Assessment of Some Biomarkers Related with Recurrent Miscarriages in Iraq

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ABSTRACT
The present study was aimed to determine some of the major biomarkers among women patients with a history of recurrent miscarriages that associate with antiphospholipid syndrome (APS), in addition, to know the positive prevalence of antiphospholipid (IgG) antibodies. The study enrolled 88 participants, including 55 aborted women with a history of recurrent miscarriages (RM) in the first trimester of pregnancy, and 33 apparently healthy subjects were selected as a control group who attend the maternity and pediatrics hospital in Babylon governorate, where their ages ranged between 18 - 35 year. Sera from all participants were tested to an assessment of some parameters related to this study. It was observed that a high positive prevalence of anticardiolipin (aCL) IgG in patients which 62.5% compared with 60.2% and 52.3% for antiphospholipid autoantibodies (aPL) IgG and anti β2-glycoprotein I (antiβ2GPI) IgG respectively, also the lower ratio was 26.1% for Lupus anticoagulant (LA) IgG at a level of statistical significance less than 0.05. The data demonstrated that elevate of odds ratio [OR] to aPL IgG which reached 59.21%, while for aCL IgG, antiβ2GPI IgG, and LA IgG which 25.47%, 16.40 %, and 9.57 % respectively. The statistical results revealed that significant difference in the mean concentration of factor V Leiden (FVL) in aborted women (28.78 ± 3.62 ng/ml) when compared with apparently healthy subjects (19.66±3.22 ng/ml). The concentration of tumor necrosis factor-alpha (TNF-α) in women with recurrent miscarriages was 75.50±6.61 pg/ml, but it was 95.59±6.35 pg/ml in the control group. In addition, the level of 25 (OH) vitamin D in patients and control groups were 37.45 ± 3.92 ng/ml and 35.96 ± 3.77 ng/ml respectively with no significant differences (P = 0.082). The ROC curve was used to evaluate the prevalence of IgG for variables of APS, where the degree of accuracy ranged from good to excellent for all IgG of variables.

INTRODUCTION
Recurrent pregnancy loss (RPL) or RM, defines according to the European Society of Human Reproduction and Embryology maintains as three or more consecutive pregnancy losses, while, American Society for Reproductive Medicine defines it as two or more consecutive pregnancy losses before 24 weeks of gestation [1,2]. It was estimated that RPL occurs in 2-4% of women of childbearing age and 50% from un-explained RPL cause physical and psychological shock to pregnant women [3,4].There are many epidemiological causes for RPL such as, genetic (2-4%), anatomic (10-15%), endocrine (17 -20%), immunologic (20%), thrombotic (>15%), infections (0.5-5%), environmental, and 40-50% unresolved etiologies, as well as, raised NK cells count and autoantibodies levels in miscarriage patients cause decrease blood flow uterine during early pregnancy [5,6]. Furthermore, numerous reports indicated that a link between autoimmune response and recurrent miscarriage [7], hence, the autoimmune has a pathologic role in causing RM in pregnant women patients [8,9]. Antiphospholipid syndrome (APS, Hughes’ syndrome), is an autoimmune disease relate to thrombosis in the veins and/or arteries, RPL and fetal death in the existence of antiphospholipid autoantibodies (aPL) against the patient’s tissues through interfering with coagulation by direct reacting against phospholipid-binding proteins expressed on, or bound to, the surface of vascular endothelial cells or platelets [10,11]. Several studies have indicated that a strong relationship with the significant increase in aPL IgG antibodies, between women with RPL, that induced by proinflammatory immune response from trophoblasts and complement activation [12,13,14]. aCL IgG antibodies regarded as an important factor for RM that their high levels were associated especially in the first trimester of pregnancy [15,16]. On the other hand, some studies showed a significant increase in the level of antiβ2GPI IgG antibodies [17,18]. LA are a heterogeneous antibody associated with an increased risk factor for RPL that bind to the negatively charged phospholipids and proteins such as β2GP1 [19]. In addition, LA constitutes a risk factor for thrombosis than aCL [20,21]. Thrombophilia’s like FVL mutation is an important risk factor responsible for recurrent miscarriages, may be associated with stillbirth in the first trimester [22]. It was reported that a higher percentage of the prevalence of FVL in different populations [23]. Various cytokines secreted by CD4 T-cells may be advantageous or harmful at different stages of pregnancy; for example, TNF-α and other inflammatory cytokines appear to be critical during the implantation process and hence causes recurrent miscarriage [24]. Therefore, the aim of this study was to determine some biomarkers related to recurrent miscarriages.
**MATERIALS AND METHODS**

**Patients and control groups**
This work was conducted on women that suffering from RM in the first trimester of pregnancy, included 55 patients, while the apparently healthy was 33. The ages of patients and control groups ranged between eighteen and thirty-five years.

