between free-hCGB protein concentrations and the detection rate for hCGB transcripts, which provides an opportunity to study the fetus at the molecular level.

This study demonstrates that tissue-specific fetal RNA sequences can be detected in maternal blood in pregnant women regardless of the genetic situation of the fetus relative to the mother.

Such noninvasive access to the gene expression pattern of fetal tissues offers new possibilities. The discovery of additional fetal RNA markers (e.g., trophoblast-produced human placental lactogen or placental growth hormones) may open new opportunities for detecting and monitoring aberrant or differentially expressed genes. Fetal RNA analysis in maternal blood may therefore provide information regarding gene expression in many physiologic and pathologic conditions, especially those involving trophoblastic tissue, even if the question of the role of such transcripts remains to be elucidated.

We are indebted to Dr. Lavergne for reviewing this manuscript.

References


Functional Hyperhomocysteinemia in Healthy Vegetarians: No Association with Advanced Glycation End Products, Markers of Protein Oxidation, or Lipid Peroxidation after Correction with Vitamin B12. Katarina Šebeková,1* Marica Krajčovičová-Kudláková,3 Pavol Blažiček,2 Vojtech Parrák,3 Reinhard Schirzel,4 and August Heidland4 (1 Institute of Preventive and Clinical Medicine, 2 Hospital of Ministry of Defense of Slovak Republic, and 3 St. Cyril and Method Hospital, 833 01 Bratislava, Slovakia; 4 University of Wuerzburg, 97070 Wuerzburg, Germany; * address correspondence to this author at: Institute of Preventive and Clinical Medicine, Limbová 14, 833 01 Bratislava, Slovakia; fax 421-2-9369-170, e-mail sebekova@upkm.sk)

Vegetarians are at risk of developing hyperhomocysteinemia (HHcy). The predominant or selective consumption of proteins of plant origin shifts homocysteine (Hcy) metabolism to the remethylation pathway (1), which requires vitamin B12 as a cofactor and methyltetrahydrofolate as a substrate. In the vegetarian diet, the intake of folic acid exceeds the recommended dietary allowance (RDA), whereas intake of vitamin B12 is inadequate or even absent (2).

HHcy represents an independent risk factor for cardiovascular disease (3). Autooxidation of Hcy produces reactive oxygen species (ROS) (4), which may stimulate lipid peroxidation and formation of advanced oxidation protein products (AOPPs) and advanced glycation end products (AGEs). Interaction of AGEs with their specific receptor, RAGE, induces formation of ROS (5). In mice, HHcy was shown to enhance the expression of RAGE (6). AGEs, AOPPs, and lipid peroxidation products are implicated in the pathogenesis of degenerative and inflammatory diseases, including atherosclerosis (7).

In vegetarians, plasma concentrations of AGEs are mildly but significantly increased compared with populations on a Western mixed diet (8). We therefore investigated (a) whether there is an association between Hcy and plasma AGE concentrations or markers of protein oxidation and lipid peroxidation, and (b) whether supplementation of vitamin B12 affects the measured analytes in vegetarians with HHcy produced by a potential vitamin B12 deficit.

The study was approved by the Institutional Ethics Board and was conducted according to the Declaration of
Helsinki. All participants gave written consent to participate.

We investigated 63 healthy vegetarians in whom HHcy had been revealed previously. The normohomocysteinemic (NHcy; Hcy <12.0 μmol/L) subgroup (with plasma folate and vitamin B12 concentrations within the appropriate reference intervals) was compared with the subgroup with functional HHcy (Hcy >12.0 μmol/L) attributable to vitamin B12 deficiency (plasma B12 <220 pmol/L) who had plasma folate and iron concentrations and blood values within the appropriate reference intervals. This subgroup was then administered intramuscular doses of vitamin B12 (Léčiva), with an initial dose of 100 μg and four 300-μg doses over the next 14 days.

