New Decision-Tree Model for Defining the Risk of Reproductive Failure

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Problem
Natural killer (NK) cells play a key role in embryo implantation and pregnancy success, whereas blood and uterine NK expansions have been involved in the pathophysiology of reproductive failure (RF). Our main goal was to design in a large observational study a tree-model decision for interpretation of risk factors for RF.

Methods of study
A hierarchical multivariate decision model based on a classification and regression tree was developed. NK and NKT-like cell subsets were analyzed by flow cytometry.

Results
By multivariate analysis, blood NK cells expansion was an independent risk factor for RF (both recurrent miscarriages and implantation failures). We propose a new decision-tree model for the risk interpretation of women with RF based on a combination of main risk factors.

Conclusions
Women with age above 35 years and >13% $CD56^{+}CD16^{+}$ NK cells showed the highest risk of further pregnancy loss (100%).

Introduction

Approximately 70% of spontaneous conceptions are lost prior to completion of the first trimester. Implantation failure and pre-clinical losses account for 85% of total pregnancy losses, and clinical miscarriage for 15%.[1] These percentages may underestimate the actual frequency of reproductive failure (RF). It has been estimated that only 22–30% of all conceptions result in a live birth.[2,3] RF is a broad term that encompasses the inability to conceive or the inability to maintain a pregnancy. It includes the following: infertility, repeated implantation failure (RIF) in women undergoing (*in vitro* fertilization) IVF, and recurrent miscarriage (RM).[4] RM is defined by three or more consecutive losses prior to 20 weeks of pregnancy.[5] The observed incidence of RM (1–2%) is much higher than that expected by

chance alone (0.34%).[6] Unlike sporadic miscarriage, RM may occur even if the fetus has a normal karyotype, as up to 50% of RM occurs in normal karyotype fetuses.[7] RIF is defined as three or more failure after the transfer of 2 embryos of good quality, in at least three IVF cycles (embryo transfers) or oocyte donations.[8] Both RM and RIF have numerous causes and clinical presentations and may occur during first trimester of pregnancy,[9] including parental chromosomal abnormalities, anatomic factors, autoimmune diseases, endocrine disorders, and infections, accounting for a 54% of all RM. Indeed, after exclusion for these causes, still half of all cases remain unexplained.[3]

A woman's risk of miscarriage is directly related to the maternal age and outcome of previous pregnancies.[9] However, there is no surrogate marker that may help to define women at risk of pregnancy loss in the next gestation. Thus, the need for biological predictive markers is critical.

Normal implantation and pregnancy requires the finely regulated interplay of reproductive, immune, endocrine, and clotting systems. RM has been defined as a disease of inflammation and coagulation.[10]

Previous studies have explored the functional status of peripheral blood natural killer (NK) cells and have reported that women with RF of unknown etiology have a significantly higher proportion of activated NK cells in peripheral blood and express up-regulated CD69 as compared with those of normal fertile controls.[11,12] Elevated numbers of peripheral blood NK cells and increased infiltration of endometrial NK cells have been reported to be related to pregnancy complications such as pre-eclampsia and miscarriage.[11] In particular, NK cells constitute the predominant leukocyte population in uterine mucosa from embryo implantation, and considerable effort has been made to investigate the phenotype and functions of NK cells at implantation and during pregnancy. Women with RM have increased NK cells in their uterine mucosa than controls,[13] and those with the highest levels have a correspondingly high rate of miscarriage in subsequent pregnancies without treatment.[7] However, the prognostic value of circulating NK cells remains to be elucidated.

There is currently no model proposed for the diagnostic and prognostic classification of women with RF. To address this goal, we show here that a decision-tree approach may define a population of risk for bad pregnancy outcome based on clinical and immunological parameters.