**Blood Samples**
Five milliliters of venous blood collected from each sample of the study, placed in tubes containing a gel, then the blood was separated by centrifuge at 3000 rpm for 15 minutes, and the remaining serum was placed in Eppendorf tubes and stored in a deep freeze at -20°C until used [25].

**Immunological assays**
aPL screen IgG and aCL IgG were estimated by ELISA kits according to the manual procedure of Aeskulisa-Company(Germany), whereas aβ2GPI IgG, LA IgG, FVL, and TNF-α were evaluated by using ELISA kits provided by Cusabio-Company (China), in addition 25 (OH) vitamin D was estimated by ELISA kit provided by Calbiotech-Company (USA).

**Statistical Analysis**
Statistical analysis was carried out using statistical package for social sciences (SPSS) statistical software for Windows version 24, and IBM SPSS statistics version 24 documentation, also MedCalc statistical software version 17.9.7. Data of all individuals were entered and analyzed with suitable statistical tests.

**RESULTS AND DISCUSSION**
Tables (1) and (2), shows a high significant increase in concentration of aCL IgG in aborted women than control group ($X^2 = 38.403, P < 0.001$). The OR in patients with a history of RM in the presence of aCL IgG was 25.47 (8.05-90.54) times more than healthy women with a highly significant difference ($P <0.001$). Moreover, the significant increase ($P<0.001$) was registered in the index value of aβ2GPI IgG ($X^2 =29.163$) for study groups, while the OR was 16.40 (5.31-50.70) with $P<0.0001$. Also, LA IgG revealed a significant increase ($X^2 =11.023, P=0.001$; OR 9.57 (2.07-44.21), $P=0.0038$) in patients compared with healthy women. In addition, the concentration of aPL screen IgG showed a significant increase in aborted women ($X^2 =51.010, P < 0.001$) in contrast to the control group.

**Table 1**: The levels of anti-phospholipid syndrome markers in women with recurrent miscarriages.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients (n=55)</th>
<th>Control (n=33)</th>
<th>Total (n=88)</th>
<th>$X^2$ Value</th>
<th>P. Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>aCL IgG U/ml</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive count (%)</td>
<td>48 (87.3%)</td>
<td>7 (12.7%)</td>
<td>55 (100.0%)</td>
<td>38.403</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>% of Total</td>
<td>54.5%</td>
<td>8.0%</td>
<td>62.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative count (%)</td>
<td>7 (21.2%)</td>
<td>26 (78.8%)</td>
<td>33 (100.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of Total</td>
<td>8.0%</td>
<td>29.5%</td>
<td>37.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>aβ2GPI IgG index value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive count (%)</td>
<td>41 (89.1%)</td>
<td>5 (10.9%)</td>
<td>46 (100.0%)</td>
<td>29.163</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>% of Total</td>
<td>46.6%</td>
<td>5.7%</td>
<td>52.3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative count (%)</td>
<td>14 (33.3%)</td>
<td>28 (66.7%)</td>
<td>42 (100.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of Total</td>
<td>15.9%</td>
<td>31.8%</td>
<td>47.7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA IgG index value</td>
<td></td>
<td></td>
<td></td>
<td>11.023</td>
<td>0.001</td>
</tr>
<tr>
<td>Positive count (%)</td>
<td>21 (91.3%)</td>
<td>2 (8.7%)</td>
<td>23 (100.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of Total</td>
<td>23.9%</td>
<td>2.3%</td>
<td>26.1%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative count (%)</td>
<td>34 (52.3%)</td>
<td>31 (47.7%)</td>
<td>65 (100.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of Total</td>
<td>39.6%</td>
<td>45.2%</td>
<td>47.9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>aPL screen IgG U/ml</strong></td>
<td></td>
<td></td>
<td></td>
<td>51.010</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Positive count (%)</td>
<td>49 (92.5%)</td>
<td>4 (7.5%)</td>
<td>53 (100.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of Total</td>
<td>55.7%</td>
<td>4.5%</td>
<td>60.2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative count (%)</td>
<td>6 (17.1%)</td>
<td>29 (82.9%)</td>
<td>35 (100.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of Total</td>
<td>6.8%</td>
<td>33.0%</td>
<td>39.8%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$x^2$: Chi square
* Significant difference at $P\leq0.05$