Hcy (9), vitamin B12, and folate concentrations (Elecsys 2010 System; Boehringer); AGE-associated fluorescence (A330/A330 nm) (10); carboxymethyllysine (CML; competitive ELISA; Roche Diagnostics) (11, 12); AOPPs (13); and lipid conjugated dienes (CDs) (14) were measured in all participants before and in the treated group 4 weeks after the last intervention. Plasma concentrations of β-carotene (15); vitamins A (15), C (16), and E (15); thyrotropin (IRMA); triiodothyronine (RIA); and thyroxine (RIA; all assays from Immunotech) were determined. Routine chemistries were performed on a COBAS Integra 700 analyzer (Roche), and blood profiles were determined on a Sysmex KX-21 analyzer.

The participants’ nutritional regimens were evaluated by use of dietary interviews and food frequency questionnaires. The intake of vitamin B12 was determined with use of the Alimenta database (Food Research Institute).

The results of the above analyses are presented as the mean (SE) in Table 1. For statistical evaluation, we used unpaired and paired Wilcoxon tests. Regression analysis was performed. P <0.05 was considered significant.

For the whole group, plasma Hcy (by 15%) was slightly increased, whereas plasma vitamin B12, folate, and iron concentrations and the blood profiles (data not given) were within the appropriate reference intervals. Mean (SE) daily intake of vitamin B12 was 2.41 (0.11) μg (RDA = 2.0 μg/day). All participants had normal kidney and thyroid gland function (data not given). Plasma albumin and vitamin concentrations indicated a balanced diet. Stepwise multiple regression analysis revealed that plasma vitamin C, vitamin B12, and iron concentrations correlated significantly with Hcy (ANOVA, F = 12.12; P <0.0001).

NHcy vegetarians had significantly higher vitamin B12 concentrations than the HHcy group because the latter were vitamin B12-deficient. Other investigated variables did not differ significantly. Stepwise multiple regression analysis suggested that CML, vitamin B12, and folate concentrations (inverse relationship) correlated significantly with Hcy (ANOVA, F = 7.399; P <0.007).

### Table 1. Pertinent data for the group as a whole, the NHcy vegetarians, and those with HHcy attributable to vitamin B12 deficiency before and after vitamin B12 administration.a  

