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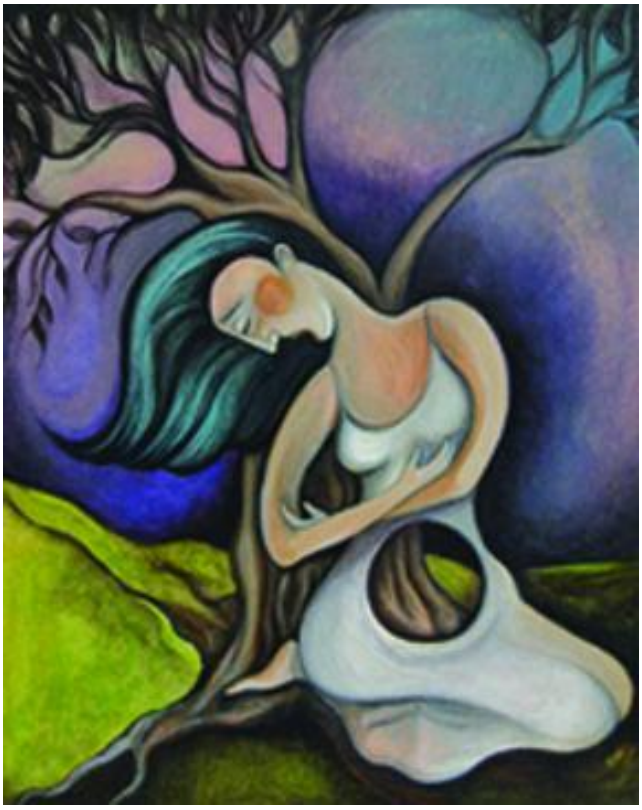
PhD thesis

Astrid Marie Kolte

Recurrent Pregnancy Loss – a family affair

Studies of genetics, epidemiology and evolution



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Recurrent Pregnancy Loss – a family affair

Studies of genetics, epidemiology and evolution

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Front page illustration
Miscarriage of Justice, Lina Scarfi. Original: Gouache. Reprint with the artist's permission

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Paper I

Kolte AM, Nielsen HS, Moltke I, Degn B, Pedersen B, Sunde L, Nielsen FC, Christiansen OB

A genome-wide scan in affected sib-pairs with idiopathic recurrent miscarriage suggests genetic linkage

Molecular Human Reproduction 17:379-385 (2011).

Paper II

Kolte AM, Steffensen RN, Christiansen OB, Nielsen HS

Maternal HY-restricting HLA class II alleles are associated with poor long term outcome in recurrent pregnancy loss after a boy

Submitted to American Journal of Reproductive Immunology, June 2016

Paper III

Kolte AM, Nielsen HS, Steffensen RN, Crespi B, Christiansen OB

The inheritance of the 8.1 ancestral haplotype in recurrent pregnancy loss

Evolution, Medicine and Public Health 16 (1): 325-331 (2015)

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At the heart of the research presented in this thesis is an early amazement of how two haploid cells can give rise to something as complex as a newborn child. In August 2004, this led me to the Recurrent Pregnancy Loss Unit the Fertility Clinic at Copenhagen University Hospital, Rigshospitalet. I was a young medical student, and I was immediately captured by the intense scientific curiosity and the openness to new ideas. During a sabbatical from medical school, I started the work presented in this thesis as *Paper I*. After the first year of residency in obstetrics and gynecology, I returned as a full time PhD fellow in April 2011, leaving for continued residency in September 2015. Those four years were filled with happiness, victories and frustration, and I am ever grateful for this period of my life.

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Abbreviations

Array CGH	Array comparative genomic hybridization
CI	Confidence interval
hCG	Human chorionic gonadotropin
HLA	Human leukocyte antigen
HR	Hazard ratio
IMH	Inherited maternal haplotype
LOD	Logarithm-of-the-odds
NGS	Next-generation sequencing
NIMH	Non-inherited maternal haplotype
OR	Odds ratio
PDFH	Paternally derived fetal haplotype
PUL	Pregnancy of unknown location
RPL	Recurrent pregnancy loss
SNP	Single nucleotide polymorphism

Introduction

Recurrent pregnancy loss (RPL) is a clinical and scientific conundrum. In 1938, Percy Malpas published a study on 'abortion sequences' (Malpas 1938) which was probably the first scientific paper on RPL. Despite massive research efforts ever since, we are seemingly still far from an explanation of why some women experience repeated demise of desired pregnancies (Saravelos *et al.* 2014).

A better understanding of RPL pathophysiology is paramount both for counselling and for developing effective treatment strategies. The studies presented in this thesis represent a continued effort to unravel some of the still unknown aspects of early pregnancy. Evolutionary medicine is an emerging field and may provide answers to why conditions develop.

The work for this thesis started with the clinical observation that a remarkably high number of patients reported that their siblings also had experienced pregnancy losses. We hypothesized that there is a genetic background for RPL in families with two or more siblings with RPL. With the publication of the Human Genome Project, relatively high-resolution single nucleotide polymorphism (SNP) microarrays enabled hypothesis-free genomic studies of conditions and disorders. In *Paper I* we describe a genome-wide linkage study of 38 affected sib-pairs. Approximately 1/3 of women with RPL have a birth before their pregnancy losses (secondary RPL). In the first pregnancy after referral for secondary RPL, women with a firstborn boy have a poorer chance of live birth, but only if they carry HY-restricting HLA class II, reviewed by (Nielsen 2011). In *Paper* II we wanted to investigate if maternal HY-restricting HLA class II alleles had a similar negative prognostic value for long-term chance of live birth, which would be more clinically and biologically relevant. It has been proposed that pregnancy is not a harmonious and symbiotic condition, and that some maternal genes may actively conspire against embryos not carrying their copies, a phenomenon called gestational drive (Haig 1996; Haig 1997). In *Paper III* we investigate if gestational drive could be an explanation for some cases of RPL.

This thesis is built up with a general introduction to RPL and a presentation of the theoretical framework for each paper. Hereafter follows a short description of materials and methods for the studies. The main findings in each study are presented, along with a discussion of the results, strengths and limitations. Finally, conclusions and perspectives for further research are reviewed.

Background

What is recurrent pregnancy loss?

Research in RPL is complicated by discrepancies in the definitions of pregnancy loss, recurrent pregnancy loss and the terminology used. In the following I present the background for the definitions used in this thesis and the accompanying papers.

Definition of a pregnancy loss

According to the latest opinion paper regarding the definition of infertility and RPL published by the American Society of Reproductive Medicine's Practice Committee, a "pregnancy is defined as a clinical pregnancy documented by ultrasonography or histopathologic examination" (ASRM Practice Committee 2013). This definition excludes all pregnancy losses which are only documented by urinary or serum hCG measurements (biochemical pregnancy losses and failed/resolved pregnancies of unknown location, collectively called non-visualized pregnancy losses (Kolte *et al.* 2014; Kolte *et al.* 2015)).

From the time of implantation until the moment when the placenta is fully functional, the developing trophoblast produces hCG which has various functions including support of the corpus luteum. Apart from the small amount of hCG produced by the pituitary and by certain rare cancers, hCG is produced exclusively in the context of a pregnancy (Cole 2010). Therefore, in the presence of a positive urinary or serum hCG test (>2 mIU/ml), a woman is pregnant.

Whether a pregnancy loss can be documented by ultrasound or histology depends on several factors apart from gestational age at the time of pregnancy demise. Accessibility of ultrasound examinations and the course of a pregnancy loss will also determine whether it can be documented. E.g. if a woman is 8 weeks pregnant and has a complete miscarriage at home, subsequent ultrasound will not be able to document the pregnancy. Thus, according to the American Society of Reproductive Medicine definition, this woman has not been pregnant.

In this thesis and in *Papers I-III*, a pregnancy loss is defined as the demise of a pregnancy documented at least by a positive urine or serum hCG, thus including both non-visualized pregnancy losses and miscarriages.

The definition of recurrent pregnancy loss

RPL is a distinct disorder defined by a number of events, namely pregnancy losses (Larsen *et al.* 2013). However, sporadic pregnancy losses are common. In a register-based study of 634,272 Danish women with a total of 1,221,546 pregnancies occurring between 1978 and 1992, 13.5% of all pregnancies ended in a miscarriage, fetal loss or stillbirth treated in a hospital setting. The risk of an adverse pregnancy outcome increased significantly with increasing age at conception (Nybo Andersen *et al.* 2000). It should be noted that only pregnancy losses leading to hospital contact were registered, i.e. the percentage of all pregnancies not ending as a live birth is presumably higher.

Using highly sensitive hCG measurements in a cohort study of healthy women, it was demonstrated that 22% of 198 pregnancies perished before the woman knew she was pregnant. Among women younger than 40 years, 95% of the women who had experienced a very early pregnancy loss had a clinically recognized pregnancy within the two following years (Wilcox *et al.* 1988). These findings suggested that very early pregnancy losses (i.e. before 4 weeks' gestation) were not associated with a poor chance of a future clinical pregnancy, but on the contrary were a sign of high fertility. Other publications, employing theoretical calculations, conclude that 30% of all conceptions fail to implant, 30% of implanted embryos end as pregnancy losses before the expected first day of the next menstrual cycle, and a further 10% (25% of recognized pregnancies) are lost after the due period (Chard 1991; Macklon *et al.* 2002). Thus, sporadic pregnancy loss is the most common early pregnancy complication, and it has been difficult to reach a consensus on how many losses and which types of pregnancy losses are required to make a diagnosis of RPL and initiate investigations and possibly treatment.

Overall, current definitions fall in two groups: Either two clinical miscarriages (not necessarily consecutive) or three or more consecutive pregnancy losses, as shown in Table 1.

Table 1 Current definitions of recurrent pregnancy loss			
Society	**N losses**	**Sequence**	**Reference**
Japan Society of Obstetrics and Gynecology	3	Consecutive	(Minakami *et al.* 2014)
National college of French Gynecologists and Obstetricians	3[a]	Consecutive	(CNGOF 2014)
European Society of Human Reproduction and Embryology	3	Consecutive	(Jauniaux *et al.* 2006)
Royal College of Obstetrics and Gynecology (UK)	3[b]	Consecutive	(RCOG 2011)
Danish Society of Obstetrics and Gynaecology	3[c]	Consecutive	(DSOG 2009)
Dutch Society of Obstetrics and Gynecology	2[d]	Non-consecutive	(NVOG 2007)
American Society of Reproductive Medicine	2[d]	Non-consecutive	(ASRM Practice Committee 2013)

[a]Before 14 weeks' gestation; [b]Before viability; [c]Of which at least one verified miscarriage; [d]Verified miscarriages

If all pregnancy losses were due to random effects or events, they should not impact the future chance of live birth, and would not be associated with an underlying pathology in the woman.

However, in terms of chance of live birth after RPL, numbers matter. Several cohort studies have demonstrated that the number of prior pregnancy losses is an important determinant for the prognosis after RPL (Parazzini *et al.* 1988; Knudsen *et al.* 1991; Quenby *et al.* 1993; Brigham *et al.* 1999; Bhattacharya *et al.* 2010; Lund *et al.* 2012; Kolte *et al.* 2014; Greenberg *et al.* 2015).

The societies and research groups who define RPL as two losses usually only include verified intrauterine miscarriages (Table 1). Yet, in a paper from the RPL Unit at Rigshospitalet reporting data from 2454 pregnancy losses, we documented that the impact of each pregnancy loss on the chance of live birth in the first pregnancy after referral was similar for non-visualized pregnancy losses and miscarriages, namely a decline in relative risk (RR) of approximately 10%. Furthermore, we showed that the mean gestational length for non-visualized pregnancy losses was approximately 6 weeks, whereas for miscarriages the mean gestational length was almost 9 weeks. This shows that while non-visualized pregnancies perish earlier than miscarriages, they do not solely comprise very early pregnancy losses (Kolte *et al.* 2014).

We therefore recommended that non-visualized pregnancy losses be included in the definitions of RPL and in the count of a couple's losses (Kolte *et al.* 2015). In the Danish RPL Unit, RPL is defined as three or more consecutive pregnancy losses before 12 weeks' gestation, as it is our opinion that this definition decreases the likelihood of random events being responsible for the pregnancy losses.

Terminology

Adding to the confusion are variations in terminology: 'Recurrent pregnancy loss' is referred to by 'recurrent spontaneous abortion', 'recurrent miscarriage', 'habitual abortion' and even 'recurrent spontaneous miscarriages'.

I believe that if we are ever to arrive at a generally accepted and evidence-based definition of RPL or hope to discover both causal factors and effective treatment strategies, the first step is to reach consensus on terminology. As the terminology regarding RPL has changed since *Paper I* was written, it should be noted that the term 'miscarriage' here covers both non-visualized pregnancy losses and verified miscarriages before 12 weeks' gestation. Otherwise, through-out the thesis and papers, 'miscarriage' describes a pregnancy loss with a verified intrauterine location (ultrasound or histology) and 'non-visualized pregnancy losses' encompass both resolved and treated pregnancies of unknown location (PUL) as well as biochemical pregnancy losses (Kolte *et al.* 2015).

What causes recurrent pregnancy loss?

RPL has been associated with a wide range of conditions, such as acquired and inherited thrombophilias; uterine malformations; endocrine disruptions such as hypo- and hyperthyroidism and polycystic ovary syndrome; parental karyotypic anomalies and overweight (Saravelos *et al.* 2014). I will not discuss these associations further, as these are areas that I have not covered in my research.

Oocyte quality – the missing link in RPL research

Pregnancy loss has been described as "natural function responsible for control not only of quantity but also of quality" (Roberts *et al.* 1975). The literature suggests that approximately 45% of sporadic pregnancy losses are due to fetal chromosomal anomalies (van den Berg *et al.* 2012).

The risk of meiotic errors and subsequent aneuploidy increases with maternal age, probably due to loss of cohesin (Jones *et al.* 2013). In a cytogenetic study of pregnancy loss tissue from 420 pregnancies from women with RPL compared with an unselected population, it was demonstrated that among women aged 18-29 years, 65% of the samples were euploid,

which was significantly more often than among controls (52%, p=0.03). Among RPL women aged 30-35 years, 63% of the samples were euploid, again significantly more often than among controls; 48%, p=0.001. For women aged ≥36 years, there was no difference in cytogenetic results (Stephenson *et al.* 2002). This is supported by a study of 43 women with RPL who were 35 years or older, showing that 78% of the pregnancy loss tissues were aneuploid, which was comparable to 137 women in the same age group with sporadic pregnancy losses (70% p=0.28) (Marquard *et al.* 2010).

In a study of tissue from 234 pregnancy losses experienced by women with RPL (≥2 pregnancy losses) 48.7% of the embryonic karyotypes were normal, which was significantly higher compared with 114 sporadic pregnancy losses (23.7%), p=0.000014, and the percentage of abnormal embryonic karyotypes decreased significantly with increasing numbers of prior losses (p=0.011) (Ogasawara *et al.* 2000). Based on a decision-analytic model, it has been proposed that karyotyping be a standard investigation of the second pregnancy loss a woman experiences (Bernardi *et al.* 2012). It is a serious limitation to clinical RPL management and research that investigations into fetal causes of pregnancy loss, such as karyotyping of pregnancy loss tissue, are rarely performed (Saravelos *et al.* 2014), also in Denmark. This is most likely due to tradition and considerations of costs. But as a result, it is not possible for us to select patients for studies of e.g. genetic or immunological causes of RPL who have experienced exclusively or predominantly euploid pregnancy losses. However, inclusion in all the studies presented in this thesis has been limited to patients who are ≤40 years of age at referral, reducing the likelihood that the majority of pregnancy losses were due to fetal chromosomal rearrangements.

A familial disposition to RPL? – *Paper I*

In the 1980s and 1990s, several studies reported that pregnancy loss or RPL was more common among RPL patients' first-degree relatives than controls. Alexander et al. found that among 100 women with RPL, 7% of their mothers and sisters also had RPL, in contrast to none of the control group's relatives (Alexander *et al.* 1988). This confirmed a finding by Johnson et al., who also described a higher frequency of RPL among first-degree relatives than controls, 12% vs. 7% (Johnson *et al.* 1988).

In 1990, Christiansen et al. published a retrospective cohort study of pregnancy outcomes among 90 RPL patients' first degree relatives. The study documented that 25.3% of the pregnancies reported by the RPL patients' 59 sisters ended as a pregnancy loss, whereas the

pregnancy loss rate in a control group was 12.6% ($p<0.001$). Among 52 brothers' wives there was a non-significant trend towards a higher risk of pregnancy loss (Christiansen *et al.* 1990). Ho et al. found that 1.4% of RPL couples' relatives experienced RPL themselves, significantly more than among fertile controls' relatives (Ho *et al.* 1991).

These findings were later supported by a Chinese age-matched case-control study which found that having a family history of pregnancy loss increased the risk of RPL, OR 2.12 (95% CI 1.28; 4.39). The OR for having a family history of pregnancy loss was higher for those patients who had 4 or more pregnancy losses (OR 3.09, 95% CI 1.51; 6.33) than those with three pregnancy losses (OR 1.90, 95% CI 1.074; 3.36) (Zhang *et al.* 2010).

Based on these studies, it seemed plausible that in some families there is a familial disposition to RPL. The publication of the sequence of the entire human genome in 2003 enabled a new type of genetic study of human disease: the high-resolution genome wide association studies (unrelated individuals) and genome wide linkage studies (relatives) (Ott *et al.* 2011). Today, exome or genome sequencing by massive, parallel sequencing (Next Generation Sequencing, NGS) is the gold standard, but ten years ago, genome-wide single nucleotide polymorphism (SNP) arrays provided an unprecedented way to perform genome wide genetic studies with relatively high marker density, enabling hypothesis-free genetic studies of complex diseases. This is what we wanted to do in *Paper I*.

If there is a hereditary component of RPL, we would also expect that RPL women's mothers have a higher prevalence of pregnancy loss. To my knowledge, this has not been formally explored. In general, intergenerational effects of pregnancy loss have only been studied sparsely. One Finnish study of intergenerational effects of reproductive patterns has been carried out (Pouta *et al.* 2005). This questionnaire-based study included 12,055 women who gave birth in 1966 and who were recruited at 22 – 28 weeks of gestation. Thirty-one years later, the researchers contacted 4523 daughters born by these women in the index pregnancy and asked them the same questions. The authors found no correlation between pregnancy loss experienced by the mothers and losses experienced by the daughters. However, of the mothers, only 7 had had three or more pregnancy losses, and likewise, only 6 of the 4523 daughters had experienced three or more pregnancy losses (Pouta *et al.* 2005). The results from this study do not indicate that sporadic pregnancy loss is inherited, but the conclusions are difficult to extrapolate to families with RPL.

Big brother is killing you – *Paper II*

The human leucocyte antigen (HLA) complex

The HLA complex (the human equivalent of the Minor Histocompatibility Complex, MHC) is a group of genes encoding surface molecules which present antigenic peptides to T cells. The gene complex is located on the human chromosome 6 and spans approximately 4 MB. The following description is simplified and not exhaustive, but a detailed description of the immune system is outside the scope of this thesis.

The HLA system is classically divided into class I (HLA-A, -B, -C) and class II (HLA-DP, -DQ, -DR) and is highly polymorphic. In addition to class I and II HLA genes, there are a few non-classical HLA gene loci. For reproductive immunology, the HLA-E and –G genes are especially interesting, as they encode molecules which are expressed by the fetally derived extravillous trophoblasts invading the endometrium. HLA-E and –G molecules have inhibitory functions on maternal NK cells, possibly ensuring the immunological non-recognition of the fetal invasion in the maternal uterine wall (Moffett *et al.* 2015).

