human reproduction update

Number and function of uterine natural killer cells in recurrent miscarriage and implantation failure: a systematic review and meta-analysis

Ee Von Woon[1,2,*], Orene Greer[1], Nishel Shah[1], Dimitrios Nikolaou[2], Mark Johnson[1], and Victoria Male[1]

[1]Department of Metabolism, Digestion and Reproduction, Institute of Developmental Reproductive and Developmental Biology, Imperial College London, London, UK [2]The Fertility Centre, Chelsea and Westminster Hospital, London, UK

Supervisor: Consultant 黃貞瑜 主任

Presenter: R3 曾美齡 醫師 Kathleen Tseng M.D.

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Introduction

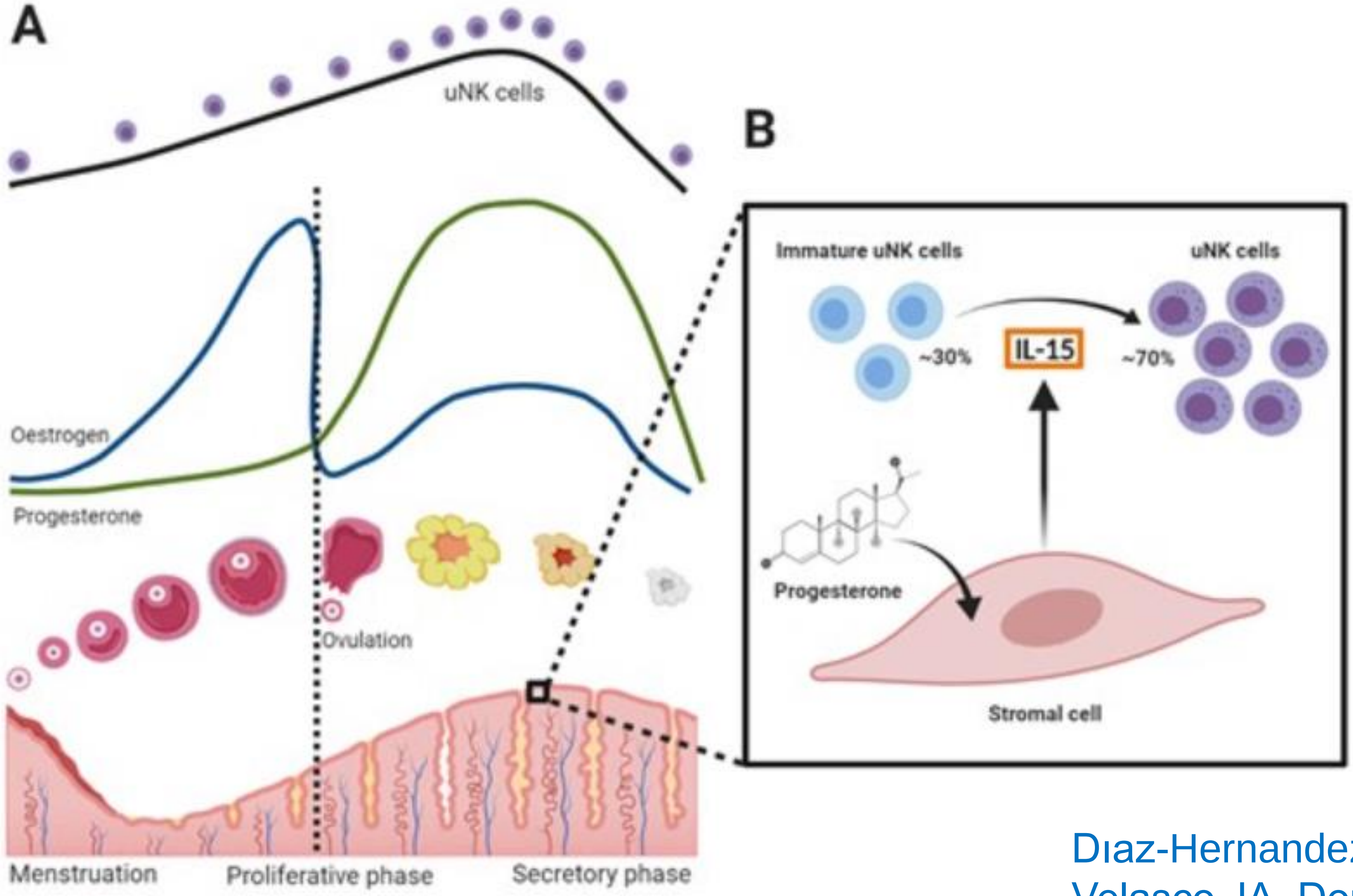
Embryo quality + Endometrium receptivity = Successful implantation

- 50% of RM and RIF cases remain unexplained
- Could it be immunological factor? **NK cells**: highest proportions of immune cells in the palcental bed during 1st trimester pregnancy

➢ In non-pregnant endometrium, inactive uNK cells undergo differentiation during menstrual cycle in preparation for pregnancy. (Manaster et al., 2008; Strunz et al., 2021)

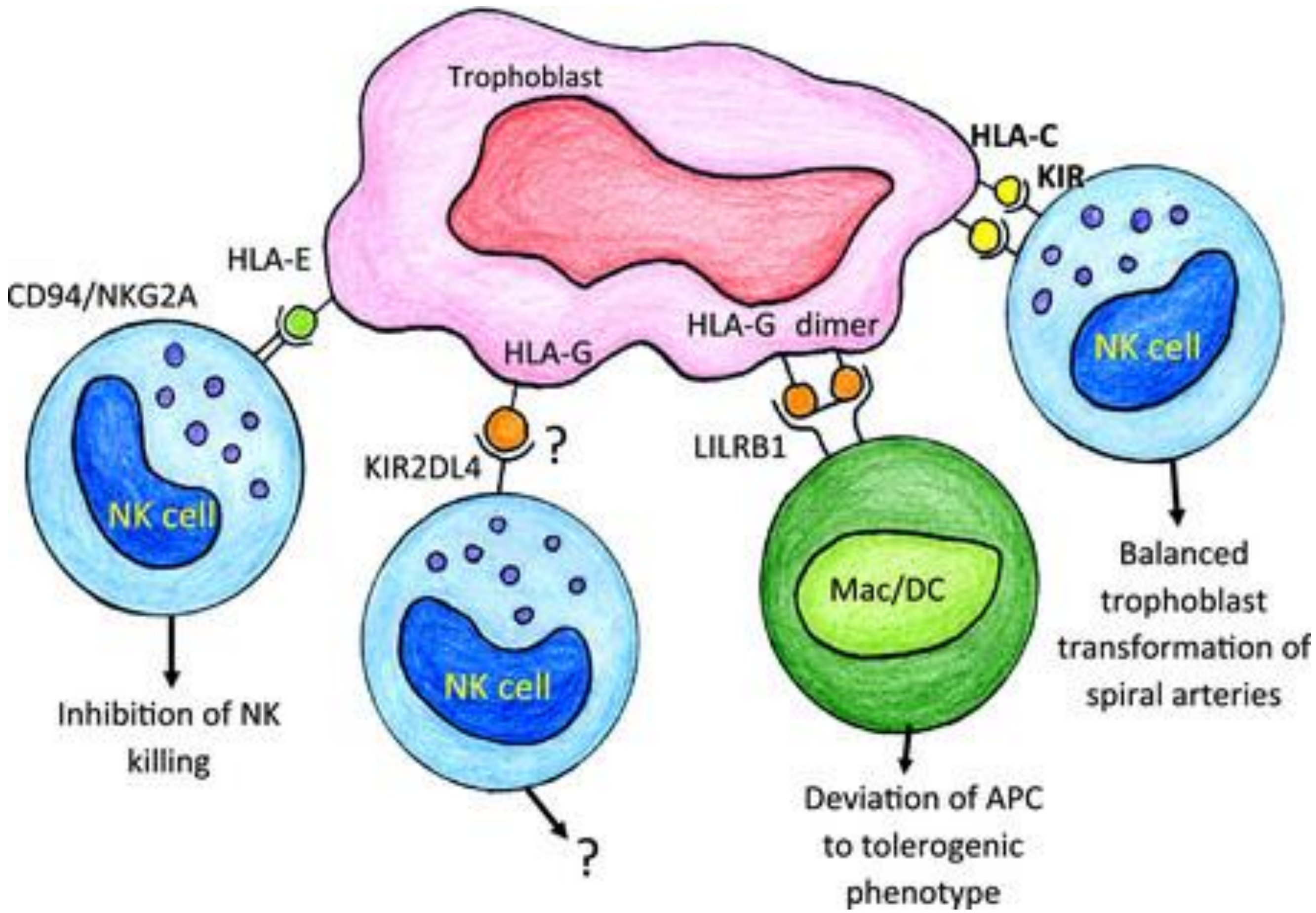
➢ Implantation of embryo → uNK → trophoblast invasion and spiral artery remodelling → placentation (Huhn et al., 2021)

➢ Balance between excessive and insufficient trophoblast invasion → miscarriage, pre-eclampsia, FGR (Brosens et al., 2011)



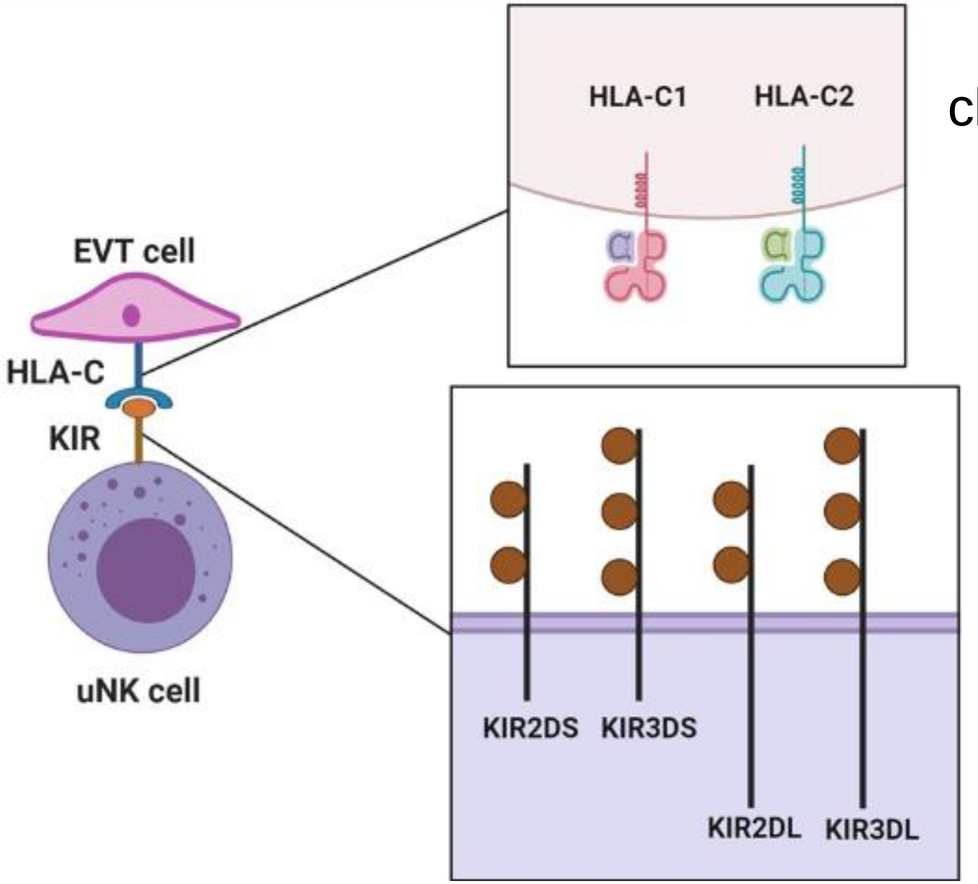
Dıaz-Hernandez I, Alecsandru D, Garcıa-Velasco JA, Domınguez F. *Uterine natural killer cells: from foe to friend in reproduction. Hum Reprod Update 2021*

Extravillous trophoblasts (EVT): Fetal-derived cells in the maternal-fetal interface, expressing MHC-I antigens



NK-cell receptors
CD94/NKG2
Leucocyte immunoglobulin-like receptor (LILR)
Killer-like immunoglobulin receptor (KIR) families

Activation of uNK → Cytokine production

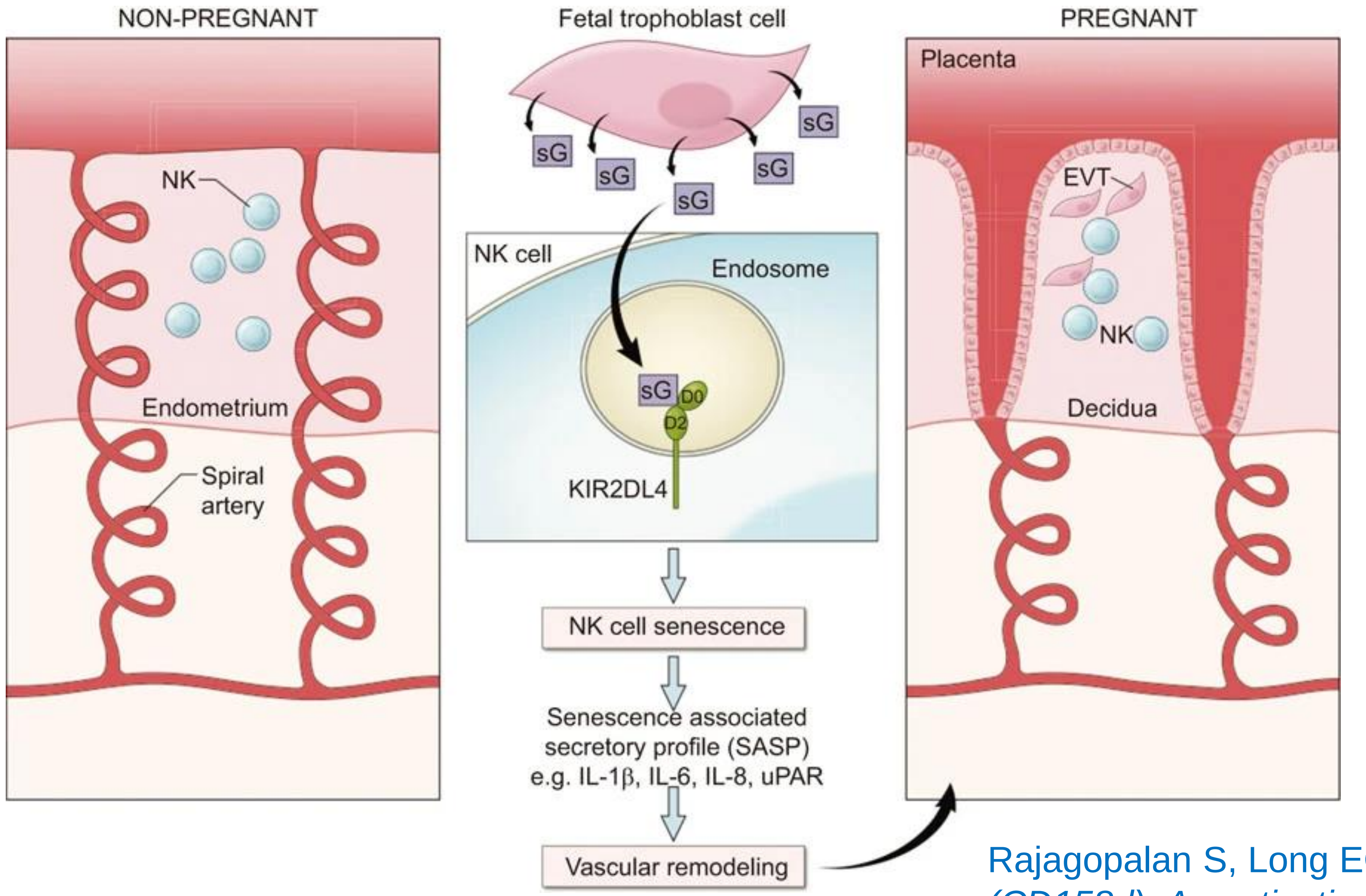


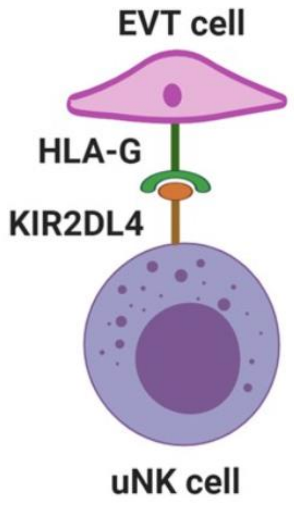
classical class I molecule

KIR2DS1 and KIR2DS4

→ Granulocyte-macrophage colony-stimulating factor secretion
→ Migration of trophoblast cells
(Xiong et al., 2013; Kennedy et al., 2016)

