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A blinded multicenter investigation: Accentuated NK lymphocyte CD335 (NKp46) expression predicts reproductive failures after IVF.

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ABSTRACT

The peripheral blood NK cell diversity is highly complex. Recent studies have described more than a thousand phenotypes sharing NK cell receptors (NKRs), across the leukocyte lineages. Previously, we have found that accentuated NK p46 phenotype has prognostic value for NK cytotoxicity status, and is characteristic for patients with recurrent implantation failure (RIF).

In a blinded investigation we studied blood samples from IVF women before embryo transfer (pre-implantation genetic tested [PGT] embryos $n = 116$; not tested embryos $n = 219$). We studied NKp46 expression by flow cytometry and anti-cardiolipin antibody (aCL) levels. aCL results were transmitted to the clinic but NKp46 expression was blinded (for us and for the clinic) and not analyzed before termination of the study (end of last pregnancy). Association of NKp46 phenotype with clinical pregnancy rate (CPR), pregnancy failure (PF) rate and life birth rate (LBR) were analyzed. aCL positive and IvIg treated cases were excluded.

IVF success was dependent on p46 NK phenotype in patients with PGT embryos. Elevated p46 expression on NK (>93%) as well as decreased (<66%) significantly reduce CPR (OR 12.7 and 3.8) without affecting pregnancy failure frequency. Both accentuations (taken together) resulted in a significant reduction of LBR (OR 3.9 $p = 0.019$) compared with non-accentuated phenotypes (p46 levels 66–93%). Elevated NK cell levels (>14.5% weakly) were associated with PF (OR 3.1 $p = 0.069$), but not significantly with reduced LBR. In contrast, numbers of NKCD335+ lymphocytes (>11.5%) were a significant predictor of PF (OR-4.0 $p<0.05$) and decreased LBR (OR 2.1 $p = 0.06$). At the same time, accentuated numbers of NKCD335neg lymphocytes (<0.7 and >4%) were also associated with decreased LBR (OR 2,65 $p = 0.05$). In patients with NKCD335++ numbers (<5 and >21%), we found a weakly association with IVF failure. We found similar associations in IVF patients without PGT -A but at lower significance levels regardless the higher number of patients. Impact of NKp46 phenotype for IVF success was significant in patients with donor's ET and almost imperceptible in patients > 35y.o. with own embryo transfer.

Accentuated increased or decreased CD335 expression on NK was associated with embryo implantation failure. Balanced CD335 levels form a condition favorable for implantation. Elevated numbers of p46+NK (CD3-CD56+CD335+) predicts pregnancy failures at higher significance levels than elevated NK cell numbers. Elevated numbers of p46negNK (CD3-CD56+CD335-) indicate reduced LBR. Accentuation of p46 expression on

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NK cells is associated with reproductive failures. In combination with PGD it provides a powerful prediction algorithm and treatment option.

1. Introduction

Innate lymphoid cells (ILCs) are functionally heterogeneous because of having high cellular plasticity, maintaining tissue homeostasis and playing important effector role during disease [1]. The most researched part of this population is Natural Killer lymphocytes subsets (CD3negCD56pos). Natural killer (NK) cells are effector and regulatory lymphocytes of the innate immune system that contribute to tumor surveillance, hematopoietic allograft rejection, control of viral and microbial infections, and reproduction [2].

The role of NK cells in human reproduction has been studied for decades. NK cells play a critical role in reproductive processes such as implantation, trophoblast invasion, and spiral artery remodeling [3]. Many of the studies demonstrate a close relationship between the accentuated parameters of peripheral blood or endometrium NK cells and both quantitative or functional and reproductive failures, such as, recurrent implantation failures (RIF) and recurrent pregnancy loss (RPL) [4–9].

Everyone agrees that the NK function is extremely important for the successful course of the reproductive process, but the main discussion revolves around whether it is possible to evaluate these functions and which one should be evaluated [10, 11].

The activity of NK cells is tightly regulated by a combination of cell surface-expressed inhibiting and activating receptors. CD335 (NKp46) is a major NK cell-activating receptor that is involved in the elimination of target cells [12, 13] and NK cell maturation [2].

Previously, we have found that a fraction of NKp46+expression has prognostic value of accentuated NK cytotoxicity status, both low and high [14]. Some studies suggest that accentuations of NKp46 expression associated with reproductive failure, such as RPL and implantation failures [15, 16]. We also fined that NKp46 expression accentuated in patients with repeated implantation failures (with pre-implantation genetic diagnosis [PGD] tested embryos) compared to fertile control [17]. But it was not clear whether accentuation of p46 was the cause of failure or the result of numerous unsuccessful attempts to implant.

In these blinded multicenter investigation, we study diagnostic prognostic value of NK p46 expression phenotype for subsequent reproductive success.

