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Abstract: The immunophenotypation of circulating lymphocyte by flow cytometry is a fast and efficient modern quantitative method and has been also used to assess immune status in normal pregnancy and pregnancy complicated by infection or with placental abnormalities or with preeclampsia. Since the literature indicates controversy in the immune etiology of abortion and in the levels of circulating lymphocyte subsets in normal pregnancy, this study aims to extend current knowledge by quantification with flow cytometry sets and subsets of circulating lymphocytes in the second trimester of normal pregnancy and pregnancy complicated by miscarriage.

Key-Words: Circulating lymphocyte, Immunophenotypation, Flow cytometry, Second trimester pregnancy, Pregnancy complicated by miscarriage, Recurrent abortion

1 Introduction
When the mechanisms allowing feto-maternal tolerance are not completely understood it is difficult to establish how their dysfunction can induce spontaneous abortion [1].

The process of human placenta implantation implies the proliferation of trophoblastic cells inside the cytotrophoblastic columns, followed by the migration of cytotrophoblasts in the uterine decidua. Besides the adhesion of the placenta to the uterus, these trophoblastic cells (named extravillous trophoblast) have another important function, by invading and destroying the walls of the uterine arteries (spiral, radial), which they convert in loose sinusoidal sacs [2]. This arterial transformation is crucial to the success of implantation, because it allows the increase in blood flow needed in the development of the feto-placental unit during the pregnancy. Inadequate vascular conversion performed by the invasive trophoblast in early pregnancy might be the cause of preeclampsia in some cases, intrauterine growth restriction and abortion, and diseases manifesting in adulthood, such as arterial hypertension and ischemic cardiopathy; it is not clear the nature of the bond between the lack of formation of adequate hemochorial placentation and the vast clinical expression, ranging from preeclampsia to intrauterine growth restriction or death of the fetus inside the uterus, or preterm labor or premature rupture of membranes and second trimester recurrent abortion [2, 3, 4].

The large granular lymphocytes (LGL) play an important role in the uniqueness of the trophoblast migration in the decidua, which implies a tight association of cells coming from two genetically different individuals that should trigger the maternal immune reaction of non-self [5]. During the implantation there is dramatic influx of LGL in the decidua. The LGL have an unusual phenotype, containing CD3-, CD16-, CD56bright+ and are uterus-specific, no other human organs containing them. Functionally speaking, the LGL have a Natural Killer (NK) cytolytic activity and produce a large range of cytokines. At the placenta level, the extravillous trophoblast is unique by expressing the Class I, non classical, monomorphic antigen, a product of HLA-G locus, instead of the classical HLA-A,B,C polymorphism, found on other somatic cells. This special trophoblastic antigen constitutes a recognition molecule for decidual LGL to which it transmits a positive or negative message [5, 3]. A positive signal would stimulate LGL to secrete cytokines that can influence the growth, differentiation and migration of the trophoblast. A negative signal would inhibit the trophoblast.
cytolysis by the LGL, such that one can consider that HLA-G functions as a protective molecule, being unanimously recognized the concept according to which Class I HLA antigens can inhibit the lysis of the target cells by the NK cells.

The trophoblast invasion during the implantation is controlled by the interaction between Class I, non classical trophoblastic antigen and an unusual population of NK decidual lymphocytes, implying an evolutionist defense system that is more primitive than the classical immune allogeneic response noticed in the case of organ transplant [5, 3].

The autoimmune explanation for a suggestive proportion of recurrent abortion that is often affecting the second trimester of pregnancy, is unanimously accepted today, on the basis of the anti phospholipid primary syndromes relating to the recurrent abortion affilinating to vein thrombosis, with a demonstration in the laboratory of anti phospholipid antibodies persistent in the peripheral blood [6].

Anti phospholipid antibodies are a family of antibodies, of which the most important ones are the anticoagulant lupus and the anti cardiolipin antibodies [7, 8, 9]. The sanguine screening test for the anti phospholipid antibodies is usually repeated in the first pregnancy weeks because some women with recurrent abortion have abnormal results in this test only during gestation [10]. The prevalence of anti phospholipid antibodies is of 15% in case of women with recurrent abortion, compared to 2% in case of those who have not suffered abortion before [11, 10], and the abortion rate in case of women with reduced obstetrical risk but carrying persistent anti phospholipid antibodies is of 50-75%, suggesting that women with recurrent abortion associated with persistent anti phospholipid antibodies have a risk of abortion (an abortion prospective rate) of 90% in pregnancies that are not pharmacologically treated [12].

