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ABSTRACT

Immune system aberrations are suggested to be an important factor in the pathophysiology of unexplained secondary recurrent pregnancy loss (sRPL). The objective was to investigate if the sex ratio of the firstborn child in sRPL patients differs from the background population and whether the sex of the firstborn child has a negative impact on the pregnancy prognosis alone and/or in combination with carriage of male-specific minor histocompatibility (H-Y) restricting HLA class II alleles. From January 2016 to October 2022, 582 patients with unexplained RPL were admitted to the RPL Center of Western Denmark and continuously followed-up. HLA-DRB1 and -DQB1 typing was performed as part of the routine diagnostic work-up. In sRPL patients, a history of a firstborn boy was significantly more frequent than in the Danish background population and was associated with significantly lower odds of a successful reproductive outcome in the first pregnancy after admission compared to a firstborn girl (OR=0.41, 95% CI: 0.20–0.83, p = 0.014). The odds of a successful reproductive outcome were enhanced in patients carrying ≥ 1 H-Y-restricting HLA class II alleles with a first-born girl compared to a firstborn boy (OR=3.33, 95% CI: 1.40–7.88, p = 0.005), while no difference in successful reproductive outcome was seen in sRPL patients not carrying these alleles (OR=1.20, 95% CI: 0.33–4.43, p = 0.781). The sex ratio of children born after RPL was similar to the Danish background population. These findings confirm previous findings and suggests that a harmful immune response triggered by H-Y-antigen exposure during a previous pregnancy in preconditioned women may cause sRPL.

1. Introduction

Recurrent pregnancy loss (RPL) is variously defined as two or three consecutive or not necessarily consecutive pregnancy losses (Atik et al., 2017; Practice Committee of the American Society for Reproductive Medicine, 2020; Regan et al., 2011). RPL can be divided into primary (pRPL) and secondary RPL (sRPL) in which the latter occurs after completion of a pregnancy beyond 24 weeks gestation. In only approximately 50% of RPL women, one or more known risk factors are found including endocrine disorders, acquired and congenital thrombophilia, anatomic abnormalities of the pelvic organs, and structural chromosomal abnormalities (Atik et al., 2017).

An underlying immunological explanation of the condition is suspected; especially in unexplained RPL patients, who more often than expected have signs of autoimmunity (Atik et al., 2017). Among sRPL patients, a previous Danish cohort study observed a significantly higher frequency of a firstborn boy and a poorer prognosis after the birth of a firstborn boy compared to a firstborn girl (Christiansen et al., 2004; Nielsen et al., 2008). However, the studies did not adjust for acknowledged confounding variables other than the number of previous miscarriages. Later, these investigators observed that the poorer prognosis after a firstborn boy only concerned women carrying male-specific minor histocompatibility (H-Y) restricting (H-Y-r) HLA class II (HYr-cII) alleles whereas no difference was observed if the patient

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carried no such alleles (Nielsen et al., 2009).

H-Y antigens are male-specific minor histocompatibility antigens found in all male cells which are encoded by genes on the Y chromosome. At least 5 different H-Y antigens of clinical relevance have been identified (H.S. Nielsen et al., 2010; H. Nielsen et al., 2010). For every H-Y molecule there is a corresponding H-X molecule expressed in female cells. The DBY antigen for example has a DBX homologous protein that is 91% identical with the DBY protein with regard to the amino acid sequence. H-Y antigens are expressed in or on the surface of cells (including trophoblast cells) or vesicles derived from a male infant that have crossed the fetomaternal barrier into the maternal circulation (Vogt et al., 2002). The peptides derived from H-Y peptides can be presented by specific HLA class I and class II molecules resulting in a cellular and/or humoral immune response. In the present study we focus on the immune responses mediated by HLA class II molecules since a previous study found that only maternal carriage of H-Y-r HLA class II but not HLA class I alleles had a negative prognostic impact in RPL patients with a firstborn boy (Nielsen et al., 2009). In this case, the H-Y antigens may be processed intracellularly by maternal antigen-presenting cells (APCs) and bound to HLA class II molecules on the cell surface enabling the APCs to trigger a cellular or humoral immune response directed against the fetal antigen(s): but only if the woman carries an HYr-cII allele. When such immunization has occurred, it is suggested that it may cause a harmful cytotoxic T-cell response and often also a humoral response against the allograft, as seen in graft versus host disease (GVHD) (Dzierzak-Mietla et al., 2012; Spierings et al., 2013). In relation to women trying to conceive, it could potentially cause a rejection of the following pregnancies rather than a normal tolerogenic response.

To our knowledge, no study has confirmed this theory since presented by Nielsen et al. (Nielsen et al., 2009). As it potentially explains the yet unknown immunological pathology causing a rejection of the semi-allogeneic fetus in sRPL patients, we find it critical to reexamine this hypothesis. Understanding the pathology is fundamental for finding or developing a successful treatment.

The aim of the present study was to test the hypothesis that the birth of a firstborn boy before RPL is more frequent than the birth of a girl, that it affects the pregnancy prognosis negatively after adjustment for relevant confounders, and that this association is connected to maternal carriage of HYr-cII alleles.