2. Methods

2.1. Blind research design

Patients were tested on anticardiolipin (aCL) IgG antibody and NKp46 phenotype. The results of aCL antibodies were returned to the clinic and patients with high levels (> 20GPL) were excluded from further analysis. 16 patients with a level of ACL, which was > 20GPL, were excluded. The NKp46 phenotype results were remained in the primary data files (CellQest BDBioscience) and were not analyzed until the end of the study, staying blinded. The end of the study was the end of the pregnancy of the last patient admitted to the study. After the end of the clinical phase, the results of NKp46 were analyzed and sent to medical institutions. In parallel, the results were sent to independent experts (Dosenko VE., OscovskyVI., Veselovsky VV). Similarly, the clinical results of IVF results and pregnancy ongoing were sent to the laboratory and independent experts for analysis.

Patients, younger than 39 years old, were negative for anticardiolipin antibodies, with normal karyotype and without endometrial insufficiency and thrombotic disorders. Patients did not obtain any additional treatment. (In PGT-A tested group and Donors Oocytes patients were younger than 42 years old)

We analyzed implantation rate (IR) per ET as a part of hCG positive cases per ET (>50 u/ml on the 14th day after ET). Biochemical, ectopic, and anembryonic pregnancies were included in IR and annualized as separate groups and were not included in Clinical Pregnancy rate calculation. Biochemical pregnancies were calculated as hCG positive cases that were not confirmed as an egg in the uterus by ultrasound scans (US) on the fifth week.

Clinical Pregnancy rate (CPR) was calculated as percentages of the ongoing pregnancy (gestational sac in uterus by US on 5 wk.) per ET.

Pregnancy failures were calculated as a part of the failed pregnancy of the confirmed ongoing pregnancy.

Live birth rate (LBR) is as part of births of alive baby after 24 w/g per ET.

We did not obtain clinical information about subsequent pregnancy ongoing in some patients. We exclude them from the calculation of CPR or LBR.

2.2. Patients groups

320 IVF patients were examined according to the scheme. Testing on p46 NK phenotype was performed on 3th – 5th days before the planned ET by single frozen/vitrificated blastocyst stage embryo. Patients, who were previously selected for study by the doctors' opinion, had a high chance of a positive IVF result and did not require additional research and treatment. (not have RPL or RIF in anamnesis, uterine anomalies and endometrium thickness >7 mm) According to the type of ET and age, we divided the patients into 4 clinical groups. Table 1**.** Patients who successfully became pregnant after PGT embryo transfer had a worse obstetric history (increased pregnancy failures in anamnesis). It's possible that in this group of PGT-A technology results to elimination of the cause of previous failures in significant part of population. While in all other groups the difference between groups was not significant, but the trends are expected. Significant better embryo quality (AB/AA embryo's ratio) was registered patients with an embryo transfer of a donor's egg with subsequent successfully gave birth.

All the study subjects had signed informed consent before they entered the study.

2.3. The protocol of endometrium preparing before the embryo transfer

A-GnRH (Dipherelin 3,75 mg, IPSEN Pharma, France) was administered in the mid luteal phase of the cycle (19th −24th day). In 14–16 days, but not earlier than 3rd day of the next cycle, estradiol valeriate (Proginova, 2 mg, Delfarm Lille, France for Schering AG, Germany) at a daily dosage of 6 mg was prescribed. In12–14 days the ultrasound examination was performed to assess the thickness of endometrium. If endometrium was less than 7 mm, the medication with estrogens was continued for one-two weeks till endometrium rich the thickness of 7 mm or more. After endometrium reached at least 7 mm, micronized progesterone (Utrogestan 200 mg, Besin Healthcare, Spain) was added in a 400 mg daily dosage. The embryo transfer was performed on the 7th day of progesterone administration. Medical support with progesterone and estradiol valerate in the same doses was continued for another 14 days after embryo transfer. Then the patient took a blood test for hCG, and if positive, hormonal support was continued for another 14 days. And an ultrasound examination was performed to detect gestational sac, embryo pale and heart beating. Later, the patients were referred to women's clinics for observation. Data on the course of pregnancy, delivery and the consequences of childbirth were monitored and recorded.

Pre-Implantation Genetic Testing for Aneuploidy (PGT-A) of the 5th days blastocyst -stage embryos was performed using next-generation

sequencing technology.

2.4. PGT-A tested group

Patients who had a transfer of the PGT-A tested embryos E ($n = 118$) were analyzed separately. As a result, in this group pregnancies did not occur in 61 patients, pregnancies were terminated in 14 patients, and 43 patients successfully gave birth to alive children. Three patients who successfully gave birth were excluded from further analysis because they were given intravenous immunoglobulin during the first trimester of pregnancy (contrary to the wishes and conditions of the study).

2.5. PGT untested groups

- Donors Oocytes group

47 patients with an embryo transfer of a donor's egg were examined according to the same protocol as the previous group. As a result, pregnancies did not occur in 21 patients, 7 pregnancies ended with miscarriage and 15 patients successfully gave birth to alive children.

- IVF groups

Patients, who had a transfer of their own PGT untested embryos were divided by age. 70 patients were younger than 35 years (IVF <35 group) at the time of ET and 71 patients were older than 35 years but younger than 39 years (IVF> 35 group). All patients had ET of their own AA or AB quality embryo.

