the use of standard laboratory equipment, such as a thermoshaker (Fig. 1).

To evaluate the compatibility of the GeneStiX system with rapid mutation screening in tumor tissue, we analyzed ovarian tumor specimens for the presence of variations in the K-*ras* gene. We did not study K-*ras* codon 61 mutations because of their reported low frequency in ovarian carcinomas *(13–15)*.

We collected 85 ovarian tumor specimens from patients seen at the Department of Obstetrics and Gynecology at