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# The role of KIR genes in women with impaired embryo implantation and unexplained infertility: an immunogenetic retrospective analysis

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**Abstract:**

**Background:** The adaption of maternal immune cells towards the semi-allogenic fetus could represent a crucial factor related to the onset of infertility and pregnancy-related disorders. Herein we report the results of an immunogenetic study in couples with fertility disorders by profiling KIR and HLA-C genetic analysis.

**Methods:** We have conducted a retrospective study recruiting women with Idiophatic Infertility (IF), Repeated Miscarriages (RM), Repeated Implantation Failure (RIF) and Ovodonation Failure (OF), Women with an history of live-birth child represented the control group. Contingency tables using Fisher's exact test was adopted for statistic calculations and a p value less than 0.05 was considered as statistically significant**.**

**Results:** A total of one hundred and twenty-nine women were recruited. AB (Bx>1) was significantly lower in women with RM, RIF and OF than control group. Conversely, AA diplotype was significantly higher in women with RIF and OF than control group. Diplotypes KIR genes did not differ between women with UI and Control groups.

**Conclusions:** Except for women with IF, this analysis confirmed the association between specific KIR genes expression and disorders related with spontaneous or medically assisted implantation. Considering the low sample, these findings should be corroborated by further analysis.

**Keywords:** KIR genes, HLA-C ligand, repeated implantation failure, recurrent miscarriage, unexplained infertility, ovodonation failure, embryo implantation

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## 1. Introduction

An increasing number of studies published in the last years suggested that the adaption of maternal immune cells towards the semi-allogenic fetus could represent a crucial factor related to the onset of pregnancy-related disorders as pre-eclampsia, fetal growth restriction and abortion which collectively account for nearly 10% of the global burden of disease [1,2].

The fetal cells in the direct contact with mother's immune dNK (decidual Natural Killer) cells, are extra villous trophoblast (EVT), the layer that surrounds the blastocyst [3]. dNK cells increase greatly in number in the mid-secretory phase under the stimulus of interleukine 15 (IL 15) in a microenvironment progesterone-rich and represent the major endometrial lymphocyte population in the late secretory phase (70% of cells) and the first trimester of pregnancy [4].

dNK (CD56bright CD16-) cells are poorly cytotoxic and greatly differ from their peripheral counterpart (pNK) both in immune-phenotype markers and functions [5]. The activation of dNK cells is primed by the binding of specific receptors expressed on the cell surface, as the maternal killer immunoglobulin-like receptors (KIRs), to definite epitopes of the HLA-C molecules expressed by EVT cells [6,7].

HLA-C detected in a population can be divided in two groups C1 and C2, according to the aminoacidic present at position 80 of the alfa domain: C1 asparagine, C2 lysine. This protein region is where KIR receptors bind to HLA-C molecules and define the KIR-HLA-C epitope [6,8].

The genes encoding KIR receptors are clustered in a region of human genome highly variable both in gene content and DNA sequence [9]. This extensive variability gives rise to haplotypes (array of different genes) diversity and an elevated number of different genotypes KIR.

KIR receptors are functionally categorized in inhibitory or activating according to the kind of signal elicited by the interaction with their cognate ligands on target cells [10].

Activating receptors enable the synthesis and release of cytokines, chemokines and growth factors from dNK cells [11,12], necessary to promote trophoblast invasion and the remodeling of maternal uterine arteries in high conductance vessels to supply the fetal growth demands of nutrients during pregnancy and in humans include KIR 2DS1, 2DS2, 2DS4 [13], 2DS5 genes(this latter for some alleles in African population) and 3DS1 [12,13]; inhibitory receptors are unable to trigger synthesis and release of trophic factors from dNK cells and are engaged in a fundamental process named "education of NK cells" [15] which promotes the functional competence of the cells and in humans include KIR 2DL1,2DL2/2DL3 genes( receptors) .

KIR2DL2/3 receptors bind to C1 epitope of HLA-C and drive a weakly inhibitory signal to dNK cell whereas KIR2DL1 and KIR2DS1 receptors bind to C2 epitope imparting respectively strong inhibitory or activating signals to NK cells.

NK cell function is determined by the overall input of activating and inhibitory signals following engagement of KIR receptors with HLA-C ligands on EVT cells.

EVT cells also express the oligomorphic HLA-E molecule that can engage inhibitory NKG2A and activating NKG2C receptor on dNK cells as well as oligomorphic HLA-G ligand which can interact intracellular with the KIR2DL4 receptor via endosome route or by trogocytosis (capture of the molecule from the cell surface) and LILRB1 (leucocyte immunoglobulin-like receptor subfamily B) receptor also expressed on dNK cells [5,16].