2.5.1. Assessment of NKp46 expression

We analyzed CD335 phenotype as described previously [14,17]. 5 ml of peripheral blood was collected and delivered to the laboratory no later than 24 h after the collection. To determine NKp46 expression, 100μl of whole blood were stained by FITC-, PE- and -Cy5-conjugated monoclonal antibodies to CD3, CD335(NKp46) and CD56 (BD Bioscience, San Jose, USA). Lysed and washed samples were analyzed by FACScan flow cytometer using CellQuest software (BD Bioscience, San Jose, USA).

2.5.2. Gating strategy for NKp46^{+}NK cells

Baseline NKp46 expression on NK cells was assessed by flow cytometry. CD3negative CD56+ NK cells were gated from the total lymphocyte population –%NK (Fig 1). In all samples, CD56bright NK cells almost totally expressed high levels of NKp46 (more 90% of CD56++ was CD335++). Three different CD56dim NK CD335(NKp46) phenotypes were revealed - NKp46high, NKp46medium, and NKp46neg predominance (Fig 1).

The percentage of NKp46^{+}NKcells (%NKp46^{+}NK) among all lymphocyte was determined as both - NKp46^{high} and NKp46^{medium} subsets (Fig. 1) (for manual gating we use CD56++ as a mark for CD335++) and CD3+ population as control for CD335negative population) a In parallel to manual gating, standard invariant gating for NKp46^{high}, NKp46^{medium}, and NKp46^{neg} enumeration were conducted (Fig. 1F) .

2.6. Anti-cardiolipin antibodies measurement

The aCL ELISA was performed according to our previous publication [18] and was sensitive to β2GP1-independent and β2GP1-dependent aCLa. Polystyrene plate (polySorp, Nunc, Roskilde, Denmark) were covered within 20 μl/well of 50 μg/ml cardiolipin (CL) (Sigma-Aldrich, Steinheim, Germany) in ethanol or ethanol alone (as control well). After that, wells were blocked by 1.0% Bovine serum albumin (BSA) in 0.05 M Trizma buffer pH-8.5. Serum samples were instilled to the well for an hour after diluting1:50 in RPMI medium with 0.5% BSA, 0.01% Tween 20. The amount of bounded aCL Ab was found out by incubation with horseradish peroxidase-conjugated goat anti-human IgG (λ chain specific) and reaction of peroxidase with TMB. Optical density (OD) was read at λ = 450 nm using a Multiscan (LabSystems, Finland). Values beloved 10 GPL for CL were considered as negative (>20GPL as positive) and were exclude from investigation.

2.7. Statistical analysis

The statistical analysis of the results was performed using Fisher's Exact Test (unpaired, non-parametric, two-sided P value) and the Spearman and Pearson correlations (In Stat version 3.0 for Windows Graph Pad Software Inc., San Diego, CA, USA).

3. Results

Majority (90.9%) of patients from all groups with subsequent successful Life Birth have NK CD335 expression in "favorable corridor" >66% but <93% (Fig 2A). In contrast, only 76.7% of patients with subsequent IVF failures have same balanced levels of CD335 expression (Fig. 2B). in PGT-A group this difference was significant (Odds ratio=

Table 1
Clinical and anamnestic parameters of investigated IVF groups.

Groups	IVF results	Age y.	BMI	IVF/ET cycle by score	AB/AA embryo's ratio	Pregnancy failures in anamnesis	Live birth in anamnesis	Secondary infertility %
PGT	implantation failure $n = 61$	36.8	22.1	1.72	1	0.83	0.52	63.6
	pregnancy failure $n = 14$	35.9	23.4	1.42	0.86	1	0.92	63.6
	live birth $n = 40$	36.5	22.1	1.45	0.93	**1.45** *	0.45	73.5
DO	implantation failure $n = 21$	39.6	23.6	1.86	0.96	1.36	0,52	65
	pregnancy failure $n = 7$	40.2	22.4	2.29	0.94	0.71	0.53	57.1
	live birth $n = 15$	37.3	24.7	1.53	**0.71***	1.46	0.6	61.7
IVF<35	implantation failure $n = 27$	29.7	22.5	1.83	1.07	0.18	0.03	55.5
	pregnancy failure $n = 8$	31.5	22.3	1.66	1.13	0.35	0	62.5
	live birth $n = 35$	30.6	22.1	1.53	0.93	0.08	0.14	57.1
IVF>35	implantation failure $n = 42$	37.3	23.9	1,7	1.26	0.23	0.16	52.3
	pregnancy failure $n = 9$	36.8	24.4	1.4	0.96	0.11	0.11	44.4
	live birth $n = 20$	38.2	23.5	1.88	0,97	0.15	0.45	60

PGT –(pre implantation genetic testing PGT-A), **DO** – (donated oocyte).
IVF<35 -(self embryo and younger 35y/o), **IVF>35** -(self embryo and older 35y/o).
BMI body mass index, AB/AA embryo's ratio- proportion of AB quality embryos that transferred to AA quality. *$P < 0.05$ compared to implantation failure group.

