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Intravenous immunoglobulin or intravenous lipid emulsion therapy in patients with elevated NK cytotoxicity improves live birth rates in different ways.

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Revised main doc clean version2.docx	Main Document - MS Word	52.3 KB	Page 4
Revised main doc Tracked Changes.docx	Main Document - Tracked Changes	52.3 KB	Page 24
Study design.jpg	Figure	251.7 KB	Page 44
TAB1.docx	Table	16.4 KB	Page 45
Tabe3.docx	Table	16.8 KB	Page 46
Table2.docx	Table	19.2 KB	Page 48
table4.docx	Table	17.8 KB	Page 49
Sup Fig Legends.docx	Supplementary Material for Review	14.8 KB	Page 50
supplemental Fig1a b .pptx	Supplementary Material for Review	1.0 MB	Page 51
Answer for Reviewers.docx	Author Response	18.4 KB	Page 53

Intravenous immunoglobulin or intravenous lipid emulsion therapy in patients with elevated NK cytotoxicity improves live birth rates in different ways.

Running title:
In patients with elevated NK cytotoxicity, IvIg improves IVF outcomes mainly by increasing the ongoing pregnancy rate, while IL also reduces pregnancy failures. IvIg or IL treatment significantly improves LBR, but does not affect the high rate of preterm birth inherent in eNKc patients.

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The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The study conformed Diagnostic and immunotropic treatment options were agreed with the patients and informed consent was obtained (approved by the Biomedical Ethics Committee of the Institute of Pediatrics, Obstetrics and Gynecology, National Academy of Medical Sciences of Ukraine, No. 6, according to the Declaration of Helsinki).

No Conflict of interest

Abstract

Problem: Many studies have demonstrated the negative impact of high rates of NK cytotoxicity (NKc) on reproductive outcomes, as well as the beneficial effects of intravenous immunoglobulin or intravenous lipid emulsion, specifically in these patients.
Method of study: NKc was measured in PBMCs against K562 by flow cytometry. For retrospective analysis, 2250 cycles of ET were selected and divided into 4 groups according to NKc levels and treatment: normal (nNKc, n=587), elevated (eNKc, n=615), eNKc with IvIg treatment (n=231), and eNKc with IL treatment (n=180). Patients were <36 years old, negative for anticardiolipin antibodies, and did not have insufficient endometrium, thrombotic disorders, uterine abnormalities, or obesity. Patients aged 36-39 years (n=359) and 39-42 years (n=279) were analyzed separately using a similar approach.
Results: Patients with eNKc, in addition to having reduced clinical pregnancy rates (CPR) 26.99%, had increased levels of pregnancy failures (PF) 36.7% and a preterm birth rate (PBR) 13.3%, compared to nNKc patients (34.07%, 23.5%, and 3.26%, respectively). As a result, patients with eNKc had a reduced live birth rate (LBR) (17.0%), compared to nNKc patients (26.06%, OR 1.720, $p<0.0002$).
Both IvIg and IL treatment resulted in increased CPR (44.15% and 39.44%) and LBR (32.03% and 32.22%) compared to patients with eNKc without treatment. IvIg treatment increased CPR even compared to patients with nNKc. However, the pregnancy failure rate

(PFR) in IvIg-treated patients was not significantly reduced compared to non-treated patients with eNKc. In contrast, IL-treated patients had significantly decreased PFR compared to patients with eNKc without treatment. As a result, both treatments (IvIg and IL) led to an LBR similar to that of nNKc. Both therapies (IvIg and IL) did not affect the Preterm birth rate, which remained relatively high (14.86% and 13.79%, respectively). However, the frequency of antenatal or prenatal fetal death was the same in all groups. In patients aged 36-39y.o. the results were quite similar, although the PFR in all groups was already significantly higher. However, in patients aged 39-42 years, the negative impact of eNKc was no longer significant, as high PFR and low LBR were also found in patients with nNKc. Nevertheless, even in this group, IvIg therapy led to higher LBR rates compared to eNKc without therapy.

Conclusions: Our findings highlight the negative impact of high levels of NK cytotoxicity on all stages of the reproductive process. Treatment with IvIg and IL in this group significantly improves LBR. It seems that the strength of IvIg is based on a strong increase in the CPR even compared with nNKc patients, whereas IL's effectiveness is more based on a significantly reduced PF rate. It also appears that the efficacy of IvIg is not limited to the group of patients with eNKc. Combining IvIg and IL treatments may enhance the results in patients with elevated NK activity.

Keywords: NK cytotoxicity, Intravenous immunoglobulin, intravenous lipid emulsion, IVF failures, Pregnancy failures, live birth rates, Preterm birth.

Introduction

Natural Killer lymphocytes (NK) play a critical role in human reproduction: blastocyst recognition, embryo implantation[1,2], trophoblast invasion[3], spiral artery remodeling[4,5,6,7], and subsequent pregnancy ongoing[8,9]. It is logical to assume that the affected NK numbers or functions influence the efficiency of these reproductive processes and form an unfavorable background for reproduction and it was shown in many studies[10,11,12,13,16]. Status of peripheral blood (PB) natural killer cells NK cell frequency and NK cytotoxicity (NKc) associated with reproductive failures (recurrent pregnancy loss (RPL) abortions, infertility of unknown etiology, and IVF failure[10,11,14,15,17].

The number and phenotype of endometrial natural killer cells are also associated with reproductive disorders, such as unexplained subfertility, recurrent implantation failures (RIF), and RPL[18,19,20].

A lot of the meta-analyses showed higher NK-cell proportions or NK cytotoxicity in RPL or RIF[21,22,23,24].

High production of pro-inflammatory cytokines by PB NK cells was registered in women with recurrent miscarriages[25] as well as elevated HLA-DR, CD69, and CD94 expression in NK and T lymphocytes[10,26]. Elevated HLA-DR on endometrial NK and CD3+CD8+lymphocytes was also found in patients with RIF[27,20].

It is well known that there is no correlation between NK numbers in endometrium and PB, but a similarity between decidual and PB in CD161 and CD158b NK expression was shown[28]. The association between high CD45+CD3+CD8+DR+ levels in peripheral blood and higher uNK numbers was also shown[27]. We previously described that CD3+CD8+DR+ peripheral lymphocytes correlated with the same population in the endometrium. We also found a correlation between endometrial NK and PB NK CD335 and CD8 expression[29,30,20]. So endometrial immunophenotype is specific, but not autonomic and isolated, from PB immunophenotype.

Elevated NK numbers, activity, or cytotoxicity in patients with reproductive failures were shown in the majority of studies. However, in recent years, studies have shown the adverse effects of reduced/suppressed NK states. So, it was shown: that the low cytotoxic activity of peripheral blood NK cells may relate to unexplained recurrent miscarriage[31] or IVF failures[16]. Increased natural killer cell subsets with inhibitory cytokines and inhibitory surface receptors in patients with recurrent miscarriage, was shown[32].

Thus, the vast majority of studies reveal an association between accentuated high levels of NK lymphocytes and NK activity with reproductive losses, as well as the effectiveness of immunomodulation therapy by Intravenous immunoglobulin or intravenous fat emulsion among such patients[33,34,35]. However, the issue of implementing these diagnostic methods and therapeutic approaches remains unresolved.

NK cytotoxic reaction, measured by the NK assay against HLA-negative K562 cell lines, is the “gold standard” for the investigation of NK activity in vitro[36]. In Kyiv, we implemented NK cytotoxicity testing of patients with reproductive failures back in 2006 and during this time have done > 28,000 tests. We planned this study back in 2010 and completed it well before the SarsCov2 pandemic. In a previous retrospective study, we showed that accentuated NK cytotoxicity (reduced or increased) is an unfavorable factor for implantation and pregnancy ongoing[16]. The current retrospective study continues the previous one and analyzes the effectiveness of immunomodulation therapy by Intravenous immunoglobulin or intravenous fat emulsion in ordinary IVF population with elevated NK cytotoxicity.

Material and Methods

Blood sample collection

NK cytotoxicity was routinely investigated in patients who had at least one incident of idiopathic failure in previous IVF cycles (2009 to 2019). In the overall analysis, we took more than 8000 determinations of NK cytotoxicity in more than 4,000 patients from the (Reproductive Medicine Clinic "IRM Dachno", Kyiv, Ukraine). The clinic is adjacent to a laboratory (800m) and guarantees the optimal time and conditions for blood sample transportation. 5.5 ml peripheral blood was collected from 8:30 to 10:00 am and delivered to the laboratory no later than at 12 am. Thus, no more than 5 hours at room temperature passed after the blood collection and onset of lymphocyte isolation. Effectors (peripheral blood mononuclear cells (PBMC)) were isolated on a density gradient (“Histopaque-1077” Sigma-Aldrich. Darmstadt. Germany).

Study design.

We select the clinical history of 2250 embryo transfer (ET) cycles for retrospective analysis from more than 8.000 ET cycles Fig1. For analysis, cycles of ET were selected according to the criteria: the ET was performed no more than one year after the last NKc examination at least 1 good-quality embryo was transferred. Patients were negative for anticardiolipin antibodies and did not have: insufficient endometrium (<7mm, chronic endometritis or endometriosis), thrombotic or acute infectious disorders, uterus abnormality, abnormal karyotype and obesity. Most patients had several reasons for performing IVF, but the dominant ones were in the tubo-peritoneal factor, the male factor and the endocrine factor.

Main study groups

In all Main study groups patient’s oocytes were obtained before 36yo. Clinical and anamnestic data are shown in **(Tabe1).**

Control groups

Patients from control groups did not obtain any additional treatment (except low-dose aspirin). In the case of elevated NKc (eNKc) or normal NKc (nNKc) results, the determination of NKc was repeated in the majority of cases (in 64% for eNKc and 61% for nNKc). For analysis, the patients from control groups were divided into 2 groups according to the NKc levels; eNKc (<36yo n=615) or nNKc (<36yo n=587) (Fig1).

IvIg group

The intravenous immunoglobulin group included patients with elevated NKc obtained intravenous immunoglobulin infusion (eNKc <36yo IvIg n=231) 100-250mg/kg (average 165mg/kg) (IvIg, Biofarma, Kyiv Ukraine), 3-5days before embryo transfer. 49 patients obtained secondary IvIg infusion after a positive hCG test and their PF and LBR were analysed separately.

IntaLipid group

Intralipid group (n=180) same-age patients with elevated NKc obtained intravenous 20% (200ml) Fat Emulsion **(“**Intralipid Fresenius Kabi”, Austria) infusion 3-5 days before embryo transfer.

Addition groups

The same treated and control patients (36-39y.o. and 39-42y.o.) were analyzed separately by similar design Fig1.

Analysis

Pregnancy rate (PR) is calculated as % of the ongoing pregnancy (egg in uterus confirmed by ultrasound on 5 weeks of gestation (w/g) per ET. Pregnancy failures are calculated as a part of the pregnancy’s that not result to live birth (failed pregnancy and fetal death) from the confirmed ongoing pregnancy (pregnancy failures refer to ongoing pregnancies that do not result in live birth, including early pregnancy loss and fetal demise).

Live birth rate (LBR) was calculated as part of births of a live baby after 24 weeks of gestation per ET. Medical abortion due to fetal malformations was included as a pregnancy success for PR and as a failure for LBR, but not as a Pregnancy failure. Pre-term Birth is calculated as % of births of a live baby after 24 w/g and before 37w/g from all Live births.

Immunotropic treatment options were agreed upon with the patients and informed consent was obtained (approved by the Biomedical Ethics Committee of the Institute of Pediatrics, Obstetrics and Gynecology, National Academy of Medical Sciences of Ukraine, No. 6, 17/12/2013, according to the Declaration of Helsinki).

Comparison of groups

(**Table1**) presents the age and reproductive history severity of the control and main study groups. Patients with normal NKc have less number of previous unsuccessful programs and predominantly have primary infertility diagnoses compared to all groups with elevated NKc. It’s a natural limitation of randomization through the accumulation of patients with eNKc in subsequent cycles. Other parameters (age, part of fresh or cryo-cycles, endometrial thickness, number of embryos transferred, AMH, FSH, LH) did not differ significantly between the groups

Determination of NK cytotoxicity

NK cell activity was investigated as described previously[16].

Target cells K562 were labeled with 5μM CellTracker™Green CMFDA (5-chloromethyl fluorescein diacetate), (Molecular Probes, Eugene, Oreg. USA) and Calcein-AM (O, O′-diacetate tetrakis (acetoxymethyl) ester) (Sigma-Aldrich. Darmstadt. Germany) for 20min at 37°C in a humidified 5%CO2 incubator. The labeled cells were washed twice in PBS, resuspended in RPMI1640 (Sigma-Aldrich. Darmstadt. Germany) with 10% NBCS (Sigma-Aldrich. Darmstadt. Germany), and counted using Flow-CountTM Fluorospheres

(Beckman Coulter, CA, USA). The effector cells PBMC were co-incubated at the E/T ratios of 30, 15, and 7.5 for 2.5h at 37°C in the atmosphere of 5% CO2 in the air. After the incubation period, the cells were mixed with 10μL of PI solution 2mg/ml (Sigma-Aldrich. Darmstadt. Germany) in PBS to stain dead cells. For each E/T ratio, the NK cytotoxicity was measured by analyzing 10000 target cells/samples using a FACScan flow cytometer (BD Bioscience, San Jose, USA) equipped with CellQuest software. Background target cell death was determined from cells incubated in the absence of effector cells.

For each E/T ratio specific NK cytotoxicity was calculated as a percentage of PI positive (dead cells) minus the level of spontaneous lysis. After, we construct a calibration curve and calculate virtual cytotoxicity under the condition of E/T ratio (E/T ratio - 10/1 and 20/1). The level of spontaneous lysis was determined as the percentage of dead cells under the same conditions of incubation but in the absence of effector cells. Less than 5% spontaneous lysis of target cells was observed in these experiments. (**supplemental Fig1 A**)

To measure the E/T ratio correctly, we labeled permeabilized by solution (0.25% Triton and 1% paraformaldehyde) PBLs with PI, and after added labeled K562 cells. The ratio of E/T, in each sample, was measured by flow cytometry (PBLs – FL3, K562 cells – FL1.) (**supplemental Fig1 B**)

For target-gate correction (FL1 lost) we used (50% permeabilized K562 culture) 100μL target cell permeabilized by 50μL 96% ethanol for 10sec. After the vortexing 100μL of PBS and 100μL of unaffected targets were added. The obtained suspension consists of 50% life and 50% permeable cells. (**supplemental Fig1 A**)

As the concept of "normal cytotoxicity" the reference values of NK cytotoxicity are favorable for reproductive prognosis[26,16].

The cytotoxicity activity >40% (E/T ratio 20/1) and >30% (E/T ratio 10/1) was considered as elevated. Criterium for Elevated NKc: NKc (20/1) >40% but in same time NKc (10/1) not less 27% or NKc (10/1) >30% but time NKc (20/1) not less 36%. Criterium for Normal NKc: all NK that not elevated but without reduced NK cytotoxicity levels NKc (10/1) less than 10% or NKc (20/1) less than 15%. (Later these levels were clinically confirmed as clinically significant in a prospective cohort study[26] and later confirmed in correlative analysis (as levels that start correlate with NK numbers)[37,38].

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).

Results

Main study groups<36yo

Control groups.

Patients with elevated NKc display downregulated ongoing pregnancy rate, and LBR as well as increased PF and pre-term birth rate compared to same-age patients with normal NKc (**Table 2**).

IvIg and IL treatment

IvIg and IL treatments result in an increased ongoing pregnancy rate, and LBR (44.1% and 32.03%) compared to patients with eNKc without treatments (26.99% $p < 0.0001$ and 17.07% $p < 0.0001$ respectively) (**Table 2**).

IvIg treatment increased ongoing pregnancy even if compared to same-age patients with normal NKc (p=0.0103). But at the same time, PF rate in IvIg-treated patients wasn't significantly downregulated compared to non-treated patients with eNKc. LBR in IvIg-treated patients didn't significantly increase compared to nNK.

In contrast, IL-treated patients have a significantly decreased PF rate (18.3%) compared to patients with eNKc without treatments (36.7%, p= 0.0007) (**Table 2**). As a result, both treatments (IvIg and IL) have similar LBR. Treatment by IvIg and IL does not decrease the levels of preterm birth compared to eNKc patients and it was still significantly increased (14.86%,13.79%) compared to women with nNKc (3.26%, p=0.0038 and p= 0.0084 respectively) .

Both treatments result in the normalization of LBR in different ways. IvIg realizes it mostly through increased PR (even compared with the nNKc group). IL realizes it through normalization PR rate with a reduction of pregnancy failure rates.

Patient's 36-39yo

NK cytotoxicity control groups.

Patients 36-39yo with elevated NKc still display downregulated ongoing pregnancy rate and LBR and increased PF rate compared to same-age patients with normal NKc (**Table3**). In patients with eNKc levels of preterm birth were significantly increased (25.0%) compared to patients with nNKc (0%, p= 0.0079).

IvIg and IvLe treatment

In patients, 36-39yo IvIg treatment still increased, ongoing pregnancy rate, and LBR compared to patients with eNKc without treatments (**Table 3**). However, PF and preterm birth rates were similar to those in non-treated patients with eNKc.

IL treatment demonstrated a similar to the IvIg effect but the difference wasn't significant due to the smaller group.

Patients >39yo

Patients >39yo with elevated NKc (n=108) did not display downregulation ongoing pregnancy rate (18.5%) and LBR (9.2%) and increased PF rate (50%) compared to same-

age patients with normal NKc (n=113) (PR-27.4% p=0.155, LBR- 15.9% p= 0.2342 and PF-41.9% p=0.7791 subsequently). But even in these age group patients with eNKc, IvIg treatment (n=58) result to significant increase PR and LBR (37.9%p=0.0150 and 22.4% p=0.0381) but not to decrease PF rate (40.9%, p=0.67) compared non treated eNKc group. In these age group we can't find negative NKc effect but we can see positive IvIg effect.

