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Frequency and impact of obstetric complications prior and subsequent to unexplained secondary recurrent miscarriage

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BACKGROUND: The chance of a live birth after a diagnosis of secondary recurrent miscarriage (SRM) is reduced in patients who, prior to the miscarriages, gave birth to a boy and carry HLA class II alleles that efficiently present male-specific (H-Y) antigens to the immune system. Information about obstetric complications in births prior and subsequent to the SRM diagnosis is limited. The relations between maternal carriage of H-Y-restricting HLA, fetal sex, obstetric complications and prognosis are unknown.

METHODS: Women with unexplained SRM referred to the Danish Recurrent Miscarriage Clinic between 1986 and 2006 ($n = 358$) were included; 213 gave birth after the diagnosis. Controls, retrieved from the Danish National Birth Registry, were all women with singleton birth of parity 0, 1982–2005 ($n = 608\,068$) and parity 1, 1986–2008 ($n = 510\,264$). Cross-linkage to the National Discharge Registry identified birth complication diagnoses related to the relevant births among patients and controls.

RESULTS: The sex ratio was 1.49 in births prior to SRM and 0.76 in birth after SRM ($P < 0.0001$). For SRM patients with only late miscarriages (>10 weeks gestation), the corresponding sex ratios were 2.31 and 0.21. Compared with the control groups, obstetric complications were more frequent both before (39% versus 24% $P \leq 0.01$) and after (19% versus 14%, $P = 0.01$) SRM diagnosis. Births were more frequently complicated when the child was a boy (44% versus 31%, $P = 0.02$) before and a girl (24% versus 13%, $P = 0.04$) after SRM diagnosis. SRM patients with H-Y-restricting HLA class II alleles and a firstborn boy gave birth to children who weighed on average 381 g less ($P = 0.006$) and were born 0.9 weeks earlier ($P = 0.06$) and their births had more obstetric complications ($P = 0.05$) than patients with the same HLA alleles but a firstborn girl.

CONCLUSIONS: Obstetric complications, sex ratios in births prior and subsequent to SRM and maternal carriage of H-Y-restricting HLA class II alleles are associated parameters. Immune responses against fetal H-Y antigens initiated in the pregnancy prior to the SRM may play a causal role in SRM.

Key words: secondary recurrent miscarriage / sex ratio / pregnancy outcome / epidemiology / H-Y-restricting HLA class II alleles

Introduction

Recurrent miscarriage defined as a minimum of three consecutive miscarriages affects 1–3% of women (Tulppala *et al.*, 1993; Katz and Kuller, 1994). Approximately 40% of the women with recurrent miscarriage have given birth to a child prior to the series of miscarriage and are thus diagnosed with secondary recurrent miscarriage (SRM) (Jivraj *et al.*, 2001). A potential cause for the miscarriages is identified in less than half of cases following standard investigation (Quenby and Farquharson, 1993). The remaining patients are diagnosed with unexplained recurrent miscarriage.

We previously observed that boys are significantly more common than girls among births prior to SRM, and the chance of a live birth after the series of miscarriages is lower in those with a firstborn boy compared with a firstborn girl (Christiansen *et al.*, 2004; Nielsen *et al.*, 2008). The reduced chance of a live birth, however, only applied to patients who, in addition to a firstborn boy, also carry H-Y-restricting HLA class II alleles (Nielsen *et al.*, 2009). These are known to efficiently

present male-specific (H-Y) antigens to the immune system. The impact of maternal carriage of H-Y-restricting HLA class II alleles on live births after SRM diagnosis is currently unknown.

SRM patients have, in contrast to primary recurrent miscarriage patients, carried a pregnancy through to the last part of pregnancy prior to the series of miscarriages. Detailed knowledge of obstetric details regarding the birth prior and subsequent to the series of miscarriages may contribute to our understanding of unexplained SRM. So far, only three studies have addressed obstetric information on births prior to SRM (Christiansen *et al.*, 1992; Weintraub *et al.*, 2005; Yang *et al.*, 2006) and only one study separately reported on obstetric outcome subsequent to an SRM diagnosis (Jivraj *et al.*, 2001).

This study presents obstetric and neonatal outcomes of births prior and subsequent to a SRM diagnosis in a 20-year cohort of patients with unexplained SRM and tests whether complications prior to the miscarriages are associated with the outcome of subsequent births. Finally, we explored whether the sex of children born prior to the SRM diagnosis and maternal carriage of H-Y-restricting HLA class II were associated with obstetric complications in births following the diagnosis of unexplained SRM.