It's the interaction between maternal KIR receptors and the variable mendelian inheritance of both maternal and paternal HLA-C alleles on placental trophoblast cells to make the epitope KIR/HLA-C specific to a particular pregnancy [17].

Clinical and functional studies have clearly evidenced that when a pregnant woman with a KIR gene content specific for the synthesis of only inhibitory receptors (diplotype AA) as 2DL1 and 2L2/2DL3, is challenged by an embryo/fetus with a paternal derived HLA-C2 gene (epitope), the risk of the onset of pregnancy-related disorders is significantly increased [18,19].

By contrast if the maternal KIR genetic repertoire also includes genes specific for activating receptors (diplotype AB) such as 2DS1 and 2DS4, the interaction with fetal allotype HLA-C2, leads to the release of GM-CSF and other trophic factors from dNK cells, which enhances trophoblast invasion, and in turn, promotes an ongoing and evolutive pregnancy [12,13,18].

An increasing demand of immunogenetic tests, particularly by couples experiencing "failed" ART cycles (of unknown clinical causes), or idiopathic abortions following natural cycles, to predict an immunogenetic risk (of adverse reproductive outcome) has been recorded in the last years, thus raising new interest for the research and diagnosis in the field [12,16,17,19].

Herein we report the results of a clinical and immunogenetic retrospective study in couples affected by unexplained infertility, repeated abortions following natural cycles, repeated implantation failure (RIF) and ovo-donation failure in IVF settings by profiling KIR and HLA-C genetic analysis.

## 2. Results

A total of one hundred and twenty-nine women were recruited: twenty-six women with RM (n = 26), twenty women with RIF (n = 20); twenty-six women with OF (n = 26); twenty-eight women with UI (n = 28). Twenty-nine women composed the control group (n = 29).

Almost all the clinical group disclosed the three KIR diplo-types [AA, AB (Bx=1; Bx>1), but with differences in their relative frequencies (Table 1, Figure 1).

**Table 1.** Diplotypes KIR Genes, Centromere and Telomer KIR Diplotypes among study population.

| DIPLOTYPES | Control (n=29) | | RM (n=26) | | RIF (n=20) | | UI (n=28) | | OF (n=26) | |
|---|---|---|---|---|---|---|---|---|---|---|
| KIR GENES | N | % | N | % | N | % | N | % | N | % |
| **AA** | 4 | 13,8% | 8 | 30,8% | 11 | 55,0% | 8 | 28,6% | 12 | 46,2% |
| **AB(x=1)** | 11 | 37,9% | 15 | 57,7% | 6 | 30,0% | 9 | 32,1% | 11 | 42,3% |
| **AB(x>1)** | 14 | 48,3% | 3 | 11,5% | 3 | 15,0% | 11 | 39,3% | 3 | 11,5% |
| **CENTROMERE KIR DIPLOTYPES** | | | | | | | | | | |
| **Cen-A/A** | 7 | 24,1% | 14 | 53,8% | 14 | 70,0% | 12 | 42,9% | 14 | 53,8% |
| **Cen-A/B** | 20 | 69,0% | 11 | 42,3% | 6 | 55,0% | 15 | 53,6% | 11 | 42,3% |
| **Cen-B/B** | 2 | 6,9% | 1 | 3,8% | 0 | 5,0% | 1 | 3,6% | 1 | 3,8% |
| **TELOMERE KIR DIPLOTYPES** | | | | | | | | | | |
| **Tel-A/A** | 13 | 44,8% | 17 | 65,4% | 14 | 70,0% | 13 | 46,4% | 22 | 84,6% |
| **Tel-A/B** | 12 | 41,4% | 8 | 30,8% | 6 | 30,0% | 12 | 42,9% | 3* | 11,5% |
| **Tel-B/B** | 4 | 13,8% | 1 | 3,8% | 0 | 0,0% | 3 | 10,7% | 1 | 3,8% |

OF: ovodonation failure; RIF: repeated implantation failure; RM: recurrent miscarriage; UI: unexplained Infertility
*only 3DS1

**Figure 1.** Diplotypes KIR Genes, Centromere and Telomer KIR Diplotypes among study population


*Repeated Miscarriage vs Controls*

Diplotypes KIR genes distribution significantly differed comparing women with RM and control group. AB (Bx>1) was significantly lower in women with RM compared with control group (3/26 versus 14/29, $p = 0.004$, OR 0.14 95% CI 95% 0.034-0.57).