2. Method and materials

2.1. Study population

From January 2016 to October 2022, patients with pRPL or sRPL (defined as RPL after ≥1 pregnancy beyond 24 weeks) with ≥ 3 consecutive pregnancy losses admitted sequentially to The Center for Recurrent Pregnancy Loss of Western Denmark were included in the study. Patients who had significant chromosomal abnormalities (n = 17), uterine malformations (n = 12), no analysis of HLA-DRB1 genotype (n = 50) or had previously given birth after GW 24 to both a girl and a boy before RPL (n = 32) were excluded. All pregnancies were confirmed with urine hCG, serum hCG, or ultrasonic examination. Confirmed ectopic and molar pregnancies were not accounted for. An obstetric and gynaecologic history and routine blood samples were obtained from all patients at the first consultation according to the ESHRE RPL guideline (Atik et al., 2017), on the basis of which the treatment plan was decided. Treatments used in our RPL Center included tender loving care with close monitoring and weekly ultrasound scans in first trimester for all patients and also, the majority received vaginal progesterone supplementation from a positive pregnancy test and until gestational week (GW) 10. Overall, if blood sample analyses indicated antiphospholipid syndrome, hypothyroidism, or an aberrant immune profile, the patient was offered low molecular weight heparin during pregnancy, levothyroxine before conception and during pregnancy, or prednisolone with or without intravenous immunoglobulin in first trimester, respectively.

HLA class II typing has been part of the routine investigations since the RPL Center was established in 2016. In the ESHRE RPL guideline, HLA class II typing is only recommended for Scandinavian women with sRPL after the birth of a boy since all studies on the issue of maternal HLA class II and RPL have been performed in Denmark. However, since a large Danish case-control study found that HLA-DRB1 * 07 was associated with both pRPL and sRPL (Thomsen et al., 2021), we found it informative to do HLA-DRB1 typing in all our patients for explanatory purposes.

All women signed an informed consent form at the first consultation before blood samples were obtained that granted the Center permission to store all relevant data in a database. Information on pregnancies after admission was obtained from follow-up consultations and documented in the hospital record. We have no information about the patients' ethnicity in our database, but it is estimated that < 2% are non-Caucasians.

Since only data from the RPL Center's database on results from the diagnostic workup and routine investigations and interventions in the RPL Center were analysed and reported in this study, no permission from the Ethics Committee was required.

The study population consisted of 1) all included RPL patients (called the cross-sectional sample) and 2) the sample of RPL women who had minimum one pregnancy after admission, defined as an hCG > 5 IU/L (called the prospective cohort) (Fig. 1). The cross-sectional sample was included to increase the number included in analysis of HLA allele frequencies and perinatal outcomes prior to RPL. A successful reproductive outcome was defined as pregnancy ≥ 12 weeks of gestation or birth determined at the time of follow-up.

Patients were asked to contact the RPL Center as soon as they had a positive pregnancy test and were offered monitoring to GW 18. Patients who had not returned with information about pregnancy were contacted by telephone after a year for follow-up. A successful pregnancy outcome in first pregnancy after admission or final (cumulative) reproductive outcome determined at time of follow-up was defined as a pregnancy beyond GW 12 or birth in a pregnancy confirmed with p-hCG > 5

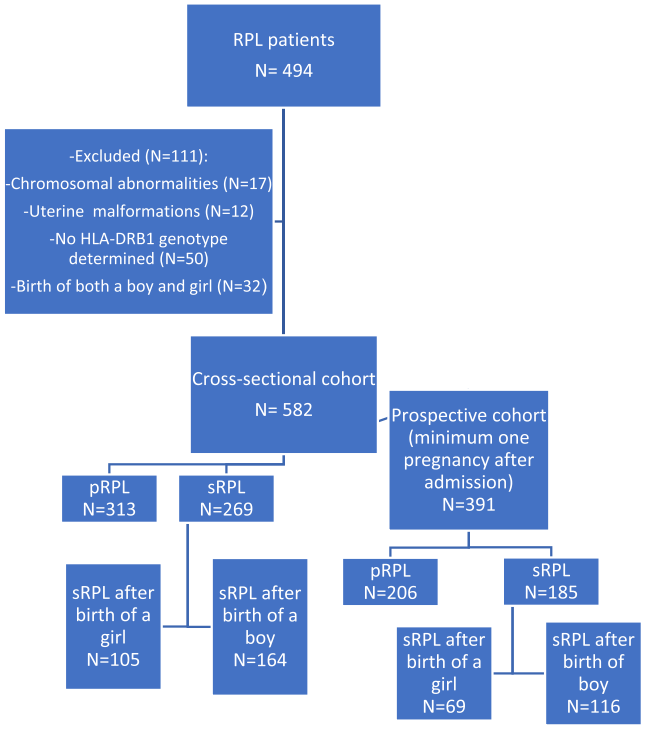

Fig. 1. A flow diagram of the number of RPL patients included in this cohort and in each of the RPL subgroups.

occurring after admission to the RPL Center. Collection of perinatal data from subsequent births was done by entering *data from* the electronic hospital records. Preeclampsia was defined as gestational hypertension and proteinuria (urine albumin/creatinine ratio >300 mg/g or urine albumin >3 g/day) presenting after GW 20 + 0.

The reference group consisted of anonymous Danish bone marrow donors on whom only information on sex, age, and HLA-DRB1 and -DQB1 genotypes at high resolution (4-digit level) was obtained (Thomsen et al., 2021).

2.2. HLA class II typing

DNA was extracted from EDTA-stabilized whole blood using the Maxwell16 Blood DNA Kit on the Maxwell RSC16 instrument according to the manufacturer's instructions (Promega, US).

HLA-DRB1 and -DQB1 allele typing (i.e., at 2-digit level) was performed by the FluoGene system, which is based on a specifically modified TaqMan probe system (Inno-train Diagnostik GmbH) and performed according to the manufacturer's instructions. The subsequent pre- and postreads were generated on a FluoVista. The HLA-DRB1 and -DQB1 genotype was concluded by the FluoGene Software.

2.3. Assignment of H-Y-r HLA class II alleles

In this study, all included patients were HLA-DRB1 typed while HLA-DQB1 was determined at the 2-digit level in 351/583 patients. At present, the known HYr-cII alleles in the HLA-DRB1 and -DQB1 loci are HLA-DRB1 * 07, HLA-DRB1 * 15 and HLA-DQB1 * 0501/0502 (Eljaafari et al., 2013; Vogt et al., 2002; Zorn et al., 2004).