Materials and Methods

Patients

The Danish Recurrent Miscarriage Clinic was established in 1986, and it is a national tertiary clinic that investigates, treats and conducts research in recurrent pregnancy losses. From 1986 up to 2006, a total of 1134 patients with recurrent miscarriage were seen in the clinic and entered into a dedicated detailed database. This study utilizes that cohort and includes all patients with unexplained SRM and, in the case of more than one previous child, preceding children of same sex ($n = 358$) (Fig. 1). SRM is defined as at least three consecutive losses of intrauterine pregnancies before the 22nd gestational week subsequent to one pregnancy of minimum 22 weeks gestation. The miscarriages were considered unexplained if the women had normal uterine anatomy evaluated by hysterosalpingography, hysteroscopy or saline hydrosonography, their menstrual cycles were regular with 21- to 35-day intervals, they were negative for lupus anticoagulant (Nielsen and Christiansen, 2005) and the couples had normal karyotypes. Among the included women, 30 (8%) had given birth to two children and 1 had three children prior to the miscarriages. The findings reported in this study relate to the birth immediately preceding the miscarriages. All reported pregnancies were confirmed by a positive urine or serum-hCG pregnancy test, ultrasonic examination and/or histology of aspirated tissue from the uterus documented in the hospital's or practitioner's records. On the basis of this information, gestational age of each previous miscarriage was available for 317 (89%) of the patients. According to this information, we classified patients as having only early losses <10 weeks ($n = 76$), only late losses ≥ 10 ($n = 43$) or both early and late losses ($n = 198$). At the first consultation, 76 (21%) of the patients reported having a different partner (who had fathered all or some of the miscarriages) than the father of the child preceding the SRM diagnosis. Demographic details of the included SRM women are shown in Table I.

By 31 December 2008, the cohort had given births to 5 twins and 213 singletons after the SRM diagnosis (only the first child delivered after the SRM diagnosis was included) (Fig. 1).

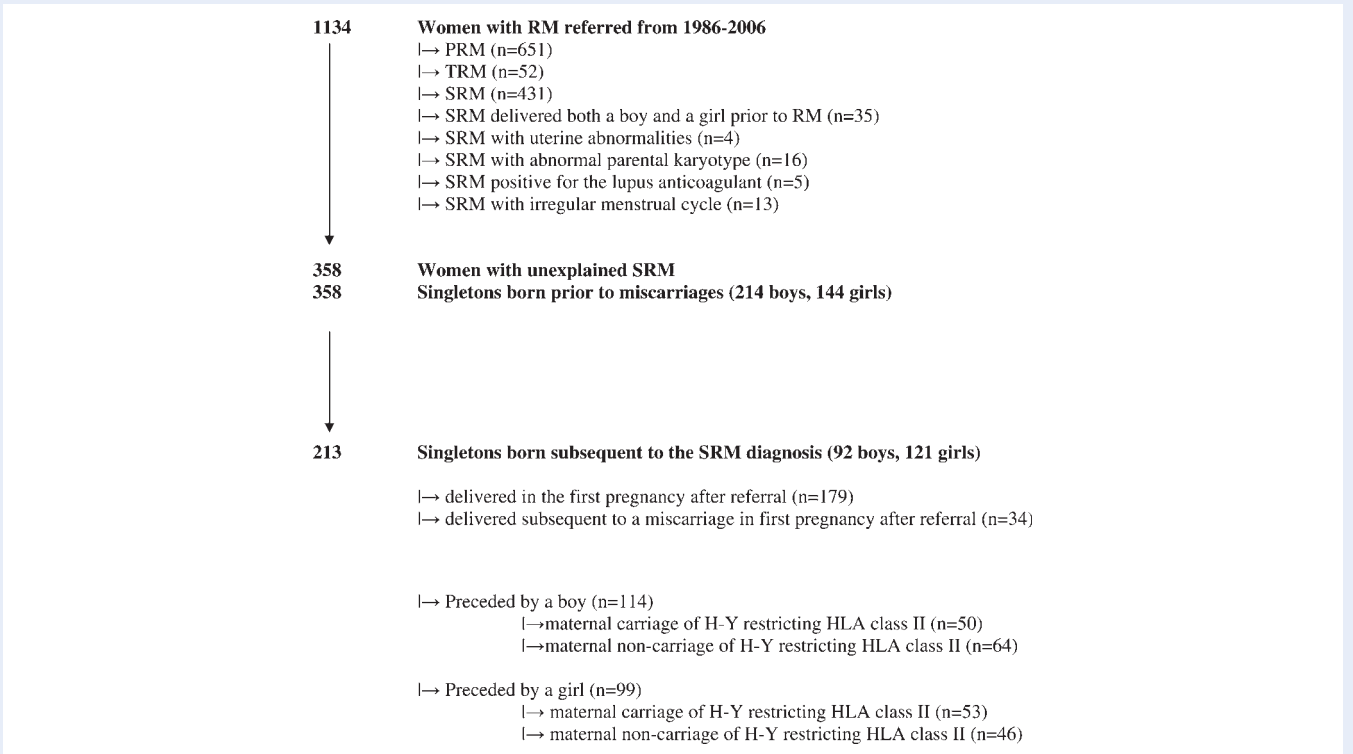

Figure 1 Flow chart illustrating the number of included unexplained SRM patients referred to the Danish Recurrent Miscarriage Clinic from 1986 to 2006. The number and the sex of children born prior and subsequent to the recurrent miscarriage and also the division according to patients carriage of H-Y-restricting HLA class II alleles are illustrated. RM, recurrent miscarriage; PRM, primary recurrent miscarriage; SRM, secondary recurrent miscarriage; TRM, tertiary recurrent miscarriage.