Dıaz-Hernandez I, Alecsandru D, Garcıa-Velasco JA, Domınguez F. *Uterine natural killer cells: from foe to friend in reproduction. Hum Reprod Update 2021*

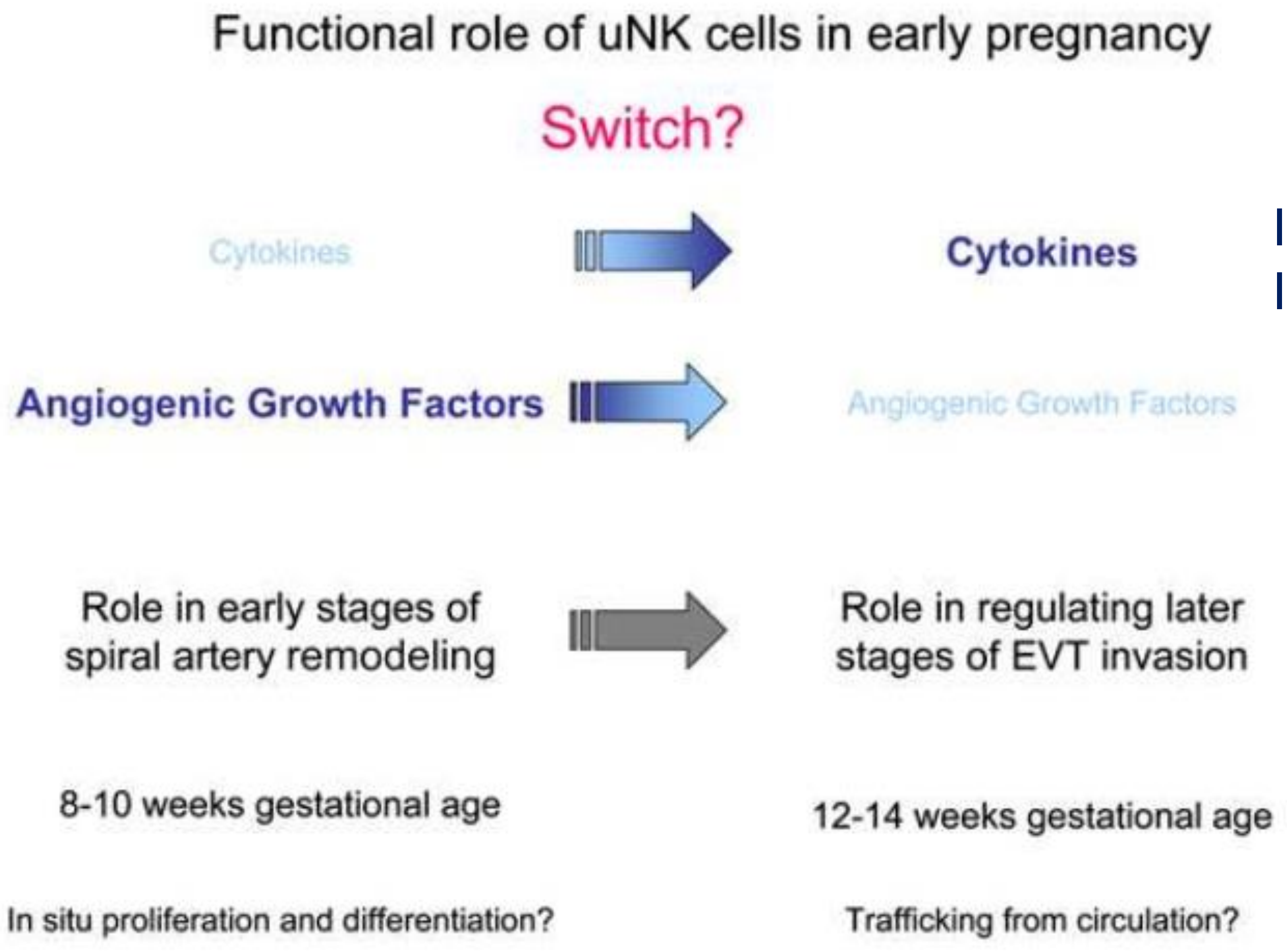




Rajagopalan S, Long EO. *KIR2DL4 (CD158d): An activation receptor for HLA-G. Front Immunol.* 2012 Aug 20;3:258

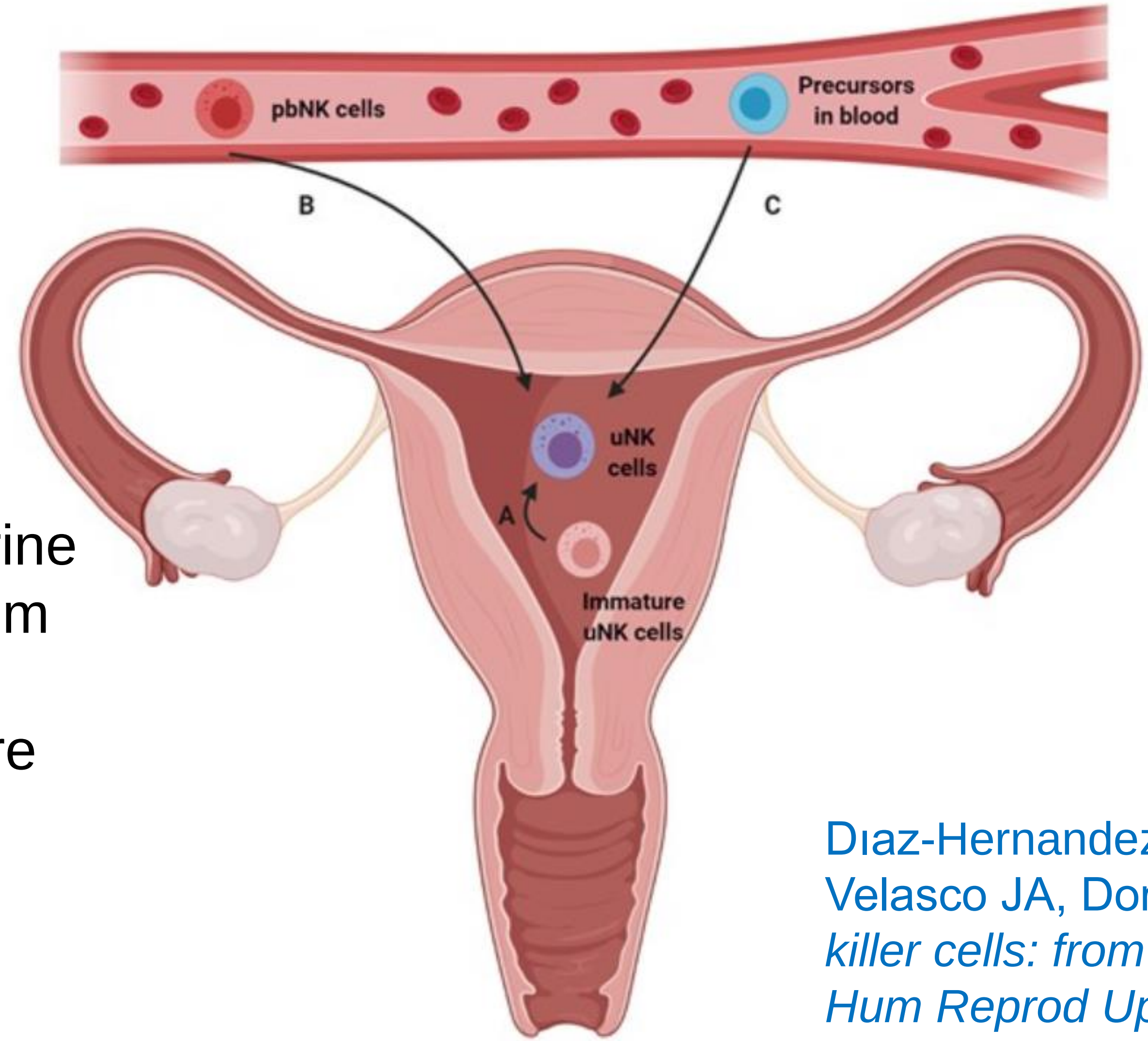
IFN-γ, IL-1β,
IL-6,IL-8

VEGF-C,
angiopoietin-1,
angiopoietin-2



Lash GE, Robson SC, Bulmer JN. *Review: Functional role of uterine natural killer (uNK) cells in human early pregnancy decidua. Placenta 2010*

The origin of uterine NK (uNK) cells



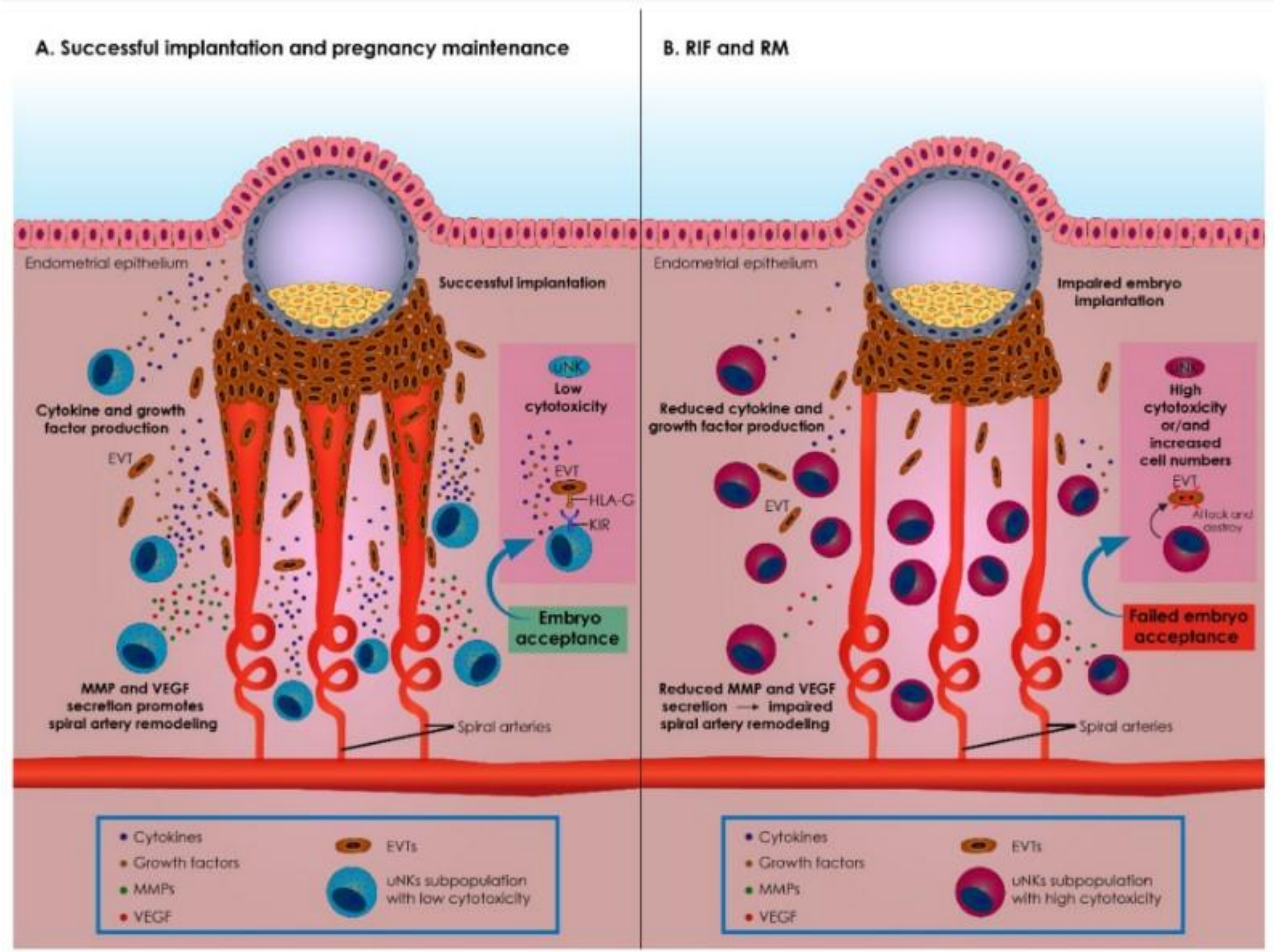
A: Differentiation from uterine resident hematopoietic stem cells
B: Recruitment from mature peripheral NK cells
C: Differentiation from circulating immature pNK cells

Dıaz-Hernandez I, Alecsandru D, Garcıa-Velasco JA, Domınguez F. *Uterine natural killer cells: from foe to friend in reproduction. Hum Reprod Update 2021*

	uNK	pNK
Phenotype	CD56bright (CD56bright CD16+) (King et al., 1991; Koopman et al., 2003)	CD56dim (CD56dimCD16-) (Caligiuri, 2008)
Tissue marker	Tissue-residence marker CD49a, subdivided into 3 subsets (Vento-Tormo et al., 2018)	not found
Cytotoxicity	Weakly cytotoxic against tumour cells and not at all against trophoblast cells (King et al., 1989)	First line defense against viruses (Horowitz et al., 2011) and malignant cells (Chiossone et al., 2018)

Pathological pregnancies

1. Higher than normal uNK level → ↑ angiogenic factors → ↑ peri-implantation flow → ↑ oxidative stress to trophoblast cells (Quenby et al., 2009; Chen et al., 2016)
2. Uterine NK cells secrete pro-inflammatory cytokines (≈ Th1-type cytokines) → dampening anti-inflammatory Th2-type cytokines to maintain healthy pregnancy (Sargent et al., 2006; Makrigiannakis et al., 2011)
3. Different combinations of parental HLA-C and maternal KIR allo-types on live birth outcome in women undergoing ART → inadequate (rather than excessive) activation of uNK may cause RM and RIF (Alecsandru et al., 2020)



Sfakianoudis, Konstantinos, et al. *"The role of uterine natural killer cells on recurrent miscarriage and recurrent implantation failure: From pathophysiology to treatment." Biomedicines 9.10 (2021): 1425*.

In the last meta-analysis

- No difference in uNK level, measured as percentage of total stromal cells (Seshadri and Sunkara, 2014)

Aims

1. Differences in uNK level in women with RM/RIF vs. controls
2. Pregnancy outcome in women with RM/RIF (high and normal uNK level)
3. Correlation between uterine and pNK in women with RM/RIF
4. Differences in uNK activity in women with RM/RIF vs. controls

Methods

Protocol registration

International Prospective Review of Systematic Reviews (PROSPERO): CRD42020175868

Study search and screen

PRISMA

MeSH keywords: Natural Killer cells, recurrent miscarriage and recurrent implantation failure
Electronic databases: MEDLINE, EMBASE, Web of Science and Cochrane Central Register of Controlled Trials

Screening process: 2 reviewers (E.V.M. and O.G.) + 3 senior authors (N.S., V.M. and M.J.)

