Circulating Chemokine Levels and Miscarriage

Brian W. Whitcomb1,2, Enrique F. Schisterman1, Mark A. Klebanoff1, Mona Baumgarten2, Alice Rhoton-Vlasak3, Xiaoping Luo3, and Nasser Chegini3

1 Epidemiology Branch, Division of Epidemiology, Statistics and Prevention Research, National Institute of Child Health and Human Development, Bethesda, MD.
2 Department of Epidemiology and Preventive Medicine, School of Medicine, University of Maryland, Baltimore, MD.
3 Department of Obstetrics and Gynecology, College of Medicine, University of Florida, Gainesville, FL.

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Evidence suggests that chemokines, proteins involved in regulation of inflammation and immune response, may have a regulatory function in pregnancy. The authors hypothesized that circulating levels of chemokines are associated with increased risk of miscarriage. Serum samples were obtained from women in the Collaborative Perinatal Project cohort who had had a miscarriage (n = 439) and controls (n = 373) matched by gestational age at sample collection. Concentrations of interleukin 8, epithelial cell-derived neutrophil-activating peptide (ENA)-78, macrophage inhibitory protein (MIP)-1α, MIP-1β, monocyte chemotactic protein 1, and RANTES (regulated upon activation, normal T-cell-expressed, and secreted) were determined by multiplex assays, and values were standardized using the standard deviation among controls. Conditional logistic regression was used to model the relation between chemokine levels and risk of miscarriage. In multivariable analysis using all available data, the authors did not observe significant associations between any of the evaluated chemokines and miscarriage risk. In analyses using subsets of the study population based on the collection-outcome interval, elevated ENA-78 levels were associated with increased risk of miscarriage as the collection-outcome interval increased; the adjusted odds ratio was 1.25 (95% confidence interval: 1.04, 1.49) for samples collected more than 35 days prior to pregnancy outcome. The observation regarding ENA-78, which has roles in regulation of angiogenesis and leukocyte recruitment, suggests a possible role for this chemokine as an early indicator of miscarriage risk.

abortion, spontaneous; angiogenesis modulating agents; chemokines; chemotaxis; cytokines; inflammation; placentation; reproduction

Abbreviations: CI, confidence interval; CPP, Collaborative Perinatal Project; ENA, epithelial cell-derived neutrophil-activating peptide; IL, interleukin; MCP, monocyte chemotactic protein; MIP, macrophage inhibitory protein; RANTES, regulated upon activation, normal T-cell-expressed, and secreted.

Human reproduction is a complex process that is prone to failures. Fecundability has been estimated to be less than 30 percent (1). Estimates of the proportion of recognized pregnancies that end in miscarriage range from 15 percent to 31 percent (1–3). Several factors, including inflammatory and immune-related cytokines, have been considered as possible mediators of pregnancy loss. Emerging evidence suggests that chemokines may also be involved in determining pregnancy outcome (4–6).

Chemokines promote the response, development, and homeostasis of the immune system, encourage stem-cell survival, and can trigger chemotaxis and angiogenesis. They are divided into four subfamilies based on structure and primary amino acid sequence: CXC, CC, C, and CX3C
(7, 8). Chemokines were originally identified as products of immune and inflammatory cells; however, their expression is not limited to these cells. Expression of chemokine receptors has been observed in the placenta and decidua (4–6, 9). Chemokines like interleukin (IL)-8 and epithelial cell-derived neutrophil-activating protein (ENA)-78 have been shown to be expressed by cells of the trophoblast and the decidua, where they may be active in angiogenesis (10). The remodeling of the endometrium in pregnancy involves local accumulation of leukocytes, including natural killer cells and macrophages, probably under the influence of chemokines (11).

There have been few epidemiologic investigations of the role of chemokines in the regulation of pregnancy. Madhappan et al. (12) investigated IL-8 levels in fetal tissue samples from cases of miscarriage and observed higher levels compared with those from an elective abortion group. Other researchers have observed lower IL-8 levels or no difference in IL-8 levels when comparing miscarriage cases with normal pregnancies (13, 14). Investigations of other chemokines, including ENA-78, monocyte chemotactic protein (MCP)-1, macrophage inhibitory protein (MIP)-1α, MIP-1β, and RANTES (regulated upon activation, normal T-cell-expressed, and secreted), in pregnancy and pregnancy loss have been largely confined to laboratory studies (5, 15–19).