Preterm birth

To study the significance of NK cytotoxicity for preterm birth, we analyzed patients with single and twin pregnancies separately. Patients with tween pregnancies had a slightly higher LBR in all groups, but not significantly (Table 4). Patients with single pregnancies and eNKc had a reduced LBR compared with those with 1 fetus and nNKc (p=0.0034). Patients with single pregnancies with eNKc who received IvIg and IL had higher LBR rates compared with eNKc without treatment (significant for IL (p=0.0111) and not quite significant for IvIg (p=0.0768)).

Patients with both normal and elevated NKc with twin pregnancies had an increased rate of preterm birth compared with those with one fetus (Table 4). However, patients with high NKc and with twin pregnancies had an increase in the rate of preterm birth compared with nNKc (p=0.0426).

Patients with single pregnancies with eNKc who received IvIg still had a higher rate of preterm birth compared to nNKc. The rate of perinatal mortality in all groups was similar.

Discussion

For more than 15 years, it has been understood that the use of immune therapy can be effective in the presence of biomarkers of immune risk factors. For IvIg and Intralipid therapy, such indicators can be emphasized by high levels of NK activity and NK cytotoxicity. A large number of studies have shown that IvIg and IL have been successful in the treatment of recurrent miscarriage and recurrent implantation failure among women with elevated NK number or NK cell activity[33,39,40,41,42,43] as well as for patients with elevated NK cell density in endometrial biopsy[44,45]**.** Such a large number of studies allowed us to hope for the rapid implementation of such approaches in the clinic- "*We believe that when laboratory results are incorporated into the diagnosis of RSA/RSAI that treatment is highly successful whereas the absence of laboratory results severely hinders the effectiveness of treatment*"[46]**.**

A lot of Systematic reviews and meta-analysis support that IvIg treatment increase LBR in patients with accentuated NK functions[24]. It was confirmed with varying levels of optimism from "*There is a strong basis for providing this treatment to patients with NK deviation*"[48] and "*Studies assessing the favoring of the intervention*"[49] to extremely cautious **"***there*

may be some benefit of immunotherapy, but paucity of high quality evidence means that it is not possible to support the use of immunotherapy even when selected based on abnormal NK cell level/activity"[50].

Sometimes authors explicitly postulate the absence of an effect even when they write in the results that "*live birth rate of the IVIG group is superior, but there was no statistical significance [OR =1.43, 95%CI (0.99, 2.07),* P=*0.05*" [51].

But it seems that all that has been done is not enough and we need more studies and larger study's groups.

In an actual retrospective study, we also showed the effectiveness of IvIg and IL in patients with high levels of NKc. We found a positive effect of both treatments at the level of pregnancy, and life-birth rates. We focused on patients with high NKc as a target group for immunotherapy. In a previous similar retrospective study, we showed a dramatic decrease in IVF efficacy in patients with high levels of NKc compared to patients with normal NK cytotoxicity[16].

That is, at certain levels of NK function begins to be unfavorable for a successful reproductive process. But what levels are favorable and why? We have shown that these reference limits for NK number and NK cytotoxicity coincide with levels where NK number and NK cytotoxicity are independent parameters and one does not determine the other[37,38]. At such levels, the function can be adjusted and regulated. While in unfavorable levels, increased or decreased, these two parameters begin to correlate significantly and the number directly determines the function. Perhaps the loss of the ability to effectively regulate is the reason for the "unfavourability" of these levels for reproduction. The difficulty of adaptation reduces the effectiveness of adaptation, but the reproductive process requires the immune system to actively participate and functional changes. We found a similar situation among participants in the Ukrainian Antarctic expeditions. Participants with high levels of NK cells and CD335+NK cells had poorer adaptation to new Antarctic conditions compared to those with balanced levels of NK cells[52,53] and the reference favorable limits in male expedition participants were similar to those in females with subsequent successful reproductive process[54].

We demonstrated that IR and PR after IvIg therapy are not only significantly higher than in patients with eNKc but also higher compared to untreated patients with normal NKc.

In addition, the positive effect of IvIg is found in the age group where high NKc no longer significantly reduces reproductive success. This is probably the result of the multi-therapeutic effects of IvIg. It has been shown that IvIg can improve the condition in the presence of auto or allo antibodies both through the idiotype-anti-idiotype system, by reducing IgG production and blocking the Fc receptor[55,56,57,58].

It also was shown that alloantibodies amplify the negative impact of high NK activity on placental function and IvIg corrects this effect[59]. In the case of inflammatory immune status, IvIg improves the Th1/Th2 balance and activates and potentiates Treg[60,61]. However, in the actual study we showed that in all age groups, the effect of IvIg did not lead to a significantly higher LBR compared to nNKc, and PFR in IvIg-treated patients was rather elevated but similar to in non-treated eNKc patients. May IvIg treatment cause hyperfertility? Previously we showed that implantation of a donor's embryos with higher implantation–invasion capacities and a lower chance of chromosomal abnormalities and genetic complications did not depend on NKc levels. Donors' embryo implantation and pregnancy rates were the same in patients with elevated and normal NKc but the incidence of pregnancy failures among those patients with elevated NKc was much higher[16]. One may speculate that elevated NKc combined with certain chromosomal, genetic, and physiological features or embryo deviation might exacerbate the negative pressure on implantation and amplify the adverse effect already at the pre-implantation and early implantation stages. IvIg treatment possibly gives an implantation chance for a "weak" or mosaic embryo with higher subsequent pregnancy failures. Is it possible to support the subsequent success of these fetuses? Or a possible that part of them had chromosomal abnormalities or genetic or physiological factors that made their further development impossible? We can only speculate about this because in the actual study embryos weren't PGT-A tested and only a small part of the aborted material has been karyotyped. It has been shown that the "normality" of unfavorable NK levels depends on the age of the patients. So, in young patients with "better embryos", the negative clinically significant levels of NK are even higher than in older patients with "poor embryos". Relatively lower levels of NK start to be unfavorable in older patients[62].

It is also difficult to exclude the placebo effect because a prolonged infusion of an expensive drug that is objectively felt by the patient may have a distinct placebo effect. (Sometimes patients with a history of multiple pregnancy failures in anamnesis even at 24-29 weeks of gestation asked to repeat the infusion). It is impossible to select a true placebo group for IvIg because patients feel the drug injection.

Therefore, it seems that IvIg therapy leads to an increase in success rates not only among patients with eNKc but also improves outcomes in patients with other immune causes.

IL therapy also led to higher levels of IR, PR, and LBR, which were statistically significantly higher compared to eNKc without treatment. The levels of PF on IL were lower compared to eNKc. Reproductive success indicators were not significantly higher compared to patients with nNKc. This means that IL therapy corrects specifically the negative effect of high NK cytotoxicity. Both therapies, IvIg and IL, improve LBR in patients with eNKc, but

neither therapy affected the high level of preterm births, which is characteristic of patients with eNKc. In both therapies, this rate remained higher than in the group with nNKc. It was shown that in women with elevated peripheral NK cells, birthweight at delivery is low but IvIg therapy significantly improved it, and IvIg therapy without NK cell elevation did not result in increased birthweight[63] and that at late gestation, premature activation of NK cells can lead to a breakdown of tolerance of the maternal-fetal interface and, subsequently, can result in preterm birth[64].

For the specific analysis of preterm births, we divided all the groups into those with single and twin's pregnancies and found that with eNK, the presence of a twin's pregnancy did not statistically increase the LBR, but it did significantly increase the risk of preterm birth compared to twin's pregnancies with normal NKc, as well as compared to high NKc and single pregnancies. However, we did not find differences between the groups in perinatal mortality.

After dividing into single and twin's pregnancies, the IvIg and IL groups became too small for statistical significance for LBR, although they remained significant for the IvIg group with single pregnancies. The increase in preterm births in eNKc patients with IvIg was statistically significant for single pregnancies, only because this group was larger compared to IL.

We focused on NK cytotoxicity (NKc) because this method has shown high sensitivity and clinical significance in previous studies[16,26]. However, it may not be convenient for widespread routine implementation because NK cytotoxicity assay is difficult to standardization, methodologically, complex to perform, and requires careful attention to the sample's blood viability. On the other hand, immunophenotyping of NK cells using flow cytometry is not a complicated method and it's easily to standardize. We demonstrated that measuring the density of CD335 (NKp46) on NK cells may be almost an alternative method to the cytotoxicity test[37]. The accentuated levels of NK CD335 expression are predictive for reproductive outcomes, both in IVF patients[54] and in patients with natural pregnancies[65]. CD335 expression in peripheral blood NK cells correlates with CD335 levels in NK cells in the endometrium[30]. This might explain why the negative impact of NK CD335 expression becomes significant even at the implantation stages for PGT-A embryos[54]. Meanwhile, high NK cytotoxicity is detrimental primarily during the pregnancy maintenance stage in donor embryos[16] because the active contact between trophoblasts and peripheral NK cells starts with the remodeling of the spiral arteries[66,18].

It explained significant first and early second-trimester pregnancy failure rates in patients with their donor embryos when peripheral blood NK cells first contact with fetal

cytotrophoblast cells. It was shown that elevated NKc leads to incorrect remodeling of the spiral arteries and subsequent pregnancy complications[66,18].

This work has demonstrated the negative impact of high levels of NK cytotoxicity and the positive effect of immunotropic treatment on reproductive process efficiency in a large group of “typical IVF patients".

Limitations of the study

The study population is 95 percent Caucasian and 85 percent Balto-Slavic. It is known that KIR repertoire and HLA-C1/2 frequencies are highly variable in populations[67] and that different combinations are differently favorable for reproduction[68]. In local populations, the number of combinations is limited, which may increase/decrease the frequency of some reproductive complications. In addition, the studied population is characterized by a fairly high level of of CMV (>85%) and HSV1 (>95%) infected and it is known that viral carriage leads to immune modifications, especially for NK lymphocytes[69,70].

All patients had ET with an embryo that was not PGT-A tested and most of the miscarriages were not tested for chromosomal abnormalities. This does not allow us to understand the phenomenon of such a high level of PR after IVIG and simultaneously high levels of PFR.

A weak point of the study is the results on hormone levels because these results were obtained from different laboratories and measured by different methods with different reference values. This is especially true for the causes of infertility, because the diagnosis was made in different medical centers with different criteria, which also changed significantly over the past 10-15 years. This could reduce the statistical significance of these parameters.

An important finding of this study is the high rate of preterm births in all patients with elevated NKc (treated and non-treated). Unfortunately, we cannot analyze the causes of preterm births, and these were mostly cesarean births of 26-32 weeks. Perhaps there were high levels of preeclampsia or other complications but we do not have documentation from the maternity hospitals. Some maternity hospitals simply do not exist physically because they were bombed by rusians, as in Mariupol or Bakhmut. In most of the maternity hospitals, all archival basements have been converted into bomb shelters. So we will not be able to analyze this anymore.

Doses and regimens of IVIG administration for patients with reproductive losses have not been studied sufficiently and actually copy the treatment regimens and doses for patients with primary immunodeficiencies. The usual dose of IGIV for antibody replacement is between 0.3 and 0.6 g/kg per month, delivered every 2 to 4 weeks [71].

The goal of this therapy is very simple: to maintain a sufficient level of serum IgG.

However, in recent years, even in such patients, the standardised administration regimen has been questioned and studies have suggested an individualised approach “The ideal immunoglobulin dose for an individual patient is the dose that keeps the patient well” [72]. Obviously, in patients with reproductive failures with high NK cytotoxicity, both doses and regimens should be developed based on favorable outcome. In addition, it should be borne in mind that IVIG contains both autoantibodies and anti-idiotypic antibodies from donors and high doses of IVIG may affect the clearance of patients anti-idiotypic antibodies [73,74].

Therefore, future studies should take into account NK cell numbers, activity, and phenotype, as well as KIR haplotypes, HLA repertoire, hormonal features, viral carriage, microbiome, and embryo karyotype.

1. Moffett A, Shreeve N. Local immune recognition of trophoblast in early human pregnancy: controversies and questions. *Nat Rev Immunol*. 2023;23(4):222-235. doi:10.1038/s41577-022-00777-2
2. Kanter J, Gordon SM, Mani S, Sokalska A, Park JY, Senapati S, Huh DD, Mainigi M. Hormonal stimulation reduces numbers and impairs function of human uterine natural killer cells during implantation. Hum Reprod. 2023;38(6):1047-1059. doi: 10.1093/humrep/dead069. PMID: 37075311; PMCID: PMC10501469.
3. Pollheimer J, Vondra S, Baltayeva J, Beristain AG, Knöfler M. Regulation of Placental Extravillous Trophoblasts by the Maternal Uterine Environment. *Front Immunol*. 2018;9:2597. Published 2018 Nov 13. doi:10.3389/fimmu.2018.02597
4. Le Bouteiller P. HLA-G in the human placenta: expression and potential functions. *Biochem Soc Trans*. 2000;28(2):208-212. doi:10.1042/bst0280208
5. Li C, Houser BL, Nicotra ML, Strominger JL. HLA-G homodimer-induced cytokine secretion through HLA-G receptors on human decidual macrophages and natural killer cells. Proc Natl Acad Sci U S A. 2009;106(14):5767-72. doi: 10.1073/pnas.0901173106. Epub 2009 Mar 20. PMID: 19304799; PMCID: PMC2667005.
6. Turco MY, Moffett A. Development of the human placenta. *Development*. 2019;146(22):dev163428. Published 2019 Nov 27. doi:10.1242/dev.163428
7. Robson A, Harris LK, Innes BA, Lash GE, Aljunaidy MM, Aplin JD, Baker PN, Robson SC, Bulmer JN. Uterine natural killer cells initiate spiral artery remodeling in human pregnancy. *FASEB J*. 2012;26(12):4876-85. doi: 10.1096/fj.12-210310. Epub 2012 Aug 23. PMID: 22919072.
8. Rao VA, Kurian NK, Rao KA. Cytokines, NK cells and regulatory T cell functions in normal pregnancy and reproductive failures. *Am J Reprod Immunol*. 2023;89(2):e13667. doi:10.1111/aji.13667
9. Smith GC. First-trimester determination of complications of late pregnancy. *JAMA*. 2010;303(6):561-562. doi:10.1001/jama.2010.102

10. Ntrivalas EI, Kwak-Kim JY, Gilman-Sachs A, Chung-Bang H, Ng SC, Beaman KD, Mantouvalos HP, Beer AE. Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. *Hum Reprod.* 2001;16(5):855-61. doi: 10.1093/humrep/16.5.855. PMID: 11331628.
11. Gilman-Sachs A, DuChateau BK, Aslakson CJ, Wohlgemuth GP, Kwak JY, Beer AE, Beaman KD. Natural killer (NK) cell subsets and NK cell cytotoxicity in women with histories of recurrent spontaneous abortions. *Am J Reprod Immunol*. 1999;41(1):99-105. doi: 10.1111/j.1600-0897.1999.tb00081.x. PMID: 10097793.
12. Kuon RJ, Weber M, Heger J, Santillán I, Vomstein K, Bär C, Strowitzki T, Markert UR, Toth B. Uterine natural killer cells in patients with idiopathic recurrent miscarriage. *Am J Reprod Immunol*. 2017;78(4). doi: 10.1111/aji.12721. Epub 2017 Jun 21. PMID: 28639334.
13. Zhu L, Aly M, Wang H, et al. Decreased NK cell immunity in kidney transplant recipients late post-transplant and increased NK-cell immunity in patients with recurrent miscarriage. *PLoS One*. 2017;12(10):e0186349. Published 2017 Oct 17. doi:10.1371/journal.pone.0186349
14. Mahajan D, Sharma NR, Kancharla S, et al. Role of Natural Killer Cells during Pregnancy and Related Complications. *Biomolecules*. 2022;12(1):68. Published 2022 Jan 4. doi:10.3390/biom12010068
15. Emmer PM, Nelen WL, Steegers EA, Hendriks JC, Veerhoek M, Joosten I. Peripheral natural killer cytotoxicity and CD56(pos)CD16(pos) cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. *Hum Reprod*. 2000;15(5):1163-9. doi: 10.1093/humrep/15.5.1163. PMID: 10783371.
16. Dons'koi B, Onyshchuk O, Kononenko I, et al. Accentuated Peripheral Blood NK Cytotoxicity Forms an Unfavorable Background for Embryo Implantation and Gestation. *Diagnostics (Basel)*. 2022;12(4):908. Published 2022 Apr 6. doi:10.3390/diagnostics12040908
17. Salazar MD, Wang WJ, Skariah A, He Q, Field K, Nixon M, Reed R, Dambaeva S, Beaman K, Gilman-Sachs A, Kwak-Kim J. Post-hoc evaluation of peripheral blood natural killer cell cytotoxicity in predicting the risk of recurrent pregnancy losses and repeated implantation failures. *J Reprod Immunol*. 2022;150:103487. doi: 10.1016/j.jri.2022.103487. Epub 2022 Jan 19. PMID: 35078138.
18. El-Azzamy H, Dambaeva SV, Katukurundage D, et al. Dysregulated uterine natural killer cells and vascular remodeling in women with recurrent pregnancy losses. *Am J Reprod Immunol*. 2018;80(4):e13024. doi:10.1111/aji.13024
19. Chiokadze M, Bär C, Pastuschek J, Dons'koi BV, Khazhylenko KG, Schleußner E, Markert UR, Favaro RR. Beyond Uterine Natural Killer Cell Numbers in Unexplained Recurrent Pregnancy Loss: Combined Analysis of CD45, CD56, CD16, CD57, and CD138. *Diagnostics (Basel).* 2020;29;10(9):650. doi: 10.3390/diagnostics10090650. PMID: 32872526; PMCID: PMC7555015.
20. Sudoma I, Goncharova Y, Dons'koy B, Mykytenko D. Immune phenotype of the endometrium in patients with recurrent implantation failures after the transfer of genetically tested embryos in assisted reproductive technology programs. *J Reprod Immunol*. 2023;157:103943. doi: 10.1016/j.jri.2023.103943. Epub 2023 Mar 22. PMID: 36966647.
21. Cavalcante MB, da Silva PHA, Carvalho TR, Sampaio OGM, Câmara FEA, Cavalcante CTMB, Barini R, Kwak-Kim J. Peripheral blood natural killer cell cytotoxicity in recurrent miscarriage: a systematic review and meta-analysis. *J*

Reprod Immunol. 2023;158:103956. doi: 10.1016/j.jri.2023.103956. Epub 2023 May 18. PMID: 37236061.