Study selection

All observational studies on humans until **December 2020**

RM: ≥ 2 previous pregnancy loss (Bender Atik et al., 2018)

RIF: inability to achieve clinical pregnancy after ≥ 2 fresh or frozen transfers of high-quality embryos (Polanski et al., 2014)

Control group: Women with no history of reproductive problems, including those undergoing ART because of male factor infertility

- Exclusion criteria: usage of immunotherapy, studies on immunogenetics, nonstandardized usage of hormonal therapy or no control group

Outcomes measured

1. Primary outcome: uNK level measured in absolute count, or percentage of stromal cells or lymphocytes in women with RM and RIF
2. Secondary outcome (pregnancy outcome): live births, or clinical pregnancy rate (CPR), defined as GS+ and FHB+
3. Tertiary outcome: correlation coefficient between pNK and uNK levels in women with RM and RIF
4. Final outcome: uNK activity grouped as uNK regulation and receptors, cytotoxicity, effect on uterine vasculature and cytokine production

Data extraction

Independently by E.V.W. and O.G., uploaded as template on Covidence, extracted with online software WebPlotDigitizer

Quality assessment

- Risk Of Bias In Non-randomised Studies of Intervention (ROBINS-I) tool
- Publication bias: funnel plot and Egger's test

Data synthesis

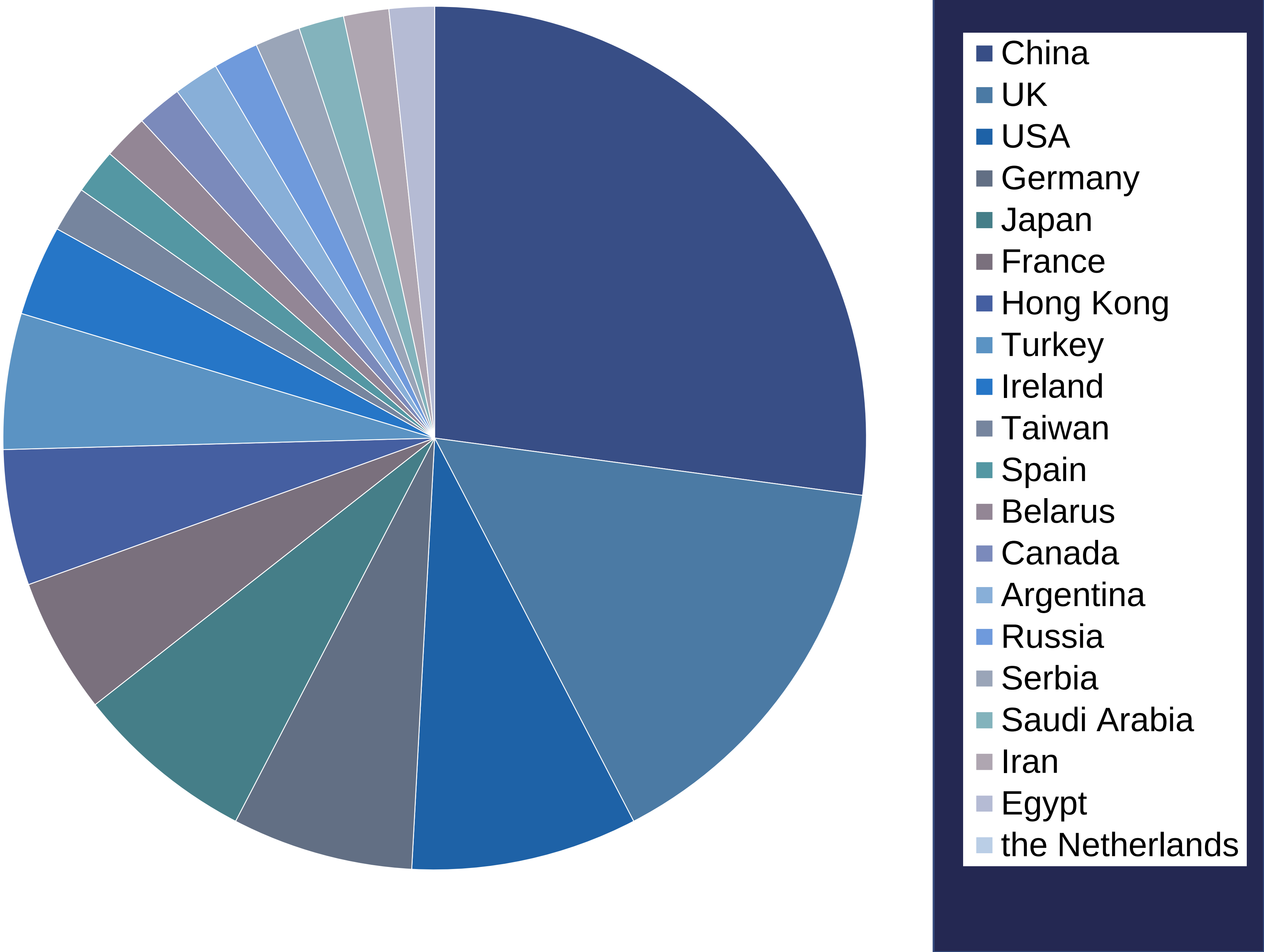
- Meta-analysis: RevMan 5.3

✓ Standardized mean difference (SMD) of uNK level in women with RM and RIF

✓ Risk ratio of clinical pregnancy and live birth rate, correlation coefficient: uNK and pNK phenotypes

- Narrative synthesis for uNK activity; 1 favours case, 0 favours control, - no difference

Results

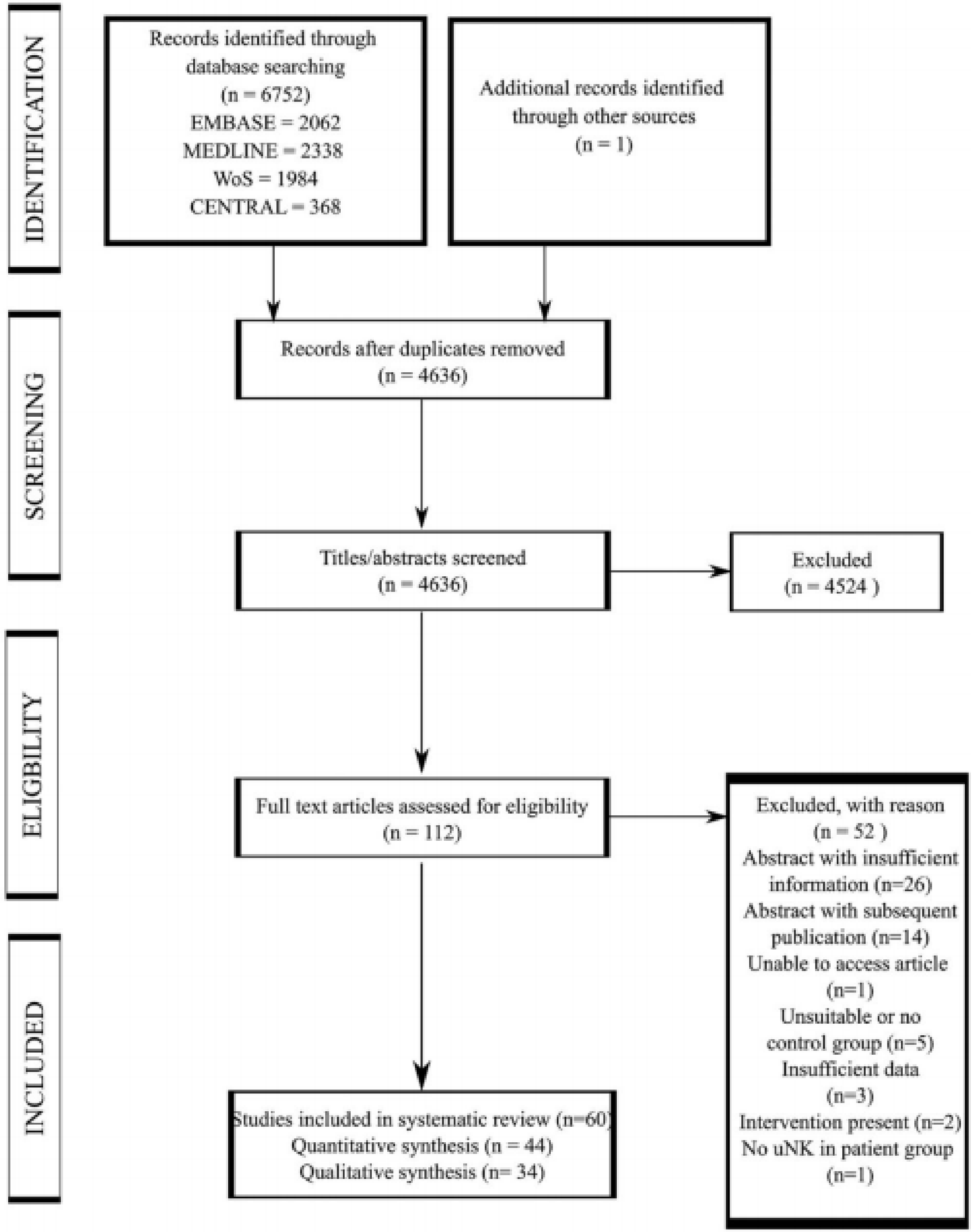

China
UK
USA
Germany
Japan
France
Hong Kong
Turkey
Ireland
Taiwan
Spain
Belarus
Canada
Argentina
Russia
Serbia
Saudi Arabia
Iran
Egypt
the Netherlands

Study selection and characteristics

Eligible: 60 articles from 20 countries

44 articles for meta-analyses
34 articles for qualitative synthesis

- uNK level, activity and correlation with pNK: all case-control studies
- Pregnancy outcomes: 6 prospective studies, 1 retrospective cohort study



Study characteristics

- Heterogeneity in definitions

✓ RM: 18 studies (≥ 3 previous miscarriages), 14 studies (≥ 2 previous miscarriages), 6 studies did not state number

✓ RIF: 6 studies (≥ 3 previous failures to achieve clinical pregnancies after ET), 4 studies (≥ 2 previous failures)

✓ Control: 16 (previous successful livebirths), 5 (male factor infertility), 10 (no history of previous miscarriages or failed IVF), 15 (healthy pregnancy for elective termination), 6 studies (no statement on pregnancy history)

- Samples studied

✓ Endometrial tissue: non-pregnant women at mid-luteal phase, but timing method varied (18 studies by urine LH, 3 studies by estrogen-progesterone therapy, 2 by LMP, 2 by histological dating, 1 by basal body temperature and ultrasound)

✓ Decidual tissue: obtained at surgery (GA 4~12 weeks)

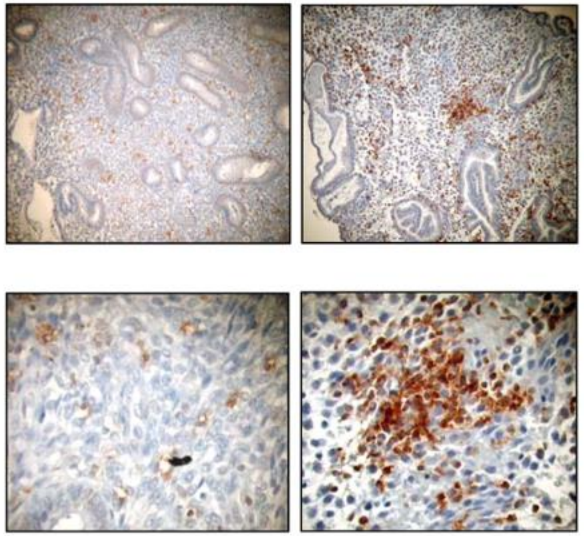
Study characteristics

- Methods of analysis

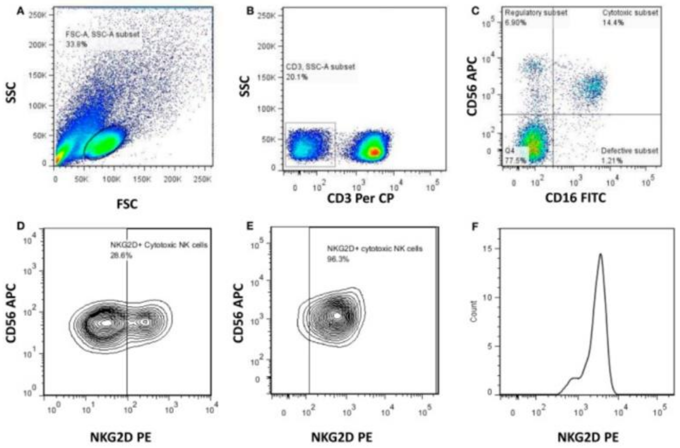
✓ Immunohistochemistry (23 studies): uNK level as total stromal cell %, absolute count or staining intensity

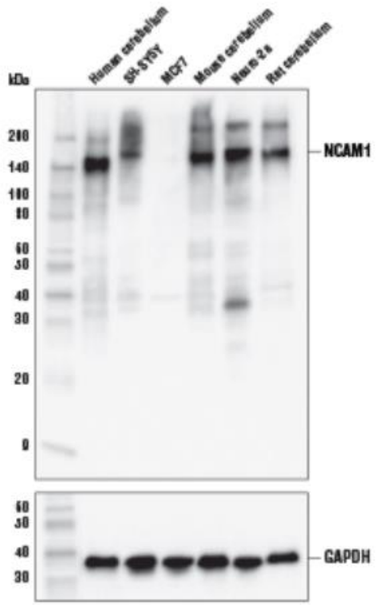
✓ Flow cytometry (14 studies): variations in gating strategy, presenting their data as total CD56+, CD56+CD16-, CD56 brightCD16-, CD56+CD16+ or CD57+ uNK

✓ Western blot (1 study): CD56 protein expression



Proliferative phase Secretory phase





Quality assessment

No significant publication bias for studies in the meta-analyses of uNK level (Egger's test, P=0.15)

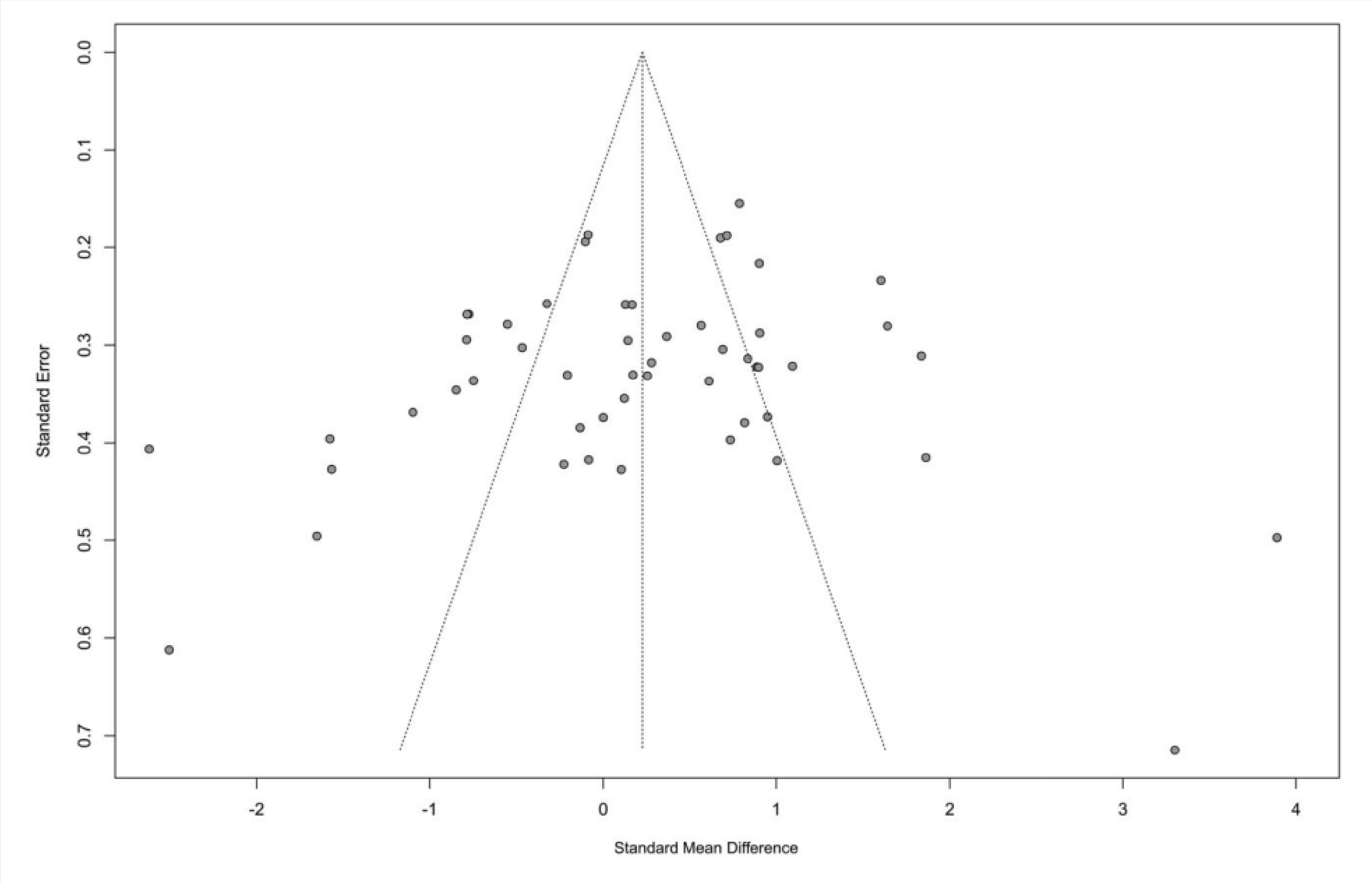


Figure 2. Funnel plot of all the studies included in the meta-analyses of uterine natural killer level.