Materials and methods

Study Subjects

Three-hundred and eighteen patients with RF diagnosis (152 with RM and 166 with RIF) were consecutively studied at the participating centers. In parallel, 50 women with proved fertility and regular menstrual cycles were studied as healthy controls (HCs), in days 1–3 and 14 of their menstrual cycles. A full fertility screening was performed for all patients and their partners. This included complete clinical history, physical examination, hormonal analysis, cytology, partner spermiogram, chromosomal abnormality screening, and pelvic ultrasound scan to assess ovarian morphology and the uterine cavity and hysterosalpingography, followed by hysteroscopy or laparoscopy, if indicated. Genetic evaluation included karyotype of parents and test for inherited thrombophilic disorders (factor V Leiden, prothrombin G20210A mutation, serum homocysteine, and deficiencies of the anticoagulant protein C, protein S, and antithrombin III). Hormonal analysis was performed for thyroid-stimulating hormone (TSH), free thyroxin (T4L), prolactin, and progesterone, and immunological screening included measurement of antinuclear antibodies, anticardiolipin and anti-beta-2-glycoprotein I (IgG or IgM) antibodies and lupus anticoagulant, antithyroid antibodies, anti-transglutaminase-2, and NK ($CD3^-CD56^{bright}CD16^-$, $CD3^-CD56^+CD16^+$) and NKT-like ($CD3^+CD56^+$) cell subsets proportions in blood. The Ethics Committee of the institutions approved the protocol, and all subjects provided their written informed consent. We excluded women with immunological disorders like antiphospholipid syndrome.

NK Cell Subset Analysis

Lymphocyte subsets were analyzed using multiparametric flow cytometry, single-platform analysis (TruCount®; FACScalibur BD Biosciences, San Jose, CA, USA). Peripheral blood lymphocytes were stained with the following monoclonal antibodies according to the manufacturer recommendations: CD3-fluorescein isothiocyanate (FITC); CD4-allophycocyanin (APC); CD8-phycoerythrin (PE); cocktail with CD16-PE/CD56-PE; CD19-APC; and CD45-peridinin chlorophyll protein (PerCP; BD Biosciences).

Simultaneously, NK cell subsets were analyzed in a separate tube. Briefly, fresh whole-blood samples

(100 μL and 1 × 10^6 cells, respectively) were direct stained with fluorescence-conjugated CD69-(FITC) (BD Biosciences), CD16-(PE) (BD Biosciences), CD3-(PerCP) (BD Biosciences), and CD56-(APC) (BD Biosciences). The cells were incubated in dark at room temperature (RT) for 20 min. In the next step, red blood cells were lysed by the addition of 2 mL of lysing solution (FACS™-Lysing Solution; Becton Dickinson, San Jose, CA, USA), incubated for 15 min in dark, and then removed and washed with 2 mL phosphate-buffered saline (PBS). In the last step, a 4-color analysis was carried out using FACScalibur flow cytometer (Becton Dickinson), using a CellQuest research (Becton Dickinson) and FlowJo (Tree Star, Ashland, OR, USA) softwares. The gate was set for both FSC and SCC and included lymphocytes. A total of 20,000 events in the lymphocyte gate were acquired for each sample. NKT-like cells were analyzed in parallel on total lymphocytes, measured as the percentage of $CD3^+CD56^+$. After further gating on CD3-negative cells, the percentage of $CD3^-CD56^{bright}$ $CD16^-$ and $CD3^-CD56^{dim}CD16^+$ (double positive, DP) NK cell subsets in gate R2 were measured in the region of R3 and R4 (Fig. 1). The control and study sample were stained and analyzed at the same time.

Statistical Analysis

Proportions were compared using the two-tailed test or Fisher's exact test for expected values below 5. Quantitative variables were expressed as means ± standard deviation, median, and interquartile range (IQR) and compared using the Student's *t*-test, ANOVA, or the non-parametric Wilcoxon rank sum test. Receiver operating characteristic (ROC) curves were used to select the optimal cutoff values of significant variables for predicting the development of pregnancy loss based on the optimum sensitivity and specificity.[14] The best cutoff value provides both the highest combination of sensitivity and specificity. Data were analyzed with SPSS v.19 software (Chicago, IL, USA). A *P* value of 0.05 was considered as statistically significant.