HLA-DRB1-DQB1 haplotypes were assigned to the patients based on the known linkage disequilibria among Caucasians (Knipper et al., 2000). When HLA-DQB1 was not determined, HLA-DRB1 * 01/10/16 positivity was considered as a surrogate biomarker for HLA-DQB1 * 0501/0502 positivity since these alleles are in strong positive linkage disequilibrium in Caucasians (Knipper et al., 2000) and the DRB1 * 01/10/16 alleles are rarely associated with other DQB1 * 05 subtypes. Using this surrogate biomarker for DQB1 * 0501/0502 will only lead to 3–5% of the patients with DRB1 * 01/10/16 and no DQB1 determination being falsely considered as carrying an HYr-cII allele, i.e., 1–2 patients. Patients with HLA-DRB1 * 01/10/16 who were HLA-DQB1 typed but did not carry the HLA-DQB1 * 05, were not considered to have an HYr-cII allele.

HLA-DRB1 * 14 is in almost 100% positive linkage disequilibrium with DQB1 * 0503 and therefore, patients carrying an HLA-DRB1 * 14-DQB1 * 05 haplotype were not considered to carry an HYr-cII allele (Knipper et al., 2000). Only rarely (approximately 0.4%) of HLA-DRB1 * 11 alleles in Caucasians are associated with HLA-DQB1 * 0502 (Knipper et al., 2000) and therefore, DRB1 * 11 positive patients with no DQB1 determination were considered as not carrying a HYr-cII allele while patients with an HLA-DRB1 * 11 -DQB1 * 05 haplotype were considered to carry an HYr-cII allele. Thus, only an exceedingly small risk of false assignment of carrying 0 HYr-cII alleles existed.

2.4. Statistics

Data was collected from the RPL Center's database (Data Protection Agency of The North Denmark Region, Approval Number 2018–5) and the statistical analysis was performed in Stata (MP 15.0 for Mac, revision 19, June 2017).

When comparing pRPL and sRPL or sRPL subgroups, χ^2 test was used for categorical variables and when less than 5 patients were expected, Fisher's exact test was used. T-test or Wilcoxon rank-sum test was used for comparison of continuous variables depending on data distribution. A binomial test was used to compare the sex ratio (boy:girl) of firstborn children with the ratio in the Danish background population of 51% boys (Denmark Statistics, 2021). The impact of previous birth of a child (0 =pRPL, 1 =sRPL), sex of the firstborn child (0 =girl, 1 =boy), and HYr-cII allele carriage on the odds ratio (OR) for the first pregnancy after admission being successful (beyond GW 12 or birth at time of follow-up) was determined with univariate and an adjusted logistic regression with adjustment for body mass index (BMI), and maternal age at referral as continuous variables, and current smoking (0 =no, 1 =yes) as these variables were identified as being associated with pregnancy outcome using directed acyclic graphs. The number of previous pregnancy losses was not included as a confounding variable as this variable was considered to depend on the other confounding variables and the exposure; however, a sensitivity analysis with the variable included was performed to check for any significant changes.

Mean and 95% confidence interval (CI) was reported for parametric variables, while median and 25th and 75th percentile (IQR) was reported for non-parametric variables. Frequency for categorical variables was reported as percentage. A probability value of $p < 0.050$ was considered significant.

3. Results

3.1. The study sample and subgroups

The cross-sectional sample included 582 RPL patients while the prospective cohort included 391 RPL patients with minimum one pregnancy after admission (Fig. 1).

In the cross-sectional sample, 61.0% (n = 164) of sRPL patients had a firstborn boy which differed from the expected percentage of a firstborn boy seen in the Danish background population being 51.3% (p = 0.001) (Denmark Statistics, 2021). The male:female sex ratio of firstborn children was 1.56 in the cross-sectional sample.

In sRPL patients with no HYr-cII alleles, 67.7% had a firstborn boy which differed significantly from the background population (p = 0.004) and from the sRPL patients with ≥ 1 HYr-cII alleles (p = 0.049).

3.2. H-Y-restricting HLA class II phenotype frequency

In the cross-sectional sample, 213 RPL patients (36.6%) carried no HYr-cII allele while the remaining 369 RPL patients (63.4%) were considered to carry minimum one HYr-cII allele. Among the 391 RPL patients in the prospective cohort, 245 RPL patients (62.7%) carried an HYr-cII allele.

The prevalence of carrying ≥ 1 HYr-cII allele did not differ significantly between pRPL and sRPL patients. When compared to the external reference group, pRPL and sRPL patients had a similar prevalence of carriage of the HYr-cII alleles (Table 1). The prevalence of HLA-DRB1 * 15 was significantly higher in sRPL patients with a firstborn girl compared to those with a firstborn boy (p = 0.018).

The sex ratio of the firstborn children, the distribution of the HYr-cII alleles, and the frequency of carrying ≥ 1 HYr-cII alleles did not differ between the cross-sectional sample and the prospective cohort.

3.3. Obstetric and perinatal data in prior births

In the cross-sectional sample, sRPL patients with a firstborn boy who carried ≥ 1 minimum one HYr-cII allele had significantly more often experienced a history of pre-eclampsia compared to women with a firstborn boy who did not carry HYr-cII alleles (p = 0.004). Also, the birthweight of the firstborn boy was significantly lower if the mother carried ≥ 1 HYr-cII allele than in mothers who did not carry an HYr-cII allele (p = 0.030) (Table 2).