Meta-analysis: uNK cell level

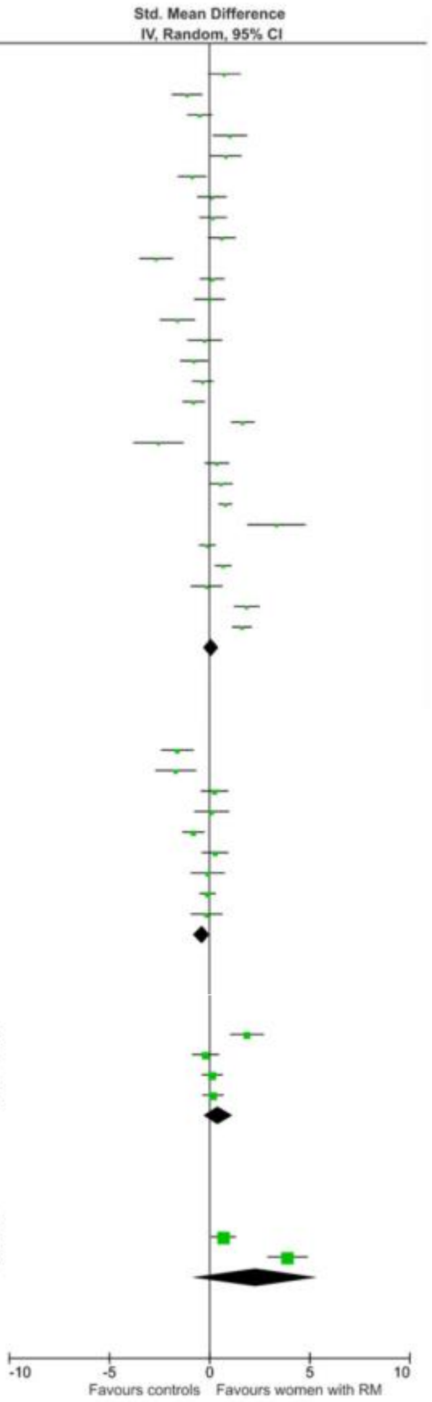
Recurrent Miscarriage

- 33 studies in total

Different phenotypes of uNK cells

(A) 28 studies on total CD56+ cells (both uNK and pNK cells in the uterus)
(B) 9 studies on CD56+CD16- cells (predominantly uNK)
(C) 4 studies on CD56+CD16+ cells (pNK in the uterus)
(D) 3 studies on CD57+ cells (mature circulating NK cells)

	Women with RM			Control				Std. Mean Difference	
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	Year
(A) CD56+ uNK									
Chao 1995	53.5	17.4	10	40.7	16.8	21	3.5%	0.73 [-0.04, 1.51]	1995
Lachapelle 1996	76	7	20	83	5	15	3.5%	-1.10 [-1.82, -0.37]	1996
Lea 1997	10.8	8.15	23	17.2	17.35	22	3.7%	-0.47 [-1.06, 0.13]	1997
Quenby, 1999	10.05	5.7	22	4.77	3.11	9	3.4%	1.00 [0.18, 1.83]	1999
Clifford 1999	146	71	29	94	19	10	3.5%	0.82 [0.07, 1.56]	1999
Quack 2001	43.8	17.7	17	56.55	11.6	20	3.6%	-0.85 [-1.53, -0.17]	2001
Michimata 2002	43.5	15.9	17	41.7	12.1	15	3.6%	0.12 [-0.57, 0.82]	2002
Shimada 2004	18.3	15.2	20	15.9	11.3	17	3.6%	0.17 [-0.47, 0.82]	2004
Tuckerman, 2007	11.2	8.4	87	6.2	4.4	10	3.6%	0.61 [-0.05, 1.27]	2007
Qu 2008	39.79	14	22	130.45	44.73	25	3.4%	-2.62 [-3.42, -1.82]	2008
Ozcimen, 2009	3.42	10.31	23	2.14	6.81	23	3.7%	0.14 [-0.43, 0.72]	2009
Bohlmann 2010	2.4	0.62	25	2.4	0.62	10	3.5%	0.00 [-0.73, 0.73]	2010
Parkin 2011	15.9	5.3	24	23.4	2.4	10	3.4%	-1.57 [-2.41, -0.73]	2011
Giuliani 2014	18.3	14.6	13	22.3	19.9	10	3.4%	-0.23 [-1.05, 0.60]	2013
Fu 2013	56.34	14.5	11	66.06	12.5	56	3.6%	-0.75 [-1.41, -0.09]	2013
Sotnikova 2014	80.07	8.26	26	82.87	8.7	37	3.8%	-0.32 [-0.83, 0.18]	2014
Wang 2014	51.8	20.8	30	66.7	16.86	30	3.8%	-0.78 [-1.30, -0.25]	2014
Almasry 2015	4.98	1.83	40	2.1	1.6	30	3.7%	1.64 [1.09, 2.19]	2015
Eskicioglu 2016	22,283.4	12,855.61	10	52,760.78	10,516.98	11	2.8%	-2.50 [-3.70, -1.30]	2016
Radovic 2016	85.03	23.3	30	77.6	13.3	20	3.7%	0.37 [-0.20, 0.94]	2016
Kuon 2017	257	212	58	148	73	17	3.7%	0.57 [0.02, 1.12]	2017
Chen 2017	3.95	1.65	97	2.86	0.98	84	4.0%	0.79 [0.48, 1.09]	2017
El-Azzamy 2018	107.27	30.3	15	19.4	6.7	7	2.6%	3.30 [1.90, 4.70]	2018
Wei 2019	9.14	7.2	58	9.83	6	49	3.9%	-0.10 [-0.48, 0.28]	2019
Marron 2019	4.8	2.2	155	3.39	1.36	35	3.9%	0.68 [0.30, 1.05]	2019
Liu 2019	43.7	7.2	10	45	10.44	21	3.5%	-0.13 [-0.89, 0.62]	2019
Zhao, 2020	13.85	4.75	30	6.83	2.43	30	3.7%	1.84 [1.23, 2.45]	2020
Lyzikova 2020	150	68.1	39	50.4	57.4	63	3.8%	1.60 [1.14, 2.06]	2020
Subtotal (95% CI)			**961**			**707**	**100.0%**	**0.11 [-0.26, 0.47]**	
Heterogeneity: $Tau^2 = 0.84$; $Chi^2 = 280.75$, df = 27 ($P < 0.00001$); $I^2 = 90\%$									
Test for overall effect: Z = 0.58 (P = 0.56)									
(B) CD56+CD16- uNK									
Lachapelle 1996	16	6	20	24	3	15	10.4%	-1.58 [-2.35, -0.80]	1996
Yamamoto 1999	26.1	17.1	9	49.9	11.7	15	8.7%	-1.65 [-2.62, -0.68]	1999
Shimada 2004	15	13.3	20	12	8.9	17	11.6%	0.26 [-0.39, 0.90]	2004
Hosseini 2014	56.5	20.1	14	54.4	17.5	9	9.8%	0.11 [-0.73, 0.94]	2014
Wang 2014	41.5	17.97	30	55.7	17.68	30	12.8%	-0.79 [-1.31, -0.26]	2014
Dong 2017	49.6	17	20	45	15.2	20	11.9%	0.28 [-0.34, 0.90]	2017
Guo 2017	56	14.5	11	57.5	19.5	12	10.0%	-0.08 [-0.90, 0.73]	2017
Marron 2019	41.8	15.2	155	43.05	8.75	35	14.2%	-0.09 [-0.45, 0.28]	2019
Liu 2019	43.7	7.2	10	45	10.44	21	10.6%	-0.13 [-0.89, 0.62]	2019
Subtotal (95% CI)			**289**			**174**	**100.0%**	**-0.37 [-0.79, 0.05]**	
Heterogeneity: $Tau^2 = 0.29$; $Chi^2 = 29.90$, df = 8 ($P = 0.0002$); $I^2 = 73\%$									
Test for overall effect: Z = 1.71 (P = 0.09)									
(C) CD56+CD16+ uNK									
Lachapelle 1996	11	3	20	6	2	15	22.1%	1.86 [1.05, 2.68]	1996
Shimada 2004	3.33	2.61	20	3.83	2.07	17	24.6%	-0.21 [-0.85, 0.44]	2004
Wang 2014	12.1	6.27	30	11.1	8.74	30	26.7%	0.13 [-0.38, 0.64]	2014
Gao 2015	24.92	47.3	30	17.89	34.2	30	26.7%	0.17 [-0.34, 0.68]	2015
Subtotal (95% CI)			**100**			**92**	**100.0%**	**0.44 [-0.28, 1.16]**	
Heterogeneity: $Tau^2 = 0.44$; $Chi^2 = 17.19$, df = 3 ($P = 0.0006$); $I^2 = 83\%$									
Test for overall effect: Z = 1.20 (P = 0.23)									
(D) CD57+ uNK									
Quenby, 1999	0.24	0.26	22	0	0	9		Not estimable	1999
Ozcimen, 2009	3.42	2.15	23	2.14	1.42	23	50.8%	0.69 [0.09, 1.29]	2009
Radovic 2016	95.3	26	30	15.2	2.6	20	49.2%	3.89 [2.91, 4.86]	2016
Subtotal (95% CI)			**75**			**52**	**100.0%**	**2.27 [-0.87, 5.40]**	
Heterogeneity: $Tau^2 = 4.94$; $Chi^2 = 30.07$, df = 1 ($P < 0.00001$); $I^2 = 97\%$									
Test for overall effect: Z = 1.42 (P = 0.16)									



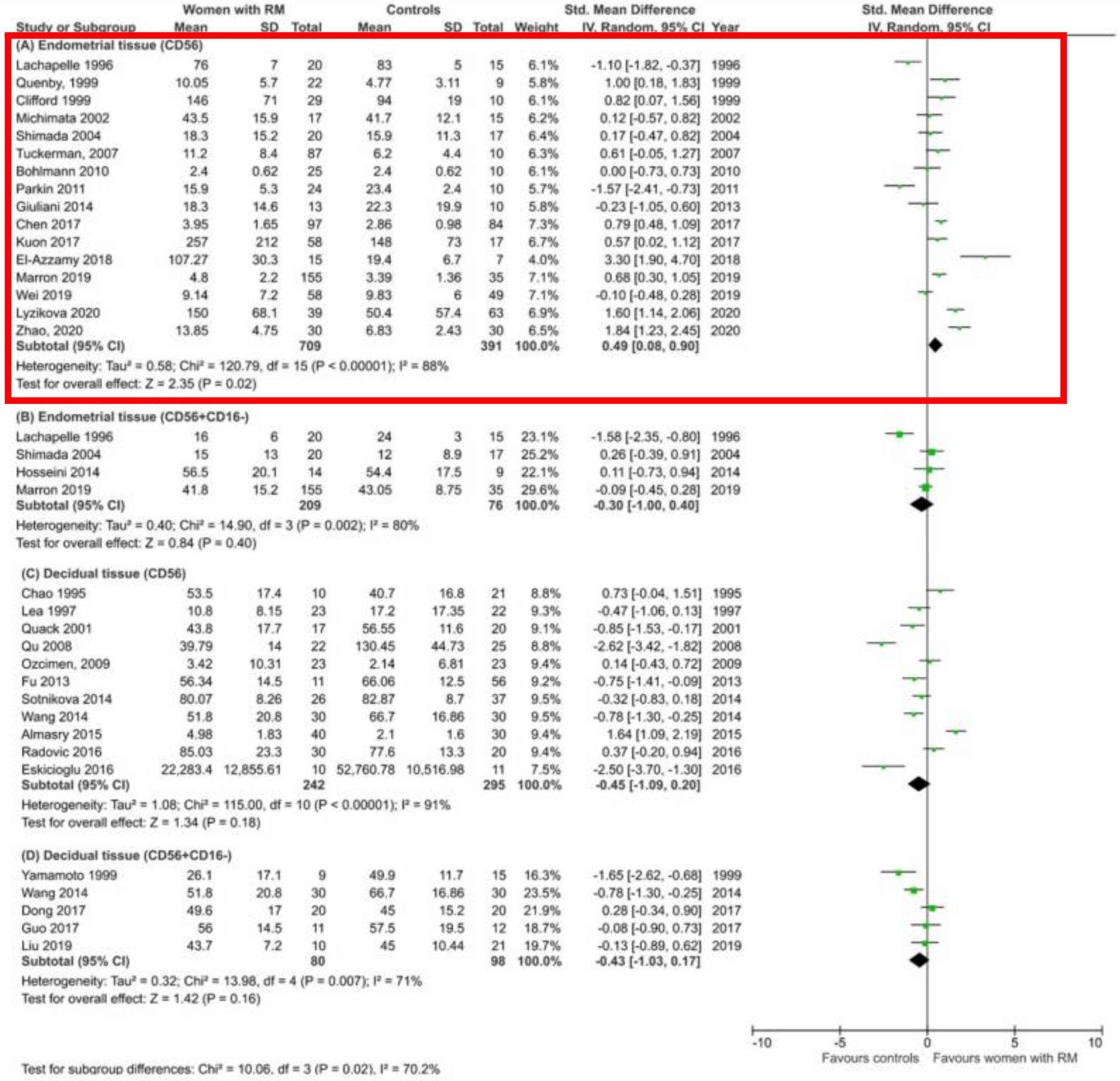
Meta-analysis: uNK cell level

Recurrent **M**iscarriage

- Subgroup analysis

➢ **Significantly higher** total CD56+ uNK in women with RM compared with controls in endometrial samples (A) from mid-luteal phase only, not replicated in decidual tissue (C)

➢ No significant difference in CD56+CD16- cells in either endometrial (B) or decidual tissue (D)