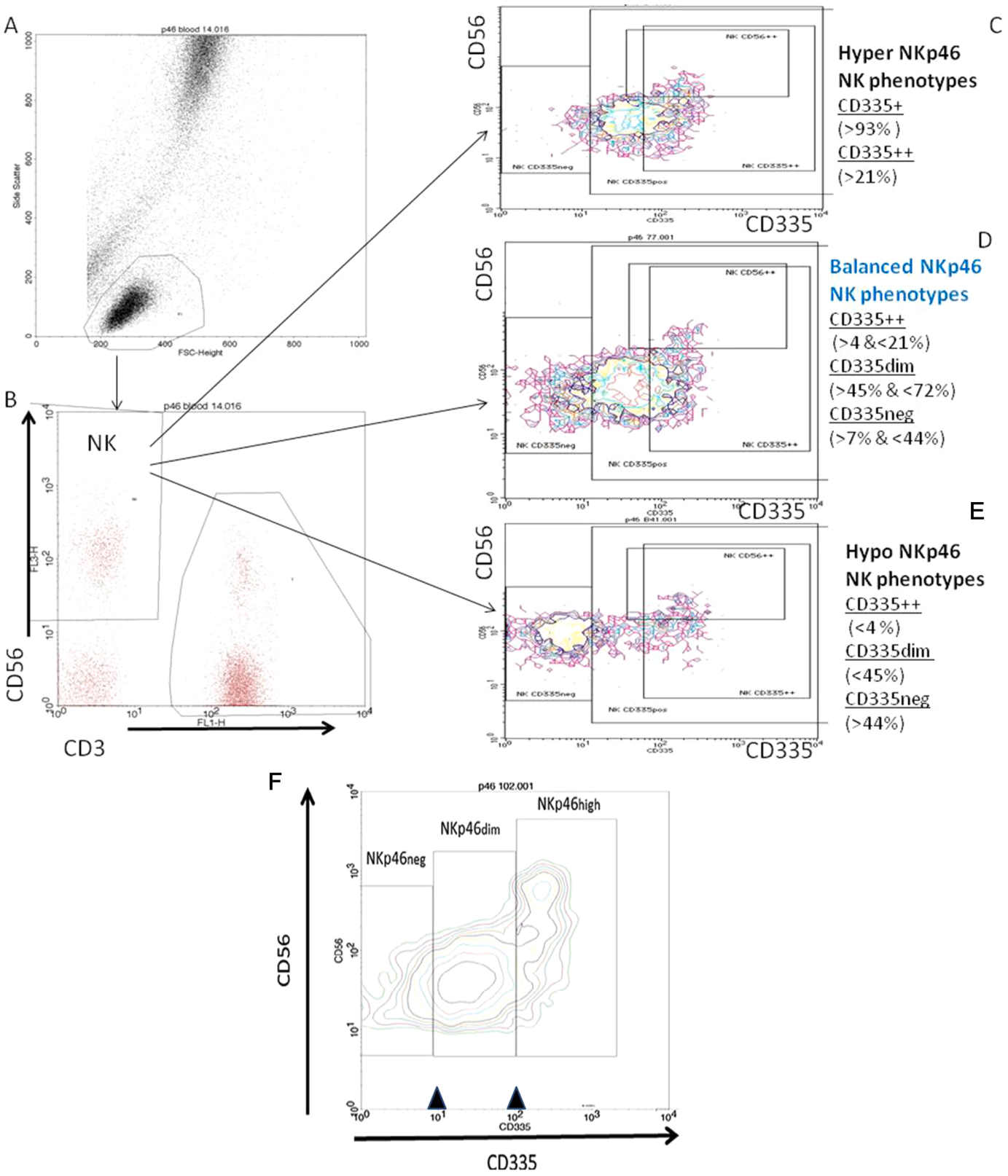


Fig. 1. Gating strategy used to identify NK cells and NKp46 NK cell subsets. Representative flow cytometry plots from the peripheral blood of 3 study participants (with contrast type of CD335 expression). The lymphocytes were live gated during acquisition using the side and forward scatter dot plot display (A). Furthermore, by using the negative gating strategy, CD3negative lymphocyte population was identified (B). The NK cell population was further identified as CD3−CD56+− (%NK) and differentiated into NKp46high, NKp46dim and NKp46neg subsets(C, D, E). The NKp46high subsets was also separated on CD56++ and CCD56dim. In parallel to manual gating, standard invariant gating for NKp46^{high}, NKp46^{medium}, and NKp46^{neg} enumeration were conducted (F).

