Secondary recurrent miscarriage and H-Y immunity

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TABLE OF CONTENTS

- Introduction
- Methods
- H-Y antigens, pregnancy and transplantation
  - Donor sex and transplantation outcome
  - H-Y antigens
  - Clinical relevance of H-Y antigens in transplantation
  - Immune priming against H-Y antigens in pregnancy
  - HLA restriction of H-Y antigen presentation
- SRM patients
- SRM patients and H-Y immunity
  - Epidemiologic characteristics of SRM
  - Immunogenetic characteristics of patients with SRM
  - Immunological characteristics of SRM patients
- Sex of prior children and subsequent reproductive performance in the background population
- The H-Y hypothesis in SRM
- Conclusion

BACKGROUND: Approximately half recurrent miscarriage (RM) cases remain unexplained after standard investigations. Secondary RM (SRM) is, in contrast to primary RM, preceded by a birth, which increases the transfer of fetal cells into the maternal circulation. Mothers of boys are often immunized against male-specific minor histocompatibility (H-Y) antigens, and H-Y immunity can cause graft-versus-host disease after stem-cell transplantation. We proposed the H-Y hypothesis that aberrant H-Y immunity is a causal factor for SRM.

METHODS: This is a critical review of the H-Y hypothesis based on own publications and papers identified by systematic PubMed and EMBASE searches.

RESULTS: SRM is more common after the birth of a boy and the subsequent live birth rate is reduced for SRM patients with a firstborn boy. The male:female ratio of children born prior and subsequent to SRM is 1.49 and 0.76 respectively. Maternal carriage of HLA-class II alleles presenting H-Y antigens to immune cells is associated with a reduced live birth rate and increased risk of obstetric complications in surviving pregnancies in SRM patients with a firstborn boy. In early pregnancy, both antibodies against HLA and H-Y antigens are increased in SRM patients compared with controls. Presence of these antibodies in early pregnancy is associated with a lower live birth rate and a low male:female ratio in subsequent live births, respectively. Births of boys are also associated with subsequent obstetric complications in the background population.

CONCLUSIONS: Epidemiological, immunogenetic and immunological studies support the hypothesis that aberrant maternal H-Y immune responses have a pathogenic role in SRM.

Key words: secondary recurrent miscarriage / H-Y antigen / pregnancy outcome / parity / pregnancy complications
Introduction

Recurrent miscarriage (RM) defined as three or more consecutive miscarriages affect 1–3% of females (Tulipala et al., 1993; Katz and Kuller, 1994). Approximately half of the cases remain unexplained following standard investigation (Quenby and Farquharson, 1993; Stephenson, 1994). Random chromosome errors such as trisomy, monosomy and polyplody, are responsible for 50–80% of miscarriages in the general reproductive population (Jacobs et al., 1987; Ohno et al., 1991; Morales et al., 2008) and these random events complicate the research of the etiology of RM. Important for the search of non-random causes of RM are the findings that the frequency of abnormal chromosomes in miscarriages decreases with increasing number of miscarriages (Ogasawara et al., 2000) and the risk for having a chromosomally normal miscarriage is increased after one chromosomally normal miscarriage (Warburton et al., 1987; Stephenson et al., 2002).

Distinguishing between primary RM (PRM) and secondary RM (SRM) may reduce the heterogeneity of RM patient populations. Approximately 40% of the women with RM have given birth to a child prior to the series of miscarriages and accordingly they are diagnosed with SRM (Jviraj et al., 2001; Christiansen et al., 2006). During pregnancy fetal cells enter the maternal circulation (Evans et al., 1999; Adams and Nelson, 2004) and in late pregnancy apoptotic syncytiotrophoblast debris is normally shed in large quantities (several grams per day) from the placenta (Huppertz et al., 2002). SRM is hence preceded by a possible priming of the immune system of the mothers that may theoretically lead to harmful immunological reactions against the semi-allogeneic fetus. A well-known example of immunization in an ongoing pregnancy that can cause harm in subsequent pregnancies is the production of maternal antibodies against the rhesus antigens on the fetus’ red cells causing fetal erythroblastosis. Similarly, it is possible that maternal immunization against male-specific minor histocompatibility (H-Y) antigens carried by a male fetus in a pregnancy that went to the third trimester may harm (in particular male) embryos and fetuses in subsequent pregnancies.

Maternal immune recognition of H-Y antigens has been demonstrated following pregnancies with boys (Verdijk et al., 2004; Piper et al., 2007; van Halteren et al., 2009). Anti H-Y immunity is held responsible for the increased risk of graft-versus-host disease (GvHD) in male recipients of stem-cell transplantation with female donors (Flowers et al., 1990; Gratwohl et al., 2001). Two placebo-controlled, randomized trials testing intravenous immunoglobulin treatment for RM in consecutive, eligible patients with four or more miscarriages, carried out at our clinic, found 34 (74%) of SRM patients had given birth to a boy prior to the miscarriages (Christiansen et al., 2002) suggesting that birth of a boy predisposes to SRM. On the basis of these findings, we proposed the H-Y hypothesis that aberrant H-Y immunity initiated in a prior long-lasting male fetus pregnancy is a causal factor for SRM. To test our H-Y hypothesis, we initiated a series of relevant studies in 2003 based on the SRM patients from the Danish Recurrent Miscarriage Clinic in collaboration with Dutch and US laboratories specialized in H-Y immunity as well as The National Institute of Public Health, Denmark. This review presents and critically discusses: (i) studies addressing the association between female donor pregnancy history and the risk of GvHD in allogeneic stem-cell recipients and the immunological priming of females against H-Y antigens as a result of pregnancy; (ii) epidemiological, immunogenetic and immunologic studies in patients with SRM and population-based studies testing the H-Y hypothesis; (iii) detailing the hypothesis followed by a discussion of strengths, limitations and perspectives of the findings.

Methods

A critical review of the current literature forming and testing the H-Y hypothesis in SRM patients was performed. The review is based on own publications and papers from other groups identified by systematic searches of the PubMed (1968–2010) and EMBASE (1980–2010) databases and identifying relevant studies published in English. In addition abstracts from ESHRE meetings were checked and reference lists of identified papers. The latest search was done August 2010. The following MeSH terms were used: H-Y antigen, female, sex factors, risk factors, transplantation, GvHD, pregnancy, parity, pregnancy complications, abortion habitual, abortion placentae, birth order and siblings. This review is based mainly on human studies and animal studies when human studies were lacking.