High circulating levels of chemokines have been observed in many pathologic processes and may serve as early indicators of adverse pregnancy outcomes. To test this hypothesis, we used serum samples obtained from women in the Collaborative Perinatal Project (CPP) cohort to evaluate levels of circulating chemokines and risk of miscarriage. The chemokines evaluated in this study included those of the CC type (MIP-1α, MIP-1β, MCP-1, and RANTES) and those of the CXC type (IL-8 and ENA-78).

**MATERIALS AND METHODS**

**Study design and population**

Study participants were derived from the CPP, a multisite US study of pregnancy and pediatric outcomes conducted from 1959 to 1974 (20). CPP participants were enrolled while seeking prenatal care. Repeat study participation was allowed, and 48,197 women representing 54,390 pregnancies were prospectively recruited for participation (21). Data on demographic factors and medical history were collected at entry into the study. Serum samples were collected at study entry and at subsequent bimonthly visits and were stored at −20°C.

Studies of miscarriage are well-known to face challenges related to the fact that many of the earliest pregnancy losses go undetected. To address this concern, we limited our case definition to clinically recognized pregnancies and used gestational age matching, which yielded similar distributions of gestational age at study entry in cases and controls. For the current study, we defined miscarriage as involuntary loss of a clinically recognized intrauterine pregnancy at less than 140 days of gestation, confirmed by review of original study records. Of the 830 women who experienced miscarriage in the CPP, 704 provided serum samples (22). When selecting cases, in order to ensure that cytokine measurements were performed on biologic specimens collected prior to the actual pregnancy loss, we excluded subjects whose serum samples had been collected less than 10 days prior to miscarriage (n = 229). Participants with unavailable serum samples (n = 36) were also excluded. After exclusions, 439 cases of miscarriage were selected for this study, each contributing a single serum sample for analysis.

**Selection of controls**

Control selection followed a nested case-control design, modified to allow for a case-crossover analysis in addition to the standard case-control comparison. Women whose pregnancies did not end in miscarriage and who had available serum samples provided during the first 18 weeks of gestation were eligible for inclusion. Control serum samples were matched to those of cases by gestational age at sample collection, most frequently corresponding to the time of entry into the CPP. This matching approach helped us to address underlying biologic variability in cytokine levels across gestation.

Some women who experienced miscarriage also had normal pregnancies in the CPP. Serum samples from normal pregnancies that were provided by women who were selected as cases were included as control samples. Inclusion of serum samples collected in miscarriage and normal pregnancy from women who experienced both allowed for a case-crossover analysis to address time-invariant confounding (23). The case-control analysis included only one pregnancy per woman—thereby including all miscarriage samples and excluding the samples from the normal pregnancies of the 86 crossover women. The case-control analysis included a single serum sample per pregnancy for 439 cases and 373 controls. The case-crossover approach depended upon the availability of multiple pregnancies per woman. This analysis included 186 serum samples from 86 pregnancies that ended in miscarriage and 100 serum samples from the control pregnancies of the same 86 women.

**Exposure assessment**

Serum chemokine levels were measured using the Fluorokine MAP Multiplex Human Cytokine Panel A detection system (R&D Systems, Inc., Minneapolis, Minnesota) and the Luminex 100IS platform (Luminex Corporation, Austin, Texas) as previously described (24). Briefly, the assays use 96-well plates with 50 μl of sera in duplicates in a sandwich approach based on enzyme-linked immunosorbent assay. The solid phase consists of fluorescent beads covalently linked with cytokine-specific monoclonal antibodies allowing capture of each cytokine and corresponding biotinylated antibody. After addition of streptavidin-phcoerythrin, intensity is measured using the Luminex 100 IS system. To evaluate assay reliability, we performed an evaluation of these assays in which we measured chemokine levels in serum samples from the CPP and in serum samples freshly collected from pregnant women seen for prenatal care at Shands Hospital at the University of Florida (Gainesville,
Potential confounders

Maternal age, race, and cigarette smoking status were considered as possible confounders based on suspected associations with miscarriage and with cytokine levels (3, 25–27). Smoking was self-reported as number of cigarettes smoked per day. Information on previous miscarriage was abstracted from medical records; however, to avoid bias related to adjustment for prior outcomes, this variable was excluded from multivariable models, as suggested by Weinberg (28) and others (29–31). For factors measured at multiple time points, values were taken from the visit concurrent with sample collection, usually corresponding to the initial visit.