Table I Demographic details of 358 consecutive, unexplained SRM patients referred to the Danish Miscarriage Clinic (1986–2006) stratified by sex of the child born prior to the series of miscarriages.

Demographic data of patients	Total $n = 358$	Sex of child born prior to recurrent miscarriages		
		♂, $n = 214$	♀, $n = 144$	*P*-value
Age, median (range)	33 (22–45)	33 (22–45)	32 (23–42)	0.10
Miscarriages, median (range)	4 (3–13)	4 (3–13)	4 (3–9)	0.09
≥5 miscarriages, *n* (%)	90 (25)	64 (30)	26 (18)	0.01
Change of partner, *n* (%)	76 (21)	45 (21)	31 (21)	0.91

Control groups

The Danish Birth Registry was the source of our control groups. This registry is population covering. The control group for the pregnancies prior to the miscarriages was defined as all Danish nullipara women giving birth to singleton children in the period from 1 January 1982 until 31 December 2004 ($n = 608\,068$) (corresponding to the time period that the patients delivered the child preceding the miscarriages). The control group for the pregnancies following the recurrent miscarriage was defined as all Danish women giving birth to a second-born singleton child in the period from 1 January 1986 until 31 December 2007 ($n = 510\,264$).

Complication variables

Offspring sex, birthweight and gestational age were retrieved from The Danish Birth Registry. Gestational age was estimated from the last menstrual period in the beginning of the study period. In 2004, ultrasound examination for nuchal translucency measurement was made available to all pregnant women in gestational week 12 in Denmark, and this examination was used from then on to estimate gestational age. Preterm birth was defined as delivery before the 37 completed weeks of gestation. Before 1 April 2004, stillbirth was defined as a child born without signs of life and with gestational age $>27 + 6$ weeks; and after this date, the defining gestational age was lowered to $>21 + 6$ weeks. Small for gestational age (SGA) was defined according to a Canadian sex-stratified reference based on singletons (Kramer *et al.*, 2001). The following ICD8/ICD10 codes were used to identify: stillbirth: 634.63, 77909, 779.99/DO364, DP95; pre-eclampsia: 637.03,04,09; 661.30-39; 762.19/DO14; placental abruption: 632.19; 651.5x; 770.10-19/DO45; severe haemorrhage: 653.1x, 653.9x/DO67; and hypoxia: 764-9.4x, 765-8.9x, 776.39,49,90,91,98,99/ DO68 DP20 DP21. Caesarean section was identified using operating codes: in the period from 1982 to 1996: 66020, 66040 and from 1996 to 2008: KMCA10A-E, KMCA12A-B, KMCA10-11.

Identification of obstetric and neonatal complications among patients and controls was obtained through data-linkage to the National Discharge Registry based on each person's unique ID number. Data from the registry were compared with information obtained from the patients at the initial consultation regarding the birth(s) preceding the series of miscarriages. As standard procedure in the clinic, complications reported by the patients were confirmed or rejected by requesting primary hospital records before being entered into the database. For seven patients, registry data were missing as they had given birth to their first child abroad. Information about these patients' birth was based on patients' reports and their copies of medical records. Registry data regarding the birth after the miscarriages were compared with questionnaire data. Patients who become pregnant after referral were followed at the clinic either until they miscarried again or until an ongoing pregnancy was assured (usually until gestational week 18). Patients with an ongoing pregnancy were given a questionnaire regarding their upcoming birth and asked to return it after delivery. A reminder was sent to patients who had not returned the questionnaire, ~2 months after expected delivery. Two patients failed to respond to the reminder and their data were based solely on their registry records. In the registry, we identified 12 cases of severe bleeding and 1 case of pre-eclampsia unreported by the patients. One case of placental abruption and six cases of pre-eclampsia were reported by the patients and confirmed in primary records, however not registered in the National Discharge Registry. The seven complications not reported in the Registry were all related to the births prior to SRM and the patients were all registered with minimum one other complication relating to that birth.

H-Y-restricting HLA class II

The term 'H-Y-restricting HLA class II' is used to describe those HLA alleles reported to date that functionally present H-Y peptides to CD4+ T-lymphocytes. These include the following: HLA-DRB1*15, -DQB1*0501/2, -DRB3*0301 (Vogt *et al.*, 2002; Spierings *et al.*, 2003; Zorn *et al.*, 2004). DNA extraction and HLA typing is described in detail elsewhere (Nielsen *et al.*, 2009).

Statistical analysis

Frequencies were compared by the χ^2 test and Fisher's exact test. The *t*-test and Mann–Whitney *U*-test compared means and medians of continuous data, respectively.

Ethics

The National Data Protection Agency approved the data-linkage to The National Birth and Discharge Registry (2008-41-2666). DNA for HLA-typing was taken from all patients referred to our clinic as a standard procedure. In 2004, the Danish Central National Committee on Biomedical Research Ethics approved (Approval no. 2004-7041-12) the analysis of the impact of H-Y-restricting HLA. From then on, patients included prospectively gave both oral and written consent to participate.

