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Fresh *vs.* frozen embryo transfers in patients with KIR Bx haplotype: impact on reproductive outcomes

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Abstract. – OBJECTIVE: A controversy persists over whether or not the type of embryo transfer (ET) influences reproductive outcomes. This study aimed to evaluate the reproductive outcomes of pregnant patients undergoing their first *in vitro* fertilization procedure and explore the influence of various KIR genotypes on these reproductive outcomes.

PATIENTS AND METHODS: Prospective enrollment of patients with infertility who sought treatment at Origyn Fertility Center in Iasi, Romania, was conducted between January 2019 and March 2023. Descriptive statistics and average treatment effects (ATE) using propensity-score matching were employed to analyze our data.

RESULTS: Our results indicated that both groups were homogenous regarding baseline characteristics. When we evaluated the ATE of fresh *vs.* frozen ET on the main outcomes, we discovered that only frozen ET significantly improved the pregnancy rates (ATE: 0.17, 95% CI: 0.04-0.30, *p*=0.011) and live birth rates (ATE: 0.36, 95% CI: 0.02-1.19, *p*=0.03). The miscarriage rates were similar between the two groups. None of the evaluated KIR genotypes had a significant influence on the ATE corresponding to fresh and frozen ET.

CONCLUSIONS: KIR screening is not necessary before an IVF cycle, except for specific situations such as recurrent pregnancy loss or recurrent implantation failure.

Key Words:
In vitro fertilization, Fresh embryo transfer, Frozen embryo transfer, KIR haplotype, Reproductive outcomes.

Introduction

The inability to achieve a clinical pregnancy following 12 months of regular, unprotected sexual activity defines infertility as an illness[1]. In contemporary times, there has been a trend towards the delayed onset of parenthood, particularly in developed countries, where individuals opt to commence childbearing at a later stage in their lives. A recent report[2], published in 2023 by the World Health Organization (WHO), indicated some variation in infertility prevalence across regions, with the highest prevalence of lifetime infertility encountered in the Western Pacific region (23.2%), followed by the region of the Americas (20.0%) and the European region (16.5%). Moreover, the same report outlined that approximately one in six people worldwide will experience a form of infertility in their lifetimes.

There are several factors cited in the literature that have an important impact on infertility rates, such as advanced maternal age, smoking, obesity, ovulatory dysfunction (especially due to polycystic ovary syndrome), male factor infertility, tubal disease, etc.[3-5]. However, it is important to explore and quantify the influence of other risk factors that can influence reproductive outcomes in specific populations undergoing assisted reproductive techniques (ART).

The processes of physiological implantation and placentation play a crucial role in ensuring

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favorable reproductive outcomes[6]. The process entails the preservation and safeguarding of the intervillous space, which serves as the interface between the maternal and fetal systems. The successful migration of extravillous trophoblasts requires the negotiation between maternal/decidual killer-cell immunoglobulin-like receptors (KIRs) and their ligands, extravillous trophoblast Major Histocompatibility Complex (MHC) Class-I antigens[7,8].

Inflammation at the level of the maternal-fetal interface may result in compromised implantation and placentation, as well as adverse obstetrical outcomes such as intrauterine growth restriction (IUGR), preterm delivery, preeclampsia, and pregnancy loss[9-11].

The genetic loci for KIR molecules are situated on chromosome 19[12]. Within a set of 15 KIR genes exhibiting high levels of polymorphism, it has been observed[13] that 9 of these genes encode inhibitory KIRs, while the remaining 6 genes encode activating KIRs.

The nomenclature of each KIR gene is determined by the quantity of Ig-like domains present in its extracellular region and the length of its cytoplasmic tail. Receptor families possessing elongated tails, referred to as "L" KIRs, are predominantly inhibitory in nature14. Conversely, receptor families possessing shortened tails, known as "S" KIRs, are primarily activating in function.

The complexity associated with KIR genetic polymorphism is managed through two distinct haplotypes, namely KIR A and KIR B. These haplotypes differ primarily in the presence of additional activating KIRs on haplotype B. The KIR A haplotype is characterized by the presence of three inhibitory KIRs, three framework genes, and one activating KIR (KIR2DS4), which is typically considered to be non-functional[13,14]. According to reports, mothers who possessed the telomeric end of the KIR B haplotype, which includes activating KIR2DS1, exhibited a lower incidence of obstetric complications[15].

We have recently explored, in a prospective study[16], the influence of maternal KIR haplotype on the reproductive outcomes after single embryo transfer in IVF (*in vitro* fertilization) cycles in patients with recurrent pregnancy loss (RPL) and recurrent implantation failure (RIF). The findings of our study suggest that individuals possessing a KIR AA haplotype exhibit a statistically significant increase in the likelihood of experiencing a miscarriage following an *in vitro* fertilization (IVF) procedure, thus outlining the importance of KIR haplotype determination in this category of patients before an IVF procedure.

To the best of our knowledge, there are no studies that have evaluated the results of fresh and frozen embryo transfers in pregnant patients with the KIR Bx haplotype. Thus, the aim of this prospective study was to evaluate the reproductive outcomes of pregnant patients undergoing their first IVF procedure, and to explore the potential influence of various KIR genotypes on these reproductive outcomes.

Patients and Methods

The present study prospectively enrolled patients diagnosed with infertility who underwent their initial *in vitro* fertilization (IVF) cycle at Origyn Fertility Center located in Iasi, Romania, during the period that extended from January 2019 to March 2023. The study received ethical approval from the Institutional Ethics Committee of the University of Medicine and Pharmacy, 'Grigore T. Popa' (No. 143/18.03.2019). All patients who participated in the study provided informed consent.