HLA class I molecules present internal antigens, such as viruses, to CD8+ cytotoxic T cells (T_{CTL}). Almost all cell types carry HLA class I molecules. HLA class II molecules present peptides to CD4+ T cells and are located primarily on professional antigen-presenting cells such as macrophages, dendritic cells and B cells. With presentation of an antigen, the CD4+ T cells initiate an immunologic reaction partly by cytokine production leading to cytotoxic cellular responses or differentiation of B lymphocytes into immunoglobulin-producing plasma cells (humoral response) (Parham 2015)

Early works on RPL showed that immunological disturbances may cause RPL (Christiansen 1996; Coulam 2000). If this is the case, an association with certain HLA class II alleles would be expected, as the majority of autoimmune diseases are associated with specific HLA alleles (Klein *et al.* 2000). And indeed, it has been shown that female relatives of RPL patients had a higher risk of pregnancy loss if they were carriers of HLA-DRB1*01 or –DRB1*03 compared with those who were not (Christiansen *et al.* 1995). Furthermore, HLA-DRB1*03 is associated with RPL, especially in women with secondary RPL and higher numbers of pregnancy losses (Kruse *et al.* 2004). HLA-DRB1*15 has also been found to predispose to secondary RPL (Takakuwa *et al.* 2003).

A number of genes on the Y chromosome encode T cell epitopes known as HY antigens. HY antigens are presented by both HLA class I and class II alleles (Popli *et al.* 2014). HY

immunity in the form of female donor-derived T_{CTL} in the graft is an important source of graft-versus-host disease in HLA identical hematopoietic stem cell transplantation with a parous female donor and a male recipient (Spierings *et al.* 2003; Popli *et al.* 2014), but also decreased rates of relapse – a graft-versus-leukemia effect (Loren *et al.* 2006).

The HY hypothesis for secondary RPL

The HY hypothesis suggests that in the first pregnancy which leads to a live- or stillborn boy, women who later experience RPL develop an immune response against HY antigens if they are carriers of HY-restricting HLA class II alleles. In a series of studies, Nielsen et al explored this hypothesis (reviewed in (Nielsen 2011). The studies were initiated based on a finding in a randomized controlled trial of IVIg for unexplained secondary RPL which showed that 34 (74%) of the firstborn children were boys (Christiansen *et al.* 2002). This was later confirmed in two larger cohort studies of women with secondary RPL from our center, where the chance of live birth in the first pregnancy after referral (Nielsen *et al.* 2008) as well as in the long-term (Christiansen *et al.* 2004) was significantly lower for those with a firstborn boy compared with a firstborn girl.

When comparing 358 Danish women with unexplained secondary RPL to the general Danish population, the sex ratios were shown to be significantly skewed in the RPL population: sex-ratio (boy vs girl) of the children born prior to secondary RPL was 1.49 vs 1.05 in the general population (p=0.001). The sex-ratio of live born children in the first pregnancy after referral was 0.76, in the general population 1.06 (p=0.02), which is a significantly changed sex ratio from firstborn to the first pregnancy after referral (p<0.0001) (Nielsen *et al.* 2010). Obstetric complications were significantly more frequent in the RPL population than in the control group (39% versus 24%, p≤0.01)). In the group of women with subsequent RPL, 44% of the male births were obstetrically complicated, compared with 31% of the female births (p=0.01). After the series of pregnancy losses, 24% of the female births were obstetrically complicated, compared with 13% of the male births (p=0.04) (Nielsen *et al.* 2010).

In an Irish study of 85 women with secondary RPL, sex-ratios prior to secondary RPL was 1.66 (p=0.002), but there was no significant differences in chances of live birth according to sex of the firstborn (Ooi *et al.* 2011). In a study of 170 women with secondary RPL, Li et al only found a skewed sex ratio for first stillborn children but not liveborn children (Li *et al.* 2014). Chromosomal analysis or other investigations of possible fetal causes of pregnancy loss have not been performed in the studies mentioned above. However, when examining only women with losses after gestational week 9 (where the likelihood of lethal fetal

chromosomal abnormalities is lower), the sex ratios were even more skewed, 2.3 before the series of losses versus 0.21 in the first pregnancy after referral (p=0.009) (Nielsen *et al.* 2010).

In a study of HLA types in 286 women with secondary RPL, Nielsen et al demonstrated that maternal carriage of HY restricting HLA class II alleles and especially HLA-DRB1*15 and HLA-DQB1*0501/02 decreased the chance of live birth in the first pregnancy after referral, but only when the firstborn child was a boy (Nielsen *et al.* 2009). Recently, HLA-DRB1*07 has been shown to restrict HY in vitro (Eljaafari *et al.* 2013) and has been shown to be more prevalent among women with secondary RPL than controls (Gharesi-Fard *et al.* 2014). In *Paper II* we investigated whether maternal carriage of HY-restricting HLA class II alleles was associated with long-term chance of live birth after secondary RPL.

Selfish genes and conflicts in pregnancy – *Paper III*

Mother-offspring conflict and human life history theory

In human life history, resources have been limited and women have faced an evolutionary trade-off between the investment of many resources in a small number of children or fewer resources in a large number of children. A child benefits from maternal survival, but the child is more related to himself than to any siblings, in contrast to the mother who is equally related to all her children, as regards to autosomes. Therefore, the resource expenditure in pregnancy and beyond, which maximizes the mother's fitness (fitness here describes the number of gene replicas propagated to the next generation, a measure of fecundity) may be less than what maximizes the individual child's fitness (Haig 1996). This mother-offspring conflict has been compared to a tug-of-war. In a tug-of-war, each team tries to move a flag a small distance, but the tension in the rope is high. If either team stops pulling, the system collapses (Haig 1993). Likewise there can be an intense conflict between mother and child even if the difference in optimum is small.

Human hemochorial placentas, which are fetal in origin, are characterized by a high degree of invasiveness. The placenta invades the endometrium and prompts a transformation of the spiral arteries. As a consequence of this transformation, the spiral arteries lose their contractile properties and significantly increase in diameter, thus gaining free access to maternal blood flow for the fetus in the second and third trimester (Sadler 2014). The growing embryo/fetus is crucially dependent on the invasive properties of the placenta, but both mother and child are likely to perish with unlimited invasion beyond the decidua

basalis as in the case of placenta accreta/increta/percreta. The same goes for birthweight. A growth-restricted child faces significantly increased risk of neonatal morbidity and mortality, though the relationship is complex (Wilcox 2001). With a large-for-gestation fetus, the risk of fetopelvic disproportion increases, which poses significant risk of prolonged labor, excessive bleeding, and subsequent intra/peripartum morbidity and mortality for both mother and child, especially when there is limited or no access to assisted childbirth (Das *et al.* 2004). This Goldilocks situation – not too small and not too big, but just right – illustrates that maternal-fetal conflict may play a significant role for the outcome of a pregnancy, given that the mother and fetus do not have identical optima (Haig 1993).

Maternal-paternal-fetal conflict

As the fetus' genome is composed of equal contributions from the mother and the father, there are three interacting genetic compartments present in every pregnancy, the paternally derived fetal haplotypes (PDFHs), inherited maternal haplotypes (IMHs) and non-inherited maternal haplotypes (NIMHs) (Figure 1).

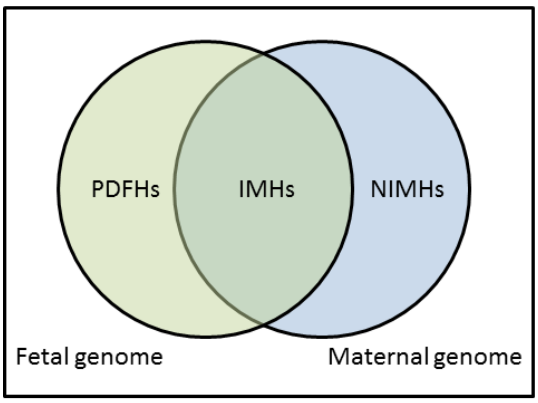

Figure 1 Mother-offspring conflict in viviparous pregnancy.
PDFHs: Paternally derived fetal haplotypes
IMHs: Maternally derived fetal haplotypes
NIMHs: Non-inherited maternal haplotypes.
PDFHs and IMHs benefit directly from the survival of the child, NIMHs do not.
Adapted from (Kolte *et al.* 2015)

The mother is equally related to all her children, and therefore, all maternal genes have the same likelihood of being present in a fetus in all pregnancies – 50%. As a woman can have pregnancies by several partners, the paternally derived fetal haplotypes (PDFHs) have a higher likelihood of being propagated in the present pregnancy (100%) than in a subsequent pregnancy (<50%). Therefore, the PDFHs will seek to maximize the likelihood of survival for the present fetus or child (Haig 2004).

As mentioned in the section above, a large and well-nourished child is more likely to survive pregnancy and early childhood.In this respect, it is interesting that a large number of imprinted genes are involved in growth with paternally derived active alleles promoting growth. The maternally derived genes reduce growth, decreasing the fetus/child's demands on the mother's resources (Tilghman 1999).

Gestational drive

David Haig has proposed that certain maternal genes or haplotypes have the ability to recognize whether a given fetus has inherited the same gene or haplotype from a heterozygous mother. NIMHs have no benefit from the survival of the fetus, and it has therefore been speculated if some NIMHs can be abortifacient, a process called *gestational drive* (Haig 1996; Haig 1997). As the interval between an uncomplicated early pregnancy loss and the next pregnancy will be shorter than after a live birth, the NIMH will have a better chance of propagation into the next generation if non-carrier fetuses are miscarried. If reproductive compensation is present, the gene would persist in the population. The term 'reproductive compensation' classically refers to a tendency by parents to 'replace' offspring that were lost to genetic disorders, and was originally proposed as a theory to explain the perpetuation of lethal or fitness reducing recessive genes with a high prevalence in a population, e.g. the cystic fibrosis gene in Western Europe (Hastings 2001). The alleles most likely to exhibit gestational drive are longer stretches of DNA which survive for generations without recombination (Haig 1997). One example is the 8.1 ancestral haplotype (8.1AH). The 8.1AH (HLA-A1, C7, B8,C4AQ0, C4B1, DRB1*0301, DQ2) is an extraordinarily conserved haplotype (>99.9%), spanning 2.9 MB. Approximately 10% of Caucasian Europeans are carriers of this haplotype (Aly *et al.* 2006). The haplotype displays effects consistent with antagonistic pleiotropy, the phenomenon of a trait having both beneficial and maleficent effects (Williams 1957). In *Paper III* we investigated the inheritance of the 8.1AH in unexplained RPL under the theoretical framework of gestational drive.

Objectives

Paper I

- Is the prevalence of pregnancy loss among RPL patients' siblings higher than in the background population?
- Are some genetic regions more frequently shared between siblings with RPL than expected by Mendelian inheritance?

Paper II

- Does maternal carriage of HY-restricting HLA class II alleles impact long-term chance of live birth in secondary RPL after a boy vs. a girl?

Paper III

- Do live born children born by RPL women who are heterozygous for the 8.1 ancestral haplotype (8.1AH) more often inherit this haplotype than expected?

Materials and methods

Patients *Paper I-III*

All patients included in the studies had experienced at least three consecutive pregnancy losses. We included both non-visualized pregnancy losses (biochemical pregnancy losses and resolved and failed pregnancies of unknown location combined) and miscarriages (verified intrauterine pregnancy demise) (Kolte *et al.* 2015). At least one of the pregnancy losses was a miscarriage. Patients with surgically or ultrasonically confirmed ectopic pregnancies were not excluded, but contingent ectopic pregnancies were not included in the count of pregnancy losses.

Patients with secondary RPL had given birth to a maximum of two singleton children before the series of pregnancy losses, and all had experienced at least three consecutive pregnancy losses after the latest birth, as described above.

Except for a small subset of patients, e.g. patients with systemic lupus erythematosus, the majority of RPL patients have no definitive reason for their pregnancy losses. However, there are a number of more or less well established risk factors associated with RPL, and to minimize the risk of confounding, all patients fulfilled the following criteria:

- Regular menstrual cycle (21 – 34 days)
- Normal uterine anatomy verified by saline sonography, hysteroscopy or hysterosalpingogram
- Negativity for the lupus anticoagulant
- Anticardiolipin antibody IgG/IgM <45 IE
- Normal karyotype of both the woman and her partner
- Age at referral ≤40 years of age.

For study-specific inclusion and exclusion criteria, please refer to the included manuscripts.

Laboratory methods

DNA extraction *Paper I, II, III*

DNA extraction was performed on EDTA-treated peripheral blood either by a salting out method as previously described (Miller *et al.* 1988) or using the Maxwell 16 Blood DNA kit on the Maxwell 16 Instrument. DNA from buccal swaps was extracted using Maxwell 16 Buccal Swab LEV DNA Purification Kit on the Maxwell 16 Instrument (Promega, Madison, WI, USA). All DNA extractions

were performed at Dept. of Clinical Immunology at Aalborg University hospital, Denmark, by laboratory technician Rudi Steffensen, PhD.

Affymetrix GeneChip 50K *Xba*I *Paper I*

We used the Afymetrix ® GeneChip ® 50K *Xba*I platform, according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). The laboratory analyses were carried out in 2005 – 2006 by the author and laboratory technician Susanne Smed under the supervision of professor Finn Cilius Nielsen, MD, DMSc at the Department of Clinical Biochemistry, Copenhagen University Hospital, Rigshospitalet, Denmark.

HLA typing *Paper II, III*

HLA typing was performed using the Luminex xMAP system LABType SSO, a reverse SSO DNA typing system according to the manufacturers' instructions (One Lambda Inc., Canoga Park, CA, USA). All HLA genotyping was performed at the Department of Clinical Immunology at Aalborg University hospital, Denmark, by laboratory technician Rudi Steffensen, PhD.

Statistics

Paper I

1) Risk of pregnancy loss among patients' siblings was estimated by Mantel-Haenzel X^2 statistics calculated in the Statistical Package for Social sciences (SPSS). The analysis was performed by Henriette Svarre Nielsen, MD, DMSc.
2) Genome-wide non-parametric linkage analysis was performed by Ida Moltke, PhD, using S_{ALL} statistics for all sibling pairs; sister pairs only; pairs where the patient had experienced ≥4 pregnancy losses; one sibling pair from each family.

Paper II

1) Non-parametric median test was used to compare obstetrical characteristics stratified for sex of firstborn and number of maternal HY restricting HLA class II alleles, respectively.
2) Sex ratios were compared by Mantel-Haenzel X^2 statistics.
3) Chance of live birth after secondary RPL was presented as Cox proportional hazard ratios with time from LMP in the pregnancy leading to a birth (before the pregnancy losses) till LMP in the next pregnancy leading to live birth as survival time.

All statistical analyses were performed in SPSS version 22 (IBM SPSS, Armonk, NY, USA) by Astrid Marie Kolte.

Paper III

1) The exact binomial test was used to test if inheritance of the 8.1AH from RPL patients to their live born children differed from Mendelian inheritance.
2) Non-parametric median test was used to assess differences in birth weight and the X^2 test to test sex ratio.

All statistical analyses were performed in SPSS version 20 (IBM SPSS, Armonk, NY, USA) by Astrid Marie Kolte.

Paper I: A genome-wide scan in affected sib-pairs with idiopathic recurrent miscarriage suggests genetic linkage

Kolte AM, Nielsen HS, Moltke I, Degn B, Pedersen B, Sunde L, Nielsen FC, Christiansen OB
Molecular Human Reproduction 17:379-385 (2011).

Summary of results

Risk of pregnancy loss among RPL patients' siblings

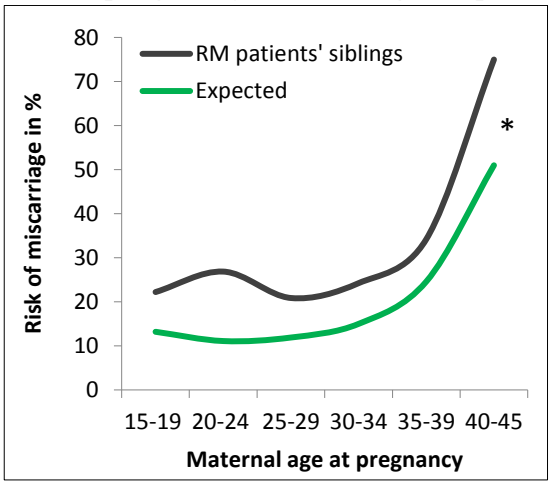

Figure 2 Risk of pregnancy loss among 268 siblings of 244 patients with RPL compared with the Danish general population (Nybo Andersen *et al.* 2000). * OR=2.04, 95% CI 1.4-3.0. Modified from (Kolte *et al*. 2011).

As demonstrated in Figure 2, the risk of pregnancy loss among RPL patients' siblings was significantly higher than in age-matched women from the general Danish population, irrespective of age at pregnancy.

The overall risk of demise per pregnancy was 23.3% among RPL patients' siblings, compared to 13.5% in the general population, OR 2.04 (95 % CI 1.4 – 3.0).

Indications of linkage to genomic regions

In the analysis of all 38 sibling pairs we found no genetic areas with a LOD score >3.0, but in subgroup analyses we identified regions with a LOD score >2.5 but <3.0, indicating suggestive linkage. In the analysis of females only (28 pairs), there was a linkage peak with a LOD score of 2.9 located near the *FHIT* gene (fragile histidine triad gene) on chromosome 3. When we restricted the analysis to sibling pairs where the proband had experienced at least 4 pregnancy losses (18 pairs), we had two linkage peaks: a) in the *hypothetical protein LOC 15297* (chromosome 9), LOD score 2.6 and b) in the *PDE2A* gene (chromosome 11), LOD score 2.9. When randomly removing one affected sibling in the sibships with three affected individuals, a LOD score of 2.5 was found in the *GRIK2* gene on chromosome 6, as shown in Figure 3.