H-Y antigens, pregnancy and transplantation

Donor sex and transplantation outcome

Allogeneic hematopoietic stem-cell transplantation has proved to be a curative therapy for patients with hematological malignancies, though associated with high morbidity and mortality. Donor factors affecting morbidity and mortality have been studied in details to improve transplantation outcomes. Female donors are reported to increase the risk of GvHD (Storb et al., 1977; Bross et al., 1984; Randolph et al., 2004) and transplant related mortality (Gratwohl et al., 1998, 2001). Pregnancy-induced alloimmunization was hypothesized as the underlying mechanisms and female donor pregnancy history and GvHD was thus investigated. Table I gives an overview of human studies addressing an association of the pregnancy history of female donors and the risk of acute and/or chronic GvHD. Despite no clear distinction between parity and gravidity, pregnancy history in female donors was shown to increase the risk of acute (Atkinson et al., 1986; Gale et al., 1987; Flowers et al., 1990; Nash et al., 1992) and chronic GvHD (Atkinson et al., 1990; Carlens et al., 1998; Kollman et al., 2001; Remberger et al., 2002; Loren et al., 2006) compared with other sex combinations of recipients and donors, except from one small study (Przepiorka et al., 1999). Atkinson et al. and Kollman et al. showed a dose–response association between increased number of pregnancies among female donors and increased risk of acute GvHD and chronic GvHD, respectively (Atkinson et al., 1986; Kollman et al., 2001). The majority of the studies referred to in Table I was performed on HLA-matched siblings so the development of GvHD was triggered by non-HLA differences between donor and recipient. Male-specific minor histocompatibility (H-Y) antigens were accordingly suggested to be such targets.

H-Y antigens

Genes on the Y chromosome encode H-Y antigens. Each of these H-Y genes has an X chromosome homolog that is more than 85% identical
Table I Human studies addressing an association between female donor pregnancy history and the risk of GvHD in allogeneic stem cell recipients.

<table>
<thead>
<tr>
<th>Author and year</th>
<th>Patient (Recipients), n</th>
<th>Female donors, n (%)</th>
<th>Pregnancy history of female donors</th>
<th>Allocation of the female donors in relation to pregnancy history</th>
<th>Previous pregnancy and risk of aGvHD</th>
<th>Results (statistical parameters)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td>Unadjusted analysis</td>
</tr>
<tr>
<td>Atkinson et al. (1986)</td>
<td>40</td>
<td>19 (48)</td>
<td>FP (n = 9, total of 37 pregnancies)</td>
<td>Data not provided</td>
<td>Increased severity with increasing number of pregnancies</td>
<td>Increased</td>
</tr>
<tr>
<td>Gale et al. (1987)</td>
<td>2036</td>
<td>925 (46)</td>
<td>FP# (n = 231), FNP# (n = 517), No pregnancy data (n = 177)</td>
<td>FNP# to F (n = 208), FNP# to M (n = 309), FP# to F (n = 112), FP# to M (n = 119)</td>
<td>Increased % aGvHD, FNP# to F 34%, FNP# to M 46%, FP# to F 39%, FP# to M 66%</td>
<td>FNP# to M compared with FNP# to F: RR 2.0, $P = 0.01$; FP# to M compared with FP# to F: RR 2.9, $P = 0.0001$</td>
</tr>
<tr>
<td>Flowers et al. (1990)</td>
<td>136</td>
<td>60 (44)</td>
<td>FP (n = 30), FNP (n = 30)</td>
<td>FNP to F (n = 10), FNP to M (n = 20), FP to F (n = 11), FP to M (n = 19)</td>
<td>Increased % aGvHD, FNP to F 20%, FNP to M 20%, FP to F 45%, FP to M 63%</td>
<td>FP compared with FNP, RR 2.54 (1.15–5.64), $P = 0.02$</td>
</tr>
<tr>
<td>Atkinson et al. (1990)</td>
<td>2534</td>
<td>1158 (46)</td>
<td>FP# (n = 354)</td>
<td>Data not provided</td>
<td>Increased</td>
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<tr>
<td>Nash et al. (1992)</td>
<td>446</td>
<td>198 (44)</td>
<td>FP (n = 106), FNP (n = 92)</td>
<td>FNP to F (n = 41), FNP to M (n = 51), FP to F (n = 47), FP to M (n = 59)</td>
<td>Increased % aGvHD, FNP to F 15%, FNP to M 28%, FP to F 32%, FP to M 54%</td>
<td>Comparing FNP to F with: FNP to M: RR 1.88 (0.72–4.89), FP to F: RR 2.41 (0.93–6.22), FP to M: RR 3.85 (1.58–9.37)</td>
</tr>
<tr>
<td>Carlens et al. (1998)</td>
<td>451</td>
<td>210 (47)</td>
<td>FP# (n = 61)</td>
<td>FNP# to M (n = 14), FP# to M (n = 61)</td>
<td>Increased % cGvHD, FP# to M 61%, All other recipients 40%</td>
<td>FP# to M compared with all other donor/recipient combinations, RH 1.70 (1.17–2.48), $P = 0.006$</td>
</tr>
<tr>
<td>Przepiorka et al. (1999)</td>
<td>160</td>
<td>79 (49)</td>
<td>FP# (n = 62)</td>
<td>FNP# to M (n = 13), FP# to M (n = 30)</td>
<td>No difference in relation to pregnancy history</td>
<td></td>
</tr>
<tr>
<td>Kollman et al. (2001)</td>
<td>6798</td>
<td>2945 (43)</td>
<td>FNP (n = 1140), FP 1 pregnancy, (n = 416), FP ≥ 2 pregnancies (n = 1358), No pregnancy data (n = 31)</td>
<td>Data not provided</td>
<td>No difference in relation to pregnancy history</td>
<td>Increased % cGvHD when donor: M 44%, FNP 47%, FP1 pregnancy 51%, FP ≥ 2 pregnancies 54%</td>
</tr>
<tr>
<td>Remberger et al. (2002)</td>
<td>679</td>
<td>312 (46)</td>
<td>Data not provided</td>
<td>FNP# to M (n = 94), FP# to M (n = 81)</td>
<td>Increased % cGvHD, FP# to M 17%, All other combinations 8%</td>
<td>FP# to M compared with all other donor/recipient combinations, RH 2.16 (1.14–4.11), $P = 0.02$</td>
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</table>
at the amino acid level (Lahn and Page, 1997). H-Y antigens were described when it was observed that male skin grafts were rejected by syngenic female mice (Eichwald and Silmser, 1955). The H-Y antigen is expressed as early as the 8-cell stage in mouse embryos (Krco and Goldberg, 1976). Generally, the H-Y antigens are ubiquitously expressed in male cells, including fetal and trophoblast cells (Warren et al., 2000).

**Clinical relevance of H-Y antigens in transplantation**

H-Y-specific CD8+ cytotoxic T lymphocytes (T_{CTL}) were first described in humans more than three decades ago. A strong post-transplant T_{CTL} response specific for male donor HLA-matched target cells was found in the peripheral blood lymphocytes of a female patient who rejected the bone marrow of her HLA-identical brother (Goulmy et al., 1976). Subsequently, H-Y-specific T_{CTL} were found to increase during GvHD in sex-mismatched stem-cell transplants (Mutis et al., 1999). In the latter study, donors were also tested and some were observed to have substantial levels of anti-H-Y T_{CTL}. All were female bone marrow donors and the relatively high levels of circulating H-Y-specific T_{CTL} were suggested to be the result of either pregnancies with boys or prior blood transfusions. Also, H-Y-specific CD4+ T cells have been found to be crucial in females rejecting male grafts (Zelenika et al., 1998; Spierings et al., 2003), as well as in sex–mismatched GvHD (van Els et al., 1990a; Faber et al., 1995). A potent B cell response against the H-Y antigen DBY has been shown in 50% of male patients who received stem cells from female donors as they developed a high-titer antibody response to the HY-protein DBY (Miklos et al., 2004). Evidence that the immunogenicity of H-Y antigens results in a coordinated response involving B cells and T cells was subsequently provided (Zorn et al., 2004). Finally, the presence of H-Y antibodies to one or more of five recombinant H-Y antigens correlated with chronic GvHD in male patients with female donors (Miklos et al., 2005). Thus, both cellular and humoral H-Y immunity is associated with GvHD in male recipients of female donors.