The inclusion criteria comprised the following: age $\geq$ 18, with a diagnosis of infertility and no previous IVF procedure, who gave their informed consent for enrollment in this study. In 2017, the International Committee for Monitoring Assisted Reproductive Technologies (ICMART), along with multiple international scientific societies, including ESHRE, revised the definition of infertility as a disease characterized by the failure to establish a clinical pregnancy after 12 months of regular, unprotected sexual intercourse or due to an impairment of a person's capacity to reproduce either as an individual or with his/her partner[17].

Patients were excluded if they had congenital uterine abnormalities, contraindications for IVF or pregnancy (uncontrolled hypertension or diabetes, severe heart, liver, or kidney disease, history of gynecological cancers, psychiatric disorders), or were unable to give their informed consent.

Maternal peripheral blood (5 ml) was harvested on EDTA, and 200 µl were further used for DNA extraction by a silica adsorption columns-based method (QIAamp DNA Blood Mini Kit, Qiagen, Germany). After quantification, according to the manufacturer's specifications, a proper amount of DNA was further used for KIR genotyping by the PCR-SSP (polymerase chain reaction – sequence-specific primers) technique with an Inno Train commercially available kit (Inno Train Diagnostik GmbH, Kronberg, Germany).

The KIR typing was performed for the following genes: 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS4N, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1, 2DP1 and 3DP1. If any of the genes, 2DL2, 2DL5, 3DS1, 2DS1, 2DS2, 2DS3, and 2DS5, were present, the genotype was accepted as Bx. Baseline characteristics such as the patient's age, body mass index (BMI), place of residence, personal history of disease, and adverse pregnancy outcomes were recorded.

The patients underwent standard IVF procedures using either fresh (group 1, n=102 patients) or frozen embryos (group 2, n=48 patients). All patients were stimulated with recombinant follicle-stimulating hormone (rFSH), from day 2 of the menstrual cycle (Gonal F®- Merck Europe B.V., Amsterdam, The Netherlands, Puregon®- N.V. Organon, Oss, The Netherlands, or Rekovelle®- Ferring Pharmaceuticals A/S, Kastrup, Denmark), and the dosage was adjusted by clinicians taking into consideration the patients' age, weight, and serum level of anti-müllerian hormone (AMH).

All patients underwent a short gonadotropin-releasing hormone (GnRH) antagonist regimen (Orgalutran®- N.V. Organon, Oss, The Netherlands, 0.25 mg) from the 6th day of controlled ovarian hyperstimulation (COH). Patients were monitored with transvaginal ultrasound, and when at least 3 ovarian follicles reached a minimum of 17 mm in diameter, oocyte maturation was induced using human chorionic gonadotropin (hCG), followed up by oocyte retrieval after 34-36 hours.

Oocytes were fertilized, cultured in a continuous medium using a timelapse system (EmbryoScope®, Vitrolife Inc, Gothenburg, Sweden), and examined by an experienced embryologist. The Istanbul consensus grading system for embryo quality assessment was used as we previously described, and a good-quality embryo was considered to have a 4:1:1 grading[16,18]. Only embryos at the blastocyst stage that met the quality assessment criteria were used for fresh embryo transfer or were cryopreserved by vitrification. Pre-implantation genetic testing was not performed in our cases. All patients underwent a single embryo transfer.

Patients who underwent fresh embryo transfer received luteal phase support with progesterone administered intravaginally (Utrogestan®- Besins Healthcare, London, United Kingdom, 200 mg t.i.d.) from oocyte retrieval until 12 weeks after conception. Patients who underwent frozen embryo transfer received oral estradiol (Cyclo-progynova®- Bayer Pharma AG, Berlin, Germany, 2 mg t.i.d.) from the first day of the menstrual cycle and were evaluated by vaginal ultrasound and hormonal panel after 8-10 days of treatment.

The following criteria were used to start luteal phase support with progesterone: a) endometrial thickness of at least 7 mm; b) minimum serum estradiol levels of 150 pg/ml; c) serum progesterone under 1 ng/ml. For luteal phase support in this group of patients, we used progesterone administered intravaginally (Utrogestan®- Besins Healthcare, London, United Kingdom, 200 mg t.i.d.) and subcutaneously (Prolutex®- IBSA Farmaceutici Italia Srl, Lodi, Italy, 25 mg). On the fifth day of the cycle, an embryo transfer was performed. In patients with a high risk of ovarian hyperstimulation syndrome and a serum value of progesterone higher than 1.5 ng/ml on the trigger's day, we chose to perform a frozen ET, as stated in the international guidelines[19].

We followed up with all patients, and we recorded the following primary outcomes: live birth after the transfer of the first embryo, pregnancy rate, and miscarriage rate. We also followed the secondary outcomes: gestational age at birth, newborn gender, weight, Apgar score at 5 minutes, type of birth, and neonatal complications (acute respiratory distress syndrome, need for invasive ventilation, neonatal intensive care unit admission, intraventricular hemorrhage, necrotizing enterocolitis).

The estimated sample size and power analysis for conducting this study were calculated a priori using G*Power software (version 3.1.9.6, Heinrich Heine Universität Düsseldorf, Düsseldorf, Germany) and considering the following input parameters: effect size (w) of 0.3, alpha error probability of 0.05, 1-beta error probability of 0.8, and degrees of freedom 5. The calculated sample size to achieve a statistical power of 0.80 was 143. Figure 1 represents the central and non-central a priori distributions for the calculated sample size.

Statistical Analysis

The statistical technique of Pearson's Chi-squared test was employed to ascertain the presence of a significant difference between the anticipated frequencies and the actual frequencies in one or more groups of clinical attributes. The statistical analysis of continuous variables involved the presentation of mean and standard deviation (SD) values, and the evaluation of inter-group differences was conducted through *t*-tests.