22. Kolanska K, Suner L, Cohen J, Ben Kraiem Y, Placais L, Fain O, Bornes M, Selleret L, Delhommeau F, Feger F, Mathieu d'Argent E, Darai E, Chabbert-Buffet N, Antoine JM, Kayem G, Mekinian A. Proportion of Cytotoxic Peripheral Blood Natural Killer Cells and T-Cell Large Granular Lymphocytes in Recurrent Miscarriage and Repeated Implantation Failure: Case-Control Study and Meta-analysis. *Arch Immunol Ther Exp (Warsz)*. 2019;67(4):225-236. doi: 10.1007/s00005-019-00546-5. Epub 2019 May 30. PMID: 31147723.
23. Von Woon E, Greer O, Shah N, Nikolaou D, Johnson M, Male V. Number and function of uterine natural killer cells in recurrent miscarriage and implantation failure: a systematic review and meta-analysis. *Hum Reprod Update*. 2022;28(4):548-582. doi:10.1093/humupd/dmac006
24. Seshadri S, Sunkara SK. Natural killer cells in female infertility and recurrent miscarriage: a systematic review and meta-analysis. *Hum Reprod Update*. 2014;20(3):429-438. doi:10.1093/humupd/dmt056
25. Guerrero B, Hassouneh F, Delgado E, Casado JG, Tarazona R. Natural killer cells in recurrent miscarriage: An overview. *J Reprod Immunol*. 2020;142:103209. doi:10.1016/j.jri.2020.103209
26. Chernyshov VP, Dons'koi BV, Sudoma IO, Goncharova YO. Favorable immune phenotype predicts successful implantation and pregnancy. *Immunol Lett*. 2014;162(2 Pt B):217-221. doi:10.1016/j.imlet.2014.10.022
27. Kuon RJ, Vomstein K, Weber M, Müller F, Seitz C, Wallwiener S, Strowitzki T, Schleussner E, Markert UR, Daniel V, Toth B. The "killer cell story" in recurrent miscarriage: Association between activated peripheral lymphocytes and uterine natural killer cells. *J Reprod Immunol*. 2017;119:9-14. doi: 10.1016/j.jri.2016.11.002. Epub 2016 Nov 6. PMID: 27865124.
28. El-Badawy O, Helmy AS, Abbas AM, Zahran AM, Afifi NA, Abdel-Rahim MH. Concordance between peripheral and decidual NK cell subsets and killer immunoglobulin-like receptors in women with recurrent spontaneous miscarriages. *J Reprod Immunol*. 2020;140:103130. doi:10.1016/j.jri.2020.103130

29. Chernyshov VP, Dons'koi BV, Sudoma IO, Goncharova YO. Comparison of T and NK lymphocyte subsets between human endometrial tissue and peripheral blood. *Cent Eur J Immunol*. 2019;44(3):316-321. doi:10.5114/ceji.2019.89610
30. Anoshko Y, Dons'koi B, Sudoma I, Khazhylenko K, Zabara D, Goncharova Y. Changes in the immunophenotype of endometrium during implantation window receptivity formation in healthy fertile women. *Placenta*. 2023;143:62-68. doi:10.1016/j.placenta.2023.10.002
31. Zhang Y, Huang C, Lian R, Xu J, Fu Y, Zeng Y, Tu W. The low cytotoxic activity of peripheral blood NK cells may relate to unexplained recurrent miscarriage*. Am J Reprod Immunol.* 2021;85(6):e13388. doi: 10.1111/aji.13388. Epub 2021 Jan 27. PMID: 33410220.
32. Zhu L, Aly M, Wang H, Karakizlis H, Weimer R, Morath C, Kuon RJ, Toth B, Ekpoom N, Opelz G, Daniel V. Increased natural killer cell subsets with inhibitory cytokines and inhibitory surface receptors in patients with recurrent miscarriage and decreased or normal subsets in kidney transplant recipients late post-transplant. *Clin Exp*

Immunol. 2018;193(2):241-254. doi: 10.1111/cei.13142. Epub 2018 May 31. PMID: 29679490; PMCID: PMC6046488.
33. Coulam CB, Acacio B. Does immunotherapy for treatment of reproductive failure enhance live births?. *Am J Reprod Immunol*. 2012;67(4):296-304. doi:10.1111/j.1600-0897.2012.01111.
34. Mu F, Huo H, Wang M, Liu L, Wang F. Intravenous immunoglobulin improves live birth rates in patients with unexplained recurrent pregnancy loss. *J Reprod Immunol.* 2024;166:104322. doi: 10.1016/j.jri.2024.104322. Epub 2024 Aug 26. PMID: 39216166.
35. Marchand GJ, Masoud AT, Ulibarri H, Arroyo A, Coriell C, Goetz S, Moir C, Moberly A, Gonzalez D, Blanco M, Craig HR. Effect of a 20% intravenous fat emulsion therapy on pregnancy outcomes in women with RPL or RIF undergoing IVF/ICSI: a systematic review and meta-analysis. *J Clin Transl Res*. 2023;12;9(4):236-245. PMID: 37564251; PMCID: PMC10411978.
36. Cholujová, D.; Jakubíková, J.; Kubes, M.; Arendacká, B.; Sapák, M.; Ihnatko, R.; Sedlák, J. Comparative study of four fluorescentprobes for evaluation of natural killer cell cytotoxicity assays. *Immunobiology* 2008;213, 629–640.
37. Dons'koi BV, Osypchuk DV, Chernyshov VP. Enumeration of peripheral blood NKp46 positive NK lymphocytes reflects NK cytotoxic activity in vitro. *J Immunol Methods*. 2019;474:112639. doi:10.1016/j.jim.2019.112639
38. Dons'koi BV, Chernyshov VP, Osypchuk DV, et al. Natural killer frequency determines natural killer cytotoxicity directly in accentuated zones and indirectly in "moderate-to-normal frequency" segment. *Cent Eur J Immunol*. 2020;45(3):315-324. doi:10.5114/ceji.2020.101263
39. Moraru M, Carbone J, Alecsandru D, et al. Intravenous immunoglobulin treatment increased live birth rate in a Spanish cohort of women with recurrent reproductive failure and expanded CD56(+) cells. *Am J Reprod Immunol*. 2012;68(1):75-84. doi:10.1111/j.1600-0897.2012.01135.x
40. Ramos-Medina R, García-Segovia A, Gil J, et al. Experience in IVIg therapy for selected women with recurrent reproductive failure and NK cell expansion. *Am J Reprod Immunol*. 2014;71(5):458-466. doi:10.1111/aji.12217
41. Cohen BM, Machupalli S. Use of Gammaglobulin to Lower Elevated Natural Killer Cells in Patients with Recurrent Miscarriage. *J Reprod Med*. 2015;60(7-8):294-300.
42. Chernyshov VP, Dons'koi BV, Sudoma IO, Goncharova YO. Multiple immune deviations predictive for IVF failure as possible markers for IVIG therapy. *Immunol Lett*. 2016;176:44-50. doi:10.1016/j.imlet.2015.12.010
43. Yamada H, Deguchi M, Saito S, et al. High doses of intravenous immunoglobulin stimulate regulatory T cell and suppress natural killer cell in women with recurrent pregnancy loss. *J Reprod Immunol*. 2023;158:103977. doi:10.1016/j.jri.2023.103977
44. Coulam CB. Intralipid treatment for women with reproductive failures. *Am J Reprod Immunol*. 2021;85(4):e13290. doi:10.1111/aji.13290
45. Lédée N, Vasseur C, Petitbarat M, Chevrier L, Vezmar K, Dray G, Chenière S, Lobersztajn A, Vitoux D, Cassuto GN, Chaouat G. Intralipid® may represent a new hope for patients with reproductive failures and simultaneously an over-immune endometrial activation. *J Reprod Immunol.* 2018;130:18-22. doi: 10.1016/j.jri.2018.09.050. Epub 2018 Sep 24. PMID: 30286362.

46. Beaman KD, Ntrivalas E, Mallers TM, Jaiswal MK, Kwak-Kim J, Gilman-Sachs A. Immune etiology of recurrent pregnancy loss and its diagnosis. *Am J Reprod Immunol*. 2012;67(4):319-325. doi:10.1111/j.1600-0897.2012.01118.x
47. Kumar P, Philip CE, Eskandar K, Marron K, Harrity C. Effect of intravenous immunoglobulin therapy in recurrent implantation failure: A Systematic review and meta-analysis. *J Reprod Immunol*. 2024;166:104323. doi:10.1016/j.jri.2024.104323
48. Yang X, Meng T. Is there a Role of Intravenous Immunoglobulin in Immunologic Recurrent Pregnancy Loss?. *J Immunol Res*. 2020;2020:6672865. Published 2020 Dec 27. doi:10.1155/2020/6672865
49. Polanski LT, Barbosa MA, Martins WP, et al. Interventions to improve reproductive outcomes in women with elevated natural killer cells undergoing assisted reproduction techniques: a systematic review of literature. *Hum Reprod*. 2014;29(1):65-75. doi:10.1093/humrep/det414
50. Woon EV, Day A, Bracewell-Milnes T, Male V, Johnson M. Immunotherapy to improve pregnancy outcome in women with abnormal natural killer cell levels/activity and recurrent miscarriage or implantation failure: A systematic review and meta-analysis. *J Reprod Immunol*. 2020;142:103189. doi: 10.1016/j.jri.2020.103189. Epub 2020 Aug 22. PMID: 32889304.
51. Ling Q, Xu J, Tian Y, Chen D, Mo C, Peng B. Effect of IVIG therapy on pregnant women with unexplained recurrent spontaneous abortion: a systematic review and meta-analysis. *Front Endocrinol (Lausanne*). 2024;14;15:1381461. doi: 10.3389/fendo.2024.1381461. PMID: 39205682; PMCID: PMC11349655.
52. Dubrovskyi, I., Zabara, D., Anoshko, Y. et al. Immune accentuations as a prognostic factor for adaptation in Antarctic conditions. *Polar Biol* 2025;48, 53. https://doi.org/10.1007/s00300-025-03359-9
53. Zabara D, Kozeretska I, Deineko I, Anoshko Y, Shapovalenko N, Stamboli L, Dons'koi, B. Immune factors and health of Antarctic explorers. *Ukrainian Antarctic Journal*. 2021;2, 94-105. https://doi.org/10.33275/1727-7485.2.2021.680
54. Dons'Koi BV, Osypchuk DV, Baksheev SM, Sudoma IO, Goncharova YO, Palyha IE, Sirenko VY, Khazhylenko KG, Onyshchuk O, Anoshko Y, Shapovalenko NO. A blinded multicenter investigation: Accentuated NK lymphocyte CD335 (NKp46) expression predicts reproductive failures after IVF. *Immunol Lett.* 2022;251-252:47-55. doi: 10.1016/j.imlet.2022.10.003. Epub 2022 Oct 18. PMID: 36265608.
55. Chernyshov V, Dons'Koi B, Vodyanik M. Clinical immunologyAnti-idiotypic regulation of cofactor-independent antiphospholipid antibodies. *Central European Journal of Immunology.* 2011;36(4):220-225.
56. Skariah A, Sung N, Salazar Garcia MD, Wu L, Tikoo A, Gilman-Sachs A, Kwak-Kim J. Low-dose prednisone and immunoglobulin G treatment for woman at risk for neonatal alloimmune thrombocytopenia and T helper 1 immunity. *Am J Reprod Immunol.* 2017;77(6). doi: 10.1111/aji.12649. Epub 2017 Feb 27. PMID: 28240400
57. Blank M, Anafi L, Zandman-Goddard G, Krause I, Goldman S, Shalev E, Cervera R, Font J, Fridkin M, Thiesen HJ, Shoenfeld Y. The efficacy of specific IVIG anti-idiotypic antibodies in antiphospholipid syndrome (APS): trophoblast invasiveness and APS animal model. *Int Immunol*. 2007;19(7):857-65. doi: 10.1093/intimm/dxm052. Epub 2007 Jun 18. PMID: 17576752.
58. Yuan X, Zhang W, Wang T, Jiang P, Wang ZK, Li CQ. Use of intravenous immunoglobulin in antiphospholipid antibody positive patients with high risk of miscarriage: a systematic review and meta-analysis. *PeerJ.* 2024;31;12:e18419. doi: 10.7717/peerj.18419. PMID: 39494295; PMCID: PMC11531756.

59. Yougbaré I, Tai WS, Zdravic D, Oswald BE, Lang S, Zhu G, Leong-Poi H, Qu D, Yu L, Dunk C, Zhang J, Sled JG, Lye SJ, Brkić J, Peng C, Höglund P, Croy BA, Adamson SL, Wen XY, Stewart DJ, Freedman J, Ni H. Activated NK cells cause placental dysfunction and miscarriages in fetal alloimmune thrombocytopenia. *Nat Commun.* 2017;8(1):224. doi: 10.1038/s41467-017-00269-1. PMID: 28794456; PMCID: PMC5550461.
60. Park JS, Song AY, Bae JY, Han JW, Kim TH, Kim CJ, Lee SK. IL-17 Producing T to Foxp3^{+}CD4^{+} Regulatory T Cell Ratio as a Diagnostic and Prognostic Marker in Women With Recurrent Pregnancy Loss and Its Implications for Intravenous Immunoglobulin Therapy. *Am J Reprod Immunol*. 2024;92(5):e70020. doi: 10.1111/aji.70020. PMID: 39584290.
61. Yamada H, Deguchi M, Saito S, Takeshita T, Mitsui M, Saito T, Nagamatsu T, Takakuwa K, Nakatsuka M, Yoneda S, Egashira K, Tachibana M, Matsubara K, Honda R, Fukui A, Tanaka K, Sengoku K, Endo T, Yata H. High doses of intravenous immunoglobulin stimulate regulatory T cell and suppress natural killer cell in women with recurrent pregnancy loss. *J Reprod Immunol*. 2023;158:103977. doi: 10.1016/j.jri.2023.103977. Epub 2023 Jun 14. PMID: 37354783.
62. Ramos-Medina R, García-Segovia Á, León JA, Alonso B, Tejera-Alhambra M, Gil J, Caputo JD, Seyfferth A, Aguarón Á, Vicente Á, Ordoñez D, Alonso J, de Albornoz EC, Carbone J, Caballero P, Fernandez-Cruz E, Ortiz-Quintana L, Sánchez-Ramón S. New decision-tree model for defining the risk of reproductive failure. *Am J Reprod Immunol*. 2013;70(1):59-68. doi: 10.1111/aji.12098. Epub 2013 Mar 11. PMID: 23480226.
63. Reed JL, Winger EE. IVIg therapy increases delivery birthweight in babies born to women with elevated preconception proportion of peripheral blood (CD56+/CD3-) natural killer cells. *Clin Exp Obstet Gynecol*. 2017;44(3):384-391. PMID: 29949278.
64. Zhang X, Wei H. Role of Decidual Natural Killer Cells in Human Pregnancy and Related Pregnancy Complications. *Front Immunol.* 2021;26;12:728291. doi: 10.3389/fimmu.2021.728291. PMID: 34512661; PMCID: PMC8426434.
65. Dons'koi BV, Baksheev SM, Sudoma IO, Palyha IE, Khazhylenko KG, Zabara DV, Anoshko YI, Dosenko VE, Dubrovsky EI. A Blinded Investigation: Accentuated NK Lymphocyte CD335 (NKp46) Expression Predicts Pregnancy Failures. *Diagnostics (Basel)*. 2023;25;13(11):1845. doi: 10.3390/diagnostics13111845. PMID: 37296696; PMCID: PMC10252685.
66. Koo HS, Kwak-Kim J, Yi HJ, Ahn HK, Park CW, Cha SH, Kang IS, Yang KM. Resistance of uterine radial artery blood flow was correlated with peripheral blood NK cell fraction and improved with low molecular weight heparin therapy in women with unexplained recurrent pregnancy loss. *Am J Reprod Immunol*. 2015;73(2):175-84. doi: 10.1111/aji.12316. Epub 2014 Oct 23. PMID: 25339113.
67. Hiby SE, Ashrafian-Bonab M, Farrell L, Single RM, Balloux F, Carrington M, Moffett A, Ebrahimi Z. Distribution of killer cell immunoglobulin-like receptors (KIR) and their HLA-C ligands in two Iranian populations. Immunogenetics. 2010;62(2):65-73. doi: 10.1007/s00251-009-0408-5. *Erratum in: Immunogenetics*. 2010;62(4):261. Ebrahimi, Zahra [added]. PMID: 19936734; PMCID: PMC2814031.
68. Boudreau JE, Hsu KC. Natural killer cell education in human health and disease. *Curr Opin Immunol*. 2018;50:102-111. doi: 10.1016/j.coi.2017.11.003. Epub 2018 Feb 3. PMID: 29413815; PMCID: PMC5958620.
69. Farzamikia N, Hejazian SM, Haghi M, Hejazian SS, Zununi Vahed S, Ardalan M. Evaluation of telomeric KIR genes and their association with CMV infection in kidney transplant recipients. *Immunogenetics*. 2022;74(2):207-212. doi: 10.1007/s00251-021-01245-2. Epub 2022 Jan 29. PMID: 35091752.