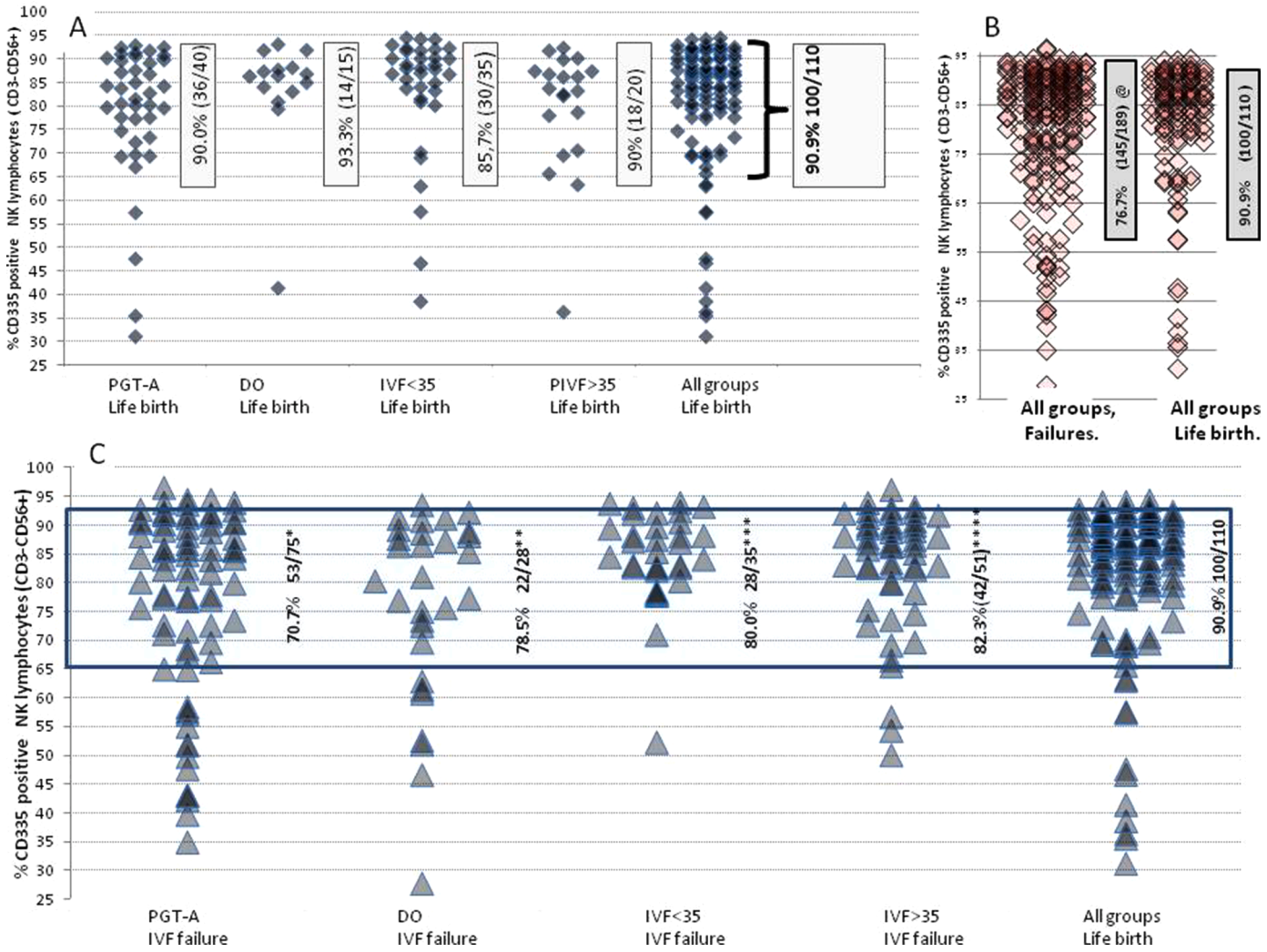


Fig. 2. Frequency of "balanced" CD335 positivity on NK Lymphocytes **(A)** was similar high in patients from different clinical groups with subsequent successful Life Birth. Majority of them 85–93% (average 90.9%) get into favorable corridor >66 <93% CD335 positivity on NK. **(B)** In contrast, in all patients with reproductive failures after IVF (both implantation failures and pregnancy failures) frequency of patients with favorable levels was significantly reduced 76.7% (@Odds ratio= 3.034, The two-sided P value is 0.0018). **(C)** Separately, in PGT-A group this difference was significant (* Odds ratio= 0.2409 The two-sided P value is 0.0006, considered extremely significant) in DO groups weakly significant (**Odds ratio= 0.3667, The two-sided P value is 0.0946, considered not quite significant) and not significant in IVF<35 and IVF>35 groups (***Odds ratio= 0.4000, The two-sided P value is 0.126 and (**** Odds ratio= 0.4667, The two-sided P value is 0.1245) compared to all patients with successful Life Birth.

0.2409 The two-sided P value is 0.0006, considered extremely significant) in DO groups weakly significant (Odds ratio= 0.3667, The two-sided P value is 0.0946, considered not quite significant) and not significant in IVF<35 and IVF>35 groups (Odds ratio= 0.4000, The two-sided P value is 0.126 and (Odds ratio= 0.4667, The two-sided P value is 0.1245) compared to all patients with successful Life Birth.**(Fig 2C)**

3.1. PGT embryo transfer

Embryo implantation outcomes and pregnancy success were particularly dependent on the NKp46 phenotype in the IVF group with PGT-embryos.

Thus, an accentuated increased level of p46 expression > 93% was found in 6 (9.8%) out of 61 patients with failed embryo-implantation and 0 out of 54 women with a clinical pregnancy (OR 12.7 The two-sided P value = 0.0289) Fig 1. Table 2. This accentuation did not affect the subsequent success of pregnancy but was still associated with a certain reduction of LBR (OR 7.6 The two-sided P value = 0.09 considered not quite significant).