Centromere AA diplotype frequency resulted significantly higher in RM vs control group (14/26 vs 7/29, $p = 0.03$, OR 3.67 CI 95% 1.16-11.5).

Telomere KIR diplotypes distribution did not significantly differ comparing women with RM and control group.

*Repeated Implantation Failure vs Controls*

Diplotypes KIR genes frequency significantly differed comparing women with RIF and control group. AA diplotype was significantly higher in RIF vs control group (11/20 vs 4/29, $p = 0.04$, OR 7.64 CI 95% 1.93-30.2). Furthermore, AB (Bx>1) was significantly lower in RIF vs control group (3/20 vs 14/29, $p = 0.03$, OR 0.19 CI 95% 0.04-0.79).

Centromere AB was significantly lower in RIF vs Control group (6/20 vs 20/29, $p = 0.008$, OR 0.19 CI 95% 0.06-0.66) while Cen AA was significantly higher in RIF vs control group (14/20 vs 7/29, $p = 0.003$, OR 7.33 CI 95% 2.04-26.37).

Telomere KIR diplotypes distribution did not significantly differ comparing women with RIF and control group.

*Ovodonation failure vs Controls*

Diplotypes KIR genes distribution significantly differed comparing women with OF and Control group. AA diplotype was significantly higher in women with OF vs Control group (12/26 vs 4/29, $p = 0.009$, OR 5.36 CI 95% 1.45-19.8). Furthermore, AB (Bx>1) was significantly lower in women with OF vs Control group (3/26 vs 14/29, $p = 0.003$, OR 0.14 CI 95% 0.03-0.57).

Centromere AA diplotype was significantly higher in OF vs Control group (14/26 vs 7/29, $p = 0.03$, OR 3.66 CI 95% 1.16-11.56).

Telomere AA diplotype was significantly higher in women with OF vs Control group (22/26 vs 13/29, $p = 0.004$, OR 6.77 CI 95% 1.86-24.65), Telomere AB diplotype was significantly lower in women with OF vs Control group (3/26 vs 12/29, $p = 0.02$, OR 0.18 CI 95% 0.45-0.76).

*Unexplained infertility vs Controls*

Diplotypes KIR genes and Centromere/Telomere KIR diplotypes frequencies did not differ comparing women with UI and Control groups.

*Maternal KIR genes analysis*

The 2DL2 and 2DS2 gene were significantly lower in RIF vs Control group (6/20 vs 19/29, $p = 0.021$, OR 0.23 CI 95% 0.07-0.77).

2DS1 (3/26 vs 15/29, $p = 0.02$, OR 0.12 CI 95% 0.03-0.49), 3DS1(5/26 vs 15/29, p 0.01, OR 0.22 CI 95% 0.66-0.75), and 2DS5 (2/26 vs 11/29, $p = 0.01$, OR 0.14 CI 95% 0.03-0.69) gene frequencies were significantly lower in OF vs Control group.

No other significant difference of maternal KIR gene frequencies was observed among the clinical groups.

Distribution of maternal KIR genes is reported in Table 2 and illustrated in Figure 2.

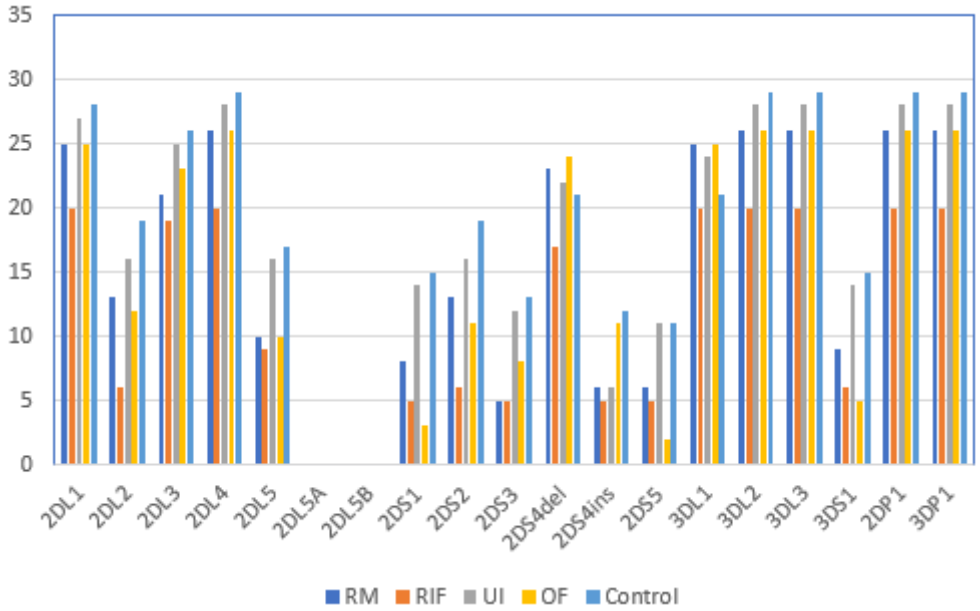
**Table 2.** Maternal KIR genes distribution among study population