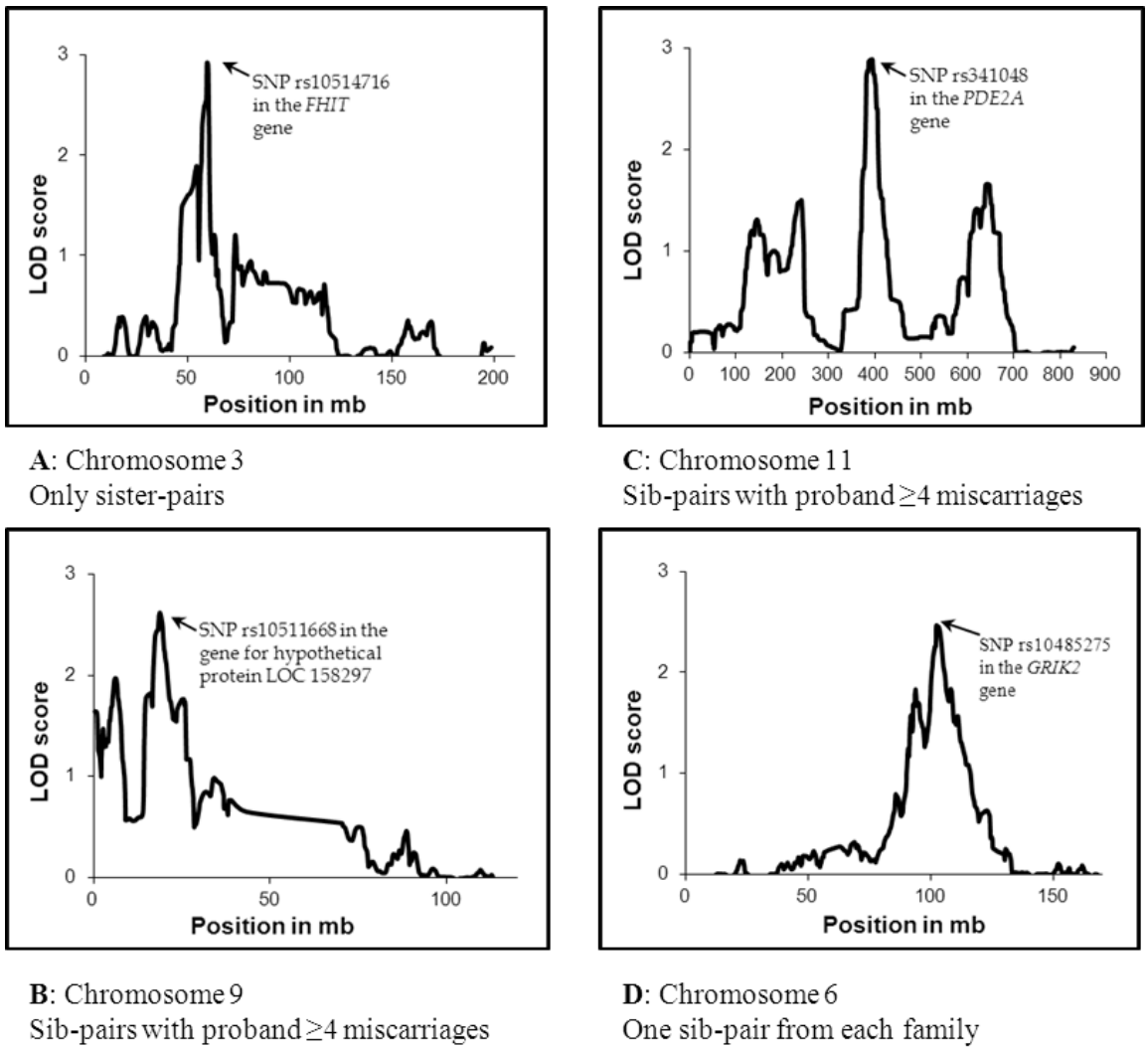

Figure 3 Areas with LOD scores between 2.5 and 3.0 in subgroup analyses, adapted from (Kolte *et al.* 2011)

Discussion

We confirmed that RPL patients' siblings have a higher risk of pregnancy loss compared with the general population. It strengthens the conclusion that the risk of pregnancy loss follows the same curve as reported for the general population (Nybo Andersen *et al.* 2000). The recruitment of siblings was dependent on patients (probands). Thus, patients who knew that their siblings had experienced pregnancy losses may have been more inclined to participate, and siblings who had experienced pregnancy losses themselves may also have been more interested in the study. This issue is difficult to ascertain with certainty, but register studies coupling sisters via their mothers would perhaps alleviate some of these concerns.

In the genome-wide linkage analysis, the major problem is lack of power. A sample size of 38 sibling pairs in a genome-wide study of a multifactorial, heterogeneous condition is very small. Though the statistical analyses did not find any significantly linked regions, we did find regions

which may be interesting subjects for future studies. Finally, it is worth considering that if inherited genetic factors play a role in the pathogenesis of RPL, there is a risk of selection bias by design, as the most severely affected RPL patients' mothers may also have decreased fecundity, limiting the number of multi-case families for inclusion in the study.

Paper II: Maternal HY-restricting HLA class II alleles are associated with poor long term outcome in recurrent pregnancy loss after a boy

Kolte AM, Steffensen RN, Christiansen OB, Nielsen HS
Submitted to American Journal of Reproductive Immunology, June 2016

Summary of results

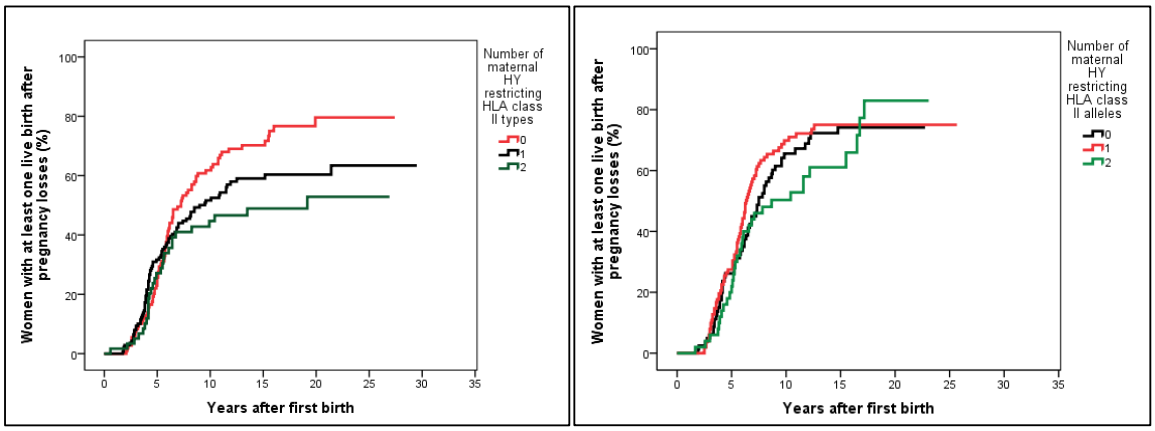

Figure 4 Kaplan-Meier plot of the percentage of women with secondary RPL after a boy (left) or a girl (right) with at least one live birth after referral, stratified by number of maternal HY-restricting HLA class II alleles. Adapted from (Kolte *et al.* 2016).

Among the 307 women with a firstborn boy, the long-term chance of live birth after RPL was poorer if the woman carried the HY-restricting HLA class II alleles. As demonstrated in Figure 4, the chance of live birth followed a dose-dependent pattern. Women (n=139) with one HY-restricting HLA class II allele had an adjusted hazard ratio (HR) for live birth of 0.75 (95% CI 0.55 – 1.02) compared with those with no HY-restricting HLA class II alleles (n=109). The women with two HY-restricting HLA class II alleles (n=59) had an adjusted HR for live birth of 0.62 (0.40 – 0.94). The only individual alleles which had a significant impact on the chance of live birth were HLA-DQB1*05:01/02, adj. HR 0.64 (0.42 – 0.97) and HLA-DRB1*15, adj. HR 0.64 (0.45 – 0.93). We found no impact by HY-restricting HLA class II alleles on the long-term chance of live birth among women with a first born girl (Figure 4).

When comparing women without HY restricting HLA class II alleles, there was not difference in the chance of live birth according to sex of the firstborn, adj. HR 1.00 (95% CI 0.73 – 1.41).

When comparing single allele status for women with firstborn boys vs. firstborn girls, none of the alleles had a statistically significant impact on the chance of live birth, but the carriage of any HY-restricting HLA class II allele significantly decreased the chance of live birth, adj. HR 0.72 (95% CI 0.55 – 0.98).

Discussion

We extended our previous findings regarding HY-restricting HLA class II alleles to long-term chance of live birth in *Paper II*. The cohort of 540 women with RPL is the largest published on secondary RPL and sex-specific effects. The findings support the hypothesis that HY immunity plays an important role in secondary RPL after a boy, and the study urgently needs replication in other populations.

It is a limitation that we do not have complete follow-up on live births after referral. Ideally, follow-up should be performed via the Danish Medical Birth Register, but despite intensive efforts, we were unable to do this at this stage.

Paper III The inheritance of the 8.1 ancestral haplotype in recurrent pregnancy loss

Kolte AM, Nielsen HS, Steffensen RN, Crespi B, Christiansen OB
Evolution, Medicine and Public Health 16 (1): 325-331 (2015)

Summary of results

In our cohort of 110 mother-child pairs, we found that 61 (55%) of the children had inherited the 8.1AH, p=0.29. There was a non-significant trend towards higher an inheritance of the 8.1AH by girls (63%, p=0.11) than boys (50%, p=1.00). We saw no differences in birthweight according to inheritance, nor differences according to type of RPL or number of pregnancy losses, see Table 2.

Table 2 Inheritance of the 8.1 ancestral haplotype from recurrent pregnancy loss (RPL) women to their live born children. Adapted from (Kolte *et al.* 2015).

	Inherited	**Did not inherit**	**p-value**
All live born children (N=110) *N (%)*	61 (55)	49 (45)	0.29[a]
Type of RPL *N (%)*			
Secondary RPL (n=87)	48 (55)	39 (45)	0.39[a]
Primary RPL (n=23)	13 (57)	10 (43)	0.68[a]
Median birth weight (range)[b]			
Boys (n=53)	3320 (1992; 4270)	3481 (1000; 5300)	0.90[c]
Girls (n=37)	3300 (1240; 4800)	3100 (2440; 3675)	0.37[c]
Sex of live born *N (%)*			
Boys	31 (50)	31 (50)	1.0[a]
Girls	30 (63)	18 (37)	0.11[a]
Pregnancy losses before referral, *N (%)*			
3	25 (58)	18 (39)	0.36[a]
4	13 (52)	12 (48)	1[a]
5 or more	23 (55)	19 (45)	0.64[a]

[a]Exact binomial test
[b]We did not have information on birth weight for all live born children.
[c]Non-parametric median test

Discussion

In *Paper III* we formally tested the gestational drive theory with the 8.1AH as the driving haplotype, and even though we did not reach statistical significance, the study is interesting. A negative, or not statistically significant result is always more difficult to discuss, but we found indications that gestational drive by the 8.1AH may play a role for the birth of girls born by women

with RPL. The gestational drive theory does not predict sex-specific effects, but competition between siblings of the same sex may be more intense.

In this study it is a serious limitation that we do not have access to pregnancy loss tissue. As women with RPL have more pregnancy losses than live born children, genotyping pregnancy loss tissue for the 8.1AH would have substantially strengthened the study.

General discussion

The results from *Paper I* confirmed our hypothesis that RPL patients' siblings have a higher risk of pregnancy loss per pregnancy. It would be interesting to investigate whether a family history of pregnancy loss has an impact on the chance of live birth after RPL. In the affected sib-pair analysis, we were unable to unequivocally identify genetic regions linked to RPL. It would be interesting to redo the study with more affected sib-pairs.

However, if there is a genetic background in some cases of RPL, it is likely that the mechanisms vary between patients or have a complex nature, such as we have seen for maternal carriage of HY-restricting HLA class II alleles (Nielsen 2011), making such a study difficult to design. It stands to reason that a genetic background for RPL could lead to a significant decrease in inclusive fitness, and it would be expected that the genes either have 1) pleiotropic effects which would increase inclusive fitness by other means; or 2) genomic conflicts were present, so that some genomic party / parties would stand to benefit, even if the mother's inclusive fitness was decreased. Presently, the lifetime fecundity of couples with RPL is unknown. Our group has previously shown that five years after referral for RPL 66.7% (95% CI 63.7–69.7) had achieved at least one live birth, (Lund *et al.* 2012), but cumulative live birth rates for couples with RPL have not been published.

In *Paper II* we showed that maternal HY-restricting HLA class II alleles confer a poorer chance of livebirth for women with RPL after the birth of a boy. This relationship is clinically important when counselling patients. Since Sir Peter Medawar first addressed the immunological paradox of viviparous pregnancy (Medawar 1952), the fetus has been viewed as a semi-allograft – comparable to an organ transplant with maternal immune suppression as a condition for a successful pregnancy outcome. However, there is no convincing evidence that pregnant women have a suppressed immune system and an overall higher risk of infection (Kourtis *et al.* 2014), and it has been proposed that the placenta functions more like an invasive tumor than a transplant (Mor *et al.* 2015). This is supported by the fact that mammals with highly invasive placentas, like the dog, cat and humans, have a higher risk of metastatic cancer (Murray *et al.* 1999). The evolutionary background for this theory is not established, as it could be due to both antagonistic pleiotropy and positive pleiotropy between the degree of trophoblast invasiveness and metastasis (D'Souza *et al.* 2014).

The results in *Paper III* did not confirm the gestational drive hypothesis. However, I agree with the view that the theory of parent-offspring conflict and the view of pregnancy and childrearing – not as a state of peaceful cohabitation but a scene of intense intra- and inter-individual conflict –

deserves much more scrutiny in future research (Crespi 2014; Haig 2014). Theories of evolutionary medicine may bring insights into mechanisms of RPL and early pregnancy in general. Evolutionary medicine is a nascent scientific field and evolutionary biology is largely ignored by medical research and clinical practice (Crespi 2014). This is at odds with the very essence of basic and translational medicine, i.e. the study of how things are connected and why people become ill. It is my opinion that evolutionary medicine should be part of medical scientific thinking, to the benefit of hypothesis generation and patients.

It is a major obstacle in early pregnancy research that pregnancy loss tissue is rarely investigated. As early pregnancy loss is common and often caused by fetal chromosomal abnormalities, this is also likely to be a common cause of RPL, especially for women older than 36 years (Ogasawara *et al.* 2000; Stephenson *et al.* 2002; Marquard *et al.* 2010). The non-significant results in *Papers I* and *III* may partly be a reflection of this. If a couple experience RPL due to fetal chromosomal anomalies it is unlikely that we would be able to identify other causes. We have classified patients according to age, number of pregnancy losses, parental karyotype and acquired and inherited trombophilias, but we have no information on the characteristics of the lost embryos/fetuses and no information on the endometrium.

A few studies have been conducted on the endometrium. Traditionally, the focus has been on the uterine specific NK cells, but a recent genomics forward study compared endometrial gene expression pattern with histological findings. The study demonstrated that endometrial samples which were out-of-phase or in-phase but with high cyclin E levels had distinctive expression patterns, but only of Eutherian evolved genes, i.e. present in placental mammals. The group with in-phase endometrium and normal cyclin E levels did not have a distinctive expression pattern, probably reflecting a heterogeneous group (Kosova *et al.* 2015). This type of study seems promising, not only for women with RPL but also for our understanding of endometrial receptivity and the necessary conditions for successful implantation. Still, this type of study does not allow us to directly understand what happens in the endometrium during early pregnancy – be it a successful pregnancy or a pregnancy loss. Studies of pregnancy loss tissue, both the fetus proper but also the placenta, are difficult as the tissue investigated will have been dead for an unknown period before study.

There can be no doubt that studies of RPL are difficult and fraught by the limits of our current understanding. It is my belief that only well-conceived studies in collaborative settings with

rigorous criteria for diagnosis, classification of pregnancy losses and a clear outcome and thorough follow-up will bring this field forward.

Future directions

"I contain multitudes" – Walt Whitman

Early in gestation the embryo implants in the uterus and already at this stage maternal blood flows to the uterus, and the maternal hormonal profile and blood glucose levels change to accommodate the growing embryo. Through the fetally derived placenta, the woman and the fetus are intimately coupled and there is evidence that substantial transplacental flow takes place, not only of nutrients and waste products, but also cytokines, hormones and even cells. These cells can colonize the recipient and for decades be detected in both the circulation and solid organs, a process known as microchimerism (Nelson 2012). Endometrial fetal microchimerism is frequent among parous women (Hromadnikova *et al.* 2014) and cells from an older sibling will here be perfectly situated to interfere with the implantation or growth of a subsequent pregnancy. Hereafter, two different scenarios are conceivable: Firstly, the microchimeric cells may work indiscriminately to delay the implantation or growth of any subsequent embryo, clinically recognized as secondary infertility or recurrent pregnancy loss. Secondly, more severe effects are possible if the fetally derived cells conspire against those fetuses not carrying a particular genotype. This may happen directly or via the maternal immune system (e.g. through maternal HLA alleles). This process resembles gestational drive and the hypothesis explored in *Paper III*, but in this case the driving gene is fetal in origin and not maternal (Haig 2014).

The data presented in *Paper II* and by Nielsen et al. suggest that a first born boy has fitness costs for the mother and subsequent siblings. The chance of live birth after RPL is lower if the mother carries HY-restricting HLA class II alleles (Nielsen *et al.* 2009; Kolte *et al.* 2016). In the general population, birth weight of younger siblings is reduced after a firstborn boy, particularly for younger brothers (Nielsen *et al.* 2008). The evolutionary background is unexplored, but it would be interesting to explore the importance of HY immunity and microchimerism under the theoretical framework of parent-offspring conflict. In a population with a younger mean age at first birth than in the Western world today, secondary RPL may serve as a mechanism to increase interbirth intervals, leading to a higher inclusive fitness for the firstborn and perhaps also the mother, though the optimum interbirth interval is probably not identical for mother and child (Haig 2014). Both HY immunity and microchimerism may play a role in this situation.

Technological breakthroughs such as NGS will enable us to gain more detailed insight into genetic and fetal causes of pregnancy loss. Array CGH and NGS are replacing traditional karyotyping for ultrasonically discovered structural fetal malformations (Filges *et al.* 2015), and a few papers have already been published on exome sequencing in recurrent pregnancy loss, potentially opening the door for the identification of genetic causes of pregnancy loss beyond the large numerical and structural anomalies identified by traditional karyotyping (Shamseldin *et al.* 2013; Qiao *et al.* 2016).

Cell free fetal DNA (cffDNA) – small fragments of cell-free fetal DNA in the maternal blood stream – would also be interesting to study in relation to RPL. cffDNA is already used clinically to detect chromosomal aneuploidies, primarily chromosome 15, 18, 21 and sex chromosomes from Gestational Week 10. After delivery, cffDNA is cleared very quickly from the maternal blood stream, but the half-life after a delayed miscarriage is to my knowledge not known. An increased fraction of cffDNA in the maternal blood stream at 14 – 20 weeks' gestation has been linked to the risk of preterm birth (Dugoff *et al.* 2016). As a proof-of-concept study, it would be interesting to investigate how early cffDNA could be discovered in the maternal blood stream in women with RPL and whether this is related to the outcome of pregnancy.

Concluding remarks

We have to be humble. A proportion of RPL patients do not have a pathological condition. Reproductively speaking, they are too old or just unlucky. These couples need emotional support and recognition of their loss (Kolte *et al.* 2015) as well as realistic counselling of their chances of a having a liveborn child. But some couples and families with RPL have an underlying disorder and before we have a better understanding of the physiology of early pregnancy, our chances of discovering effective treatments are poor. The studies presented in this thesis may help to answer some of our questions and lay the foundation for new questions and endeavors into early pregnancy research.

Summary

Recurrent pregnancy loss (RPL) is a condition defined as three or more consecutive pregnancy losses. The disorder affects 1-3% of couples attempting to have a child. The etiology is mostly unknown. The work for this thesis stems from a wish to unravel aspects of epidemiology, genetics and evolution in RPL.

A number of early studies found an increased prevalence of pregnancy loss among patients' siblings. In *Paper I* we conducted an epidemiological study and showed that female RPL patients' siblings experienced pregnancy loss in 23.3% percent of their pregnancies, significantly more than in the background population where 13.5% of pregnancies end in a pregnancy loss, OR 2.04 (1.4 – 3.0). In a genome-wide linkage analysis of 38 affected sib-pairs, we identified a number of genomic regions with a LOD score between 2.5 and 3.0, thus not unequivocally identifying a linkage to genomic regions. This part of the study was limited by the small number of affected sibling pairs.

It has been shown that women with secondary RPL after the birth of a boy have a poorer chance of live birth in the first pregnancy after referral to a dedicated RPL unit if they are carriers of HY-restricting HLA class II alleles. However, the long term chance of live birth is both clinically and biologically a more important outcome measure, which is what we investigated in *Paper II* in a cohort of 540 women with unexplained secondary RPL. We found that the chance of live birth was significantly poorer for women who were carriers of HY-restricting HLA class II alleles, but only if their firstborn was a boy. Thus we could extend our results regarding first pregnancy after referral to long term outcome.