Decreased expression of NKp46 > 66% was found in 15 (24.5%) out of 61 patients who did not become pregnant. This is significantly higher compared to patients with occurred pregnancy (5 (9.2%) out of 54 women (OR3.19 The two-sided P value = 0.04) Table 2. This accentuation also did not affect the success of pregnancy and was statistically insignificant as a result of the decrease in LBR (OR2.4 The one-sided P value = 0.0998, considered not quite significant)

Accentuated increased or decreased NKp46 expression (<66% or >93%) significantly associated with reduced LBR if compared to patients with moderate/unaccented NKp46 expression levels 66–93% (OR3.7 The two-sided P valueis 0.01) Table 2. Fig 2

Accentuations of individual NK phenotypes CD335dim and CD335++ were also associated with IVF results.

Patients with balanced proportion of CD335dim subset (45% - 72%) had significantly higher LBR (32 out of 40 women, 80%) while patients with accentuated levels were successful only in 66% of cases (42 out of 75 women) (Odds ratio = 3.143 The two-sided P value is 0.0138, considered significant) Table 2.

Also balanced level of NK CD335++ expression had a weakly reliable association with the best LBR. Thus, women with 5% - 21% level of

Table 2
Significance of favorable NKp46 phenotypes for IVF success in patients with different type of embryo transfers and age. (*standard invariant gating), (ns - no significant).

	IVF result	%p46 pos on NK (>66% and <93%)	% of p46dim from NK (>45% and <72%)	% of p46++ from NK (>5% <21%)*	NKp46negLymph.(>0.7 and <4%)	NKp46posLymph. (<11.5%)
PGT-A	IF $n = 61$	65.6% (40/61)	52,5% (29/61)	77.0% (47/61)	59% (36/61)	62.3% (38/61)
	PF $n = 14$	92.8% (13/14)	71,4% (10/14)	92.9% (13/14)	85.7% (12/14)	42.% (6/14)
	LB $n = 40$	90.0% (36/40)	80,0%(32/40)	92.5% (37/40)	82.5% (33/40)	75% (30/40)
Significance for	implantation	OR 5.15 $p = 0.0015$ 95%CI 1.74- 13.23	OR 3.17 $p = 0.0062$ 95%CI 1.41- 6.91	OR 3.172 $p = 0.037$ 95%CI 1.14- 12.13	OR 3.4 $p = 0.007$ 95%CI 1.4- 8.3	ns
	Successful pregnancy	ns	ns	ns	ns	OR 4.0 $p = 0.047$ 95%CI 1.11- 14.36
	LB	OR 3.74 $p = 0.0199$ 95%CI 1.27- 10.63	OR 3.14 $p = 0.0138$ 95%CI 1.28- 7.72	OR 3.08 $p = 0.094$ 95%CI 0.83- 11.38	OR 2,65 $p = 0.05$ 95%CI 1.03- 6.8	OR 2,11 $p = 0.06$ 95%CI 0.90- 4.95
DO	IF $n = 21$	90.5% (19/21)	57.1% (12/21)	81% (17/21)	85.7% (18/21)	52.4% (11/21)
	PF $n = 7$	42.9% (3/7)	71.4% (5/7)	85.7% (6/7)	28.6% (2/7)	71.4% (5/7)
	LB $n = 15$	93.3% (14/15)	73.7% (11/15)	93.3% (14/15)	86.7% (13/15)	60% (9/15)
Significance for	implantation	ns	ns	ns	ns	ns
	Successful pregnancy	OR 18.6 $p = 0.02$ 95%CI 1.55- 239	ns	ns	OR 16.25 $p = 0.013$ 95%CI 1.99- 107	ns
	LB	ns	ns	ns	ns	ns
IVF<35	IF $n = 27$	77.8% (21/27)	70.4% (19/27)	74.1% (20/27)	63% (17/27)	77.8% (21/27)
	PF $n = 8$	87.5 (7/8)	37.5% (3/8)	75% (6/8)	100% (8/8)	50% (4/8)
	LB $n = 35$	85,7% (30/35)	68.6% (24/35)	85.7% (30/35)	82,9% (29/35)	91.4% (32/35)
Significance for	implantation	ns	ns	ns	OR 3.63 $p = 0.039$ 95%CI 1.11- 12.6	ns
	Successful pregnancy	ns	OR 3.6 $p = 0.1$ 95%CI 0.7- 15.2	ns	ns	OR 11 $p = 0.014$ 95%CI 2.02- 54
	LB	ns	ns	ns	ns	OR 4.4 $p = 0.034$ 95%CI 1.2- 15.8
IVF>35	IF $n = 42$	85.7%(36/42)	69% (29/42)	88.1% (37/42)	69% (29/42)	78.6% (33/42)
	PF $n = 9$	88.9% (8/9)	88.9% (8/9)	100% (9/9)	66.7% (6/9)	66.7% (6/9)
	LB $n = 20$	90% (18/20)	60% (12/20)	85% (17/20)	75% (15/20)	75.0% (15/20)
Significance for	implantation	ns	ns	ns	ns	ns
	Successful pregnancy	ns	ns	ns	ns	ns
	LB	ns	ns	ns	ns	ns

NK CD335 ++ had an overall success rate 77.5% (31 out of 40 women) while patients with accentuations had an overall success rate 61.4% (46 out of 75 women) (The two-sided P value is 0.0974. Odds ratio = 2.171).