<table>
<thead>
<tr>
<th></th>
<th>All (n = 63)</th>
<th>NHcyb (n = 36)</th>
<th>Basal (n = 14)</th>
<th>Posttreatmentd (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, F/M</td>
<td>38/25</td>
<td>25/11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>38.5 (1.8)</td>
<td>39.8 (2.6)</td>
<td>42.1 (3.0)</td>
<td></td>
</tr>
<tr>
<td>BMIa, kg/m²</td>
<td>22.2 (0.4)</td>
<td>22.1 (0.5)</td>
<td>22.5 (1.0)</td>
<td></td>
</tr>
<tr>
<td>Time on diet, years</td>
<td>8.2 (0.6)</td>
<td>8.3 (0.8)</td>
<td>7.6 (1.0)</td>
<td></td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td>53.4 (0.4)</td>
<td>53.6 (0.5)</td>
<td>52.2 (0.7)</td>
<td></td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>96.4 (1.8)</td>
<td>95.6 (2.4)</td>
<td>101.0 (4.4)</td>
<td></td>
</tr>
<tr>
<td>Iron, μmol/L</td>
<td>17.3 (0.8)</td>
<td>16.6 (1.1)</td>
<td>17.8 (1.3)</td>
<td></td>
</tr>
<tr>
<td>Hcy, μmol/L</td>
<td>13.8 (0.8)</td>
<td>9.7 (0.3)</td>
<td>18.6 (1.6)</td>
<td></td>
</tr>
<tr>
<td>Vitamin B12, pmol/L</td>
<td>243.5 (12.5)</td>
<td>287.2 (16.7)</td>
<td>151.7 (9.7)</td>
<td>281.1 (18.3)</td>
</tr>
<tr>
<td>Folate, nmol/L</td>
<td>29.7 (1.3)</td>
<td>31.7 (1.8)</td>
<td>30.3 (2.7)</td>
<td></td>
</tr>
<tr>
<td>AGE-Fl, AU</td>
<td>213.6 (6.0)</td>
<td>218.6 (6.0)</td>
<td>231.5 (17.8)</td>
<td></td>
</tr>
<tr>
<td>CML, μg/L</td>
<td>403.5 (13.1)</td>
<td>408.3 (17.0)</td>
<td>364.5 (31.7)</td>
<td>384.8 (22.8)</td>
</tr>
<tr>
<td>Vitamin C, μmol/L</td>
<td>67.4 (2.3)</td>
<td>70.5 (3.3)</td>
<td>64.9 (4.0)</td>
<td>ND</td>
</tr>
<tr>
<td>Vitamin E, μmol/L</td>
<td>26.0 (0.8)</td>
<td>26.3 (1.2)</td>
<td>25.2 (1.30)</td>
<td>ND</td>
</tr>
<tr>
<td>Vitamin A, μmol/L</td>
<td>1.78 (0.06)</td>
<td>1.77 (0.73)</td>
<td>1.89 (0.13)</td>
<td>ND</td>
</tr>
<tr>
<td>β-Carotene, μmol/L</td>
<td>0.56 (0.05)</td>
<td>0.61 (0.06)</td>
<td>0.56 (0.12)</td>
<td>ND</td>
</tr>
<tr>
<td>AOPPs, μmol/L</td>
<td>43.9 (2.0)</td>
<td>42.3 (2.4)</td>
<td>43.7 (4.3)</td>
<td>44.2 (5.4)</td>
</tr>
<tr>
<td>CDs, A233</td>
<td>1.15 (0.06)</td>
<td>1.12 (0.07)</td>
<td>1.11 (0.13)</td>
<td>1.30 (0.16)</td>
</tr>
</tbody>
</table>

a Data are given as the mean (SE).
b Hcy <12.0 μmol/L.
c Hcy >12.0 μmol/L.
d Four weeks after treatment.
e BMI, body mass index; AGE-Fl, AGE-associated fluorescence; AU, arbitrary units; ND, not determined.

f,h,g Compared with NHcy: f P <0.01; g P <0.05.
h P <0.01 vs pretreatment values.
Hcy concentrations were >12.0 μmol/L (HHcy) in 27 of the vegetarians. HHcy was not associated with a vitamin B₁₂ deficiency in 6 of these individuals, whereas in the remaining 21, the daily intake of vitamin B₁₂ was 1.48 (0.09) μg. Seven individuals were not evaluated in the clinical study (refusal of participation or exclusion because of concomitant anemia and iron deficiency). Plasma albumin and vitamin concentrations and body mass index were within the appropriate reference intervals, thus allowing the exclusion of protein energy malnutrition as a source of HHcy.

In vegetarians with HHcy attributable to vitamin B₁₂ deficiency, HHcy was not associated with increases in plasma AGE, AOPP, or CD concentrations. However, the pretreatment Hcy concentrations did correlate with AOPP and CD concentrations (r = 0.598; P < 0.03 and r = 0.575; P < 0.03, respectively).

In individuals with a vitamin B₁₂ deficiency, plasma vitamin B₁₂ concentrations normalized and Hcy concentrations decreased to within reference values by 4 weeks after initiation of vitamin B₁₂ treatment. The higher the pretreatment Hcy value, the more profound the observed decrease (r = 0.872; P < 0.001). None of the other investigated variables was affected significantly. Posttreatment Hcy concentrations and changes in Hcy during treatment did not correlate with any of the investigated data.