| | Control (n=29) | | RM (n=26) | | RIF (n=20) | | UI (n=28) | | OF (n=26) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | N | % | N | % | N | % | N | % | N | % |
| 2DL1 | 28 | 96,6% | 25 | 96,2% | 20 | 100,0% | 27 | 96,4% | 25 | 96,2% |
| 2DL2 | 19 | 65,5% | 13 | 50,0% | 6 | 30,0% | 16 | 57,1% | 12 | 46,2% |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 2DL3 | 26 | 89,7% | 21 | 80,8% | 19 | 95,0% | 25 | 89,3% | 23 | 88,5% |
| 2DL4 | 29 | 100,0% | 26 | 100,0% | 20 | 100,0% | 28 | 100,0% | 26 | 100,0% |
| 2DL5 | 17 | 58,6% | 10 | 38,5% | 9 | 45,0% | 16 | 57,1% | 10 | 38,5% |
| 2DL5A | 0 | 0,0% | 0 | 0,0% | 0 | 0,0% | 0 | 0,0% | 0 | 0,0% |
| 2DL5B | 0 | 0,0% | 0 | 0,0% | 0 | 0,0% | 0 | 0,0% | 0 | 0,0% |
| 2DS1 | 15 | 51,7% | 8 | 30,8% | 5 | 25,0% | 14 | 50,0% | 3 | 11,5% |
| 2DS2 | 19 | 65,5% | 13 | 50,0% | 6 | 30,0% | 16 | 57,1% | 11 | 42,3% |
| 2DS3 | 13 | 44,8% | 5 | 19,2% | 5 | 25,0% | 12 | 42,9% | 8 | 30,8% |
| 2DS4del | 21 | 72,4% | 23 | 88,5% | 17 | 85,0% | 22 | 78,6% | 24 | 92,3% |
| 2DS4ins | 12 | 41,4% | 6 | 23,1% | 5 | 25,0% | 6 | 21,4% | 11 | 42,3% |
| 2DS5 | 11 | 37,9% | 6 | 23,1% | 5 | 25,0% | 11 | 39,3% | 2 | 7,7% |
| 3DL1 | 21 | 72,4% | 25 | 96,2% | 20 | 100,0% | 24 | 85,7% | 25 | 96,2% |
| 3DL2 | 29 | 100,0% | 26 | 100,0% | 20 | 100,0% | 28 | 100,0% | 26 | 100,0% |
| 3DL3 | 29 | 100,0% | 26 | 100,0% | 20 | 100,0% | 28 | 100,0% | 26 | 100,0% |
| 3DS1 | 15 | 51,7% | 9 | 34,6% | 6 | 30,0% | 14 | 50,0% | 5 | 19,2% |
| 2DP1 | 29 | 100,0% | 26 | 100,0% | 20 | 100,0% | 28 | 100,0% | 26 | 100,0% |
| 3DP1 | 29 | 100,0% | 26 | 100,0% | 20 | 100,0% | 28 | 100,0% | 26 | 100,0% |

OF: ovodonation failure; RIF: repeated implantation failure; RM: recurrent miscarriage; UI: unexplained Infertility

**Figure 1.** Maternal KIR gene distribution among study population




Maternal KIR – HLA C (2DL1-2DL2-2DL3)

Frequencies of Maternal KIR genes/HLA-C epitopes in the Clinical groups investigated are reported in Table 3. No significant differences emerge comparing Study groups and Control group.