Thereby, the presence of all subpopulations of NK is important for overall reproductive success. All p46 phenotypes of NK should be in certain balanced proportions.

High levels of NK lymphocytes (> 14%) (not reliable enough) were more common in patients with miscarriage (57% 8/14) than in women whose pregnancy ended successfully (12/40 30%) (Odds ratio = 3.111The one-sided P values 0.0696, considered not quite significant). Whereas the level of NKp46 + cells (CD3-CD335+ CD56+ > 11.6% of all lymphocytes) was significantly increased in women with miscarriage (8/14 57.1%) compared to those who successfully gave birth (10/40, 25%) (OR 4 The two-sided P values 0.045). The general level of live birth was not sufficiently influenced by this accentuation (Odds ratio = 2.114The one-sided P values 0.0607, considered not quite significant) but more than just the level of NK (CD3-CD56+).

The number of NKp46 negative cells CD3-CD335-CD56+ (> 4% <0.7% of all lymphocytes) worsened the onset of implantation, did not increase the incidence of miscarriage and as a result decreased LBR (OR 2.65 $p = 0.05$) Table 2.

3.2. Donor's embryo patients

In the group of patients with transfer of PGD untested donor embryo, all tendencies of association between accentuations of NKp46 expression and IVF success remained, but due to the smaller group, the levels of reliability were lower. Among those who successfully gave birth the levels of NKp46 expression 66–93% and levels of p46 negative NK cells were significantly less common (13.3% 2/15 and 6.6% 1/15) than among the patients with miscarriages (71.4% 5/7 and 57.1% 4/7). The effect of these parameters on LBR did not reach statistical significance.

3.3. Untested own embryo

In patients who had transfer with own embryo and were younger than 35 years, accentuated NKp46 expression levels were also more common in those who had IVF failure, but the difference was not statistically significant. However, high levels of NKp46+ NK lymphocytes were significantly associated with both miscarriage and low LBR. Patients who failed IVF had accentuated levels of high levels of NKp46+ NK (28.5% 10/35) compared to those who successfully gave birth (8.8% 3/35) (OR 4.4 Two-sided $p = 0.0346$).

The number of NKp46 negative cells CD3-CD335-CD56+ (> 4% <0.7% of all lymphocytes) worsened the onset of implantation OR 3.6 $p = 0.039$) but did not increase the incidence of pregnancy loss Table 2.

All previously described trends were also characteristic of this group, but the level of reliability did not reach statistical significance (considered not quite significant).

In the similar group of patients who had transfer with own embryo but were older than 35 years, no significant associations between the expression of p46 and IVF success rates were found. Even the trends described in the previous groups were often invisible. Thus, the embryonic factor in these women resulted in very low LBR and actually leveled out the NK phenotype significance.

4. Discussion

It was shown that NK46 expression was associated with enhanced target recognition [19], NK activation [20] maturation [21] CD107a

degranulation [22] IFN-c secretion [23] and cytotoxicity [14]. High NKp46 expression defines a specific NK-cell subset that may be involved in both the suppression of HCV replication and HCV-associated liver damage underpinning the role of NK cells in the immunopathogenesis of HCV [23].

Patients with increased NKp46 levels have better overall survival in AML [24] decreased viral loud in HCV [23] but in same tame have an unfavorable prognosis of INFa HCV treatment [22].

Peripheral blood (PB) NKp46 expression increased in women RIF [17] at the same time endometrial NKp46+ cells were markedly decreased in women with RSA [25]. Controversy can be explained by difficult NKp46 expression phenotype. Increase or decrease of NKp46 is too simple of an approach. PB NK can be divided by NKp46 expression density into at least 4 different subsets (p46neg, p46dim and p46++). Subsequently NK46++ subset consist CD56++ and CD56dim). Our approach to NKp46 subsets structure we presented in [17,14] but almost the same approach was previously proposed by Krämer at al [23] and also showed that NKp46 subsets have different functional activities. In actual investigation we have shown that all NKp46 subsets in balanced proportion form favorable backgrounds for the implantation of an embryo and pregnancy outcome. Accentuation of one subset affects the balance and forms an unfavorable phenotype for reproduction. Previously we showed that balanced NK frequency [26] NK activation [20], CD158a [27] and CD8 [28] expression on NK and NK cytotoxicity were favorable for reproduction compared to increased or decreased unbalanced-accentuated levels. It was reported that similar unbalanced NK phenotypes form unfavorable condition in Acute Myeloid Leukemia (AML patients) [29, 30] and rheumatoid arthritis [31].