**Immune priming against H-Y antigens in pregnancy**

Fetal cells can cross the placenta during pregnancy and male DNA is detectable in the maternal circulation both during and after an ongoing pregnancy and can persist in the circulation up to 27 years post-partum (Bianchi et al., 1996, 2001). Early reports identified H-Y-specific T_{CTL} in women in whom pregnancy was the only possible exposure to H-Y antigens (Singal et al., 1981; Tekolf and Shaw, 1983). Further support for pregnancy as a source of inducing H-Y-specific T_{CTL} has been given recently (Table II). James et al. (2003) detected measurable levels of circulating H-Y-specific T_{CTL} that readily expanded in vitro in a multiparous female donor who had been exposed to H-Y antigens in her three pregnancies with male fetuses compared with a nullipara woman. Verdijk et al. (2004) found functional H-Y-specific T_{CTL} of a memory phenotype in two of six healthy female donors who had given birth to boys 20 and 22 years previously; H-Y microchimerism was also observed in one of these females. Piper et al. (2007) tested 35 female donors and demonstrated functional H-Y-specific T_{CTL} responses in 37% of the women who had given birth to boys and the prevalence increased to 50% in
women with two or more prior births of boys. Thus, pregnancies with male fetuses can prime for H-Y-specific immunity. However, not all women with a previous birth of a boy developed cytolytic activity against H-Y antigens (Verdijk et al., 2004; Piper et al., 2007). A recent study focused on whether natural exposure to fetal minor histocompatibility alloantigens from prior male fetus pregnancies induces different T cells in healthy parous female donors. The presence of functionally different types of H-Y-specific CD8+ T cells, i.e. T regulatory cells (TREG) and T cells was studied (van Halteren et al., 2009). Indeed, H-Y-specific TREG were identified in five of 10 female donors. Functional H-Y-specific TREG were detected in four of the remaining healthy female donors with a previous birth of a boy and these women were classified as ‘tolerant to H-Y’ in contrast to females with predominantly TCTL, were classified as ‘H-Y sensitized’ (van Halteren et al., 2009). It remains to be established what causes some women to be sensitized instead of tolerant to H-Y antigens. A recent study found maternal immunity against antigens in the seminal fluid but not in semen (Moldenhauer et al., 2009), whether unprotected sexual intercourse can prime anti H-Y responses is unknown.

**HLA restriction of H-Y antigen presentation**

Isolation of H-Y-specific T cell clones has identified HLA class I and II alleles that restrict the presentation of the epitopes. To date, the following HLA alleles have been reported to functionally present H-Y peptides and will in the following be referred to as H-Y-restricting HLA class I alleles: HLA-A*01, -A*02, -A*33, -B*07, -B*08, -B*52, -B*60 and H-Y-restricting HLA class II alleles: HLA-DRB1*15, -DQB1*0501/2, -DRB3*03 (Hambach et al., 2007).

**SRM patients**

Differentiation between PRM and SRM seems relevant, as there are several significant differences between these two subsets of RM. The frequency of abnormal embryonic karyotypes is significantly lower in patients with SRM compared with PRM (Coulam et al., 1996). Immunotherapy with intravenous immunoglobulin has had no demonstrable effect for PRM patients but has improved live birth rates in SRM (Hutton et al., 2007). However, a new randomized placebo-controlled trial on intravenous immunoglobulin in unexplained SRM did not find a significant higher live birth rate in the treated group (Stephenson et al., 2010). This difference in immunotherapy efficacy suggests that immunologic disturbances are more pronounced in SRM, or that immunological disturbances in the two subsets of patients are different. Disturbances in adaptive immunity may play a role in SRM while disturbances in innate immunity may be of importance in PRM (Christiansen et al., 2008). This is supported by a few large studies. SRM patients, in contrast to PRM, carry the immunologically high responder alleles HLA-DRB1*03 (Kruse et al., 2004) and HLA-DRB1*15 (Takakuwa et al., 2003) more frequently. These alleles may present trophoblast-derived peptide to maternal autoreactive T cells or the association to SRM may be caused by linkage disequilibrium to alleles in other loci in the HLA region predisposing to hypersecretion of cytokines with embryo-toxic or trophoblast-inhibiting activity (Raghupathy, 1997). Alternatively, the association is a result of HLADRBI*03 in linkage disequilibrium with

<table>
<thead>
<tr>
<th>Table II</th>
<th>Human immunological studies investigating whether pregnancies with male fetuses primes immune responses directed against H-Y antigens.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author and Year</strong></td>
<td><strong>Study population</strong></td>
</tr>
<tr>
<td>James et al. (2003)</td>
<td>Six female donors who had given birth to boys and one non-pregnant woman</td>
</tr>
<tr>
<td>Verdijk et al. (2004)</td>
<td>Six HLA-A2 positive female donors, of whom four had given birth to boys and two were non-pregnant women</td>
</tr>
<tr>
<td>Piper et al. (2007)</td>
<td>35 female donors: 4 nulliparous, 4 only given births to girls, 19 given birth to one boy and, 8 given birth to minimum 2 boys. Last delivery 2 month-8 years before testing</td>
</tr>
<tr>
<td>van Halteren et al. (2009)</td>
<td>10 female donors with H-Y-mismatched offspring</td>
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</table>
a 14-base pair sequence polymorphism in the HLA-G gene, which is associated with RM (Hviid and Christiansen, 2005; Kolte et al., 2010).

**SRM patients and H-Y immunity**

Table III gives an overview of the studies testing (or relevant for testing) the H-Y hypothesis that aberrant maternal H-Y immunity is a causal factor in SRM. These studies are described and discussed in details in the following sections.

**Epidemiologic characteristics of SRM**

*Impact of the sex of children born prior to the SRM diagnosis*

Two studies on strictly unexplained SRM patients with three or more miscarriages have found SRM more frequently preceded by birth of a boy than a girl, which suggests that firstborn boys or associated factors represent a risk factor for SRM (Christiansen et al., 2004; Nielsen et al., 2008a). One smaller study found that 27 (47%) boys preceded two or more consecutive miscarriages (Weintraub et al., 2005). The latter study included patients not categorized as unexplained and with only two prior losses which may ‘dilute’ the estimate of risk factors in case–control studies (Christiansen et al., 2005). The studies by Christiansen et al. and Nielsen et al. also explored whether the sex of children born prior to the SRM diagnosis impacts the chance of a live birth after SRM. Patients who gave birth to a boy compared with a girl prior to SRM had a significantly reduced chance of a live birth both in the first pregnancy after referral (Nielsen et al., 2008a) and long-term cumulative chance (Christiansen et al., 2004). Thus prior birth of a boy is a risk factor for unexplained SRM and the negative association to the subsequent chance of a live birth suggests a causal relationship.