We analyzed and compared the data and studied the predictive value of maternal age and total NK

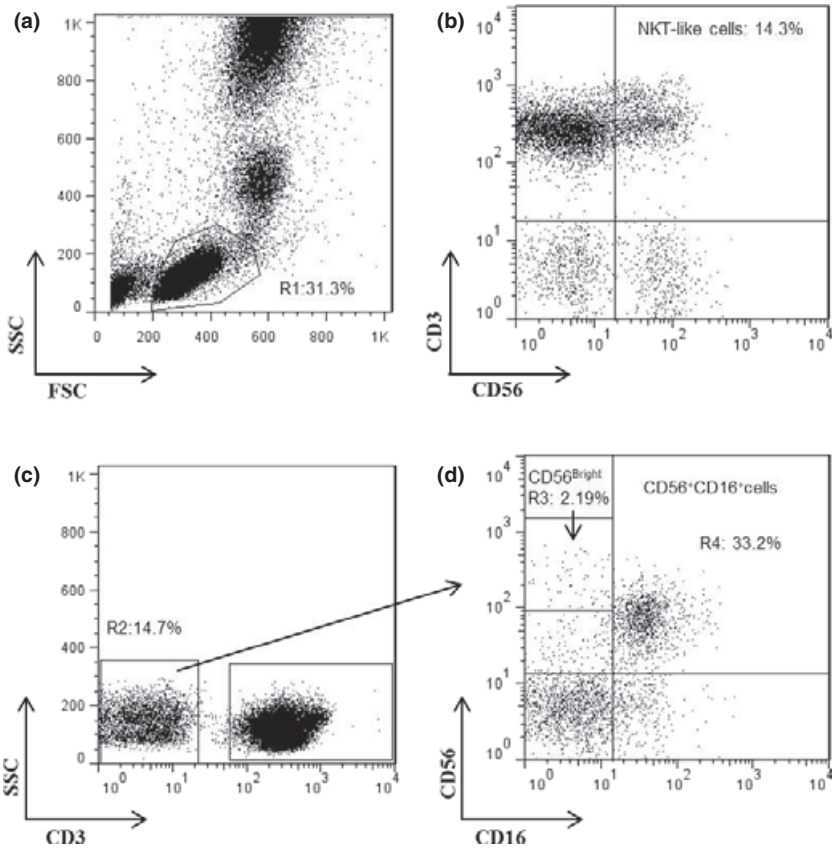


Fig. 1 Gating strategy for natural killer (NK) cell subsets. (a) Peripheral blood events were measured against forward and side scatter parameters. R1 contains total lymphocytes. (b) NKT-like cells were analyzed gating on lymphocytes cells (R1). (c) Cells negative for CD3 lie in R2 ($CD3^-$ gate). (d) Cells contained within R2 were further displayed on a plot of CD16 versus CD56 expression. Cells negative for CD16 and positive for CD56 are CD3-$CD56^{bright}$ CD16- NK cells lying within R3. Those CD3-$CD56^{dim}$CD16+ NK cells lie within R4.

cells, NK cell subsets, and NKT-like cells for the risk of recurrent gestational failures. We developed a hierarchical multivariate decision model based on a binary classification and regression tree, which attempts to maximize within-node homogeneity, measured by the Gini index. This method allowed us to take into account also the observations with missing values for some of the predictive variables, using surrogate classification rules. We assumed a base prevalence of miscarriage of 10% of the population.[15]

Results

We studied 318 women with RF (mean maternal age, 37.5 ± 3.54 years). Women with a history of RM ($n = 152$) (mean maternal age, 36.48 ± 3.63 years) and RIF ($n = 166$) (mean maternal age, 37.55 ± 3.36 years), who were consecutively studied at the Clinical Immunology Unit at Hospital General Universitario Gregorio Marañón and at Clinica Tambre, comprise the study group. A control group of 50 fertile women with regular menstrual cycles were studied at days 1–3 and at day 14 (ovulation) of their menstrual cycle and most of them on both of these days (mean maternal age, 30.75 ± 6.65 years).

NK Cell Changes Within the Menstrual Cycle

Compatible with previously reported data,[16] no significant differences neither for the values of the total NK cells nor for the NK cell subsets between the days 1–3 and 14 of the menstrual cycle of the control group of normal fertile women were found (Fig. 2).[16] In parallel, according to van den Heuvel et al.,[17] no statistically significant differences in the percentage of NKT-like cells during the two phases of menstrual cycle were observed. Therefore, we inferred that NK cell subsets could be measured at any time point of the menstrual cycle without significant modifications on these cells and that NK cells in the group of patients can be compared without any bias prior to conception.