70. Dons'koi BV, Tutchenko TM, Chernyshov VP, Stepaniuk KS. HCMV seropositivity is associated with specific proinflammatory immune phenotype in women with implantation failure. *Immunol Lett.* 2020;217:84-90. doi: 10.1016/j.imlet.2019.11.008. Epub 2019 Nov 19. PMID: 31756347.
71. Orange JS, Hossny EM, Weiler CR, Ballow M, Berger M, Bonilla FA, Buckley R, Chinen J, El-Gamal Y, Mazer BD, Nelson RP Jr, Patel DD, Secord E, Sorensen RU, Wasserman RL, Cunningham-Rundles C; Primary Immunodeficiency Committee of the American Academy of Allergy, Asthma and Immunology. Use of intravenous immunoglobulin in human disease: a review of evidence by members of the Primary Immunodeficiency Committee of the American Academy of Allergy, Asthma and Immunology. J Allergy Clin Immunol. 2006 Apr;117(4 Suppl):S525-53. doi: 10.1016/j.jaci.2006.01.015. Erratum in: J Allergy Clin Immunol. 2006 Jun;117(6):1483. Dosage error in article text. PMID: 16580469.
72. *Wasserman RL. Personalized Therapy: Immunoglobulin Replacement for Antibody Deficiency. Immunol Allergy Clin North Am. 2019 Feb;39(1):95-111. doi: 10.1016/j.iac.2018.08.001. PMID: 30466775.*
73. *Reams V, Emtiazjoo AM, Gries C, Rackauskas M, Saha BK. Does Intravenous Immunoglobulin Administration Affect the Clearance of Monoclonal Antibodies in Transplant Recipients? Transplantation. 2024 May 1;108(5):e69-e71. doi: 10.1097/TP.0000000000004921. Epub 2024 Jan 26. PMID: 38277265.*
74. *Miyamoto T, Fukunaga Y, Ogasawara A, Munakata A, Murai K. Autoantibody profiles in intravenous immunoglobulin preparations: A possible cause of mistaken autoimmunity diagnosis. Transfusion. 2024 Apr;64(4):597-605. doi: 10.1111/trf.17766. Epub 2024 Feb 23. PMID: 38400628.*

Intravenous immunoglobulin or intravenous lipid emulsion therapy in patients with elevated NK cytotoxicity improves live birth rates in different ways.

Running title:
In patients with elevated NK cytotoxicity, IvIg improves IVF outcomes mainly by increasing the ongoing pregnancy rate, while IL also reduces pregnancy failures. IvIg or IL treatment significantly improves LBR, but does not affect the high rate of preterm birth inherent in eNKc patients.

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The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The study conformed Diagnostic and immunotropic treatment options were agreed with the patients and informed consent was obtained (approved by the Biomedical Ethics Committee of the Institute of Pediatrics, Obstetrics and Gynecology, National Academy of Medical Sciences of Ukraine, No. 6, according to the Declaration of Helsinki).

No Conflict of interest

Abstract

Problem: Many studies have demonstrated the negative impact of high rates of NK cytotoxicity (NKc) on reproductive outcomes, as well as the beneficial effects of intravenous immunoglobulin or intravenous lipid emulsion, specifically in these patients.
Method of study: NKc was measured in PBMCs against K562 by flow cytometry. For retrospective analysis, 2250 cycles of ET were selected and divided into 4 groups according to NKc levels and treatment: normal (nNKc, n=587), elevated (eNKc, n=615), eNKc with IvIg treatment (n=231), and eNKc with IL treatment (n=180). Patients were <36 years old, negative for anticardiolipin antibodies, and did not have insufficient endometrium, thrombotic disorders, uterine abnormalities, or obesity. Patients aged 36-39 years (n=359) and 39-42 years (n=279) were analyzed separately using a similar approach.
Results: Patients with eNKc, in addition to having reduced clinical pregnancy rates (CPR) 26.99%, had increased levels of pregnancy failures (PF) 36.7% and a preterm birth rate (PBR) 13.3%, compared to nNKc patients (34.07%, 23.5%, and 3.26%, respectively). As a result, patients with eNKc had a reduced live birth rate (LBR) (17.0%), compared to nNKc patients (26.06%, OR 1.720, p<0.0002).
Both IvIg and IL treatment resulted in increased CPR (44.15% and 39.44%) and LBR (32.03% and 32.22%) compared to patients with eNKc without treatment. IvIg treatment increased CPR even compared to patients with nNKc. However, the pregnancy failure rate

(PFR) in IvIg-treated patients was not significantly reduced compared to non-treated patients with eNKc. In contrast, IL-treated patients had significantly decreased PFR compared to patients with eNKc without treatment. As a result, both treatments (IvIg and IL) led to an LBR similar to that of nNKc. Both therapies (IvIg and IL) did not affect the Preterm birth rate, which remained relatively high (14.86% and 13.79%, respectively). However, the frequency of antenatal or prenatal fetal death was the same in all groups. In patients aged 36-39y.o. the results were quite similar, although the PFR in all groups was already significantly higher. However, in patients aged 39-42 years, the negative impact of eNKc was no longer significant, as high PFR and low LBR were also found in patients with nNKc. Nevertheless, even in this group, IvIg therapy led to higher LBR rates compared to eNKc without therapy.

Conclusions: Our findings highlight the negative impact of high levels of NK cytotoxicity on all stages of the reproductive process. Treatment with IvIg and IL in this group significantly improves LBR. It seems that the strength of IvIg is based on a strong increase in the CPR even compared with nNKc patients, whereas IL's effectiveness is more based on a significantly reduced PF rate. It also appears that the efficacy of IvIg is not limited to the group of patients with eNKc. Combining IvIg and IL treatments may enhance the results in patients with elevated NK activity.

Keywords: NK cytotoxicity, Intravenous immunoglobulin, intravenous lipid emulsion, IVF failures, Pregnancy failures, live birth rates, Preterm birth.

Introduction

Natural Killer lymphocytes (NK) play a critical role in human reproduction: blastocyst recognition, embryo implantation[1,2], trophoblast invasion[3], spiral artery remodeling[4,5,6,7], and subsequent pregnancy ongoing[8,9]. It is logical to assume that the affected NK numbers or functions influence the efficiency of these reproductive processes and form an unfavorable background for reproduction and it was shown in many studies[10,11,12,13,16]. Status of peripheral blood (PB) natural killer cells NK cell frequency and NK cytotoxicity (NKc) associated with reproductive failures (recurrent pregnancy loss (RPL) abortions, infertility of unknown etiology, and IVF failure[10,11,14,15,17].

The number and phenotype of endometrial natural killer cells are also associated with reproductive disorders, such as unexplained subfertility, recurrent implantation failures (RIF), and RPL[18,19,20].

A lot of the meta-analyses showed higher NK-cell proportions or NK cytotoxicity in RPL or RIF[21,22,23,24].

High production of pro-inflammatory cytokines by PB NK cells was registered in women with recurrent miscarriages[25] as well as elevated HLA-DR, CD69, and CD94 expression in NK and T lymphocytes[10,26]. Elevated HLA-DR on endometrial NK and CD3+CD8+lymphocytes was also found in patients with RIF[27,20].

It is well known that there is no correlation between NK numbers in endometrium and PB, but a similarity between decidual and PB in CD161 and CD158b NK expression was shown[28]. The association between high CD45+CD3+CD8+DR+ levels in peripheral blood and higher uNK numbers was also shown[27]. We previously described that CD3+CD8+DR+ peripheral lymphocytes correlated with the same population in the endometrium. We also found a correlation between endometrial NK and PB NK CD335 and CD8 expression[29,30,20]. So endometrial immunophenotype is specific, but not autonomic and isolated, from PB immunophenotype.

Elevated NK numbers, activity, or cytotoxicity in patients with reproductive failures were shown in the majority of studies. However, in recent years, studies have shown the adverse effects of reduced/suppressed NK states. So, it was shown: that the low cytotoxic activity of peripheral blood NK cells may relate to unexplained recurrent miscarriage[31] or IVF failures[16]. Increased natural killer cell subsets with inhibitory cytokines and inhibitory surface receptors in patients with recurrent miscarriage, was shown[32].

Thus, the vast majority of studies reveal an association between accentuated high levels of NK lymphocytes and NK activity with reproductive losses, as well as the effectiveness of immunomodulation therapy by Intravenous immunoglobulin or intravenous fat emulsion among such patients[33,34,35]. However, the issue of implementing these diagnostic methods and therapeutic approaches remains unresolved.

NK cytotoxic reaction, measured by the NK assay against HLA-negative K562 cell lines, is the “gold standard” for the investigation of NK activity in vitro[36]. In Kyiv, we implemented NK cytotoxicity testing of patients with reproductive failures back in 2006 and during this time have done > 28,000 tests. We planned this study back in 2010 and completed it well before the SarsCov2 pandemic. In a previous retrospective study, we showed that accentuated NK cytotoxicity (reduced or increased) is an unfavorable factor for implantation and pregnancy ongoing[16]. The current retrospective study continues the previous one and analyzes the effectiveness of immunomodulation therapy by Intravenous immunoglobulin or intravenous fat emulsion in ordinary IVF population with elevated NK cytotoxicity.

Material and Methods

Blood sample collection

NK cytotoxicity was routinely investigated in patients who had at least one incident of idiopathic failure in previous IVF cycles (2009 to 2019). In the overall analysis, we took more than 8000 determinations of NK cytotoxicity in more than 4,000 patients from the (Reproductive Medicine Clinic "IRM Dachno", Kyiv, Ukraine). The clinic is adjacent to a laboratory (800m) and guarantees the optimal time and conditions for blood sample transportation. 5.5 ml peripheral blood was collected from 8:30 to 10:00 am and delivered to the laboratory no later than at 12 am. Thus, no more than 5 hours at room temperature passed after the blood collection and onset of lymphocyte isolation. Effectors (peripheral blood mononuclear cells (PBMC)) were isolated on a density gradient (“Histopaque-1077” Sigma-Aldrich. Darmstadt. Germany).

Study design.

We select the clinical history of 2250 embryo transfer (ET) cycles for retrospective analysis from more than 8.000 ET cycles Fig1. For analysis, cycles of ET were selected according to the criteria: the ET was performed no more than one year after the last NKc examination at least 1 good-quality embryo was transferred. Patients were negative for anticardiolipin antibodies and did not have: insufficient endometrium (<7mm, chronic endometritis or endometriosis), thrombotic or acute infectious disorders, uterus abnormality, abnormal karyotype and obesity. Most patients had several reasons for performing IVF, but the dominant ones were in the tubo-peritoneal factor, the male factor and the endocrine factor.

Main study groups

In all Main study groups patient’s oocytes were obtained before 36yo. Clinical and anamnestic data are shown in **(Tabe1).**

Control groups

Patients from control groups did not obtain any additional treatment (except low-dose aspirin). In the case of elevated NKc (eNKc) or normal NKc (nNKc) results, the determination of NKc was repeated in the majority of cases (in 64% for eNKc and 61% for nNKc). For analysis, the patients from control groups were divided into 2 groups according to the NKc levels; eNKc (<36yo n=615) or nNKc (<36yo n=587) (Fig1).

IvIg group

The intravenous immunoglobulin group included patients with elevated NKc obtained intravenous immunoglobulin infusion (eNKc <36yo IvIg n=231) 100-250mg/kg (average 165mg/kg) (IvIg, Biofarma, Kyiv Ukraine), 3-5days before embryo transfer. 49 patients obtained secondary IvIg infusion after a positive hCG test and their PF and LBR were analysed separately.

IntaLipid group

Intralipid group (n=180) same-age patients with elevated NKc obtained intravenous 20% (200ml) Fat Emulsion **(“**Intralipid Fresenius Kabi”, Austria) infusion 3-5 days before embryo transfer.

Addition groups

The same treated and control patients (36-39y.o. and 39-42y.o.) were analyzed separately by similar design Fig1.

Analysis

Pregnancy rate (PR) is calculated as % of the ongoing pregnancy (egg in uterus confirmed by ultrasound on 5 weeks of gestation (w/g) per ET. Pregnancy failures are calculated as a part of the pregnancy’s that not result to live birth (failed pregnancy and fetal death) from the confirmed ongoing pregnancy (pregnancy failures refer to ongoing pregnancies that do not result in live birth, including early pregnancy loss and fetal demise).

Live birth rate (LBR) was calculated as part of births of a live baby after 24 weeks of gestation per ET. Medical abortion due to fetal malformations was included as a pregnancy success for PR and as a failure for LBR, but not as a Pregnancy failure. Pre-term Birth is calculated as % of births of a live baby after 24 w/g and before 37w/g from all Live births.

Immunotropic treatment options were agreed upon with the patients and informed consent was obtained (approved by the Biomedical Ethics Committee of the Institute of Pediatrics, Obstetrics and Gynecology, National Academy of Medical Sciences of Ukraine, No. 6, 17/12/2013, according to the Declaration of Helsinki).

Comparison of groups

(**Table1**) presents the age and reproductive history severity of the control and main study groups. Patients with normal NKc have less number of previous unsuccessful programs and predominantly have primary infertility diagnoses compared to all groups with elevated NKc. It’s a natural limitation of randomization through the accumulation of patients with eNKc in subsequent cycles. Other parameters (age, part of fresh or cryo-cycles, endometrial thickness, number of embryos transferred, AMH, FSH, LH) did not differ significantly between the groups

Determination of NK cytotoxicity

NK cell activity was investigated as described previously[16].

Target cells K562 were labeled with 5μM CellTracker™Green CMFDA (5-chloromethyl fluorescein diacetate), (Molecular Probes, Eugene, Oreg. USA) and Calcein-AM (O, O′-diacetate tetrakis (acetoxymethyl) ester) (Sigma-Aldrich. Darmstadt. Germany) for 20min at 37°C in a humidified 5%CO2 incubator. The labeled cells were washed twice in PBS, resuspended in RPMI1640 (Sigma-Aldrich. Darmstadt. Germany) with 10% NBCS (Sigma-Aldrich. Darmstadt. Germany), and counted using Flow-CountTM Fluorospheres

(Beckman Coulter, CA, USA). The effector cells PBMC were co-incubated at the E/T ratios of 30, 15, and 7.5 for 2.5h at 37°C in the atmosphere of 5% CO2 in the air. After the incubation period, the cells were mixed with 10μL of PI solution 2mg/ml (Sigma-Aldrich. Darmstadt. Germany) in PBS to stain dead cells. For each E/T ratio, the NK cytotoxicity was measured by analyzing 10000 target cells/samples using a FACScan flow cytometer (BD Bioscience, San Jose, USA) equipped with CellQuest software. Background target cell death was determined from cells incubated in the absence of effector cells.

For each E/T ratio specific NK cytotoxicity was calculated as a percentage of PI positive (dead cells) minus the level of spontaneous lysis. After, we construct a calibration curve and calculate virtual cytotoxicity under the condition of E/T ratio (E/T ratio - 10/1 and 20/1). The level of spontaneous lysis was determined as the percentage of dead cells under the same conditions of incubation but in the absence of effector cells. Less than 5% spontaneous lysis of target cells was observed in these experiments. (**supplemental Fig1 A**)

To measure the E/T ratio correctly, we labeled permeabilized by solution (0.25% Triton and 1% paraformaldehyde) PBLs with PI, and after added labeled K562 cells. The ratio of E/T, in each sample, was measured by flow cytometry (PBLs – FL3, K562 cells – FL1.) (**supplemental Fig1 B**)

For target-gate correction (FL1 lost) we used (50% permeabilized K562 culture) 100μL target cell permeabilized by 50μL 96% ethanol for 10sec. After the vortexing 100μL of PBS and 100μL of unaffected targets were added. The obtained suspension consists of 50% life and 50% permeable cells. (**supplemental Fig1 A**)

As the concept of "normal cytotoxicity" the reference values of NK cytotoxicity are favorable for reproductive prognosis[26,16].

The cytotoxicity activity >40% (E/T ratio 20/1) and >30% (E/T ratio 10/1) was considered as elevated. Criterium for Elevated NKc: NKc (20/1) >40% but in same time NKc (10/1) not less 27% or NKc (10/1) >30% but time NKc (20/1) not less 36%. Criterium for Normal NKc: all NK that not elevated but without reduced NK cytotoxicity levels NKc (10/1) less than 10% or NKc (20/1) less than 15%. (Later these levels were clinically confirmed as clinically significant in a prospective cohort study[26] and later confirmed in correlative analysis (as levels that start correlate with NK numbers)[37,38].

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).

Results

Main study groups<36yo

Control groups.

Patients with elevated NKc display downregulated ongoing pregnancy rate, and LBR as well as increased PF and pre-term birth rate compared to same-age patients with normal NKc (**Table 2**).

IvIg and IL treatment

IvIg and IL treatments result in an increased ongoing pregnancy rate, and LBR (44.1% and 32.03%) compared to patients with eNKc without treatments (26.99% p < 0.0001 and 17.07% p < 0.0001 respectively) (**Table 2**).