Significant number of women with reproductive failures have accentuated NK cell parameters reflecting high immunological activity [10] but increased NKp46 levels is a favorable prognostic and predictive biomarker in AML patients [24]. The old question "is high NK cell activity good for health and bad for reproduction?" [10] should be paraphrased. Accentuated unbalanced NK function forms a highly specific immunophenotype that may be favorable for specific challenge but is unfavorable for adaptation for all other challenges. We show that in healthy men elevated count of NK lymphocytes and NK cytotoxicity was an unfavorable factor of the overall health during the 1-year Antarctic expedition [32].

The accentuated idea similar to the situation that was described in the study KIR-HLA recognition model [33] "when responding to any specific challenge, an individual with a narrow range of possible NK cell behaviors that is entirely optimized for that challenge will do better than an individual with a wider range of possible NK cell behaviors. However, the individual with narrow NK cell responses will, of course, perform badly against any challenge that requires NK cell responses outside of its range" [33] . We show that the zone (moderate levels) where NKc does not correlate with NK frequency [34] is identical to NKc levels favorable for reproduction. "Normal-moderate" level of NK cytotoxicity is a favorable value of the parameter where the function can be generated depending on the actual needs of the organism. In contrast, elevated or reduced of NK cytotixicyty correlated directly with NK frequency and at same time increased reproductive failures [35].

This work demonstrated the negative impact of accentuated levels of NKp46 expression on the efficiency of the reproductive process "in real time" in actual IVF cycle.

Our results support the statement that misbalance of NKp46 subsets forms an unfavorable background for embryo implantation and if combined with general NK number - for pregnancy outcome.

We show that association of NKp46 phenotype with reproductive failures is especially noticeable in patients with PGT embryo transfer. However, the same association was not so contrast in the entire IVF population with not PGD tested embryo transfers patients. In investigated groups we excluded uterine abnormalities and anticardiolipin antibodies. High levels of Embryo aneuploidy in patients >35yo mismatch the immune NK factor to less it significance. However, in a similar group, if transferred embryos were donated (from young women with low risk of chromosomal embryo abnormalities) the pressure of chromosomal factor decreased and the NKp46 significance increased.

Embryo implantation is regulated by fetal-endometrial NK recognition. But endometrial NK population and peripherals blood NK cell have different phenotypes and functions. NK and T cell numbers in peripheral blood do not correlate with endometrial NK and T frequencies. How do NK phenotypes or cytotoxicity in blood represent local endometrium conditions? And how does it affect embryo implantation? Previously we showed that NK and T lymphocyte populations in the endometrium tissue were autonomic but not independent from the general immune condition. Expression of CD8aa and p46(CD335) in the peripheral blood and endometrial NK correlated significantly [36,37]. It also was shown that the PB NK cell levels reflect changes in decidual NK cell phenotypes. The number of decidual CD16(+) cells was significantly higher in women with elevated peripheral blood NK levels [38, 39].At the same time number of endometrial CD16(+) NK cells was significantly higher in women with RPL. Endometrial NK levels in PRL patients are often increased but, in some patients, decreased NK levels were also registered [40]. It is also possible that PB NK phenotype and functional condition influence the local immune environment formation and affect receptivity of endometrium.

We describe an easy and highly standardized flow cytometry method for NK p46 phenotype investigation. This method is accessible for routine diagnostic. It predicts subsequent reproductive failures after IVF. In combination with PGT investigation of NKp46 phenotype is highly predictive for IVF failures and open the way for target treatment. It is also possible that determination of KIR-HLA-C mother/fetal combination with NKp46 testing can provide a more powerful diagnostic approach for prediction reproduction complications.

An important feature of this study is that we analyzed patients with unburdened history or without history of reproductive failure. In previous work, we have shown that NKp46 levels had been retrospectively accentuated in patients with multiple IVF failures compared to their fertility control [17] - however, whether this was the factor leading to numerical losses or whether p46 accentuation was the result of numerical losses could not be identified. In this blind study, we demonstrated that NKp46 accentuations led to an actual risk of reproductive failure in unburdened patients. However, patients without a history of reproductive failure with accentuated NKp46 phenotype have a higher risk of implantation failure and miscarriage.

In our previous retrospective study, we showed that patients with RIF had a higher frequency of NKp46 expression accentuations [17]. In this paper, we confirmed the previous conclusions, but the limits of favorable values were slightly changed. Thus, numbers of CD335posNK lymphocytes >13% in the previous study were considered unfavorable. In this blind study, unfavorable levels of CD335posNK lymphocytes were >11.5%. In this study also compare the manual gating and standard invariant gating of different populations and found that it affects the results, however, only the limits of the level of disadvantage and not the sense of the accentuation. Thus, for manual application of the NKp46 ++ subset, the significant unfavorable limit was <16%, while for the standard fixed gate it was <7%. However, in both cases, the accentuation on this parameter was significantly unfavorable for the reproductive process. Manual gating of minor populations led to its magnification.

At this stage, we have demonstrated a lot of NKp46 populations whose accentuation leads to an unfavorable prognosis or whose balance leads to a normal physiological prognosis. However, it is not entirely clear which populations physiologically affect the next course because an increase in one automatically leads to a decrease in the other. Therefore, it is necessary to conduct a deeper and more detailed study of the physiological and phenotypic properties of different NKp46 populations.

Declaration of Competing Interest

Authors not have conflict of interest.

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