Peripheral NK Cell Subsets in Women with RF and Controls

We then compared whether there were differences in total NK and NKT-like cell subsets between RF patients and HCs. We observed that total NK cells were significantly higher in RF patients compared with the controls ($P < 0.0001$) (Fig. 3a,b). Similarly,

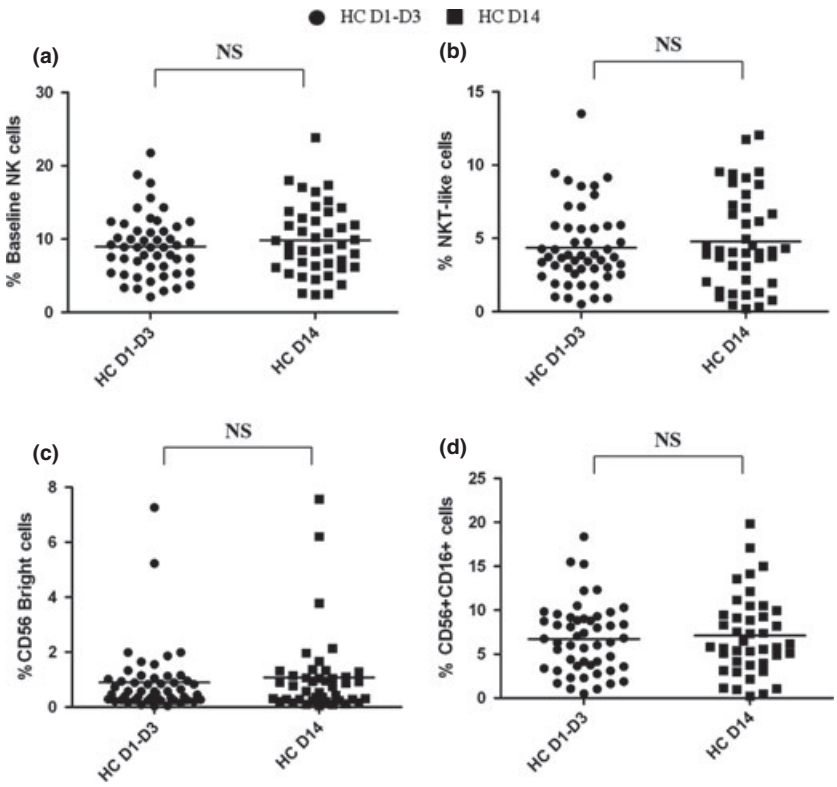


Fig. 2 No differences were noted either in total natural killer (NK) cells or NKT cells or in NK cell subsets between the days 1–3 and 14 of the menstrual cycle. Box plot showing the percentage of (a) baseline NK cells, (b) NKT-like cells, (c) $CD56^{bright}$ NK subset, and (d) $CD56^{+}CD16^{+}$ NK subset in peripheral blood from healthy control. Healthy control D1–D3 (HC D1) and HC D14 (HC D14). Each dot represents a woman of control group. The bar represents the median value for (a, c and d), and the geometrical mean for (b).

(a)

	RF (n = 318)	RM (n = 152)	RIF (n = 166)	HC (n = 50)
NK cells	13.11±5.98*** 12 (8.56)	13.35±5.60*** 13 (7.65)	12.88±6.31*** 12 (9)	9.37±4.43 8.90 (5.9)
CD56+CD16+ cells	10.58±5.67*** 9.46 (7.98)	9.21±4.67* 8.77 (7.77)	11.26±6.02*** 10.05 (9.96)	6.90±4.21 6.34 (5.56)
CD56 bright cells	0.71±0.50 0.70 (0.65)	0.79±0.63 0.80 (0.64)	0.67±0.43 0.61 (0.65)	0.98±1.35 0.58 (0.83)
NKT-like cells	4.80±4.03 4 (4)	5.11±4.09 4 (4.9)	4.58±3.99 3.34 (4.42)	4.55±2.92 3.90 (3.7)

