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High levels of NK cells in the peripheral blood of patients affected with anti-phospholipid syndrome and recurrent spontaneous abortion: a potential new hypothesis

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Objectives. No data regarding phenotypic assets of circulating lymphocytes in anti-phospholipid syndrome (APS) are reported in the literature. Role of anti-phospholipid antibodies (aPL) in recurrent spontaneous abortion (RSA) remains uncertain, while natural killer (NK)-cells are involved in RSA pathogenesis. In this study, patients affected with APS without RSA, APS with RSA and RSA without aPL were studied for NK-cell subpopulation to evaluate its role in abortive events typical of APS.
Methods. NK-cell levels in peripheral blood of APS patients without RSA ($n=28$) and in APS-RSA patients ($n=25$) were evaluated by means of flow cytofluorimetry. NK-cells levels were evaluated also in RSA without aPL associated with either endocrine ($n=86$), anatomic ($n=30$) or idiopathic ($n=77$) conditions and in 42 healthy women.
Results. High NK levels were found in 14/25 (56%) APS-RSA patients. Among these patients, all except one aborted before the 10th gestational week (GW), while among the remaining patients all except one aborted after the 10th GW. NK mean levels were significantly higher in APS-RSA than in all the other conditions studied, including healthy subjects, except idiopathic RSA.
Conclusions. Our results demonstrate that the numbers and proportions of NK-cells are significantly higher in patients with RSA with APS than in APS without RSA. Increased numbers of NK-cells correlate with reduced gestational age at abortion in patients with APS-RSA. These data lead to a hypothesis that NK-cells contribute to the development of RSA in patients with APS. NK-cells might precipitate damage initiated by aPL or they might cause pathology in RSA independent of aPL.

Key words: APS, anti-phospholipid syndrome, NK, recurrent spontaneous abortion, aPL.

Introduction

Anti-phospholipid syndrome (APS) is an autoimmune disease characterized by the presence of one or more laboratory findings of anti-phospholipid antibodies (aPL) and at least one clinical manifestation besides deep venous and/or arterial thrombosis and/or recurrent spontaneous abortion (RSA), with or without thrombocytopenia [1–4].

RSA is a heterogeneous condition defined by three or more consecutive spontaneous abortions. RSA has numerous causes and clinical presentations, and may occur during any stage of pregnancy [5]. The association between APS and RSA is well known, so that RSA represents one of the clinical diagnostic criteria for APS [6, 7]. The risk of pregnancy loss in women with APS is higher from the tenth gestational week (GW) onward (fetal period) [8]. The Sapporo criteria, and the revised criteria for APS, underline this situation by considering only patients with one or more unexplained fetal losses (of a morphologically normal fetus) beyond the 10th GW, or three or more unexplained consecutive spontaneous abortions before the 10th GW [6, 7]. A number of investigators demonstrated that 2–16% of women with reproductive failure during the first trimester were aPL positive [9–11]. Several pathogenic mechanisms responsible for fetal loss in patients with APS have been described, such as an enhanced adhesion molecule expression by endothelial cells [12]; the intrinsic prothrombotic properties of anticardiolipin antibodies (aCL)/β_2GP-I [13]; the induction of tissue factor release leading to micro-vascular thrombi; increased syncitial knotting and premature aging of the *villi* with necrosis upon exposure to anti-β_2GP-I; decreased human Chorionic Gonadotropin (hCG) secretion by the trophoblast [14, 15]. However, the exact role of the aPL in the genesis of RSA in APS patients is still not completely understood.

Although there are conditions associated with RSA other than APS (e.g. genetic, endocrine, anatomic, pharmacological, infectious, haematological) [4, 16], sometimes, notwithstanding extensive studies, the cause of recurrent abortion remains unexplained [17]. A deeper investigation of apparently unexplained RSA offers increasing evidence to support that an alloimmunologic mechanism, a condition in which natural killer (NK) cells may play a relevant role, is involved [18].

