Rivard et al. (1) are not comparable to those reported by Walker et al. (7), especially because the latter group did not measure Hcy in women after delivery. Malinow et al. (8) hypothesized in their publication that Hcy, an amino acid that is not a constituent of proteins, crosses the maternal-placental-fetal interphases and is taken up by the fetus. To test this hypothesis, Malinow et al. (8) determined the concentration of plasma Hcy in the maternal vein and neonatal umbilical vessels at the time of delivery. Their findings demonstrated a progressive decrease in the concentration of plasma Hcy going from the maternal vein to the umbilical vein and to the umbilical artery. Their data support the hypothesis that Hcy is sequestered by the fetus.

In my view this study had a proper design for the time of specimen collection. They sampled the blood at the time of delivery but not 48 h postpartum. Pagan et al. (9) studied serum Hcy in smoking and non-smoking pregnant women (18–30 weeks of gestation only). They found that the mean (SD) Hcy in smokers [5.7 (3.4) \( \mu \text{mol/L} \)] was not different from that in the nonsmokers [4.9 (1.6) \( \mu \text{mol/L} \)]. Interestingly, the difference in Hcy concentrations between the two groups was almost the same as the one reported by Infante-Rivard et al. (1); however, Pagan et al. (9) did not report the difference as significant.

In conclusion, additional data are required to support the unexpected relationship observed by Infante-Rivard et al. (1) between plasma Hcy and intrauterine growth restriction. In addition, the roles of vitamin B$_{12}$ and folic acid should also be investigated.

References


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Drs. Infante-Rivard and Rivard respond:

To the Editor:

In his letter, Moridani raises a few points about our most recent report (1) on the relationship between maternal and newborn plasma total homocysteine (tHcy) and intrauterine growth restriction (IUGR), and about a previous report showing perinatal references values for tHcy (2). It is not clear to us why, as stated by Moridani, “specimen collection at various points during pregnancy . . . [T]o determine whether the difference between the obtained tHcy values is meaningful and statistically significant” is more “proper” than what we have done. Our comparison between cases and controls, whose measures of tHcy were taken at the same time, is completely proper and valid. Our conclusions were about perinatal measures of tHcy; if others want to measure and compare tHcy at other times during, or even before, pregnancy, that is another matter. Validity of results has nothing to do with timing of measurements but with quality of measures and appropriateness of the comparisons. Our study meets both.

Another point of Moridani’s letter is confusing to us. It seems to oppose the description of means, or of data, with the notion of “individuals at risk”. As a simple descriptive analysis, Table 2 of our report (1) shows mean tHcy values and their confidence intervals. For the comparison of means, elementary statistical theory informs us that confidence intervals that do not overlap are equivalent to a statistically significant difference. As stated in our report, the mean tHcy was different between case and control mothers, but not between case and control newborns. However, the study’s main objective was not to compare tHcy between cases and controls, but to determine whether tHcy is a risk factor for IUGR, accounting for established IUGR risk factors. Moridani seems to have missed this perspective. Our statistical analysis, in which we used unconditional logistic regression and linear regression, which may not be familiar to the author, was completely appropriate for the study’s goal. A box plot is not, to our knowledge, a way to estimate risk, whereas contribution to the probability of disease, i.e., risk, is readily estimated by odds ratios in a multivariable model.

Finally, Moridani proposes an apparently new mechanism for our findings, which relates to a better diet, to be studied along with weight gain during pregnancy. We have already shown in our previous report that a better diet (folate-rich foods) reduces the tHcy concentration (2), and in the present study (1), we measured and adjusted for weight gain during pregnancy. Although Moridani’s suggestion does not seem fruitful, we agree that additional data are required to support the observation of an inverse relationship between tHcy and IUGR, including the roles of vitamin B$_{12}$ and folic acid.
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Time Is an Important Factor when Processing Samples for the Detection of Disseminated Tumor Cells in Blood/Bone Marrow by Reverse Transcription-PCR

To the Editor:

Despite histopathologic evidence of tumor-free margins, many breast cancer patients suffer from recurrence of their disease. This may reflect the presence of disseminated, premetastatic tumor cells at the time of initial diagnosis and therapy. The detection of disseminated tumor cells in bone marrow/blood has been associated with increased mortality in breast cancer patients (1). Reverse transcription-PCR (RT-PCR) for cytokeratin 19 (CK19) may be a useful alternative or complement to immunocytochemical detection (2). Unfortunately, results of RT-PCR studies vary widely. The reasons for this are conceptual as well as technical.

Because RNAses in samples can degrade RNA before analysis, we measured mRNA by RT-PCR in 15 sets of four samples each that were processed at different time points after collection and addition of a known amount of MCF-7 cells. One sample was processed immediately (<5 min after blood drawing); the other three blood samples were processed after being left in their original tubes at room temperature (10 sets of samples) and on ice (5 sets of samples) for 1, 2, and 8 h, respectively, to simulate routine handling. After mRNA extraction, RT-PCR for CK19 was performed on the LightCycler® System. Primers and probes were specific for the CK19 gene (Tib Molbiol) and avoided amplification of the CK19 pseudogene (3). We used a calibration curve and the Fit Points Method of the LightCycler quantification software. Experimental details are available in the Data Supplement that accompanies the online version of this letter at http://www.clinchem.org/content/vol50/issue4/. We repeated this set-up 10 times for experiments with samples kept at room temperature and 5 times for experiments with samples kept on ice.

The apparent concentration of tumor cells decreased with increasing time of storage (Fig. 1). In 10 of 15 cases, the “1 h” samples yielded results that showed fewer or an equivalent amount of cells compared with the “0 h” results. In 12 of 15 cases, fewer cells were amplified in the “2 h” samples than in the “1 h” samples. Recovery of any cells was possible in only 4 of 15 “8 h” samples. We observed no difference in terms of recovery for samples that were kept at room temperature and those that were kept on ice at each time point.

Our results demonstrate that the time between sample collection and RNA stabilization is an important factor when the amounts of cellular RNA to be detected are small, as in the case of disseminated tumor cells. Despite our expectations, results from experiments on samples stored on ice did not differ from results for those kept at room temperature. Detection of very small amounts of specific mRNA species, i.e., at the limits of detectability, might therefore be highly sensitive to even small amounts of RNase activity.

Most research groups use enrichment methods such as density gradient centrifugation over Ficoll or epithelial antigen magnetic bead enrichment before RNA isolation and/or freezing. These are lengthy procedures, which take at least 60–90 min before cells can be added to a RNA-stabilizing environment, and

Fig. 1. Degradation over time of mRNA from MCF-7 cells isolated from 5 mL of blood. The y axis indicates the mean number of cells detected in 15 experiments (whiskers, 2SD); the x axis represents the four time points (0, 1, 2, and 8 h) at which samples were processed.