Statistical analysis

Characteristics of the 812 women comprising the study sample were assessed using mean values for continuous variables and proportions for categorical variables. Relations of factors with miscarriage were evaluated using Student t tests for continuous variables and chi-squared tests for categorical variables.

To obtain standardized measures, we divided chemokine values by their standard deviation within the controls. Conditional logistic regression models matched on gestational age at sample collection were utilized to estimate unadjusted odds ratios. Multivariable models that included all chemokines with covariates were used for estimating adjusted odds ratios. Potentially confounding variables were evaluated in these multivariable models. Variables whose inclusion in the model resulted in a change of at least 10 percent in the adjusted odds ratio for any of the primary study variables were considered confounders (32). Reported adjusted odds ratios correspond to a one-standard-deviation change in the level of the biomarker. For the case-crossover analysis, conditional logistic regression matched on individual woman was used. In order to address time trends in chemokine levels in case-crossover analysis, we included gestational age at sample collection in multivariable models, in addition to maternal age.

Samples collected within 10 days of pregnancy outcome were excluded; however, it is possible that diagnosis may have followed actual loss by more than 10 days in some instances. In situations where serum specimens were collected after actual pregnancy loss but prior to diagnosis, chemokine levels may have reflected a response to the miscarriage, rather than a risk factor for it. To address this issue of timing, we created “virtual inclusion criteria”: Observations were classified into five overlapping categories—no restriction/more than 10 days (n = 812), more than 14 days (n = 723), more than 21 days (n = 632), more than 28 days (n = 568), and more than 35 days (n = 506) from sample collection to pregnancy loss—and these categories were utilized for conditional logistic regression models.

RESULTS

Main analyses

Characteristics of the study population are shown in Table 1 by case-control status. Maternal age at the time of sample provision was significantly higher among cases than among controls (27.0 years vs. 25.5 years; p < 0.001). In addition, cases were more likely than controls to have a history of three or more miscarriages (6.6 percent vs. 1.6 percent; p < 0.001). Gestational age at sample provision was equal in cases and controls by design, with an average of 10.7 weeks and a range of 5–18 weeks in both groups.

Table 2 displays the results of both the unadjusted and multivariable conditional logistic regression models of miscarriage risk. In unadjusted analyses, risk was significantly associated with higher levels of MIP-1α; the odds ratio for a one-standard-deviation increase was 1.15 (95 percent confidence interval (CI) 1.00, 1.34). Using the previously described criteria, only age was observed to be a possible confounder of the relation of interest; none of the other factors considered, including prior history of miscarriage, were observed to affect estimates for the main study variables. Multivariable models thus included terms for all six chemokines and maternal age. Estimates were similar to those from the unadjusted analyses, and none were statistically significant.

Table 3 shows the results from multivariable models with restrictions defined by the interval between sample collection and outcome. In the unrestricted study population (interval of >10 days), adjusted odds ratios greater than 1 were observed for MIP-1α and MIP-1β. However, as the interval increased, estimates for these chemokines moved closer to 1; among participants with samples provided more than 35 days prior to the event, the adjusted odds ratio for MIP-1α was 1.00 (95 percent CI: 0.89, 1.12) and that for MIP-1β was 1.02 (95 percent CI: 0.84, 1.25). Conversely, estimates moved further from the null with increasing interval for RANTES and ENA-78. Among participants with an interval greater than 35 days, the adjusted odds ratio for RANTES was 1.24 (95 percent CI: 1.02, 1.52) and the adjusted odds ratio for ENA-78 was 1.25 (95 percent CI: 1.04, 1.49).

Case-crossover analysis

The case-crossover analysis compared chemokine levels in 186 case and control pregnancies contributed by 86 women who experienced both miscarriage and normal
pregnancy in the CPP. Of the 100 control pregnancies, 63 occurred prior to the matched miscarriage, while 37 occurred subsequent to the woman’s miscarriage. As table 4 shows, in unadjusted analyses, none of the odds ratio estimates were statistically significant. All adjusted odds ratio estimates were closer to the null than the unadjusted estimates. Although some of the chemokines had elevated adjusted odds ratios, 95 percent confidence intervals were wide and all included 1.