Results

Obstetric and neonatal complications prior and subsequent to the SRM diagnosis

Table II reports the occurrence of stillbirth, pre-eclampsia, placental abruption, severe haemorrhage, birthweight <2500 g, preterm birth and hypoxia in births preceding the SRM diagnosis and in the control cohort of nullipara women with singleton births in the same time period. All complications, except hypoxia, were more frequent in births preceding the SRM diagnosis than in the control group ($P < 0.02$). At least one of the selected complications affected 39% of the births prior to the SRM diagnosis, compared with 24% of the births in the control group ($P < 0.0001$). Among the patients, 44% of the births involving a male child were complicated compared with 31% of births involving a female child ($P = 0.01$).

Table III reports the frequency of obstetric and neonatal complications related to the first birth after the SRM diagnosis and to the second singleton birth in control women. Hypoxia, preterm delivery

Table II Obstetric and neonatal complications associated with the birth prior to the miscarriages in patients with unexplained SRM compared with the frequency of complications in the first birth in the control cohort (Danish Birth Registry para 0 1982–2005).

	Unexplained secondary recurrent miscarriage patients				Controls				Patients versus controls *P*-value
	Birth prior to the miscarriages				First birth				
	Total	Boys	Girls	*P*-value	Total	Boys	Girls	*P*-value	
Number (sex ratio)	358 (1.49)	214 (59.8%)	144 (40.2%)		608 068 (1.05)	311 977 (51.3%)	296 091 (48.7%)		0.001
	n (%)				*n* (%)				
Stillbirth[a]	19 (5.4)	11 (5.3)	8 (5.6)	0.92	2585 (0.43)	1308 (0.42)	1277 (0.43)	0.47	0.000
Pre-eclampsia	23 (6.4)	16 (7.5)	7 (4.9)	0.32	25 500 (4.2)	13 380 (4.3)	12 120 (4.1)	0.000	0.035
Placental abruption	8 (2.2)	7 (3.3)	1 (0.7)	0.15	5617 (0.9)	3152 (1.0)	2465 (0.8)	0.000	0.020
Severe haemorrhage	22 (6.1)	14 (6.5)	8 (5.6)	0.70	8644 (1.4)	4316 (1.4)	4328 (1.5)	0.01	0.000
Birthweight <2500 g[a]	46 (12.8)	33 (15.4)	13 (9.0)	0.06	32 248 (5.3)	15 998 (5.1)	16 250 (5.5)	0.000	0.000
Gestational age <37 weeks[a]	46 (12.8)	34 (15.9)	12 (8.3)	0.03	33 835 (5.6)	18 751 (6.0)	15 084 (5.1)	0.000	0.000
Hypoxia	36 (10.1)	22 (10.3)	14 (9.7)	0.86	61 871 (10.2)	34 160 (10.9)	27 711 (9.4)	0.000	0.941
All complications[b]	140 (39.1)	95 (44.4)	45 (31.3)	0.01	142,770 (23.5)	76 203 (24.4)	66 567 (22.5)	0.000	0.000
C-section	59 (16.5)	37 (17.3)	22 (15.3)	0.62	81.547 (13.4)	44 134 (14.1)	37 413 (12.6)	0.000	0.09
Mean birthweight, g (SD)		3216 (830)[a]	3273 (653)	0.49		3460 (581)	3351 (543)	0.000	Boys: 0.0001,[a] girls: 0.08

[a]Additional seven firstborn boys were stillborn with gestational week 24–27 and therefore excluded from this analysis.
[b]Complicated pregnancy or birth (stillbirth, pre-eclampsia, placental abruption, severe haemorrhage, birthweight <2500 g, gestational age <37 weeks and hypoxia).

and birthweight <2500 g more frequently affected the births of patients with SRM, whereas none of the other complications differed in frequency between patients and controls. More patients than controls were delivered by Caesarean section in their subsequent births ($P < 0.001$). In contrast to the preceding births, a complicated birth was more frequent if the patient delivered a girl (25%) than a boy (13%) ($P = 0.03$).

Sex ratio among children born prior and subsequent to SRM

We observed significantly more boys born to patients prior to SRM than in the control group. The sex ratio (male:female ratio) was 1.49 in the births prior to SRM diagnosis compared with 1.05 among the children delivered by the control group ($P = 0.001$). In contrast, more girls were born after the SRM diagnosis compared with the children born in the control cohort. The sex ratio after SRM was 0.76 compared with 1.06 in the control cohort ($P = 0.02$) and compared with the sex ratio among children born prior to the SRM diagnosis (0.76 versus 1.49, $P < 0.001$).

Birthweight

The mean birthweight of the boys born prior to the SRM diagnosis was not different from that of the preceding girls ($P = 0.49$) and was on average 244 g less than the mean birthweight of the boys born in the control group ($P < 0.0001$). Girls delivered to patients prior to the SRM diagnosis weighed on average 78 g less than the girls born in the control group ($P = 0.08$; Table II). Among the children born prior to SRM diagnosis, 44 (21%) of the boys and 21 (15%) of the girls were SGA (<10th percentile). Fourteen of the SGA boys and 11 of the girls did not suffer any of the other obstetric or neonatal complications.