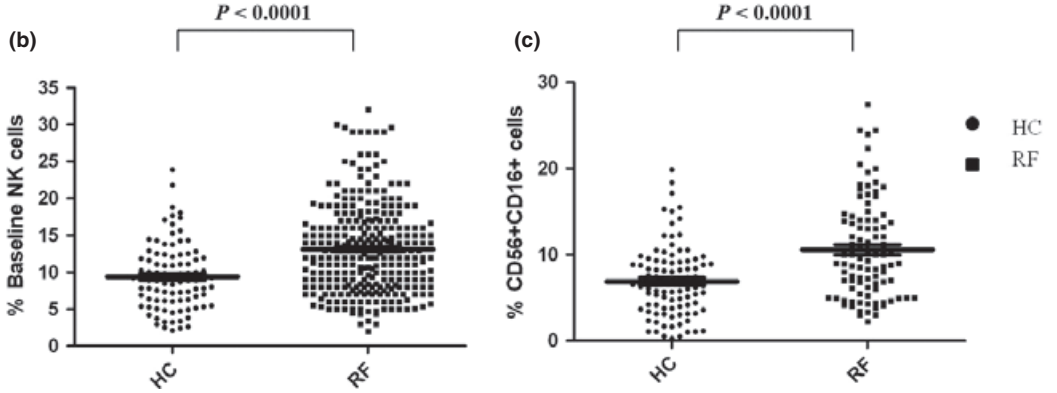


Fig. 3 (a) Percentages of total natural killer (NK) cells, NKT-like cells, and NK subsets in women with reproductive failure (RF), recurrent miscarriages (RM), recurrent implantation failure (RIF), and healthy controls. Values are expressed as mean ± S.D. and median (IQR). *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ with respect to HC. (b and c) Proportions of baseline NK cells and double-positive NK subset in women with RF and HC. Statistical analysis was made by Student's *t*-test between the study and the control groups. Each dot represents a woman of the study (RF) and control groups. The bar represents the median value. $P < 0.05$ was considered to be statistically significant.

DPNK cell subsets were significantly higher in a RF compared with controls ($P < 0.0001$) (Fig. 3c). By contrast, no significant differences were observed in $CD56^{bright}$ and in NKT-like cells between both groups.

To confirm whether the differences observed in RF patients were similar in patients with RM and RIF, we compared women with RM and RIF separately with the control group. The % NK cells were significantly higher in women with RM and/or RIF ($P < 0.0001$ and $P < 0.0001$, respectively) than in the control group.

When compared by separate groups, the RIF group and the RM showed higher DPNK cells than HC ($P < 0.0001$ and $P < 0.05$, respectively, Fig. 3a). No differences were obtained for either $CD56^{bright}$ or NKT-like cells in patients with RM and RIF.

Risk Factors for RF

Using ROC curves, the statistically chosen cutoff value for NK cells in our RF population was 18% of total lymphocytes, with sensitivity for the occurrence of pregnancy loss of 19% and specificity of 95.7%. Patients with NK cells >18% had 5.1-fold (hazard ratio, HR 95% CI: 1.81–14.62) higher risk of having pregnancy loss (Fig. 4). By Cox multiple regression analysis, NK cells were independently associated with increased risk of pregnancy loss after adjustment for maternal age.

When we analyzed all the variables by bivariant analysis, maternal age, NK and DP cells proportions through ROC curves showed a highly significant predictive value for gestational failures, with an increasing risk of higher values, while NKT and $CD56^{bright}$ showed a significant predictive value with an increasing risk of lower values (Table I).

New Model of Decision Tree for RF

The best performing diagnostic and prognostic algorithm is shown in Fig. 5. The tree thus begins with 318 patients and 92 HCs at the root. As expected,

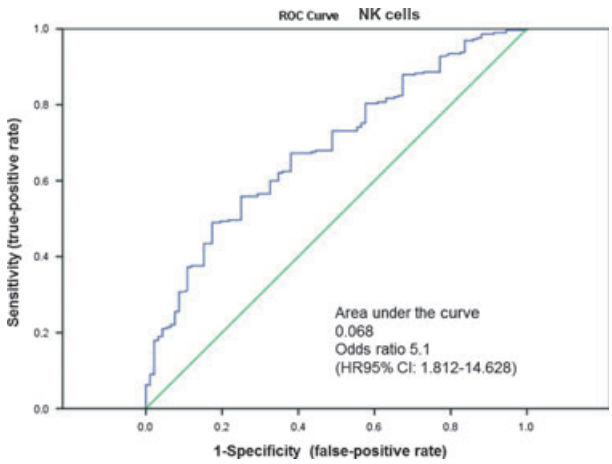


Fig. 4 Receiver operator characteristic (ROC) curve for total circulating natural killer (NK) cells as predictor of further pregnancy loss. The curve describes the association between sensitivity and specificity. The curve shows the tradeoff between sensitivity and specificity. An increase in sensitivity will be accompanied by a decrease in specificity. The accuracy of the prediction increases as the curve approaches the left hand of the top portions of the ROC space. The area under the curve (AUC) is the percentage of randomly drawn pairs for which the prediction is true.