**Table 2**: Odds ratio and their relationship with anti-phospholipid syndrome markers.

<table>
<thead>
<tr>
<th>Variables</th>
<th>OR (95% CI)</th>
<th>P. Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>aCL IgG U/ml</strong></td>
<td>25.47 (8.05 - 80.54)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>aβ2GPI IgG index value</strong></td>
<td>16.40 (5.31-50.70)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>LA IgG index value</strong></td>
<td>9.57 (2.07-44.21)</td>
<td>0.0038</td>
</tr>
<tr>
<td><strong>aPL screen IgG U/ml</strong></td>
<td>59.21 (15.41 - 227.46)</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

CI: Confidence Interval
OR: Odds Ratio
*P≤0.05
The data pointed out that there was a significant difference (P < 0.001) in the concentration of FVL in sera of aborted women (28.78 ± 3.62 ng/ml) when compared with control group (19.66±3.22 ng/ml), also all aborted women have shown a high significance difference (P<0.001) in the level of TNF-α in comparison with the healthy women. Although, vitamin D was implicated in cases of recurrent miscarriages in pregnant women, but the results of the current study revealed no significant difference (P = 0.082) in the concentration of vitamin D between aborted women (37.45 ± 3.92 ng/ml) and control group (35.96 ± 3.77 ng/ml), as illustrated in Table (3).

Table 3: The levels of FV Leiden, TNF-α, and 25 (OH) vitamin D in aborted women and control groups.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients (n=55) Mean ± S.D.</th>
<th>Control (n=33) Mean ± S.D.</th>
<th>P. Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVL ng/ml</td>
<td>28.78 ± 3.62</td>
<td>19.66 ± 3.22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TNF-α pg/ml</td>
<td>78.50 ± 6.61</td>
<td>59.50 ± 6.35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>25 (OH) Vitamin D ng/ml</td>
<td>37.45 ± 3.92</td>
<td>35.96 ± 3.77</td>
<td>0.082</td>
</tr>
</tbody>
</table>

S.D: Standard deviation
* Significant difference at P≤0.05

The results of this study showed that a significant difference (P<0.001) for area under ROC curve (AUC) for all variables, in which the AUC of aPL screen IgG was 0.98(0.93-1.00) with sensitivity and specificity 100.00(93.5-100.0) and 87.88(71.8 - 96.6) respectively, but when compared with aβ2GPI IgG [AUC = 0.88(0.79-0.94)] the sensitivity and specificity were 92.73 (82.4-98.0) and 81.82 (64.5-93.0) respectively (Table 4). Hence, aPL screen IgG was more sensitive than aβ2GPI IgG in the diagnosis of immunological causative which associated with recurrent miscarriages, as illustrated in Figure (1). Also, aβ2GPI IgG regarded more sensitive than aCL IgG [AUC = 0.94(0.86-0.98), in which the sensitivity and specificity were 87.27(75.5-94.7) and 90.91(75.7-98.1) respectively, while for LA IgG [AUC = 0.87(0.78-0.93)], it was 83.64(71.2-92.2) and 78.79(61.1-91.0) for sensitivity and specificity respectively, therefore, it was less sensitive than all IgG for variables.

Table 4: The sensitivity and specificity of biomarkers for patients with anti-phospholipid syndrome.

<table>
<thead>
<tr>
<th>Variables</th>
<th>AUC (95% CI)</th>
<th>P. Value</th>
<th>Sensitivity (95%CI)</th>
<th>Specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCL IgG</td>
<td>0.94 (0.86-0.98)</td>
<td>&lt; 0.001</td>
<td>87.27 (75.5-94.7)</td>
<td>90.91 (75.7-98.1)</td>
</tr>
<tr>
<td>aβ2GPI IgG</td>
<td>0.88 (0.79-0.94)</td>
<td>&lt; 0.001</td>
<td>92.73 (82.4-98.0)</td>
<td>81.82 (64.5-93.0)</td>
</tr>
<tr>
<td>LA IgG</td>
<td>0.87 (0.78-0.93)</td>
<td>&lt; 0.001</td>
<td>83.64 (71.2-92.2)</td>
<td>78.79 (61.1-91.0)</td>
</tr>
<tr>
<td>aPL screen IgG</td>
<td>0.98 (0.93-1.00)</td>
<td>&lt; 0.001</td>
<td>100.00 (93.5-100.0)</td>
<td>87.88 (71.8 - 96.6)</td>
</tr>
</tbody>
</table>

AUC: Area under ROC curve
CI: Confidence Interval
ROC: Receiver Operating Characteristic
* Significant difference at P≤0.05