IvIg treatment increased ongoing pregnancy even if compared to same-age patients with normal NKc (p=0.0103). But at the same time, PF rate in IvIg-treated patients wasn't significantly downregulated compared to non-treated patients with eNKc. LBR in IvIg-treated patients didn't significantly increase compared to nNK.

In contrast, IL-treated patients have a significantly decreased PF rate (18.3%) compared to patients with eNKc without treatments (36.7%, p= 0.0007) (**Table 2**). As a result, both treatments (IvIg and IL) have similar LBR. Treatment by IvIg and IL does not decrease the levels of preterm birth compared to eNKc patients and it was still significantly increased (14.86%,13.79%) compared to women with nNKc (3.26%, p=0.0038 and p= 0.0084 respectively) .

Both treatments result in the normalization of LBR in different ways. IvIg realizes it mostly through increased PR (even compared with the nNKc group). IL realizes it through normalization PR rate with a reduction of pregnancy failure rates.

Patient's 36-39yo

NK cytotoxicity control groups.

Patients 36-39yo with elevated NKc still display downregulated ongoing pregnancy rate and LBR and increased PF rate compared to same-age patients with normal NKc (**Table3**). In patients with eNKc levels of preterm birth were significantly increased (25.0%) compared to patients with nNKc (0%, p= 0.0079).

IvIg and IvLe treatment

In patients, 36-39yo IvIg treatment still increased, ongoing pregnancy rate, and LBR compared to patients with eNKc without treatments (**Table 3**). However, PF and preterm birth rates were similar to those in non-treated patients with eNKc.

IL treatment demonstrated a similar to the IvIg effect but the difference wasn't significant due to the smaller group.

Patients >39yo

Patients >39yo with elevated NKc (n=108) did not display downregulation ongoing pregnancy rate (18.5%) and LBR (9.2%) and increased PF rate (50%) compared to same-

age patients with normal NKc (n=113) (PR-27.4% p=0.155, LBR- 15.9% p= 0.2342 and PF-41.9% p=0.7791 subsequently). But even in these age group patients with eNKc, IvIg treatment (n=58) result to significant increase PR and LBR (37.9%p=0.0150 and 22.4% p=0.0381) but not to decrease PF rate (40.9%, p=0.67) compared non treated eNKc group. In these age group we can't find negative NKc effect but we can see positive IvIg effect.

Preterm birth

To study the significance of NK cytotoxicity for preterm birth, we analyzed patients with single and twin pregnancies separately. Patients with tween pregnancies had a slightly higher LBR in all groups, but not significantly (Table 4). Patients with single pregnancies and eNKc had a reduced LBR compared with those with 1 fetus and nNKc (p=0.0034). Patients with single pregnancies with eNKc who received IvIg and IL had higher LBR rates compared with eNKc without treatment (significant for IL (p=0.0111) and not quite significant for IvIg (p=0.0768)).

Patients with both normal and elevated NKc with twin pregnancies had an increased rate of preterm birth compared with those with one fetus (Table 4). However, patients with high NKc and with twin pregnancies had an increase in the rate of preterm birth compared with nNKc (p=0.0426).

Patients with single pregnancies with eNKc who received IvIg still had a higher rate of preterm birth compared to nNKc. The rate of perinatal mortality in all groups was similar.

Discussion

For more than 15 years, it has been understood that the use of immune therapy can be effective in the presence of biomarkers of immune risk factors. For IvIg and Intralipid therapy, such indicators can be emphasized by high levels of NK activity and NK cytotoxicity. A large number of studies have shown that IvIg and IL have been successful in the treatment of recurrent miscarriage and recurrent implantation failure among women with elevated NK number or NK cell activity[33,39,40,41,42,43] as well as for patients with elevated NK cell density in endometrial biopsy[44,45]**.** Such a large number of studies allowed us to hope for the rapid implementation of such approaches in the clinic- "*We believe that when laboratory results are incorporated into the diagnosis of RSA/RSAI that treatment is highly successful whereas the absence of laboratory results severely hinders the effectiveness of treatment*"[46]**.**

A lot of Systematic reviews and meta-analysis support that IvIg treatment increase LBR in patients with accentuated NK functions[24]. It was confirmed with varying levels of optimism from "*There is a strong basis for providing this treatment to patients with NK deviation*"[48] and "*Studies assessing the favoring of the intervention*"[49] to extremely cautious **"***there*

may be some benefit of immunotherapy, but paucity of high quality evidence means that it is not possible to support the use of immunotherapy even when selected based on abnormal NK cell level/activity"[50].

Sometimes authors explicitly postulate the absence of an effect even when they write in the results that "*live birth rate of the IVIG group is superior, but there was no statistical significance [OR =1.43, 95%CI (0.99, 2.07),* P=*0.05*" [51].

But it seems that all that has been done is not enough and we need more studies and larger study's groups.

In an actual retrospective study, we also showed the effectiveness of IvIg and IL in patients with high levels of NKc. We found a positive effect of both treatments at the level of pregnancy, and life-birth rates. We focused on patients with high NKc as a target group for immunotherapy. In a previous similar retrospective study, we showed a dramatic decrease in IVF efficacy in patients with high levels of NKc compared to patients with normal NK cytotoxicity[16].

That is, at certain levels of NK function begins to be unfavorable for a successful reproductive process. But what levels are favorable and why? We have shown that these reference limits for NK number and NK cytotoxicity coincide with levels where NK number and NK cytotoxicity are independent parameters and one does not determine the other[37,38]. At such levels, the function can be adjusted and regulated. While in unfavorable levels, increased or decreased, these two parameters begin to correlate significantly and the number directly determines the function. Perhaps the loss of the ability to effectively regulate is the reason for the "unfavourability" of these levels for reproduction. The difficulty of adaptation reduces the effectiveness of adaptation, but the reproductive process requires the immune system to actively participate and functional changes. We found a similar situation among participants in the Ukrainian Antarctic expeditions. Participants with high levels of NK cells and CD335+NK cells had poorer adaptation to new Antarctic conditions compared to those with balanced levels of NK cells[52,53] and the reference favorable limits in male expedition participants were similar to those in females with subsequent successful reproductive process[54].

We demonstrated that IR and PR after IvIg therapy are not only significantly higher than in patients with eNKc but also higher compared to untreated patients with normal NKc.

In addition, the positive effect of IvIg is found in the age group where high NKc no longer significantly reduces reproductive success. This is probably the result of the multi-therapeutic effects of IvIg. It has been shown that IvIg can improve the condition in the presence of auto or allo antibodies both through the idiotype-anti-idiotype system, by reducing IgG production and blocking the Fc receptor[55,56,57,58].

It also was shown that alloantibodies amplify the negative impact of high NK activity on placental function and IvIg corrects this effect[59]. In the case of inflammatory immune status, IvIg improves the Th1/Th2 balance and activates and potentiates Treg[60,61]. However, in the actual study we showed that in all age groups, the effect of IvIg did not lead to a significantly higher LBR compared to nNKc, and PFR in IvIg-treated patients was rather elevated but similar to in non-treated eNKc patients. May IvIg treatment cause hyperfertility? Previously we showed that implantation of a donor's embryos with higher implantation–invasion capacities and a lower chance of chromosomal abnormalities and genetic complications did not depend on NKc levels. Donors' embryo implantation and pregnancy rates were the same in patients with elevated and normal NKc but the incidence of pregnancy failures among those patients with elevated NKc was much higher[16]. One may speculate that elevated NKc combined with certain chromosomal, genetic, and physiological features or embryo deviation might exacerbate the negative pressure on implantation and amplify the adverse effect already at the pre-implantation and early implantation stages. IvIg treatment possibly gives an implantation chance for a "weak" or mosaic embryo with higher subsequent pregnancy failures. Is it possible to support the subsequent success of these fetuses? Or a possible that part of them had chromosomal abnormalities or genetic or physiological factors that made their further development impossible? We can only speculate about this because in the actual study embryos weren't PGT-A tested and only a small part of the aborted material has been karyotyped. It has been shown that the "normality" of unfavorable NK levels depends on the age of the patients. So, in young patients with "better embryos", the negative clinically significant levels of NK are even higher than in older patients with "poor embryos". Relatively lower levels of NK start to be unfavorable in older patients[62].

It is also difficult to exclude the placebo effect because a prolonged infusion of an expensive drug that is objectively felt by the patient may have a distinct placebo effect. (Sometimes patients with a history of multiple pregnancy failures in anamnesis even at 24-29 weeks of gestation asked to repeat the infusion). It is impossible to select a true placebo group for IvIg because patients feel the drug injection.

Therefore, it seems that IvIg therapy leads to an increase in success rates not only among patients with eNKc but also improves outcomes in patients with other immune causes.

IL therapy also led to higher levels of IR, PR, and LBR, which were statistically significantly higher compared to eNKc without treatment. The levels of PF on IL were lower compared to eNKc. Reproductive success indicators were not significantly higher compared to patients with nNKc. This means that IL therapy corrects specifically the negative effect of high NK cytotoxicity. Both therapies, IvIg and IL, improve LBR in patients with eNKc, but

neither therapy affected the high level of preterm births, which is characteristic of patients with eNKc. In both therapies, this rate remained higher than in the group with nNKc. It was shown that in women with elevated peripheral NK cells, birthweight at delivery is low but IvIg therapy significantly improved it, and IvIg therapy without NK cell elevation did not result in increased birthweight[63] and that at late gestation, premature activation of NK cells can lead to a breakdown of tolerance of the maternal-fetal interface and, subsequently, can result in preterm birth[64].

For the specific analysis of preterm births, we divided all the groups into those with single and twin's pregnancies and found that with eNK, the presence of a twin's pregnancy did not statistically increase the LBR, but it did significantly increase the risk of preterm birth compared to twin's pregnancies with normal NKc, as well as compared to high NKc and single pregnancies. However, we did not find differences between the groups in perinatal mortality.

After dividing into single and twin's pregnancies, the IvIg and IL groups became too small for statistical significance for LBR, although they remained significant for the IvIg group with single pregnancies. The increase in preterm births in eNKc patients with IvIg was statistically significant for single pregnancies, only because this group was larger compared to IL.

We focused on NK cytotoxicity (NKc) because this method has shown high sensitivity and clinical significance in previous studies[16,26]. However, it may not be convenient for widespread routine implementation because NK cytotoxicity assay is difficult to standardization, methodologically, complex to perform, and requires careful attention to the sample's blood viability. On the other hand, immunophenotyping of NK cells using flow cytometry is not a complicated method and it's easily to standardize. We demonstrated that measuring the density of CD335 (NKp46) on NK cells may be almost an alternative method to the cytotoxicity test[37]. The accentuated levels of NK CD335 expression are predictive for reproductive outcomes, both in IVF patients[54] and in patients with natural pregnancies[65]. CD335 expression in peripheral blood NK cells correlates with CD335 levels in NK cells in the endometrium[30]. This might explain why the negative impact of NK CD335 expression becomes significant even at the implantation stages for PGT-A embryos[54]. Meanwhile, high NK cytotoxicity is detrimental primarily during the pregnancy maintenance stage in donor embryos[16] because the active contact between trophoblasts and peripheral NK cells starts with the remodeling of the spiral arteries[66,18].

It explained significant first and early second-trimester pregnancy failure rates in patients with their donor embryos when peripheral blood NK cells first contact with fetal

cytotrophoblast cells. It was shown that elevated NKc leads to incorrect remodeling of the spiral arteries and subsequent pregnancy complications[66,18].

This work has demonstrated the negative impact of high levels of NK cytotoxicity and the positive effect of immunotropic treatment on reproductive process efficiency in a large group of “typical IVF patients".

Limitations of the study

The study population is 95 percent Caucasian and 85 percent Balto-Slavic. It is known that KIR repertoire and HLA-C1/2 frequencies are highly variable in populations[67] and that different combinations are differently favorable for reproduction[68]. In local populations, the number of combinations is limited, which may increase/decrease the frequency of some reproductive complications. In addition, the studied population is characterized by a fairly high level of of CMV (>85%) and HSV1 (>95%) infected and it is known that viral carriage leads to immune modifications, especially for NK lymphocytes[69,70].

All patients had ET with an embryo that was not PGT-A tested and most of the miscarriages were not tested for chromosomal abnormalities. This does not allow us to understand the phenomenon of such a high level of PR after IVIG and simultaneously high levels of PFR.

A weak point of the study is the results on hormone levels because these results were obtained from different laboratories and measured by different methods with different reference values. This is especially true for the causes of infertility, because the diagnosis was made in different medical centers with different criteria, which also changed significantly over the past 10-15 years. This could reduce the statistical significance of these parameters.

An important finding of this study is the high rate of preterm births in all patients with elevated NKc (treated and non-treated). Unfortunately, we cannot analyze the causes of preterm births, and these were mostly cesarean births of 26-32 weeks. Perhaps there were high levels of preeclampsia or other complications but we do not have documentation from the maternity hospitals. Some maternity hospitals simply do not exist physically because they were bombed by rusians, as in Mariupol or Bakhmut. In most of the maternity hospitals, all archival basements have been converted into bomb shelters. So we will not be able to analyze this anymore.

Doses and regimens of IVIG administration for patients with reproductive losses have not been studied sufficiently and actually copy the treatment regimens and doses for patients with primary immunodeficiencies. The usual dose of IGIV for antibody replacement is between 0.3 and 0.6 g/kg per month, delivered every 2 to 4 weeks [71].

The goal of this therapy is very simple: to maintain a sufficient level of serum IgG.

However, in recent years, even in such patients, the standardised administration regimen has been questioned and studies have suggested an individualised approach “The ideal immunoglobulin dose for an individual patient is the dose that keeps the patient well” [72]. Obviously, in patients with reproductive failures with high NK cytotoxicity, both doses and regimens should be developed based on favorable outcome. In addition, it should be borne in mind that IVIG contains both autoantibodies and anti-idiotypic antibodies from donors and high doses of IVIG may affect the clearance of patients anti-idiotypic antibodies [73,74].

Therefore, future studies should take into account NK cell numbers, activity, and phenotype, as well as KIR haplotypes, HLA repertoire, hormonal features, viral carriage, microbiome, and embryo karyotype.

1. Moffett A, Shreeve N. Local immune recognition of trophoblast in early human pregnancy: controversies and questions. *Nat Rev Immunol*. 2023;23(4):222-235. doi:10.1038/s41577-022-00777-2
2. Kanter J, Gordon SM, Mani S, Sokalska A, Park JY, Senapati S, Huh DD, Mainigi M. Hormonal stimulation reduces numbers and impairs function of human uterine natural killer cells during implantation. Hum Reprod. 2023;38(6):1047-1059. doi: 10.1093/humrep/dead069. PMID: 37075311; PMCID: PMC10501469.
3. Pollheimer J, Vondra S, Baltayeva J, Beristain AG, Knöfler M. Regulation of Placental Extravillous Trophoblasts by the Maternal Uterine Environment. *Front Immunol*. 2018;9:2597. Published 2018 Nov 13. doi:10.3389/fimmu.2018.02597
4. Le Bouteiller P. HLA-G in the human placenta: expression and potential functions. *Biochem Soc Trans*. 2000;28(2):208-212. doi:10.1042/bst0280208
5. Li C, Houser BL, Nicotra ML, Strominger JL. HLA-G homodimer-induced cytokine secretion through HLA-G receptors on human decidual macrophages and natural killer cells. Proc Natl Acad Sci U S A. 2009;106(14):5767-72. doi: 10.1073/pnas.0901173106. Epub 2009 Mar 20. PMID: 19304799; PMCID: PMC2667005.
6. Turco MY, Moffett A. Development of the human placenta. *Development*. 2019;146(22):dev163428. Published 2019 Nov 27. doi:10.1242/dev.163428
7. Robson A, Harris LK, Innes BA, Lash GE, Aljunaidy MM, Aplin JD, Baker PN, Robson SC, Bulmer JN. Uterine natural killer cells initiate spiral artery remodeling in human pregnancy. *FASEB J*. 2012;26(12):4876-85. doi: 10.1096/fj.12-210310. Epub 2012 Aug 23. PMID: 22919072.
8. Rao VA, Kurian NK, Rao KA. Cytokines, NK cells and regulatory T cell functions in normal pregnancy and reproductive failures. *Am J Reprod Immunol*. 2023;89(2):e13667. doi:10.1111/aji.13667
9. Smith GC. First-trimester determination of complications of late pregnancy. *JAMA*. 2010;303(6):561-562. doi:10.1001/jama.2010.102