During normal pregnancy, peripheral NK cell activity and percentages tend to increase in the first trimester and then tend to decline from the second trimester with a second fall in the third trimester of pregnancy [19, 20]. Recent studies suggest that the increase of NK cells in peripheral blood may be considered a causal and prognostic indicator for sterility, infertility and miscarriage [21–23]. However, other studies did not fully confirm these findings [24], and even if NK cell activity and levels are altered during RSA, it remains unclear whether the difference is a cause or an effect of reproductive failure [25].

No data regarding peripheral NK cell levels in APS patients have been reported in the literature. This consideration, as well as the uncertain pathogenic role of aPL in RSA, induced us to evaluate the phenotypic assets of circulating lymphocytes, particularly the NK cell subpopulation, in patients affected with APS without RSA, APS with RSA and RSA without aPL.

Patients and methods

Twenty-eight Caucasian APS patients without RSA who presented only thrombotic events in their clinical history, referred to the Department of Rheumatology, Policlinico Tor Vergata, Rome, Italy. This population was composed of 28 non-pregnant females of reproductive age, in which APS diagnosis was made in

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accordance with international guidelines (all patients completely fulfilled Sapporo's clinical and laboratory criteria, and the revised criteria for APS diagnosis) [6, 7]. None of the women showed any infectious or parasitic disease or the presence of other autoantibodies except aPL. None was using any medication at the time of the study. The patient's written consent was obtained according to the Declaration of Helsinki.

Two hundred and eighteen Caucasian RSA patients were included in this study. Patients were included only if they had three or more previous consecutive pregnancy losses. Study protocol for the evaluation of the abortive causes was performed in all patients and included clinical and abortive anamnesis, gynaecological exams and kariotype study. Autoantibody screening included anti-nuclear antibodies, anti-extractable nuclear antigens, anti-mitochondrial antibodies, anti-smooth muscle antibodies, anti-thyroperoxidase and thyroglobulin antibodies, anti-neutrophil cytoplasmic antibodies and aPL dosage. We performed coagulation assays, endocrine evaluation, thyroid functionality evaluation, including thyroid ecography, hysteroscopy and pelvic ecography, colposcopy, thrombophilic evaluations and cultural assays for the research of common germs, *Mycoplasma* spp. and *Chlamydiae* spp.

Only patients who underwent the complete screening, who were not in therapy during the time of the study, who did not show any infectious or parasitic disease and who tested negative for all of the aforementioned conditions except APS ($n = 25$), except clinical or subclinical hypothyroidism ($n = 86$), except anatomic alterations sufficient to explain abortions ($n = 30$) and except nothing (idiopathic RSA) ($n = 77$), were admitted.

Forty-two healthy Caucasian women of reproductive age served as controls. No significant differences were observed among the age distribution of each group. All subjects provided informed consent.

Laboratory evaluation

All tests comparing the patients and controls were conducted outside pregnancy during the second phase of the menstrual cycle. Patients were screened for the presence of aPL (aCL and β_2GP-I immunoglobulin M and immunoglobulin G class) according to international guidelines using previously described methods through ELISA assays [7, 26, 27]. Tests were considered positive when anti-cardiolipin antibodies of the IgG and/or IgM isotype at a medium or high titre (> 40), and/or anti-β_2GP-I antibodies of IgG and/or IgM isotype in titre > 99th percentile, were present in blood on two or more occasions at least 12 weeks apart. Lupus anticoagulant (LAC) was detected by coagulation assays in accordance with the International Society of Thrombosis and Haemostasis [28]. Tests were repeated at least 12 weeks apart.

Lymphocytes immunophenotypic profiles

To assess lymphocyte subpopulations, a peripheral blood immunophenotype assay was performed by means of flow cytofluorimetry in all subjects. Peripheral blood was collected in heparinized tubes. Cells were incubated with anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD56 and anti-CD16 monoclonal antibodies. The instrument, manufactured by Instrumentation Laboratory-Beckman Coulter, was provided with a He–Ne laser-ray, which recognizes wavelengths of four different fluorochromes (FITC, PE/RD1, ECD, PC5/PE). Representative flow cytometry dot plots of circulating NK cells are shown in Fig. 1.