The 8.1 ancestral haplotype (AH) (HLA-A1, C7, B8, C4AQ0, C4B1, DR3, DQ2) is a remarkably long and conserved haplotype in the human major histocompatibility complex. The haplotype has been associated with both beneficial and detrimental effects. Theoretically, the survival of haplotypes such as the 8.1AH may be due to gestational drive, i.e. selective miscarriage of embryos/fetuses who have not inherited the haplotype from a heterozygous mother, leading to a higher percentage of surviving carriers of the haplotype than expected by Mendelian inheritance. In *Paper III* we tested the gestational drive theory for the 8.1AH in 82 women with RPL and 110 of their live-born children. In the group overall, 55% of the live-born children had inherited the 8.1AH, which was not significantly more than the expected 50% (p=0.25). However, we found a non-significant trend towards a higher inheritance of the 8.1AH in the 48 girls, 63%, p=0.11 as

opposed to the 62 boys, 50%, p=1.00. The gestational drive theory does not predict sex-specific effects but competition between siblings may be more intense among same-sex siblings.

The studies presented in this thesis answer some of our outstanding questions in RPL, but the studies are constrained by the limits of our current understanding of early pregnancy in general and RPL in particular. However, the studies have generated new hypotheses and laid the foundation for future undertakings in the mesmerizing world of early pregnancy research.

Dansk resumé

Abortus habitualis er defineret som tre eller flere konsekutive ufrivillige graviditetstab. Tilstanden rammer 1-3% af alle par, som forsøger at få et barn. Ætiologien er i de fleste tilfælde ukendt. De studier, som er præsenteret i denne phd-afhandling, bygger på et ønske om at klarlægge aspekter af epidemiologi, genetik og evolution i abortus habitualis.

Et antal tidlige studier fandt en øget forekomst af graviditetstab blandt abortus habitualis-patienters søskende. I *Studie I* udførte vi et epidemiologisk studie, hvor vi fandt, at kvindelige abortus habitualis-patienters søskende oplevede graviditetstab i 23,3% af deres graviditeter, hvilket var signifikant oftere end i baggrundsbefolkningen, hvor 13,5% af alle graviditeter ikke ender med et levende barn; OR 2,04 (95% CI 1,4 – 3,0). I en fuld-genom-undersøgelse af 38 afficerede søskendepar fandt vi et antal genomiske områder med en LOD score mellem 2,5 og 3,0. Vi kunne således ikke vise en signifikant kobling mellem abortus habitualis og genomiske regioner. Denne del af studiet var begrænset af det lave antal informative søskendepar.

Det er tidligere vist, at kvinder med abortus habitualis efter en førstefødt dreng har en dårligere chance for at få et levende barn i første graviditet efter henvisning til en specialiseret abortus habitualis enhed, hvis de er bærere af HY-præsenterende HLA klasse II alleler. Både klinisk og biologisk er langtidsprognosen for et levende barn mere relevant, hvilket vi undersøgte i *Studie II* i en kohorte af 540 kvinder med uforklaret sekundær abortus habitualis. Vi fandt, at langtidsprognosen for et levende barn var signifikant dårligere for kvinder med HY-præsenterende HLA klasse II alleler, men kun hvis de havde en førstefødt dreng. Således kunne vi udvide vores tidligere resultater til også at omhandle langtidsprognose.

8.1 ancestrale haplotype (8.1AH) (HLA-A1, C7, B8, C4AQ0, C4B1, DR3, DQ2) er en bemærkelsesværdigt lang og evolutionært konserveret haplotype i det humane histokompatibilitetskompleks. Haplotypen er blevet koblet til både positive og negative effekter. Teoretisk set kunne overlevelsen af haplotyper som 8.1AH skyldes 'gestational drive', dvs. selektiv abort af fostre, som ikke har arvet haplotypen fra en heterozygot mor. Dette ville føre til en højere andel af overlevende børn, som var bærere, end forventet ud fra mendelsk arvegang. I *Studie III* testede vi gestational drive-hypotesen for 8.1AH blandt 82 kvinder med abortus habitualis og 110 af deres levendefødte børn. I gruppen som helhed havde 55% af børnene arvet 8.1AH fra deres mor, hvilket ikke var signifikant flere end de forventede 50% (p=0.29). Blandt de 48 piger fandt vi en ikke-signifikant trend mod højere nedarvning af 8.1AH, 63%, p=0.11, i modsætning til de 62

drenge, 50%, p=1.0. Gestational drive-hypotesen forudsiger ikke kønsspecifikke effekter, men konkurrence blandt søskende af samme køn kan være mere intens.

Studierne præsenteret i denne afhandling afdækker nogle af de hidtil ubesvarede spørgsmål vedrørende abortus habitualis, men studierne er begrænset af vores manglende forståelse for processerne i tidlig graviditet og særligt for abortus habitualis. Studierne har genereret nye hypoteser og banet vejen for nye ekspeditioner i den fascinerede verden af tidlig graviditetsforskning.

References

Alexander, S. A., D. Latinne, M. Drebruyere, E. Dupont, W. Gottlieb and K. Thomas (1988). Belgian experience with repeat immunisation in recurrent spontaneous abortions. Early Pregnancy Loss: Mechanisms and Treatment. Eds. R. W. Beard and F. Sharp. London, UK, Springer Verlag**:** 355-363.

Aly, T. A., E. Eller, A. Ide, K. Gowan, S. R. Babu, H. A. Erlich, M. J. Rewers, G. S. Eisenbarth and P. R. Fain (2006). Multi-SNP analysis of MHC region: remarkable conservation of HLA-A1-B8-DR3 haplotype. Diabetes **55**(5): 1265-1269.

ASRM Practice Committee (2013). Definitions of infertility and recurrent pregnancy loss: a committee opinion. Fertil Steril **99**(1): 63.

Bernardi, L. A., B. A. Plunkett and M. D. Stephenson (2012). Is chromosome testing of the second miscarriage cost saving? A decision analysis of selective versus universal recurrent pregnancy loss evaluation. Fertil Steril **98**(1): 156-161.

Bhattacharya, S., J. Townend and S. Bhattacharya (2010). Recurrent miscarriage: Are three miscarriages one too many? Analysis of a Scottish population-based database of 151,021 pregnancies. Eur J Obstet Gynecol Reprod Biol **150**(1): 24-27.

Brigham, S. A., C. Conlon and R. G. Farquharson (1999). A longitudinal study of pregnancy outcome following idiopathic recurrent miscarriage. Hum Reprod **14**(11): 2868-2871.

Chard, T. (1991). Frequency of implantation and early pregnancy loss in natural cycles. Baillieres Clin Obstet Gynaecol **5**(1): 179-189.

Christiansen, O. B. (1996). A fresh look at the causes and treatments of recurrent miscarriage, especially its immunological aspects. Hum Reprod Update **2**(4): 271-293.

Christiansen, O. B., H. H. Andersen, M. Hojbjerre, T. A. Kruse, S. L. Lauritzen and N. Grunnet (1995). Maternal HLA class II allogenotypes are markers for the predisposition to fetal losses in families of women with unexplained recurrent fetal loss. Eur J Immunogenet **22**(4): 323-334.

Christiansen, O. B., O. Mathiesen, J. G. Lauritsen and N. Grunnet (1990). Idiopathic recurrent spontaneous abortion. Evidence of a familial predisposition. Acta Obstet Gynecol Scand **69**(7-8): 597-601.

Christiansen, O. B., B. Pedersen, H. S. Nielsen and A. M. Nybo Andersen (2004). Impact of the sex of first child on the prognosis in secondary recurrent miscarriage. Hum Reprod **19**(12): 2946-2951.

Christiansen, O. B., B. Pedersen, A. Rosgaard and M. Husth (2002). A randomized, double-blind, placebo-controlled trial of intravenous immunoglobulin in the prevention of recurrent miscarriage: evidence for a therapeutic effect in women with secondary recurrent miscarriage. Hum Reprod **17**(3): 809-816.

CNGOF. (2014). Recommandations pour la pratique clinique "Les pertes de grossesse" (texte court) (French). Retrieved March 17, 2015, from http://www.cngof.asso.fr/data/RCP/CNGOF_2014_pertes_grossesse.pdf.

Cole, L. A. (2010). Biological functions of hCG and hCG-related molecules. Reprod Biol Endocrinol **8**: 102.

Coulam, C. B. (2000). Understanding the immunobiology of pregnancy and applying it to treatment of recurrent pregnancy loss. Early Pregnancy **4**(1): 19-29.

Crespi, B. (2014). Medical, ethical and personal dimensions of parent-offspring conflicts. Evol Med Public Health **2014**(1): 51-53.

D'Souza, A. W. and G. P. Wagner (2014). Malignant cancer and invasive placentation: A case for positive pleiotropy between endometrial and malignancy phenotypes. Evol Med Public Health **2014**(1): 136-145.

Das, U. G. and G. D. Sysyn (2004). Abnormal fetal growth: intrauterine growth retardation, small for gestational age, large for gestational age. Pediatr Clin North Am **51**(3): 639-654, viii.

DSOG. (2009). Abortus Habitualis, clinical guideline (Danish). Retrieved March 17, 2015, from http://gynobsguideline.dk/hindsgavl/Abortus%20habitualis%20Hindsgavl%20guideline%202007ret09.pdf.

Dugoff, L., A. Barberio, P. G. Whittaker, N. Schwartz, H. Sehdev and J. A. Bastek (2016). Cell-free DNA fetal fraction and preterm birth. Am J Obstet Gynecol.

Eljaafari, A., O. Yuruker, C. Ferrand, A. Farre, C. Addey, M. L. Tartelin, X. Thomas, P. Tiberghien, E. Simpson, D. Rigal and D. Scott (2013). Isolation of human CD4/CD8 double-positive, graft-versus-host disease-protective, minor histocompatibility antigen-specific regulatory T cells and of a novel HLA-DR7-restricted HY-specific CD4 clone. J Immunol **190**(1): 184-194.

Filges, I. and J. M. Friedman (2015). Exome sequencing for gene discovery in lethal fetal disorders--harnessing the value of extreme phenotypes. Prenat Diagn **35**(10): 1005-1009.

Gharesi-Fard, B., R. Askarinejad-Behbahani and S. Behdin (2014). The effect of HLA-DRB1 sharing between the couples with recurrent pregnancy loss on the pregnancy outcome after leukocyte therapy. Iran J Immunol **11**(1): 13-20.

Greenberg, T., L. Tzivian, A. Harlev, R. Serjienko, M. Mazor and A. Bashiri (2015). Index pregnancy versus post-index pregnancy in patients with recurrent pregnancy loss. J Matern Fetal Neonatal Med **28**(1): 63-67.

Haig, D. (1993). Genetic conflicts in human pregnancy. Q Rev Biol **68**(4): 495-532.

Haig, D. (1996). Gestational drive and the green-bearded placenta. Proc Natl Acad Sci U S A **93**(13): 6547-6551.

Haig, D. (1997). Maternal-fetal interactions and MHC polymorphism. J Reprod Immunol **35**(2): 101-109.

Haig, D. (2004). Evolutionary conflicts in pregnancy and calcium metabolism--a review. Placenta **25 Suppl A**: S10-15.

Haig, D. (2014). Interbirth intervals: Intrafamilial, intragenomic and intrasomatic conflict. Evol Med Public Health **2014**(1): 12-17.

Haig, D. (2014). Troubled sleep: A response to commentaries. Evol Med Public Health **2014**(1): 57-62.

Hastings, I. M. (2001). Reproductive compensation and human genetic disease. Genet Res **77**(3): 277-283.

Ho, H. N., T. J. Gill, 3rd, C. Y. Hsieh, Y. S. Yang and T. Y. Lee (1991). The prevalence of recurrent spontaneous abortions, cancer, and congenital anomalies in the families of couples with recurrent spontaneous abortions or gestational trophoblastic tumors. Am J Obstet Gynecol **165**(2): 461-466.

Hromadnikova, I., K. Kotlabova, P. Pirkova, P. Libalova, Z. Vernerova, B. Svoboda and E. Kucera (2014). The occurrence of fetal microchimeric cells in endometrial tissues is a very common phenomenon in benign uterine disorders, and the lower prevalence of fetal microchimerism is associated with better uterine cancer prognoses. DNA Cell Biol **33**(1): 40-48.

Jauniaux, E., R. G. Farquharson, O. B. Christiansen and N. Exalto (2006). Evidence-based guidelines for the investigation and medical treatment of recurrent miscarriage. Hum Reprod **21**(9): 2216-2222.

Johnson, P. M., K. V. Chia, J. M. Risk, R. M. Barnes and J. C. Woodrow (1988). Immunological and immunogenetic investigation of recurrent spontaneous abortion. Dis Markers **6**(3): 163-171.

Jones, K. T. and S. I. Lane (2013). Molecular causes of aneuploidy in mammalian eggs. Development **140**(18): 3719-3730.

Klein, J. and A. Sato (2000). The HLA system. Second of two parts. N Engl J Med **343**(11): 782-786.

Knudsen, U. B., V. Hansen, S. Juul and N. J. Secher (1991). Prognosis of a new pregnancy following previous spontaneous abortions. Eur J Obstet Gynecol Reprod Biol **39**(1): 31-36.

Kolte, A. M., L. A. Bernardi, O. B. Christiansen, S. Quenby, R. G. Farquharson, M. Goddijn, M. D. Stephenson and E. P. Eshre Special Interest Group (2015). Terminology for pregnancy loss prior to viability: a consensus statement from the ESHRE early pregnancy special interest group. Hum Reprod **30**(3): 495-498.

Kolte, A. M., L. A. Bernardi, O. B. Christiansen, S. Quenby, R. G. Farquharson, M. Goddijn and M. D. Stephenson (2015). Terminology for pregnancy loss prior to viability: a consensus statement from the ESHRE early pregnancy special interest group. Hum Reprod **30**(3): 4.

Kolte, A. M., H. S. Nielsen, I. Moltke, B. Degn, B. Pedersen, L. Sunde, F. C. Nielsen and O. B. Christiansen (2011). A genome-wide scan in affected sibling pairs with idiopathic recurrent miscarriage suggests genetic linkage. Mol Hum Reprod **17**(6): 379-385.

Kolte, A. M., H. S. Nielsen, R. Steffensen, B. Crespi and O. B. Christiansen (2015). Inheritance of the 8.1 ancestral haplotype in recurrent pregnancy loss. Evol Med Public Health **2015**(1): 325-331.

Kolte, A. M., L. R. Olsen, E. M. Mikkelsen, O. B. Christiansen and H. S. Nielsen (2015). Depression and emotional stress is highly prevalent among women with recurrent pregnancy loss. Hum Reprod **30**(4): 777-782.

Kolte, A. M., R. Steffensen, O. B. Christiansen and H. S. Nielsen (2016). HY-restricting HLA class II alleles are associated with poor long term outcome in recurrent pregnancy loss after a boy. Submitted to Am J Reprod Immunol June 2016.

Kolte, A. M., R. H. van Oppenraaij, S. Quenby, R. G. Farquharson, M. Stephenson, M. Goddijn, O. B. Christiansen and E. S. I. G. E. Pregnancy (2014). Non-visualized pregnancy losses are prognostically important for unexplained recurrent miscarriage. Hum Reprod **29**(5): 931-937.

Kosova, G., M. D. Stephenson, V. J. Lynch and C. Ober (2015). Evolutionary forward genomics reveals novel insights into the genes and pathways dysregulated in recurrent early pregnancy loss. Hum Reprod **30**(3): 519-529.

Kourtis, A. P., J. S. Read and D. J. Jamieson (2014). Pregnancy and infection. N Engl J Med **370**(23): 2211-2218.

Kruse, C., R. Steffensen, K. Varming and O. B. Christiansen (2004). A study of HLA-DR and -DQ alleles in 588 patients and 562 controls confirms that HLA-DRB1*03 is associated with recurrent miscarriage. Hum Reprod **19**(5): 1215-1221.

Larsen, E. C., O. B. Christiansen, A. M. Kolte and N. Macklon (2013). New insights into mechanisms behind miscarriage. BMC Med **11**: 154.

Li, J., L. Liu, B. Liu, S. Saravelos and T. Li (2014). Recurrent miscarriage and birth sex ratio. Eur J Obstet Gynecol Reprod Biol **176**: 55-59.

Loren, A. W., G. R. Bunin, C. Boudreau, R. E. Champlin, A. Cnaan, M. M. Horowitz, F. R. Loberiza and D. L. Porter (2006). Impact of donor and recipient sex and parity on outcomes of HLA-identical sibling allogeneic hematopoietic stem cell transplantation. Biol Blood Marrow Transplant **12**(7): 758-769.

Lund, M., M. Kamper-Jorgensen, H. S. Nielsen, O. Lidegaard, A. M. Andersen and O. B. Christiansen (2012). Prognosis for live birth in women with recurrent miscarriage: what is the best measure of success? Obstet Gynecol **119**(1): 37-43.

Macklon, N. S., J. P. Geraedts and B. C. Fauser (2002). Conception to ongoing pregnancy: the 'black box' of early pregnancy loss. Hum Reprod Update **8**(4): 333-343.

Malpas, P. (1938). A study of abortion sequences. Journal of obstetrics and gynaecology of the British Empire **45**(6): 18.

Marquard, K., L. M. Westphal, A. A. Milki and R. B. Lathi (2010). Etiology of recurrent pregnancy loss in women over the age of 35 years. Fertil Steril **94**(4): 1473-1477.

Medawar, P. B. (1952). Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. Symp Soc Exp biol **7**: 19.

Miller, S. A., D. D. Dykes and H. F. Polesky (1988). A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res **16**(3): 1215.

Minakami, H., T. Maeda, T. Fujii, H. Hamada, Y. Iitsuka, A. Itakura, H. Itoh, M. Iwashita, T. Kanagawa, M. Kanai, Y. Kasuga, M. Kawabata, K. Kobayashi, T. Kotani, Y. Kudo, Y. Makino, S. Matsubara, H. Matsuda, K. Miura, T. Murakoshi, J. Murotsuki, A. Ohkuchi, Y. Ohno, Y. Ohshiba, S. Satoh, A. Sekizawa, M. Sugiura, S. Suzuki, T. Takahashi, Y. Tsukahara, N. Unno and H. Yoshikawa (2014). Guidelines for obstetrical practice in Japan: Japan Society of Obstetrics and Gynecology (JSOG) and Japan Association of Obstetricians and Gynecologists (JAOG) 2014 edition. J Obstet Gynaecol Res **40**(6): 1469-1499.

Moffett, A. and F. Colucci (2015). Co-evolution of NK receptors and HLA ligands in humans is driven by reproduction. Immunol Rev **267**(1): 283-297.

Mor, G. and J. Y. Kwon (2015). Trophoblast-microbiome interaction: a new paradigm on immune regulation. Am J Obstet Gynecol **213**(4 Suppl): S131-137.

Murray, M. J. and B. A. Lessey (1999). Embryo implantation and tumor metastasis: common pathways of invasion and angiogenesis. Semin Reprod Endocrinol **17**(3): 275-290.

Nelson, J. L. (2012). The otherness of self: microchimerism in health and disease. Trends Immunol **33**(8): 421-427.

Nielsen, H. S. (2011). Secondary recurrent miscarriage and H-Y immunity. Hum Reprod Update **17**(4): 558-574.