**Sex ratio**

The sex ratio (male:female ratio) prior and subsequent to SRM has been investigated in a 20-year cohort of unexplained SRM patients (Nielsen et al., 2010b). The sex ratio among children born prior to SRM was 1.49 compared with 0.76 in subsequent births, \( P < 0.0001 \). Both sex ratios differed significantly from the sex ratios in the control populations. The sex ratios were even more skewed in patients expected to have a low incidence of aneuploid conceptions as all their miscarriages were at gestational Week 10 or more. The sex ratio of births prior and subsequent to this subgroup of SRM patients was 2.31 and 0.21, respectively. These data suggest the existence of a male-specific factor triggering SRM and making pregnancies with a male fetus more likely to be miscarried after the first birth.

This is at odds with results from several studies which examined the chromosome results of miscarriages from RM patients (not differentiating between SRM and PRM) that found an excess of female miscarriage products (Halder and Fauzdar, 2006; Kano et al., 2009). The excess of female miscarriage products in these studies is most likely the result of maternal contamination, which is supported by a recent large scale study where microsatellite testing were undertaken in all miscarriage samples with 46 XX and the corresponding maternal DNA leaving only true fetal samples. This normalized the sex ratios among the miscarried embryos from patients with RM (no differentiation between PRM and SRM) (Stephenson et al., 2009). To support the belief that euploid male embryos are at an increased risk of miscarriage compared with female embryos are the results from a large study of the anatomic sex ratio of 662 miscarried singleton embryos and fetuses that found the sex ratio was 1.30 (299 boys:230 girls) among miscarried fetuses with normal anatomy whereas the sex ratio was 0.92 (59 boys:64 girls) among malformed miscarried fetuses (Byrne and Warburton, 1987).

Sex ratio as high as observed prior to the series of miscarriages is reported from countries with a strong tradition of preference for sons, for example China (Zhu et al., 2009). Low sex ratios have been observed in populations exposed to severe stress such as severe preconceptional life events (Hansen et al., 1999) although not replicated in a recent study in a stable western population (Khoshhal et al., 2009). Emotional stress imposed by repeated pregnancy losses (Bagsh and Fridman, 1999) may explain the low sex ratio after SRM. We found the sex ratio subsequent to unexplained PRM 1.18 in a recent study on pregnancy outcome according to thrombophilia in RM ([Lund et al., 2010], sex ratio data not shown in article), which speaks against the stress hypothesis explaining the low sex ratio in births subsequent to SRM. The different sex ratio after PRM and SRM suggests different mechanisms behind the two types of RM.

**Obstetric characteristics of birth prior and subsequent to the SRM diagnosis**

Obstetric details regarding births prior and subsequent to SRM may contribute to the understanding of unexplained SRM. Four studies have reported on obstetric characteristics of the birth preceding the SRM diagnosis. Birth prior to SRM were characterized by lower than expected birthweight (Christiansen et al., 1992), a higher than expected frequency of pre-eclampsia (Weintraub et al., 2005) and fetal death (Yang et al., 2006). The largest study found stillbirth, pre-eclampsia, placental abruption, severe hemorrhage, birthweight \(<2500 \text{ g}\) and preterm birth more frequent in births prior to unexplained SRM than in firstborn singleton controls \( (P < 0.04; \text{Nielsen et al., 2010b}) \). Among SRM patients, significantly more births were complicated if the child was a boy compared to a girl (44% versus 31%, \( P = 0.01 \)). The higher frequency of obstetric complications in male fetus pregnancies preceding the miscarriages was also reflected in the mean weight of boys born prior to SRM which was 244 g lower \((P = 0.0001)\) than the mean birthweight of boys born in the control group while this difference was 78 g for girls (Nielsen et al., 2010b). Thus, a large proportion of births prior to SRM and particularly those of boys are severely complicated. Prior obstetric complications potentially influences subsequent pregnancies as the complications are associated with increased production of inflammatory cytokines, systemically or locally in uterus (Gerber et al., 2005; Girardi et al., 2006; Germain et al., 2007). Fetal antigens in the maternal circulations are thus to be presented to the maternal immune system under inflammatory conditions possible causing maternal sensitization instead of tolerance towards the paternally inherited antigens (Steinman et al., 2003).

Obstetric complications in births after SRM have been described in two studies. Jivraj et al. observed preterm birth, perinatal mortality and small for gestational age to be more frequent among 67 children born after the SRM diagnosis although only the latter reached statistical significance (Jivraj et al., 2001). The other study found increased frequencies of hypoxia, preterm delivery and birthweight \(<2500 \text{ g}\) among 213 births after unexplained SRM compared with second-born singleton controls (Nielsen et al., 2010b). In contrast to pregnancies before SRM, births of girls after SRM were more often complicated than
### Table III  Studies in SRM patients testing (or relevant for testing) the H-Y hypothesis that aberrant maternal H-Y immunity is a causal factor for SRM.

<table>
<thead>
<tr>
<th>Author and year</th>
<th>Study design</th>
<th>Included subjects</th>
<th>Male:Female ratio</th>
<th>Objectives</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epidemiological observation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jivraj et al. (2001)</td>
<td>Case–control study</td>
<td>67 unexplained and explained</td>
<td></td>
<td>No information</td>
<td>Compare the frequency of obstetric complication subsequent to RM to controls in the background population</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95 explained and unexplained PRM 24 699 singleton deliveries</td>
<td>1.53 0.84 (110:72) (54:64)</td>
<td>Live birth in follow-up period (2–16 years) after SRM according to sex of child born prior to SRM</td>
<td>After SRM: preterm birth 8% versus 4%, P = 0.19, SGA 13% versus 2%, P = 0.001, perinatal mortality 3% versus 1%, P = 0.15</td>
</tr>
<tr>
<td>Christiansen et al. (2004)</td>
<td>Cohort study</td>
<td>182 unexplained</td>
<td></td>
<td></td>
<td>Reduced chance of a live birth if boy compared with girl before SRM Adjusted Hazard Ratio: 0.59 (0.41–0.86)</td>
</tr>
<tr>
<td>Wentraub et al. (2005)</td>
<td>Case–control study</td>
<td>58 unexplained and explained. Included patients with only two miscarriages</td>
<td>0.87 (27:31)</td>
<td></td>
<td>Pregnancy complications in live birth prior to SRM</td>
</tr>
<tr>
<td>Yang et al. (2006)</td>
<td>Cohort study</td>
<td>675 unexplained and explained</td>
<td>539 PRM, 431 female controls</td>
<td>No information</td>
<td></td>
</tr>
<tr>
<td>Nielsen et al. (2008a)</td>
<td>Cohort study</td>
<td>305 unexplained</td>
<td>1.52 0.85 (184:121) (71:84)</td>
<td></td>
<td>Live birth in first pregnancy after referral for SRM according to sex of child born prior to SRM</td>
</tr>
<tr>
<td>Nielsen et al. (2010b)</td>
<td>Cohort study</td>
<td>358 unexplained [358 birth prior to SRM, 213 singleton birth after SRM (by 2008)]</td>
<td>1.49 0.76 (214:144) (92:121)</td>
<td></td>
<td>Compare the frequency of obstetric complications prior and subsequent to SRM to controls in the background population</td>
</tr>
<tr>
<td></td>
<td>All Danish women given birth to a first born singleton 1982–2005 (608,068)</td>
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<td>Complicated birth more frequent before SRM 39% versus 24% in firstborn singletons, P &lt; 0.0001</td>
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<td>All Danish women given birth to a second born singleton 1986–2008 (510,264)</td>
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<td>Complicated birth more frequent after SRM 20% versus 14% in second born singletons, P = 0.01</td>
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<td>Complicated birth more frequent if boys before SRM 44% versus 31%, P = 0.02 and girls after SRM 25% versus 13%, P = 0.03</td>
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<td>Sex ratio 2.31 before and 0.21 after SRM for patients with all losses ≥ gestational Week 10</td>
<td></td>
</tr>
</tbody>
</table>
### Immunogenetic observations