Anti phospholipid antibodies cause abortion in the second trimester, but also in the first trimester of pregnancy, by thrombosis of utero-placental vessels, followed by extensive placental infarction (anti phospholipid antibodies would block the transport of annexin V to the apical membrane of the syncytiotrophoblast, which thus becomes a thrombogenic surface, because annexin V is the component of a phospholipid binding protein family, strong physiologic anticoagulants [13]), but also by adverse effects on the embryonic implantation and placentation, forasmuch as the binding of the anti phospholipid antibodies to the phospholipids on the cytotrophoblast surface causes direct damage to these cells, inhibiting the syncytiotrophoblast formation.

In an attempt to increase the rate of births with viable fetuses in women with primary anti phospholipid syndrome there were used various treatments including corticosteroids, aspirin, heparin and immunoglobulins, but up to now there are no prospective randomized studies, powerful enough, to determine a significant difference between two therapeutic protocols, with any of the above mentioned pharmacological agents [14]. On the other hand, corticotherapy during pregnancy presents risks because it associates a significant amount of premature births and preeclampsia [15]. Following a prospective randomized study [12] which compared the efficiency of low doses of aspirin with the aspirin-heparin association (the only therapies accepted nowadays in the treatment of primary anti phospholipid syndrome in pregnancy [10]) on an number of 90 pregnant women with persistent anti phospholipid antibodies, including anticoagulant lupus and anti cardiolipin antibodies, demonstrated unequivoacally that therapy based only on low doses of aspirin (75mg/day) has a success rate (births after 34 weeks) of 40%, compared to a 70% success rate in case of women treated with both aspirin and heparin (5000 U sc x 2 / day).

The concept of alloimmune cause in case of an abortion is debatable [16, 17]. The first authors who have proposed this theory suggested that women with recurrent abortion have in common an increased proportion of human leukocyte antigen alleles with their partners, and therefore they are incapable of an adequate immune response to the semi allograft represented by the conception product, theory that is rejected nowadays and has suffered numerous modifications on a 10 year period, during which researchers have tried to identify the deceiving nature of the protective immune response [5, 3, 18, 19, 20].

Despite the lack of specific data sustaining this hypothesis, deliberate immunization of the mother with paternal or donor lymphocytes (immunotherapy) was introduced in many centers with the hope of inducing an adequate maternal immune response, that would prevent rejection of the fetus [21].

Out of all studies on the efficiency of this immunotherapy (but all being based on a small number of observations, usually not blinded [22]) only one randomized study managed to demonstrate a significant benefit following application of immunotherapy to pregnant women with recurrent abortions.

The majority of centers practicing immunotherapy rely on the absence of antipaternal
cytotoxic antibodies (APCA) to identify the women fit for immunotherapy, although longitudinal studies on the incidence and natural history of APCA in pregnancy show that detection of APCA is rare before 28 weeks of gestation and that APCA disappear from the serum between pregnancies, making the APCA an unusable marker for identification in due time of the women with an alloimmune basis of recurrent abortion [23].

Two recent metaanalyses, that included mixed quality randomized studies, suggested only a marginal benefit derived from immunotherapy, being necessary to immunize 10 to 13 women to obtain one supplementary birth with viable fetus compared to the placebo [24].

One important drawback in the interpretation of a meta analysis is the fact that the result can be significantly influenced by the lack of objectivity of the publications and by the fact that not all negative results are reported, but on the other hand, a meta analysis can offer inexact results if it only relies on small studies, which differ in protocol and design. The two above mentioned metaanalyses, on the efficiency of the immunotherapy, were based on 8 respectively 10 randomised studies, some including only 11 subjects, and could not prove the significant benefit of the immunotherapy (the highest success with respect to placebo was of 28 respectively 17%). Moreover, any marginal benefit which is believed to be the result of immunotherapy must be weighed with respect to the risks of this therapy, such as hepatitis (infectious and autoimmune) with its secondary results, anaphylactic shock, viral transmission and transfusion reactions [25].

It was demonstrated that full term delivery is possible in 75% of cases without immunotherapy, by means of a prospective study on a number of 106 women examined consecutively, with a history of at least three spontaneous abortions and who had a normal peripheral blood karyotype, a normal LH secretion, tested negative for anti phospholipid antibodies and moreover, APCA negative (therefore eligible for immunotherapy, in the conditions of an exhaustive investigation which included thrombophilic defects), but who were monitored in the first 3 months of pregnancy by weekly repeated ultrasounds and were applied sustained psychotherapy [22].