Multinomial logistic regression was employed to conduct a multivariate analysis of intervention

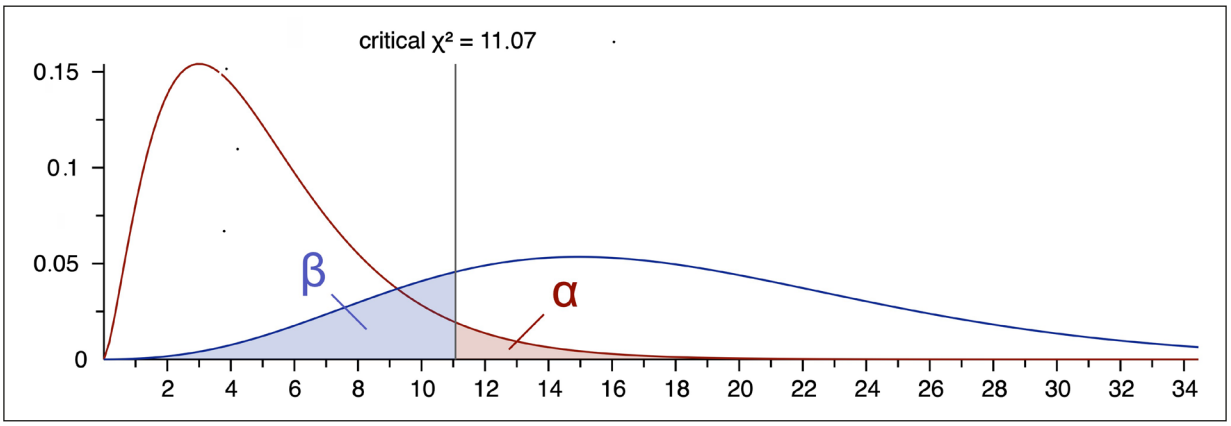

Figure 1. Central and non-central distributions for the calculated sample size.

groups, with adjustments made for maternal age, BMI, and smoking status. The study computed the values of relative risk (RR) and 95% confidence interval (CI) for binary outcomes.

We calculated the average treatment effects (ATE) of fresh *vs.* frozen embryo transfer over the main outcomes using propensity-score matching. ATE measures the difference in mean (average) outcomes between patients who were included in the frozen ET compared to patients included in the fresh ET group.

The potential influence of individual KIR genotypes on the average treatment effects was additionally assessed. A significance level of 0.05 was used to determine statistical significance, whereby any *p*-value below this threshold was deemed significant. The statistical analyses were carried out utilizing STATA SE software (version 17, 2022, StataCorp LLC, College Station, TX, USA).

Results

This prospective study included 150 patients who underwent their first *in vitro* fertilization, using either fresh embryos (group 1, n=102 patients) or frozen embryos (group 2, n=48 patients). Their clinical characteristics are presented in Table I. Moreover, we described the distribution of KIR genotypes using a heatmap (Figure 2), which showed a relatively homogenous distribution of alleles between groups segregated using the type of ET criteria.

The distribution of risk factors for infertility was relatively homogenous among groups, and we could not find a statistically significant difference between them. It is notable that tubal disease and ovulatory dysfunction were responsible for most cases of infertility in both groups.

Also, we could not find any statistically significant difference between groups regarding age, medium distribution, and smoking status. However, patients who underwent fresh embryo transfer had a significantly higher BMI compared to their counterparts (24.40±4.29 *vs.* 23.76±2.47, $p<0.001$).

The pregnancy rate for fresh ET was 57.8% (n=59 patients), and for frozen ET was 79.1% (n=38 patients). We could find a statistically significant difference between groups regarding pregnancy rates ($p=0.01$).

Table I. Clinical characteristics of the patients included in the main groups.

Patients' characteristics	Group 1 (Fresh ET, n=102)	Group 2 (Frozen ET, n=48)	*p*-value
Age, years (mean ± SD)	33.40± 4.66	32.19±4.09	0.06
Place of residence (n/ %)	Urban=50 (49%) Rural=52 (51%)	Urban=31 (64.6%) Rural=17 (35.4%)	0.11
BMI, kg/m² (mean ± SD)	24.40± 4.29	23.76±2.47	<0.001
Smoking (n/%)	Yes=10 (9.8%)	Yes=9 (18.8%)	0.12
Ovulatory disfunction (n/%)	Yes=41 (40.2%)	Yes=22 (45.2%)	0.51
Tubal disease (n/%)	Yes=54 (52.9%)	Yes=23 (47.91%)	0.41
Male infertility (n/%)	Yes=21 (20.6%)	Yes=8 (16.7%)	0.57

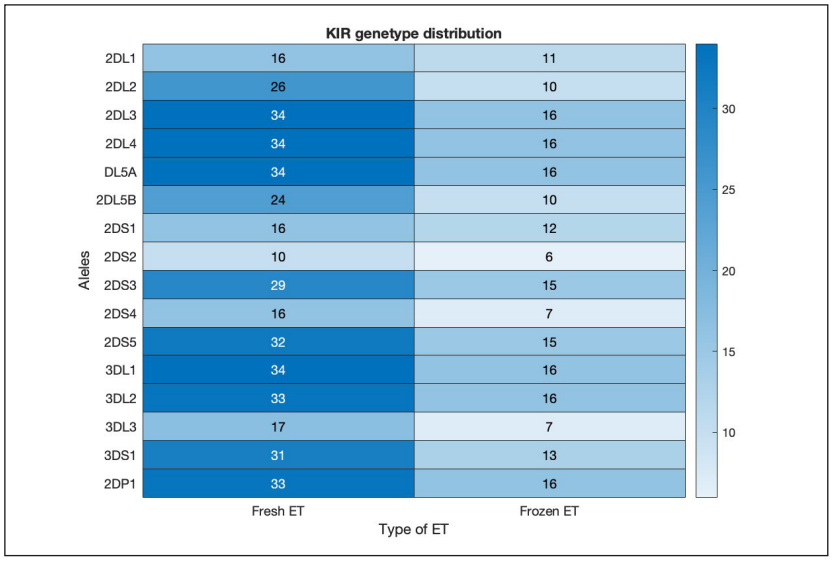

Figure 2. Heatmap representing KIR genotype distribution in the evaluated groups.