the maternal age was the strongest predictor and determinates also the first split in the multivariate classification mode. Based on maternal age, the RF patients and controls were classified as women below 35 years of age, represented in node 1, or above 35 years included in node 2. We found a risk of RF more than fivefold higher for the women aged more than 35 years (i.e., after the 35th birthday) at node 2 than for the younger ones at node 1.

First of all, we focus our attention in node 1. For the younger women, total NK and DP cells appear as equivalent predictors due to their strong correlation. To facilitate the reading of the tree, we have removed node 3 and we have represented a triple branch comprising a range of values for the NK% between 13 and 24%. The same is true for the DP% whose range is between 11 and 22%. Among the 170 observations below 35 years, we observed very low risk for NK $\leq$ 13% and/or $CD56^+CD16^+ \leq 11\%$ in node 7. Node 8 represents an intermediate risk with a percentage similar to the general population (12%). Node 4 defines the population of women with the higher risk, with 8 registers (100%) belonging to this group of patients with recurrent gestational failures with NK >24%/$CD56^+CD16^+$ >22%.

For women above 35 years (node 2), the DP subset was found to be a better predictor, while NK cells can be used as a surrogate marker. This could be explained by the fact that for patients with RF aged more than 35 years, % $CD56^+CD16^+$ (DPNK) cells represent the 84% of total NK, whereas only the 55.6% of total NK in HC. Node 6 defines the population with higher risk of RF (100%). We observed 50 registries belonging to the group of patients with recurrent gestational failures with $CD56^+CD16^+$ (DPNK) cells >13% and NK cells >18%.

We then designed decision trees for each clinical entity (RM and RIF) separately. The decision-tree model obtained for RIF is structurally similar to that for RF, with age, %NK, and %$CD56^+CD16^+$ cells as splitting variables.

In the case of RM, a decision-tree model was based on age with surrogate variable of % NK cells (18%). For RM women, % NKT-like cells appear as a second splitting variable, with % NKT-like cells above 10% showing higher risk of pregnancy loss (data not shown).

Discussion

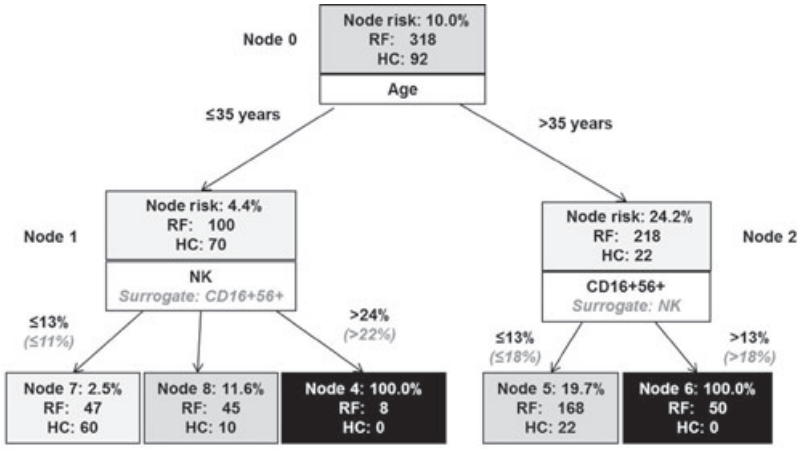
To our knowledge, this is the first decision-tree modeling to identify prognostic markers for RF. Importantly; it provides a immunological surrogated biomarker associated with RF, easy to perform and reliable. Here we show that a cutoff value for NK > 18% is the best variable to discriminate

Table I Optimal Cutoff Values for All Parameters That Have Been Studied

Predictor	Cutoff value	Area (%)	Sensitivity (%)	Specificity (%)	Odds ratio	PPV (%)	NPV (%)
Maternal Age (year)	35	79	74.3	75.3	6.9	90.7	41.6
CD56+CD16+%	13	69	31.3	91.3	4.7	89.9	56
Total NK%	18	68	19	95.7	5.1	93.2	27.2
NKT-like%	3.01	51	42.5	69.6	1.69	13.4	91.6
CD56Bright%	1.01	50	90.3	30.4	4.07	12.6	96.6

Increasing risk for large values, increasing risk for small values, Positive predictive value (PPV) and Negative predictive value (NPV).