A positive result for high levels of NK cells was defined as a percentage >15% lymphocytes (>410 cells/μl). These values were defined as the mean + 2 S.D. calculated in 200 normal subjects.

Statistical analysis

The statistical analysis was performed by means of a computer-assisted statistical analysis program. Comparison between the groups was performed by one-way analysis of variance (ANOVA) corrected with Bonferroni's multiple comparison test. Paired *t*-tests were used to compare data from the gestational week of abortion within the APS-RSA group. Two-tailed *P*-values were reported together with a 95% confidence interval (CI) of differences, and *P*-values <0.05 were reported to be statistically significant.

Results

The results of NK cell percentages and absolute numbers for each patient group are presented in Table 1. Table 2 presents the Bonferroni's multiple comparison test between the NK percentages within all groups, showing the *P*-values and the 95% CI of differences. Table 3 presents the Bonferroni's multiple comparison test between the NK cell absolute numbers within all groups, showing the *P*-values and the 95% CI of differences. NK cell

TABLE 1. NK cell percentages (%) and absolute numbers (cells/μl) found in different groups of RSA patients

	Mean ± S.D.	Mean ± S.D.	Patients with NK >15% (*n*)	Patients with NK >15% (%)
APS-RSA	15.18 ± 7.32%	477.7 ± 390.8	14/25	56.0
APS-without RSA	8.79 ± 4.68%	184.9 ± 103.4	3/28	10.71
Idiopathic RSA	23.24 ± 6.72%	499.8 ± 205.8	71/77	92.2
Hypothyroidism RSA	12.16 ± 5.41%	238.4 ± 141.7	23/86	26.8
Anatomic factors RSA	7.21 ± 3.77%	168.7 ± 95.3	2/30	6.7
Controls	8.32 ± 3.43%	184.2 ± 84.2	3/42	7.1

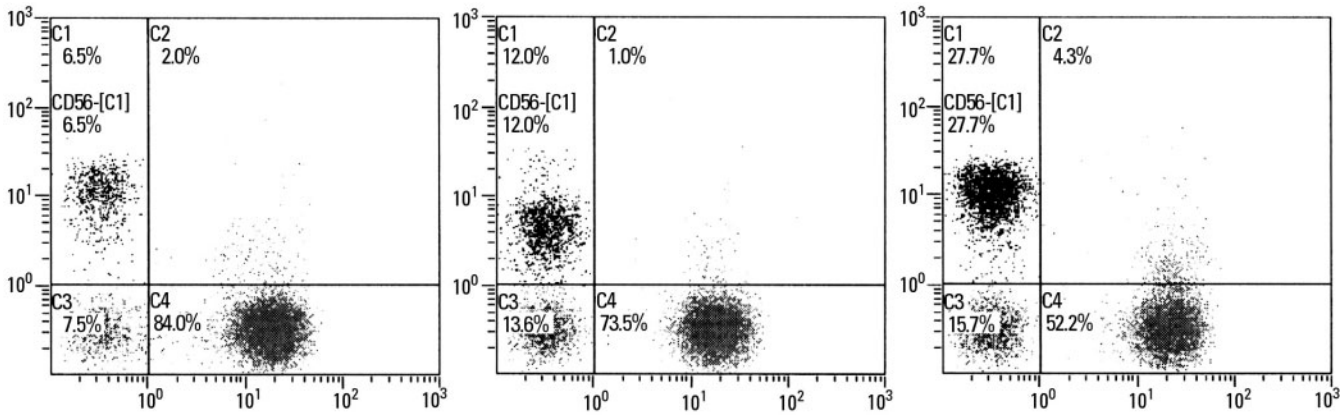

FIG. 1. Representative flow cytometry dot plots of circulating NK cells. Left: APS without RSA patient (NK-cells percentage = 6.5%). Centre: APS-RSA patient (NK-cells percentage = 12%). Right: Idiopathic RSA patient (NK-cells percentage = 27.7%).

mean percentages were significantly higher in APS-RSA than in all the other conditions studied, including healthy subjects, except idiopathic RSA. These differences, except the one between APS-RSA and hypothyroidism-RSA patients, were confirmed when employing NK cell absolute numbers. Figures 2 and 3 represent the scatter graphs of the NK cell percentages and absolute numbers (cells/microlitre) in the various subgroups.