Figure 1: The sensitivity and specificity for IgG markers among four individuals of anti-phospholipid syndrome by using ROC curves analysis.
The results of this study indicated the presence of a significant difference between patients and control groups, in which a total prevalence of the aCL IgG in patients was 62.5%, whereas it was 37.5% in healthy women. The elevated levels of these autoantibodies may be related to the occurrence of chronic infection which initiates the autoimmune disease. These data agreed with the results by [26] during their study on women with a history of RPL, they were interpreted the significant increase in the concentrations of IgG aCL, as a result of infection for long time with treatment, leads to induce the formation of antibodies that contribute to the pathogenesis of disease through the deposition of immune complexes which trigger the autoimmune reactions. The increased level of this autoantibody may be related to a non-continuing pregnancy [27]. Also, the oxidizing agents that activate the endothelial cells, hence increasing the levels of aCL IgG, which interact with phospholipid-binding proteins or with monocytes or natural anticoagulants, like protein S and, therefore lead to a blockage of blood vessels and cause abortion [28]. The OR of the aCL IgG was 25.47%, this result was agreed with the data by [29], when studying the aCL IgG in pregnant women with a history of the RM (OR=35.1, P=0.001), while [30] recorded that 12.1% of OR was highly significance (p=0.019), by their studying of patient’s women with a history of three or more consecutive abortions in the first trimester of gestation. The total positive prevalence of aβ2GPI IgG was 52.3%, whereas 47.7% in normal pregnant women under significant difference (p=0.001). In all pathological cases, it may be a chronic infection that causes rise titer of aβ2GPI IgG. These results were in agreement with certain studies [31,18], where the last study showed that 12.8% (32/250) of women with RM, their sera were positive for aβ2GPI IgG, while it was 2.5% (5/200) in healthy women with a high significance difference. A significant increase in Tunisian’s women with more than three consecutive RM, these antibodies appear to be independent risk markers of RM [32]. Meantime, 52.5% of sera were positive for aβ2GPI (IgG) in patients group, β2GPI is the main antigenic target for anti-phospholipid autoantibodies (aβ2GPI IgG), but it has a functional role in events like the ability to interact with anticoagulant proteins (activated protein C and S) were competing in their binding with phosphatidylethanolamine and phosphatidylserine; interact with the regulatory and activator proteins of complement (C4BP and MBL) [33]. These results did not agree with the study of [34], that demonstrated no significant difference in the levels of aβ2GPI IgG between study groups, where the women positive sera for IgG with RPL was 7%, while it was 6% in control group. The current study revealed a significant increase in the total positive prevalence of LA IgG (26.1%) versus healthy women (7.3%), which consistent with the study of [35], where they observed that LA had a significant difference in the positive prevalence of LA IgG in pregnant women with a history of RPL, in comparison with control group, they interpreted the reason of this result not only attributed to the primary APS, but also other etiologies, such as viral hepatitis C, bacterial (syphilis) and parasitic infections (malaria), furthermore some different drugs like Hydralazine and Guanidine, as well as Phenytoin may be related, besides some various diseases like sickle cell disease ,autoimmune thrombocytopenic purpura and autoimmune hemolytic anemia. The ratio of LA was 26% (61/231) in women with RM [36], while it was 25.6% (21/82) in another study [37]. In Iraq, LA has associated with two hundred Baghdad women that have a history of RPL with a ratio of 13.5% (27/200) [38]. LA has been implicated in thrombosis events in the placental vessels, and the OR of LA was 9.57% with a significant increase [39], So, this result was compatible with the data of [40], in which the OR was 4.4%, while the ratio of OR was 8.7% as mention by [41]. In the current study the aPL IgG kit used to screen the presence of aCL and other types of the phospholipids like phosphatidyserine (PS), phosphatidylcholine (PC), splenomyelins (SM), phosphatidylethanolamine (PE), as well as phosphatidylinositol (PI). The results highlighted that the total positive prevalence of aPL IgG was 60.2% in aborted women, compared with 39.8% in healthy women with the presence of a significant difference (P=0.05). In all pathological cases, there may be a chronic infection that has led to elevate the concentration of aPL IgG. These results have a corresponding with the study of [42], where they found that a total positive prevalence of the aPE IgG was 11.8% (31/263), whereas the prevalence of the aPS IgG was 6.5% (17/263) in patients with RM. In Iraq, 3.3% (7/210) of aborted Baghdad’s women were positive for aPS IgG [43], while there was no significant difference among women with RPL in the Mosul city compared with control group [44]. In vitro, trophoblastic cells are the main targeted by anti-phospholipid antibodies, so these cells express adhesion molecules α1 and α5 integrin’s, when they cultured for 24 hours, aPL (IgG) antibodies of patients with APS lead to reduce of α1 integrin and increased α5 integrin, also the aPL activities induced vascular endothelial cadherin down-regulation and the epithelial cadherin up-regulation [45,46]. The results of the current study did not correspond to the study of [47], where they pointed out no significant differences in the level of aPL IgG antibodies in patients with RPL compared with healthy women, this may be due to the lower their sensitivity for aPL kit.