The mean birthweight of boys born after SRM diagnosis was not different from the mean birthweight of boys born in the control group ($P = 0.35$), whereas girls born after the SRM diagnosis on average weighed 235 g less than the girls in the control group ($P < 0.0001$; Table III). Among the children born after the SRM diagnosis, 13 (11%) of the girls and 4 (4%) of the boys were SGA (<10th percentile).

Change of partner

Among the 76 patients who had changed partner, 42% had complications during birth prior to SRM diagnosis ($P = 0.51$) and 21% ($P = 0.76$) of the births after SRM. The sex ratio prior to SRM was 1.45 ($P = 0.91$) and after SRM 0.5 ($P = 0.2$) compared with those with the same partner. Accordingly, the main findings were unchanged if those who changed partner were excluded. We did not have information about change of partner for the control cohort.

Table III **Obstetric and neonatal complications associated with births after unexplained SRM compared with complications in second-born singletons in the control cohort (Danish Birth Registry para 1 1986–2008).**

	Unexplained secondary recurrent miscarriage patients				Controls				Patients versus controls, *P*-value
	Birth subsequent to the miscarriages				Second birth				
	Total	Boys	Girls	*P*-value	Total	Boys	Girls	*P*-value	
Number (sex ratio)	213 (0.76)	92 (43.2%)	121 (56.8%)		510 264 (1.06)	262 379 (51.4%)	247 885 (48.6%)		0.02
	n (%)				*n* (%)				
Stillbirth	1 (0.5)	0	1 (0.8)	1.0	1518 (0.3)	778 (0.3)	740 (0.3)	0.90	0.47
Pre-eclampsia	5 (2)	2 (2.2)	3 (2.5)	1.0	9500 (1.9)	4922 (1.9)	4578 (1.8)	0.44	0.60
Placental abruption	2 (0.9)	0	2 (1.7)	0.51	3966 (0.8)	2254 (0.9)	1712 (0.7)	0.000	0.68
Severe haemorrhage	1 (0.5)	0	1 (0.8)	1.0	5138 (1.0)	2585 (1.0)	2553 (1.0)	0.11	0.73
Birthweight <2500 g	17 (8.0)	3 (3.3)	14 (11.6)	0.03	16 108 (3.2)	7648 (2.9)	8460 (3.4)	0.000	0.000
Gestational age <37 weeks	17 (8.0)	3 (3.3)	14 (11.6)	0.03	18 972 (3.7)	10 524 (4.0)	8448 (3.4)	0.000	0.001
Hypoxia	18 (8.5)	6 (6.5)	12 (9.9)	0.38	24 158 (4.7)	13.734 (5.2)	10.424 (4.2)	0.000	0.02
All complications[a]	42 (19.7)	12 (13.0)	30 (24.8)	0.03	68 858 (13.5)	36 921 (14.1)	31 937 (12.9)	0.000	0.01
C-section	45 (21.1)	17 (18.5)	28 (23.1)	0.41	54 390 (10.7)	28 770 (11)	25 620 (10.3)	0.000	0.000
Mean birthweight, g (SD)		3572 (557)	3258 (784)	0.001		3627 (564)	3493 (535)	0.000	Boys: 0.35, girls: 0.0001

[a]Complicated pregnancy or birth (stillbirth, pre-eclampsia, placental abruption, severe haemorrhage, birthweight <2500 g, gestational age <37 weeks and hypoxia).

Impact of complicated pregnancies prior to the SRM diagnosis

As reported in Table IV, obstetric and neonatal complications were more frequent in births after SRM diagnosis if the birth prior to the miscarriages had been complicated (28%), compared with births following an uncomplicated first birth (16%) ($P = 0.03$; Table IV). Of the reported 61 complications associated with the births after the SRM diagnosis, 20 (33%) were the same complication as in the birth prior to SRM.

Obstetric complications and sex ratio in relation to gestational age of the miscarriages

Table V shows the frequency of complications and the sex ratios in births prior and subsequent to the SRM diagnosis according to the gestational age of the miscarriages. Patients with only late miscarriages delivered children with a sex ratio of 0.21 after the SRM diagnosis compared with a sex ratio of 1.0 in children delivered by patients with only early losses ($P = 0.009$).

Obstetric complications in births subsequent to SRM, sex of the firstborn child and maternal carriage of H-Y-restricting HLA class II

Among the 213 patients who gave birth after the series of miscarriages, 114 (54%) had a boy prior to the miscarriages; 110 patients (52%) carried no H-Y-restricting HLA class II alleles, 85 (40%) carried one allele and 18 (8%) carried two alleles. Among the children born after the miscarriages, 48 (42%) were boys with an older brother and 44 (44%) were boys with an older sister. Table VI compares obstetric details of births after SRM diagnosis according to the sex of the child preceding the diagnosis and further stratifies for whether the patient carried H-Y-restricting HLA class II alleles or not. Patients who carried H--Y-restricting HLA class II alleles and gave birth to a boy rather than to a girl prior to the miscarriages had an infant with an average 381 g lower mean birthweight ($P = 0.006$), mean gestation was 0.9 weeks shorter ($P = 0.06$) and stillbirth, placental abruption or pre-eclampsia affected 8% more births ($P = 0.05$). None of these comparisons differed in H-Y-restricting HLA class II-negative patients with a preceding boy compared with a girl (Table VI).