No significant differences were found among and within the patient groups for the other lymphocyte subpopulations studied (CD3, CD4, CD8 and CD19 percentages and absolute numbers).

Subsequently, we studied patients with APS and RSA by dividing them into two categories: those with NK levels higher than 15% (14/25, 56%), and those with normal NK levels (11/25, 44%), in order to evaluate any possible difference in the week of abortion in the two groups (Table 4). Interestingly, all except one of the patients with APS, RSA and NK cell levels >15% had their last abortions within the first 10 GWs, with a mean of 8.28 ± 2.33 weeks. Only one patient had her last abortion at the 13th GW. This was fully confirmed by a retrospective analysis of these patients, considering the mean week of abortion between all the previous abortions of each patient. Each patient tended to abort within a similar period of time, demonstrating that all patients, except one, with APS, RSA and NK cells >15% had their mean week of abortion within the first 10 GWs with a mean of 8.80 ± 2.23 weeks.

In contrast, all except one of the 11 patients with APS, RSA and NK cell levels <15% had their last abortions beyond the 10th GW, with a mean of 15.63 ± 4.74. In this category only one patient had her last abortion at the fifth GW. This was fully confirmed by a retrospective analysis of these patients, considering the mean week of abortion between all the previous abortions of each patient. Each patient tended to abort at a similar GW, demonstrating that all except one of the patients with APS, RSA and NK cells <15% had their mean week of abortion after the 10th GW with a mean of 14.62 ± 4.34 weeks.

Thus, when considering the week of abortion in these two groups there is a noticeable difference ($P < 0.001$), which clearly distinguishes these two populations (Table 4). Linear regression analysis further demonstrated a significant correlation ($P = 0.006$ and $P = 0.0011$ when considering, respectively, the GW of the last abortion or the mean of the abortions) between NK cell percentages and the week of abortion in APS-RSA patients (Fig. 4). No significant difference was observed within the other RSA groups (i.e. hypothyroidism RSA, idiopathic RSA and anatomic factor RSA) among patients showing NK cell levels >15% and those showing NK cell levels <15% concerning gestational week of abortion. In fact, the large majority of these patients tended to abort early (within the first 10 GWs). GW of abortion in these groups is reported in Table 5.

Table 2. Bonferroni's multiple comparison test between the NK cell percentages within all groups

	Mean diff.	*P*-value		95% CI of diff.
APS-RSA *vs* APS-without RSA	6.395	< 0.001	Sign.	1.874 to 10.92
APS-RSA *vs* anatomic factors RSA	7.974	< 0.001	Sign.	3.438 to 12.51
APS-RSA *vs* hypothyroidism RSA	3.027	> 0.05	NS	−0.779 to 6.833
APS-RSA *vs* idiopathic RSA	8.056	< 0.001	Sign.	4.201 to 11.91
APS-RSA *vs* controls	6.86	< 0.001	Sign.	2.629 to 11.09
APS-without RSA *vs* hypothyroidism RSA	3.368	> 0.05	NS	−0.2068 to 6.942
APS-without RSA *vs* anatomic factors RSA	−1.579	> 0.05	NS	−5.896 to 2.738
APS-without RSA *vs* idiopathic RSA	14.45	< 0.001	Sign.	10.83 to 18.08
APS-without RSA *vs* controls	0.466	> 0.05	NS	−3.543 to 4.474
Idiopathic RSA *vs* hypothyroidism RSA	11.08	< 0.001	Sign.	8.455 to 13.71
Idiopathic RSA *vs* anatomic factors RSA	16.03	< 0.001	Sign.	12.43 to 19.64
Idiopathic RSA *vs* controls	14.92	< 0.001	Sign.	11.70 to 18.13
Hypothyroidism RSA *vs* anatomic factors RSA	4.947	< 0.001	Sign.	1.395 to 8.499
Hypothyroidism RSA *vs* controls	3.833	< 0.01	Sign.	0.6801 to 6.986
Anatomic factors RSA *vs* controls	−1.114	> 0.05	NS	−5.118 to 2.890

Sign., Significant; NS, Non-Significant.