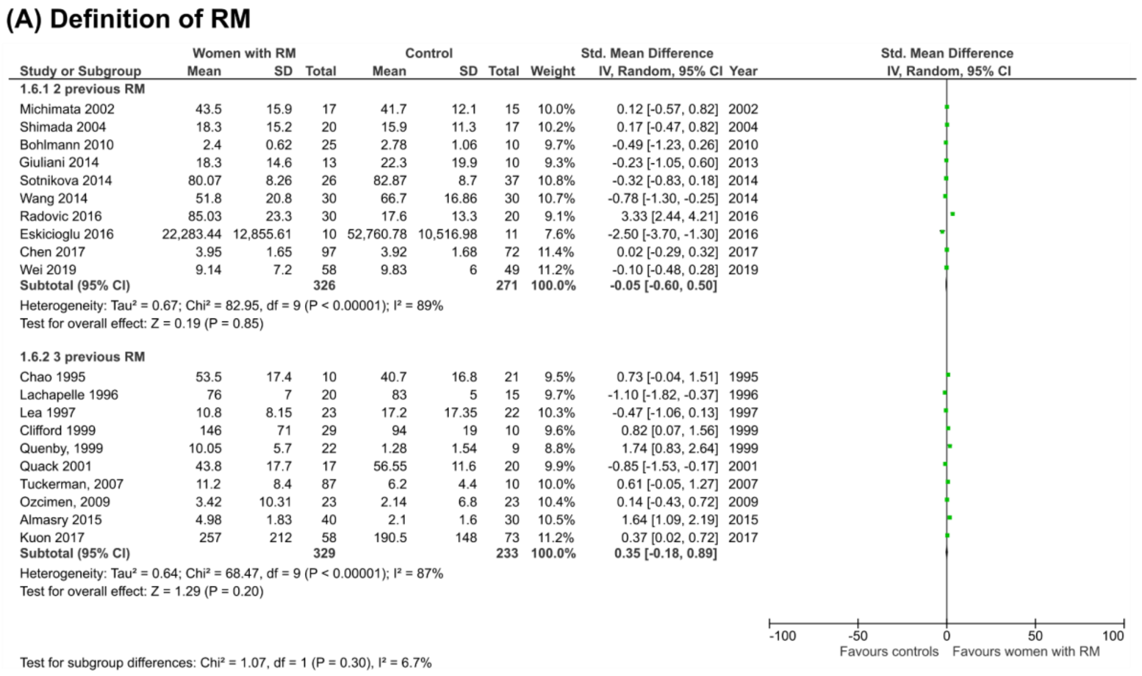
Study or Subgroup	Women with RM Mean	SD	Total	Controls Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI	Year
(A) Endometrial tissue (CD56)									
Lachapelle 1996	76	7	20	83	5	15	6.1%	-1.10 [-1.82, -0.37]	1996
Quenby, 1999	10.05	5.7	22	4.77	3.11	9	5.8%	1.00 [0.18, 1.83]	1999
Clifford 1999	146	71	29	94	19	10	6.1%	0.82 [0.07, 1.56]	1999
Michimata 2002	43.5	15.9	17	41.7	12.1	15	6.2%	0.12 [-0.57, 0.82]	2002
Shimada 2004	18.3	15.2	20	15.9	11.3	17	6.4%	0.17 [-0.47, 0.82]	2004
Tuckerman, 2007	11.2	8.4	87	6.2	4.4	10	6.3%	0.61 [-0.05, 1.27]	2007
Bohlmann 2010	2.4	0.62	25	2.4	0.62	10	6.1%	0.00 [-0.73, 0.73]	2010
Parkin 2011	15.9	5.3	24	23.4	2.4	10	5.7%	-1.57 [-2.41, -0.73]	2011
Giuliani 2014	18.3	14.6	13	22.3	19.9	10	5.8%	-0.23 [-1.05, 0.60]	2013
Chen 2017	3.95	1.65	97	2.86	0.98	84	7.3%	0.79 [0.48, 1.09]	2017
Kuon 2017	257	212	58	148	73	17	6.7%	0.57 [0.02, 1.12]	2017
El-Azzamy 2018	107.27	30.3	15	19.4	6.7	7	4.0%	3.30 [1.90, 4.70]	2018
Marron 2019	4.8	2.2	155	3.39	1.36	35	7.1%	0.68 [0.30, 1.05]	2019
Wei 2019	9.14	7.2	58	9.83	6	49	7.1%	-0.10 [-0.48, 0.28]	2019
Lyzikova 2020	150	68.1	39	50.4	57.4	63	6.9%	1.60 [1.14, 2.06]	2020
Zhao, 2020	13.85	4.75	30	6.83	2.43	30	6.5%	1.84 [1.23, 2.45]	2020
Subtotal (95% CI)			**709**			**391**	**100.0%**	**0.49 [0.08, 0.90]**	
Heterogeneity: Tau² = 0.58; Chi² = 120.79, df = 15 (P < 0.00001); I² = 88%									
Test for overall effect: Z = 2.35 (P = 0.02)									
(B) Endometrial tissue (CD56+CD16-)									
Lachapelle 1996	16	6	20	24	3	15	23.1%	-1.58 [-2.35, -0.80]	1996
Shimada 2004	15	13	20	12	8.9	17	25.2%	0.26 [-0.39, 0.91]	2004
Hosseini 2014	56.5	20.1	14	54.4	17.5	9	22.1%	0.11 [-0.73, 0.94]	2014
Marron 2019	41.8	15.2	155	43.05	8.75	35	29.6%	-0.09 [-0.45, 0.28]	2019
Subtotal (95% CI)			**209**			**76**	**100.0%**	**-0.30 [-1.00, 0.40]**	
Heterogeneity: Tau² = 0.40; Chi² = 14.90, df = 3 (P = 0.002); I² = 80%									
Test for overall effect: Z = 0.84 (P = 0.40)									
(C) Decidual tissue (CD56)									
Chao 1995	53.5	17.4	10	40.7	16.8	21	8.8%	0.73 [-0.04, 1.51]	1995
Lea 1997	10.8	8.15	23	17.2	17.35	22	9.3%	-0.47 [-1.06, 0.13]	1997
Quack 2001	43.8	17.7	17	56.55	11.6	20	9.1%	-0.85 [-1.53, -0.17]	2001
Qu 2008	39.79	14	22	130.45	44.73	25	8.8%	-2.62 [-3.42, -1.82]	2008
Ozcimen, 2009	3.42	10.31	23	2.14	6.81	23	9.4%	0.14 [-0.43, 0.72]	2009
Fu 2013	56.34	14.5	11	66.06	12.5	56	9.2%	-0.75 [-1.41, -0.09]	2013
Sotnikova 2014	80.07	8.26	26	82.87	8.7	37	9.5%	-0.32 [-0.83, 0.18]	2014
Wang 2014	51.8	20.8	30	66.7	16.86	30	9.5%	-0.78 [-1.30, -0.25]	2014
Almasry 2015	4.98	1.83	40	2.1	1.6	30	9.4%	1.64 [1.09, 2.19]	2015
Radovic 2016	85.03	23.3	30	77.6	13.3	20	9.4%	0.37 [-0.20, 0.94]	2016
Eskicioglu 2016	22,283.4	12,855.61	10	52,760.78	10,516.98	11	7.5%	-2.50 [-3.70, -1.30]	2016
Subtotal (95% CI)			**242**			**295**	**100.0%**	**-0.45 [-1.09, 0.20]**	
Heterogeneity: Tau² = 1.08; Chi² = 115.00, df = 10 (P < 0.00001); I² = 91%									
Test for overall effect: Z = 1.34 (P = 0.18)									
(D) Decidual tissue (CD56+CD16-)									
Yamamoto 1999	26.1	17.1	9	49.9	11.7	15	16.3%	-1.65 [-2.62, -0.68]	1999
Wang 2014	51.8	20.8	30	66.7	16.86	30	23.5%	-0.78 [-1.30, -0.25]	2014
Dong 2017	49.6	17	20	45	15.2	20	21.9%	0.28 [-0.34, 0.90]	2017
Guo 2017	56	14.5	11	57.5	19.5	12	18.7%	-0.08 [-0.90, 0.73]	2017
Liu 2019	43.7	7.2	10	45	10.44	21	19.7%	-0.13 [-0.89, 0.62]	2019
Subtotal (95% CI)			**80**			**98**	**100.0%**	**-0.43 [-1.03, 0.17]**	
Heterogeneity: Tau² = 0.32; Chi² = 13.98, df = 4 (P = 0.007); I² = 71%									
Test for overall effect: Z = 1.42 (P = 0.16)									

Test for subgroup differences: Chi² = 10.06, df = 3 (P = 0.02), I² = 70.2%

Subgroup meta-analysis of standard mean difference of uNK level of women with RM compared to controls

(A) For primary RM (B) For secondary RM

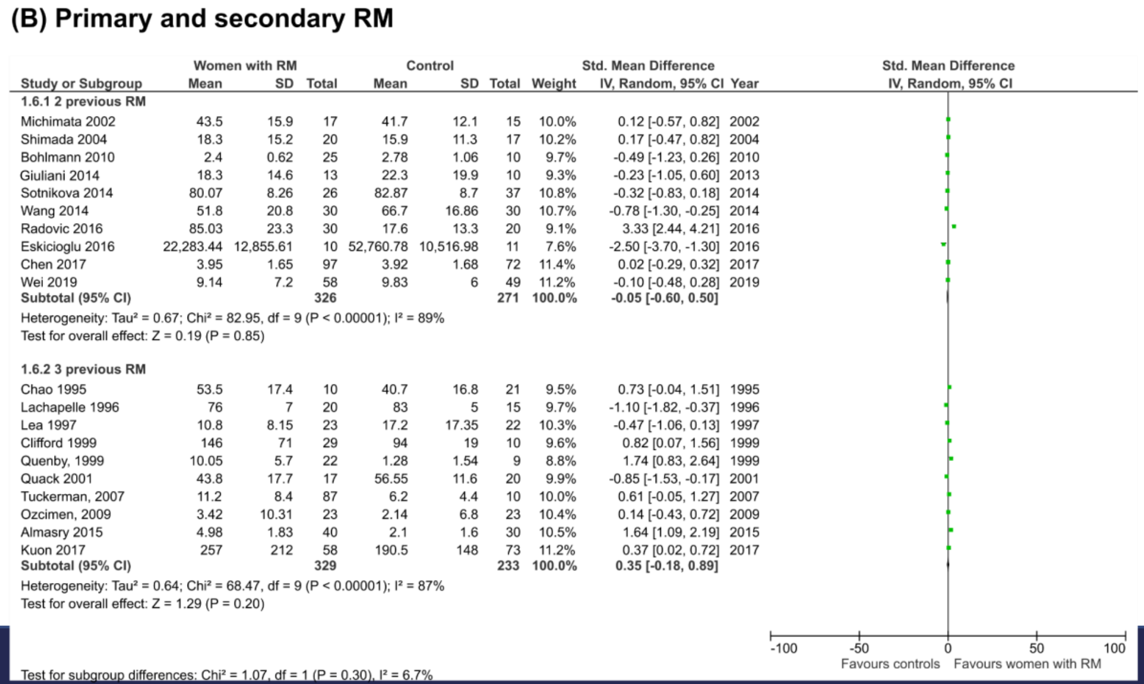
- ➢ No significant difference in subgroup analysis of CD56+ or CD56+CD16- cells level between primary or secondary RM



(A) Definition of RM

Study or Subgroup	Women with RM Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI	Year
1.6.1 2 previous RM									
Michimata 2002	43.5	15.9	17	41.7	12.1	15	10.0%	0.12 [-0.57, 0.82]	2002
Shimada 2004	18.3	15.2	20	15.9	11.3	17	10.2%	0.17 [-0.47, 0.82]	2004
Bohlmann 2010	2.4	0.62	25	2.78	1.06	10	9.7%	-0.49 [-1.23, 0.26]	2010
Giuliani 2014	18.3	14.6	13	22.3	19.9	10	9.3%	-0.23 [-1.05, 0.60]	2013
Sotnikova 2014	80.07	8.26	26	82.87	8.7	37	10.8%	-0.32 [-0.83, 0.18]	2014
Wang 2014	51.8	20.8	30	66.7	16.86	30	10.7%	-0.78 [-1.30, -0.25]	2014
Radovic 2016	85.03	23.3	30	17.6	13.3	20	9.1%	3.33 [2.44, 4.21]	2016
Eskicioglu 2016	22,283.44	12,855.61	10	52,760.78	10,516.98	11	7.6%	-2.50 [-3.70, -1.30]	2016
Chen 2017	3.95	1.65	97	3.92	1.68	72	11.4%	0.02 [-0.29, 0.32]	2017
Wei 2019	9.14	7.2	58	9.83	6	49	11.2%	-0.10 [-0.48, 0.28]	2019
Subtotal (95% CI)			**326**			**271**	**100.0%**	**-0.05 [-0.60, 0.50]**	
Heterogeneity: Tau² = 0.67; Chi² = 82.95, df = 9 (P < 0.00001); I² = 89%									
Test for overall effect: Z = 0.19 (P = 0.85)									
1.6.2 3 previous RM									
Chao 1995	53.5	17.4	10	40.7	16.8	21	9.5%	0.73 [-0.04, 1.51]	1995
Lachapelle 1996	76	7	20	83	5	15	9.7%	-1.10 [-1.82, -0.37]	1996
Lea 1997	10.8	8.15	23	17.2	17.35	22	10.3%	-0.47 [-1.06, 0.13]	1997
Clifford 1999	146	71	29	94	19	10	9.6%	0.82 [0.07, 1.56]	1999
Quenby, 1999	10.05	5.7	22	1.28	1.54	9	8.8%	1.74 [0.83, 2.64]	1999
Quack 2001	43.8	17.7	17	56.55	11.6	20	9.9%	-0.85 [-1.53, -0.17]	2001
Tuckerman, 2007	11.2	8.4	87	6.2	4.4	10	10.0%	0.61 [-0.05, 1.27]	2007
Ozcimen, 2009	3.42	10.31	23	2.14	6.8	23	10.4%	0.14 [-0.43, 0.72]	2009
Almasry 2015	4.98	1.83	40	2.1	1.6	30	10.5%	1.64 [1.09, 2.19]	2015
Kuon 2017	257	212	58	190.5	148	73	11.2%	0.37 [0.02, 0.72]	2017
Subtotal (95% CI)			**329**			**233**	**100.0%**	**0.35 [-0.18, 0.89]**	
Heterogeneity: Tau² = 0.64; Chi² = 68.47, df = 9 (P < 0.00001); I² = 87%									
Test for overall effect: Z = 1.29 (P = 0.20)									

Test for subgroup differences: Chi² = 1.07, df = 1 (P = 0.30), I² = 6.7%



(B) Primary and secondary RM

Study or Subgroup	Women with RM Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI	Year
1.6.1 2 previous RM									
Michimata 2002	43.5	15.9	17	41.7	12.1	15	10.0%	0.12 [-0.57, 0.82]	2002
Shimada 2004	18.3	15.2	20	15.9	11.3	17	10.2%	0.17 [-0.47, 0.82]	2004
Bohlmann 2010	2.4	0.62	25	2.78	1.06	10	9.7%	-0.49 [-1.23, 0.26]	2010
Giuliani 2014	18.3	14.6	13	22.3	19.9	10	9.3%	-0.23 [-1.05, 0.60]	2013
Sotnikova 2014	80.07	8.26	26	82.87	8.7	37	10.8%	-0.32 [-0.83, 0.18]	2014
Wang 2014	51.8	20.8	30	66.7	16.86	30	10.7%	-0.78 [-1.30, -0.25]	2014
Radovic 2016	85.03	23.3	30	17.6	13.3	20	9.1%	3.33 [2.44, 4.21]	2016
Eskicioglu 2016	22,283.44	12,855.61	10	52,760.78	10,516.98	11	7.6%	-2.50 [-3.70, -1.30]	2016
Chen 2017	3.95	1.65	97	3.92	1.68	72	11.4%	0.02 [-0.29, 0.32]	2017
Wei 2019	9.14	7.2	58	9.83	6	49	11.2%	-0.10 [-0.48, 0.28]	2019
Subtotal (95% CI)			**326**			**271**	**100.0%**	**-0.05 [-0.60, 0.50]**	
Heterogeneity: Tau² = 0.67; Chi² = 82.95, df = 9 (P < 0.00001); I² = 89%									
Test for overall effect: Z = 0.19 (P = 0.85)									
1.6.2 3 previous RM									
Chao 1995	53.5	17.4	10	40.7	16.8	21	9.5%	0.73 [-0.04, 1.51]	1995
Lachapelle 1996	76	7	20	83	5	15	9.7%	-1.10 [-1.82, -0.37]	1996
Lea 1997	10.8	8.15	23	17.2	17.35	22	10.3%	-0.47 [-1.06, 0.13]	1997
Clifford 1999	146	71	29	94	19	10	9.6%	0.82 [0.07, 1.56]	1999
Quenby, 1999	10.05	5.7	22	1.28	1.54	9	8.8%	1.74 [0.83, 2.64]	1999
Quack 2001	43.8	17.7	17	56.55	11.6	20	9.9%	-0.85 [-1.53, -0.17]	2001
Tuckerman, 2007	11.2	8.4	87	6.2	4.4	10	10.0%	0.61 [-0.05, 1.27]	2007
Ozcimen, 2009	3.42	10.31	23	2.14	6.8	23	10.4%	0.14 [-0.43, 0.72]	2009
Almasry 2015	4.98	1.83	40	2.1	1.6	30	10.5%	1.64 [1.09, 2.19]	2015
Kuon 2017	257	212	58	190.5	148	73	11.2%	0.37 [0.02, 0.72]	2017
Subtotal (95% CI)			**329**			**233**	**100.0%**	**0.35 [-0.18, 0.89]**	
Heterogeneity: Tau² = 0.64; Chi² = 68.47, df = 9 (P < 0.00001); I² = 87%									
Test for overall effect: Z = 1.29 (P = 0.20)									

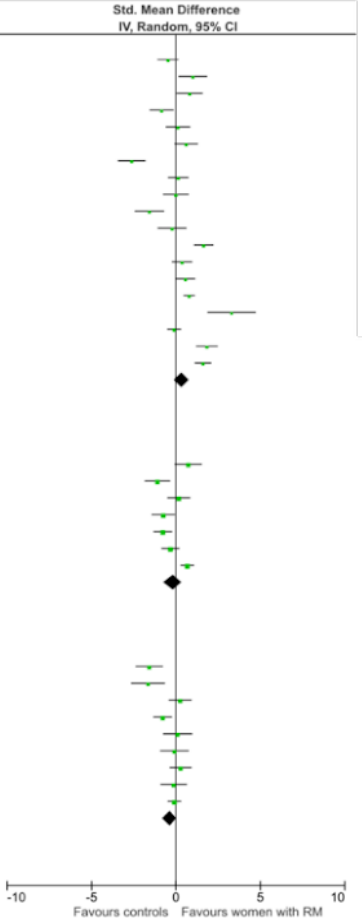
Test for subgroup differences: Chi² = 1.07, df = 1 (P = 0.30), I² = 6.7%

Subgroup meta-analysis of standard mean difference of uNK level of women with RM compared to controls.

- ➢ No significant difference (A) By method of uNK analysis (B) By unit of measurement.