3.4. Baseline characteristics of prospective cohort

In the prospective cohort, sRPL patients were older than pRPL

Table 1
The HLA-DRB1 phenotype frequency (%) in the cross-sectional cohort in subgroups based on prior birth after 24 weeks of gestation and the sex of the firstborn child delivered before RPL.

	pRPL (n = 313)	sRPL (n = 269)	P[a]	sRPL only boy (s) (n = 164)	sRPL only girl (s) (n = 105)	P[2]	Reference group (n = 2066)
HLA-DRB1 * 07 (%)	17.9	21.6	0.266	21.5	21.9	0.933	18.4
HLA-DRB1 * 15 (%)	27.8	27.5	0.939	22.1	35.2	0.018	32.3
HLA-DRB1 * 01/10/16 (%)	25.2	24.2	0.764	24.5	23.8	0.892	23.7
≥ 1 HYr-cII alleles (%)	62.3	64.7	0.552	60.1	71.4	0.059	63.6
2 HYr-cII alleles (%)	15.6	12.6	0.439	12.3	15.2	0.487	16.0

pRPL/sRPL: primary/secondary recurrent pregnancy loss, HYr-cII: H-Y-restricting HLA class II.
[a] : comparing pRPL with sRPL. [2]: comparing sRPL with boy vs sRPL with girl

Table 2
Perinatal data from births before sRPL in the cross-sectional sample according to the sex of the child before sRPL and HYr-cII allele carriage.

	sRPL with firstborn boy			sRPL with firstborn girl		
	0 HYr-cII allele (n = 65)	≥ 1 HYr-cII alleles (n = 98)	P	0 HYr-cII allele (n = 30)	≥ 1 HYr-cII alleles (n = 75)	P
Preeclampsia, n %	0	11	0.004	4	4	0.213
	0	10.5		12.5	4.8	
Birthweight Median (±SD)	3537 (±571)	3295 (±754)	0.030	3250 (±566)	3230 (±603)	0.881
Gestational age Median (IQR)	280 (275–287)	280 (270–287)	0.546	279 (266–286)	280 (272–285)	0.763
Emergency cesarean section, N %	13	23	0.601	9	8	0.015
	20.0	23.5		30.0	10.7	

IQR: interquartile rage (25th-75th percentile)

patients while pRPL patients were more often given immunomodulatory treatment and more often had a reproductive history complicated with infertility. No differences in baseline characteristics were observed between sRPL patients with a firstborn boy and girl (Table 3).

3.5. Reproductive outcome after RPL

In total, previous delivery of child ≥ 24 weeks of gestation compared to nulliparity was not associated with a change in successful reproductive outcome (OR:1.35; 95% CI 0.88–2.07; p = 0.162); however, within the sRPL subgroup, having a firstborn boy was associated with a reduced success rate in first reproductive outcome after admission compared to a having a firstborn girl (OR: 0.41; 95% CI 0.20–0.83; p = 0.012) (Table 4).

A small increase in successful reproductive outcome was seen in sRPL patients with a firstborn girl when the number of HYr-cII alleles increased, while the opposite trend was observed in sRPL patients with a firstborn boy (Fig. 2). However, no significant differences were found between patients who had 0, 1, and 2 HYr-cII alleles within each of the four subgroups; pRPL, sRPL and sRPL stratified by sex of firstborn child (p(pRPL)= 0.548, p(sRPL)= 0.846, p(sRPL w. boy)= 0.228, p(sRPL w. girl)= 0.434) (Fig. 2).

After stratification for carriage of HYr-cII alleles, a reduced successful reproductive outcome was observed in sRPL patients with ≥ 1 HYr-cII allele and a firstborn boy compared to sRPL with ≥ 1 HYr-cII allele and a firstborn girl (OR= 0.30; 95% CI: 0.13–0.71; p = 0.005) while this was not found in sRPL patients carrying no HYr-cII alleles (OR=0.83; 95% CI: 0.23–3.07; p = 0.781) (Table 4 & Fig. 2). Moreover, a significantly higher proportion of sRPL patients with a firstborn girl had a successful reproductive outcome than pRPL patients (OR:2.44; 95%CI 1.25–4.76; p = 0.009) which was enhanced in patients with ≥ 1 HYr-cII alleles (OR: 3.11; 95% CI 1.39–6.94; p = 0.006) but absent in patients with 0 HYr-cII alleles (OR: 1.38; 95% CI: 0.40–4.71; p = 0.612). sRPL patients with a firstborn boy had a similar frequency of a successful reproductive outcome as pRPL with and without stratification for HYr-cII carriage.

The adjusted OR (aOR) for a successful pregnancy in first pregnancy after admission was significantly decreased in women with a firstborn boy relative to a firstborn girl (aOR=0.37, 95% CI 0.18–0.80, p = 0.011). Carriage of ≥ 1 HYr-cII alleles relative to 0 HYr-cII alleles as an individual, independent variable in the analysis did not seem to be a risk factor (aOR= 0.77; 95% CI 0.37–1.60; p = 0.490). However, when

Table 3
Baseline characteristics for RPL patients with minimum one pregnancy after admission (prospective cohort) divided into subgroups based on a history of 0 or ≥ 1 births after 24 weeks of gestation and the sex of the firstborn child delivered before RPL.

	pRPL (n = 206)	sRPL (n = 185)	P[a]	sRPL only boy (s) (n = 116)	sRPL only girl (s) (n = 69)	P[2]
Age, mean (±SD)	31.9 (±5.2)	32.9 (±4.6)	0.049	33.3 (±4.5)	32.3 (±4.7)	0.144
BMI	25.1	24.0	0.110	24.1	24.0	0.395
Median (IQR)	(22.5–29.0)	(22.0–28.4)		(22.0–29.3)	(22.0–27.5)	
Number of consecutive losses, median (IQR)	3 (3–4)	3 (3–4)	0.023	3 (3–4)	3 (3–4)	0.282
Smoking, %	10.7	10.9	0.965	7.8	15.9	0.087
Immunotherapy in relation to 1st pregnancy after admission, %	58.0	47.0	0.026	50.0	42.0	0.294
Assisted reproductive technology treatment, %	44.2	22.7	< 0.001	20.7	26.1	0.397

Immunotherapy: prednisolone 5–10 mg daily with or without intravenous immunoglobulin prior to and in 1st trimester. Assisted reproductive technology treatment: including IVF, ICSI and FET.
[a] : comparing pRPL with sRPL. [2]: comparing sRPL with boy vs sRPL with girl

Table 4
The frequency of a successful reproductive outcome in first pregnancy after admission according to HLA class II phenotype and RPL subgroup.