Table 3. Bonferroni's multiple comparison test between the NK cell absolute numbers within all groups

	Mean diff.	*P*-value		95% CI of diff.
APS-RSA *vs* APS-without RSA	298.6	< 0.001	Sign.	144.7 to 452.4
APS-RSA *vs* anatomic factors RSA	309	< 0.001	Sign.	140.6 to 477.4
APS-RSA *vs* hypothyroidism RSA	239.3	< 0.001	Sign.	106.5 to 372.0
APS-RSA *vs* idiopathic RSA	22.18	> 0.05	NS	−113.1 to 157.5
APS-RSA *vs* controls	293.5	< 0.001	Sign.	147.7 to 439.3
APS-without RSA *vs* hypothyroidism RSA	59.30	> 0.05	NS	−56.68 to 175.3
APS-without RSA *vs* anatomic factors RSA	−10.44	> 0.05	NS	−164.3 to 143.4
APS-without RSA *vs* idiopathic RSA	320.7	< 0.001	Sign.	202.0 to 439.5
APS-without RSA *vs* controls	−5.083	> 0.05	NS	−135.1 to 125.0
Idiopathic RSA *vs* hypothyroidism RSA	261.4	< 0.001	Sign.	174.3 to 348.6
Idiopathic RSA *vs* anatomic factors RSA	331.2	< 0.001	Sign.	195.9 to 466.5
Idiopathic RSA *vs* controls	315.7	< 0.001	Sign.	209.7 to 421.6
Hypothyroidism RSA *vs* anatomic factors RSA	69.74	> 0.05	NS	−63.05 to 202.5
Hypothyroidism RSA *vs* controls	54.22	> 0.05	NS	−48.48 to 156.9
Anatomic factors RSA *vs* controls	−15.52	> 0.05	NS	−161.3 to 130.3

Sign., significant; NS, non-significant.

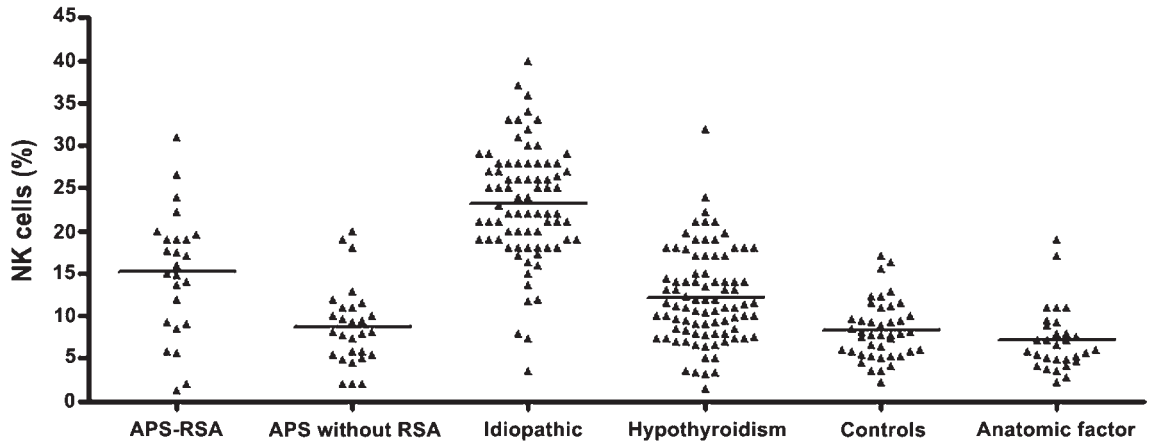

Fig. 2. NK cells percentages in the various subgroups.