The cumulative miscarriage rate for fresh ET was 25% (n=26 patients), and for frozen ET was 20.8% (n=10 patients). We could not find any statistically significant difference between groups regarding cumulative miscarriage rates (p=0.53).

The cumulative live birth rate for fresh ET was 33.3% (n=34 patients), and for frozen ET was 56.2% (n=27 patients). We could find a statistically significant difference between groups regarding live birth rates (p=0.007).

The results from our multinomial logistic regression are presented in Table II. Our results indicated that the chances of obtaining a pregnancy (RR: 1.36, 95% CI: 1.09-1.70, p=0.005) and a live birth (RR: 1.68, 95% CI: 1.16-2.44, p=0.005) were higher in the group of patients who underwent frozen ET. We could not find any statistically significant difference regarding the relative risk of the occurrence of reproductive outcomes determined by various KIR genotypes. Overall, the pregnancy and live birth rates were higher, while the miscarriage rate was lower in the group of patients who underwent frozen ET.

We evaluated the average treatment effects of frozen *vs.* fresh embryo transfer on the main outcomes, and the results are presented in Table III. When individually compared, only frozen ET significantly improved the pregnancy rates (ATE: 0.17, 95% CI: 0.04-0.30, p=0.011) and live birth rates (ATE: 0.36, 95% CI: 0.02-1.19, p=0.03). When considering the additional influence of a specific KIR genotype, we could not find any statistically significant improvement in the evaluated outcomes.

Graphical representations of the ATE of fresh *vs.* frozen ET on the main outcomes are included in Figures 3-5. Although the KIR genotypes did not have a significant impact on the pregnancy, miscarriage, and live birth rates, we observed a relatively homogenous influence of these genotypes.

We analyzed and compared pregnancy and neonatal outcomes between the evaluated groups (Table IV). The mean gestational age at birth and standard deviation in the fresh ET group were 37.45 and 1.73 weeks of gestation, and in the frozen ET group, were 37.48 and 1.85 weeks of gestation, their comparison not being statistically significant (p=0.47) Our results indicated that patients who underwent an IVF cycle using fresh embryos had significantly higher odds of giving birth to a neonate that had a small for gestational age weight (aOR: 1.81, 95% CI: 0.67-3.47, p=0.007) or a weight higher than the 95th percentile for its gestational age (aOR: 2.65, 95% CI: 1.55-4.55, p<0.001). We identified a case of preeclampsia in the fresh ET group, and none in the frozen ET group.

On the other hand, we could not find any statistically significant differences between groups regarding other obstetrical or neonatal outcomes, such as the rates of singletons, twins, cesarean delivery, low Apgar scores at 1 or 5 minutes, and NICU admission (p>0.05).

Discussion

In this prospective study, we evaluated the main reproductive outcomes of 150 pregnant patients

Table II. Calculated relative risks for two types of embryo transfer and KIR genotypes in relationship with the evaluated reproductive outcomes.

	Pregnancy rate (n=97 patients, 64.7%)			Miscarriage rate (n=36 patients, 24%)			Live birth rate (n=61 patients, 40.7%)		
Parameter	**RR**	**95% CI**	***p*-value**	**RR**	**95% CI**	***p*-value**	**RR**	**95% CI**	***p*-value**
Frozen ET *vs.* Fresh ET	1.36	1.09-1.70	0.005	0.66	0.27-1.61	0.37	1.68	1.16-2.44	0.005
2DL1*	0.21	0.15-9.57	0.85	0.29	0.27-1.62	0.82	0.11	0.14-8.75	0.92
2DL2*	0.83	0.25-2.72	0.76	0.10	0.14-11.60	0.89	0.78	0.25-2.45	0.68
2DL3*	0.19	0.36-3.87	0.67	0.31	0.28-4.32	0.67	0.75	0.24-2.30	0.61
2DL4*	0.81	0.19-3.32	0.77	0.22	0.13-6.57	0.89	0.12	0.05-2.59	0.94
2DL5A*	0.90	0.01-2.41	0.96	0.13	0.42-5.83	0.36	0.36	0.50-3.73	0.31
2DL5B*	0.82	0.26-2.60	0.74	0.50	0.11-3.24	0.31	0.33	0.43-4.08	0.62
2DS1*	0.88	0.13-5.83	0.90	0.53	0.13-1.93	0.66	0.60	0.09-3.93	0.60
2DS2*	0.74	0.25-2.14	0.58	0.38	0.23-10.43	0.15	0.43	0.50-4.07	0.50
2DS3*	0.72	0.29-10.21	0.54	0.67	0.10-1.41	0.68	0.34	0.21-8.60	0.76
2DS4*	0.46	0.40-5.35	0.56	0.97	0.15-8.49	0.97	0.49	0.42-5.26	0.54
2DS5*	0.54	0.04-6.24	0.62	0.56	0.20-4.72	0.73	0.71	0.06-8.15	0.78
3DL1*	0.57	0.09-3.41	0.54	0.98	0.02-4.33	0.77	0.67	0.14-2.37	0.45
3DL2*	0.72	0.16-3.14	0.66	0.72	0.56-6.44	0.54	0.48	0.11-1.99	0.31
3DL3*	0.51	0.02-9.86	0.65	0.40	0.31-9.75	0.59	0.89	0.04-8.10	0.94
3DS1*	0.23	0.30-5.05	0.77	0.71	0.01-4.82	0.66	0.70	0.42-6.84	0.46
2DP1*	0.10	0.02-5.69	0.96	0.16	0.16-3.21	0.94	0.06	0.02-7.03	0.98
3DP1*	0.67	0.04-9.24	0.56	0.29	0.02-5.05	0.95	0.53	0.01-4.17	0.78

RR-relative risk; CI-confidence interval; ET-embryo transfer. *Considering the influence of KIR alleles.