Nielsen, H. S., A. M. Andersen, A. M. Kolte and O. B. Christiansen (2008). A firstborn boy is suggestive of a strong prognostic factor in secondary recurrent miscarriage: a confirmatory study. Fertil Steril **89**(4): 907-911.

Nielsen, H. S., L. Mortensen, U. Nygaard, O. Schnor, O. B. Christiansen and A. M. Andersen (2008). Brothers and reduction of the birth weight of later-born siblings. Am J Epidemiol **167**(4): 480-484.

Nielsen, H. S., R. Steffensen, M. Lund, L. Egestad, L. H. Mortensen, A. M. Andersen, O. Lidegaard and O. B. Christiansen (2010). Frequency and impact of obstetric complications prior and subsequent to unexplained secondary recurrent miscarriage. Hum Reprod **25**(6): 1543-1552.

Nielsen, H. S., R. Steffensen, K. Varming, A. G. Van Halteren, E. Spierings, L. P. Ryder, E. Goulmy and O. B. Christiansen (2009). Association of HY-restricting HLA class II alleles with pregnancy outcome in patients with recurrent miscarriage subsequent to a firstborn boy. Hum Mol Genet **18**(9): 1684-1691.

NVOG (2007). Guideline recurrent miscarriage.

Nybo Andersen, A. M., J. Wohlfahrt, P. Christens, J. Olsen and M. Melbye (2000). Maternal age and fetal loss: population based register linkage study. BMJ **320**(7251): 1708-1712.

Ogasawara, M., K. Aoki, S. Okada and K. Suzumori (2000). Embryonic karyotype of abortuses in relation to the number of previous miscarriages. Fertil Steril **73**(2): 300-304.

Ooi, P. V., N. Russell and K. O'Donoghue (2011). Secondary recurrent miscarriage is associated with previous male birth. J Reprod Immunol **88**(1): 38-41.

Ott, J., Y. Kamatani and M. Lathrop (2011). Family-based designs for genome-wide association studies. Nat Rev Genet **12**(7): 465-474.

Parazzini, F., B. Acaia, O. Ricciardiello, L. Fedele, P. Liati and G. B. Candiani (1988). Short-term reproductive prognosis when no cause can be found for recurrent miscarriage. Br J Obstet Gynaecol **95**(7): 654-658.

Parham, P. (2015). The Immune System. New York, Garland Publishing.

Popli, R., B. Sahaf, H. Nakasone, J. Y. Lee and D. B. Miklos (2014). Clinical impact of H-Y alloimmunity. Immunol Res **58**(2-3): 249-258.

Pouta, A., M. R. Jarvelin, E. Hemminki, U. Sovio and A. L. Hartikainen (2005). Mothers and daughters: intergenerational patterns of reproduction. Eur J Public Health **15**(2): 195-199.

Qiao, Y., J. Wen, F. Tang, S. Martell, N. Shomer, P. C. Leung, M. D. Stephenson and E. Rajcan-Separovic (2016). Whole exome sequencing in recurrent early pregnancy loss. Mol Hum Reprod **22**(5): 364-372.

Quenby, S. M. and R. G. Farquharson (1993). Predicting recurring miscarriage: what is important? Obstet Gynecol **82**(1): 132-138.

RCOG. (2011). Recurrent Miscarriage, Investigation and Treatment of Couples (Green-top Guideline No. 17). RCOG Green Top Guideline Retrieved March 17, 2015, from https://www.rcog.org.uk/en/guidelines-research-services/guidelines/gtg17/.

Roberts, C. J. and C. R. Lowe (1975). Where have all the conceptions gone? Lancet **305**(7905): 2.

Sadler, T. W. (2014). Langman's Medical Embryology. Philadelphia, Lippincott Williams & Wilkins.

Saravelos, S. H. and L. Regan (2014). Unexplained recurrent pregnancy loss. Obstet Gynecol Clin North Am **41**(1): 157-166.

Shamseldin, H. E., A. Swaid and F. S. Alkuraya (2013). Lifting the lid on unborn lethal Mendelian phenotypes through exome sequencing. Genet Med **15**(4): 307-309.

Spierings, E., C. J. Vermeulen, M. H. Vogt, L. E. Doerner, J. H. Falkenburg, T. Mutis and E. Goulmy (2003). Identification of HLA class II-restricted H-Y-specific T-helper epitope evoking CD4+ T-helper cells in H-Y-mismatched transplantation. Lancet **362**(9384): 610-615.

Stephenson, M. D., K. A. Awartani and W. P. Robinson (2002). Cytogenetic analysis of miscarriages from couples with recurrent miscarriage: a case-control study. Hum Reprod **17**(2): 446-451.

Takakuwa, K., H. Adachi, I. Hataya, K. Ishii, M. Tamura and K. Tanaka (2003). Molecular genetic studies of HLA-DRB1 alleles in patients with unexplained recurrent abortion in the Japanese population. Hum Reprod **18**(4): 728-733.

Tilghman, S. M. (1999). The sins of the fathers and mothers: genomic imprinting in mammalian development. Cell **96**(2): 185-193.

van den Berg, M. M., M. C. van Maarle, M. van Wely and M. Goddijn (2012). Genetics of early miscarriage. Biochim Biophys Acta **1822**(12): 1951-1959.

Wilcox, A. J. (2001). On the importance--and the unimportance--of birthweight. Int J Epidemiol **30**(6): 1233-1241.

Wilcox, A. J., C. R. Weinberg, J. F. O'Connor, D. D. Baird, J. P. Schlatterer, R. E. Canfield, E. G. Armstrong and B. C. Nisula (1988). Incidence of early loss of pregnancy. N Engl J Med **319**(4): 189-194.

Williams, G. C. (1957). Pleiotropy, Natural Selection, and the Evolution of Senescence. Evolution **11**(4): 14.

Zhang, B. Y., Y. S. Wei, J. M. Niu, Y. Li, Z. L. Miao and Z. N. Wang (2010). Risk factors for unexplained recurrent spontaneous abortion in a population from southern China. Int J Gynaecol Obstet **108**(2): 135-138.

Appendix

Paper I, II & III

Authorship declarations

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MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

A genome-wide scan in affected sibling pairs with idiopathic recurrent miscarriage suggests genetic linkage

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ABSTRACT: Previously, siblings of patients with idiopathic recurrent miscarriage (IRM) have been shown to have a higher risk of miscarriage. This study comprises two parts: (i) an epidemiological part, in which we introduce data on the frequency of miscarriage among 268 siblings of 244 patients with IRM and (ii) a genetic part presenting data from a genome-wide linkage study of 38 affected sibling pairs with IRM. All IRM patients (probands) had experienced three or more miscarriages and affected siblings two or more miscarriages. The sibling pairs were genotyped by the Affymetrix GeneChip 50K *Xba*I platform and non-parametric linkage analysis was performed via the software package Merlin. We find that siblings of IRM patients exhibit a higher frequency of miscarriage than population controls regardless of age at the time of pregnancy. We identify chromosomal regions with LOD scores between 2.5 and 3.0 in subgroups of affected sibling pairs. Maximum LOD scores were identified in four occurrences: for rs10514716 (3p14.2) when analyzing sister-pairs only; for rs10511668 (9p22.1) and rs341048 (11q13.4) when only analyzing families where the probands have had four or more miscarriages; and for rs10485275 (6q16.3) when analyzing one sibling pair from each family only. We identify no founder mutations. Concluding, our results imply that IRM patients and their siblings share factors which increase the risk of miscarriage. In this first genome-wide linkage study of affected sibling pairs with IRM, we identify regions on chromosomes 3, 6, 9 and 11 which warrant further investigation in order to elucidate their putative roles in the genesis of IRM.

Key words: recurrent miscarriage / affected sibling pair analysis / risk of miscarriage / epidemiology / genome-wide linkage study

Introduction

Recurrent miscarriage (RM) is defined as three or more consecutive intrauterine miscarriages. RM affects 1–3% of couples who wish to reproduce. Uterine abnormalities, abnormal karyotypes in the couple, endocrine disorders and the presence of the lupus anticoagulant or antinuclear antibodies are relatively well-documented risk factors for miscarriage (Stephenson, 1996; Baek *et al.*, 2007). However, in about 50% of couples suffering from RM none of these factors can be found, and the condition is thus termed idiopathic RM (IRM). It has been proposed that RM has a multifactorial etiology (Christiansen *et al.*, 2008).

Further, the risk of miscarriage has earlier been reported to be enhanced for siblings of IRM patients (Christiansen *et al.*, 1990). This led to our hypothesis that IRM has a partially genetic etiology.

Various maternal genotypes have been reported to be associated with RM. These include the thrombophilic genetic variants factor II and factor V Leiden, which have been associated with both early and late RM (Rey *et al.*, 2003). The discovery that sisters who are HLA identical by descent (IBD) with probands with RM exhibit a higher risk of miscarriage than those who are not HLA identical, suggests that genes in the HLA region are involved in RM (Christiansen *et al.*, 1989). An association with HLA-DRB1*03 has been reported (Christiansen *et al.*, 1994; Kruse *et al.*, 2004), especially for patients with four or more miscarriages or secondary RM. Hviid *et al.*, 2004 found that homozygosity for a 14-bp insertion in exon 8 of the *HLA-G* gene is correlated with an increased risk of RM. High cytokine-producing genotypes of *IFN-γ* (Daher *et al.*, 2003) as well as a specific haplotype of *IL10* promoter polymorphisms have been associated with RM (Zammiti *et al.*, 2006), although there is no consensus on this (Babbage *et al.*, 2001). Low levels of plasma mannose-binding lectin (MBL) in the plasma (Kilpatrick *et al.*, 1995; Kruse *et al.*, 2002) and carriage of *MBL2* genotypes disposing to MBL deficiency have also been associated with RM (Christiansen *et al.*, 2009b).

Recently, in a genome-wide association study, 430 microsatellites were investigated in 44 patients with two or more idiopathic miscarriages and 44 unrelated controls. The authors found significant associations between 6q27, 9q33.1 and Xp22.11 and IRM (Li et al., 2010).

In this paper we present results from a study with an epidemiological part confirming that siblings of IRM patients exhibit a higher risk of miscarriage. In the genetic part of the study we investigate a multicase cohort using affected sibling pair analysis (Risch 1990) and attempt to identify genetic markers shared among the affected siblings with IRM.

Materials and Methods

Participants

Hospital records of all RM patients referred to the Danish Miscarriage Clinic between 1986 and 2004 (709 patients) were evaluated in 2004–2005. *A priori* the following patients were excluded: those with no living, full siblings in Denmark ($n = 70$); incomplete medical investigation ($n = 52$); less than three confirmed miscarriages ($n = 25$); abnormal parental karyotype ($n = 6$); abnormal uterine anatomy ($n = 14$); irregular menstrual cycle ($n = 8$) and lupus anticoagulant in the plasma ($n = 6$), and we also excluded non-Danish-speaking or non-Caucasian patients ($n = 12$); those who had emigrated ($n = 6$) or were deceased ($n = 4$). Thus, the eligible patients (probands) fulfilled the criteria for IRM, having had at least three consecutive miscarriages and no established risk factors.

Five hundred and six probands were contacted and asked for an update on their reproductive history and the names and addresses of their full siblings. Of these, 102 did not wish to participate and 160 patients did not reply despite a reminder, leaving 244 patients (48%) and 381 full siblings in the study group. From the 381 siblings we obtained reproductive data from 268 sisters and brothers' wives.

Twenty-three of the probands had one responding sibling who had had at least two miscarriages, and three had two responding siblings with two or more miscarriages.

The siblings' miscarriages were not necessarily consecutive. For both probands and siblings, second trimester miscarriages and biochemical pregnancies were included. In a few instances, karyotype data were available, but not in the majority of cases. Ectopic pregnancies were not counted as miscarriages for probands or siblings. From 2005 to 2008, an additional five multicase families were included, applying the protocol indicated above. In one of these families there were two affected siblings. In the remaining four there was one sibling with two or more miscarriages. From these 31 families, blood samples from the proband and the affected sibling(s) were collected for DNA chip analysis.

All probands and siblings in multicase families were also HLA typed as part of a larger cohort, the results of which are previously published (Kolte et al., 2010).

Laboratory work

The DNA was extracted using a salting-out method, as described elsewhere (Miller et al., 1988). For the genome-wide linkage study we used the Affymetrix® GeneChip® 50K *Xba*I (Affymetrix, Santa Clara, CA, USA) platform, according to the manufacturer's instructions. Briefly: First, the DNA was digested for 2 h at 37°C with *Xba*I (New England BioLabs, Ipswich, MA, USA). After ligation with a 31-bp adaptor (Affymetrix), the DNA was amplified by one-primer PCR using Platinum *Pfx* polymerase (Invitrogen, Carlsbad, CA, USA). The amplicons were then fractionated to less than 180 bp by DNaseI (Affymetrix). We increased fragmentation time from 35 to 40 min. After labeling via TUNEL assay, the amplicons were hybridized to GeneChips (Affymetrix). The hybridized amplicons were visualized with streptavidin R-phycoerythrin (SAPE) (Invitrogen), biotinylated anti-streptavidin (Vector Labs, Burlingame, CA, USA) and SAPE. Finally the chips were scanned in an Affymetrix® GeneChip® scanner.

Data processing and statistics

The prevalence of miscarriage among the probands' siblings was evaluated using the Mantel Haenzel χ^2 test for linear association at a 95% significance level, inputted in SPSS. Only pregnancies where the woman's age was known were considered.

The SNPs were annotated using the following database: genome version: ucsc: hg18, ncbi build 36.1; snp version: dbsnp 126; annotation version: netaffx build 27. For genotype calling, the CRLMM algorithm for base calling was used as implemented in the 'oligo' R package. All CELL files were called in the same batch. In total, 58 959 SNPs were called. Of these 336 did not have annotation for either physical or genetic mapping position. Two SNPs mapped to the same position and thus a total of 338 SNPs were removed. Using the method of moments estimator from the 'plink' software, IBD sharing between individuals was determined. Based on this, one sample was declared misspecified and the family containing this sample was removed from further analysis. Analysis for sex misspecification and inbreeding showed no problems. Further SNP cleaning: 5404 SNPs had a minor allele frequency lower than 1% and were removed; 3563 SNPs had more than 5% missing data and were removed; 31 275 SNPs were in LD with adjacent markers ($r^2 > 0.1$ and LOD score >2) and were removed. The method we use for the linkage analysis is based on the assumption that there is no LD in the analyzed data. Therefore r^2 is set at a particularly low level of 0.1. A total of 18 379 SNPs remained after cleaning. Complying with the MIAME standards, raw data are available through the ArrayExpress Archive: http://www.ebi.ac.uk/arrayexpress/, accession number E-MEXP-2942.

Based on these SNPs, genome-wide non-parametric linkage analysis was performed using the S_{ALL} statistic (Whittemore and Halpern, 1994) as implemented in Merlin (Abecasis et al., 2002).

This was done both with all sibling pairs and with three different subsets of these: (i) sister pairs only (not including brothers), (ii) pairs containing probands with four or more miscarriages only and (iii) one sibling pair from each family only (randomly removing one of the siblings in all families with two affected siblings).

We also performed a relatedness analysis on the same data using the software package Relate (Albrechtsen et al., 2009) in order to look for founder mutations. Relate was run on all pairs of individuals between families assuming none of the individuals share more than one chromosome IBD and assuming that the rate of IBD state change along the genome is a function of the IBD sharing.

For both the linkage analyses and the relatedness analysis, we used genetic distances inferred from the Kong map (Kong et al., 2002).

Results

Epidemiological data

In 2005, former patients in the Danish Recurrent Miscarriage Clinic and their siblings were contacted for evaluation of reproductive history. Data were obtained from 244 patients with IRM (probands) and 268 siblings.

Frequency of miscarriage among the probands' siblings is 25.3 versus 13.1% in the general population (Nybo Andersen et al., 2000). Figure 1 illustrates that the increased risk among patients'

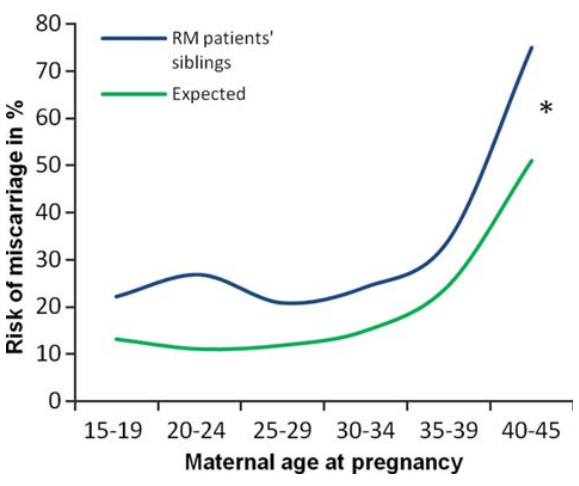

Figure 1 Risk of miscarriage among IRM patients' sisters and brothers' wives compared with the expected rate of miscarriage in the Danish population (34). *OR = 2.04, CI 1.4–3.0; *P*-value <0.001.

siblings is regardless of age at the time of pregnancy (OR = 2.04, confidence interval: 1.4–3.0; *P*-value <0.001).

Genome-wide linkage study

The families where a sister or brother's partner had had two or more miscarriages were included in the genetic part of the study. Thirty probands, 25 sisters and 9 brothers fulfilled the criteria, shown in Table I.

In a genome-wide linkage study using an SNP platform, the cut-off value for significance is usually set at LOD score 3.0 to compensate for multiple testing.

Applying a significance level of LOD score ≥3.0, no significantly linked markers were identified. However, in subanalyses, we did identify markers with LOD score >2.5 but <3.0, which suggests linkage. When studying sister pairs (28 pairs) exclusively, a LOD score of 2.9 was calculated for the marker rs10514716 (3p14.2). In sibling pairs where the proband had had four or more miscarriages (18 pairs), two peaks were found. Maximum LOD scores were found for rs10511668 (9p22.1) and rs341048 (11q13.4). When looking only at one sibling pair in each family (30 pairs), the highest LOD score was for rs10485275 (6q16.3) (see Table II for a description of the identified peaks in the subanalyses and Fig. 2 for an illustration

Table I The distribution of affected siblings and probands in the 30 families.

Number of families	Probands ≥3 miscarriages	Sisters ≥2 miscarriages	Brothers' spouses: ≥2 miscarriages	Sibling pairs
18	1	1	0	18
3	1	2	0	9
8	1	0	1	8
1	1	1	1	3

In total, 30 probands, 25 affected sisters and 9 brothers affected through their spouses were included, which gave 38 affected sibling pairs.

Table II Chromosomal areas with LOD score ≥2.5.

Chromosomal position	Marker[a]	Physical position[b]	LOD score	*P*-value	Gene containing the SNP	Borders of linkage peak	Width[d]
Only sister pairs							
3p14.2	rs10514716	59 456 628	2.9	0.00012	*FHIT*[c]	Chr3: 58 166 473–60 246 217	208
Sibling pairs with proband ≥4 miscarriages							
9p22.1	rs10511668	19 012 470	2.6	0.0003	*Hypothetical protein LOC 158297*, part of FAM154A	Chr9:18 244 023–19 583 084	1339
11q13.4	rs341048	71 975 928	2.9	0.00013	*PDE2A*	Chr11:68,435,538–74 753 514	6317
One sibling pair per family							
6q16.3	rs10485275	102 272 746	2.5	0.0004	*GRIK2*	Chr6:102 022 335–104 005 966	1983

In the analysis of all 38 sibling pairs, no markers had an LOD score ≥2.5.
[a]The SNP with the highest LOD score.
[b]In base pairs.
[c]SNP located 254 kb centromeric to AUG in *FHIT*.
[d]In kilobase.