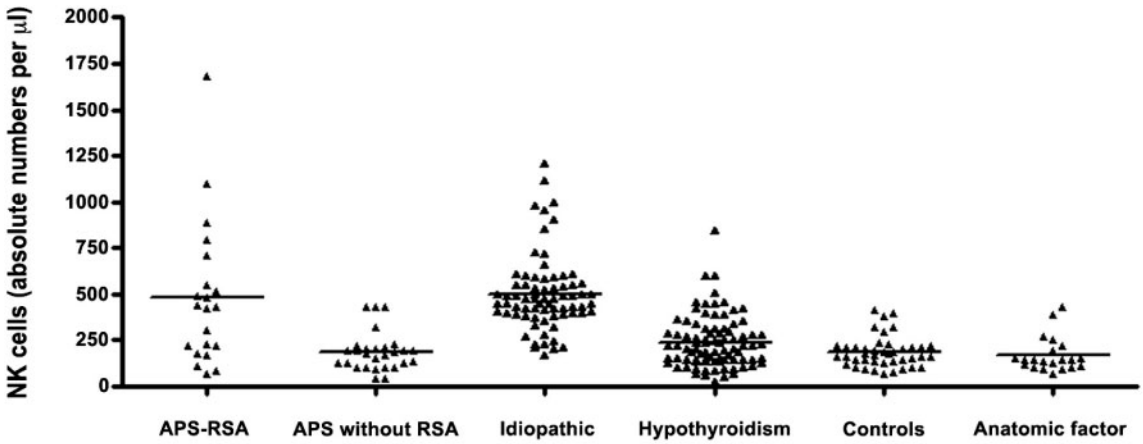

FIG. 3. NK cells (absolute numbers per microlitre) in the various subgroups.

TABLE 4. NK-cell percentages found in APS patients and gestational week of abortion (GWA) in APS-RSA patients (Means ± S.D.)

	APS-without RSA ($n=15$)	APS-RSA NK<15% ($n=11$)	APS-RSA NK>15% ($n=14$)
NK, %	8.79 ± 4.68	8.74 ± 4.67	20.25 ± 4.39
GWA (last abortion)		15.63 ± 4.74	8.28 ± 2.33
GWA (mean of the abortions)		15.03 ± 4.67	8.73 ± 1.95

GWA (last abortion) (APS-RSA NK<15% *vs* APS-RSA NK>15%) $P<0.001$.
GWA (mean of the abortions) (APS-RSA NK<15% *vs* APS-RSA NK>15%) $P<0.001$.

Evaluation of aPL levels in APS patients, either without or with RSA, did not show any difference within the groups (not IgG or IgM aCL, not IgG or IgM β_2GP-I, not LAC). Even when the APS-RSA patients with NK levels < 15% and those with NK levels >15% were considered separately, there were no differences in aPL levels ($P>0.05$).

Discussion

The results demonstrate that NK cell numbers and proportions that are found in APS-RSA patients are significantly higher when compared with NK cell levels found in patients affected by APS without RSA. This finding is particularly evident in a specific population of APS-RSA patients, recognized in 56% of these patients (Tables 1–3). It is noteworthy that this population can also be distinguished from the other 44% of APS-RSA patients with normal levels of NK cells in the clinical picture presented. In fact, it was demonstrated that the patients who have APS, RSA and high levels of NK cells tend to abort earlier, generally within the first 10 GWs as compared to the other APS-RSA patients with NK cell levels <15% (Table 4). This same population has a typical spectrum of clinical manifestations, and presents in the majority of the cases a later onset, usually beyond the 10th GW. Linear regression demonstrated that NK cell levels were strongly correlated to week of abortion, showing a trend of earlier onset of abortive events related to higher levels of NK cells (Fig. 4). No significant difference was observed in the other RSA groups (i.e. hypothyroidism RSA, idiopathic RSA and anatomic factor RSA) among patients showing NK cell levels > 15% and those showing NK cell levels <15% with regard to GW of abortion. These results differed from the APS-RSA group. Interestingly, a large majority of all patients in the other RSA groups tended to abort early (within the first ten gestational weeks). Therefore, the (minimal) conditions that can be related to early abortion are high NK cells, hypothyroidism and anatomic alterations, but not aPL.

FIG. 4. NK cells percentages correlation with GWA (mean of the abortions) in APS-RSA patients.