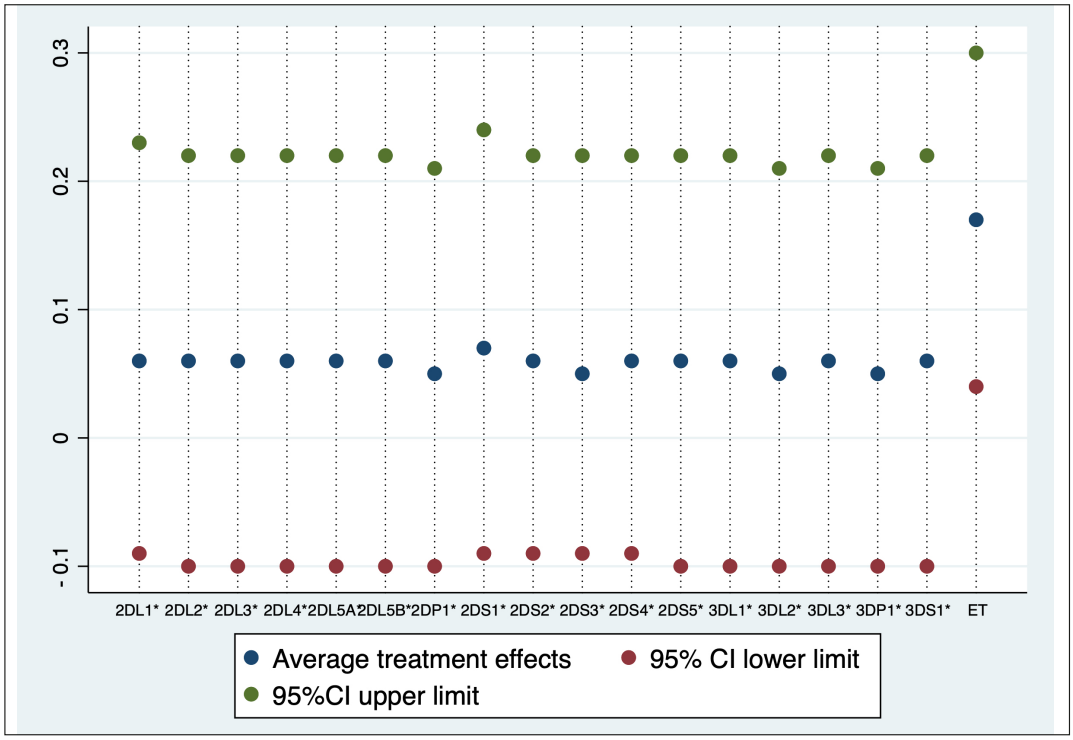

Figure 3. Graphical representation of the average treatment effects of frozen *vs.* fresh ET on pregnancy rates considering KIR genotypes.

Table III. Average treatment effects of fresh vs. frozen embryo transfer considering KIR genotypes influence.

	Pregnancy rate (n=97 patients, 64.7%)				Miscarriage rate (n=36 patients, 24%)				Live birth rate (n=61 patients, 40.7%)			
Treatment	ATE	95% CI lower bound	95% CI upper bound	*p*-value	ATE	95% CI lower bound	95% CI upper bound	*p*-value	ATE	95% CI lower bound	95% CI upper bound	*p*-value
Frozen *vs.* fresh ET	0.17	0.04	0.30	0.011	0.15	-0.01	0.32	0.07	0.36	0.02	1.19	0.03
Frozen *vs.* fresh ET considering 2DL1 influence	0.06	-0.09	0.23	0.42	-0.03	-0.19	0.11	0.61	0.10	-0.06	0.28	0.23
Frozen *vs.* fresh ET considering 2DL2 influence	0.06	-0.10	0.22	0.46	-0.04	-0.19	0.10	0.55	0.10	-0.06	0.27	0.22
Frozen *vs.* fresh ET considering 2DL3 influence	0.06	-0.10	0.22	0.46	-0.04	-0.18	0.09	0.52	0.10	-0.06	0.27	0.22
Frozen *vs.* fresh ET considering 2DL4 influence	0.06	-0.10	0.22	0.46	-0.04	-0.18	0.09	0.52	0.10	-0.06	0.27	0.22
Frozen *vs.* fresh ET considering 2DL5A influence	0.06	-0.10	0.22	0.46	-0.04	-0.18	0.09	0.52	0.10	-0.06	0.27	0.22
Frozen *vs.* fresh ET considering 2DL5B influence	0.06	-0.10	0.22	0.46	-0.04	-0.18	0.10	0.54	0.10	-0.06	0.27	0.22
Frozen *vs.* fresh ET considering 2DS1 influence	0.07	-0.09	0.24	0.39	-0.04	-0.19	0.11	0.59	0.11	-0.06	0.29	0.21
Frozen *vs.* fresh ET considering 2DS2 influence	0.06	-0.09	0.22	0.43	-0.03	-0.18	0.10	0.62	0.10	-0.06	0.26	0.24
Frozen *vs.* fresh ET considering 2DS3 influence	0.05	-0.09	0.22	0.49	-0.05	-0.19	0.07	0.39	0.11	-0.05	0.28	0.18
Frozen *vs.* fresh ET considering 2DS4 influence	0.06	-0.09	0.22	0.43	-0.04	-0.18	0.09	0.53	0.10	-0.06	0.27	0.21
Frozen *vs.* fresh ET considering 2DS5 influence	0.06	-0.10	0.22	0.46	-0.04	-0.18	0.09	0.52	0.10	-0.06	0.27	0.21
Frozen *vs.* fresh ET considering 3DL1 influence	0.06	-0.10	0.22	0.46	-0.04	-0.18	0.09	0.52	0.10	-0.06	0.27	0.21
Frozen *vs.* fresh ET considering 3DL2 influence	0.05	-0.10	0.21	0.48	-0.03	-0.18	0.10	0.58	0.10	-0.06	0.27	0.21
Frozen *vs.* fresh ET considering 3DL3 influence	0.06	-0.10	0.22	0.46	-0.04	-0.18	0.09	0.53	0.10	-0.06	0.27	0.22
Frozen *vs.* fresh ET considering 3DS1 influence	0.06	-0.10	0.22	0.46	-0.04	-0.20	0.08	0.40	0.12	-0.05	0.29	0.16
Frozen *vs.* fresh ET considering 2DP1 influence	0.05	-0.10	0.21	0.48	-0.03	-0.18	0.10	0.40	0.12	-0.04	0.29	0.15
Frozen *vs.* fresh ET considering 3DP1 influence	0.05	-0.10	0.21	0.48	-0.03	-0.18	0.10	0.40	0.12	-0.04	0.29	0.15