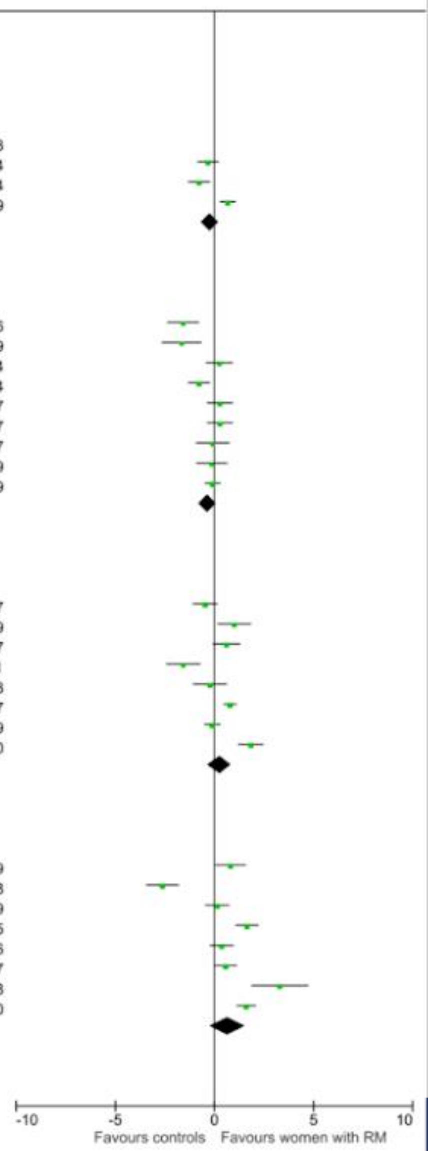
(A) Method of analysis

Study or Subgroup	Women with RM Mean	SD	Total	Controls Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI	Year
1.2.1 Immunohistochemistry (CD56)									
Lea 1997	10.8	8.15	23	17.2	17.35	22	5.4%	-0.47 [-1.06, 0.13]	1997
Quenby, 1999	10.05	5.7	22	4.77	3.11	9	5.0%	1.00 [0.18, 1.83]	1999
Clifford 1999	146	71	29	94	19	10	5.2%	0.82 [0.07, 1.56]	1999
Quack 2001	43.8	17.7	17	56.55	11.6	20	5.3%	-0.85 [-1.53, -0.17]	2001
Michimata 2002	43.5	15.9	17	41.7	12.1	15	5.3%	0.12 [-0.57, 0.82]	2002
Tuckerman, 2007	11.2	8.4	87	6.2	4.4	10	5.3%	0.61 [-0.05, 1.27]	2007
Qu 2008	39.79	14	22	130.45	44.73	25	5.1%	-2.62 [-3.42, -1.82]	2008
Ozcimen, 2009	3.42	10.31	23	2.14	6.81	23	5.5%	0.14 [-0.43, 0.72]	2009
Bohlmann 2010	2.4	0.62	25	2.4	0.62	10	5.2%	0.00 [-0.73, 0.73]	2010
Parkin 2011	15.9	5.3	24	23.4	2.4	10	5.0%	-1.57 [-2.41, -0.73]	2011
Giuliani 2014	18.3	14.6	13	22.3	19.9	10	5.0%	-0.23 [-1.05, 0.60]	2013
Almasry 2015	4.98	1.83	40	2.1	1.6	30	5.5%	1.64 [1.09, 2.19]	2015
Radovic 2016	85.03	23.3	30	77.6	13.3	20	5.5%	0.37 [-0.20, 0.94]	2016
Kuon 2017	257	212	58	148	73	17	5.5%	0.57 [0.02, 1.12]	2017
Chen 2017	3.95	1.65	97	2.86	0.98	84	5.8%	0.79 [0.48, 1.09]	2017
El-Azzamy 2018	107.27	30.3	15	19.4	6.7	7	3.8%	3.30 [1.90, 4.70]	2018
Wei 2019	9.14	7.2	58	9.83	6	49	5.7%	-0.10 [-0.48, 0.28]	2019
Zhao, 2020	13.85	4.75	30	6.83	2.43	30	5.4%	1.84 [1.23, 2.45]	2020
Lyzikova 2020	150	68.1	39	50.4	57.4	63	5.6%	1.60 [1.14, 2.06]	2020
Subtotal (95% CI)			**669**			**464**	**100.0%**	**0.34 [-0.11, 0.80]**	
Heterogeneity: Tau² = 0.91; Chi² = 202.65, df = 18 (P < 0.00001); I² = 91%									
Test for overall effect: Z = 1.48 (P = 0.14)									
1.2.2 Flow cytometry (CD56)									
Chao 1995	53.5	17.4	10	40.7	16.8	21	12.9%	0.73 [-0.04, 1.51]	1995
Lachapelle 1996	76	7	20	83	5	15	13.3%	-1.10 [-1.82, -0.37]	1996
Shimada 2004	18.3	15.2	20	15.9	11.3	17	14.0%	0.17 [-0.47, 0.82]	2004
Fu 2013	56.34	14.5	11	66.06	12.5	56	13.9%	-0.75 [-1.41, -0.09]	2013
Wang 2014	51.8	20.8	30	66.7	16.86	30	14.9%	-0.78 [-1.30, -0.25]	2014
Sotnikova 2014	80.07	8.26	26	82.87	8.7	37	15.1%	-0.32 [-0.83, 0.18]	2014
Marron 2019	4.8	2.2	155	3.39	1.36	35	16.0%	0.68 [0.30, 1.05]	2019
Subtotal (95% CI)			**272**			**211**	**100.0%**	**-0.19 [-0.75, 0.37]**	
Heterogeneity: Tau² = 0.47; Chi² = 39.78, df = 6 (P < 0.00001); I² = 85%									
Test for overall effect: Z = 0.66 (P = 0.51)									
1.2.3 Flow cytometry (CD56+CD16-)									
Lachapelle 1996	16	6	20	24	3	15	10.4%	-1.58 [-2.35, -0.80]	1996
Yamamoto 1999	26.1	17.1	9	49.9	11.7	15	8.7%	-1.65 [-2.62, -0.68]	1999
Shimada 2004	15	13.3	20	12	8.9	17	11.6%	0.26 [-0.39, 0.90]	2004
Wang 2014	41.5	17.97	30	55.7	17.68	30	12.8%	-0.79 [-1.31, -0.26]	2014
Hosseini 2014	56.5	20.1	14	54.4	17.5	9	9.8%	0.11 [-0.73, 0.94]	2014
Guo 2017	56	14.5	11	57.5	19.5	12	10.0%	-0.08 [-0.90, 0.73]	2017
Dong 2017	49.6	17	20	45	15.2	20	11.9%	0.28 [-0.34, 0.90]	2017
Liu 2019	43.7	7.2	10	45	10.44	21	10.6%	-0.13 [-0.89, 0.62]	2019
Marron 2019	41.8	15.2	155	43.05	8.75	35	14.2%	-0.09 [-0.45, 0.28]	2019
Subtotal (95% CI)			**289**			**174**	**100.0%**	**-0.37 [-0.79, 0.05]**	
Heterogeneity: Tau² = 0.29; Chi² = 29.90, df = 8 (P = 0.0002); I² = 73%									
Test for overall effect: Z = 1.71 (P = 0.09)									
Test for subgroup differences: Chi² = 5.24, df = 2 (P = 0.07), I² = 61.8%									



(B) Unit of measurement

Study or Subgroup	Women with RM Mean	SD	Total	Controls Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI	
1.7.1 Percentage of lymphocytes (CD56)									
Chao 1995	53.5	17.4	10	40.7	16.8	21	10.0%	0.73 [-0.04, 1.51]	
Lachapelle 1996	76	7	20	83	5	15	10.4%	-1.10 [-1.82, -0.37]	
Quack 2001	43.8	17.7	17	56.55	11.6	20	10.7%	-0.85 [-1.53, -0.17]	
Michimata 2002	43.5	15.9	17	41.7	12.1	15	10.6%	0.12 [-0.57, 0.82]	
Shimada 2004	18.3	15.2	20	15.9	11.3	17	10.9%	0.17 [-0.47, 0.82]	
Fu 2013	56.34	14.5	11	66.06	12.5	56	10.9%	-0.75 [-1.41, -0.09]	2013
Sotnikova 2014	80.07	8.26	26	82.87	8.7	37	11.9%	-0.32 [-0.83, 0.18]	2014
Wang 2014	51.8	20.8	30	66.7	16.86	30	11.8%	-0.78 [-1.30, -0.25]	2014
Marron 2019	4.8	2.2	155	3.39	1.36	35	12.7%	0.68 [0.30, 1.05]	2019
Subtotal (95% CI)			**306**			**246**	**100.0%**	**-0.23 [-0.69, 0.24]**	
Heterogeneity: Tau² = 0.41; Chi² = 44.98, df = 8 (P < 0.00001); I² = 82%									
Test for overall effect: Z = 0.94 (P = 0.35)									
1.7.2 Percentage of lymphocytes (CD56+CD16-)									
Lachapelle 1996	16	6	20	24	3	15	10.2%	-1.58 [-2.35, -0.80]	1996
Yamamoto 1999	26.1	17.1	9	49.9	11.7	15	8.6%	-1.65 [-2.62, -0.68]	1999
Shimada 2004	15	13.3	20	12	8.9	17	11.4%	0.26 [-0.39, 0.90]	2004
Wang 2014	51.8	20.8	30	66.7	16.86	30	12.5%	-0.78 [-1.30, -0.25]	2014
Dong 2017	49.6	17	20	45	15.2	20	11.6%	0.28 [-0.34, 0.90]	2017
Dong 2017	49.6	17	20	45	15.2	20	11.6%	0.28 [-0.34, 0.90]	2017
Guo 2017	56	14.5	11	57.5	19.5	12	9.8%	-0.08 [-0.90, 0.73]	2017
Liu 2019	43.7	7.2	10	45	10.44	21	10.4%	-0.13 [-0.89, 0.62]	2019
Marron 2019	41.8	15.2	155	43.05	8.75	35	13.9%	-0.09 [-0.45, 0.28]	2019
Subtotal (95% CI)			**295**			**185**	**100.0%**	**-0.34 [-0.76, 0.08]**	
Heterogeneity: Tau² = 0.30; Chi² = 31.98, df = 8 (P < 0.0001); I² = 75%									
Test for overall effect: Z = 1.57 (P = 0.12)									
1.7.3 Percentage of total endometrial cells (CD56)									
Lea 1997	10.8	8.15	23	17.2	17.35	22	12.8%	-0.47 [-1.06, 0.13]	1997
Quenby, 1999	10.05	5.7	22	4.77	3.11	9	11.5%	1.00 [0.18, 1.83]	1999
Tuckerman, 2007	11.2	8.4	87	6.2	4.4	10	12.4%	0.61 [-0.05, 1.27]	2007
Parkin 2011	15.9	5.3	24	23.4	2.4	10	11.4%	-1.57 [-2.41, -0.73]	2011
Giuliani 2014	18.3	14.6	13	22.3	19.9	10	11.5%	-0.23 [-1.05, 0.60]	2013
Chen 2017	3.95	1.65	97	2.86	0.98	84	14.0%	0.79 [0.48, 1.09]	2017
Wei 2019	9.14	7.2	58	9.83	6	49	13.7%	-0.10 [-0.48, 0.28]	2019
Zhao, 2020	13.85	4.75	30	6.83	2.43	30	12.7%	1.84 [1.23, 2.45]	2020
Subtotal (95% CI)			**354**			**224**	**100.0%**	**0.26 [-0.36, 0.87]**	
Heterogeneity: Tau² = 0.68; Chi² = 68.36, df = 7 (P < 0.00001); I² = 90%									
Test for overall effect: Z = 0.82 (P = 0.41)									
1.7.4 Absolute cell count (CD56)									
Clifford 1999	146	71	29	94	19	10	12.5%	0.82 [0.07, 1.56]	1999
Qu 2008	39.79	14	22	130.45	44.73	25	12.3%	-2.62 [-3.42, -1.82]	2008
Ozcimen, 2009	3.42	10.31	23	2.14	6.81	23	12.9%	0.14 [-0.43, 0.72]	2009
Almasry 2015	4.98	1.83	40	2.1	1.6	30	13.0%	1.64 [1.09, 2.19]	2015
Radovic 2016	85.03	23.3	30	77.6	13.3	20	12.9%	0.37 [-0.20, 0.94]	2016
Kuon 2017	257	212	58	148	73	17	13.0%	0.57 [0.02, 1.12]	2017
El-Azzamy 2018	107.27	30.3	15	19.4	6.7	7	10.2%	3.30 [1.90, 4.70]	2018
Lyzikova 2020	150	68.1	39	50.4	57.4	63	13.2%	1.60 [1.14, 2.06]	2020
Subtotal (95% CI)			**256**			**195**	**100.0%**	**0.68 [-0.21, 1.57]**	
Heterogeneity: Tau² = 1.50; Chi² = 111.23, df = 7 (P < 0.00001); I² = 94%									
Test for overall effect: Z = 1.50 (P = 0.13)									
Test for subgroup differences: Chi² = 5.76, df = 3 (P = 0.12), I² = 47.9%									



Meta-analysis of standard mean difference of **CD16+ leucocytes** in women with (A) RM and (B) RIF compared to controls

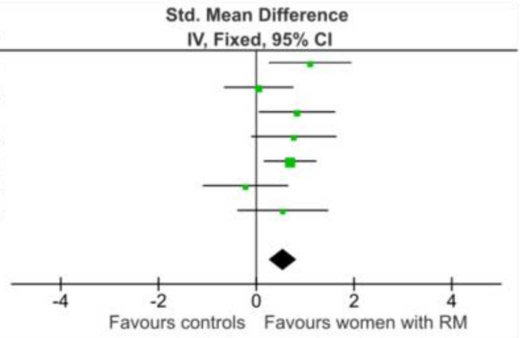
CD16+ leucocytes: mixture of pNK, monocytes and macrophages

- **Significantly higher** level in women with RM compared with controls

(A) RM

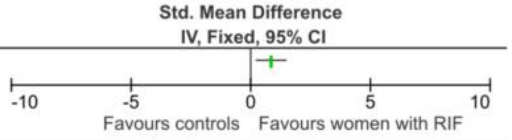
Study or Subgroup	Women with RM Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Fixed, 95% CI	Year
Quenby, 1999	4.65	3.85	22	0.9	0.93	9	11.4%	1.10 [0.27, 1.93]	1999
Michimata 2002	9.5	3.9	17	9.3	3.85	15	16.2%	0.05 [-0.64, 0.74]	2002
Parkin 2011	5.6	1.6	24	4.3	1.3	10	13.3%	0.83 [0.07, 1.60]	2011
Giuliani 2014	7.9	3.2	13	5.6	2.4	10	10.6%	0.77 [-0.09, 1.63]	2013
Wang 2014	10.8	8.55	30	6.1	4.1	30	28.7%	0.69 [0.17, 1.21]	2014
Eskicioglu 2016	59,072.33	18,077.07	10	62,651.56	12,927.87	11	10.6%	-0.22 [-1.08, 0.64]	2016
El-Azzamy 2018	14.8	8.2	15	10.1	8.6	7	9.3%	0.54 [-0.37, 1.46]	2018
Total (95% CI)			**131**			**92**	**100.0%**	**0.55 [0.27, 0.83]**	

Heterogeneity: Chi² = 7.84, df = 6 (P = 0.25); I² = 23%
Test for overall effect: Z = 3.86 (P = 0.0001)



(B) RIF

Study or Subgroup	Women with RIF Mean	SD	Total	Controls Mean	SD	Total	Std. Mean Difference IV, Fixed, 95% CI	Year
Tuckerman 2010	2.7	1.9	40	1.2	1.15	15	0.85 [0.24, 1.47]	2010



Meta-analysis: uNK cell level

Recurrent Implantation Failure

- 8 studies in total

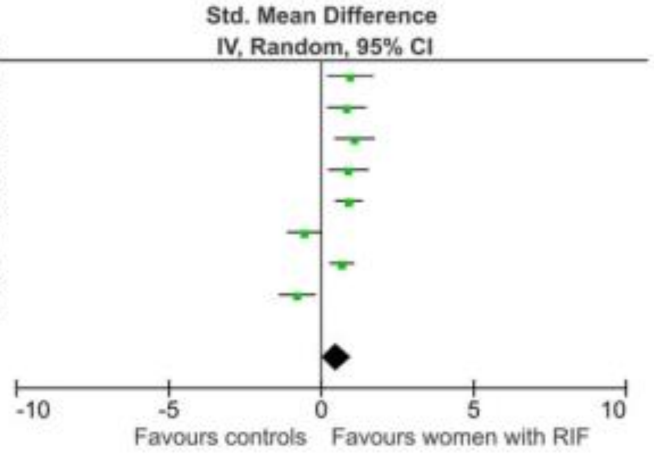
A: Significant difference in total CD56+ uNK in endometrium in women with RIF compared with controls