<table>
<thead>
<tr>
<th>Authors</th>
<th>Study Design</th>
<th>Description</th>
<th>Frequencies</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nielsen et al.</td>
<td>Case-control</td>
<td>Eight consecutive patients with recurrent (≥2) placental abruption (RPA) identified in a national cohort (1986–2005) of SRM patients or patients with second-trimester losses or stillbirth</td>
<td>37 fertile women from couples with a firstborn boy and two or more children and a history of no miscarriages and no PA</td>
<td>Compare the frequency of HY-r HLA class II&lt;sup&gt;a&lt;/sup&gt; in patients with RPA and healthy fertile controls (7.0:1) and (1.33:4.3)</td>
</tr>
<tr>
<td>Nielsen et al.</td>
<td>Cohort</td>
<td>358 unexplained + 203 children born before SRM</td>
<td>1.49</td>
<td>Live birth in first pregnancy after referral for SRM according to maternal presence or absence of HY-r HLA and sex of child born prior to SRM (214:144)</td>
</tr>
<tr>
<td>Nielsen et al.</td>
<td>Cohort</td>
<td>213 unexplained who gave birth after SRM</td>
<td></td>
<td>Frequency of obstetric complications in birth after SRM according to maternal presence or absence of HY-r HLA and sex of child born prior to SRM</td>
</tr>
<tr>
<td>Nielsen et al.</td>
<td>Case-control</td>
<td>84 unexplained PRM</td>
<td>12 unexplained PRM 37 non-RM healthy women</td>
<td>Compare the frequency of H-Y antibodies and correlate the presence of these antibodies in early pregnancy to pregnancy outcome</td>
</tr>
</tbody>
</table>

### Immunological observations

<table>
<thead>
<tr>
<th>Authors</th>
<th>Study Design</th>
<th>Description</th>
<th>Frequencies</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nielsen et al.</td>
<td>Case-control</td>
<td>84 unexplained</td>
<td></td>
<td>Patients who tested H-Y antibody positive in early pregnancy gave birth to 12% boys compared with 44% boys in H-Y antibody negative patients, <em>P</em> = 0.03</td>
</tr>
</tbody>
</table>

Continued
Table III

<table>
<thead>
<tr>
<th>Author and year</th>
<th>Study design</th>
<th>Included subjects</th>
<th>Other patients/controls</th>
<th>SRM patients</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nielsen et al. (2010c)</td>
<td>Case-control study</td>
<td>56 unexplained RM</td>
<td>24 non-RM healthy parous women</td>
<td>37 controls with no pregnancy losses</td>
<td>HLA antibodies were more frequent in patients with SRM than in the control group (P = 0.002) and among patients who tested HLA antibody positive in early pregnancy had a reduced chance of a live birth compared with HLA-antibody negative (41% versus 76%) OR: 0.22 (0.07–0.68), P = 0.008</td>
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</table>

Impact of H-Y-restricting HLA

H-Y peptides can be presented to CD8⁺ T~\text{CTL}~ by HLA class I (present in almost all cells) resulting in direct destruction of the cell to which it is bound. Alternatively, H-Y peptides are presented to CD4⁺ T helper lymphocytes by HLA class II on antigen presenting cells resulting in the T cell secreting lymphokines followed by both antibody formation and activation of T~\text{CTL}~. Three studies have explored the hypothesis of aberrant maternal H-Y immunity as an underlying mechanism in SRM and recurrent placental abruptions based on patient carriage of HLA class I and II alleles known to present H-Y antigens (Nielsen et al., 2007, 2009, 2010b). The first study identified eight patients who had experienced recurrent severe placental abruptions in an 18-year national cohort of patients with SRM or repeated second-trimester losses (Nielsen et al., 2007). The patients had a total of 22 placental abruptions in 18 of which the fetus died. Fifteen (68%) of the placental abruptions involved male fetuses. Seven of the patients had a firstborn boy. The frequency of H-Y-restricting HLA class II alleles among patients were compared with healthy parous controls. Haplotype with H-Y-restricting class II alleles comprised 64% of the HLA haplotypes in the seven patients compared with only 28% among 37 controls with no pregnancy losses (P = 0.009). Thus, carriage of H-Y-restricting HLA class II is associated with the rare and distressing condition of recurrent severe placental abruption in addition to recurrent pregnancy losses.

The second study associated carriage of H-Y-restricting HLA to the prospective chance of a live birth after referral for unexplained SRM (Nielsen et al., 2009). Maternal carriage of H-Y-restricting HLA class II alleles significantly reduced the chance of a live birth in SRM patients with a firstborn boy [odds ratio (OR): 0.17 (0.1–0.4), P = 0.001] compared with those with a firstborn girl. Among patients with a boy prior to the miscarriages the chance of a live birth was reduced in a dose–response manner; thus, maternal carriage of one H-Y-restricting HLA class II alleles reduced the chance of a live birth [OR: 0.46 (0.2–0.9), P = 0.02], while carriage of two alleles resulted in an even further reduction [OR: 0.21 (0.1–0.7), P = 0.02] compared with those with no H-Y-restricting HLA class II alleles. Live birth rate was not different according to the sex of the child born prior to SRM in patients without H-Y-restricting HLA class II alleles. No reduction in chance of a live birth was found when limiting the analysis to the cases where the firstborn child but not the mother carried a H-Y-restricting HLA class II allele (Nielsen et al., 2009).

The last study tested maternal carriage of H-Y-restricting HLA class II and obstetric complications in 213 births after unexplained SRM (Nielsen et al., 2010b).

The mean birthweight was 381 g lower (P = 0.006), the gestation 0.9 week shorter (P = 0.06), and the risk of stillbirth, pre-eclampsia and placental abruption increased (P = 0.05) in SRM patients with births of boys, 25% versus 13%, P = 0.03, which is also reflected in the mean birthweight of girls born after the miscarriages being 235 g less (P = 0.0001) compared with the control group. The RM population represents a population at high risk of obstetric problems with a need for close surveillance and girls are more likely to survive albeit with a higher frequency of complications.
H-Y-restricting HLA class II alleles and a boy rather than a girl before the miscarriages. No difference in frequency of obstetric complications were found according to sex of the first child in patients without H-Y-restricting HLA class II alleles (Nielsen et al., 2010b). Thus, maternal carriage of H-Y-restricting HLA class II is associated with a reduced chance of a subsequent live birth in a dose–response manner among SRM patients with firstborn boys. If a subsequent birth is obtained after SRM maternal carriage of H-Y-restricting HLA class II is associated with obstetric complications in patients who, prior to the miscarriages, gave birth to a boy.