ET-embryo transfer; ATE-average treatment effects; CI-confidence interval.

Table IV. Pregnancy and neonatal outcomes in the evaluated groups.

Outcome	Group 1 (Fresh ET)		Group 2 (Frozen ET)	
	aOR and 95% CI	*p*-value	aOR and 95% CI	*p*-value
Singletons	2.11 (0.77-3.14)	0.21	0.44 (0.02-1.14)	0.35
Twins	1.06 (0.18-6.02)	0.94	0.63 (0.22-3.79)	0.88
Cesarean delivery	0.94 (0.63-1.34)	0.84	0.91 (0.66-2.25)	0.74
Small for gestational age	1.81 (0.67-3.47)	0.007	1.34 (1.41-5.27)	0.73
Large for gestational age	2.65 (1.55-4.55)	<0.001	1.21 (0.45-5.58)	0.72
Apgar score at 1 min <7	0.96 (0.67-1.38)	0.84	0.44 (0.16-1.85)	0.79
Apgar score at 5 min <7	0.71 (0.45-3.38)	0.43	0.65 (0.25-3.65)	0.31
NICU admission	0.87 (0.24-3.34)	0.65	0.56 (0.16-4.23)	0.71
Necrotizing enterocolitis	0.54 (0.16-2.75)	0.79	0.38 (0.11-2.67)	0.42
Invasive ventilation	0.68 (0.14-3.89)	0.43	0.52 (0.23-4.10)	0.47
ARDS	1.11 (0.17-7.23)	0.91	1.28 (0.34-5.44)	0.95

ET-embryo transfer; aOR-adjusted OR; CI-confidence interval; NICU-neonatal intensive care unit; ARDS-acute respiratory distress syndrome.

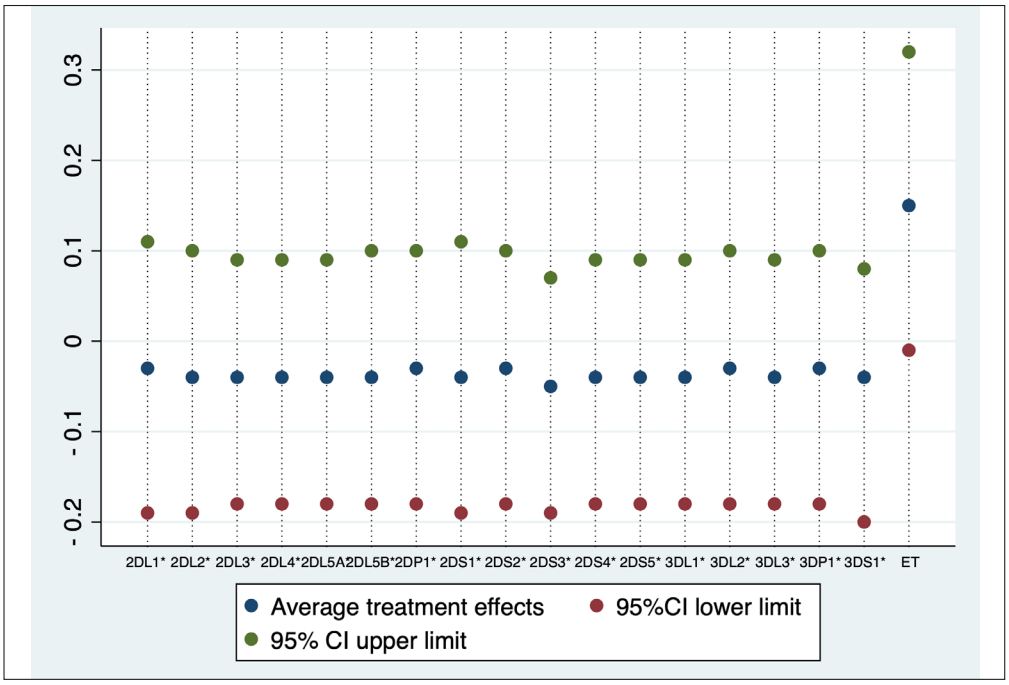

Figure 4. Graphical representation of the average treatment effects of frozen *vs.* fresh ET on miscarriage rates considering KIR genotypes.

undergoing their first IVF procedure and explored the influence of various KIR genotypes on these reproductive outcomes. Our results indicated that both groups were homogenous regarding baseline characteristics. When we evaluated the average treatment effects of fresh *vs.* frozen embryo transfer on the main outcomes, we discovered that only frozen ET significantly improved the pregnancy rates (ATE: 0.17, 95% CI: 0.04-0.30, p=0.011) and live birth rates (ATE: 0.36, 95% CI: 0.02-1.19, p=0.03). The miscarriage rates were similar between the two groups.