**Table 3** Maternal KIR – HLA C (2DL1-2DL2-2DL3) among study population

| | **Control (n=27)** | | **RM (n=23)** | | **RIF (n=20)** | | **UI (n=21)** | | **OF (n=18)** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **N** | **%** | **N** | **%** | **N** | **%** | **N** | **%** | **N** | **%** |
| **2DL1-C1/C1** | 7 | 25,9% | 3 | 13,0% | 7 | 35,0% | 5 | 23,8% | 7 | 38,9% |
| **2DL1-C1/C2** | 14 | 51,9% | 13 | 56,5% | 7 | 35,0% | 12 | 57,1% | 7 | 38,9% |
| **2DL1-C2/C2** | 6 | 22,2% | 7 | 30,4% | 6 | 30,0% | 4 | 19,0% | 4 | 22,2% |
| | **Control (n=18)** | | **RM (n=13)** | | **RIF (n=6)** | | **UI (n=13)** | | **OF (n=8)** | |
| **2DL2-C1/C1** | 5 | 27,8% | 1 | 7,7% | 3 | 50,0% | 2 | 15,4% | 5 | 62,5% |
| **2DL2-C1/C2** | 8 | 44,4% | 7 | 53,8% | 1 | 16,7% | 7 | 53,8% | 3 | 37,5% |
| **2DL2-C2/C2** | 5 | 27,8% | 5 | 38,5% | 2 | 33,3% | 4 | 30,8% | 0 | 0,0% |
| | **Control (n=25)** | | **RM (n=19)** | | **RIF (n=19)** | | **UI (n=19)** | | **OF (n=17)** | |
| **2DL3-C1/C1** | 6 | 24,0% | 3 | 15,8% | 6 | 31,6% | 5 | 26,3% | 6 | 35,3% |
| **2DL3-C1/C2** | 13 | 52,0% | 11 | 57,9% | 7 | 36,8% | 10 | 52,6% | 7 | 41,2% |
| **2DL3-C2/C2** | 6 | 24,0% | 5 | 26,3% | 6 | 31,6% | 4 | 21,1% | 4 | 23,5% |

Maternal and Paternal HLA-C

No significant statistical differences were disclosed by comparing maternal and paternal HLA-C in the three disomic mendelian combinations HLA-C1C1/C1C2/C2C2, among Control and Clinical Groups investigated

## 3. Discussion

In this study we have examined the frequencies of KIR diplotypes in different groups of patients affected by impaired implantation processes and reduced fertility. We involved both women with multiple miscarriage after spontaneous conception (RM) and women with multiple failed embryos implantation after medically assisted technique (RIF). Furthermore, we have also included study groups that are still under-investigated such as women idiopathic infertility (IF) and those with repeated failed ovo-donation cycles (OF).

We observed a statistically significant higher frequency of KIR AA diplotype in all clinical groups apart from women with RM and UI. This finding is further corroborated by the higher significant frequency of centromeric AA diplotype in RIF and OF groups that supports an underlying immunogenetic determinant in these clinical conditions [23].

Notably, the unique profile of frequency distribution of the three diplotypes recorded in OF group with higher Tel AA diplotype and the lower frequency of Telomere AB and Telomere BB diplotypes. Telomere KIR AA diplotype is characterized by the presence of only the inhibitory KIR 3DL1 gene and activating KIR gene 2DS4del (harbours a 22 base pairs deletion in exon 5 making this gene devoid of any biological function) in disomic

asset (homozygous) while Telomere AB include also an array of activating KIR genes such as 3DS1, 2DS5 and 2DS1 in close linkage.

In case of Telomere AB diplotype, the activating KIR gene(s) with proven protective reproductive function is the 2DS1 KIR gene since 2DS5 KIR gene alleles detected, so far, in Europe have shown negligible immunological positive effects compared to those detected in African populations [12] and KIR 3DS1 (as its inhibitory counterpart 3DL1) can't trigger the functional activation of dNK cells because its ligand HLA-B w4 isn't expressed by EVT cells [6]. Women who experienced failed ovo-donation showed in our population a statistical significantly lower 2DS1 KIR gene frequency stressing the importance of undertaking the immunogenetic KIR analysis in these patients in order to assess a priori the potential risk of reproductive failure associated with maternal telomere KIR AA and telomere AB diplotypes, devoid of KIR 2DS1 gene, for supportive therapies.

Patients affected by IF shown a pattern of KIR diplotypes frequency quite different from other clinical groups, likely due to the heterogeneous aetiology driving this condition.

A peculiar finding of our study points, thus, to the absence, or reduced frequency of activating KIR genes of telomere B region, and significantly of the KIR gene 2DS1, in OF group (11.5%) and to lesser extent in RIF group (25%) compared to Control group (51,7%) as a preeminent risk factor for the negative reproductive outcome.