The influence of pregnancy on immune function is poorly understood [17, 19, 26]. Although most women do not present immunodeficiency during the pregnancy the frequency and the severity of some infections suggest that some immune responses are modified [27].

So, in normal pregnancy, in contrast with the pregnancy complicated by infection with human immunodeficiency virus type1 (HIV-1), Burns and his coworkers [27] found that the lymphocytes T helper (CD4+), which are low in the first part of gestation, are increasing progressively from the third trimester, while the CD8+ cells (suppressor / cytolytic lymphocytes T) are increasing in the moment of birth which disagrees with the observations, more limited though, on the pregnancy with no complications [28].

Natural cells killer (NK) are also changing insignificantly during pregnancy compared to negative population [29, 30].

In addition to the immune changes of the uterus [5, 3, 31], the immunology of pregnancy is difficult to interpret at the peripheral blood level. There are great individual variations of normal limits in terms of maternal circulating lymphocyte subsets [28] and the insufficient knowledge of the role of mediators of cytokines in cellular events related with the installation and maintenance of pregnancy [32, 33, 34, 35, 36, 37, 38]. It is difficult to interpret the immunology of pregnancy because are well demonstrated nowadays early feto-maternal transfusion [39, 40], the activation of the fetal immune system in utero [41, 42] and the influence of the maternal immune response to genital infection with Ureaplasma urealyticum that is responsible both for recurrent miscarriage and the polyclonal B lymphocyte activation by increasing titres of different antibodies, like the antiphospholipid [43, 44]. The immunophenotypation of circulating lymphocyte by flow cytometry is a fast and efficient modern quantitative method and has been also used to assess immune status in normal pregnancy [27, 28] and pregnancy complicated by infection [27] or with placental abnormalities [45] or with pre eclampsia [20, 46].

The same method has been exploited in the study of miscarriage [23, 47, 48, 49, 50, 51] in conditions where independent immune disturbances and infection-induced are frequently incriminated in the etiology of abortion, especially the recurrent one [6, 8, 10, 11, 13, 14, 18, 43, 51, 52, 54, 55], which has repercussions on therapeutic efficiency of recurrent immune abortion [11, 12, 24, 25, 56, 57, 58, 59, 60].

Because the literature indicates controversy in the immune etiology of abortion and in the levels of circulating lymphocyte subsets in normal pregnancy, this study aims to extend current knowledge by quantification with flow cytometry sets and subsets of circulating lymphocytes in the second trimester of normal pregnancy and pregnancy complicated by miscarriage.
2 Material and Methods

This prospective and controlled study has selected 47 pregnant women with gestational age (transabdominal ultrasound confirmed) between 13-27 weeks. They were evaluated either ambulatory or interned in departments of obstetrics gynecology clinic from University of Craiova for normal pregnancy or complicated pregnancy with threat of miscarriage (diagnosis established by uterine bleeding +/- painful uterine contractions + clinical and paraclinical exclusion of placenta praevia, gestational trophoblastic disease, ectopic pregnancy and uterine and fetal malformations [14]).

After obtaining the consent of the 47 pregnant women, the study subjects were classified in the following categories:

(a) 12 pregnant women with normal pregnancy between 13-26 weeks of gestation, identified during prenatal consultation. Their results of lymphocytic Immunophenotyping were used as control for the group of pregnant women with complicated pregnancy. On the other hand, the results were stratified analyzed (< 20 weeks of gestation or >20 weeks of gestation) to exclude the possible influences of gestational age on serum levels of lymphocyte population during the second trimester of pregnancy;

(b) 35 pregnant women in second trimester of pregnancy with threat of miscarriage that had not evolved to the expel of the product concept in the first 48 hours from the debut of uterine bleeding. They were also divided in a group with gestational age <20 weeks and another with gestational age >20 weeks.

Each of the 2 subgroups included, respectively, 8 and 5 pregnant women with history of recurrent abortions, diagnosis based on the presence of at least 3 consecutive spontaneous abortions.

The pregnant women (n=47) admitted in our study for lymphocytic Immunophenotyping were used as control for the group of pregnant women with complicated pregnancy. The percentages of circulating lymphocyte populations in normal pregnancy, reveals no statistically significant differences.