DISCUSSION

In models that included data from all serum samples (collected more than 10 days prior to pregnancy outcome), we did not observe significant associations between circulating chemokine levels and miscarriage risk. When the study population was limited to serum samples collected more than 35 days prior to pregnancy outcome, statistically significant increased risks were observed for ENA-78 and RANTES. Odds ratio estimates for MIP-1α and MIP-1β decreased as the time between sample collection and pregnancy outcome increased. This suggests that the role these chemokines play in miscarriage is probably a consequence of unrecognized pregnancy loss. These CC chemokines are involved in macrophage recruitment and activation and direct cell-mediated immune responses. Additionally, plasma levels of MIP-1β have been shown to increase substantially with placental infection (33). Our observation of elevated levels of MIP-1β only within 14 days of report of miscarriage

<table>
<thead>
<tr>
<th>Cases (n = 439)</th>
<th>Controls (n = 373)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean maternal age (years)</td>
<td>27.0 (0.3)*</td>
<td>25.5 (0.3)</td>
</tr>
<tr>
<td>Mean maternal body mass index†</td>
<td>22.6 (0.2)</td>
<td>22.5 (0.2)</td>
</tr>
<tr>
<td>Mean gestational age at study entry (range)</td>
<td>10.3 (5–18)‡</td>
<td>10.3 (5–18)</td>
</tr>
<tr>
<td>Annual family income§</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>&lt;$1,000</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>$1,000–$2,999</td>
<td>72</td>
<td>21.8</td>
</tr>
<tr>
<td>$3,000–$4,999</td>
<td>120</td>
<td>36.4</td>
</tr>
<tr>
<td>$5,000–$6,999</td>
<td>66</td>
<td>20.0</td>
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<td>38</td>
<td>11.5</td>
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<tr>
<td>≥$9,000</td>
<td>31</td>
<td>9.4</td>
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<tr>
<td>Smoking (cigarettes/day) at study entry</td>
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<tr>
<td>0</td>
<td>202</td>
<td>50.9</td>
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<tr>
<td>1–10</td>
<td>93</td>
<td>23.4</td>
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<tr>
<td>11–20</td>
<td>72</td>
<td>18.1</td>
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<tr>
<td>≥21</td>
<td>30</td>
<td>7.6</td>
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<td>Maternal race</td>
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<tr>
<td>White</td>
<td>270</td>
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<tr>
<td>Black</td>
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<td>33.0</td>
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<td>Puerto Rican, Asian, or other</td>
<td>24</td>
<td>5.5</td>
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<tr>
<td>Prior clinically diagnosed pregnancy losses</td>
<td>&lt;0.001</td>
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<td>0</td>
<td>294</td>
<td>67.0</td>
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<td>1</td>
<td>73</td>
<td>16.6</td>
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<tr>
<td>2</td>
<td>43</td>
<td>9.8</td>
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<tr>
<td>≥3</td>
<td>29</td>
<td>6.6</td>
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</table>

* Numbers in parentheses, standard error.
† Weight (kg)/height (m)².
‡ Numbers in parentheses, range.
§ The median US family income in 1961 was $5,700.
may reflect an acute risk of pregnancy loss or might indicate an analogous inflammatory response to tissue damage and the retained abortus.

Conversely, estimates for MCP-1, RANTES, and ENA-78 increased in magnitude with increases in the interval between sample collection and pregnancy outcome. Although MCP-1, RANTES, and ENA-78 have been examined in reproductive tissues, cultured cells, and animal models, their involvement in human pregnancy is unclear. Since these cytokines are involved in recruitment of inflammatory and immune cells during embryo implantation (6), a protective effect of MCP-1 may be related to increased production and maintenance of levels of specialized uterine natural killer cells recruited during pregnancy, which constitute a large proportion of the uterine cellular population. Other β chemokines, including RANTES, MIP-1α, and MIP-1β, are also chemoattractants for natural killer cells; however, they may be less potent in this capacity than MCP-1 (34).

ENA-78 acts as a potent neutrophil chemoattractant and activator (6, 16). Neutrophil recruitment is prominent in response to infection and, as such, ENA-78 has been investigated in the context of infection. Keelan et al. (16) observed increased levels of this chemokine in fetal tissue and amniotic fluid during intraamniotic infection among women with preterm delivery. Other investigators have observed increased ENA-78 levels in circulation in response to an infectious stimulus (35). There is conflicting evidence with regard to the role of infection in miscarriage (3). Nevertheless, we cannot rule out the possibility that the increased risk of miscarriage we observed for ENA-78 is infection-related.