Notably we have also detected similar frequencies of KIR 2DS1 and 3DS1 genes in Control group in agreement with genetic studies so far accomplished reporting a strong linkage disequilibrium between these two genes in the telomere B region [16].

The impact of some diplotypes AB (Bx=1) and more specifically of partial Cen AB on the reproductive outcome is, to some extent, "undefined" from a functional point of view. This diplotype is characterized by the presence of the only "weak" activating KIR gene 2DS2 in heterozygous asset and no significant difference of frequencies of this diplotype, was recorded among the clinical groups investigated, except in RIF patients showing a statistically lower frequency of Cen AB (Bx=1).

In OF group this partial diplotype accounts for more than 40% of patients and, accordingly, its detection seems not predictive for a positive reproductive outcome in spite of a postulated "neutral" effect of KIR 2DS2 gene [4,16].

It seems reasonable to infer that the presence of the single activating KIR 2DS2 gene, together with strong inhibitory KIR genes as 2DL1,2DL2,2DL3 in dNK cells of women with CACB/TATA partial diplotype, elicit overall inhibitory signals to dNK cells, leading to impaired immune reproductive functions.

This possibility is, to some extent, supported by the results of a study on the dynamic and functional activity of dNK cells and ligand receptor complex involved in maternal-fetal interactions during early pregnancy by single-cell transcriptomics which disclosed a low level of KIR 2DS2 gene expression of maternal decidual NK cells during first trimester of pregnancy [24].

Three main dNK (dNK1, dNK2, dNK3) cell populations with distinctive immunomodulatory and chemokine profiles were, in facts, identified in this study. dNK1cells, the larger dNK cell population detected, expressed higher levels of KIR transcripts specific for inhibitory KIR2DL1, 2DL2 and 2DL3 and activating KIR2DS1 and 2DS4 compared to the other dNK cell subsets.

How these seminal immunogenetics findings may be translated in a clinical risk assessment of repeated reproductive failures for women carrying the specific Cen KIR AB diplotype assect, particularly in OF group, is a matter of further basic and epidemiologic studies in larger cohorts of patients.

A mere "functional assessment" of the KIR genes detected, independently of the KIR haplotype/diplotype classification, might result more appropriated to predict the risk of reproductive failure in some patients displaying KIR activating gene(s) of "unproved" immunomodulating functions in reproduction as KIR activating 3DS1 gene, detected as

single activating gene in a woman of OF group with partial diplotype CACA/TATB in this study (tab 1).

Notably, the diplotype AB with the only activating KIR 3DS1 gene was detected in nearly 1.3% of patients undergoing KIR diplotype analysis for infertility so far investigated (data not shown) and testifies to the extreme variability of assortment of KIR genes in human populations.

The ubiquitous presence of KIR 3DS1 gene within human populations, suggests, further, an evolutionary pressure to maintain this activating KIR in strong linkage disequilibrium with a gene like the KIR 2DS1 with a proven protective role in reproduction.

Another relevant issue which deserves, in our opinion, more clinical and experimental investigative efforts, pertains to the influence of maternal HLA-C on the functional competence of dNK cells in the immunological maternal-fetal crosstalk.

Pioneering studies undertaken previously [15], have clearly demonstrated the influence of maternal HLA-C alleles on the expression of inhibitory KIR 2DL1 and 2DL3 receptors of dNK cells in decidual tissue.

In presence of maternal C2 allele(s) the expression of the cognate receptor KIR 2DL1 is decreased in dNK cells while for women bearing HLA-C1 allele the expression of KIR 2DL2/3 receptors resulted increased in dNK cells, thus disclosing contrasting effects of maternal HLA-C alleles in placentation.

It seems reasonable assess that the reduced expression of KIR 2DL1 receptors in presence of maternal C2, as also pointed previously (14), is an evolutive adaption to reduce the burden of pregnancy disorders in mothers with homozygous KIR AA in presence of an HLA-C2 fetus.

A more comprehensive approach to the education of dNK cells, recently accomplished, identifies NKG2A receptor as a main driver of the process and protective in pregnancy by modulating appropriate maternal vascular adaption, fetal growth, and normal placental gene expression [16].

The pivotal role of dNK cells in human reproduction is, also, highlighted by the detection in multipara women of a unique subset of human decidual NK cells expressing higher levels of NKG2C and LILRB1activating receptors and secreting higher amounts of IFN-Y(interferon -gamma) ,VEGF(vascular endothelial growth factor)when activated by interactions with HLA-E and HLA-G antigens expressed by EVT cells .