The exclusion criteria of our study consisted of:

(a) lupus erythematosus manifested; (b) general or local infection apparent on evaluation or most recent; (c) izoimunisation Rh; (d) serum hemolysison sample of peripheral blood taken for Immunophenotypation; (e) immunomodulatory therapy influence on sampling for flow cytometry; (f) refusal of a complex evaluation before enrollment in the study of peripheral lymphocyte subpopulations in pregnant women; (g) other causes of uterine bleeding than the threat of miscarriage; (h) other causes of recurrent abortion (genetic, anatomical, fetal, endocrine, microbiological- others that Ureaplasma urealyticum and Chlamidia) and multiple pregnancy [54, 55, 62, 63, 64].

Flow cytometry [28] is a modern method, inexpensive, relatively simple technique, through it can be determined simultaneously more physical characteristics (and morphology, the existence of specific structures recognized by the fluorochrome) of one cell moving in a liquid in conditions where cell analysis rate is between 500 and 4000 cells / second).

A value of $p<0.05$ was statistically significant at the comparing the results of the investigation by Student’s and Mann whitney U tests, according to the argumentative principles of Welch & Gable [65].

3 Results and Discussions

Demographic analysis rendered in Table I, Figure 1 and Figure 2 shows, in addition to the number of subjects in the control group and test group and the comparability of the lots and sub-lots of pregnant investigated about the average age and gestational age, while the apparent incompatibilities related to parity and gestation number may not affect the results of this immune research because it is widely agreed that after conception expel the peripheral Immunocyte levels return to preconceptional status [27, 28]. The comparison between sub-lots of healthy pregnant with gestational age until 20 weeks and over, about the evolution in the major subsets of peripheral lymphocytes during the second trimester of normal pregnancy, reveals no statistically significant differences.

The percentages of circulating lymphocyte subpopulations in pregnant women with threatening abortion does not change strongly after 20 weeks of gestation over the period up to 20 weeks, in the second trimester of pregnancy.

No notable differences occur between subsets of peripheral Immunocyte in normal pregnancy reported to the complicated pregnancy with the threat of abortion in second trimester of pregnancy up to 20 weeks and after 20 weeks.
Table 1  The demography of pregnant groups investigated by flow cytometry

<table>
<thead>
<tr>
<th></th>
<th>Gestational age</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>13-20 weeks</td>
<td>21-27 weeks</td>
<td></td>
</tr>
<tr>
<td>Number of subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group (normal pregnancy)</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Test group (with/without history of recurrent miscarriage)</td>
<td>20 (12/8)</td>
<td>15 (10/5)</td>
<td></td>
</tr>
<tr>
<td>Maternal age (average +/-standard error of average)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>23 + - 1,4</td>
<td>22,6 + - 1,76</td>
<td></td>
</tr>
<tr>
<td>Test group</td>
<td>23,7 + - 0,9</td>
<td>23 + - 0,95</td>
<td></td>
</tr>
<tr>
<td>Pregnancy ( number of subjects)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Primigravida</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Multigravida</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Test group</td>
<td>11</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Parity ( number of subjects)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>9</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Multiparous</td>
<td>16</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Test group</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Gestational age ( average +/- standart error of average)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>17,66 + - 0,57</td>
<td>23,6 + - 1,66</td>
<td></td>
</tr>
<tr>
<td>Test group</td>
<td>16,75 + - 0,59</td>
<td>24,2 + - 0,47</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1

Figure 2
Table 2 A comparison between the percentages of lymphocyte subpopulations in pregnant women with normal pregnancy evolving without history of recurrent miscarriages and those of pregnant women with a history of recurrent miscarriages