TABLE 5. Gestational week of abortion (GWA) in RSA patients (means ± S.D.)

	GWA (last abortion)	*P*-value
Anatomic factor NK<15% ($n=28$)	8.76 ± 2.72	
Anatomic factor RSA NK>15% ($n=2$)	7.21 ± 2.08	>0.05
Hypothyroidism RSA NK<15% ($n=63$)	8.90 ± 4.73	
Hypothyroidism RSA NK>15% ($n=23$)	8.22 ± 3.44	>0.05
Idiopathic RSA NK<15% ($n=6$)	9.33 ± 5.72	
Idiopathic RSA NK>15% ($n=71$)	8.03 ± 7.2	>0.05

The results of this study suggest that natural immunity, specifically NK cells, plays a role in the pathogenesis of abortive events in a subpopulation of APS-RSA patients, that previously has been explained in terms of autoimmune specific reactions (aPL mediated).

As a number of controversies plague the current understanding of APS and RSA, several authors have reviewed the role of APS and specifically of aPL in RSA and they have underlined the existence of different subclasses of clinical subsets of RSA in APS patients. Indeed it is intrinsic to APS definition that there is

a dichotomy regarding patients who have multiple (>3) abortions within the first 10 GWs and those who have at least one abortion beyond the 10th GW. Moreover, in a recent paper Branch [29] raises the question as to how aPL affects recurrent early pregnancy loss in some patients while in others there is an effect on second or third trimester complications. However, no one, to our knowledge, could give any explanation regarding such a dichotomy. It is possible to think of aPL as not directly causative of reproductive failure, but rather as acting as markers or intermediaries of an underlying abnormal activation of aspecific cellular immunity, particularly NK cells. It has been hypothesized that high levels of aPL in RSA patients can be associated with high levels of activated NK cells, suggesting a fundamental predisposition to immune-mediated rejection of the fetus by these patients [30]. However, we did not find elevated NK cells in either the APS-without RSA or in the 44% of the APS-RSA patients studied, all of whom have what was formerly considered high aPL levels (>40). Thus, high levels of NK cells cannot be considered a consequence of high aPL levels.

Events that lead to an RSA condition are extremely various and largely unknown. As observed previously, even if altered levels or activity of NK cells were found in patients with RSA, it is uncertain whether these changes are the result of multiple abortions or whether they work directly with a pathogenic mechanism in RSA.

The first hypothesis is not supported by our data. In such situation, it would be expected that the NK cells would be elevated in the majority of all RSA patients. In actuality, they are not elevated in patients suffering from anatomic- or endocrine-mediated RSA nor in a certain percentage (44%) of patients with APS-RSA. We can instead propose a subcategory of APS-RSA patients, recognizing a new, separate clinical entity where APS is associated with RSA despite being strictly its triggering cause, and where elevated NK cell levels, together with aPL, may contribute to the development of early RSA.

This new category, in which it is possible that higher numbers of NK cells trigger/precipitate/accelerate damage initiated by aPL antibodies, is represented by the 56% of the APS-RSA patients studied who showed high NK cell levels and fetal losses within the first 10 GWs, two features that are often associated with idiopathic RSA.

Taken together, the data show the existence of two subpopulations of APS-RSA patients distinguishable by the NK cell levels and the clinical behaviour, in terms of GW of abortion. These data lead to a hypothesis that NK cells contribute to the development of RSA in patients with APS. The NK cells might precipitate damage initiated by aPL or they might cause pathology in RSA independent of aPL. Therefore, in all APS patients, dosage of NK cell might prove useful for the evaluation of the possible pregnancy outcome. Further, studies may clarify whether there is a difference in diagnosis and management of the subpopulation with APS, RSA and high NK cell levels as compared to the other APS subpopulations.

Rheumatology key messages

- We report two populations of patients with concomitant APS and RSA distinguishable in terms of NK cell levels and gestational week of abortion.
- We recommend a prospective study to evaluate whether NK cell numbers will serve as a marker of pregnancy outcome in patients with RSA with APS.

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