These dNK cells, also named Pregnancies Trained decidual NK cells (PTDNK), mimic “immune memory cells” that “remember pregnancy”, detected previously in peripheral NK cells of people infected by Cytomegalovirus [25,26] and might account for the low frequency of “deficient placentation” in women with many pregnancies at term.

Another finding of the study that deserves further clinical and immunogenetic investigations, in our opinion, relates to the relevant frequencies of the activating KIR 2DS1 and 2DS2 genes recorded in the UI group.

Since a great part of women, and particularly those affected by UI enrolled in the study, had experienced a previous long story of failed attempts in PMA settings, it’s conceivable to infers that altered epigenetic mechanisms such as DNA methylation at CpG dinucleotides within promoter regions of KIR genes or post-translational histone modifications (e.g.phosphorilation, methylation, or acetylation) could drive reduced expression of these genes ,thus impairing uNK activation and function .This hypothesis is supported by the results of a recent study [26] reporting a low expression of KIR2DL1/S1 and LILRB1 receptors in total uNK and uNK1, uNK2 and uNK3 cell subsets in endometrial tissue from women affected by Recurrent Miscarriages, RIF and Unexplained Infertility.

Epigenetic mechanisms based upon miRNAs molecules have, so far, also been shown to orchestrate KIR gene expression during ontogenic development and differentiation of dNK cells from more immature progenitor cells [27]. More specifically miR30e, miR 340-3p/5p; miR-486-3p; miR-141-3p/5p; miR-24 as well as Lnc-CD56 [28,29] have been implied in dNK cell function at maternal/fetal interface as well as in the etiogenesis of

infertility [30] ,while miR 146a-5p (31) has been, specifically, involved in down-regulation of KIR genes in peripheral NK cells [31].

The limitation of our analysis resides in the low sample size per each group investigated and retrospective collection of the data, that makes our findings more prone to bias.

These findings open a new avenue for research in the field to uncover how epigenetic mechanisms [32] mediated by altered methylation of CG residues in promoter of KIR genes as well as by different expression of specific miRNAs/Long noncoding RNAs may shape at maternal-fetal interface the immunocompetence of epitopes KIR-HLA-C in couples affected by deficient implantation process, unexplained infertility and pregnancy-related disorders.

## 4. Materials and Methods

We included women referred to Institute Genetic Research (IRG) in Naples from January 2019 to December 2023 affected by the following conditions: a) recurrent miscarriages (RM; patients experiencing >2 or more pregnancies loss following natural cycles); b) repeated implantation failure (RIF; women experiencing > 3 failed IVF cycles with good quality embryos), and PGT-A negative (for maternal age>37 years old) in ART setting, Beta HCG positive); c) Ovodonation failure (OF; > 2 failed donor oocyte donation cycles in couples with a story of IF and /or RIF) d) Unexplained Infertility (UI) defined as infertile couples with apparently normal reproductive function [21].

Control group included 29 women with at least 1 natural pregnancy with birth at term undergoing MAR procedures for IF and/or repeated abortions.

The present study was conducted according to the Helsinki Declaration principles. Data were anonymous and informed consent was obtained from all participants. The Institutional Review Board of IRG approved the study.

All couples attending to the study were negative to chromosomal structural rearrangements and thrombophilia disorders (MTHFR C677T/ A1298C genetic variants, Factor V Leiden and Prothrombin variant G20210A).

We did not include couples with the following exclusion criteria: abnormal TORCH; abnormal gynecological pelvic examination; abnormal immunological measurements of lupus anticoagulant, anti-cardiolipin and anti Beta-2-glycoprotein (IgG or IgM antibodies); endocrine and immunologic disorders (i.e. thyroid disorders, diabetes type I and II).; abnormal coagulation function and D-dimer.

Study subjects and samples:

Genomic DNA for KIR typing and HLA-C1C2 allelic analysis was purified from patient's whole blood by QIA amp kit (Qiagen) according to the instruction of the manufacturer.

For KIR typing a kit (Milteny Biotech,Italy) which allows the detection of all known15 human KIR genes and two pseudogenes by PCR -SSPs(sequencing specific priming approach) was employed.