Discussion

We found increased frequencies of obstetric and neonatal complications in births both prior and subsequent to unexplained SRM. Boys were more frequent than girls born prior to SRM and these male births were significantly more complicated than birth of females. In contrast, girls were more frequent after the SRM diagnosis, and these births were more frequently complicated. These data suggest the existence of a male-specific factor triggering SRM and making male fetus pregnancies more likely to be miscarried after the first birth. A candidate for such a factor could be an abnormal

Table IV Obstetric and neonatal complications associated with the first birth after the SRM diagnosis stratified for complications associated with the birth prior to the series of miscarriages.

	Complications associated with births after unexplained secondary recurrent miscarriage					
	BW < 2500 g	**GA < 37 weeks**	**Placental abruption, pre-eclampsia or stillbirth**	**Hypoxia**	**Haemorrhage**	**At least one complication**
Birth prior to unexplained secondary recurrent miscarriage	*n* (%)					
Complicated, $n = 71$	11 (16)	10 (14)	4 (3)	8 (11)	0	20 (28)
Uncomplicated, $n = 142$	6 (4)	7 (5)	4 (6)	10 (7)	1 (0.7)	22 (16)
P-value	0.004	0.02	0.31	0.31	1.0	0.03

BW, birth weight; GA, gestational age.

Table V Obstetric and neonatal complications and sex (male:female) ratios associated with births prior and subsequent to the diagnosis of unexplained SRM stratified for the gestational age of the miscarriages.

Gestational age of all miscarriages	**Birth prior to SRM**	**Complicated birth prior to SRM**	**Sex ratio in births prior to SRM**	**Live birth subsequent to SRM**	**Complicated birth subsequent to SRM**	**Sex ratio in birth subsequent to SRM**
	n	***n* (%)**	**Boys/girls**	***n* (%)**	***n* (%)**	**Boys/girls**
Only early miscarriages <10 weeks	76	31 (41)	1.45	46 (61)	9 (20)	1.0*
Only late miscarriages ≥10 weeks	43	16 (37)	2.31	23 (53)	7 (30)	0.21*
Mix of early and late miscarriages	198	75 (38)	1.36	125 (63)	25 (20)	0.81
Incomplete data on gestation	41	18 (44)	1.56	19 (46)	1 (5)	0.90
All	358	140 (39)	1.48	213	42 (20)	0.76

*Only early miscarriages versus only late miscarriages, $P = 0.009$.

Table VI Obstetric complications associated with births subsequent to unexplained SRM related to the sex of the child prior to the diagnosis and maternal carriage of H-Y-restricting HLA class II.

	Sex of child born prior to miscarriages	Birthweight			Gestational age			Stillbirth, pre-eclampsia or placental abruption		
		Mean birthweight, g (SD)	Difference in g (95% CI)	*P*-value	Mean gestational age weeks (SD)	Difference in weeks (95% CI)	*P*-value	*n* (%)	Difference in % (95% CI)	*P*-value
Characteristics of the first birth following unexplained secondary recurrent miscarriage										
	BOY, $n = 114$	3304 (720)	194 (3, 385)	0.05	38.6 (2.5)	0.4 (−0.3, 1.1)	0.31	6 (5)	3 (−2, 11)	0.29
	GIRL, $n = 99$	3498 (689)			39.0 (2.4)			2 (2)		
Stratified for maternal carriage of H-Y-restricting HLA class II										
+HY-r	Boy, $n = 50$	3238 (783)	381 (113, 649)[a]	0.006	38.6 (2.6)	0.9 (0.02, 1.7)	0.06	4 (8)	8 (0, 19)	0.05
	Girl, $n = 53$	3619 (576)			39.5 (1.6)			0		
−HY-r	Boy, $n = 64$	3356 (669)	−3 (−280, 286)[b]	0.98	38.6 (2.3)	−0.2 (−1.2, 0.8)	0.65	2 (3.1)	1.2 (−7, 12)	1.0
	Girl, $n = 46$	3359 (786)			38.4 (2.9)			2 (4.3)		

+/− HY-r: maternal carriage or non-carriage of HLA class II alleles restricting anti-H-Y reactions.
[a]Difference in mean birthweight for girls 477 g, $P = 0.02$ and for boys 228 g, $P = 0.12$.
[b]Difference in mean birthweight for girls −2 g, $P = 0.99$ and for boys −12 g, $P = 0.94$.

immune reaction against male-specific minor histocompatibility H-Y antigens. We observed that patients carrying HLA alleles that restrict immunologic reactions against H-Y antigens who had delivered a boy prior to the miscarriages had increased frequency of obstetric complications in births after SRM compared with patients with a firstborn girl, which support the hypothesis of an underlying male-specific factor in SRM patients.