ENA-78 has been studied for its role in angiogenesis. The protein structure of the CXC chemokines confers an ability to bind neutrophils and to play a role in angiogenesis. In the context of rheumatoid arthritis, ENA-78 has been demonstrated to have potent angiogenic effects (10). Given the importance of angiogenesis in placenta and fetal development, one might speculate that ENA-78 regulates vascularization in normal pregnancy. While increased blood cell

### TABLE 3. Adjusted* odds ratios for miscarriage according to levels of various chemokines among women from the Collaborative Perinatal Project, according to the length of the interval between sample collection and pregnancy outcome (n = 812), 1959–1974

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>OR†</th>
<th>95% CI†</th>
<th>OR</th>
<th>95% CI</th>
<th>OR</th>
<th>95% CI</th>
<th>OR</th>
<th>95% CI</th>
<th>OR</th>
<th>95% CI</th>
<th>OR</th>
<th>95% CI</th>
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<tbody>
<tr>
<td><strong>CC type</strong></td>
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<tr>
<td>Monocyte chemotactic protein 1</td>
<td>0.92</td>
<td>0.79, 1.07</td>
<td>0.84</td>
<td>0.67, 1.06</td>
<td>0.85</td>
<td>0.68, 1.08</td>
<td>0.86</td>
<td>0.67, 1.10</td>
<td>0.87</td>
<td>0.66, 1.13</td>
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<tr>
<td>Macrophage inhibitory protein 1α</td>
<td>1.12</td>
<td>0.97, 1.30</td>
<td>1.09</td>
<td>0.93, 1.29</td>
<td>1.07</td>
<td>0.90, 1.28</td>
<td>1.12</td>
<td>0.93, 1.35</td>
<td>1.00</td>
<td>0.89, 1.12</td>
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<tr>
<td>Macrophage inhibitory protein 1β</td>
<td>1.07</td>
<td>0.94, 1.21</td>
<td>1.06</td>
<td>0.92, 1.22</td>
<td>1.02</td>
<td>0.87, 1.20</td>
<td>1.03</td>
<td>0.87, 1.23</td>
<td>1.02</td>
<td>0.84, 1.25</td>
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<tr>
<td>RANTES†</td>
<td>1.08</td>
<td>0.93, 1.25</td>
<td>1.10</td>
<td>0.94, 1.28</td>
<td>1.12</td>
<td>0.96, 1.32</td>
<td>1.18</td>
<td>0.99, 1.41</td>
<td>1.24</td>
<td>1.02, 1.52</td>
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<td><strong>CXC type</strong></td>
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<tr>
<td>Epithelial cell-derived neutrophil-activating peptide 78</td>
<td>1.07</td>
<td>0.93, 1.22</td>
<td>1.12</td>
<td>0.97, 1.29</td>
<td>1.18</td>
<td>1.02, 1.37</td>
<td>1.19</td>
<td>1.01, 1.40</td>
<td>1.25</td>
<td>1.04, 1.49</td>
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<tr>
<td>Interleukin 8</td>
<td>1.02</td>
<td>0.91, 1.14</td>
<td>1.02</td>
<td>0.89, 1.17</td>
<td>1.03</td>
<td>0.90, 1.19</td>
<td>1.02</td>
<td>0.87, 1.19</td>
<td>1.06</td>
<td>0.89, 1.25</td>
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</tbody>
</table>

* Odds ratio estimates were obtained from conditional logistic regression models. The adjusted model included all chemokines and maternal age.
† OR, odds ratio; CI, confidence interval; RANTES, regulated upon activation, normal T-cell-expressed, and secreted.

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TABLE 4. Odds ratios for miscarriage according to levels of various chemokines in a case-crossover analysis of women from the Collaborative Perinatal Project who experienced both case and control study pregnancies (n = 186), 1959–1974