These results indicate that an aberrant maternal immune reaction against fetal H-Y antigens plays a role in SRM. Of note is the observation that birth of a boy prior to SRM only seems to impact future pregnancy outcome if the patient carries the H-Y-restricting HLA class II as no difference in outcome was observed according to sex of first child in patients without these alleles. HLA class II but not class I H-Y-restricting alleles impact the pregnancy prognosis which may reflect the participation of CD4+ T cells providing help for the CD8+ cytotoxic T-cells in their response against H-Y antigens (Fig. 1). Presence of CD4+ T cells with anti-recipient activity rather than CD8+ T cells was earlier reported to increase the risk of GvHD or graft rejection (van Els et al., 1990b; Zelenika et al., 1998; Spierings et al., 2003). Future studies may identify more HLA class I and II alleles, that may be able to present the various H-Y antigens. Those included in the analysis cannot be considered exhaustive and identification of other H-Y-restricting HLA alleles may alter the results. However, it is possible that the number of H-Y-restricting HLA class II alleles is limited and that the currently identified are the dominant ones (van Els et al., 1992; Hambach et al., 2007).

### Immunological characteristics of SRM patients

#### HLA antibodies

Maternal recognition of fetal (paternal) antigens reflected by the presence of HLA antibodies in maternal blood are found in approximately one-third of normal successful pregnancies (Ahrons, 1971; Balasch et al., 1981; Regan et al., 1991) while patients with PRM are found to have a prevalence of HLA antibodies of maximum 10% (Beard et al., 1983; Power et al., 1983; Johnson et al., 1984). This difference in prevalence has been taken as an indirect proof of the hypothesis that a failure to produce HLA antibodies was part of the underlying cause of PRM (Beard et al., 1983). SRM patients on the other hand may have high prevalence of HLA antibodies (McIntyre et al., 1984). Recent studies by Steinborn et al. found an association between an increased prevalence of HLA antibodies and both gestational diabetes and placental abruption, suggesting increased humoral immune response of the mother against the fetus as part of the pathogenesis of the conditions (Steinborn et al., 2004, 2006). A recent study investigated HLA-antibodies in patients with SRM and controls (Nielsen et al., 2010c). HLA class I and/or class II antibody responses were significantly more frequent in SRM patients with a boy prior to the series of miscarriages (62%) compared with SRM patients with a firstborn girl (29%, \( P = 0.03 \)) and compared with PRM (23%, \( P = 0.02 \)) and healthy female controls (25%, \( P = 0.005 \)). Among SRM patients HLA-antibodies were significantly more frequent if the births prior to SRM were obstetrically complicated compared with those who had uncomplicated births prior to SRM. Of the pregnant RM patients who were HLA antibody positive in early pregnancy, 41% had a live birth compared with 76% of those with no HLA antibodies. Adjusting for the number of prior miscarriages the chance of a live birth in RM

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**Figure 1** A possible scenario at the feto-maternal interface in an HLA-DRB1*15 positive woman with previous RM due to harmful anti-HY immunity. The figure depicts the possible afferent phase of immunization where the DDX3Y protein from male fetal cells and trophoblast debris is engulfed and processed to peptides by maternal macrophages. Their surface HLA-DRB1*15 molecules present the peptide to maternal CD4+ lymphocytes, which provide help for maternal CD8+ and B-cells. These induce through cellular and humoral mechanisms (that might not be HY antigen-specific) cell death in the fetus that induced the afferent reaction or in subsequent fetuses. Mat MФ, maternal macrophage; Mat Th cell, maternal T helper cell.
patients with HLA antibodies was reduced compared with patients without these antibodies \( [OR = 0.22 \ (0.07–0.68), P = 0.008] \). This study showed a remarkable higher frequency of HLA antibody positive SRM patients with firstborn boys compared with other RM patients and parous controls. This emphasizes that subdivision of RM patients is important not only into PRM or SRM patients but also according to sex of the child born prior to SRM. This study is the first showing an association with HLA-antibodies in early pregnancy and a reduced chance of a live birth in patients with RM. The mechanisms behind this association are unknown. It remains to be investigated whether HLA antibodies are the direct cause of the increased miscarriage frequency or whether it is an epiphenomenon that reflects a series of immunological disturbances possible based on prior abnormal transfers of fetal cells to the maternal immune circulation. Increased microchimerism as a consequence of previous pregnancy complications (Lo et al., 1999; Leung et al., 2001; Zhong et al., 2001; Khosrotehrani et al., 2003) may play a pathogenic role.

**H-Y antibodies**

The presence of IgG antibodies to one or more of five recombinant H-Y antigens has been shown to correlate with chronic GvHD in male recipients of stem cells from female donors (Miklos et al., 2005). A recent study investigated H-Y antibodies in SRM patients and controls (Nielsen et al., 2010d). Figure 2 is a heatmap visualizing the H-Y antibody response in patients and controls using a color

**Figure 2**  
H-Y and H-X antibody responses in serum samples from patients with unexplained recurrent miscarriage and healthy women. Heatmaps visualizing the antibody response in OD units to each H-Y and H-X protein of each participating individual, grouped according to patient or control status and sorted with the highest mean OD at the top. Positivity is defined as OD ≥ 0.1. H-Y-specific responses are responses directed at the H-Y protein and not the corresponding H-X protein. For patients who were pregnant at blood sampling, pregnancy outcome is given: G, girl; B, boy; 0, miscarriage. For control women pregnancy history is given: 2B, Given birth to only two boys; 3B, Given birth to only three boys; NP, never pregnant.
code correlating to optical density (OD) measures of the ELISA analysis. The frequencies of H-Y-specific antibody positive were significantly higher in SRM patients: 39 (46%) compared with female controls: 7 (19%, \( P = 0.004 \)), and PRM patients: one (8%, \( P = 0.01 \)). H-Y-specific antibodies were detected in 33 (49%) of SRM patients with a boy and in 6 (38%) of those women who delivered a girl prior to the miscarriages (\( P = 0.33 \)). The influence of H-Y antibodies in early pregnancy was analyzed in the 77 RM patients who were in the early stages of pregnancy at the time of serum sample; 43 (56%) of these pregnancies ended with a live birth. Live birth rates were not significantly different in H-Y antibody positive patients (48%) compared with H-Y antibody negative patients (61%, \( P = 0.26 \)). Only two (12%) of the children delivered by H-Y antibody positive patients were boys, which is significantly lower than the 12 (44%) boys delivered by H-Y antibody negative patients (\( P = 0.03 \)) and the 51% boys among newborns (Khashan et al., 2009) (\( P = 0.002 \)). The frequency of H-Y-specific IgG antibodies was significantly increased in SRM compared with both control females who previously had given birth to boys and PRM patients. The presence of H-Y antibodies in early pregnancy was associated with a low sex ratio at birth but not a statistically significantly increased clinical miscarriage rate in RM patients. These results suggest a direct, early (preclinical) and male-specific embryotoxic response in H-Y antibody positive RM patients. The impact of H-Y antisera has been shown in studies aiming at non-invasive techniques for sex selection of preimplantation cattle embryos to increase profitability of dairy and beef cattle production (Ramalho et al., 2004). Between 80% and 87% of murine and bovine male embryos that are cultured in high-titer rat H-Y antisera, at the morula stage, stop their development in contrast to female embryos (Utsumi et al., 1993; Ramalho et al., 2004). Larger studies are needed to confirm the findings of this first inventory and it also remains to be investigated whether the presence of H-Y antibodies in early pregnancy or around conception of women with no history of RM correlates with the sex of the fetus.