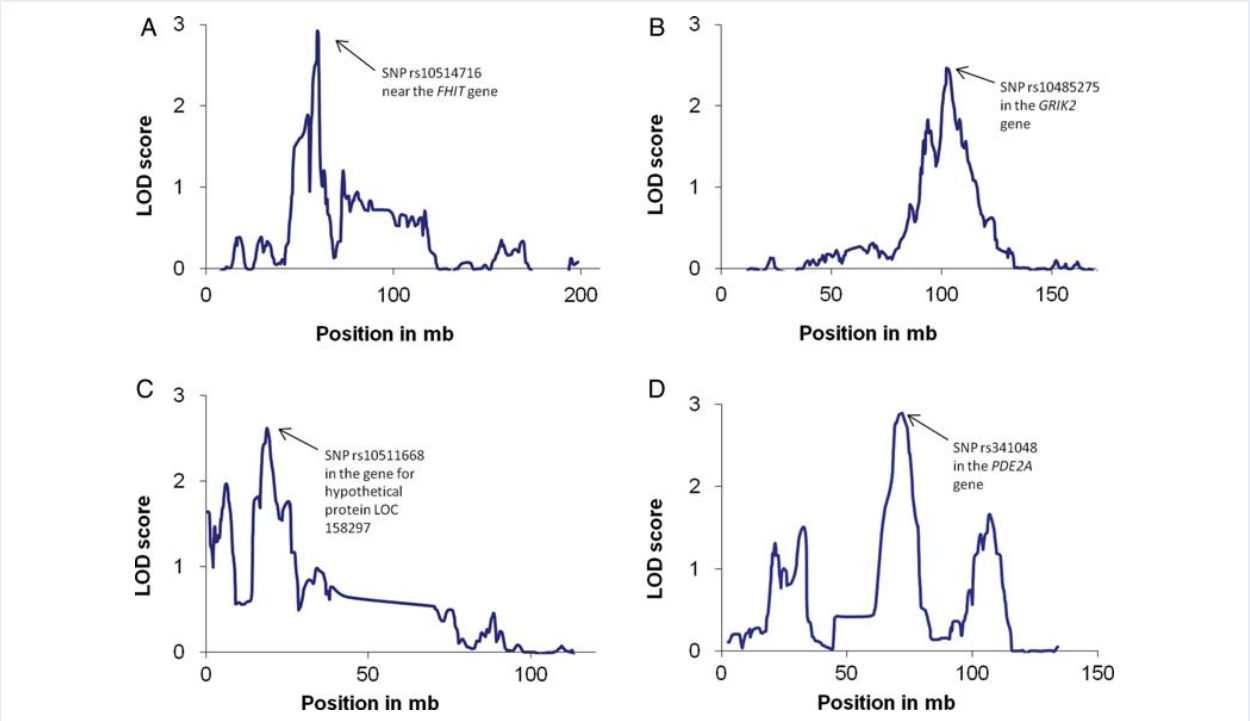

Figure 2 The four chromosomes in which peaks with LOD score >2.5 was identified in subgroups.

of the chromosomes in which we identified markers with LOD score >2.5). A full list of genes in the chromosomal areas which had an LOD score >2.5 in our analyses can be found via the UCSC genome browser at http://genome.ucsc.edu/cgi-bin/hgGateway.

We estimated the pairwise relatedness between families, but our results showed no sign of relatedness between families. Thus, no founder mutations were identified.

Discussion

The present study is the largest in which an investigation of reproductive outcome in siblings of patients with IRM has been carried out and furthermore it is the first-ever study that has carried out linkage analysis in affected sibling pairs with IRM using a genome-wide scan.

We confirm that siblings of IRM patients experience significantly higher numbers of miscarriages than the general population, and we demonstrate that this occurrence is irrespective of maternal age at the time of pregnancy. We hypothesized that some part may be genetic, and we conducted a genome-wide linkage study among 38 sibling pairs. This resulted in our identification of four genetic areas with suggestive linkage to IRM in subgroups of affected sibling pairs.

Interpretation of our results is limited by three factors.

First, there may have been a biased response rate in favor of families with affected siblings, which may tend to increase the reported miscarriage rate among siblings.

However, this would not affect the genetic part of the study as the comparisons of genetic markers were made within each family. By looking into our records obtained at the patients' first consultation (where the patient gave information about their siblings' reproductive history), we can confidently establish that the included multicase families can be considered representative for the whole IRM population, as the vast majority of multicase families have been included in the genetic part of the study.

Second, ascertainment bias may play a role, because families with two or more siblings with three or more miscarriages may preferentially be referred to our clinic. Thirteen of the 34 siblings in the genetic part of the study had had three or more miscarriages.

Finally, the present study has an inherent selection bias. If RM is indeed a condition for which genetic factors play a role it is likely that the patients' mothers also had reduced reproductive fitness. Women or couples carrying genetic variants that most severely affect reproductive fitness would therefore be expected to produce a limited number of children (one child or perhaps none). Such families would not be ascertained in the present study focusing on affected sibling pairs. This selection bias will thus result in an underestimation of the contribution of genetic variants to IRM. One could argue that miscarriage has positive effects on reproductive fitness as it prevents the birth of abnormal children and increases spacing between children, allowing the parents to improve physical and socioeconomic status. However, data from our group suggest decreased reproductive fitness due to repeated miscarriage, decreased fertility as a consequence of increasing age, salpingitis after surgical evacuation of miscarriage, abstinence from new pregnancy due to fear of miscarriage and dissolution of partnership as a consequence of stress and depression (unpublished data).

Our study is corroborated by a number of strengths. The study group is unique; the probands have been seen in the Danish RM clinic for periods of more than 20 years and as such represent the largest cohort of IRM patients worldwide to our knowledge. Furthermore, all probands had had three or more miscarriages and all probands were thoroughly investigated for known causes of RM, limiting our study group by the most rigorous criteria for IRM. Whenever possible, miscarriages reported by the participants were confirmed by hospital records. Finally, the Affymetrix GeneChip platform has been used in numerous large-scale association (Welcome Trust Case Control Consortium, 2007) and linkage studies (Romanos *et al*., 2008) can thus be considered as validated.

Epidemiology

The discovery that siblings of IRM patients have an increased frequency of miscarriage irrespective of age is important as it implies that the patient and her siblings share factors that increase susceptibility to pregnancy loss. Restricting the analysis to sisters only does not yield a significantly different result (data not shown). However, the number of reported pregnancies where the woman's age is unknown is higher in the group of brothers' wives, so caution should be taken when interpreting the results for this group. We compare the estimated risk of miscarriage among IRM patients' siblings with data from the Nybo Andersen study, which is based on information from the national discharge registry and the medical birth registry. Here, diagnoses at the time of discharge from a hospital are recorded. The data comprise information on maternal age and outcome for all pregnancies intended to be carried to birth in the period 1978–1992. Therefore, only miscarriages leading to hospital contact are registered here (Nybo Andersen *et al*., 2000). A possible bias is that we may overestimate risk of miscarriage among our patients as they may not have been admitted to a hospital following an early miscarriage. However, we estimate that only a minor number of the reported miscarriages in the group of probands' siblings have not led to a hospital contact as we have hospital documentation for the large majority. Furthermore, The Danish Recurrent Miscarriage Clinic is free of charge and women are referred to us from all over Denmark. Therefore, we have no reason to believe that our patients constitute a different socioeconomic group than the general population. Neither ultrasonic diagnosis of pregnancy nor sensitivity of urine or serum hCG has changed significantly between 1992 and 2008. Thus, we find the comparison between the estimated risk of miscarriage among our patients' siblings and the general population valid.

The hypothesis was that at least some of the factors leading to a familial clustering of miscarriages may be genetic. However, the impact of factors such as intrauterine environment, imprinting and epigenetic phenomena shared by siblings of the same mother as well as comparable early life circumstances have not, to our knowledge, been investigated in affected sibling pairs with IRM.

Genome-wide linkage study

In the linkage study based on the genome-wide scan of all affected sibling pairs, an LOD score >2.5 could not be identified in any region. Genetic variants connected with risk of miscarriage may only play a role when found in the maternal genome, e.g. genes of importance for maternal immune tolerance to the trophoblast or genes affecting maternal progesterone production. A linkage analysis restricted to affected women is therefore highly relevant. In the subanalysis of females only (probands and sisters), the peak in 3p14.2 is not located within a gene. The nearest known gene is the *FHIT* gene located 254 kb telomeric from the SNP with maximum LOD score. The *FHIT* gene (Fragile Histidine Triad gene), a member of the histidine triad gene family, encodes a diadenosine 5′,5‴-P1,P3-triphosphate hydrolase involved in purine metabolism. As the genomic region in which the *FHIT* gene located is unstable, we have checked for copy number variations and find no abnormalities; hence the peak is not due to technical difficulties. Aberrant transcripts of the *FHIT* gene have been found in about one-half of all gastro-intestinal cancers (Hassan *et al*., 2010). As *FHIT* has functions inducing apoptosis, one could speculate that aberrant expression may have an influence on for instance, trophoblast invasion and therefore risk of miscarriage, but as far as we know, this has not been investigated.

IRM patients with four or more miscarriages have a markedly increased risk of miscarrying in subsequent pregnancies and may constitute a separate disease entity (carrying more harmful genetic variants) compared with patients with fewer miscarriages (Christiansen *et al*., 1989). We identified two peaks when analyzing these probands and their affected siblings. First, in 9p22.1 the highest LOD score was observed for a SNP in the gene for hypothetical protein LOC 158297, a member of the FAM154A protein family. To our knowledge, this protein family has not been associated with specific functions or diseases. Second, linkage to an SNP in the *PDE2A* gene (11q13.4) was suggested by an LOD score of 2.9. The PDE2A (phosphodiesterase 2A, cGMP-stimulated isoform 1) protein hydrolyzes cAMP and cGMP. The gene is expressed in brain, heart, placenta, lung, skeletal muscle, kidney and pancreas.

We performed a third subgroup analysis in which we examined only one pair of affected siblings per family, removing a third affected sibling randomly to ensure independent observations. A peak was identified within the *GRIK2* gene, suggesting linkage to 6q16.2. *GRIK2* encodes the glutamate receptor, ionotropic, kainate 2. The protein functions as a ligand-activated ion channel. Mutations in the gene have been associated with autosomal recessive mental retardation (Motazacker *et al*., 2007). Affected sibling pair analysis has also linked *GRIK2* with autism (Jamain *et al*., 2002). Variations in the gene have been associated with variations in age-of-onset of Huntington's disease (MacDonald *et al*., 1999) and the expression has been found to be down-regulated in patients with schizophrenia (Choi *et al*., 2009).

None of the above-mentioned genes and their products has previously been linked with IRM or other pregnancy complications, and none of the aforementioned SNPs generated an LOD score ≥ 3.0. Thus these loci are only suggestive, but may be candidate loci in further research.

We could not confirm the loci associated with IRM in the Li *et al*., 2010 study. There may be several reasons for this. The research population differs in several aspects; the cohort in the Li *et al*. study is of Han Chinese origin and the patients had had two or more miscarriages (no information on how many had three or more miscarriages). Additionally, their study is a case–control study and they used a technique of pooled DNA and 430 microsatellites (Li *et al*., 2010). Thus,

the Li et al. study differs from the present in multiple instances. Additionally, lack of confirmation of markers associated with a condition is a well-known problem to genome-wide linkage/association studies, e.g. large differences in genetic variants associated with type 2 diabetes even in the same ethnic groups have been reported (Christiansen et al., 2009a).

We also did not find linkage to the *MBL2* gene on chromosome 10q11.2–q21 or the HLA region on chromosome 6p21.3, which have been linked to RM in previous studies (Christiansen et al., 2009b; Kolte et al., 2010). However, our previous studies have suggested that the impact on RM risk of proposed susceptibility HLA alleles is complex. HLA class II alleles restricting immunity against male-specific minor histocompatibility (HY) antigens only impact pregnancy outcome negatively in RM patients with a firstborn boy (Nielsen et al., 2009). There is also some evidence that a 14 base-pair insertion in exon 8 of the HLA-G gene may only confer susceptibility to RM when inherited from the patient's mother (Kolte et al., 2010). Furthermore, the HLA class II alleles and HLA-G variants suggested to confer susceptibility to RM are frequent and are found in >50% of the population. This complexity obviously makes it more difficult to establish significant linkage to the HLA region in a sibling pair study of limited size.

The lack of significantly linked markers can be explained by several mechanisms. First, the number of included families may be too small to generate sufficient statistical power. Second, IRM may have a polygenic inheritance pattern with relatively low size of effect of each causal variant. In a study like ours, susceptibility genes with low penetrance would not be identified. If this is the case, large, population-based studies of IRM patients and unrelated controls would be necessary, similar to what we have seen for type 2 diabetes in recent years. However, as IRM is a relatively rare condition, it does not seem feasible except in a large collaborative setting, where discrepancies in marker prevalence across different ethnicities would limit interpretation.

Third, the genetic contribution to IRM may be monogenic, but with a strong genetic heterogeneity. In this case, IRM is monogenic in individual families, but the susceptibility genes are different from family to family. It cannot be ruled out with our study design. Conversely, analysis of large pedigrees, including several generations and more distant relations, could contribute to the investigation of such a hypothesis.

Conclusions

This is the first genome-wide linkage study of affected sibling pairs with IRM. We have demonstrated that siblings of IRM patients have a higher risk of miscarriage and that this susceptibility is regardless of age at pregnancy. We did not observe LOD scores >3.0, which may be due to polygenic inheritance, genetic heterogeneity, insufficient power or lack of genetic contribution to IRM etiology. However, we identified SNPs with LOD scores very close to 3.0, thus pinpointing genetic areas which warrant further research in order to confirm or refute linkage between IRM and these genetic regions. We warmly welcome replication studies and envision a repetition of this study including more families in the future. Should linkage be confirmed, fine mapping of these chromosomal areas might provide further insight in the pathogenesis of IRM.

Ethics

The Regional Ethics Committee for Frederiksberg and Copenhagen approved the study in accordance with Danish legislation (approval number KF-01-104/04). Oral and written consent was obtained from all participants.

Authors' roles

A.M.K. was involved in study design, acquisition of patient files, collection of samples, performed the laboratory work, interpreted data and wrote the manuscript. H.S.N. did study design, interpreted data and performed statistics of epidemiological data and critically reviewed the manuscript. I.M. performed data processing and genome-wide non-parametric linkage analysis as well as critical revision of the manuscript. B.P. contributed to acquisition of patient files and collection of samples. B.D. wrote the study protocol and participated in collection of samples. L.S. participated in data interpretation and critical review of the manuscript. F.C.N. supervised A.M.K. in laboratory work and participated in data interpretation. O.B.C. participated in writing the study protocol, supervised A.M.K. in data collection, interpreted data and critically reviewed the manuscript.

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References

Abecasis GR, Cherny SS, Cookson WO, Cardon LR. Merlin—rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet* 2002;**30**:97–101.

Albrechtsen A, Sand KT, Moltke I, van Overseem HT, Nielsen FC, Nielsen R. Relatedness mapping and tracts of relatedness for genome-wide data in the presence of linkage disequilibrium. *Genet Epidemiol* 2009;**33**:266–274.

Babbage SJ, Arkwright PD, Vince GS, Perrey C, Pravica V, Quenby S, Bates M, Hutchinson IV. Cytokine promoter gene polymorphisms and idiopathic recurrent pregnancy loss. *J Reprod Immunol* 2001;**51**:21–27.

Baek KH, Lee EJ, Kim YS. Recurrent pregnancy loss: the key potential mechanisms. *Trends Mol Med* 2007;**13**:310–317.

Choi KH, Zepp ME, Higgs BW, Weickert CS, Webster MJ. Expression profiles of schizophrenia susceptibility genes during human prefrontal cortical development. *J. Psychiatry Neurosci* 2009;**34**:450–458.

Christiansen OB, Riisom K, Lauritsen JG, Grunnet N, Jersild C. Association of maternal HLA haplotypes with recurrent spontaneous abortions. *Tissue Antigens* 1989;**34**:190–199.

Christiansen OB, Mathiesen O, Lauritsen JG, Grunnet N. Idiopathic recurrent spontaneous abortion. Evidence of a familial predisposition. *Acta Obstet Gynecol Scand* 1990;**69**:597–601.

Christiansen OB, Rasmussen KL, Jersild C, Grunnet N. HLA class II alleles confer susceptibility to recurrent fetal losses in Danish women. *Tissue Antigens* 1994;**44**:225–233.

Christiansen OB, Steffensen R, Nielsen HS, Varming K. Multifactorial etiology of recurrent miscarriage and its scientific and clinical implications. *Gynecol Obstet Invest* 2008;**66**:257–267.

Christiansen J, Kolte AM, Hansen TO, Nielsen FC. IGF2 mRNA-binding protein 2: biological function and putative role in type 2 diabetes. *J Mol Endocrinol* 2009a;**43**:187–195.

Christiansen OB, Nielsen HS, Lund M, Steffensen R, Varming K. Mannose-binding lectin-2 genotypes and recurrent late pregnancy losses. *Hum Reprod* 2009b;**24**:291–299.

Daher S, Shulzhenko N, Morgun A, Mattar R, Rampim GF, Camano L, DeLima MG. Associations between cytokine gene polymorphisms and recurrent pregnancy loss. *J Reprod Immunol* 2003;**58**:69–77.

Hassan MI, Naiyer A, Ahmad F. Fragile histidine triad protein: structure, function, and its association with tumorogenesis. *J. Cancer Res Clin Oncol* 2010;**136**:333–350.

Hviid TV, Hylenius S, Lindhard A, Christiansen OB. Association between human leukocyte antigen-G genotype and success of in vitro fertilization and pregnancy outcome. *Tissue Antigens* 2004;**64**:66–69.

Jamain S, Betancur C, Quach H, Philippe A, Fellous M, Giros B, Gillberg C, Leboyer M, Bourgeron T. Linkage and association of the glutamate receptor 6 gene with autism. *Mol. Psychiatry* 2002;**7**:302–310.

Kilpatrick DC, Bevan BH, Liston WA. Association between mannan binding protein deficiency and recurrent miscarriage. *Hum Reprod* 1995;**10**:2501–2505.

Kolte AM, Steffensen R, Nielsen HS, Hviid TV, Christiansen OB. Study of the structure and impact of human leukocyte antigen (HLA)-G-A, HLA-G-B, and HLA-G-DRB1 haplotypes in families with recurrent miscarriage. *Hum Immunol* 2010;**71**:482–488.

Kong A, Gudbjartsson DF, Sainz J, Jonsdottir GM, Gudjonsson SA, Richardsson B, Sigurdardottir S, Barnard J, Hallbeck B, Masson G *et al.* A high-resolution recombination map of the human genome. *Nat Genet* 2002;**31**:241–247.

Kruse C, Rosgaard A, Steffensen R, Varming K, Jensenius JC, Christiansen OB. Low serum level of mannan-binding lectin is a determinant for pregnancy outcome in women with recurrent spontaneous abortion. *Am J Obstet Gynecol* 2002;**187**:1313–1320.

Kruse C, Steffensen R, Varming K, Christiansen OB. A study of HLA-DR and -DQ alleles in 588 patients and 562 controls confirms that HLA-DRB1*03 is associated with recurrent miscarriage. *Hum Reprod* 2004;**19**:1215–1221.

Li W, Zeng CW, Cui X, Xiao FL, Mao SY. Genome-wide screening for risk loci of idiopathic recurrent miscarriage in a Han Chinese population: a pilot study. *Reprod Sci* 2010;**17**:578–584.