A

Study or Subgroup	Women with RIF Mean	SD	Total	Controls Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI	Year
Ledee 2004	32.1	24.2	35	11.2	4.6	10	11.2%	0.95 [0.22, 1.68]	2004
Tuckerman 2010	18.9	16.2	40	6.94	4.91	15	12.2%	0.83 [0.22, 1.45]	2010
Santillan 2012	19.8	13.01	32	7.4	6.18	17	12.0%	1.09 [0.46, 1.72]	2012
Junovich 2013	8.125	6.53	26	3.38	2.28	18	12.0%	0.89 [0.26, 1.52]	2013
Chen 2017	3.95	1.65	32	2.86	0.98	84	13.5%	0.90 [0.48, 1.33]	2017
Jiang 2017	6.5	4.7	32	9.06	4.39	23	12.7%	-0.55 [-1.10, -0.01]	2017
Marron 2019	4.8	2.2	155	3.39	1.36	35	13.9%	0.68 [0.30, 1.05]	2019
Babayeva 2020	10.5	10.5	25	19.2	11.2	25	12.4%	-0.79 [-1.37, -0.21]	2020
Total (95% CI)			**377**			**227**	**100.0%**	**0.49 [0.01, 0.98]**	

Heterogeneity: Tau² = 0.41; Chi² = 45.14, df = 7 (P < 0.00001); I² = 84%

Test for overall effect: Z = 1.99 (P = 0.046)



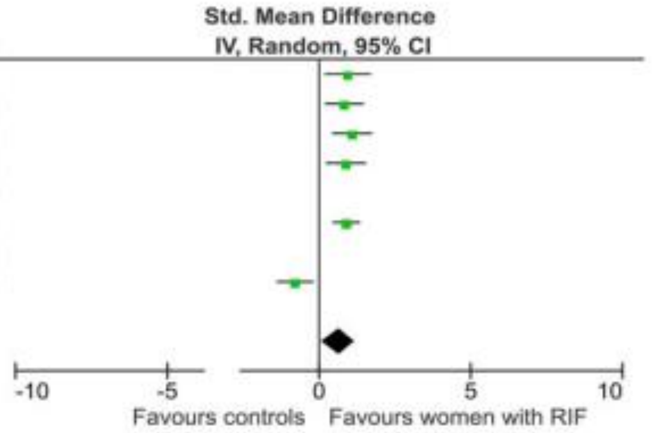
B: Sensitivity analysis of CD56+ uNK level excluding male factor → significantly higher uNK level in women with RIF compared with controls

B

Study or Subgroup	Women with RIF Mean	SD	Total	Controls Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI	Year
Ledee 2004	32.1	24.2	35	11.2	4.6	10	15.3%	0.95 [0.22, 1.68]	2004
Tuckerman 2010	18.9	16.2	40	6.94	4.91	15	16.6%	0.83 [0.22, 1.45]	2010
Santillan 2012	19.8	13.01	32	7.4	6.18	17	16.4%	1.09 [0.46, 1.72]	2012
Junovich 2013	8.125	6.53	26	3.38	2.28	18	16.4%	0.89 [0.26, 1.52]	2013
Jiang 2017	6.5	4.7	32	9.06	4.39	23	0.0%	-0.55 [-1.10, -0.01]	2017
Chen 2017	3.95	1.65	32	2.86	0.98	84	18.4%	0.90 [0.48, 1.33]	2017
Marron 2019	4.8	2.2	155	3.39	1.36	35	0.0%	0.68 [0.30, 1.05]	2019
Babayeva 2020	10.5	10.5	25	19.2	11.2	25	16.9%	-0.79 [-1.37, -0.21]	2020
Total (95% CI)			**190**			**169**	**100.0%**	**0.64 [0.07, 1.22]**	

Heterogeneity: Tau² = 0.42; Chi² = 28.66, df = 5 (P < 0.0001); I² = 83%

Test for overall effect: Z = 2.19 (P = 0.03)



However, this difference lost statistical significance following sensitivity analyses by exclusion of

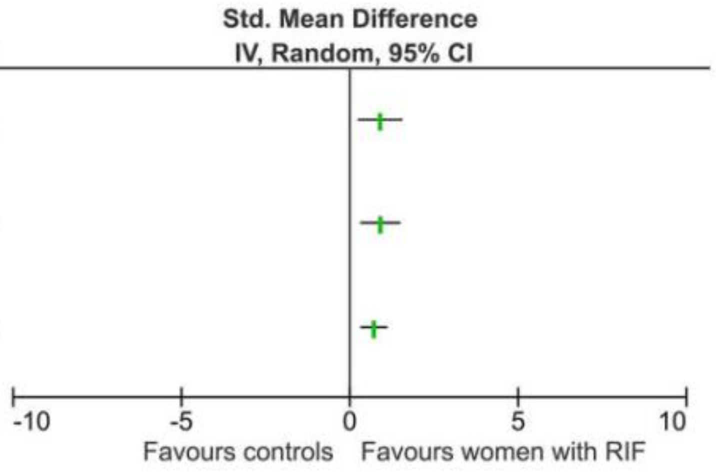
- 2 studies: did not exclusively use fertile controls
- 2 studies: included hormonal intervention
- 6 studies: serious risk of bias
- 4 studies: mean and standard deviation were converted from median and interquartile range and/or range
- 2 studies: information was extracted from the graph

Meta-analysis: uNK cell level

Recurrent Implantation Failure

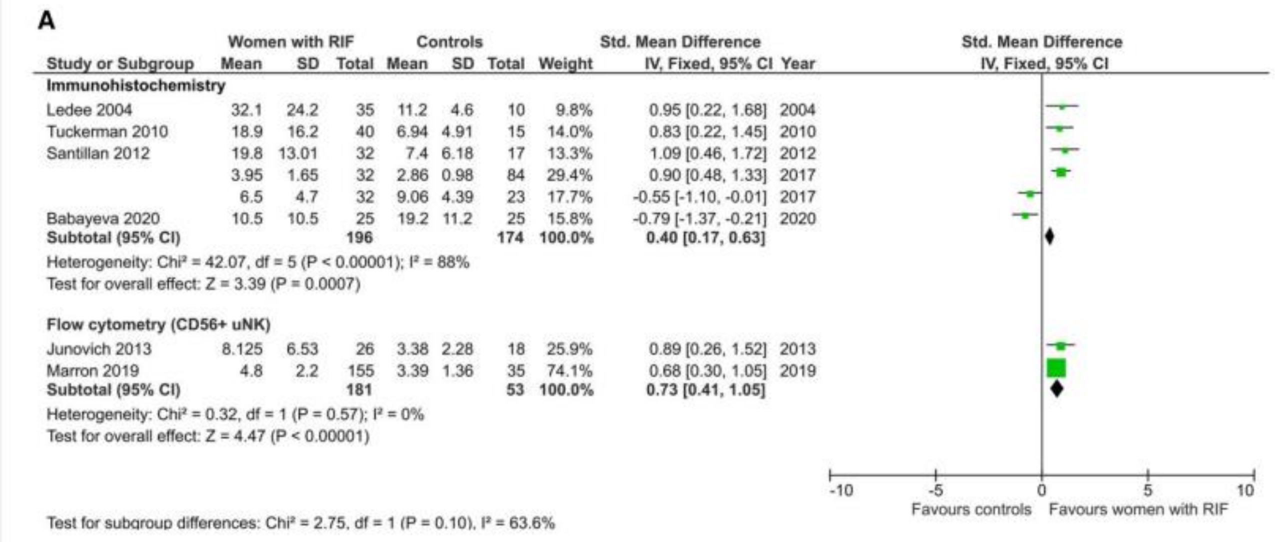
C

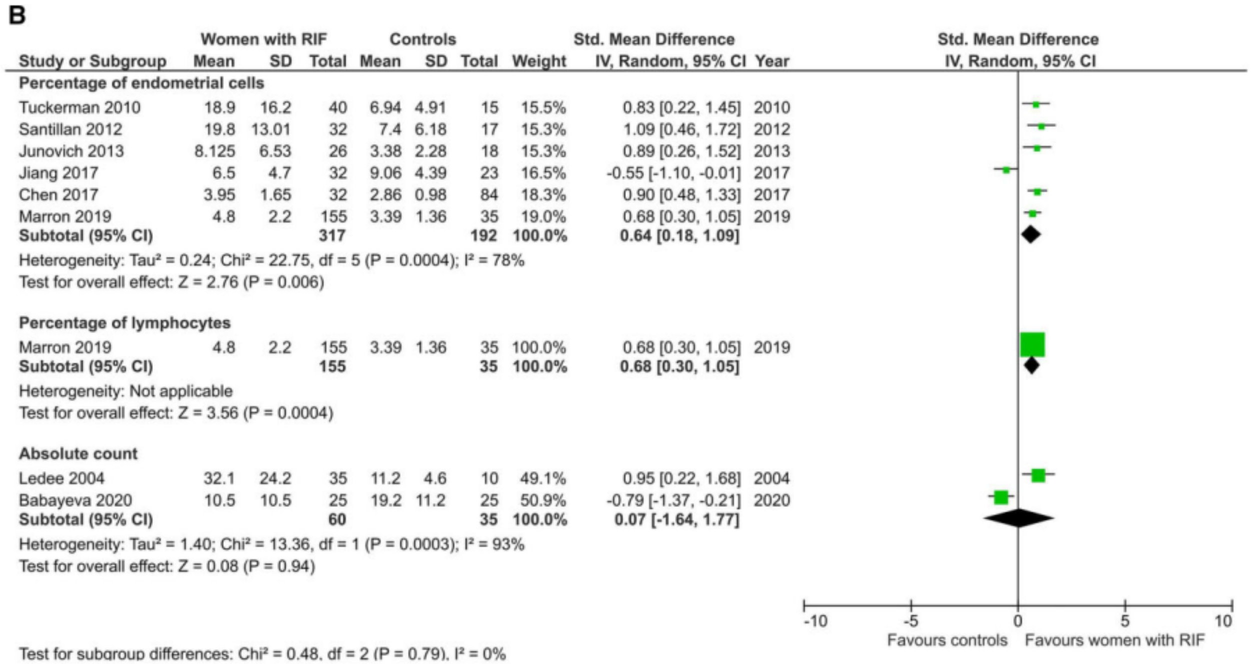
Study or Subgroup	Women with RIF Mean	Women with RIF SD	Women with RIF Total	Controls Mean	Controls SD	Controls Total	Std. Mean Difference IV, Random, 95% CI	Year
4.5.1 CD56+CD16+								
Junovich 2013	4.52	3.96	26	1.65	1.12	18	0.90 [0.27, 1.53]	2013
4.5.2 CD57+								
Jiang 2017	1.87	1.025	32	1.08	0.55	23	0.91 [0.34, 1.47]	2017
4.5.3 CD56+CD16-								
Marron 2019	52.6	14.03	181	43.05	8.75	35	0.71 [0.35, 1.08]	2019



Meta-analysis: uNK cell level

Recurrent Implantation Failure





A: by method of analysis

- ➢ Significant difference of CD56+ cells level

B: by unit of measurement

- ➢ CD56+ cells are significantly higher in women with RIF when expressed as percentage of endometrial/stromal cells, but not as absolute count

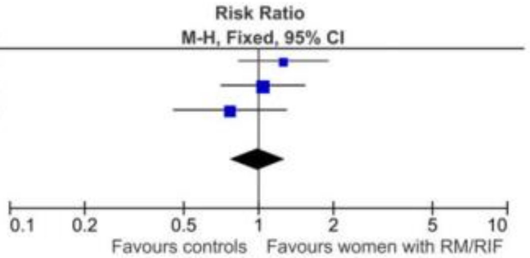
Meta-analysis: Pregnancy outcome

Pregnancy rate (high uNK vs. normal uNK)

A

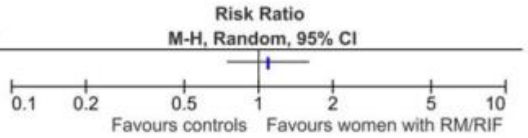
Study or Subgroup	High uNK Events	High uNK Total	Normal uNK Events	Normal uNK Total	Weight	Risk Ratio M-H, Fixed, 95% CI	Year
Tuckerman, 2007	11	15	21	36	23.5%	1.26 [0.83, 1.90]	2007
Liu, 2014	18	31	29	52	41.2%	1.04 [0.71, 1.53]	2014
Chen 2021	9	20	44	75	35.3%	0.77 [0.46, 1.29]	2020
Total (95% CI)		**66**		**163**	**100.0%**	**1.00 [0.77, 1.28]**	
Total events	38		94				

Heterogeneity: Chi² = 2.25, df = 2 (P = 0.32); I² = 11%
Test for overall effect: Z = 0.04 (P = 0.97)



B

Study or Subgroup	High uNK Events	High uNK Total	Normal uNK Events	Normal uNK Total	Risk Ratio M-H, Random, 95% CI	Year
Marron 2019b	21	58	103	311	1.09 [0.75, 1.59]	



- 7 studies following up women with RM until the next pregnancy (3 with livebirth rates, 1 reporting CPR)

A: No significant difference in livebirth rates
B: No significant difference in CPR

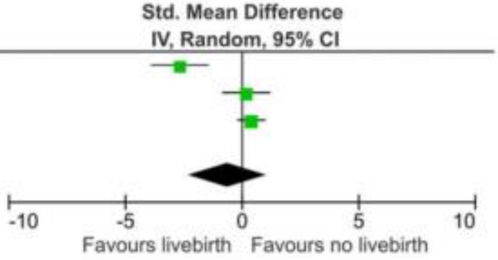
uNK levels

C: No significant difference (P=0.46) in women with RM/RIF who had **livebirth vs. miscarriage**

C

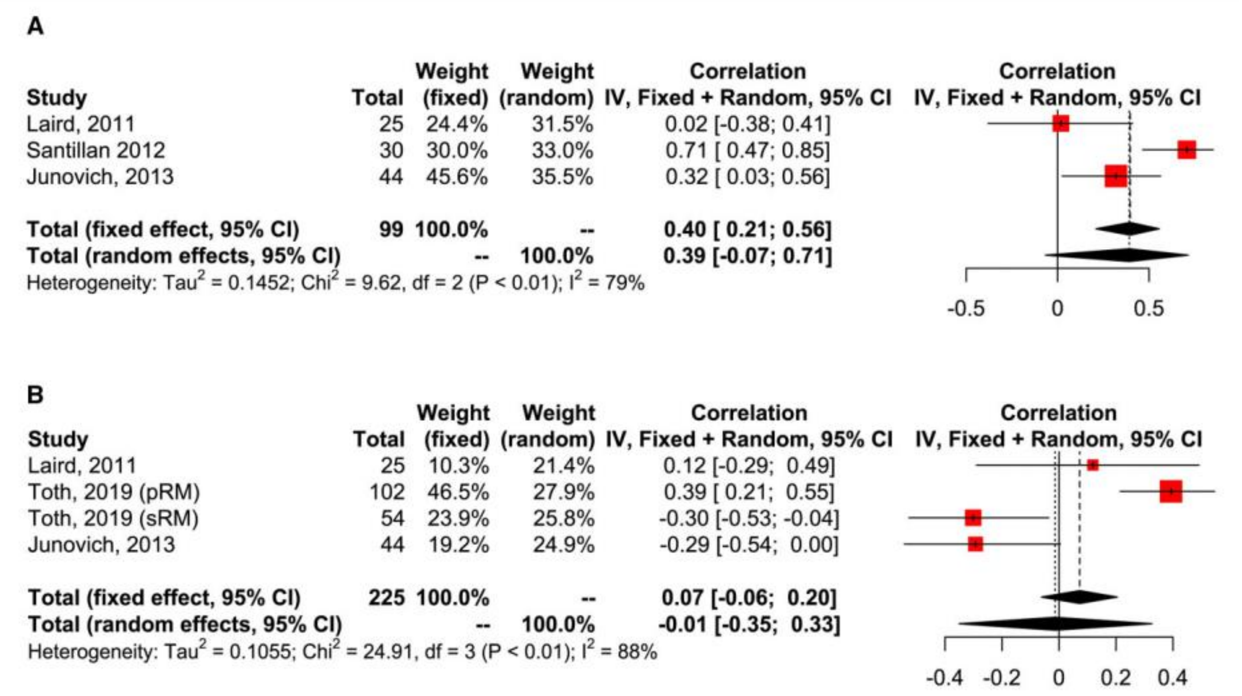
Study or Subgroup	Subsequent livebirth Mean	Subsequent livebirth SD	Subsequent livebirth Total	No subsequent livebirth Mean	No subsequent livebirth SD	No subsequent livebirth Total	Weight	Std. Mean Difference IV, Random, 95% CI	Year
Quenby, 1999	8.25	1.83	12	22	7.1	10	31.2%	-2.67 [-3.88, -1.46]	1999
Michimata 2002	63.97	21.59	11	60.17	18.27	6	33.0%	0.18 [-0.82, 1.17]	2002
Tuckerman, 2007	13.3	10.74	32	9.6	6.1	19	35.8%	0.39 [-0.18, 0.96]	2007
Total (95% CI)			**55**			**35**	**100.0%**	**-0.64 [-2.31, 1.04]**	