Sex of prior children and subsequent reproductive performance in the background population

The hypothesis of aberrant maternal immune responses against H-Y antigens as a cause of pregnancy related problems was challenged in population-covering studies exploring the association of birth of boys and subsequent obstetric complications. Population-covering studies are possible in countries like Denmark with National Birth and Discharge registries based on unique ID numbers of every citizen. Such studies cover the background population and are advantageous as these large data sets allow testing for rare conditions and small effect sizes. There is no clear biological differentiation between miscarriage and stillbirth. On the basis of the Danish Birth Registry, it was tested whether delivery of boys increased the risk of a subsequent stillbirth between 558 314 second to fifth-born children of whom 1952 were stillborn. The risk of stillbirth increased by 12% after deliveries of boys compared with girls, relative risk = 1.12 (95% CI 1.02–1.23) (Nielsen et al., 2010a). Births of boys are thus associated with both subsequent miscarriage and stillbirth. Also based on the Danish Birth Registry, differences in birthweight of 545 839 second to fourth-born children were noted in relation to sex of older siblings. One or two preceding boys, respectively, reduced the mean birthweight of later-born boys by 29 g (\( P = 0.0001 \)) and 38 g (\( P = 0.0001 \)) and later-born girls by 17 g (\( P = 0.0001 \)) and 21 g (\( P = 0.0001 \)) compared with later-born siblings with no preceding boys (Nielsen et al., 2008b). Similar findings have been reported from the Norwegian Birth Registry, where it was an unexpected finding in a study with a different aim (Magnus et al., 1985). These differences are 10–20 times less than the differences in children born by H-Y-restricting HLA class II positive SRM patients with a firstborn boy compared with a girl. Combining data from the Danish and Swedish Birth Registrries regarding second-born children only, preterm birth was more common in second borns with an older brother compared with an older sister, hazard ratio = 1.10 (1.07, 1.13) < 0.0001. The results were similar in both data sets, the use of two large population-covering registries lower the possibility of selection bias, and focusing only on second borns reduces confounding (Mortensen et al., 2011). In all three studies the associations did not appear to be confounded by maternal age, interpregnancy interval, or by maternal characteristics that do not vary from one pregnancy to the next. In line with these data, two recent studies have found that having either older brothers or a twin brother reduces the lifetime reproductive success of their siblings (Lummaa et al., 2007; Rickard et al., 2007).

Prior birth of boys is associated with a decrease in birthweight, an increased risk of stillbirth and preterm birth, and reduced reproductive success among subsequently born children in the background population. These findings support the hypothesis of non-tolerated maternal immune responses against H-Y-antigens as a possible mechanism behind SRM and also some so far unexplained obstetric complications in the background population.

The H-Y hypothesis in SRM

Detailing the H-Y hypothesis

On the basis of the knowledge obtained reviewing the evidence for the H-Y hypothesis, we propose the following pathophysiologic mechanisms for the maternal non-acceptance of the fetal allograft in the SRM patient due to anti H-Y immunity. During the patients first ongoing pregnancies fetal cells enter the maternal circulation (Evans et al., 1999; Adams and Nelson, 2004) and in late pregnancy apoptotic syncytiotrophoblast debris is normally shed in large quantities (several grams per day) from the placenta. After being processed by maternal dendritic cells, peptides derived from fetal antigens, e.g. HLA or H-Y are presented in local lymph nodes to CD4+ and CD8+ T cells as recently suggested (Adams et al., 2007). In normal pregnancies, this presentation takes place under non-inflammatory conditions resulting in T lymphocytes becoming tolerant to fetally-derived peptides (Steinman et al., 2003). A significant proportion of the pregnancies and especially those involving a male fetus prior to SRM are associated with obstetric and neonatal complications (Nielsen et al., 2010b). These complications are associated both with an increased transfer of fetal cells into the maternal circulation (Lo et al., 1999; Leung et al., 2001; Zhong et al., 2001; Khosrotehrani et al., 2003) and with increased
production of inflammatory cytokines systemically or locally in uterus (Gerber et al., 2005; Girardi et al., 2006; Germain et al., 2007). Circumstances for sensitization of the adaptive immune system against fetal or trophoblast antigens are accordingly often present in the first ongoing pregnancy of SRM patients and may be further increased in patients who are genetically predisposed to anti-H-Y immune responses, e.g. those carrying the H-Y-restricting HLA class II alleles. A recent study found some healthy female donors sensitized against H-Y while others were H-Y tolerant (van Halteren et al., 2009). Murine studies have demonstrated tolerogenic mechanisms involved in pregnancy. Female mice sensitized (recognizing and destroying) known paternal antigens before pregnancy became tolerant to the same antigens during pregnancy (Zhang et al., 1992; Tafuri et al., 1995; Jiang and Vacchio, 1998). Suppression of these tolerogenic mechanisms may leave the H-Y immunization harmful to male fetuses as demonstrated when blocking $T_{REG}$ in a murine study (Kahn and Baltimore, 2010). $T_{REG}$ are found to be decreased by numbers in addition to be functionally deficient in RM patients that have undergone a standardized investigation program. All of prior losses.

Economic status (data not available). Such selection biases are not a strong tendency for earlier referral if living closer to the clinic and of higher socioeconomic status (data not available). Such selection biases are not a strong tendency for earlier referral if living closer to the clinic and of higher socioeconomic status (data not available).

The H-Y hypothesis in SRM: Strengths, limitations and perspectives

**Strengths**

The majority of our studies testing the H-Y hypothesis in SRM patients is based on patients referred to the Danish Recurrent Miscarriage Clinic. This is a national, tertiary clinic that investigates, treats and conducts research in recurrent pregnancy losses. The clinic is publicly funded and there is access for all eligible patients. There may be a tendency for earlier referral if living closer to the clinic and of higher socioeconomic status (data not available). Such selection biases are not likely to influence the results reported here. Later referral results in more prior miscarriages so all analysis are adjusted for the number of prior losses.

The H-Y hypothesis in SRM is based on data from a large group of patients that have undergone a standardized investigation program. All pregnancies before referral are confirmed and described in records from hospitals or practitioners and follow-up after referral is almost 100%. Pregnancy outcome after referral according to the sex of the firstborn was almost identical in two independent cohorts of SRM patients referred before and after 2000 (Nielsen et al., 2008a).

The observed association between a firstborn boy and subsequent low birthweight and obstetric and neonatal complications in SRM patients was also detected, although attenuated, in registry studies based on the background populations (Magnus et al., 1985; Nielsen et al., 2008b, 2010a; Mortensen et al., 2011) emphasizing the credibility of the epidemiologic results in SRM patients.

Our immunological studies testing the H-Y hypothesis have focused on antibodies. Antibody testing has methodological advantage compared with investigation of cellular factors. The laboratory methods are often more reproducible because they are simpler, and the presence of antibodies in the peripheral blood is expected to affect immunological conditions in the uterus. Full-length recombinant H-Y proteins and the H-X homologs were used as targets in the anti-H-Y-antibody ELISA—an assay that has been validated in previous studies of GvHD (Miklos et al., 2005). Only ELISA responses that were specific to H-Y proteins were considered a measure of H-Y antibodies.