HYr-cII phenotype	pRPL	sRPL	P[a]	sRPL with only boy (s)	sRPL with only girl (s)	P[2]
DRB1 * 07	17/35	26/39	0.115	13/23	13/16	0.107
n/N, %	48.6	66.7		56.5	81.3	
DRB1 * 15	29/48	41/54	0.092	19/27	22/27	0.340
n/N, %	60.4	75.9		70.4	81.5	
DRB1 * 01/ 10/16	39/57	32/48	0.848	15/29	17/19	0.007
n/N, %	68.4	66.7		51.7	89.5	
0 HYr-cII	56/84	43/61	0.625	32/46	11/15	0.781
n/N, %	66.7	70.5		69.6	73.3	
≥ 1 HYr-cII	75/	87/	0.151	42/70	45/54	0.005
n/N, %	122	124		60.0	83.3[b]	
	61.5[b]	70.2				
2 HYr-cII	20/32	19/28	0.664	8/15	11/13	0.077
n/N, %	62.5	67.9		53.3	84.6	
Total	131/	130/	0.162	74/116	56/69	0.012
	206	185		56.9	81.2[c]	
	63.6[c]	70.3				

n/N: Number with pregnancy > 12 weeks or birth (n)/ total number with the respective HLA class II phenotype within the respective recurrent pregnancy loss (RPL) subgroup (N).
[a] : comparing pRPL with sRPL. [2]: comparing sRPL with boy vs sRPL with girl
[b] Comparing pRPL and sRPL with a firstborn girl: p = 0.004
[c] Comparing pRPL and sRPL with a firstborn girl: p = 0.009

evaluating the impact of the combination of sex of firstborn child and carriage/non-carriage of HYr-cII alleles with reference to sRPL with a firstborn girl and ≥ 1 HYr-cII alleles, the aOR for a successful reproductive outcome was significantly decreased for women with a firstborn boy and ≥ 1 HYr-cII alleles (aOR=0.28; 95% CI 0.11–0.69; p = 0.006) while a trend in the same direction was seen for women with a boy and no HYr-cII alleles (aOR=0.47; 95% CI 0.17–1.28; p = 0.139). When compared to sRPL patients with a firstborn girl and no HYr-cII alleles, no clear difference in successful reproductive outcome was seen (aOR=0.58, 95% CI 0.15–2.36, p = 0.456). In a sensitivity analysis including the number of pregnancy losses before admission as an independent variable, the aORs and p-values did not change notably in both logistic regression analyses.

When the final reproductive outcome assessed at the time of data collection was compared between subgroups in the prospective cohort, the incidence of a cumulative successful reproductive outcome differed significantly with the highest rate in sRPL patients with a firstborn girl and no HYr-cII allele (93.3%) and the lowest rate in sRPL patients with a firstborn boy and > 1 HYr-cII allele (72.9%). Only when comparing the successful reproductive outcome between sRPL patients with ≥ 1 HYr-cII alleles and a firstborn boy to those with a firstborn girl carrying ≥ 1 HYr-cII alleles, the association was significant (OR=0.27, 95% CI 0.09–0.79, p = 0.017) (Fig. 3). The time from admission to final follow-up within each subgroup did not differ significantly between the four subgroups.

Within the cross-sectional sample of RPL patients who were admitted more than 180 days before the final follow-up, the frequency of secondary infertility (no pregnancy after admission) differed significantly between sRPL subgroups ranging from 15.5% to 40.0% (Fig. 3).

Overall, the percentage of women giving birth to a boy after admission was 55.8% in pRPL and 51.2% in sRPL patients and this did not differ from the Danish background population. When dividing patients into subgroups based on HYr-cII carriage and sex of firstborn child, no significant difference from the background population was found (Fig. 4).

4. Discussion

Significantly more sRPL patients had a firstborn boy compared to the Danish background population. This is in agreement with findings in previous studies of sRPL patients (Christiansen et al., 2004; Nielsen et al., 2008; Ooi et al., 2011), which observed sex ratios before sRPL similar to this study; 1.52–1.65 versus 1.56. It is speculated whether the greater prevalence of a firstborn boy should be viewed simply as a direct risk factor for pregnancy losses in subsequent pregnancies or as a confounder for exposures differentially distributed by sex of firstborn child. We do not consider selection bias as a cause of the screwed sex ratio since criteria for referral to our Center are independent of the sex of firstborn child and also, we do not believe primary care providers are considering this factor over the referral criteria when deciding whether or not to refer a patient. However, it is not known whether the higher incidence of obstetric and perinatal complication including gestational diabetes, pre-eclampsia, placenta previa, preterm birth, low birth weight, instrumental delivery (caesarian section) and fetal morbidity, which is associated with carrying a male compared to a female fetus (DiPietro and Voegtline, 2017) are the true risk factor for subsequent RPL rather than the hypothesis regarding anti-H-Y immunization proposed in this study.

The sex ratio of children born after RPL in the present study was similar to that in the Danish background population while a previous cohort study found a slightly higher rate of girls after RPL (54.0% girls) (Nielsen et al., 2008). Nevertheless, it should be born into mind that only small numbers of newborns after RPL were included in these analyses.