MacDonald ME, Vonsattel JP, Shrinidhi J, Couropmitree NN, Cupples LA, Bird ED, Gusella JF, Myers RH. Evidence for the GluR6 gene associated with younger onset age of Huntington's disease. *Neurology* 1999; **53**:1330–1332.

Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**:1215.

Motazacker MM, Rost BR, Hucho T, Garshasbi M, Kahrizi K, Ullmann R, Abedini SS, Nieh SE, Amini SH, Goswami C *et al.* A defect in the ionotropic glutamate receptor 6 gene (GRIK2) is associated with autosomal recessive mental retardation. *Am J Hum Genet* 2007; **81**:792–798.

Nielsen HS, Steffensen R, Varming K, Van Halteren AG, Spierings E, Ryder LP, Goulmy E, Christiansen OB. Association of HY-restricting HLA class II alleles with pregnancy outcome in patients with recurrent miscarriage subsequent to a firstborn boy. *Hum Mol Genet* 2009; **18**:1684–1691.

Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal age and fetal loss: population based register linkage study. *BMJ* 2000;**320**:1708–1712.

Rey E, Kahn SR, David M, Shrier I. Thrombophilic disorders and fetal loss: a meta-analysis. *Lancet* 2003;**361**:901–908.

Risch N. Linkage strategies for genetically complex traits. II. The power of affected relative pairs. *Am J Hum Genet* 1990;**46**:229–241.

Romanos M, Freitag C, Jacob C, Craig DW, Dempfle A, Nguyen TT, Halperin R, Walitza S, Renner TJ, Seitz C *et al.* Genome-wide linkage analysis of ADHD using high-density SNP arrays: novel loci at 5q13.1 and 14q12. *Mol Psychiatry* 2008;**13**:522–530.

Stephenson MD. Frequency of factors associated with habitual abortion in 197 couples. *Fertil Steril* 1996;**66**:24–29.

Welcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 2007;**447**:661–678.

Whittemore AS, Halpern J. A class of tests for linkage using affected pedigree members. *Biometrics* 1994;**50**:118–127.

Zammiti W, Mtiraoui N, Cochery-Nouvellon E, Mahjoub T, Almawi WY, Gris JC. Association of -592C/A, -819C/T and -1082A/G interleukin-10 promoter polymorphisms with idiopathic recurrent spontaneous abortion. *Mol Hum Reprod* 2006;**12**:771–776.

Maternal HY-restricting HLA class II alleles are associated with poor long term outcome in recurrent pregnancy loss after a boy

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Running title

Chance of live birth after secondary RPL

Abstract

Problem

Women with secondary recurrent pregnancy loss (RPL) after a boy have a reduced chance of live birth in the first pregnancy after referral if they carry HY-restricting HLA class II alleles, but long term chance of live birth is unknown.

Methods of study

Live birth was compared for 540 women with unexplained secondary RPL according to firstborn's sex and maternal carriage of HLA-DRB3*03:01; HLA-DQB1*05:01/02; HLA-DRB1*15; and HLA-DRB1*07. The groups were compared by Cox proportional hazard ratios.

Results

For women with at firstborn boy, maternal carriage of HY-restricting HLA class II alleles decreased chance of live birth: 0 vs 1: Hazard ratio 0.75 (95% CI 0.55 – 1.02); 0 vs. 2: HR 0.62 (0.40 – 0.94). Carriage of HY-restricting HLA class II alleles decreased chance of live birth only if the firstborn was a boy: boy vs. girl: HR 0.72 (95% CI 0.55 – 0.98).

Conclusion

Maternal carriage of HY-restricting HLA class II alleles decreases long term chance of live birth in women with RPL after a boy.

Keywords

Cohort study, HY-restricting HLA class II alleles, Immunogenetics, Recurrent pregnancy loss

Introduction

The immunology of pregnancy is only poorly understood[1]. The human hemochorial placenta has a high degree of invasiveness, providing an intrinsic link between the growing embryo/fetus and the mother. What was once believed to be a barrier separating the two is now recognized to be a place of extensive cross talk. Besides nutrient-, waste- and gas exchange, it is now clear that there is a significant exchange of cells, endocrine and immunological components across the placenta. The fetally derived placenta itself is an active organ and has been compared to a tumor in its ability to evade destruction by the maternal immune system[2]. The seeming immunological paradox of viviparous mammals has prompted extensive research into the human leucocyte antigen (HLA) system and its putative role in recurrent pregnancy loss (RPL). Our group has previously shown an association between maternal carriage of HLA-DRB1*03 and RPL, especially among women with four or more pregnancy losses or RPL after a birth (secondary RPL)[3]. Secondary RPL accounts for approximately 1/3 of couples with RPL, and firstborn children are significantly more often boys than girls[4, 5]. Due to the Y chromosome, male fetuses are more immunologically foreign than female fetuses, a condition normally well tolerated as males account for 51% of all newborn children.

However, the Y chromosome encodes a number of male-specific minor antigens known as HY antigen[6]. Certain HLA class I and II molecules are known to present HY antigens to T lymphocytes, the so-called HY-restricting HLA alleles[7]. We have previously shown that the chance of a live born child in the first pregnancy after referral to a tertiary RPL unit was significantly lower for women who were carriers of HY-restricting HLA class II alleles, but only if they had a firstborn boy[7]. The adjusted odds ratio (OR) for live birth was 0.46 (95% CI 0.2 – 0.9) and 0.21 (95% CI 0.1 – 0.7) for women carrying one or two HY-restricting HLA class II alleles compared with non-carriers. There was no association between maternal carriage of HY-restricting HLA class II alleles and chance of live birth if the firstborn child was a girl[7].

It has not been investigated if maternal carriage of HY-restricting HLA class II alleles also has a negative impact on chance of live birth in the long term. From a patient's point of view, this is what matters. Therefore, we decided to investigate long term live birth rates among patients with

secondary RPL in a cohort with up to 30 years of follow up according to maternal carriage of HY-restricting HLA class II alleles.

Materials and methods

Participants

RPL was defined as three or more consecutive pregnancy losses, including both non-visualized pregnancy losses (biochemical pregnancy losses and pregnancies of unknown location, combined) and confirmed intrauterine miscarriages[8]. For the present study we only considered Caucasian women who had given birth to either 1-2 singleton boys or girls (but not both sexes) prior to at least three pregnancy losses (secondary RPL). Furthermore, all women were negative for the lupus anticoagulant; younger than 41 years of age at referral; had IgG anticardiolipin antibody <45 GPL-U; normal uterine anatomy; regular menstrual cycles with 21 – 35 days cycle length; <3 pregnancy losses before the first child.

We included 540 women with secondary RPL seen in the Danish RPL Unit from 1986 to 2015.

As the primary outcomes was chance of live birth, we only included women for whom we had reliable information on date of birth, birth weight, gestational age in weeks at birth and sex of the live- or still born child or children. If the woman had given birth to two children before her consecutive pregnancy losses, follow-up was started at last menstrual period (LMP) in the pregnancy leading to the last birth before her pregnancy losses to LMP in a pregnancy, which led to a live born child. Follow-up was censored at first live birth after referral, the woman's 45th birthday or time of data collection (April 15, 2016), whichever came first.
A total of 58 women were excluded due to lack of information about pregnancies before or after referral.

HLA typing

The term 'HY-restricting HLA class II alleles' covers the HLA class II alleles, which based on in-vitro studies have been reported to present HY antigens: HLA-DRB3*03:01[9]; HLA-DQB1*05:01/02[10]; and HLA-DRB1*15[11]. In 2014, HLA-DRB1*07 was described as HY-restricting and was included in the present study[12].

HLA class II typing is routinely performed as part of routine diagnostic work-up at the Danish RPL Unit. HLA typing was performed using the Luminex xMAP system LABType SSO, a reverse SSO DNA typing system (One Lambda Inc., Canoga Park, CA, USA) according to the manufacturer's instructions.

Statistics

Demographics

Age at LMP, gestational age and birth weight in pregnancies leading to a birth both before and after the pregnancy losses were compared for women who carried none, one or two HY-restricting HLA class II alleles, stratified according to sex of the firstborn. Comparisons were made by non-parametric median test. Differences in sex ratios were calculated by X^2 testing.

Chance of live birth

Chance of live birth was graphically depicted as Kaplan-Meier curves. To quantify the relative difference in live birth rates, Cox proportional hazard ratios and corresponding 95% confidence intervals (95% CI) was calculated with outcome live birth vs. no live birth and follow-up time as survival time. Number of maternal HY-restricting HLA class II alleles was added as a categorical variable with 0 as the reference and survival curves were stratified according to these. Analyses were adjusted for maternal age at time of LMP in the first pregnancy which led to a live birth, and number of pregnancy losses after the first birth.

All statistical analyses were performed in SPSS version 22 (IBM SPSS, Armonk, NY, USA).

Ethics

Maternal HLA class II typing was performed as part of standard clinical investigation at referral to the RPL Unit and anonymised data were drawn from a dedicated database in the RPL Unit. According to Danish legislation, approval by the regional ethics committee or the Danish Data Protection Agency was not needed.

Results

Demographics

Of the 540 women, 307 (57%) had a firstborn boy and 233 (43%) had a firstborn girl. Among the 341 children born after the consecutive pregnancy losses, significantly more were girls: 184 girls (54%) and 157 boys (46%), p=0.036.

As shown in tables I and II, there were no statistically significant differences in obstetrical characteristics according to maternal carriage of HY-restricting HLA class II alleles in the group with a first born boy. Among women with a firstborn girl, gestational age at first birth was significantly higher for women with two HY-restricting HLA class II alleles than women with none or one.

Median age at LMP in the first pregnancy for the whole group was 27 (IQR 23 – 31) and at LMP in the pregnancy leading to a live birth after the losses was 35 (IQR 31 – 38). The median number of pregnancy losses overall was 4 (IQR 3 – 5).

Chance of live birth

Firstborn boy

For the women with a firstborn boy there was a dose-response effect of number of maternal HY-restricting HLA class II alleles and chance of live birth (figure 1 and table III). The adjusted HR for live birth among women with one HY-restricting HLA class II allele was 0.75 (0.55 – 1.02). Women with two HY-restricting HLA class II alleles had an adjusted HR 0.62 (0.40 – 0.94) for live birth compared with women without HY-restricting HLA class II alleles. Carriage of HLA-DQB1*05:01/02 or HLA-DRB1*15 conferred a significantly poorer chance of live birth compared with non-carriers, adj. HR 0.64 (0.42 – 0.97) and 0.64 (0.45 – 0.93), respectively (Table III).

Firstborn girl

Among women with a firstborn girl, there was no significant association between chance of live birth and maternal carriage of HY-restricting HLA class II alleles, as depicted in figure 2 and table III.

Impact of maternal HY restricting HLA class II alleles dependent on sex of the firstborn

Among the women with no HY-restricting HLA class II alleles, there was not difference in the chance of live birth according to sex of the firstborn (adj. HR 1.00 (95% CI 0.73 – 1.41) (table IV).

When comparing single allele status for women with firstborn boys vs. firstborn girls none of the alleles decreased the chance of live birth, but carriage of any HY-restricting HLA class II allele significantly decreased the chance of live birth: adj. HR 0.72 (95% CI 0.55 – 0.98).

Discussion

In the present study we found that maternal carriage of HY-restricting HLA class II alleles decreases the chance of live birth after secondary RPL following the birth of a boy. The decrease follows a dose-response pattern, increasing the likelihood of a biological association. This extends our previous findings regarding maternal HY-restricting HLA class II alleles in the first pregnancy after referral[7]. We also found that women with a first born boy vs. first born girl and no HY-restricting HLA class II alleles had similar chances of live birth in the follow-up period. The negative impact of HLA-DQB1*05:01/02 and HLA-DRB1*15 may be stronger than for the two other HY-restricting HLA alleles (table III). This would not be surprising: in studies of associations between HLA alleles and autoimmune diseases, a hierarchy of strong, moderate and weak susceptibility alleles is often found[13, 14]. However, the size of our study does not allow us to make inferences about the impact of each individual HY-restricting allele.

The role of maternal HLA alleles in RPL is still unclear, as shown by a recent systematic review[15]. However, in several of the individual meta-analyses, due to small numbers of selected studies, the authors combined different ethnic groups and women with primary RPL and secondary RPL, which may have confounded the results[15].

Follow-up in this cohort is based on information provided by patients as well as data from Statistics Denmark from 2010. Complete follow-up based on register data is preferable, but this was not possible. The primary shortcoming of our approach is that we may underestimate live birth rates in the cohort. We do not have reason to believe that either of the groups was more likely to sever contact with the RPL Unit which would have biased the results. The cohort presented here is the largest secondary RPL cohort published, and all patients underwent an extensive investigative protocol to ensure that their pregnancy losses were not due to known risk factors. It is a limitation that we do not have cytogenetic information on the women's pregnancy

losses, thus enabling us to select women with exclusively/predominantly euploid losses. Yet, the women in the cohort were relatively young and had experienced a high number of pregnancy losses, both factors decreasing the likelihood of fetal chromosomal aberrations being the cause of pregnancy loss[16, 17].

Sporadic pregnancy loss is the most common early pregnancy complication, with almost 15% of all recognized pregnancies ending in miscarriage[18]. Contrary to this, RPL is a relatively rare condition, affecting between 1-3% of couples, depending on the definition used[19]. For the affected women, the condition is associated with significant emotional distress[20]. RPL was first described by Percy Malpas in 1938[21], but despite intensive research efforts, there are still only few universally recognized risk factors (e.g. lupus anticoagulant) and even fewer documented effective treatments[19]. It is clear that RPL is a heterogeneous and multifactorial condition[22], where a substantial proportion of patients meet the arbitrary definition of three or more consecutive pregnancy losses due to bad luck or advanced maternal age[19]. This makes it extremely difficult to identify truly causative risk factors, as these will vary among patients. That we are able to extend our previous findings to cumulative live birth rates strengthens our hypothesis that HY immunity plays a significant role in the pathogenesis of RPL after a boy[23]. The results from the present study support that maternal genotyping for HLA class II alleles is a valuable diagnostic test in secondary RPL after a boy in order to estimate prognosis for live birth.

Perspectives

The results of this study confirm the negative prognostic impact of HY restricting HLA class II alleles in women with a firstborn boy and subsequent RPL. Furthermore, we now have documentation that the negative impact of HY restricting HLA class II alleles is not only a temporary phenomenon, but probably decreases life time fecundity. In future studies it would be interesting to investigate this directly for women with different types of RPL compared with the general population.

Conflicts of interests

None declared.

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References

1 Mor G, Cardenas I: The immune system in pregnancy: a unique complexity. Am J Reprod Immunol 2010;63:425-433.

2 Mor G, Kwon JY: Trophoblast-microbiome interaction: a new paradigm on immune regulation. Am J Obstet Gynecol 2015;213:S131-137.

3 Kruse C, Steffensen R, Varming K, Christiansen OB: A study of HLA-DR and -DQ alleles in 588 patients and 562 controls confirms that HLA-DRB1*03 is associated with recurrent miscarriage. Hum Reprod 2004;19:1215-1221.

4 Christiansen OB, Pedersen B, Rosgaard A, Husth M: A randomized, double-blind, placebo-controlled trial of intravenous immunoglobulin in the prevention of recurrent miscarriage: evidence for a therapeutic effect in women with secondary recurrent miscarriage. Hum Reprod 2002;17:809-816.

5 Nielsen HS, Mortensen L, Nygaard U, Schnor O, Christiansen OB, Andersen AM: Brothers and reduction of the birth weight of later-born siblings. Am J Epidemiol 2008;167:480-484.

6 de Bueger M, Bakker A, Van Rood JJ, Van der Woude F, Goulmy E: Tissue distribution of human minor histocompatibility antigens. Ubiquitous versus restricted tissue distribution indicates heterogeneity among human cytotoxic T lymphocyte-defined non-MHC antigens. J Immunol 1992;149:1788-1794.

7 Nielsen HS, Steffensen R, Varming K, Van Halteren AG, Spierings E, Ryder LP, Goulmy E, Christiansen OB: Association of HY-restricting HLA class II alleles with pregnancy outcome in patients with recurrent miscarriage subsequent to a firstborn boy. Hum Mol Genet 2009;18:1684-1691.

8 Kolte AM, Bernardi LA, Christiansen OB, Quenby S, Farquharson RG, Goddijn M, Stephenson MD, Eshre Special Interest Group EP: Terminology for pregnancy loss prior to viability: a consensus statement from the ESHRE early pregnancy special interest group. Hum Reprod 2015;30:495-498.

9 Spierings E, Vermeulen CJ, Vogt MH, Doerner LE, Falkenburg JH, Mutis T, Goulmy E: Identification of HLA class II-restricted H-Y-specific T-helper epitope evoking CD4+ T-helper cells in H-Y-mismatched transplantation. Lancet 2003;362:610-615.

10 Vogt MH, van den Muijsenberg JW, Goulmy E, Spierings E, Kluck P, Kester MG, van Soest RA, Drijfhout JW, Willemze R, Falkenburg JH: The DBY gene codes for an HLA-DQ5-restricted human male-specific minor histocompatibility antigen involved in graft-versus-host disease. Blood 2002;99:3027-3032.

11 Zorn E, Miklos DB, Floyd BH, Mattes-Ritz A, Guo L, Soiffer RJ, Antin JH, Ritz J: Minor histocompatibility antigen DBY elicits a coordinated B and T cell response after allogeneic stem cell transplantation. J Exp Med 2004;199:1133-1142.

12 Eljaafari A, Yuruker O, Ferrand C, Farre A, Addey C, Tartelin ML, Thomas X, Tiberghien P, Simpson E, Rigal D, Scott D: Isolation of human CD4/CD8 double-positive, graft-versus-host disease-protective, minor histocompatibility antigen-specific regulatory T cells and of a novel HLA-DR7-restricted HY-specific CD4 clone. J Immunol 2013;190:184-194.

13 Thomson G, Robinson WP, Kuhner MK, Joe S, MacDonald MJ, Gottschall JL, Barbosa J, Rich SS, Bertrams J, Baur MP, et al.: Genetic heterogeneity, modes of inheritance, and risk estimates for a joint study of Caucasians with insulin-dependent diabetes mellitus. Am J Hum Genet 1988;43:799-816.

14 Thomson G, Valdes AM, Noble JA, Kockum I, Grote MN, Najman J, Erlich HA, Cucca F, Pugliese A, Steenkiste A, Dorman JS, Caillat-Zucman S, Hermann R, Ilonen J, Lambert AP, Bingley PJ, Gillespie KM, Lernmark A, Sanjeevi CB, Ronningen KS, Undlien DE, Thorsby E, Petrone A, Buzzetti R, Koeleman BP, Roep BO, Saruhan-Direskeneli G, Uyar FA, Gunoz H, Gorodezky C, Alaez C, Boehm BO, Mlynarski W, Ikegami H, Berrino M, Fasano ME, Dametto E, Israel S, Brautbar C, Santiago-Cortes A, Frazer de Llado T, She JX, Bugawan TL, Rotter JI, Raffel L, Zeidler A, Leyva-Cobian F, Hawkins BR, Chan SH, Castano L, Pociot F, Nerup J: Relative predispositional effects of

HLA class II DRB1-DQB1 haplotypes and genotypes on type 1 diabetes: a meta-analysis. Tissue Antigens 2007;70:110-127.

15 Meuleman T, Lashley LE, Dekkers OM, van Lith JM, Claas FH, Bloemenkamp KW: HLA associations and HLA sharing in recurrent miscarriage: A systematic review and meta-analysis. Hum Immunol 2015;76:362-373.