In a general population on a Western mixed diet, folate deficiency represents the main risk factor for development of HHcy. Its supplementation produces only a mild decrease in Hcy (17). In long-term vegetarians, vitamin B₁₂ deficiency plays a key role in the pathogenesis of HHcy because it is lacking in the vegan diet (2). In vegans, the only sources of vitamin B₁₂ are bacteria in the lower intestinal tract. Intake of food of animal origin (milk, dairy products, and eggs) contributes to vitamin B₁₂ concentration in lacto-ovo-vegetarians. In HHcy vegetarians, the estimated intake of vitamin B₁₂ (74% of RDA) was insufficient to maintain a balance of this vitamin.

The high plasma folate observed in vegetarians might be attributable to higher folate intake. Moreover, the “methyl folate trap” might be a contributing factor. In vitamin B₁₂ deficiency, 5-methyltetrahydrofolate and folic acid may accumulate (18). Cobalamin deficiency renders folate largely biologically ineffective, although its plasma concentrations and distribution appear sufficient. In the present study, plasma folate concentrations did not differ between the NHcy and pre- and posttreatment HHcy individuals. Treatment with vitamin B₁₂ decreased Hcy by 41.8%, much more than might be expected in mild HHcy. Thus, the methyl folate trap explains the rapid and substantial correction of HHcy after vitamin B₁₂ supplementation in vegetarians with vitamin B₁₂ deficiency.

In NHcy vegetarians with sufficient plasma vitamin B₁₂ and folate, CML appeared to correlate with Hcy concentrations. This relationship is of particular interest because this chemically defined AGE results not only from the classic pathway of AGE formation via Amadori products but also from autooxidation of glucose and from lipid peroxidation (5). The direct relationship between CML and Hcy could be of interest with regard to the mild increase in circulating AGE in healthy vegetarians (8). However, the other markers associated with oxidative stress (fluorescent AGEs, AOPPs, and CDs) showed no correlation with Hcy concentrations. There are limited data available on the association of Hcy and oxidative stress in the NHcy population. Powers et al. (19) found a correlation between plasma Hcy and malondialdehyde in young men, but because a methionine-load test produced a significant increase in Hcy but not malondialdehyde, they considered it unlikely that the oxidative stress could be a direct effect of Hcy.

In contrast to NHcy vegetarians, in the subgroup with functional HHcy, Hcy correlated directly with AOPPs and CDs. These data agree with the findings of Voutilainen et al. (20), who reported an association between Hcy and F₃-isoprostanes in men with HHcy. In spite of its long duration, HHcy in our study group was not associated with increased AOPP or CD concentrations. It is conceivable that the high plasma concentrations of antioxidants in vegetarians may partially abrogate the potentially enhanced formation of ROS induced by HHcy and AGEs.

Even the short-term intervention with vitamin B₁₂ effectively normalized Hcy concentrations in vegetarians with functional HHcy. This action was not associated with a change in plasma AGE, AOPP, or CD concentrations, indicating that high Hcy concentrations in vegetarians do not support the concept of a causal link to markers of oxidative stress. It should be emphasized that long-term maintenance of normohomocysteinemia in long-term vegetarians may be achieved only by their adherence to a recommended consumption of food of animal origin or by supplementation with vitamin B₁₂ preparations sufficient to saturate the body. Otherwise, vitamin B₁₂ concentrations decrease and hyperhomocysteinemia is reestablished within the next 6 months (21).

It must be considered that the HHcy state is a reflection of metabolic events within a cell. Thus, our in vivo investigation carries limitations in that measurements in plasma may not accurately reflect intracellular oxidative modifications.

In summary, this study provides the first data indicating that, in vegetarians with functional HHcy attributable to vitamin B₁₂ deficiency, increases in Hcy may be paralleled by increases in markers of lipid peroxidation and oxidation of proteins, without their overt increase. Additional studies are needed to elucidate the relationship between Hcy and AGEs, AOPPs, and lipid oxidation products in a population on a standard Western mixed diet and with various disease states.