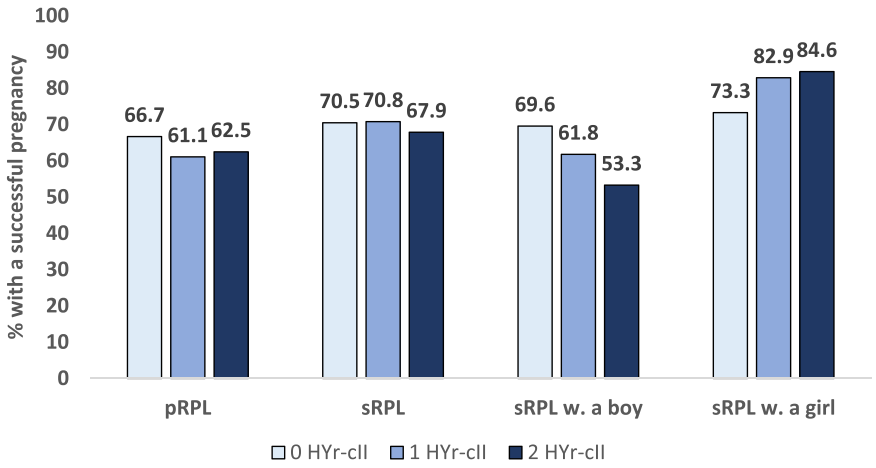

Fig. 2. The frequency of a successful reproductive outcome in first pregnancy after admission according RPL subgroup and carriage of HYr-cII alleles *HYr-cII: H-Y-restricting HLA class II* No statistically significant differences were found between the successful reproductive outcome rates in the different columns.

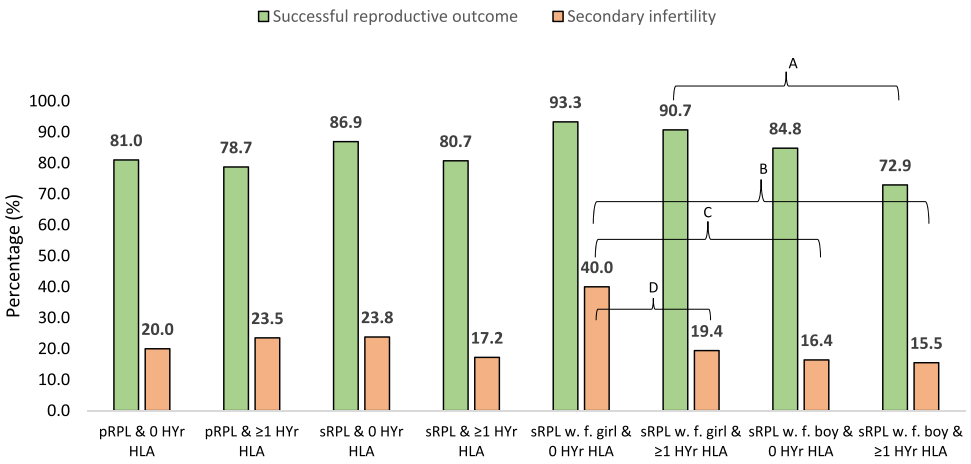

Fig. 3. The reproductive outcome on the day of final follow-up (cumulative reproductive outcome): the percentage with a successful reproductive outcome among RPL patients in the prospective cohort (green) and the percentage of RPL patients with secondary infertility in the cross-sectional sample (orange) separated according to the RPL history and carriage of HYr-cII alleles.*A, Comparing cumulative successful reproductive outcome rate in sRPL patients carrying ≥ 1 HYr-cII alleles having a firstborn girl with sRPL patients carrying ≥ 1 HYr-cII alleles having a firstborn boy; B-D, comparing secondary infertility in each sRPL subgroup with reference to sRPL patients carrying 0 HYr-cII alleles having a firstborn girl. A: OR= 0.27, 95% CI 0.09–0.79, p = 0.017 B: OR= 0.27, 95% CI 0.10–0.74, p = 0.011 C: OR= 0.29, 95% CI 0.10–0.86, p = 0.021 D: OR= 0.36, 95% CI 0.13–0.98, p = 0.042.*

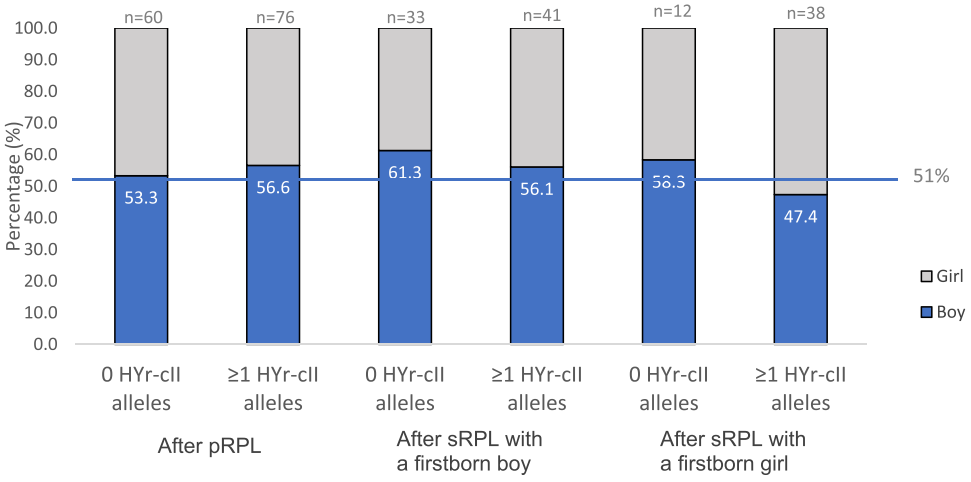

Fig. 4. Sex of the child after admission according to RPL subgroup and carriage HYr-cII alleles. The horizontal line at 51% represent the percentage of new-born boys in the Danish background population and to which the RPL subgroups are compared.

The frequency of carrying an HYr-cII allele did not differ between the total RPL cohort and the reference group and neither between the RPL subgroups.