A recent systematic review and meta-analysis by Roque et al[20], on 11 randomized controlled trials, evaluated the advantages of elective frozen ET in comparison with fresh ET in IVF/ICSI cycles regarding reproductive outcomes. The authors reported significantly higher live birth rates for pregnant patients who underwent frozen ET compared with fresh embryo transfer in the ove-

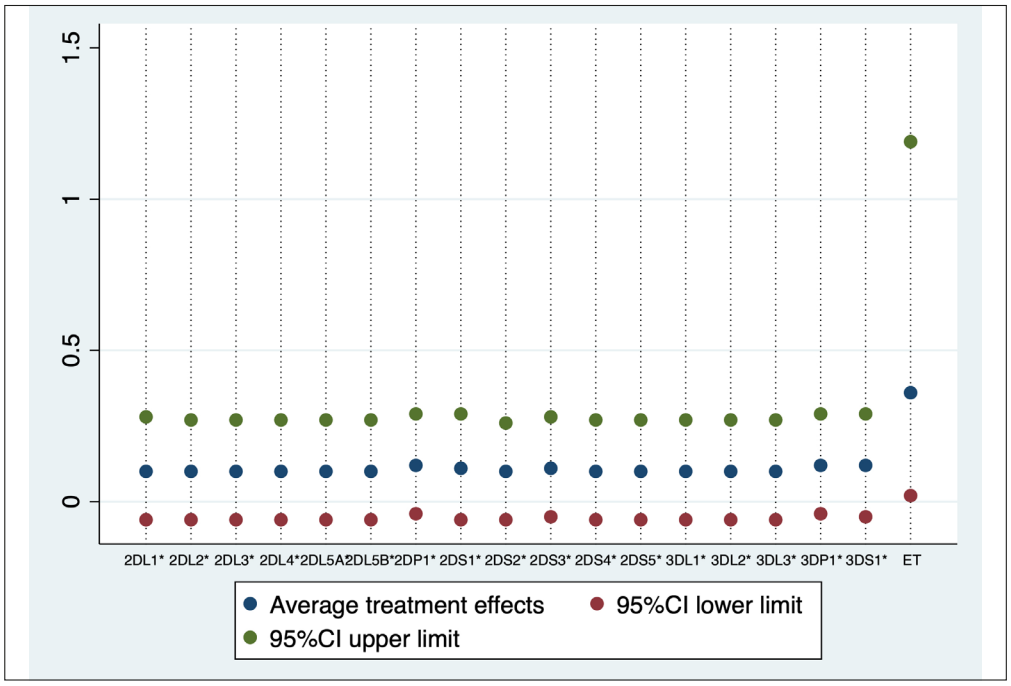

Figure 5. Graphical representation of the average treatment effects of frozen *vs.* fresh ET on live birth rates considering KIR genotypes.

rall IVF/ICSI population (risk ratio = 1.12; 95% CI: 1.01-1.24). No statistical differences were noted in this study regarding secondary outcomes such as the rate of miscarriage.

On the other hand, a Cochrane systematic review and meta-analysis, published in 2021, that evaluated the effectiveness of the freeze-all strategy compared to the conventional IVF/ICSI strategy, demonstrated no significant difference in cumulative live birth rates between the two strategies (OR: 1.08, 95% CI: 0.95-1.22; I^2=0%), based on moderate-quality evidence[21]. The authors suggested that for a cumulative live birth rate of 58% for fresh ET, the corresponding values in frozen ET patients would vary between 57% and 63%. In our cohort of patients, the cumulative live birth rate for fresh ET was 33.3%, and for frozen ET was 56.2%, and the difference between the two groups was statistically significant.

Another Cochrane systematic review and meta-analysis by Wong et al[22], on 4 randomized controlled trials, evaluated the effectiveness of the freeze-all strategy compared to conventional IVF/ICSI and found no statistically significant difference regarding cumulative live birth rates between the evaluated strategies (OR: 1.09, 95% CI: 0.91-1.31; I^2=0%).

In this study, the transferred embryos were at the blastocyst stage. A multicenter, randomized controlled trial, conducted by Wei et al[23], that aimed to compare the live pregnancy rates of 1,650 patients after fresh and frozen ET at the stage of blastocyst, indicated significantly higher rates of singleton livebirth for the frozen ET than did fresh single blastocyst transfer (50% *vs.* 40%, p<0.0001).

A recent Cochrane systematic review and meta-analysis[24], which included 32 randomized controlled trials, evaluated and compared the live birth rates per fresh transfer for the blastocyst-stage embryo transfer and cleavage-stage embryo transfer. The results of this meta-analysis were based on low-quality evidence and outlined a higher live birth rate following fresh transfer in the blastocyst-stage transfer group (OR: 1.27, 95% CI: 1.06-1.51; I^2=53%).

In this study, we also aimed to evaluate the influence of various KIR genotypes on the average treatment effects corresponding to fresh and frozen ET. Although we found a tendency toward reduction for the main evaluated reproductive outcomes, none of the KIR genotypes had a significant impact. We hypothesize that the rather small sample size of our cohort of patients may have been accountable for the heterogeneity of our results. On the other hand, similar studies that evaluated the influence of various KIR genotypes on reproductive outcomes had conflicting results.