10. Ntrivalas EI, Kwak-Kim JY, Gilman-Sachs A, Chung-Bang H, Ng SC, Beaman KD, Mantouvalos HP, Beer AE. Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. *Hum Reprod.* 2001;16(5):855-61. doi: 10.1093/humrep/16.5.855. PMID: 11331628.
11. Gilman-Sachs A, DuChateau BK, Aslakson CJ, Wohlgemuth GP, Kwak JY, Beer AE, Beaman KD. Natural killer (NK) cell subsets and NK cell cytotoxicity in women with histories of recurrent spontaneous abortions. *Am J Reprod Immunol*. 1999;41(1):99-105. doi: 10.1111/j.1600-0897.1999.tb00081.x. PMID: 10097793.
12. Kuon RJ, Weber M, Heger J, Santillán I, Vomstein K, Bär C, Strowitzki T, Markert UR, Toth B. Uterine natural killer cells in patients with idiopathic recurrent miscarriage. *Am J Reprod Immunol*. 2017;78(4). doi: 10.1111/aji.12721. Epub 2017 Jun 21. PMID: 28639334.
13. Zhu L, Aly M, Wang H, et al. Decreased NK cell immunity in kidney transplant recipients late post-transplant and increased NK-cell immunity in patients with recurrent miscarriage. *PLoS One*. 2017;12(10):e0186349. Published 2017 Oct 17. doi:10.1371/journal.pone.0186349
14. Mahajan D, Sharma NR, Kancharla S, et al. Role of Natural Killer Cells during Pregnancy and Related Complications. *Biomolecules*. 2022;12(1):68. Published 2022 Jan 4. doi:10.3390/biom12010068
15. Emmer PM, Nelen WL, Steegers EA, Hendriks JC, Veerhoek M, Joosten I. Peripheral natural killer cytotoxicity and CD56(pos)CD16(pos) cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. *Hum Reprod*. 2000;15(5):1163-9. doi: 10.1093/humrep/15.5.1163. PMID: 10783371.
16. Dons'koi B, Onyshchuk O, Kononenko I, et al. Accentuated Peripheral Blood NK Cytotoxicity Forms an Unfavorable Background for Embryo Implantation and Gestation. *Diagnostics (Basel)*. 2022;12(4):908. Published 2022 Apr 6. doi:10.3390/diagnostics12040908
17. Salazar MD, Wang WJ, Skariah A, He Q, Field K, Nixon M, Reed R, Dambaeva S, Beaman K, Gilman-Sachs A, Kwak-Kim J. Post-hoc evaluation of peripheral blood natural killer cell cytotoxicity in predicting the risk of recurrent pregnancy losses and repeated implantation failures. *J Reprod Immunol*. 2022;150:103487. doi: 10.1016/j.jri.2022.103487. Epub 2022 Jan 19. PMID: 35078138.
18. El-Azzamy H, Dambaeva SV, Katukurundage D, et al. Dysregulated uterine natural killer cells and vascular remodeling in women with recurrent pregnancy losses. *Am J Reprod Immunol*. 2018;80(4):e13024. doi:10.1111/aji.13024
19. Chiokadze M, Bär C, Pastuschek J, Dons'koi BV, Khazhylenko KG, Schleußner E, Markert UR, Favaro RR. Beyond Uterine Natural Killer Cell Numbers in Unexplained Recurrent Pregnancy Loss: Combined Analysis of CD45, CD56, CD16, CD57, and CD138. *Diagnostics (Basel).* 2020;29;10(9):650. doi: 10.3390/diagnostics10090650. PMID: 32872526; PMCID: PMC7555015.
20. Sudoma I, Goncharova Y, Dons'koy B, Mykytenko D. Immune phenotype of the endometrium in patients with recurrent implantation failures after the transfer of genetically tested embryos in assisted reproductive technology programs. *J Reprod Immunol*. 2023;157:103943. doi: 10.1016/j.jri.2023.103943. Epub 2023 Mar 22. PMID: 36966647.
21. Cavalcante MB, da Silva PHA, Carvalho TR, Sampaio OGM, Câmara FEA, Cavalcante CTMB, Barini R, Kwak-Kim J. Peripheral blood natural killer cell cytotoxicity in recurrent miscarriage: a systematic review and meta-analysis. *J*

Reprod Immunol. 2023;158:103956. doi: 10.1016/j.jri.2023.103956. Epub 2023 May 18. PMID: 37236061.

22. Kolanska K, Suner L, Cohen J, Ben Kraiem Y, Placais L, Fain O, Bornes M, Selleret L, Delhommeau F, Feger F, Mathieu d'Argent E, Darai E, Chabbert-Buffet N, Antoine JM, Kayem G, Mekinian A. Proportion of Cytotoxic Peripheral Blood Natural Killer Cells and T-Cell Large Granular Lymphocytes in Recurrent Miscarriage and Repeated Implantation Failure: Case-Control Study and Meta-analysis. *Arch Immunol Ther Exp (Warsz)*. 2019;67(4):225-236. doi: 10.1007/s00005-019-00546-5. Epub 2019 May 30. PMID: 31147723.
23. Von Woon E, Greer O, Shah N, Nikolaou D, Johnson M, Male V. Number and function of uterine natural killer cells in recurrent miscarriage and implantation failure: a systematic review and meta-analysis. *Hum Reprod Update*. 2022;28(4):548-582. doi:10.1093/humupd/dmac006
24. Seshadri S, Sunkara SK. Natural killer cells in female infertility and recurrent miscarriage: a systematic review and meta-analysis. *Hum Reprod Update*. 2014;20(3):429-438. doi:10.1093/humupd/dmt056
25. Guerrero B, Hassouneh F, Delgado E, Casado JG, Tarazona R. Natural killer cells in recurrent miscarriage: An overview. *J Reprod Immunol*. 2020;142:103209. doi:10.1016/j.jri.2020.103209
26. Chernyshov VP, Dons'koi BV, Sudoma IO, Goncharova YO. Favorable immune phenotype predicts successful implantation and pregnancy. *Immunol Lett*. 2014;162(2 Pt B):217-221. doi:10.1016/j.imlet.2014.10.022
27. Kuon RJ, Vomstein K, Weber M, Müller F, Seitz C, Wallwiener S, Strowitzki T, Schleussner E, Markert UR, Daniel V, Toth B. The "killer cell story" in recurrent miscarriage: Association between activated peripheral lymphocytes and uterine natural killer cells. *J Reprod Immunol*. 2017;119:9-14. doi: 10.1016/j.jri.2016.11.002. Epub 2016 Nov 6. PMID: 27865124.
28. El-Badawy O, Helmy AS, Abbas AM, Zahran AM, Afifi NA, Abdel-Rahim MH. Concordance between peripheral and decidual NK cell subsets and killer immunoglobulin-like receptors in women with recurrent spontaneous miscarriages. *J Reprod Immunol*. 2020;140:103130. doi:10.1016/j.jri.2020.103130

29. Chernyshov VP, Dons'koi BV, Sudoma IO, Goncharova YO. Comparison of T and NK lymphocyte subsets between human endometrial tissue and peripheral blood. *Cent Eur J Immunol*. 2019;44(3):316-321. doi:10.5114/ceji.2019.89610
30. Anoshko Y, Dons'koi B, Sudoma I, Khazhylenko K, Zabara D, Goncharova Y. Changes in the immunophenotype of endometrium during implantation window receptivity formation in healthy fertile women. *Placenta*. 2023;143:62-68. doi:10.1016/j.placenta.2023.10.002
31. Zhang Y, Huang C, Lian R, Xu J, Fu Y, Zeng Y, Tu W. The low cytotoxic activity of peripheral blood NK cells may relate to unexplained recurrent miscarriage*. Am J Reprod Immunol.* 2021;85(6):e13388. doi: 10.1111/aji.13388. Epub 2021 Jan 27. PMID: 33410220.
32. Zhu L, Aly M, Wang H, Karakizlis H, Weimer R, Morath C, Kuon RJ, Toth B, Ekpoom N, Opelz G, Daniel V. Increased natural killer cell subsets with inhibitory cytokines and inhibitory surface receptors in patients with recurrent miscarriage and decreased or normal subsets in kidney transplant recipients late post-transplant. *Clin Exp*

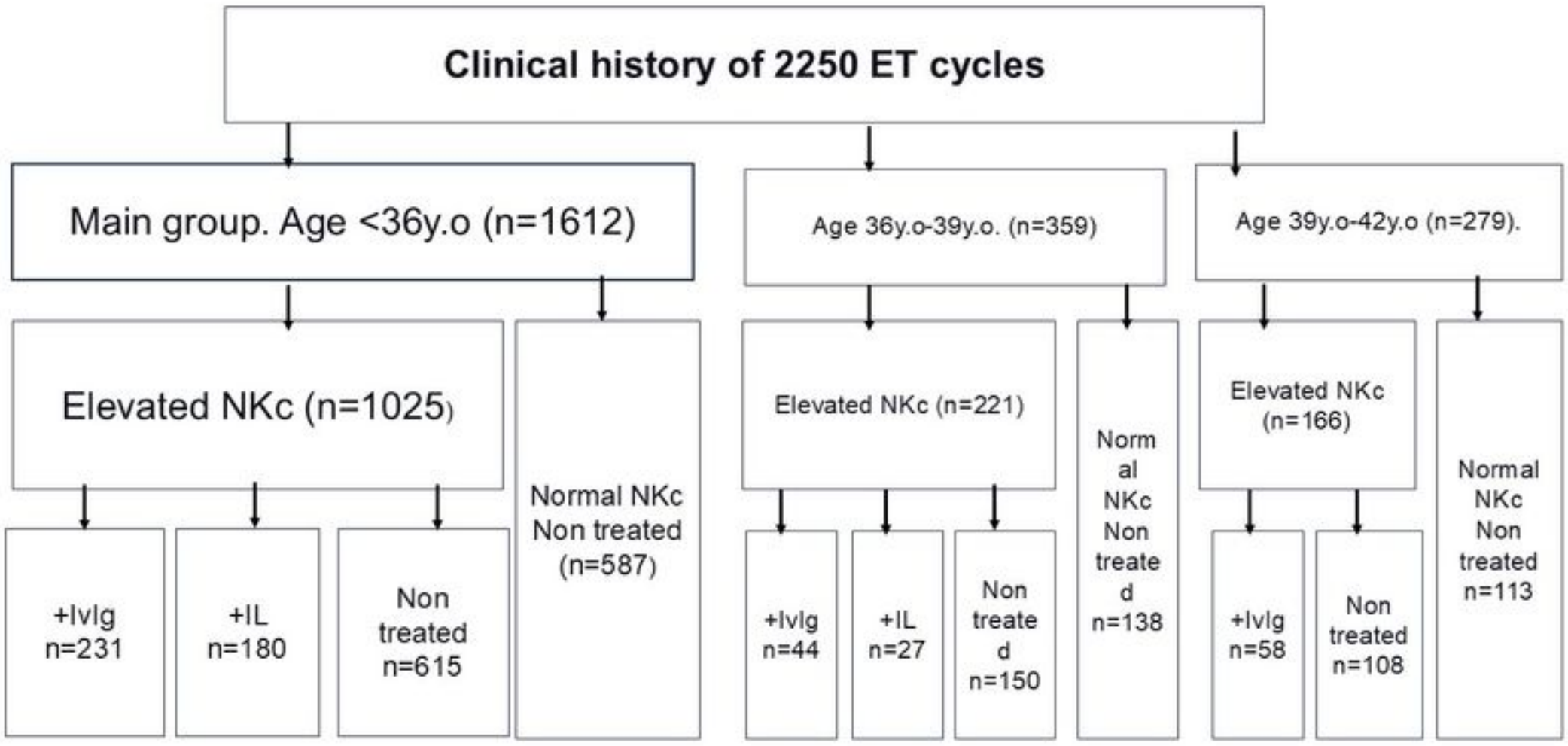
Immunol. 2018;193(2):241-254. doi: 10.1111/cei.13142. Epub 2018 May 31. PMID: 29679490; PMCID: PMC6046488.
33. Coulam CB, Acacio B. Does immunotherapy for treatment of reproductive failure enhance live births?. *Am J Reprod Immunol*. 2012;67(4):296-304. doi:10.1111/j.1600-0897.2012.01111.
34. Mu F, Huo H, Wang M, Liu L, Wang F. Intravenous immunoglobulin improves live birth rates in patients with unexplained recurrent pregnancy loss. *J Reprod Immunol.* 2024;166:104322. doi: 10.1016/j.jri.2024.104322. Epub 2024 Aug 26. PMID: 39216166.
35. Marchand GJ, Masoud AT, Ulibarri H, Arroyo A, Coriell C, Goetz S, Moir C, Moberly A, Gonzalez D, Blanco M, Craig HR. Effect of a 20% intravenous fat emulsion therapy on pregnancy outcomes in women with RPL or RIF undergoing IVF/ICSI: a systematic review and meta-analysis. *J Clin Transl Res*. 2023;12;9(4):236-245. PMID: 37564251; PMCID: PMC10411978.
36. Cholujová, D.; Jakubíková, J.; Kubes, M.; Arendacká, B.; Sapák, M.; Ihnatko, R.; Sedlák, J. Comparative study of four fluorescentprobes for evaluation of natural killer cell cytotoxicity assays. *Immunobiology* 2008;213, 629–640.
37. Dons'koi BV, Osypchuk DV, Chernyshov VP. Enumeration of peripheral blood NKp46 positive NK lymphocytes reflects NK cytotoxic activity in vitro. *J Immunol Methods*. 2019;474:112639. doi:10.1016/j.jim.2019.112639
38. Dons'koi BV, Chernyshov VP, Osypchuk DV, et al. Natural killer frequency determines natural killer cytotoxicity directly in accentuated zones and indirectly in "moderate-to-normal frequency" segment. *Cent Eur J Immunol*. 2020;45(3):315-324. doi:10.5114/ceji.2020.101263
39. Moraru M, Carbone J, Alecsandru D, et al. Intravenous immunoglobulin treatment increased live birth rate in a Spanish cohort of women with recurrent reproductive failure and expanded CD56(+) cells. *Am J Reprod Immunol*. 2012;68(1):75-84. doi:10.1111/j.1600-0897.2012.01135.x
40. Ramos-Medina R, García-Segovia A, Gil J, et al. Experience in IVIg therapy for selected women with recurrent reproductive failure and NK cell expansion. *Am J Reprod Immunol*. 2014;71(5):458-466. doi:10.1111/aji.12217
41. Cohen BM, Machupalli S. Use of Gammaglobulin to Lower Elevated Natural Killer Cells in Patients with Recurrent Miscarriage. *J Reprod Med*. 2015;60(7-8):294-300.
42. Chernyshov VP, Dons'koi BV, Sudoma IO, Goncharova YO. Multiple immune deviations predictive for IVF failure as possible markers for IVIG therapy. *Immunol Lett*. 2016;176:44-50. doi:10.1016/j.imlet.2015.12.010
43. Yamada H, Deguchi M, Saito S, et al. High doses of intravenous immunoglobulin stimulate regulatory T cell and suppress natural killer cell in women with recurrent pregnancy loss. *J Reprod Immunol*. 2023;158:103977. doi:10.1016/j.jri.2023.103977
44. Coulam CB. Intralipid treatment for women with reproductive failures. *Am J Reprod Immunol*. 2021;85(4):e13290. doi:10.1111/aji.13290
45. Lédée N, Vasseur C, Petitbarat M, Chevrier L, Vezmar K, Dray G, Chenière S, Lobersztajn A, Vitoux D, Cassuto GN, Chaouat G. Intralipid® may represent a new hope for patients with reproductive failures and simultaneously an over-immune endometrial activation. *J Reprod Immunol.* 2018;130:18-22. doi: 10.1016/j.jri.2018.09.050. Epub 2018 Sep 24. PMID: 30286362.

46. Beaman KD, Ntrivalas E, Mallers TM, Jaiswal MK, Kwak-Kim J, Gilman-Sachs A. Immune etiology of recurrent pregnancy loss and its diagnosis. *Am J Reprod Immunol*. 2012;67(4):319-325. doi:10.1111/j.1600-0897.2012.01118.x
47. Kumar P, Philip CE, Eskandar K, Marron K, Harrity C. Effect of intravenous immunoglobulin therapy in recurrent implantation failure: A Systematic review and meta-analysis. *J Reprod Immunol*. 2024;166:104323. doi:10.1016/j.jri.2024.104323
48. Yang X, Meng T. Is there a Role of Intravenous Immunoglobulin in Immunologic Recurrent Pregnancy Loss?. *J Immunol Res*. 2020;2020:6672865. Published 2020 Dec 27. doi:10.1155/2020/6672865
49. Polanski LT, Barbosa MA, Martins WP, et al. Interventions to improve reproductive outcomes in women with elevated natural killer cells undergoing assisted reproduction techniques: a systematic review of literature. *Hum Reprod*. 2014;29(1):65-75. doi:10.1093/humrep/det414
50. Woon EV, Day A, Bracewell-Milnes T, Male V, Johnson M. Immunotherapy to improve pregnancy outcome in women with abnormal natural killer cell levels/activity and recurrent miscarriage or implantation failure: A systematic review and meta-analysis. *J Reprod Immunol*. 2020;142:103189. doi: 10.1016/j.jri.2020.103189. Epub 2020 Aug 22. PMID: 32889304.
51. Ling Q, Xu J, Tian Y, Chen D, Mo C, Peng B. Effect of IVIG therapy on pregnant women with unexplained recurrent spontaneous abortion: a systematic review and meta-analysis. *Front Endocrinol (Lausanne*). 2024;14;15:1381461. doi: 10.3389/fendo.2024.1381461. PMID: 39205682; PMCID: PMC11349655.
52. Dubrovskyi, I., Zabara, D., Anoshko, Y. et al. Immune accentuations as a prognostic factor for adaptation in Antarctic conditions. *Polar Biol* 2025;48, 53. https://doi.org/10.1007/s00300-025-03359-9
53. Zabara D, Kozeretska I, Deineko I, Anoshko Y, Shapovalenko N, Stamboli L, Dons'koi, B. Immune factors and health of Antarctic explorers. *Ukrainian Antarctic Journal*. 2021;2, 94-105. https://doi.org/10.33275/1727-7485.2.2021.680
54. Dons'Koi BV, Osypchuk DV, Baksheev SM, Sudoma IO, Goncharova YO, Palyha IE, Sirenko VY, Khazhylenko KG, Onyshchuk O, Anoshko Y, Shapovalenko NO. A blinded multicenter investigation: Accentuated NK lymphocyte CD335 (NKp46) expression predicts reproductive failures after IVF. *Immunol Lett.* 2022;251-252:47-55. doi: 10.1016/j.imlet.2022.10.003. Epub 2022 Oct 18. PMID: 36265608.
55. Chernyshov V, Dons'Koi B, Vodyanik M. Clinical immunologyAnti-idiotypic regulation of cofactor-independent antiphospholipid antibodies. *Central European Journal of Immunology.* 2011;36(4):220-225.
56. Skariah A, Sung N, Salazar Garcia MD, Wu L, Tikoo A, Gilman-Sachs A, Kwak-Kim J. Low-dose prednisone and immunoglobulin G treatment for woman at risk for neonatal alloimmune thrombocytopenia and T helper 1 immunity. *Am J Reprod Immunol.* 2017;77(6). doi: 10.1111/aji.12649. Epub 2017 Feb 27. PMID: 28240400
57. Blank M, Anafi L, Zandman-Goddard G, Krause I, Goldman S, Shalev E, Cervera R, Font J, Fridkin M, Thiesen HJ, Shoenfeld Y. The efficacy of specific IVIG anti-idiotypic antibodies in antiphospholipid syndrome (APS): trophoblast invasiveness and APS animal model. *Int Immunol*. 2007;19(7):857-65. doi: 10.1093/intimm/dxm052. Epub 2007 Jun 18. PMID: 17576752.
58. Yuan X, Zhang W, Wang T, Jiang P, Wang ZK, Li CQ. Use of intravenous immunoglobulin in antiphospholipid antibody positive patients with high risk of miscarriage: a systematic review and meta-analysis. *PeerJ.* 2024;31;12:e18419. doi: 10.7717/peerj.18419. PMID: 39494295; PMCID: PMC11531756.