A main finding was that sRPL patients with a firstborn boy carrying an HYr-cII allele had a significantly lower odds of a successful reproductive outcome than sRPL patients with a firstborn girl whereas their reproductive outcome was similar to patients with pRPL. A previous study also reported that sRPL patients with a firstborn boy carrying an HYr-cII allele exhibit lower odds of subsequent livebirth than sRPL patients with a firstborn boy without these HLA class II alleles or those with a firstborn girl (Kolte et al., 2016; Nielsen et al., 2009). It is important to notice that the cohort from these studies has no overlap to our cohort.

In the previous study, the odds of a successful pregnancy in patients with sRPL with a firstborn boy or firstborn girl carrying an HYr-cII allele was 44% and 82%, respectively, which are both comparable to our present observations. This strengthens the possibility that the associations are not found by chance.

How can these epidemiological and immunogenetic findings be combined in a common theory explaining the pathophysiologic background for sRPL? Having no experimental studies of our own, we have to find possible explanations in other studies especially in the field of transplantation immunology.

The hypothesis previously put forward was that the poorer reproductive outcome in sRPL women with a firstborn boy - and especially those carrying HYr-cII alleles - may be due to exposure of maternal immune cells to H-Y antigens expressed on allogeneic cells from the male fetus and placenta that had entered the maternal circulation during her previous pregnancy. After such exposure, a cellular and humoral immune response directed against H-Y antigens may have been induced and a subsequent re-exposure may cause a harmful response similar to that in GvHD (Nielsen et al., 2009; Tan et al., 2008).

Zorn et al. (2004) reported that a T cell clone isolated from a male patient with chronic GvHD after bone-marrow transplantation from his HLA identical sister in addition to reactivity to DBY peptides, also exhibited equally strong reactivity against the homologous DBX peptide, which must be considered an autoimmune reaction since the effector cells derived from the female donor (Zorn et al., 2004). However, as the patient also produced anti-DBY antibodies but no anti-DBX antibodies 12 months after bone-marrow transplantation, this humoral response must be considered alloimmune. It was proposed that the combination of T cell-mediated autoimmune responses against DBY/DBX minor antigens and alloimmune anti-DBY humoral responses were the background for chronic GVHD in their patient (Zorn et al., 2004). In contrast, in another study (Vogt et al., 2002), it was found that a T cell clone with reactivity against another DBY-derived peptide restricted by HLA-DQ5 only reacted against the DBY peptide but not the homologous DBX peptide, which is more similar to an isolated alloimmune response than the response against the peptide studied by Zorn et al. (2004). The results from the two studies illustrate that immune reactions against H-Y minor antigens are complex and vary a lot depending on the H-Y derived

peptide involved.

Quite few studies have been published on the prevalence and clinical impact of anti-H-Y antibodies. However, one study found that 52% of male patients who received an allogeneic hematopoietic stem cell transplant from an HLA-identical female developed anti-DBY antibodies whereas such antibodies were found in 17% of healthy women, although in a lower concentration (Miklos et al., 2004). The antibodies in the latter group are thought to be produced as a consequence of a previous pregnancy with a male fetus or blood transfusions. Since none of 30 healthy men had produced anti-DBY antibodies these antibodies must be considered a purely allo-immune phenomenon (Miklos et al., 2004).

In a previous study of RPL patients, five specific anti-H-Y antibodies with no anti-H-X reactivity were found significantly more often in sRPL patients (in 49% and 38% of patients with a firstborn boy or girl, respectively) than in women with pRPL and in a control group of women with no pregnancy or normal pregnancies with boys (H. Nielsen et al., 2010). The clinical significance of the anti-H-Y antibodies in RPL was stressed by the finding that patients with these antibodies subsequently gave birth to a significant excess of girls (88%) compared with antibody-negative patients suggesting that presence of these antibodies could be responsible for the loss of mainly male fetuses.

Thus, it is possible that in sRPL patients with firstborn boys and HYr-cII alleles, anti-H-Y antibodies are the main pathogenic factor whereas sRPL patients with firstborn girls with HYr-cII alleles may primarily mount T cell responses to H-Y/H-X antigens with weaker or absent humoral anti-H-Y responses resulting in a low miscarriage rate.

However, we agree that more basic research is needed especially about the clinical impact of anti-H-Y antibodies in RPL patients.

If HYr-cII alleles indeed play a role in the pathogenesis of sRPL, we would expect that the prevalence of the alleles would be higher in sRPL patients with a firstborn boy than among patients with a firstborn girl or in the background population. In the former Danish study, no such differences of HYr-cII allele frequencies between sRPL with firstborn girls or boys could be detected (Nielsen et al., 2009) whereas in the present study we found, to our surprise, that the frequency of HYr-cII alleles was close to being significantly increased in sRPL patients with a firstborn girl when compared to both sRPL patients with a firstborn boy and to the reference group. It could represent a chance finding as these multiple comparisons were for explorative purposes and not the primary objective of the study. Thus, with multiplicity adjustment, e.g., the Bonferroni correction, no trend was present. However, if it is real, we have no reasonable explanation for it and further testing is needed. With regard to the approximately 10% lower prevalence of sRPL patients with a firstborn boy and HLA-DRB1 * 15 in our cross-sectional sample, it is possible that carriage of HYr-cII alleles in some women with a firstborn boy results in such strong immunization against H-Y minor antigens that the embryos are rejected in the uterus at a very early stage before it is possible to detect hCG in the plasma (preclinical losses). Thus, these patients will be considered as secondary infertile and will not be referred to our RPL clinic. A study of HLA-DRB1 polymorphism in patients with secondary infertility after the birth of a boy will help to clarify this issue.