Heterogeneity: Tau² = 1.95; Chi² = 20.47, df = 2 (P < 0.0001); I² = 90%
Test for overall effect: Z = 0.75 (P = 0.46)

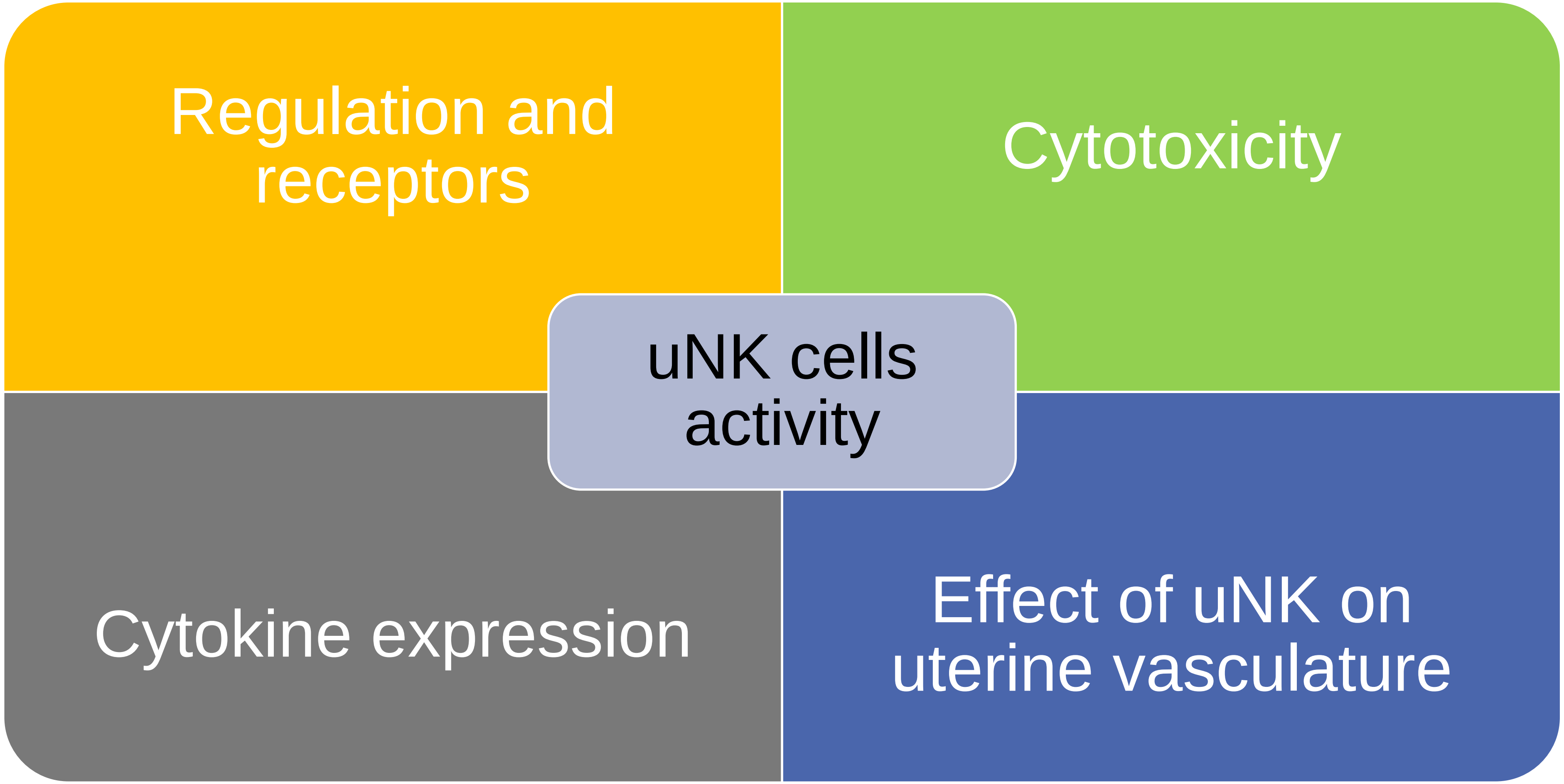


Meta-analysis: Correlation between peripheral and uNK cells

No significant positive coefficient correlation in either
A: total CD56+ pNK and uNK (P= 0.10),
or
B: CD56+CD16+ pNK and uNK (P=0.08).



Narrative synthesis on uNK cell activity



Regulation and receptors

- 16 studies on RM and 1 on RIF

- Trafficking of pNK in the response to chemokine production from uterine stromal cells (Kitaya et al., 2003; Hanna et al., 2004; Jones et al., 2004)
- Decidual cells during pregnancy → chemokines (e.g. CXCL10, CXCL12, Chemerin) → pNK migration through endothelial and stromal cells (Carlino et al., 2008, 2012)
- Preferential recruitment of CD56+CD16+ pNK to the uterus by higher expression of CCR7 on $CD56_{dim}$ pNK (Hosseini et al. 2014)
- In women with RM: trophoblast-derived CXCL12 → $CD56_{dim}$ uNK ↑adhesive ability (Lu et al., 2020)

↔ No significant higher level of $CD56_{dim}$ dNK in women with RM in our meta-analysis

Regulation and receptors

- The interaction between uNK and trophoblast cells → early placentation (activation or inhibition of uNK leading to reproductive failure??)

1. uNK activation → successful pregnancy (Hiby et al., 2010; Xiong et al., 2013; Long et al., 2015; Kennedy et al., 2016)
2. Women with RM: lower expression of inhibitory receptors (**KIR2DL4**, NKG2A, KIR2DL1) → overactivation of uNK (Yan et al., 2007; Sotnikova et al., 2014; Guo et al., 2017)
3. HLA-G (secreted by fetal trophoblasts) activates KIR2DL4 → remodeling of maternal vasculature (Rajagopalan and Long, 2012)
4. Insufficient activation of uNK cells in women with RM: Low expression of KIR2DL4 → ↓ activation of uNK, ↓ cytokine expression, ↓ trophoblast invasive ability and tube formation (Guo et al. 2017)

Regulation and receptors

1. ↑ IFN-γ, Granzyme B secretion by CD56+ uNK → reduced migration of trophoblast cells (Sotnikova et al. 2014)
2. NK cells expressing miR30e → HLA-G on trophoblast cell line, HTR-8/SVneo → ↓ pro-angiogenic cytokine secretion by dNK and ↓ trophoblast invasion and migration (Guo et al. 2017)
3. Upregulation of miR30e → ↓ NK cell cytotoxicity against K562 target cells, ↑ pro-angiogenic cytokines (IL-4, IL-10, VEGF, Ang-2), ↓ pro-inflammatory cytokines (IFN-γ, TNF-α) by uNK (Huang et al. 2019)
4. Women with RM: higher CD56dim, ↓CD82, ↑CD29 expression → regulation in trophoblast adhesion (Lu et al. 2020)

Regulation and receptors

- Cross-talk between uNK cell and other immune cells in the endometrium → homeostasis in the early pregnancy placental bed

1. ↓ regulatory T (Treg) cells (maintaining homeostasis at the maternal-fetal interface) in the endometrium of women with subinfertility (Sauerbrun-Cutler et al., 2021)
2. No correlation between CD57:CD56 ratio and Treg numbers (Jiang et al., 2017) ↔ Positive correlation between CD56+ cells and Treg numbers (Lyzikova et al., 2020)
3. CD14+ macrophage interacts with uNK → produce indoleamine 2,3-dioxygenase (IDO) that induces Tregs (Vacca et al., 2010)
   ↓ IDO expression in women with RM (Ban et al., 2013; Wei et al., 2020); but the exact regulatory relation between uNK and IDO???
4. Reduced CD27+ NK : Th17 and dNK (from women with RM) unable to suppress Th17 expansion under different cytokines (IL-15, IL-12, IL-18) (Fu et al., 2013)
5. Positive correlation between CD56+ uNK and CD68+ macrophages (Zhao et al., 2020)

Cytotoxicity

- ➢ uNK does not possess the same cytotoxicity ability as pNK. (Trundley and Moffett, 2004) dNK unable to form activating synapses → perforin release when interacting with K562 target cells (myeloid leukemic cancer cells) (Koopman et al., 2003)

Why using pNK cytotoxicity to assume uNK activity??

- Higher lysis of target cells (K562 leukemic cells) in women with RM compared with controls when co-incubated with dNK. (Chao et al., 1995; Bao et al., 2012; Li et al., 2019) However, K562 cells are more susceptible to cytotoxicity by dNK than trophoblast cells.

i. More pNK in the endometrium of RM patients

ii. uNK in RM patients may be more activated → ↑ ability to kill K562 cancer cells

Cytotoxicity

1. Expression of granzyme B and perforin ↑ in RM patients (Sotnikova et al., 2014; Li et al., 2019)
2. 3 types of pNK cytotoxicity receptors (NCR): NKp46, NKp30 and NKp44; significant ↓ expression of NKp46 in uNK of women with RM (Fukui et al., 2017) but ↑ in those with RIF (Giuliani et al., 2014)
   → interpreted with caution as NKp46+ is universally expressed in all NK cells regardless of activation status (Barrow et al., 2019)
3. Expression of NCR on uNK ≠ cytotoxicity

i. Inhibitory receptor (NKp46/NKG2A) controls uNK (El Costa et al., 2009)

ii. Different cytokine expression profiles for NKp46 between pNK and uNK (Yokota et al., 2013)

Cytokine expression

- 9 studies on RM (7 sampled 1st trimester decidua and 2 used endometrium samples) and 1 on RIF

dNK1: dNK2 ratio significantly higher in women with RM vs. control (Dong et al., 2017; Liu et al., 2019, 2020), not strictly controlled for gestational age

Most studies reported ↑ IFN-γ expression (measured by flow cytometry, ELISA, RT-PCR) in women with RM.

However, IFN-γ secretion can be found physiologically after 1st trimester to inhibit EVT invasion, and one study (Sotnikova et al., 2014) showed no elevated IFN-γ mRNA expression in dNK when co-cultured with trophoblasts in RM group.

➢ Equivocal results on predominant cytokine expression in RM/RIF, as cytokine production by uNK varies with gestational age, method of purification, activation and interaction with trophoblasts

Effect of uNK on uterine vasculature

- 4 studies on RIF and 3 on RM

i. Higher expression of proangiogenic cytokines (angiogenin, b FGF, VEGF-A) in the endometrium (Chen et al., 2018)

ii. Impaired vascular remodelling associated with ↑ uNK (Almasry et al., 2015)

iii. Positive correlation between vascular smooth muscle cells and CD56+ uNK (El-Azzamy et al., 2018)

➢ **Excessive angiogenesis → earlier peri-implantation blood flow→ oxidative stress to fetal trophoblasts → cellular injury**

- ↓ Angiogenic cytokine VEGF production and ↓IL-6 expression → ↑ cytotoxic response by CD56+CD16+ uNK (Junovich et al., 2013)

➢ **Low production of angiogenic factors → insufficient trophoblast invasion**

Effect of uNK on uterine vasculature

- Dysregulated cytokine signalling → either insufficient or excessive NK cell recruitment to endometrium → impairment of vascular remodelling

(Ledee et al. 2004, 2005, 2008)

Discussion

Key findings

1. Significantly higher total CD56+ cells in the uterus in women with RIF compared with controls.
2. Focused on endometrial samples from mid-luteal phase → significant difference between RM and control
3. Heterogeneity of studies on uNK activity
4. uNK derived from women with RM/RIF produce more Type 1 cytokines (e.g. IFN-γ and TNF-α) compared with Type 2 cytokines (e.g. IL-4 and IL-10).
5. ↓ Inhibitory receptors and ↑ Angiogenesis

Strengths

- Meticulous meta-analysis of u NK: different phenotypes, subgroup and sensitivity analyses
- Quality assessed by ROBINS-I tools (observational studies)
- Reliability: serious risk of bias was excluded

Limitations

- Clinical heterogeneity: different definitions of RM/RIF and control groups
- Exclusion of studies not published in English, derivation of mean and standard deviation from median, extraction of data from graphs (skewing of data)
- Complexity of studies on uNK activity and their interactions with surrounding decidual and immune cells → not possible to fit all studies into categories

Measurement of uNK level

1. Variability in definitions:

- RM: 2 (Bender Atik et al., 2018; Practice Committee of the American Society for Reproductive Medicine, 2020) or 3 (Green Top Guideline, Royal College of Obstetricians and Gynaecologists, 2011) previous consecutive miscarriages

- Not all studies excluded parental or fetal chromosomal abnormalities

A systematic review (Smits et al., 2020): Incidence of chromosomal abnormalities, which accounted for 46% of RM ≈ sporadic miscarriage

- RIF: failure to achieve clinical pregnancy after “minimum of 3 fresh or frozen cycles” (Coughlan et al., 2014) or “2 consecutive cycles” (Polanski et al., 2014) or based on the previous number of embryos transferred irrespective of the number of cycles (Ledee et al., 2008)

Measurement of uNK level

2. Case-controlled observational studies: not all confounding factors entirely eliminated

- Maternal age: ≥ 40 y/o, 100 times more likely to have RM (Saravelos and Li, 2012)
- Hormonal therapy might influence uNK numbers.

3. No uniformity in the inclusion criteria for controls

4. Tissue analyzed regarding RM: endometrium, decidua from 1st trimester pregnancy or menstrual blood

- uNK level fluctuation at different gestational ages, and through menstrual cycle from 26% during late proliferative up to 83% in late secretory phase (Pace et al., 1989; Flynn et al., 2000; Williams et al., 2009)
- Unified method: timing it accurately at 7 days post-ovulation by the urine LH surge

Measurement of uNK level

5. Heterogeneity in techniques to measure uNK: immunohistochemistry or flow cytometry

- Immunohistochemistry is influenced by subjectivity between observers and indeed within a single observer (Mariee et al., 2012), different techniques of tissue fixation, embedding and sectioning, selection of area for assessment, definition of immune-positive cells and inclusion/exclusion of blood vessels (Lash et al., 2016).

6. Variation in reference range of uNK level can be the source of heterogeneity in the meta-analysis for livebirth outcome (no difference in high or normal uNK level).

- uNK cannot be used as prognostic indicator for subsequent pregnancy and suggests difference observed in uNK level may be an effect of RM/RIF.

Measurement of uNK activity

1. Conflicting findings due to confounding factors
2. Measurement of cytotoxicity against cancer cell lines ≠ uNK activity *in vivo*
3. Poor understanding of uNK function in women with RM and RIF → more studies required

Future research Implications

- ✓ Measurement of uNK level: endometrium during mid-luteal phase (avoid secretory phase due to rapid change of uNK level); flow cytometry with standardized gating strategy
- ✓ Do not use CD16 as a sole marker to define uNK (unable to discern uNK from other immune cells).
- ✓ Set the baseline of uNK activity in normal pregnancies before proceeding to evaluate abnormal behavior in pathological pregnancies.
- ✓ Single cell RNA sequencing in the first trimester pregnancies: 3 new subpopulations of CD56bright dNK (Vento-Tormo et al., 2018) , with dNKI (central role in trophoblast interaction) (Huhn et al., 2020)

Future research Implications

- ✓ The role of other immune cells (innate lymphoid cells, macrophages and T cells) present in the decidua → cytokines produced by uNK cells?
- ✓ Interactions between uNK and trophoblast cells: certain combinations of parental HLA-C and maternal KIR genotype → better pregnancy outcome in ART (improved outcome in women with RIF when donor eggs are used)
- ✓ Immunogenetic screening for RM or RIF??
- ✓ Unexplained RM or RIF: lifestyle factors, BMI, subclinical chronic endometritis, or low testosterone levels?

Clinical Implications

- ✓ Measuring pNK level cannot predict uNK level or activity.
- ✓ Peripheral blood immune cells→ uNK (implying there is a circulating progenitor, but what is it?)
- ✓ A standardized reference range should be established before uNK measurement can be clinically utilized.
- ✓ Elevated CD56+ uNK in the endometrium of women with RM and RIF: Cause or effect of the underlying pathology?
- ✓ Complexity of interaction between NK cells and other immune milieu of the decidua → immunotherapy to correct altered uNK function rather than uNK number

Conclusion

1. Over the past 30 years, we are only at cusp of beginning to understand the role of NK cells in early pregnancy.
2. Complexity of their interaction with other cells in the uterine milieu → Impossible to draw conclusions from single cells or molecules
3. Novel technology e.g. single cell RNA sequencing → decoding the role of uNK cells in physiological/ pathological pregnancies
4. Measurement of uNK and immunotherapy should be performed in research setting.