(a)



(b)

Terminal node	Node risk	Accumulative (when adding the line´s node to the positive prediction)					
		Error rate	PPV	NPV	Odds ratio	Confidence interval	
7	2.5%	90%	10%	No NP	No negative prediction		
8	12.6%	32.8%	21.4%	97.5%	10.81	6.37	18.35
4	100%	8.2%	100%	91.7%	No false positive		
5	19.7%	24.4%	24.8%	95.9%	7.82	4.57	13.37
6	100%	8.4%	100%	91.4%	No false positive		

Fig. 5 Decision tree for predicting the risk of reproductive failure (calculated on 318 patients with data obtained before a new pregnancy). (a) Decision-tree model. (b) We show in the table the mean features of terminal node. Node risk represents the percentage of general population that meets the specifications of the node could suffer RF. Positive predictive value (PPV) and negative predictive value (NPV).

women with RF and normal controls, and we suggest a cutoff for $CD56^+CD16^+$ (DPNK) cells above 13% in women older than 35 years defining a subgroup of patients with highest risk of a further pregnancy loss.

In our large cohort of RF patients, NK cells were an independent risk factor for RF, further confirming the previously described association between high blood NK and RF prognosis.[11,18–20] During the last years, different cutoff levels of blood % NK cells have been proposed: In 1995, Coulam et al. established >12% value of peripheral NK ($n = 96$).[21–23] Later on, in 2003, Yamada et al. used <16.4% pNK ($n = 113$) as the normal range for optimal discrimination between miscarriage and live birth.[24,25] In 2007, Perricone et al. showed that >15% of peripheral NK ($n = 77$) were associated with increased risk of RM.[20,26,27] We have settled a cutoff level of 18% of total NK cells ($n = 318$) as the best marker defining risk of RF in the Spanish population.

Most previous studies have mainly focused on the total NK population. However, we believe that the subsets of NK and the $CD56^+CD16^+$ NK (DPNK) cells subset in particular may be a more accurate biomarker for RF. We have defined a value of % $CD56^+CD16^+$ cells above 13% defining a subgroup of patients with higher risk of a further pregnancy loss. Our results support the concept that $CD56^+CD16^+$ cytotoxic cell subsets are the main risk biomarker for predicting RF as already described by Lu et al.[18].

During the past two decades, clinicians who manage women with RF have used different clinical assays to determine a possible diagnosis and treatment. Among these, immunological tests are performed to discard the presence of antinuclear antibodies, antiphospholipid antibodies, antithyroid antibodies, and serology for celiac disease. Recently, tests for KIR receptors and HLA-C ligands, NK cell counts and NK cytotoxicity assays, and Th1/Th2 cytokine ratios have been proposed.[28] Despite the intense interest in this potential immune-based pathophysiology for RF, there is no consensus on the appropriate diagnostic workup or therapy.

Tree decision analysis is considered the best tool for integrating the most relevant variables in evidence-based medicine. When we investigated the main variables for the diagnosis of women with RF, our decision tree proposes for women with ≤ 35 years, a gradation of risk groups with an interval of values 13–24% of NK cells. However, age above 35 years defines a more accurate 'yes' versus 'no' option that seems to be more practical. Our findings indicate that being younger than 35 years

and having more than 24% of NK cells and being above 35 years and having more than 18% of NK cells seem to define the highest risk groups of RF. Both groups may have an underlying immunological alteration associated with RF. Moreover, this subgroup of patients might define good candidates for immunomodulatory therapeutic strategy.