Previous studies have focused on the genetic side of FVL in aborted women patients, but this study based on measuring the concentration of the FVL in the sera of aborted women. The results have shown that a significant increase in their level in patients with a history of RM compared with healthy women. The reason for this increase may be as a result of an inherited or acquired mutation in factor V that led to the formation of another type form FVL, whose symptoms did not appear until the pregnancy occurred and therefore had a share in the outcome of negative pregnancy. Factor V G1691A is also known “Factor V Leiden”, and in both types of FVL mutants (FVLG1691A and A506G) the replacement occurs in amino acids (G→A and A→G) this result lead to the formation of a protein which is resistant to the action of activated protein C that acts on removing the suppression effect of protein C on the coagulation mechanism and increases the conversion of prothrombin to thrombin, which lead to the formation of clots [48,49]. All aborted women that had congenital or acquired FVL mutation were activated protein C resistance, therefore, ninety- five percent of the cases of activated protein C resistance as a result of FVL mutation [50,51]. In addition, the prevalence of the FVL G1691A was 7.42% (21/283) in patients group, while it was % (5/100) for FVL A506G in another group of patients, thus the genotype of the FVL G1691A was more frequently than that genotype of FVL A506G [52]. Furthermore, FVL was regarded as a risk factor for hereditary thrombophilia and...
can act either in the first half of gestation or later in gestation, leading to possible recurrent abortions [53]. This study showed that a significant increase in the level of the TNF-α in aborted women than in healthy individuals, these results are agreeing with the study of [54], that whom indicate elevated levels of this cytokine in peripheral blood in 22 women at first trimester- threatened miscarriage with the precedent adverse outcome, and 31 of women in the first trimester- threatened miscarriage with successful outcome compared with 22 healthy women. A significant difference in the concentration of this cytokine in both women with less and more than five RM in comparison with pregnant and non-pregnant women at first trimester, where higher levels of TNF-α may be resulting from free radicals (oxidative stress), so TNF-α lead to major disturbances to embryo in the early stage of pregnancy [55]. The current study demonstrated that no significant difference in the concentration of 25 (OH) Vitamin D among women of study groups, which is opposed to most previous studies, that shown a significant decrease of this parameter in aborted women [56,57,58]. This is probably due to the application of APS criteria to patients when selecting the study samples, or perhaps the uses of vitamin D drugs by most women patients. The ROC curve was used to evaluate a performance of IgG for variables of APS, where the AUC for all variables showed a significant difference (P<0.001). The sensitivity of aCL IgG to APS patients was 72.9% which is higher than that the sensitivity of aβ2GPI IgG, which was 64.8% [AUC 0.83, CI 95% (0.78-0.89); AUC 0.92, CI 95% (0.89-0.94) respectively] [59]. One hundred and sixty of women patients with APS has sensitivity 27.3 % for both aCL IgG and aβ2GPI IgG [AUC 0.626, CI 95% (0.487–0.764); AUC 0.679, CI 95% (0.459–0.899)] respectively which less than the sensitivity of LA which was 81·8 % (AUC 0.898, CI 95% (0.778–1.0]) [60]. Furthermore, women with recurrent miscarriages and APS showed a sensitivity of IgG aCL that reached 40% [AUC 0.613, CI 95% 0.479-0.746], while it was 12% for LA sensitivity [AUC 0.558 with CI 95% 0.376-0.740] [61]. Another study relied on AUC to evaluate the efficiency of diagnosis, where they indicated that the higher values of AUC represent the best diagnostic efficiency of the test (i.e. better sensitivity of the test) in which the AUC of the aCL IgG and aβ2GPI IgG was 0.88%, and 0.82% respectively [62]. Also, AUC was 0.541% for both lupus anticoagulant and aCL IgG, whereas it was 0.492 % for IgG aβ2GPI [30].

CONCLUSION
This study suggested that all women patients showed "triple a positive results" of the autoantibodies IgG for aCL, aβ2GPI and lupus anticoagulant (AL). Also, aCL IgG autoantibody was more prevalence in patients than healthy group. In addition, aPL IgG constitutes a high OR among anti-phospholipid antibodies in women with RM, furthermore, the current data underlined that women with RM has associated with APS.

REFERENCES