Although our cohort is the largest cohort of unexplained SRM patients described to date, numbers limit our results. Most of the addressed obstetric and neonatal complications are rare and the findings need to be confirmed or rejected in other cohorts. Furthermore, non-significant results in the comparisons related to firstborn boys and girls in H-Y-restricting HLA class II-negative patients may be due to lack of statistical power (Table VI). Neither can we exclude that chance could have contributed to some of the many significant comparisons. Another weakness of the present study is the lack of chromosome testing of the miscarriages, as this would have allowed even more precise categorizing of patients with true unexplained miscarriages. This is underscored by our main findings being amplified in patients with only late miscarriages—a subgroup most likely to have true unexplained miscarriages as chromosomal abnormalities decrease with increasing gestational age (Stephenson *et al.*, 2002); underscoring the need for chromosome testing of the miscarriages. Another limitation of this study is that reported outcomes are unadjusted. We cannot rule out differences between the patients and the controls that could influence our findings. However, the clinic is a nation-covering tertiary clinic and is the only clinic in Denmark solely focusing on women with recurrent miscarriage. It is a public clinic where all investigations and treatments are publicly funded and there is no charge to be paid by the patients. We therefore think that the patients derive from the same population as the controls in terms of urban/rural lifestyle, social class, ethnicity, education and occupation. The age of the patients giving birth after SRM is likely to be higher than the controls as patients had become older suffering recurrent miscarriage. The lack of age adjustment related to second births (Table III) may slightly influence our results (Altman *et al.*, 2002). Lastly, we compared all birth prior to SRM to the first birth of the control cohort, despite the fact that in 9% of the patients, the birth immediately prior to SRM diagnosis was not their first birth, which is expected to decrease the frequency of complications. However, 14 (45%) of these births were complicated (data not shown).

The strength of the present study is that it comprises a large well-characterized national cohort of patients referred to the Danish Recurrent Miscarriage Clinic with unexplained SRM with at least three consecutive miscarriages. The primary source of identifying complications in patients and controls was based on the unique personal ID number in the National Birth and Discharge Registry. All registry-based complications in patients were compared with primary record-confirmed complications reported by the patients. The National Discharge Registry has recently been shown to have a high specificity (99.5%) but a lower sensitivity (70%) in relation to the diagnosis of pre-eclampsia (Klemmensen *et al.*, 2007). In accordance with the findings of the latter study, we found only 23 of the 29 (80%) reported and confirmed pre-eclampsia cases in the National Discharge Registry.

Our observation that obstetric and neonatal complications in the first pregnancy are likely to reoccur in subsequent pregnancies, and

also predispose to the other complications in subsequent pregnancies, has been described in several obstetric cohorts (Black et al., 2008; Hernandez-Diaz et al., 2009; Lykke, et al., 2009; Rasmussen and Irgens, 2009). In contrast, the observed high proportions of boys born prior to and girls born subsequent to SRM diagnosis have not, to our knowledge, been reported previously. A sex ratio as high as the one we found prior to the series of miscarriages is to our knowledge only reported from countries with a strong tradition of preference for sons as in China (Zhu et al., 2009). Low sex ratios have been observed in populations exposed to severe stress such as severe peri-conceptional life events (Hansen et al., 1999), although not replicated in a recent study (Khashan et al., 2010). One explanation for the low sex ratio observed in children born subsequent to SRM could be emotional stress imposed by repeated pregnancy losses (Bagshi and Fridman, 1999).

Non-tolerated or aberrant immune responses against male-specific (H-Y) antigens on male fetal or trophoblast cells may explain our main findings. H-Y antigens are expressed as early as the 8-cell stage in mouse embryos (Krco and Goldberg, 1976). The antigens are ubiquitously expressed in human male cells including fetal and trophoblast cells (Warren et al., 2000) and maternal immune recognition of H-Y antigens can be demonstrated in women following pregnancies with boys (Verdijk et al., 2004). In the physiological situation of normal pregnancy, maternal immune recognition of H-Y antigens from male fetuses is generally considered to result in development of immune tolerance against H-Y antigens. In normal pregnancy, the maternal immune system is exposed to fetal cells as apoptotic trophoblast debris which is shed in large quantities (several grams per day) from the placenta in the last part of pregnancy (Huppertz et al., 2006; Adams et al., 2007). After being processed by macrophages, it is transported to local lymph nodes and presented to maternal lymphocytes (Adams et al., 2007). This presentation is thought to take place under non-inflammatory conditions, resulting in T lymphocytes being tolerized against fetal antigens (Steinman et al., 2003). However, in some situations, harmful immune responses against H-Y antigens are known to develop. The increased risk of graft-versus-host disease in the non-physiological situation of female-to-male stem cell transplantation is believed to be due to the frequent development of a cytotoxic immune response against H-Y antigens (Gratwohl et al., 2001). We speculate that some women in their first pregnancy with a boy are sensitized rather than tolerized to H-Y antigens and therefore subsequently experience recurrent miscarriage. If conditions at the feto-maternal interface and uterine regional lymph nodes are predominantly inflammatory (excess of inflammatory cytokines) in the first ongoing pregnancy with a boy, then presentation of fetal antigens to maternal antigen presenting cells may result in a harmful immunization instead of tolerance against H-Y antigens (Steinman et al., 2003). This study provides evidence that these first ongoing pregnancies with boys, in patients who later develop SRM, are influenced by inflammatory reactions. More than 40% of these births were associated with at least one of the obstetric or neonatal complications listed in Table II. These obstetric complications have been reported to be associated with increased maternal production of inflammatory cytokines (Gerber et al., 2005; Girardi et al., 2006; Germain et al., 2007) and increased shedding of apoptotic trophoblast cells (Goswami et al., 2006). Patients carrying the H-Y-restricting HLA class II alleles may be further sensitized against H-Y antigens. Such sensitization established in the first pregnancy with a boy may result in complete fetal rejection (miscarriage) or inflammatory reactions in the placenta or fetal membranes leading to obstetric complications in subsequent pregnancies.