59. Yougbaré I, Tai WS, Zdravic D, Oswald BE, Lang S, Zhu G, Leong-Poi H, Qu D, Yu L, Dunk C, Zhang J, Sled JG, Lye SJ, Brkić J, Peng C, Höglund P, Croy BA, Adamson SL, Wen XY, Stewart DJ, Freedman J, Ni H. Activated NK cells cause placental dysfunction and miscarriages in fetal alloimmune thrombocytopenia. *Nat Commun.* 2017;8(1):224. doi: 10.1038/s41467-017-00269-1. PMID: 28794456; PMCID: PMC5550461.
60. Park JS, Song AY, Bae JY, Han JW, Kim TH, Kim CJ, Lee SK. IL-17 Producing T to Foxp3^{+}CD4^{+} Regulatory T Cell Ratio as a Diagnostic and Prognostic Marker in Women With Recurrent Pregnancy Loss and Its Implications for Intravenous Immunoglobulin Therapy. *Am J Reprod Immunol*. 2024;92(5):e70020. doi: 10.1111/aji.70020. PMID: 39584290.
61. Yamada H, Deguchi M, Saito S, Takeshita T, Mitsui M, Saito T, Nagamatsu T, Takakuwa K, Nakatsuka M, Yoneda S, Egashira K, Tachibana M, Matsubara K, Honda R, Fukui A, Tanaka K, Sengoku K, Endo T, Yata H. High doses of intravenous immunoglobulin stimulate regulatory T cell and suppress natural killer cell in women with recurrent pregnancy loss. *J Reprod Immunol*. 2023;158:103977. doi: 10.1016/j.jri.2023.103977. Epub 2023 Jun 14. PMID: 37354783.
62. Ramos-Medina R, García-Segovia Á, León JA, Alonso B, Tejera-Alhambra M, Gil J, Caputo JD, Seyfferth A, Aguarón Á, Vicente Á, Ordoñez D, Alonso J, de Albornoz EC, Carbone J, Caballero P, Fernandez-Cruz E, Ortiz-Quintana L, Sánchez-Ramón S. New decision-tree model for defining the risk of reproductive failure. *Am J Reprod Immunol*. 2013;70(1):59-68. doi: 10.1111/aji.12098. Epub 2013 Mar 11. PMID: 23480226.
63. Reed JL, Winger EE. IVIg therapy increases delivery birthweight in babies born to women with elevated preconception proportion of peripheral blood (CD56+/CD3-) natural killer cells. *Clin Exp Obstet Gynecol*. 2017;44(3):384-391. PMID: 29949278.
64. Zhang X, Wei H. Role of Decidual Natural Killer Cells in Human Pregnancy and Related Pregnancy Complications. *Front Immunol.* 2021;26;12:728291. doi: 10.3389/fimmu.2021.728291. PMID: 34512661; PMCID: PMC8426434.
65. Dons'koi BV, Baksheev SM, Sudoma IO, Palyha IE, Khazhylenko KG, Zabara DV, Anoshko YI, Dosenko VE, Dubrovsky EI. A Blinded Investigation: Accentuated NK Lymphocyte CD335 (NKp46) Expression Predicts Pregnancy Failures. *Diagnostics (Basel)*. 2023;25;13(11):1845. doi: 10.3390/diagnostics13111845. PMID: 37296696; PMCID: PMC10252685.
66. Koo HS, Kwak-Kim J, Yi HJ, Ahn HK, Park CW, Cha SH, Kang IS, Yang KM. Resistance of uterine radial artery blood flow was correlated with peripheral blood NK cell fraction and improved with low molecular weight heparin therapy in women with unexplained recurrent pregnancy loss. *Am J Reprod Immunol*. 2015;73(2):175-84. doi: 10.1111/aji.12316. Epub 2014 Oct 23. PMID: 25339113.
67. Hiby SE, Ashrafian-Bonab M, Farrell L, Single RM, Balloux F, Carrington M, Moffett A, Ebrahimi Z. Distribution of killer cell immunoglobulin-like receptors (KIR) and their HLA-C ligands in two Iranian populations. Immunogenetics. 2010;62(2):65-73. doi: 10.1007/s00251-009-0408-5. *Erratum in: Immunogenetics*. 2010;62(4):261. Ebrahimi, Zahra [added]. PMID: 19936734; PMCID: PMC2814031.
68. Boudreau JE, Hsu KC. Natural killer cell education in human health and disease. *Curr Opin Immunol*. 2018;50:102-111. doi: 10.1016/j.coi.2017.11.003. Epub 2018 Feb 3. PMID: 29413815; PMCID: PMC5958620.
69. Farzamikia N, Hejazian SM, Haghi M, Hejazian SS, Zununi Vahed S, Ardalan M. Evaluation of telomeric KIR genes and their association with CMV infection in kidney transplant recipients. *Immunogenetics*. 2022;74(2):207-212. doi: 10.1007/s00251-021-01245-2. Epub 2022 Jan 29. PMID: 35091752.

70. Dons'koi BV, Tutchenko TM, Chernyshov VP, Stepaniuk KS. HCMV seropositivity is associated with specific proinflammatory immune phenotype in women with implantation failure. *Immunol Lett.* 2020;217:84-90. doi: 10.1016/j.imlet.2019.11.008. Epub 2019 Nov 19. PMID: 31756347.
71. Orange JS, Hossny EM, Weiler CR, Ballow M, Berger M, Bonilla FA, Buckley R, Chinen J, El-Gamal Y, Mazer BD, Nelson RP Jr, Patel DD, Secord E, Sorensen RU, Wasserman RL, Cunningham-Rundles C; Primary Immunodeficiency Committee of the American Academy of Allergy, Asthma and Immunology. Use of intravenous immunoglobulin in human disease: a review of evidence by members of the Primary Immunodeficiency Committee of the American Academy of Allergy, Asthma and Immunology. J Allergy Clin Immunol. 2006 Apr;117(4 Suppl):S525-53. doi: 10.1016/j.jaci.2006.01.015. Erratum in: J Allergy Clin Immunol. 2006 Jun;117(6):1483. Dosage error in article text. PMID: 16580469.
72. *Wasserman RL. Personalized Therapy: Immunoglobulin Replacement for Antibody Deficiency. Immunol Allergy Clin North Am. 2019 Feb;39(1):95-111. doi: 10.1016/j.iac.2018.08.001. PMID: 30466775.*
73. *Reams V, Emtiazjoo AM, Gries C, Rackauskas M, Saha BK. Does Intravenous Immunoglobulin Administration Affect the Clearance of Monoclonal Antibodies in Transplant Recipients? Transplantation. 2024 May 1;108(5):e69-e71. doi: 10.1097/TP.0000000000004921. Epub 2024 Jan 26. PMID: 38277265.*
74. *Miyamoto T, Fukunaga Y, Ogasawara A, Munakata A, Murai K. Autoantibody profiles in intravenous immunoglobulin preparations: A possible cause of mistaken autoimmunity diagnosis. Transfusion. 2024 Apr;64(4):597-605. doi: 10.1111/trf.17766. Epub 2024 Feb 23. PMID: 38400628.*

Fig1 Study design. Patients separated according age son a 3 group and in each age groups separated according NK cytotoxicity. Patients with elevated NKc separated according treatment (non treated, treated by intravenous Ig (Ivlg) or lipid emulsion (IL).



Implantation rate (IR) per ET as a part of hCG-positive cases per ET (>50u/ml day)
Pregnancy rate (PR) is calculated as % of the ongoing pregnancy (egg in uterus confirmed by ultrasound on 5 wg) per ET. Pregnancy failures are calculated as a part of the failed pregnancy from the confirmed ongoing pregnancy.
Live birth rate (LBR) was calculated as part of births of a live baby after 24w/g per ET.
Pre-term Birth is calculated as % of births of a live baby after 24 w/g and before 37w/g from all Live births.

Table 1. Clinical, anamnesis and hormonal characteristic of Main study groups ($p < 0.05$ * compared to “elevated NKc group)

	eNKc <36yo	nNK <36yo	eNKc <36yo Ivlg	eNKc <36yo IL
Age years	31.5±3.3	31.7±3.02	31.5±3.01	31.1±2.91
Number IVF failures in anamnesis (% of patients with 3 or more IVFf)	2.7±1.9 (23.12%)	1.9±1.3* (6.5%)*	2.96±2.25 (23.07%)	3.12±1.65 (31.0%)
Number of Transferred Embryos (in study cycle)	1.8±0.4	1.79±0.48	1.83±0.36	1.92±0.3
Endometrial thickness (mm)	10.9±2.5	11.1±2.1	11.1±2.02	11.05±1.9
Cryo cycles %	56.1%	61.4%	60.5%	68.3%
Part of secondary infertility %	49%	32.7%*	57.1%	50%
Infertility years	5.1±3.5	5.3±3.29	6.9±4,6	4.7±3.66
AMH (ng/mL) 2–3 Day of Cycle	2.6±2.1	3.4±2.4	3.04±2.6	3.29±1.9
FSH, (Mmo/Ml) 2–3 Day of Cycle	8.5±9.5	7.8±6.1	8.03±7.1	6.88±2.67
LH, (mMO/mL) 2–3 Day of Cycle	7.0±9,6	7.1 ±3.8	6.1±4.04	6.78±3.66
NK cytotoxicity at E/T ratio 20/1	54.1±15.1	27.9± 6.6	63.1±17.7	59.5±15.5

TABLE 3. Results of IVF outcome in patients (36-39yo) with normal NK cytotoxicity (nNKc), elevated NK cytotoxicity (eNKc), elevated NK cytotoxicity with intravenous immunoglobulin treatment (eNKcIvIg) and elevated NK cytotoxicity with intravenous lipid emulsion treatment (eNKc IL)
* p=0.0247 OR= 19.32 compared to normal NKc

	Ongoing Uterine Pregnancy (US) %	PF per all OP %	LBR %	Preterm Birth (% per all birth)
	OR 95%CI:	OR 95%CI:	OR 95%CI:	OR 95%CI:
nNK **n=** 138	51/138 **36.95%**	17/51 **33.33%**	34/138 **24.6%**	0/34
eNKc / **n=** 150	36/150 *24%*	20/36 **55.55%**	16/150 **(10.6%)**	4/16 (**25.0%)**
eNKc Significant decreased compared to **nNKc**	**p=0.0207** OR 1.85 1.11 –3.09	**p=0.0489** OR 0.40 0.166 - 0.96	**p=0.0019** OR 2.738 1.433- 5.23	**p= 0.0079** OR 0.04026 0.002-0.803
eNKc **IvIg** **n=** 44	19/44 **43.18**	9/19 47.36%	10/44 **22.72%**	5/10 50%
eNKc **IL** **n=**27	11/27 *40.74%*	6/11 54.54%	5/27 18.51%	0/5
IvIg Significant increased compared to **eNKc**	**p= 0.0214** OR 2.407 1.19-4.8	p= 0.58 OR 0.72 0.2360- 2.196	**p= 0.0467** OR 2.463 1.026- 5.9	p= 0.234 OR 0.3333 0.06-1.786
IL Significant increased compared to **eNKc**	p= 0.0958 OR 2.177 0.9264-5.116	p= 1.0 OR 0.96 0.2471- 3.729	p= 0.327 OR 1.9 0.6329-5.724	p= 0.53 OR 3.96 0.18-87.0

Table 2. Results of IVF outcome in patients (< 36years old) with; normal NK cytotoxicity (nNKc), elevated NK cytotoxicity (eNKc), eNKc cytotoxicity with intravenous immunoglobulin treatment (eNKcIvIg) and elevated NK cytotoxicity with intravenous lipid emulsion treatment (eNKc IL). (PF per all OP) Pregnancy failures are calculated as a part of the pregnancy's that not result to live birth (failed pregnancy and fetal death) per all OP(Ongoing Uterine Pregnancy)

Patient's groups	Ongoing Uterine Pregnancy (US) %	PF per all OP %	LBR %	Preterm Birth (% per all birth)
	OR 95%CI:	OR 95%CI:	OR 95%CI:	OR 95%CI:
nNKc n=587	200/587 **34.07%**	47/200 **23.5**	153/587 **26.06%**	5/153 **3.26%**
eNKc n=615	166/615 **26.99%**	61/165 **36.7%**	105/615 **17.07%**	14/105 **13.3%**
eNKc significant decreased compared to **nNKc**	p=0.0084 OR 1.398 95%CI: 1.09- 1.78	p=0.0057 OR 0.5237 95%CI: 0.33-0.82	p= 0.0002 OR 1.78 95%CI: 1.29-2.259	p= 0.0032 OR 4.554 95%CI: 1.58- 13.0
eNKc **IvIg** n=231	102/231 **44.15%**	26/102 **25.49%**	74/231 **32.03%**	11/74 **14.86%**
eNKc **IL** 180	71/180 **39.44%**	13/71 **18.3%**	58/180 **32.22%**	8/58 **13.79%**
eNKc IvIg significant increased compared to **eNKc**	p < 0.0001 OR 2.106 95%CI: 1.53-2.88	p= 0.0610 OR 0.5889 95%CI: 0.34-1.016	p < 0.0001 OR 2.261 95%CI: 1.59-3.19	p= 0.828 OR 1.135 95%CI: 0.48- 2.66
eNKc IL significant increased compared to **eNKc**	p= 0.0016 OR 1.762 95%CI: 1.24 -2.49	p= **0.0007** OR **0.3259** 95%CI**:0.166-0.638**	p < 0.0001 OR 2.309 95%CI: 1.58-3.36	p= 1.0 OR 1.04 95%CI: 0.408-2.649
eNKc **IvIg** significant increased compared to **nNKc**	p=0.0103 OR 1.507	p= 0.77 OR 1.11 95%CI: 0.64-1.9	p= 0.099 OR 1.340 95%CI: 0.96-1.868	p=0.0038 OR 5.168
eNKc **IL** significant increased compared to **nNKc**	p= 0.21 OR 1.26 95%CI: 0.89-1.7	p= 0.20 OR 0.63 95%CI: 0.32-1.24	p= 0.1057 OR 1.35 95%CI: 0.94-1.943	p= 0.0084 OR 4.736

TABLE 4. Results of single and tween pregnancy outcome in patients with normal NK cytotoxicity (nNKc), elevated NK cytotoxicity (eNKc), elevated NK cytotoxicity with intravenous immunoglobulin treatment (eNKcIvIg) and elevated NK cytotoxicity with intravenous lipid emulsion treatment (eNKc IL)

	pregnancy	LBR per ongoing pregnancy	Pre-term LB per all LB	Perinatal mortality
nNKc	single	76.8% (123/160)	1.6% (2/123)	3
	twin	85.1% (23/27)	13% (3/23)[C]	1
eNKc	single	60.4% (81/134)[A]	6.2% (5/81)	2
	twin	73% (19/26)	42.1% (8/19) [D E]	0
eNKc IvIg	single	73.1 % (60/82)	10.0 % (6/60)[F]	0
	twin	80% (16/20)	25% (4/16)	1
eNKc IL	single	80.3% (45/56)[B]	6.6% (3/45)	0
	twin	84.6% (11/13)	27.2% (3/11)	1

A-significant compared nNKc single pregnancy (p=0.0034, OR 2.17) B- significant compared eNKc single pregnancy (p=0.0111, OR 2.67). C- significant compared nNKc single pregnancy (p=0.027, OR 9.07). D- significant compared eNKc single pregnancy (p=0.0003, OR 11.05). E- significant compared nNKc twins pregnancy (p=0.0426, OR 4.84). F-significant compared nNKc single pregnancy (p=0.0159, OR 6.72)

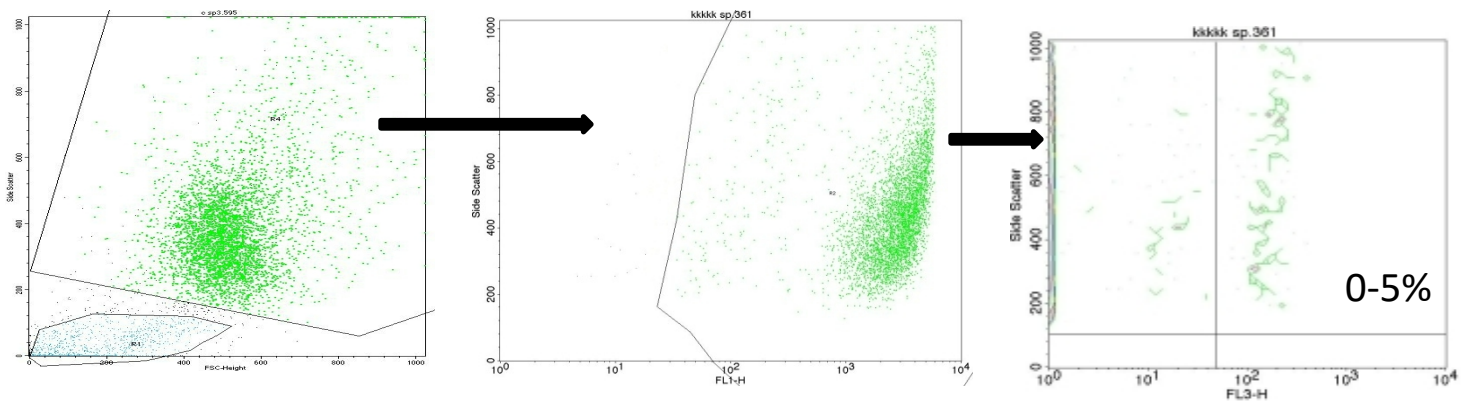
Supplemental Fig1

A- Gate ant test strategy for spontaneous levels, 50% Lysis and individuals NK cytotoxic activity investigation.