<table>
<thead>
<tr>
<th>Lymphocytic population</th>
<th>Percent (percentili median with 25 and 27)</th>
<th>Statistically significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without recurrent abortions</td>
<td>With recurrent abortions</td>
</tr>
<tr>
<td>Total T lymphocytes</td>
<td>66 (65-72.5)</td>
<td>64 (55-69)</td>
</tr>
<tr>
<td>Total B lymphocytes</td>
<td>19 (14,25-22)</td>
<td>13 (10,75-16,75)</td>
</tr>
<tr>
<td>T-helper lymphocytes</td>
<td>43 (37,75-46,5)</td>
<td>44 (33,5-49,75)</td>
</tr>
<tr>
<td>Cytholytic T lymphocytes</td>
<td>32 (27,25-40,5)</td>
<td>31 (36,25-44,75)</td>
</tr>
<tr>
<td>T-helper/ T cytholytic</td>
<td>1,253 + - 0,235</td>
<td>1,230 + - 0,291</td>
</tr>
<tr>
<td>Activated B lymphocytes</td>
<td>13 (11,75-14)</td>
<td>15 (14,25-16,75)</td>
</tr>
<tr>
<td>Activated T lymphocytes</td>
<td>10 (8-10)</td>
<td>4 (2-10)</td>
</tr>
<tr>
<td>Natural killer cells</td>
<td>18 (16,25-20)</td>
<td>9 (6,5-19,75)</td>
</tr>
</tbody>
</table>

Figure 3 A comparison between the percentages of lymphocyte subpopulations

When one views in parallel the percentages of circulating lymphocyte subpopulations in the second trimester of, respectively, normal pregnancy and pregnancy complicated by threat of miscarriage, regardless of the gestational age, what is opposites all pregnant women with painful uterine contractions (11 from 35 abortion threats) of the test group to those of the control group (painless contractile), the threat of abortion in second trimester of pregnancy is characterized by a significant increase of CD4 + (T helper) cells and by a decrease in statistical evidence of total B lymphocytes compared to normal pregnancy in second trimester of pregnancy.

The comparison, rendered in Table 2, of the entire sub-lot of recurrent miscarriages under threat of abortion regardless of gestational age in terms of percentage levels of the main peripheral blood lymphocyte subpopulation, indicate a significant growth of the B lymphocytes activated only among pregnant women with recurrent miscarriages compared to those with uncomplicated pregnancy by abortion in the second trimester of pregnancy.

The results show that the fluctuation levels and peripheral lymphocyte subpopulation percentages during the normal pregnancy in second trimester of pregnancy are consistent with most previous studies [27, 28, 29] by flow cytometry in normal pregnancy. The fact that no CD4 + cell rise beginning at the end of second trimester of pregnancy, or CD8 + population growth are associated to fetal expulsion [27] are not apparent in normal pregnant women.
studied by us is explained by the maximum limit in the control group of 26 weeks gestational age and by the fact that no normal pregnant and with threat of abortion not expelled the fetus in the first hours after sampling the reading profile of peripheral cellular immune.

Significantly increasing of the percentage of T-helper lymphocytes near by the reducing the level of total peripheral B lymphocytes in the threat of abortion in second trimester of pregnancy, noticed by us, is not a simple redistribution of these sets of immunocyte [66], which was detected from normal reporting of the groups with fewer pregnant women with threatening of late miscarriage. Is rather the immunomodulating effect associated to the release of prostaglandin E2 during painful uterine contractions that accompany the hicercorticolemy [67].

The only difference between the normal pregnant women of same gestational age from group with pregnancy with threat of miscarriage and a history of recurrent abortions was the significant increasing of circulating activated B lymphocyte subset. That means that by one measure, using the Immunophenotypation of peripheral lymphocytes we can make the screening of the group of recurrent abortion generate by the presence of the antiphospholipid antibodies with or without genital infection with Ureaplasma urealyticum [43] which separates it from the rest of recurrent genetic abortions associated with other defects thrombophilia [12].

Reporting our results to those from the literature [27] one could show that fenotipation of peripheral blood by flow cytometry in dynamic of pregnant women in the second trimester of pregnancy can serve as screening simple, inexpensive, practical to detect HIV - 1 infection associated with pregnancy.

On the other hand, specific therapy in case of infection with Ureaplasma urealyticum associated to the pregnancy, the fenotipation by flow cytometry in dynamic could be a way to monitor the therapy efficiency.

By comparing our results obtained by flow cytometry for shaping the peripheral immune profile of pregnancy complicated with recurrent miscarriage and abortion threat in the second trimester of pregnancy with the observations on serum C-reactive protein (CRP) in the second trimester of pregnancy complicated with the same things [68, 69] we can say that the most common mechanism of recurrent miscarriage in the study population is expressed through another variant of the cytokine cascade than the one that trains the interleukin – 6 (the main inductor of serum CRP).

4 Conclusion

Our study highlights that the fenotipation by flow cytometry of the peripheral lymphocytes can be a practical screening, at least for recurrent abortion of immune cause in the second trimester of pregnancy.

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