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Unadjusted models</th>
<th>Adjusted model†</th>
<th>OR‡</th>
<th>95% CI‡</th>
<th>OR‡</th>
<th>95% CI‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CC type</strong></td>
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<tr>
<td>Monocyte chemotactic protein 1</td>
<td>2.60</td>
<td>0.94, 7.19</td>
<td>2.68</td>
<td>0.78, 9.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophage inhibitory protein 1α</td>
<td>5.81</td>
<td>0.91, 37.2</td>
<td>2.77</td>
<td>0.27, 28.6</td>
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<tr>
<td>Macrophage inhibitory protein 1β</td>
<td>1.45</td>
<td>0.98, 2.14</td>
<td>0.87</td>
<td>0.44, 1.69</td>
<td></td>
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<tr>
<td>RANTES†</td>
<td>0.93</td>
<td>0.66, 1.32</td>
<td>0.75</td>
<td>0.47, 1.17</td>
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<td><strong>CXC type</strong></td>
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<tr>
<td>Epithelial cell-derived neutrophil-activating peptide 78</td>
<td>1.63</td>
<td>0.80, 3.35</td>
<td>1.66</td>
<td>0.69, 3.97</td>
<td></td>
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<tr>
<td>Interleukin 8</td>
<td>1.15</td>
<td>0.88, 1.51</td>
<td>0.98</td>
<td>0.74, 1.29</td>
<td></td>
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</tbody>
</table>

* Odds ratio estimates were obtained from conditional logistic regression models.
† The adjusted model included all chemokines, maternal age, and gestational age at sample collection.
‡ OR, odds ratio; CI, confidence interval; RANTES, regulated upon activation, normal T-cell-expressed, and secreted.

Our study was limited by certain factors. One such factor was the age and storage conditions of the samples. In preliminary analyses, levels were comparable between samples from the CPP and fresh samples collected from patients in the first trimester and at term (see Appendix for details). Assay reliability, as measured by the intraclass correlation coefficient, for RANTES was high for samples from the CPP (intraclass correlation coefficient = 0.890) but lower for fresh samples taken in the first trimester (intraclass correlation coefficient = 0.235), and levels were substantially higher among samples from the main study than among samples from the preliminary study. It is unlikely that degradation of sample content would have occurred differentially between cases and controls; however, any nondifferential effects of storage on chemokine concentrations would have biased results toward the null. Additionally, because of use of the previously collected CPP data set, there was limited information available on certain variables, such as perinatal infection, which was not recorded longitudinally, and smoking status, which was self-reported. In regard to the latter, there was less of a stigma attached to smoking (which might have affected reporting) at the time the data were collected, and the accuracy of self-reported smoking in the CPP has been demonstrated previously (39). Lastly, despite the large sample size overall, some adjusted analyses, such as the sensitivity analysis and the case-crossover comparison in particular, may have been affected by small cell sizes.

We did not observe an association between miscarriage risk and circulating levels of IL-8, MCP-1, MIP-1α, MIP-1β, RANTES, and ENA-78; however, when consideration was restricted to serum samples collected more than 3 weeks prior to miscarriage, higher levels of ENA-78 were associated with miscarriage. The results suggest possible utility for this protein as a biomarker providing an early indication of risk of pregnancy failure, as well as providing a basis for future studies investigating molecular causes of miscarriage.

ACKNOWLEDGMENTS

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Statistics and Prevention Research at the National Institute of Child Health and Human Development.
Conflict of interest: none declared.

REFERENCES

Evaluation of assay reliability

Because of the age and storage conditions of serum samples from the Collaborative Perinatal Project, the authors conducted an analysis to evaluate the feasibility of their use for the current study. To this end, 20 samples obtained during the third trimester in Project women with normal pregnancies (hereafter referred to as “frozen samples”) were randomly selected for inclusion in the feasibility study. Additionally, among women seen at Shands Hospital at the University of Florida for routine prenatal-care visits, 20 samples from women in the first trimester and 20 samples from women at term were also obtained (hereafter referred to as “early fresh samples” and “term fresh samples,” respectively). The sera were collected after approval was obtained from the University of Florida institutional review board. All samples were assayed in duplicate for cytokine levels using the Fluorokine MAP Multiplex Human Cytokine Panel A detection system (R & D Systems, Inc., Minneapolis, Minnesota) and the Luminex 100IS platform (Luminex Corporation, Austin, Texas). Samples were also tested for interleukin 6 using standard enzyme-linked immunosorbent assays (R & D Systems, Inc.). Fresh and frozen samples were compared regarding the proportion of samples above the assay sensitivity limits, absolute values for each analyte, and assay reliability (test-retest agreement).