We wish to acknowledge support from Léčiva SK (Bratislava, Slovakia) and the Verein zur Bekämpfung der Hochdruck-und Nierenkrankheiten (Wuerzburg, Germany) as well as the excellent assistance of André Klassen in preparing the manuscript. A portion of this study was presented as a poster at the 2002 AACC Annual Meeting (Orlando, FL).
Bilirubin in Amniotic Fluid Does Not Interfere with the Abbott TDX FLM II Asssay, Rohit Carippa, Curtis A. Parvin, and Ann M. Gronowski (Department of Pathology and Immunology, Division of Laboratory Medicine, Washington University School of Medicine, 660 South Euclid Ave., Box 8118, St. Louis, MO 63110; * author for correspondence: fax 314-362-1461, e-mail gronowski@pathology.wustl.edu)

Bilirubin, a breakdown product of lysed red blood cells, is present in amniotic fluid in very small concentrations relative to serum (1). In an uncomplicated pregnancy, bilirubin in amniotic fluid peaks at ~19–22 weeks of gestation at concentrations of 1.6–1.8 mg/L (1). Rh isoimmunization and its severe manifestation, erythroblastosis fetalis, are associated with intrauterine hemolysis, which leads to increases in amniotic fluid bilirubin concentrations (2) up to 9.6 mg/L (3, 4). The assessment of fetal lung maturity (FLM) in these pregnancies is sometimes necessary, but the ability to utilize current methods in the presence of increased bilirubin concentrations is unclear. The commercial TDX FLM II assay (Abbott Laboratories), which is based on fluorescence polarization technology and the dye PC-16 (5), is the most commonly used method to assess FLM (6). Although the manufacturer does not recommend testing icteric amniotic fluid samples, nothing has been published about the nature or quantification of their interference with the TDX FLM II assay. Our objective was to examine the effect of bilirubin on TDX FLM II concentrations.

Frozen leftover amniotic fluid samples sent to the laboratory for physician-ordered FLM testing were sorted and combined posttesting to obtain six pools with FLM values of 23, 31, and 36 mg/g (immature, <39 mg/g); 44 mg/g (intermediate); and 60 and 77 mg/g (mature, >55 mg/g). Each pool contained amniotic fluid from at least four women. Amniotic fluid samples with even minimal visual evidence of hemolysis or meconium were excluded from the study. Each pool was analyzed for total bilirubin concentration on the Hitachi 747 analyzer. The analytical sensitivity for bilirubin measurements in amniotic fluid is poor on this analyzer at concentrations <1.0 mg/L (Table 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.chem.org/content/vol49/issue6/); therefore, this assay is not used routinely to measure bilirubin in amniotic fluid. This method was suitable, however, for the purpose of confirming that none of the pooled amniotic fluid samples had pre-addition bilirubin concentrations >1.0 mg/L. Human studies committee approval was obtained for this study.

A stock solution of unconjugated bilirubin (2500 µg/mL) was prepared by dissolving bilirubin (cat. no. 101018; ICN Biomedicals Inc.) in laboratory-grade dimethyl sulfoxide (DMSO; cat. no. D-5879; Sigma Chemical Company; final concentration, 40 mL/L) at alkaline pH, and then neutralizing the solution with acid. The DMSO concentration in the sample with the highest concentration of bilirubin in our experiments (15 mg/L) was 1 mL/L. Addition of 2 mL/L DMSO to amniotic fluid samples caused no change in FLM concentrations.

The stock solution of unconjugated bilirubin was added to an aliquot of pooled sample, vortex-mixed, and serially diluted to create samples with decreasing concentrations of bilirubin. The FLM concentrations of these samples and the pre-addition pool were measured in duplicate in the TDX FLM II assay. Experiments were performed on 2 separate days (three pools each day). Validation studies were performed with serum to establish that there was no matrix effect when measuring bilirubin at this concentra-