We speculate that in pregnancies affected by H-Y immune responses, male fetuses are most severely affected resulting in the observed low sex ratio in births subsequent to SRM. However, were this response completely anti-H-Y-specific, we would expect all pregnancies subsequent to SRM diagnosis and following a firstborn boy, to be of a female infant. However, as this is not the case, it seems that the initial anti-H-Y response also affects subsequent female fetuses to some degree. Determinant spreading, a recognized and common feature in autoimmune diseases, might be responsible for the possible impact on pregnancies with girls (Lehmann et al., 1993; Ott et al., 2004). The H-Y-specific reaction may lose specificity with exposure or time and become directed towards non-sex-specific proteins on the fetus or trophoblast that have achieved immunogenicity due to the inflammatory process initiated by the anti-H-Y reaction, resulting in an increased incidence of complicated but surviving pregnancies with girls after SRM.

Patients with only late miscarriages are expected to have a lower frequency of aneuploid miscarriages than patients with only early miscarriages (Stephenson et al., 2002) and are thus more likely to have a non-chromosomal (for example immunological) aetiology for their miscarriages. SRM patients with only late miscarriages were characterized by a very high sex ratio (2.31) in the births before and a very low sex ratio (0.21) after the miscarriages, which may reflect that, especially in this subset of patients, anti-H-Y immunity may be the dominant pathogenic factor. In concordance with this theory is the finding that the sex ratios before and after the series of miscarriages in patients with a mixture of early and late miscarriages are intermediate compared with patients with exclusively early or late miscarriages (Table V). Three studies have previously reported on obstetric complications prior to the SRM diagnosis with main findings in accordance with this study (Christiansen et al., 1992; Weintraub et al., 2005; Yang et al., 2006). Weintraub et al. (2005), however, reported lower frequencies of placental abruption, vaginal haemorrhage and the sex ratio prior to the miscarriages, which may be due to the inclusion of patients with only two miscarriages, thereby increasing the chance of including patients with repeated aneuploid miscarriages and thus 'diluting' the estimate of a maternal risk factor in case–control studies (Christiansen et al., 2005).

Five studies have reported on obstetric and neonatal complications in pregnancies subsequent to the recurrent miscarriage diagnosis (Reginald et al., 1987; Hughes et al., 1991; Tulppala et al., 1993; Jivraj et al., 2001; Sheiner et al., 2005). Only one of these studies reported separately on the obstetric outcomes in SRM patients (Jivraj et al., 2001). Among 67 SRM patients, including those with explainable causes, the incidence of preterm birth was 8%, identical to our finding. Overall, the above-mentioned studies all report a high incidence of obstetric and neonatal complications subsequent to a series of miscarriages and convincingly conclude that the recurrent miscarriage population represent a population at high risk of obstetric problems with a need for close surveillance in late pregnancy. This study supports that conclusion.

In our control groups, pregnancies with boys compared with girls were more frequently complicated with placental abruption, pre-

eclampsia (only among firstborn children), preterm birth, hypoxia and Cesarean section, in accordance with previously reported observations (Kramer *et al.*, 1997; Basso and Olsen, 2001; Di Renzo *et al.*, 2007). This may suggest that the same factors that have been discussed as responsible for the obstetric problems related to firstborn boys among SRM patients also play a role in the background population, although in a lower scale.

In conclusion, we found that obstetric and neonatal complications are frequent in pregnancies preceding SRM and appear to be associated with an increased risk of complications in on-going pregnancies following the SRM diagnosis. Hereto, we found a high sex ratio prior and a low sex ratio subsequent to the SRM diagnosis. Maternal carriage of H-Y-restricting HLA class II and a history of firstborn boys, in contrast to girls, were associated with obstetric complications in births after the miscarriages. No such impact was found in relation to sex of the preceding child in SRM patients not carrying the H-Y-restricting HLA class II alleles. These novel findings need to be confirmed in other cohorts of unexplained SRM patients. Further exploring these possible underlying mechanisms may be decisive for immune treatment in patients with recurrent miscarriage.

Authors' roles

H.S.N. planned and designed the study, collected and analysed data, and drafted the manuscript. R.S. was involved in HLA typing and data analysis. M.L. and L.E. were involved in data collection and obtaining relevant permissions to perform the study. Ø.L. was involved in designing and extraction data on the control groups; A.-M.N.A. and L.H.M. were involved in data analysis and interpretation. O.B.C. was involved in study design, data collection and analysis as well as writing of the manuscript. All co-authors critically commented on the manuscript.

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