significant increase as a function of time was observed (4.9% between 15 min and 2 h in both media). The difference in tHcy measurement by FPIA between the two collection media was not significant (P > 0.05). Such an absence of difference was opposite to data obtained with HPLC in the present study and in a previous study (9). It could be explained by less selectivity of FPIA than HPLC methods.

In conclusion, tCys, tHcy, and tGSH concentrations measured with HPLC remain stable for 6 h in whole blood, whatever the anticoagulant used: EDTA at 0 °C or acidic citrate at room temperature. This latter approach is a good alternative when the use of EDTA medium collection is not possible, in particular in epidemiological surveys involving several centers. However, because of the significant differences between the mean concentrations in these two media, reference values need to be established that take into account the anticoagulant used. For CysGly, a different strategy must be defined to keep its concentration stable over the same blood storage period. The slight increase in tHcy as a function of time in both collection media that we observed only when using FPIA cannot be fully explained at the present time. The priority is thus to define optimal collection and treatment conditions and to keep them constant in an overall study.

We are indebted to the pr eclectic staff and laboratory department of the Centre de Médecine Préventive (Vandoeuvre-lès-Nancy, France) and to the participating subjects who made this study possible. We particularly wish to thank Dominique Aguillon, Maryvonne Chaussard, Chantal Laforue, and Marie-Pierre Recouvreur. We also thank Abbott Laboratories (Abbott Park, IL) for providing FPIA IMx reagents free of charge.

### Table 1. Plasma concentrations of tHcy measured by FPIA as a function of blood anticoagulant and delay times between collection and centrifugation.

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>15 min</th>
<th>2 h</th>
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<tbody>
<tr>
<td>EDTA</td>
<td>9.2 ± 3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.6 ± 3.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acidic citrate</td>
<td>9.4 ± 2.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.9 ± 2.8&lt;sup&gt;c&lt;/sup&gt;</td>
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</table>

<sup>a</sup> Mean ± SD; n = 14.<br>
<sup>b</sup> EDTA at 0 °C; acidic citrate at room temperature.<br>
<sup>c</sup> Significant medium and time effects (ANOVA for repeated measurements, statistical significance accepted at P < 0.05).

### References


### Active Ceruloplasmin in Cervicovaginal Secretions: Its Association with Term Premature Rupture of the Membranes, Mitsuharu Ogino,<sup>1</sup> Shuichi Hiymuta,<sup>3</sup> Akiko Kadota,<sup>1</sup> Yuko Io,<sup>2</sup> and Makoto Hanazono<sup>3</sup>

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Cervicovaginal secretions (CVSs) contain cytokines of maternal origin (1, 2), inflammatory cells, and proteolytic enzymes derived from leukocytes (3, 4) and act to protect the intraamniotic compartment from infectious agents that may ascend along the birth canal. CVSs have been found to contain ceruloplasmin (Cp) (5), a protein formed predominantly in the liver. The potential functions of Cp have been divided into two categories: (a) transportation of copper to tissue sites, and (b) functions such as oxidase activity of aromatic amines and serum antioxidation. In this setting, Cp acts as an extracellular scavenger of free radicals and superoxide ions and endogenously modulates inflammatory responses (6). The former function uses inactive Cp, and the latter function is carried out by active Cp. Hiymuta et al. (7), using an original ELISA method that they established, found both inactive and...
active Cp in human serum. In a preliminary screening, active Cp was shown to be present in several external body secretions, such as in tears, salivary excretions, and nasal discharge. In this study, we used this assay system to measure active Cp in CVS in term pregnancy, and we found that increased active Cp in CVS was associated with premature rupture of the membranes (PROM).

We enrolled 80 women at term pregnancy. With an informed consent from these 80 women, CVS was obtained from the cervical canal at ~36 weeks of pregnancy, using a pair of sterilized cotton swabs (Japan Medical Service) at the time of regular antepartum checking. The total volume of collected CVS was weighed, and the mean CVS collected was ~20 mg. One of the paired sterilized cotton swabs was used routinely for microbial culture. The other sterilized cotton swab was transferred to a silicon-coated polypropylene tube containing phosphate-buffered saline (1.0 mL) and rinsed vigorously. These samples were frozen and were kept at ~85 °C until the assay. A 10-μL aliquot of this solution was used for the measurement of interleukin (IL)-8 by a human IL-8 ELISA kit (Endogen). An aliquot of this solution was diluted 100-fold and was used to measure active Cp. PROM was diagnosed when a clinically apparent leakage of amniotic fluid was confirmed in the patients who arrived thinking fluid was leaking without a preceding uterine contraction. Among the 80 women enrolled, 21 women developed PROM (PROM cases) and 59 women did not develop PROM (non-PROM cases). The diagnosis of PROM was made without knowledge of the active Cp results. At the

![Fig. 1. Immunoblotting analysis of CVSs with human active Cp monoclonal antibody (A) and respective active Cp values in CVS in PROM and non-PROM cases (B).](image-url)

(A), lanes 1 and 2, CVS from pregnant women; lane 3, purified active Cp as a positive control. (B), active Cp concentrations in the CVS (μg/L) for each case of PROM (n = 21) or non-PROM (n = 59). The horizontal bars indicate the mean value in each group. ○, PROM cases in which microbial cultures of leaked amniotic fluid were positive (11 of 21). ●, non-PROM cases and PROM cases in which microbial cultures of leaked amniotic fluid were negative.
time of admission, leaked amniotic fluid was obtained from all women with PROM and was used for microbial culture.