Supporting this theory of an overreactive immune response initiated in relation to a prior pregnancy is that patients with a firstborn boy carrying one or more HYr-cII alleles more often suffered from pre-eclampsia and had a lower birth weight in their prior pregnancy (Table 2). Preeclampsia is associated with a higher level of free circulating syncytiotrophoblast microparticles able to stimulate peripheral blood mononuclear cells to upregulate their production of proinflammatory cytokines (Germain et al., 2007). These microparticles also express H-Y antigens and their release in a proinflammatory environment may lead to a high risk of anti-H-Y immunization especially in patients carrying HYr-cII alleles. However, these findings of an increased proportion of HYr-cII positive sRPL patients affected by pre-eclampsia while carrying their firstborn boys and giving birth to boys with a lower birth weight were not a primary objective of this study but rather an explorative finding. Thus, these results should be considered with caution. If the Bonferroni correction was applied, only the difference in prevalence of pre-eclampsia remained significant, However, since these analyses were included to generate hypothesis rather than confirm them, no correction for multiplicity testing was applied.

If cellular and/or humeral immunity against H-Y antigens are causing pregnancy loss in RPL patients, we would expect that patients who are suspected to have the highest risk of anti-H-Y immunization (i.e., sRPL patients with a firstborn boy carrying HYr-cII alleles) would give birth to an excess of girls after RPL, since male pregnancies may be preferably attacked by the immune system. However, as shown in Fig. 4, this seems not to be the case. A possible explanation may be the well-known phenomenon epitope spreading (Lehmann et al., 1993) meaning that the initial very specific alloimmune reactions against H-Y antigens with time becomes more unspecific and autoimmune. The reaction may spread to e.g. the homologous H-X antigens which may lead to an immune system opposing and killing embryos irrespective of sex.

The present study has some limitations. HLA-DRB3 assessment was not performed. The DRB3 * 0301 allele has in in-vitro experiments been shown to act as an H-Y-r allele (Spierings et al., 2013). However, the expression of HLA-DRB3 molecules in peripheral blood lymphocytes is only 1/4 of that of HLA-DRB1 molecules(Yamamoto et al., 2020) and alleles of the HLA-DRB3 locus are not or only weakly associated with autoimmune diseases (Kim et al., 2016) emphasizing their limited clinical impact. In support of its absent clinical role, HLA-DRB3 * 0301 carriage did not have any impact on the odds of a subsequent live birth in sRPL patients with firstborn boys (Nielsen et al., 2009), the allele is rare and therefore lack of HLA-DRB3 assessment is not expected to have had noteworthy impact on the present results.

Another limitation was that HLA-DQB1 was only determined in 53.7% of our patients. However, using the HLA-DRB1 * 01/10/16 as a surrogate biomarker for the presence of HLA-DQB1 * 0501/0502 knowing the strong positive linkage disequilibrium between these DRB1 and DQB1 alleles could potentially have led to maximally 2 patients considered falsely as HYr-cII carriers. This would not have changed the significance of the results.

Also, a cytogenetic analysis of the miscarriages would have enabled us to selectively compare reproductive outcome between groups with euploid pregnancies, which would strengthen the results. However, this is rarely possible because the huge majority of our patients are not undergoing uterine evacuation but rather prostaglandin-induced abortion outside the hospital with limited chance of karyotyping the product of conception. The risk of aneuploid pregnancy loss is expected to be independent on the sex of first-born children and maternal HLA class II alleles and thus we expect that the differences in subsequent successful pregnancy outcome in the relevant patient subgroups would remain even if pregnancies with aneuploid embryos were excluded.

For future elaboration of the hypothesis presented in this manuscript, several analyses are needed. First of all, an international study investigating sex ratio of the firstborn children and HYr-cII alleles in RPL patients from centers from different geographical regions having similar condition of life, diagnostic work-up, and access to treatments, as well as good quality data would be useful. It would have the potential to explore whether the findings in this study are indeed associated directly with the pathogenesis of sRPL or alternatively whether it is caused by a confounder only present in the Danish population of RPL patients. At present, we are collaborating with two RPL Centers outside Denmark on this topic. Secondary, a laboratory-based research study is needed to test the tolerance towards paternal and placental antigens by the decidual and peripheral blood immune cells in each of the subgroups presented in this study. Since epitope spreading may be involved, the analysis cannot be restricted to reactions against H-Y antigens only. The T-helper cells, cytotoxic T-cells, regulatory T-cells, B-cells, and antigen-presenting cells as well as memory cells are all among the primarily suspected actors in this hypothesis but at present other immunological mechanisms cannot be excluded.

5. Conclusions

A firstborn boy is more frequent than expected in sRPL patients, and our findings strongly support the theory that a firstborn boy compared to a firstborn girl has a negative impact on reproductive outcome in sRPL patients; especially if the sRPL patient carries ≥ 1 HYr-cII alleles. This suggests that immunity against male-specific H-Y antigens is involved in the pathogenesis of sRPL and confirms the findings in a previous study; however, the results have not until now been validated in a new independent group of sRPL patients. The authors therefore suggest that future studies including sRPL patients should consider this issue when they report baseline characteristics and make adjusted analyses of reproductive prognosis.

In conclusion, an immunologic pathogenesis of unexplained sRPL is highly suspected but the mechanism(s) counteracting maternal tolerance to the fetal allograft is still unknown. These findings do suggest HY-immunity as an important risk factor for RPL; possibly in women with certain genetic and/or environmental factors or being in an inflammatory state when presented to H-Y antigens increasing her risk of developing H-Y immunization.

This knowledge should lead to the development of more efficient treatments especially for sRPL in cases where anti-H-Y immunity is suggested to be a pathogenetic factor.

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Contribution

CNP & OBC: data collection, result interpretation, collecting consent forms. CNP: statistical data analyses, drafting the manuscript, OBC: manuscript revisions, USK: statistical support, RS: HLA analyses. All authors critically revised and approved the final version of the manuscript.

Declaration of interest

None

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