**Limitations**

One weakness in the studies of the negative prognostic impact of a firstborn boy in SRM patients is the fact that a substantial proportion of the patients were treated with intravenous immunoglobulin (Ivlg), which may decrease the miscarriage risk (Christiansen et al., 2002). However, the sex of the firstborn child was not a determinant for offering Ivlg treatment to the patients. Furthermore, the two cohorts that were monitored prospectively showed similar higher live birth rate among patients with a firstborn girl compared with a firstborn boy in spite of the fact that in the first cohort 15% were offered Ivlg, and in the second cohort 45% (Nielsen et al., 2008a).

Except for women with complicated pregnancies prior to the miscarriages (Nielsen et al., 2009) and women with severe recurrent placental abruptions (Nielsen et al., 2007) our immunogenetic studies found no differences in H-Y-restricting HLA class II allele frequency in women with SRM and firstborn boys compared with firstborn girls. If H-Y-restricting HLA class II alleles truly confer susceptibility to RM they are expected to show increased frequencies in this population. The heterogeneous background of SRM may explain the observed similar frequencies. Patients suffering RMs due to aneuploid embryos are of course not expected to exhibit any HLA associations. Women with a previous aneuploid miscarriage display a better prognosis than those with previous euploid miscarriage (Boue et al., 1975; Stephenson et al., 2002). Accordingly, patients with immunological may manifest themselves most clearly through a poor prognosis in the next pregnancy.

The conclusions from our immunological studies may be limited due to relatively low numbers. In support of the H-Y hypothesis these studies found the presence of HLA and H-Y antibodies to be associated with a lower live birth rate and a low male/female ratio in subsequent live births, respectively (Nielsen et al., 2010c, d), consistent with a direct cytotoxic capacity as in allogeneic hematopoietic stem-cell transplantation (Miklos et al., 2005). The frequency of H-Y antibody positivity did, however, not differ significantly between SRM patients.
with a firstborn boy compared with a girl and neither did H-Y antibody positive pregnant patients have a significantly higher miscarriage rate. Both these observations seem to be at odds with the H-Y hypothesis. However, the frequency of H-Y antibodies was 11% higher in SRM patients with a firstborn boy and the subsequent miscarriage rate was 13% higher among H-Y antibody positive patients. Future adequately powered studies need to address these issues.

**Perspectives and future studies**

The observation that the sex of the firstborn child and presence of particular HLA class II alleles and HLA and H-Y antibodies characterize SRM and subsequent pregnancy outcome provides valuable insight in the pathogenesis of many cases of SRM and may also explain some obstetric and perinatal complications in the background population. However, none of the clinical and immunological risk factors reviewed has sufficiently high specificity for predicting future obstetric and perinatal complications to be introduced in clinical practice. It is therefore important further to identify the putative immunological interactions responsible for the anti-H-Y related pregnancy complications.

HLA antibodies have until now never convincingly been found to play any pathologic role for outcome in normal women or patients with RM but have instead been regarded as a result of previous ongoing pregnancies (Sargent et al., 1988; Coulam, 1992). It remains to be investigated whether HLA antibodies in SRM patients are the direct cause of the increased miscarriage risk or whether this is an epiphenomenon that reflects a series of immunological disturbances possibly based on prior abnormal transfers of fetal cells to the maternal immune circulation. It also remains to be determined whether anti H-Y antibodies are directly causing implantation failure and early miscarriage or whether they are epiphenomena to causal cellular reactions. Most studies of anti-H-Y immunity as a cause of GvHD have focused on cellular reactions. However, studies on cellular immunologic reactions in peripheral blood in pregnant women do not necessarily reflect the conditions at the fetomaternal interface and in case of H-Y-specific T-cells, the frequency of these cells is very low (Muts et al., 1999) requiring large volumes of blood drawn. We did, however, attempt to analyze the presence of functionally different types of H-Y-specific CD8+ T cells, i.e. T_REG and T_CTL in five SRM patients (after ethical approval and informed consent) referred to the clinic and prior to their next pregnancy attempt. We used exactly the same approach as van Halteren et al. (2009) enabling us to use their healthy donors as controls. Outgrowth of H-Y-specific T cells in all five samples failed whereas van Halteren et al. (2009) had a 50% outgrowth rate. Whether the lack of H-Y-specific CD8+ T cells was due to bad luck due to few samples or whether it truly reflects a lower than expected frequency of H-Y-specific T cells in SRM patients needs to be tested in larger studies.

Future studies should aim at exploring how maternal tolerogenic mechanisms against H-Y antigens are established. The nature of CD4+ T_REG is an obvious candidate. A recent murine study found a portion of the maternal immune response to fetal antigens to be T_REG in nature. Depletion of the T_REG resulted in a lower fraction of live male offspring and a selective reduction in weight of the surviving males (Kahn and Baltimore, 2010).

It also remains to be explored how or whether the initially specific anti-H-Y response suggested to develop after the birth of boys in SRM may also affects subsequent female fetuses. Although male fetuses seem to be at the highest risk of demise after a previous birth of a boy in SRM, female fetuses also seem to be in a higher than normal risk of miscarriage. Determinant spreading, the phenomenon that an initially specific immunological reaction with time spreads to be directed against several related proteins is a recognized and common feature in autoimmune diseases (Lehmann et al., 1993; Ott et al., 2004). A spread of an initially H-Y-specific reaction to closely related H-X analogs carried by females might be responsible for the possible impact on pregnancies with girls. It is also possible that anti-H-Y responses through production of Th1 cytokines, such as TNF-α, could be one of the mechanisms inhibiting T_REG cells with specificity for non-sex-specific trophoblast antigens thereby increasing the risk of female fetuses miscarriage too.

**Conclusion**

This review has critically discussed the basis for and the testing of the H-Y hypothesis that aberrant H-Y immunity is a causal factor for SRM. A series of observations lend support to the proposed hypothesis. Sex ratios prior and subsequent to SRM suggest that birth of boys predispose to SRM and male fetuses are more likely to be miscarried. Obstetric complications are more common in pregnancies prior to SRM potentially sensitization the mother against fetal antigens. Maternal carriage of HLA-class II alleles presenting H-Y antigens to the immune cells is associated with a reduced live birth rate and with obstetric complications in surviving pregnancies in SRM patients with a firstborn boy. HLA and anti H-Y antibodies are increased in SRM patients compared with controls. Presence of these antibodies in early pregnancy is associated with a lower live birth rate and a low male:female ratio in subsequent live births, respectively. Births of boys are also associated with subsequent obstetric complications in the background population.

These first results need to be confirmed by other RM centers, and collaborative studies across medical specialties and national borders are needed independent testing our hypothesis. This can have implications for our insight into the pathogenesis of RM but may also add to our understanding of maternal–fetal interactions during both normal and pathological pregnancies.

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**References**


Stephenson M, Bossano C, Schultz P. Accurate chromosome testing of miscarriages requires conventional cytogenetic analysis and selective DNA technologies. *Hum Reprod* 2009;24(suppl 1):i, 0–018.