Monoclonal antibodies were made using the method of Köhler and Milstein (8), as modified by Hiayamuta and Ito (9), who reported the details. Partially purified Cp was used as the immunogen, and the antibody titer was checked by ELISA using Cp-fixed microtiter plates. Splenocytes were fused with P3 × 63 Ag8.6.5.3 myeloma cells and then selectively cultured in HAT medium (Sigma) followed by cloning. Ascites samples (20 μL) collected from hybridomas were incubated with 20 μg of purified Cp for 30 min at 37 °C and were electrophoresed and stained for oxidase activity. Monoclonal antibody ID2 neutralized Cp oxidase activity. Antigens in the CVS were separated electrophoretically on 7.5% polyacrylamide gels, transferred onto nitrocellulose membrane, and blocked with casein solution (Snow Brand Milk). They were then incubated with peroxidase-labeled antibody ID2 to identify active Cp in the CVS.

An original method using sandwich ELISA was used to measure active Cp in the CVS. A microtiter plate (Nunc) was coated with monoclonal antibody (2 μg/well) and blocked with casein solution (300 μL/well; Snow Brand Milk). Purified Cp (as a calibrator) and 100-fold diluted samples of CVS were added to the wells of the plates, and the mixture was incubated for 90 min at 30 °C. The wells were washed three times with phosphate-buffered saline containing 5 mL/L Tween 20. Peroxidase-labeled ID2 antibody (0.02 μg/well) was added to each well, and the plates were incubated for 90 min at 30 °C. The wells were washed, and peroxidase substrate was added to each well to detect active Cp. This procedure was duplicated in the same assay, and the results were expressed as the mean concentration (μg/L) ± SD for each patient. ROC curves were constructed to determine the cutoff value for active Cp in CVS with maximal sensitivity and specificity for PROM. The assay was performed without knowledge of the patient’s status (PROM or non-PROM) or the IL-8 results.

The gestational age of sampling CVS was 36.2 ± 0.3 weeks (mean ± SD; n = 21) in the PROM cases and 36.4 ± 0.6 weeks (n = 59) in the non-PROM cases. The gestational age at delivery was 39.5 ± 1.1 weeks in the PROM cases and 39.3 ± 1.0 weeks in the non-PROM cases. Microbial culture of CVS was positive in two of the PROM cases (10%) and in six of the non-PROM cases (10%). However, microbial cultures of the leaked amniotic fluid at the time of admission were positive in 11 of 21 PROM cases (52%).

The concentration of IL-8 in the CVS varied greatly among the patients in both PROM (range, 133-6260 ng/L) and non-PROM (range, 71–8957 ng/L) cases. Active Cp in CVS was identified by Western blotting. Immunoreactive substances with anti-active Cp monoclonal antibody in CVS showed the same electrophoretic mobility as that of the purified human serum active Cp (Fig. 1A). The active Cp concentration in the CVS was 2454 ± 812 μg/L (mean ± SD; range, 1429–4656 μg/L; n = 21) in the PROM cases and 729 ± 404 μg/L (range, 178-1622 μg/L; n = 59) in the non-PROM cases. Active Cp in the CVS was significantly (P < 0.001) higher in the PROM cases than in the non-PROM cases (Table 1). The results are plotted for both PROM and non-PROM cases (Fig. 1B). A cutoff of 1420 μg/L provided a sensitivity of 100% and specificity of 95%.

Hiayamuta and Ito (9) established an original sensitive ELISA system to measure active Cp. In this study, we used this assay system to detect and measure active Cp in CVS in term pregnancy, and we found that increased active Cp in CVS was associated with term PROM. This is the first report that indicates the presence of active Cp in CVS. Furthermore, this study suggests that increased active Cp in the CVS would be a reliable predictive PROM marker. Active Cp in CVS appeared more useful than IL-8 in CVS. In addition, this method is easy and uses noninvasive sampling. From our observations of PROM cases, only 2 of 21 (10%) microbial cultures collected at the same time as the CVS sampling were positive, whereas 11 of 21 (52%) microbial cultures of the leaked amniotic fluid were positive. Although the mechanism to regulate the appearance of active Cp in CVS remains unclear, high concentrations of active Cp in CVS may be, at least in part, induced by a latent process of intraamniotic inflammation because local appearance of active Cp is considered closely linked to locally occurring inflammatory changes mediated by the cytokine network (10). Furthermore, the latent process of intraamniotic inflammation might be closely associated with the development of PROM (4).