Given that there were no significant differences between RM and RIF, we chose that the best tree-model representing RF delineated the predictive risk of pregnancy loss for a given woman with history of RF, considering other variables in the routine clinical practice. The first step in prevention of pregnancy loss is to define risk factors for pregnancy loss. The personal risk of a given patient will be the net result of the different obstetrical, hematological, and immunological studies that should be balanced. We propose a new diagnostic and prognostic decision-making model for RF when facing with a patient before planning a new pregnancy.

The RF associated with immunological alterations represents over 70–80% of total cases with unexplained causes.[29] In RF, an imbalance between the Th1 and Th2 immune response, resulting in a prevalent Th1 cytokine environment in the periphery, may lead to NK cell activation and proliferation, which could result in migration of cytotoxic NK cells into the uterus that in turn contributes to the inflammatory mechanisms driving to miscarriage.[30]

What are the possible roles of NK cells in pregnancy? Several studies showed that NK cells play important roles in pregnancy since the very early moment of embryo implantation, such as immunosurveillance, angiogenesis, remodeling of the spiral arteries to uteroplacental arteries, supporting proper trophoblast and placental growth, vascularization of the decidua, and production of immunomodulatory molecules. Nowadays, several pathological roles have been described for the NK cells, for instance killing cytolytic activity, inhibiting placental hCG secretion, complement activation, cytokine imbalance, failure in the generation of Th2-type responses, and activation of APCs leading to the development of pathogenic Th1 responses. However, the exact mechanism that leads to fetal lost is still unknown.

In relation to the above, we propose the following question: Is peripheral NK cell population a good biomarker for predicting women who suffer RF? NK cells have been recently identified among the relevant immunological factors for reproductive success. Nowadays, we distinguish three types of NK cells (peripheral, endometrial, and decidual).[31] Two subsets of NK cells are found in the peripheral blood: $CD56^{+}CD16^{+}$, which make up approximately 90–95% of the total NK, and $CD56^{Bright}CD16^{-}$, which make up only about 5% of peripheral blood lymphocytes. Endometrial NK (eNK) cells have not been fully characterized.[32] Human eNK cells are present in the non-gravid endometrium throughout the menstrual cycle.

Decidual NK cells (dNK) have been extensively studied. Approximately 70% of decidual lymphocytes are NK cells of the $CD56^{Bright}CD16^{-}$ phenotype.[33] The origin of decidual $CD56^{bright}$ NK cells remains unknown, but because they closely resemble the minor $CD56^{bright}$ NK cell population in the blood, one possibility was suggested that this blood NK cells population might migrate into the uterus to proliferate, differentiate, enlarge, and acquire cytoplasmic granules in the hormone-rich mucosal microenvironment.[19] The processes of implantation and placentation are closely linked to each other and involve the direct contact of fetal cells and tissues with maternal cells and tissues. When the onset of maternal blood flow into intervillous space of the placenta at the end of the first trimester finally enables the direct physical contact between maternal circulating immune cells and the placental syncytiotrophoblast,[34] so peripheral blood NK cells circulate around the implantation site and are in direct contact with trophoblast cells.[11] Park et al.[20] showed that the number of $CD56^{+}$ NK cells in decidual tissues was significantly correlated with the number of $CD56^{+}CD16^{+}$ NK cells in own peripheral blood. This indicates that peripheral NK cells reflect decidual cell changes. Therefore, if peripheral NK, and specifically the $CD56^{+}$ $CD16^{+}$ cells subset, can reflect changes in the decidual cells, the clinicians could use them as a predictive biomarker of RF, both in cases of RM and RIF.

In conclusion, we have proposed a useful tool for the classification of risk in women diagnosed of RF by a multivariate decision-tree model. Finally, we propose that NK cells and $CD56^{+}CD16^{+}$ NK cells are the main biomarkers for diagnosis of women with RF.

Authors roles

RRM and SSR designed research, analyzed data, and wrote the manuscript; RRM, BA, and MTA performed experiments and analyzed data. AGS was

involved in study design, data interpretation, and critical discussion; JAL and JA contributed to all revisions of the manuscript, supervised the data analysis, and critically revised the manuscript for important intellectual content; AS contributed to statistical analysis. JG, DO, AV, ECA, EFC, LOQ, JC, and PC participated in critical discussions. JDC contributed to sample collection. All authors revised the manuscript and approved the final version.

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Disclosures

The authors declare that they have no competing financial interest.

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