Preliminary study results

Levels of all chemokines were consistently detected in most of the samples (frozen, early fresh, and term fresh) (appendix table 1). The chemokines monocyte chemotactic protein 1, macrophage inhibitory protein (MIP)-1α, and RANTES (regulated upon activation, normal T-cell-expressed, and secreted) were detected in all samples and at similar levels in fresh and frozen samples. Levels of interleukin 8 were low in all samples and less frequently detected in term fresh samples than in early fresh samples; however, median concentrations were similar across these groups. Median concentrations of MIP-1α were also similar across groups; MIP-1α was detected in all frozen samples and in most of the term (70 percent) and early (85 percent) fresh samples. Epithelial cell-derived neutrophil-activating peptide 78 was detected in all but one term fresh sample, and levels varied between the groups. The chemokine levels observed in the frozen samples were similar to those obtained by other investigators using freshly collected serum (40).

Test-retest reliability, as measured by intraclass correlation coefficients, was above 0.95 for most analytes in all groups (appendix table 2). An exception was RANTES, which had an intraclass correlation coefficient of 0.890 in frozen samples and 0.935 in fresh term samples but only 0.235 in early fresh samples.

APPENDIX TABLE 1. Median values and interquartile ranges for serum chemokine concentrations, as determined by multiplex assays, in serum samples obtained from pregnant women from the Collaborative Perinatal Project (frozen samples) and Shands Hospital at the University of Florida (early and term samples), January 2004–January 2005

<table>
<thead>
<tr>
<th>Factor</th>
<th>Frozen samples (n = 20)</th>
<th>Term fresh samples (n = 20)</th>
<th>Early fresh samples (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Detected†</td>
<td>Median chemokine level (pg/ml)</td>
<td>IQR*</td>
</tr>
<tr>
<td>Monocyte chemotactic protein 1</td>
<td>20</td>
<td>13.5</td>
<td>6.8–22.5</td>
</tr>
<tr>
<td>Macrophage inhibitory protein 1α</td>
<td>20</td>
<td>10.4</td>
<td>6.8–19.9</td>
</tr>
<tr>
<td>Macrophage inhibitory protein 1β</td>
<td>20</td>
<td>25.1</td>
<td>19.4–42.8</td>
</tr>
<tr>
<td>RANTES*</td>
<td>20</td>
<td>5,020.0</td>
<td>3,992–6,460</td>
</tr>
<tr>
<td>Epithelial cell-derived neutrophil-activating peptide 78</td>
<td>20</td>
<td>387.2</td>
<td>210.7–618.2</td>
</tr>
<tr>
<td>Interleukin 8</td>
<td>13</td>
<td>9.3</td>
<td>0–22.1</td>
</tr>
</tbody>
</table>

* IQR, interquartile range; RANTES, regulated upon activation, normal T-cell-expressed, and secreted.
† Number of samples in which the chemokine was detected.
**APPENDIX TABLE 2.** Reliability of multiplex assays for measurement of chemokine concentrations in serum samples obtained from pregnant women from the Collaborative Perinatal Project (frozen samples) and Shands Hospital at the University of Florida (early and term samples), January 2004–January 2005

<table>
<thead>
<tr>
<th>Factor</th>
<th>Frozen samples (n = 20)</th>
<th>Term fresh samples (n = 20)</th>
<th>Early fresh samples (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICC* Detected†</td>
<td>ICC Detected</td>
<td>ICC Detected</td>
</tr>
<tr>
<td>Monocyte chemotactic protein 1</td>
<td>0.992 20</td>
<td>0.970 20</td>
<td>0.990 20</td>
</tr>
<tr>
<td>Macrophage inhibitory protein 1α</td>
<td>0.999 20</td>
<td>0.895 14</td>
<td>0.971 17</td>
</tr>
<tr>
<td>Macrophage inhibitory protein 1β</td>
<td>0.999 20</td>
<td>0.996 20</td>
<td>0.990 20</td>
</tr>
<tr>
<td>RANTES*</td>
<td>0.890 20</td>
<td>0.935 20</td>
<td>0.235 20</td>
</tr>
<tr>
<td>Epithelial cell-derived neutrophil-activating peptide 78</td>
<td>0.997 20</td>
<td>0.980 19</td>
<td>0.911 20</td>
</tr>
<tr>
<td>Interleukin 8</td>
<td>0.999 13</td>
<td>0.930 11</td>
<td>0.993 20</td>
</tr>
</tbody>
</table>

* ICC, intraclass correlation coefficient; RANTES, regulated upon activation, normal T-cell-expressed, and secreted.  
† Number of samples in which the chemokine was detected.