Shimoya et al. (11) measured both IL-8 and conventional acute phase proteins in cord sera and found that IL-8 is more closely associated with PROM than other acute phase proteins. However, in this study, the concentration of IL-8 in CVS varied greatly among the PROM cases, and it was high in the CVS in non-PROM cases. The fact that IL-8 concentrations in CVS varied greatly could be explained as follows. Pregnant women at term are under preparation for delivery. Human parturition is generally started by cervical-decidual-amniochorionic activation and is completed by myometrial contraction. The first process is particularly important for the initiation of parturition and is mainly regulated by three factors: (a) stress hormones, e.g., steroids and corticotropin-releasing hormone; (b) inflammatory cytokines, e.g., IL-1β, IL-6, and tumor necrosis factor-α; and (c) decidual hemorrhage.

<table>
<thead>
<tr>
<th>Table 1. Active Cp in CVS from PROM and non-PROM cases.</th>
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<tbody>
<tr>
<td>PROM (n = 21)</td>
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<tr>
<td>GA for CVS sampling</td>
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<tr>
<td>GA for delivery</td>
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<tr>
<td>Active Cp in CVS</td>
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<td>(1429–4656)</td>
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* GA, gestational age; data are the mean weeks ± SD.
* Data are the mean μg/L ± SD. Values in parentheses are the concentration ranges of active Cp in CVS.
* P < 0.001.
(12). These factors are variably associated with the first process, and they work together to effect the amniochorionic system that is the main source for production not only of IL-8 (13) but also of various prostanoids (14, 15). Therefore, the highly variable concentrations of IL-8 in the CVS may be attributable to the extent of and/or the stages in which these factors contribute to cervical-decidual-amniochorionic activation. However, the precise mechanism is to be investigated further. Moreover, the kinds of signals involved in increasing active Cp in CVS need to be better understood.

We are indebted to Prof. Toshiko Fujii at the Department of Pharmacology and Prof. Takashi Nakamura at the 3rd Department of Internal Medicine, Teikyo University School of Medicine, for their continuous encouragement and valuable advice. We are also grateful to the paramedical staff at the Inoue Ladies’ Clinics, who cooperated with us in collecting the samples. We thank Christopher Moreby for helping us with preparing the manuscript.

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PCR-Oligonucleotide Ligation Assay from Dried Blood Spots, Eeva-Liisa Romppanen and Ilkka Mononen* (Kuopio University Hospital, Department of Clinical Chemistry, P.O. Box 1777, FIN-70211 KUOPIO, Finland; * author for correspondence: fax 358-17-173186, e-mail ilkka.mononen@messi.uku.fi)

PCR followed by oligonucleotide ligation assay (PCR-OLA) is a molecular method that can be used for the detection of nucleotide sequence variants. During the past 10 years it has been used successfully for numerous diagnostic applications (1–15). PCR-OLA is based on the enzymatic ligation of two oligonucleotides that anneal next to each other onto the PCR-amplified target DNA. Even a single-nucleotide mismatch between the oligonucleotides and the template precludes the ligation. Automated PCR-OLA on a microplate format in combination with ELISA detection technology is a powerful method for testing numerous samples (2). One bottleneck for the effective use of PCR-OLA in population screening programs has been the time-consuming and tedious preparation of DNA samples that is necessary to eliminate PCR inhibitors (16).

Dried blood spot (DBS) specimens on filter blotters are widely used for collection, storage, and shipping of blood samples in screening programs for newborns (17), and they have been used for genotypic confirmation of positive screening tests (18). PCR inhibitors can be eliminated from DBS specimens by eluting the spots (19, 20), by fixing the spots to the disk with methanol (21, 22), or by extracting DNA from the disk (23, 24). To increase the throughput of PCR-OLA, we treated DBS specimens with methanol and used the methanol-fixed samples for the PCR-OLA analysis of the major mutation (AGUFin) (25), which is responsible for Finnish-type aspartylglycosaminuria (AGU; McKusick 208400). We tested the methanol treatment on DBS specimens collected on five different types of glass fiber or filter paper blotters. The results show that PCR-OLA from methanol-treated DBSs enables rapid and reliable detection of genetic variances.

We collected EDTA blood samples from 115 Finnish individuals with unknown genotype and 20 carriers of AGUFin, making these anonymous after collection. The genotype of the AGUFin carriers was confirmed by restriction fragment length polymorphism (25). DBS specimens were prepared by dropping 10–20 μL of fresh or previously frozen (−20 °C) blood onto Merckquant® blank strips (Merck), GF/C glass microfiber filters (Whatman), BFC 180 specimen collection paper (Whatman), Schleicher & Schuell (S&S) filter paper no. 2992, or S&S filter paper no. 903. The membranes were air-dried for 2 h, followed by application of one drop of methanol (~50 μL) to the DBS area. The methanol was allowed to evaporate in a fume hood for 1 h, and the membranes were stored desiccated at room temperature until analysis. In addition, for each membrane type, 10 DBS specimens that had not been treated with methanol were analyzed by PCR-OLA as controls.

A disk 1.5 mm in diameter, representing ~0.5–1 μL of