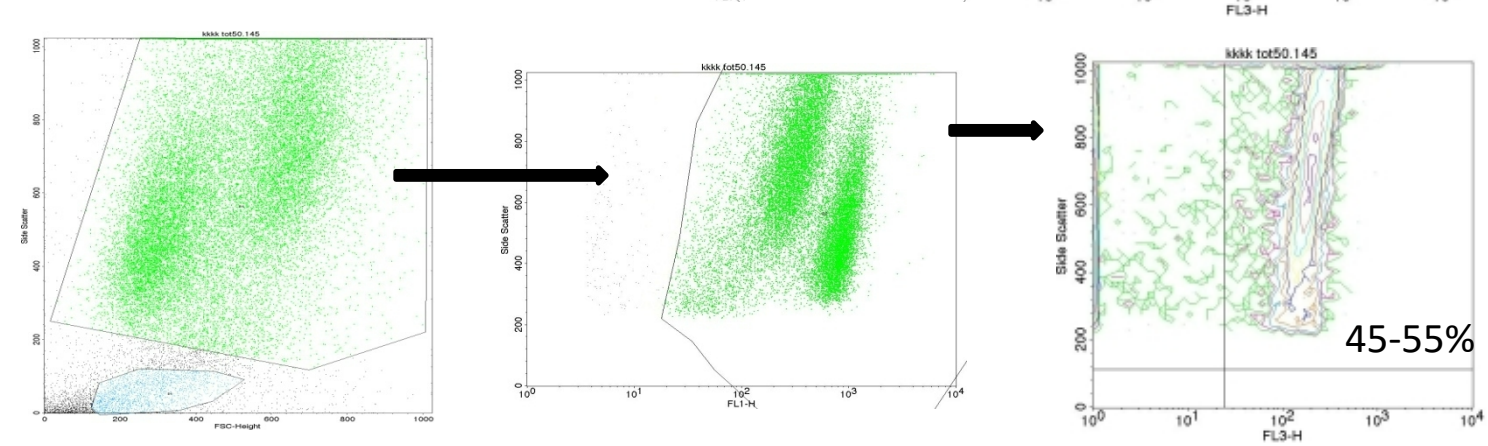
B- Gate strategy for K562 absolute counting with Flow-Count Fluorospheres and effectors/target ratios calculation.

A

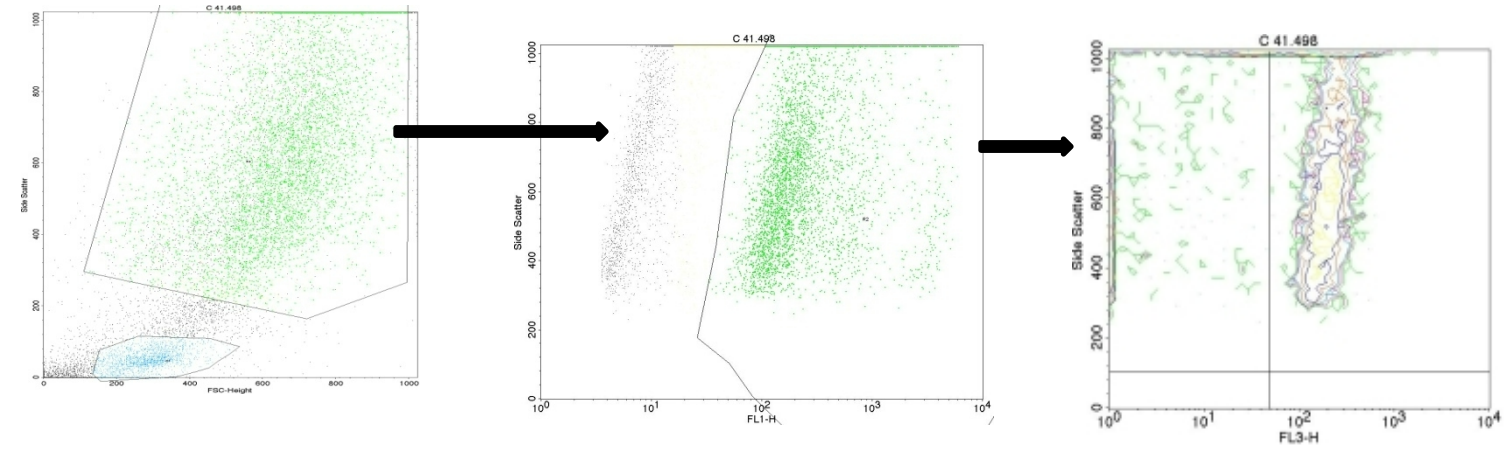
Spontaneous control



50% Lysis Gate control

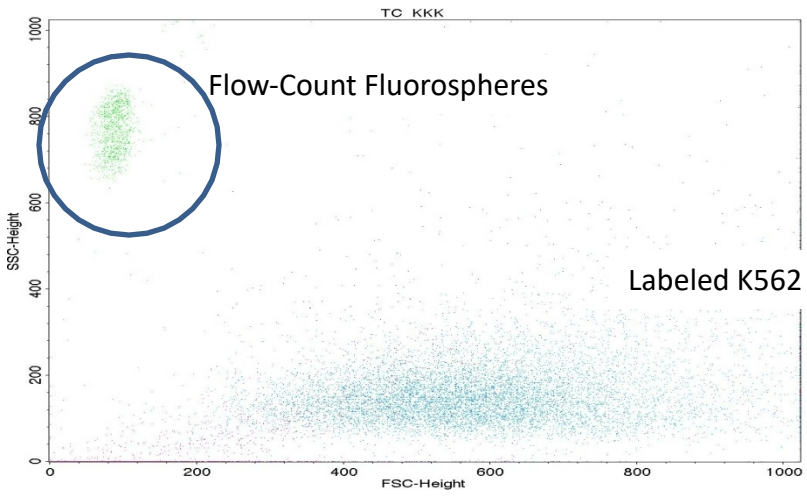


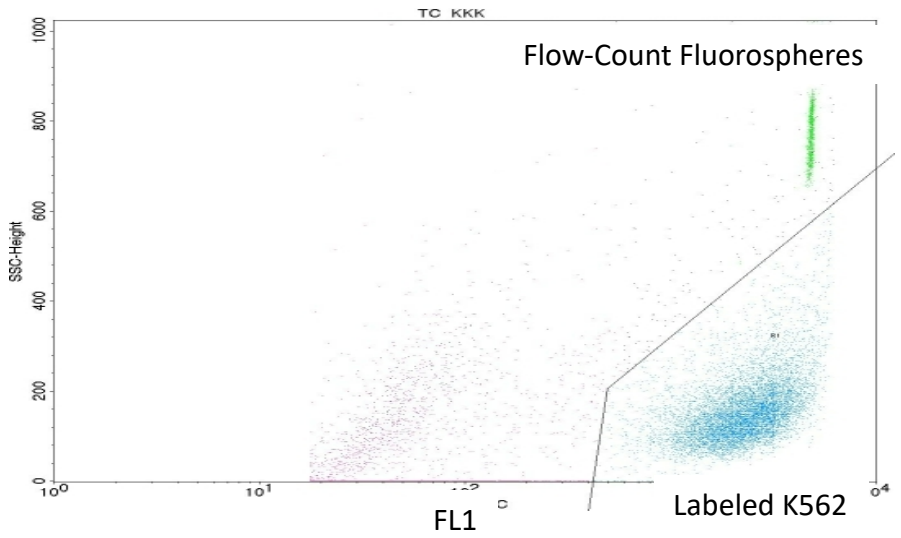
Test with PBMC E/T 30/1



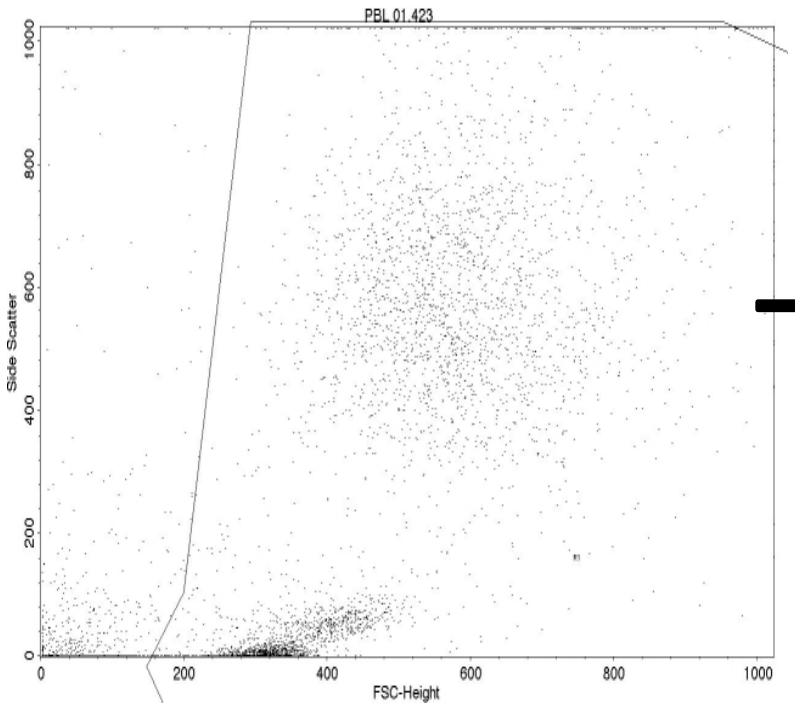
B

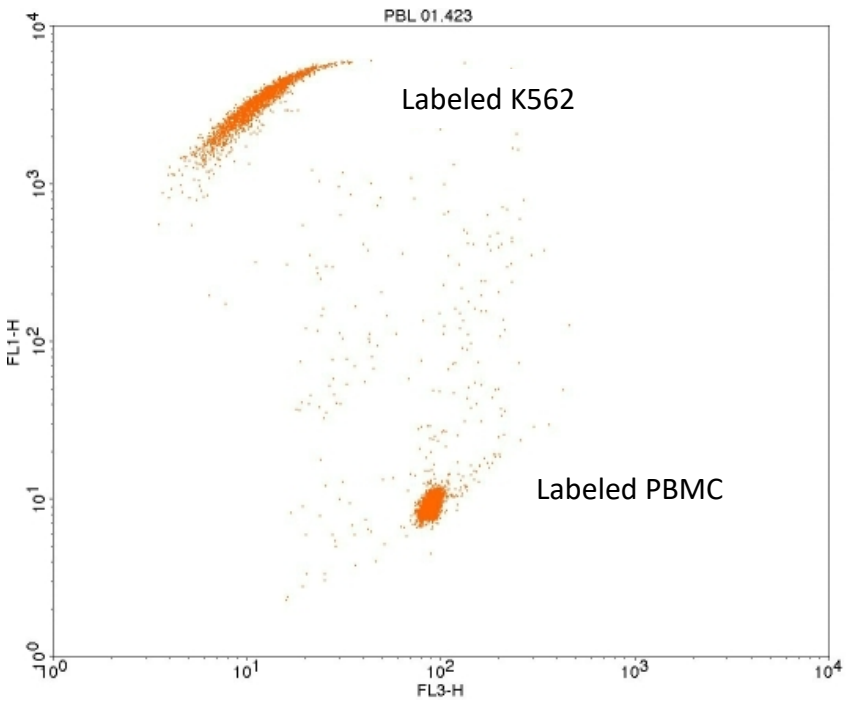
Target absolute counting with Flow-Count Fluorospheres (Becman Coulter)





Effectors /Target ratios calculation





Answer for Reviewers

Reviewer 1 Comments to the Author

This manuscript investigates the effect of a single or double infusion of IVIG around the time of embryo transfer in women with elevated NK cytotoxicity.
Although it would have been more informative if the study had included patients with recurrent implantation failure (RIF) or those who failed to achieve pregnancy after euploid embryo transfer, the data still provide valuable insight into the potential benefits of IVIG or intralipid therapy during the implantation and early pregnancy periods.

-We agree. The RIF and the PGD group could have been validated on a smaller group and this group could have been more informative. The same as karyotyping of all the aborted pregnancies. And we indicated this in the limitation. We are planning such a study, unfortunately PGD did not fully meet expectations and there are not so few women with 3 IVF failures on PGD embryos.

But on the other hand, this is also the strength of the study. It is also quite important and interesting to identify the effects of therapy on normal-ordinal IVF patients. And the fact that the therapies work on an unselected group makes the results more practically significant. And maybe we shouldn't wait for 3 IVFs or miscarriages to start doing something.

The revised version of the manuscript presents the findings more clearly than before.
Please ensure that all typographical errors are corrected and that actual p-values are provided throughout the manuscript instead of using general terms like "significant" or "not significant."

-We add it in text

In addition, since IVIG and intralipid treatment did not reduce the rate of preterm birth, it would be helpful to include a discussion on the pharmacokinetics of these agents—specifically their duration of action and half-life—to assess whether the timing of administration may have influenced the lack of effect on later pregnancy outcomes such as preterm birth.

-This is a difficult point. The goal (target) pursued by IVIG treatment in patients with immunodeficiencies is different in patients with autoimmune diseases, and in reproduction, the mechanism is not yet fully understood.

We add it in limitation

Doses and regimens of IVIG administration for patients with reproductive losses have not been studied sufficiently and actually copy the treatment regimens and doses for patients with primary immunodeficiencies. The usual dose of IGIV for antibody replacement is between 0.3 and 0.6 g/kg per month, delivered every 2 to 4 weeks [71].

The goal of this therapy is very simple: to maintain a sufficient level of serum IgG.

However, in recent years, even in such patients, the standardised administration regimen has been questioned and studies have suggested an individualised approach “The ideal immunoglobulin dose for an individual patient is the dose that keeps the patient well” [72]. Obviously, in patients with reproductive failures with high NK cytotoxicity, both doses and regimens should be developed based on favorable outcome. In addition, it should be borne in mind that IVIG contains both autoantibodies and anti-idiotypic antibodies from donors and high doses of IVIG may affect the clearance of patients anti-idiotypic antibodies [73,74].

Thank you for the detailed and insightful review

Reviewer 2 Comments to the Author

I would like to express my gratitude to the authors for their efforts in revising the paper. However, I still think it has some problems.

1. First of all, authors should think twice when describing outcomes such as CPR, IR, ongoing pregnancy, and LBR. For example, the authors stated in the abstract, "It seems that the strength of IvIg is based on a strong increase in the CPR even compared with nNKc patients, whereas IL's effectiveness is more based on a significantly reduced PF rate." However, in the results, they stated, "IvIg realizes it mostly through increased implantation and PR. IL realizes it through normalizing implantation and PR rates with a reduction in pregnancy failure rates." As you know, the implantation rate is the number of embryos among the transferred embryos that have been confirmed to have implanted. Since this paper does not describe the pregnancy rate per transferred embryo, I believe it is not appropriate to compare it with the IR. Tables 2 and 3 only describe ongoing pregnancy rates and pregnancy failure rates.

-Yes, we agree. In the previous version, we indicated the results of the IR, but the reviewer noted that the tables were too heavy and we removed this data from the tables and forgot to remove it from the text.

2. In table 2, even if there is no statistical significance in PF and LBR, please describe the exact p-value instead of ns. Likewise, please do not leave the preterm birth rate blank and provide the results.

-We add information in tab2 and tab3

3. The pregnancy outcomes described in Tables 2 and 3 also appear to contain errors. For example, Table 2 lists 200 of the 587 cycles in the nNKc group as ongoing pregnancies, with 43 resulting in PF and 153 resulting in live births. Results for four pregnancies are missing. The results for the eNKc IVIG and eNKc IL groups also appear to contain the same data errors.

These are the four patients that had prenatal deaths (initially we counted them separately... and then included them in the Pregnancy losses but forgot to put them in the table. There were 2 of them in the IvIg group, and one prenatal death in the IL group was not taken into account

-We corrected and recalculated all the data and statistics.

In the eNKc group, we excluded one patient from the PF calculation because she had fetal malformations *and had an medical abortion... but we still included her in the CPR... because she became pregnant. But in the calculation of LBR, we left her as an unsuccessful IVF because LBR is formally unsuccessful*

*We add explanation in text "*Medical abortion due to fetal malformations was included as a pregnancy success for PR and as a failure for LBR, but not calculated in Pregnancy failure rate".

And in Table 3, one twin birth at 24 weeks ended in the death of one child, but the second child survived... so formally it should be considered as a successful pregnancy. And we recalculated it.

Thank you for the detailed and insightful review