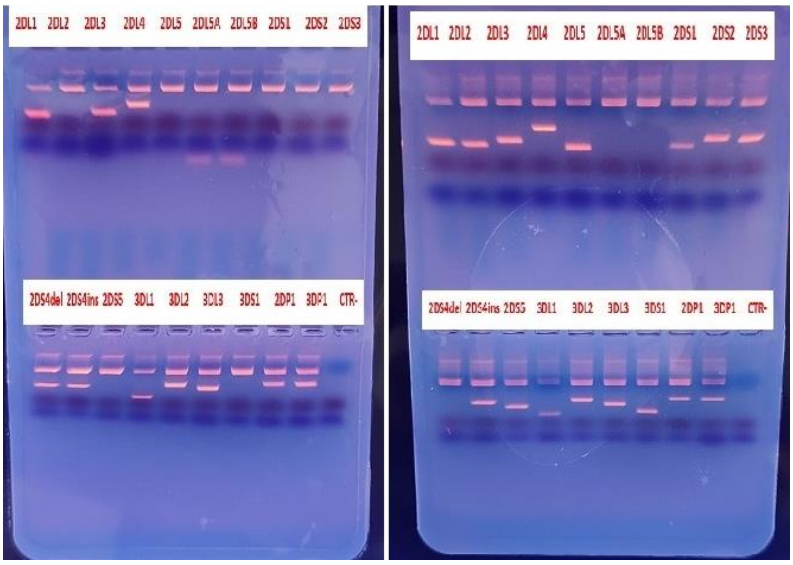
Every KIR typing PCR reaction contains an internal control, generated by a second pair of primers, thereby, two PCR fragments are generated from the sample template: one is derived from the KIR-specific primers and the second by the internal control primers.

After running PCR products on 3% agarose gel electrophoresis with Ethidium Bromide, an Evaluation Form for each patient was signed to record presence/absence of the stained KIR-specific product of the expected size in each designate field and the presence of the internal control of 400bp.

Allele specific KIR-HLA-C ligand (C1C2) assay was performed by using KIR HLA ligand kit Olerup SSP kit (Sweden) using a similar approach (PCR sequence specific primers) which allows all Known HLA-C1 alleles to be distinguished from C2 alleles by agarose gel electrophoresis with Ethidium Bromide.

For each woman enrolled in the study, KIR diplotypes AA, AB (Bx=1 ;Bx>1) BB and partial KIR haplotypes CenA/A;CenA/B,CenB/B) and Tel AA; Tel AB; Tel BB were derived following established criteria (Figure 3) [13].

**Figure 3.** Electrophoretogram of KIR diplotypes AA (left) and KIR diplotypes AB (right)




Gene studies have fixed the content of KIR genes in the chromosomal region 19q13.3 in four definite KIR clusters (array of genes closely located which tend to be transmitted and inherited together) : Centromeric A which contains only KIR inhibitory genes 2DL1 and 2DL3, and Telomeric A region including 2DS4Del (not functional form of the gene 2DS4) or its functioning counterpart 2DS4wt and inhibitory 3DL1; Centromeric B which contains mainly activating genes 2DS2 and /or 2DS3 and inhibitory 2DL2 and Telomeric B which includes activating KIR genes 2DS1,2DS5,3DS1.

Bx content identifies the number of detected B clusters with centromeric or telomeric location (from1 to 4). Bx=1 content usually refers to a single centromeric or telomeric cluster containing activating KIR gene(s).

*Statistics*

The frequencies of the different KIR genes were obtained by direct counting. The comparison between different clinical samples were performed in contingency tables using Fisher's exact test [22]. Odd ratio values with 95% confidence intervals were also calculated. SPSS (Statistical Package for the Social Sciences, IBM version 29) was adopted for statistic calculations and a bilateral p value less than 0.05 was considered as statistically significant..

## 5. Conclusions

In conclusion, this analysis demonstrated an association between specific KIR genes in women with RM, RIF and OF comparing with control. In particular, this study is one of the first analysis exploring KIR genotypes in women with OF that showed a significantly reduced activatory KIR genes 2DS1, 2DS5 and 3DS1. Considering the low sample, these findings should be corroborated by further analysis..

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Guida, Federica Cariati, Maria Campitiello, Carlo Alviggi, Thomas D'Hooghe, Elena Vaquero, Roberto Valerio and Salvatore Longobardi; Validation, Maria Campitiello; Visualization, Federica Cariati, Thomas D'Hooghe, Elena Vaquero and Roberto Valerio; Writing – original draft, Alessandro Conforti, Domenico Valerio and Valeria Saide; Writing – review & editing, Alessandro Conforti and Domenico Valerio.

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**Data Availability Statement:** We encourage all authors of articles published in MDPI journals to share their research data. In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Where no new data were created, or where data is unavailable due to privacy or ethical restrictions, a statement is still required. Suggested Data Availability Statements are available in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics.

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