For example, a retrospective study by Alecsandru et al[25], on a cohort of 291 patients with infertility due to recurrent miscarriage or recurrent implantation failure, found no statistically significant differences regarding pregnancy, miscarriage, and live birth rates among patients with KIR AA, AB, and BB haplotypes after single embryo transfer (SET). Moreover, a literature review13 outlined the need for further studies that will evaluate the influence of KIR haplotypes on various stages of blastocyst implantation.

Varla-Leftherioti et al[26] conducted a prospective study on 26 couples with at least 2 abortions and 26 couples that served as controls, and determined the presence of inhibitory (2DL1, 2DL2, and 2DL3) and activating (2DS1) KIRs. Their results indicated that fewer aborters than controls had all three inhibitory KIRs (30.77% *vs.* 69.23%, p=0.01), while some of them had only one inhibitory KIR (19.23% *vs.* 3.85%, p=0.08). No differences were found in the activating KIR repertoires between groups.

Finally, we analyzed and compared pregnancy and neonatal outcomes between the evaluated groups, and our results indicated that patients who underwent an IVF cycle using fresh embryos had significantly higher odds of giving birth to a neonate that was small or large for gestational age. On the other hand, we could not find any statistically significant differences between groups regarding other obstetrical or neonatal outcomes, such as the rates of singletons, twins, cesarean delivery, low Apgar scores at 1 or 5 minutes, and NICU admission (p>0.05). Our results are in line with previous studies27,28 that indicated a higher prevalence of weight extremes in neonates that were conceived using fresh embryos.

It was demonstrated[29] that pregnant patients with a KIR AA haplotype possess a higher risk of recurrent miscarriage, pre-eclampsia, or fetal growth restriction compared to patients with a Bx haplotype. Moreover, it appears that the absence of activating KIR increases the risk of adverse pregnancy outcomes, especially in single-fetal pregnancies[30].

Limitations and Strengths

The results of this study must be evaluated considering several limitations: the limited sample size, and the observational design. Moreover, in this prospective study, the results indicated only the presence of the Bx haplotype, and we could not assess the influence of the AA haplotype comparatively. We consider that a randomized controlled trial, with a multicentric design, could better quantify the influence of KIR genotypes over reproductive outcomes in patients who undergo fresh *vs.* frozen ET and could stratify the patients according to specific clinical characteristics.

On the other hand, this is the first prospective study in the literature to evaluate the influence of KIR genotypes over the reproductive outcomes in this type of setting. We hypothesize that KIR screening is not necessary before an IVF cycle, except for specific situations such as recurrent pregnancy loss or recurrent implantation failure. Until now, we could not find any studies that evaluated the cost-effectiveness of KIR genotyping in patients with infertility. Thus, another future direction of research could assess this aspect. In patients with specific risk factors, such as a personal history of adverse reproductive outcomes, KIR genotyping could be useful for risk stratification and individualized management.

Numerous artificial intelligence-derived methods could improve reproductive and neonatal outcomes in IVF patients, allowing better embryo selection and risk stratification based on a large number of individual parameters derived from clinical and paraclinical data[31-34]. This future direction of research could include KIR genotyping, immunophenotyping, or proteomic data, with special considerations for the ethical and legal implications, especially for patients with recurrent pregnancy loss or recurrent implantation failure[35-38]. Moreover, recent literature data[39-42] that used machine learning-based algorithms or artificial neural networks showed promising results in the field of predictive medicine.

Conclusions

Only frozen ET significantly improved the pregnancy rates and live birth rates, but other reproductive outcomes, such as miscarriage rates, were not influenced by the type of ET in our cohort of patients.

Patients who underwent an IVF cycle using fresh embryos were found to have significantly higher odds of giving birth to a neonate that was characterized by extremes of weight.

Although we found a tendency toward rate reduction for the main evaluated reproductive outcomes, none of the KIR genotypes had a significant impact.

Further interventional studies will be needed to better quantify the influence of KIR genotypes on the reproductive outcomes in patients who undergo fresh *vs.* frozen ET.

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Authors' Contributions
This manuscript is part of the doctoral research of Radu Maftei. Conceptualization was done by Radu Maftei, Bogdan Doroftei, Cristian Vaduva, Valeriu Harabor, Ana-Maria Adam, and AnaMaria Harabor. Methodology was developed by Ana-Maria Harabor, Gigi Adam, Elena Mihalceanu, Petronela Vicoveanu, and Ingrid-Andrada Vasilache. Software was provided by Anca Bivoleanu. Validation was carried out by Anca Bivoleanu, Gabriela Lunguleac, Ana-Maria Cretu, and Teodora Armeanu. Formal analysis was performed by Ana-Maria Harabor, Gigi Adam, Elena Mihalceanu, Petronela Vicoveanu, and Ingrid-Andrada Vasilache. Investigation was conducted by Radu Maftei, Bogdan Doroftei, Cristian Vaduva, Valeriu Harabor, Ana-Maria Adam, and Petru Cianga. Resources were provided by Bogdan Doroftei. Data curation was handled by Gabriela Lunguleac, Ana-Maria Cretu, Petronela Vicoveanu and Teodora Armeanu. Writing—original draft preparation was done by Radu Maftei, Bogdan Doroftei, Cristian Vaduva, Valeriu Harabor, Ana-Maria Adam, and Petru Cianga. Writing—review and editing were carried out by Radu Maftei, Bogdan Doroftei, Cristian Vaduva, Valeriu Harabor, Ana-Maria Adam, and Petru Cianga. Visualization was conducted by Radu Maftei. Supervision was provided by Petru Cianga. Project administration and final approval were performed by Petru Cianga. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest
The authors declare no conflict of interest.

Data Availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval
The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of the University of Medicine and Pharmacy, 'Grigore T. Popa' (No. 143/18.03.2019).

Informed Consent
Informed consent was obtained from all subjects involved in the study.

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