16 Ogasawara M, Aoki K, Okada S, Suzumori K: Embryonic karyotype of abortuses in relation to the number of previous miscarriages. Fertil Steril 2000;73:300-304.

17 Stephenson MD, Awartani KA, Robinson WP: Cytogenetic analysis of miscarriages from couples with recurrent miscarriage: a case-control study. Hum Reprod 2002;17:446-451.

18 Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M: Maternal age and fetal loss: population based register linkage study. BMJ 2000;320:1708-1712.

19 Saravelos SH, Regan L: Unexplained recurrent pregnancy loss. Obstet Gynecol Clin North Am 2014;41:157-166.

20 Kolte AM, Olsen LR, Mikkelsen EM, Christiansen OB, Nielsen HS: Depression and emotional stress is highly prevalent among women with recurrent pregnancy loss. Hum Reprod 2015;30:777-782.

21 Malpas P: A study of abortion sequences. Journal of obstetrics and gynaecology of the British Empire 1938;45:18.

22 Nielsen HS, Andersen AM, Kolte AM, Christiansen OB: A firstborn boy is suggestive of a strong prognostic factor in secondary recurrent miscarriage: a confirmatory study. Fertil Steril 2008;89:907-911.

23 Nielsen HS: Secondary recurrent miscarriage and H-Y immunity. Hum Reprod Update 2011;17:558-574.

Tables

Table I Comparison of obstetrical characteristics according to number of maternal HY-restricting HLA class II types in women with a firstborn boy. Values are medians (IQR)

	Number of maternal HY-restricting HLA class II alleles			p-value[1]
	0	1	2	
Birth before RPL				
Gestational age (weeks)	40 (38; 41)	40 (38; 40)	40 (38; 41)	0.77
Birth weight (grams)	3466 (2985; 3795)	3300 (2875; 3708)	3480 (2810; 3708)	0.17
Maternal age LMP (years)	27 (24; 30)	27 (23; 31)	27 (22; 31)	0.85
Number of pregnancy losses	4 (3; 5)	4 (3; 5)	4 (3; 6)	0.11
Birth after RPL				
Gestational age (weeks)	39 (38; 40)	40 (38; 40)	39 (37; 40)	0.16
Birth weight (grams)	3300 (2889; 3748)	3480 (3116; 3805)	3350 (3087; 3713)	0.27
Maternal age LMP[2] (years)	35 (31; 37)	34 (31; 38)	35 (31; 38)	0.85

[1]Non-parametric median test [2]Last menstrual period

Table II Comparison of obstetrical characteristics according to number of maternal HY-restricting HLA class II types in women with a firstborn girl. Values are medians (IQR)

	Number of maternal HY-restricting HLA class II alleles			p-value[1]
	0	1	2	
Birth before RPL				
Gestational age (weeks)	40 (39; 41)	40 (39; 40)	40 (40; 41)	0.024
Birth weight (grams)	3310 (3012; 3643)	3400 (3000; 3685)	3380 (3078; 3778)	0.87
Maternal age LMP (years)	25 (22; 29)	28 (25; 32)	26 (23; 30)	0.22
Number of pregnancy losses	4 (3;5)	4 (3; 4)	4 (3; 5)	0.37
Birth after RPL				
Gestational age (weeks)	39 (38; 40)	40 (38; 41)	39 (38; 40)	0.52
Birth weight (grams)	3590 (3288; 3915)	3596 (2986; 4015)	3373 (3058; 3764)	0.31
Maternal age LMP[2] (years)	33 (30; 37)	36 (32; 38)	35 (31; 38)	0.11

[1]Non-parametric median test; [2] Last menstrual period

Table III Hazard ratios (95% confidence intervals) for cumulative live birth after secondary RPL according to maternal carriage of HY-restricting HLA class II alleles.

	Firstborn BOY n= 307					First born GIRL n= 233				
	Total	Live birth (%)	HR[1]	95% CI	p-value	Total	Live birth (%)	HR*	95% CI	p-value
0 HY-restricting alleles	109	80 (73.4)	*Reference*			81	57 (70.4)	*Reference*		
1 HY-restricting alleles	139	81 (58.3)	0.75	0.55 - 1.02	0.064	101	74 (72.5)	1.12	0.78 – 1.57	0.57
2 H- restricting alleles	59	29 (49.2)	0.61	0.40 – 0.94	0.026	50	33 (66.0)	0.87	0.56 – 1.34	0.52
HLA-DRB3*03:01	29	18 (62.1)	0.84	0.50 – 1.40	0.49	26	21 (80.8)	1.40	0.84 – 2.32	0.19
HLA-DQB1*05:01/02	61	30 (49.2)	0.64	0.42 – 0.97	0.037	49	31 (63.3)	0.90	0.58 – 1.42	0.66
HLA-DRB1*07	67	39 (58.2)	0.77	0.52 – 1.33	0.18	50	36 (72.0)	1.12	0.73 – 1.71	0.60
HLA-DRB1*15	85	45 (52.9)	0.64	0.45 – 0.93	0.019	65	45 (69.2)	0.93	0.63 – 2.38	0.71

[1]Adjusted for number of pregnancy losses and maternal age at first birth.

Table IV Hazard ratios (95% CI) for cumulative live birth after secondary RPL according to sex of the firstborn.

	Firstborn BOY n= 307		First born GIRL n= 233		HR[1]	95% CI	p-value
	Total	Live birth (%)	Total	Live birth (%)			
0 HY-restricting alleles	109	80 (73.4)	81	57 (70.4)	1.00	0.73 – 1.41	0.99
HY-restricting alleles	198	110 (55.6)	151	107 (70.9)	0.72	0.55 – 0.98	0.015
HLA-DRB3*03:01	29	18 (62.1)	26	21 (80.8)	0.53	0.26 – 1-06	0.071
HLA-DQB1*05:01/02	61	30 (49.2)	49	31 (63.3)	0.73	0.44 – 1.22	0.24
HLA-DRB1*07	67	39 (58.2)	50	36 (72.0)	0.68	0.43 – 1.08	0.10
HLA-DRB1*15	85	45 (52.9)	65	45 (69.2)	0.73	0.48 – 1.10	0.13

[1]Hazard ratios for live birth in patients with a firstborn boy versus a firstborn girl adjusted for number of pregnancy losses and maternal age at first birth.

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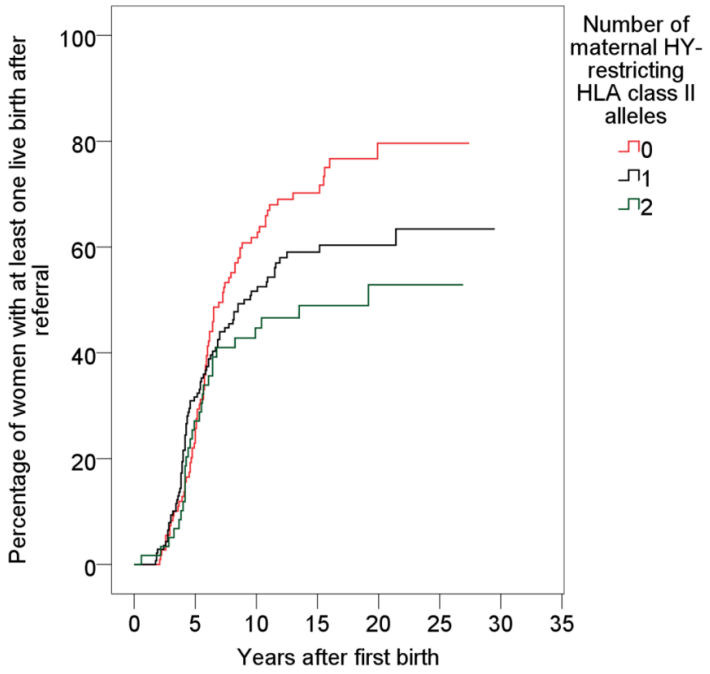
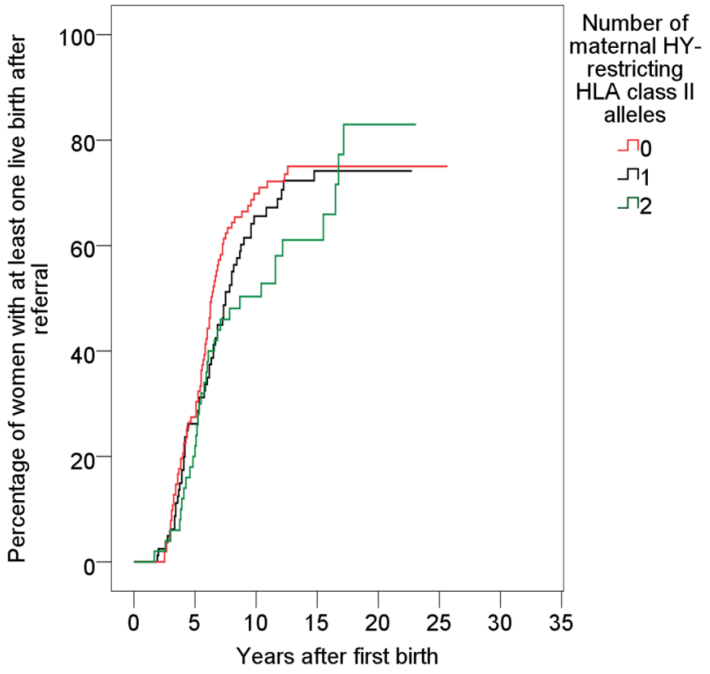
Figures

Figure 1 Kaplan-Meier plot of the percentage of women with secondary RPL after a boy with at least one live birth after referral, stratified by number of maternal HY-restricting HLA class II alleles.



302 Figure 1 Kaplan-Meier plot of the percentage of women with secondary RPL after a girl with at least one
303 live birth after referral, stratified by number of maternal HY-restricting HLA class II alleles



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ORIGINAL RESEARCH ARTICLE

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Inheritance of the 8.1 ancestral haplotype in recurrent pregnancy loss

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ABSTRACT

Background and objectives: The 8.1 ancestral haplotype (AH) (HLA-A1, C7, B8, C4AQ0, C4B1, DR3, DQ2) is a remarkably long and conserved haplotype in the human major histocompatibility complex. It has been associated with both beneficial and detrimental effects, consistent with antagonistic pleiotropy. It has also been proposed that the survival of long, conserved haplotypes may be due to gestational drive, i.e. selective miscarriage of fetuses who have not inherited the haplotype from a heterozygous mother. Recurrent pregnancy loss (RPL) is defined as three or more consecutive pregnancy losses. The objective was to test the gestational drive theory for the 8.1AH in women with RPL and their live born children.

Methodology: We investigated the inheritance of the 8.1AH from 82 heterozygous RPL women to 110 live born children. All participants were genotyped for HLA-A, -B and -DRB1 in DNA from EDTA-treated blood or buccal swaps. Inheritance was compared with a Mendelian inheritance of 50% using a two-sided exact binomial test.

Results: We found that 55% of the live born children had inherited the 8.1AH, which was not significantly higher than the expected 50% ($P=0.29$). Interestingly, we found a non-significant trend toward a higher inheritance of the 8.1AH in girls, 63%, $P=0.11$ as opposed to boys, 50%, $P=1.00$.

Conclusions and implications: We did not find that the 8.1AH was significantly more often inherited by live born children of 8.1AH heterozygous RPL women. However our data suggest that there may be a sex-specific effect which would be interesting to explore further, both in RPL and in a background population.

KEYWORDS: recurrent pregnancy loss; gestational drive; selfish gene theory; cohort study; mother–offspring conflict

INTRODUCTION

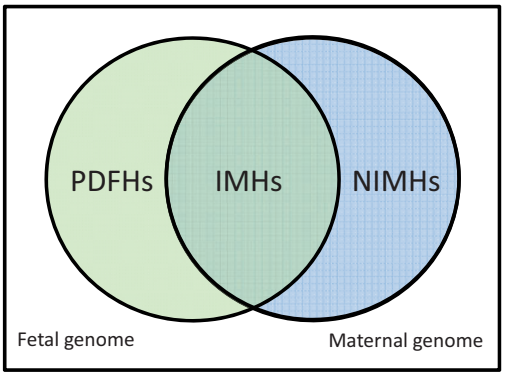
A haplotype is a contiguous set of alleles which tends to be inherited together without recombination. The 8.1 ancestral haplotype (AH) (HLA-A1, C7, B8, C4AQ0, C4B1, DRB1*0301, DQ2) is carried by approximately 10% of Northern Europeans. The 8.1AH is remarkably long, spanning 2.9 MB and has >99.9% conservation [1]. It has been suggested that the high prevalence of the haplotype in Caucasian populations is due to increased resistance to infections, as the 8.1AH has been associated with a longer time to permanent infections with bacteria in the lungs of patients with mucoviscidosis (cystic fibrosis) [2, 3] and a protective role against multi-organ failure (septic shock) in patients with blood infection (sepsis) due to bacterial lung infections (pneumonia) [4]. However, it also leads to an increased susceptibility to HIV and a higher frequency of progression to AIDS [5] and risk of field fever (leptospirosis) [6]. Furthermore, the 8.1AH is associated with a number of autoimmune diseases, such as toxic diffuse goiter (Grave's disease) and systemic lupus [7, 8]. The 8.1AH's effects on health and disease are in some cases sex-dependent. Some effects of the 8.1AH appear sex specific: female, but not male, carriers of the 8.1AH have a higher risk of cancer in the distal gut [9] and early onset myasthenia gravis, an autoimmune neuromuscular disease which leads to muscle weakness and fatigue [10]. Likewise, patients with sporadic inclusion body myositis (an inflammatory muscle disease with progressive muscle wasting and weakening) and concurrent Sjögren's syndrome (an autoimmune disease where the exocrine glands are destroyed, leading to dry eyes and mouth) were found to be predominantly female carriers of the 8.1AH [11]. Conversely, the 8.1AH seems to be associated with longevity, but only in men [12]. Of interest in reproductive immunology, fetal carriage of the 8.1AH has been associated with higher birth weight [13].

These diverse effects may be consistent with the theory of antagonistic pleiotropy; that a gene or haplotype has both beneficial and deleterious effects in the same individual [14]. If the positive effects on average outweigh the negative effects, the haplotype persists. There is evidence that the 8.1AH stems from a single ancestor rather than recombination [8, 15, 16].

Gestational drive and the human MHC

In all viviparous pregnancies, there are three interacting genetic compartments. These are the inherited maternal haplotypes (IMHs), non-inherited maternal haplotypes (NIMHs) and paternally derived fetal haplotypes (PDFHs) [17] (Fig. 1). These genetic compartments may not always have the same optimal outcome of the pregnancy. In a given pregnancy, the IMHs and PDFHs benefit directly from the survival of the fetus, as they are present in the fetus, in contrast to the NIMHs. Gestational drive is described as the ability of maternal genes or haplotypes to favor those offspring who carry their replicas [18] and it has been proposed that NIMHs may be responsible for selective miscarriage of the present fetus in order to increase the chances of their own propagation (via the next pregnancy). The abortifacient maternal haplotype would benefit if reproductive compensation is present [19]. According to Haig [18, 20], this system of 'spiteful abortion' would be more likely to occur in the large, conserved haplotypes present in the MHC. One of these is the 8.1AH.

Women with recurrent pregnancy loss (RPL) would be a suitable sub-population within which to search for evidence of gestational drive [17]. RPL is defined as three or more consecutive early pregnancy losses and is a heterogeneous condition with an unknown etiology for the majority of patients after standard evaluation [21]. The majority of women with RPL has only experienced pregnancy losses before 22 weeks' gestation (primary RPL), but 40% of women with RPL have a live- or stillborn child after 22 weeks' gestation before the series of early pregnancy losses, 'secondary RPL' [22]. Half of the pregnancy



PDFHs: Paternally derived fetal haplotypes
IMHs: Maternally derived fetal haplotypes
NIMHs: Non-inherited maternal haplotypes.
PDFHs and IMHs benefit directly from the survival of the child, NIMHs do not.

Figure 1. Mother–offspring conflict in viviparous pregnancy. PDFHs and IMHs benefit directly from the survival of the child, NIMHs do not. See also Ref. [17]

losses are aneuploid [23], the frequency of which decreases with increasing number of pregnancy losses in the history [24] and among younger patients [23]. These findings suggest that non-chromosomal causes dominate in younger patients and patients with multiple (> 4) pregnancy losses.

Polymorphisms in both classical and non-classical HLA loci have been reported to play a role in RPL pathogenesis [25–27]. We have found that women with secondary RPL or with ≥4 pregnancy losses were significantly more often carriers of the HLA-DRB1*03 allele compared with controls [26]. HLA-DRB1*0301 is part of the 8.1AH but in this earlier study, typing of HLA class I alleles was not performed, nor were live born children or miscarried fetuses HLA typed. In the present study, the objective was to investigate whether there was a preferential inheritance of the 8.1AH from heterozygous women to their live born children. We investigated this hypothesis within a 30-year national RPL cohort.

METHODOLOGY

RPL was defined as three or more consecutive pregnancy losses, including both non-visualized pregnancy losses (biochemical pregnancy losses and pregnancies of unknown location combined) and confirmed intrauterine miscarriages [28]. The inclusion criteria for the study were regular menstrual cycles (21–34 days), normal uterine anatomy, at least one of the pregnancy losses had to be a verified intrauterine miscarriage, normal karyotype (also the partner), negative test for lupus anticoagulant and IgG anticardiolipin antibody <45 GPL-U. Furthermore, patients had to be younger than 40 years of age at referral, of Caucasian descent and have at least one live born child at the time of the study. Data about subsequent pregnancy outcome were collected by questionnaires returned by the patients after they had given birth and/or from the Danish national birth register.

As part of standard clinical evaluation, all patients in the Danish RPL Unit are genotyped for HLA-DRB1.

In a previous study of women with unexplained RPL after the birth of a live- or still born child (secondary RPL), 358 women and 203 of their firstborn children were genotyped for HLA-A, -B and -DRB1 [27]. Of these 203 mother–child pairs, 35 women were heterozygous for the 8.1AH. These 35 mother–child dyads were included in the present study and formed the basis for the power calculation.

We went through all files of patients seen from January 1990 to April 2015 in the Danish RPL Unit (approximately 2000 patients) and identified, in addition to the 35 women mentioned above, women who were homo- or heterozygous for HLA-DRB1*03 and had had at least one live born child either before or after their series of pregnancy losses ($n = 185$). Patients who were heterozygous or homozygous for HLA-DRB1*03 were genotyped for HLA-A and -B. Of these, 55 were heterozygous for the 8.1AH. These women were invited to participate in the study and 47 accepted. They had a total of 80 children, from whom we were able to collect samples from 75. Combined with the previous cohort, we included 110 mother–child pairs. We gathered information on birth weight from 90 of the live born children (82%). In all dyads, identity by descent of the 8.1AH was unequivocally ascertained without the need for paternal karyotyping, which was not performed. One child was homozygous for the 8.1AH, but otherwise, none of the included children had inherited the 8.1AH from their fathers.

Written informed consent was obtained. The study was approved by the Regional Ethics Committee for the Capital Region of Denmark, with approval number H-2-2011-055 and by the Danish Data Protection Agency, file number 2007-58-0015.

Laboratory methods

EDTA-treated peripheral blood (women and adult children) or buccal swaps (children younger than 18 years of age) were collected. DNA from blood was extracted either using a salting-out method as previously described [29] or using the Maxwell 16 Blood DNA kit on the Maxwell 16 Instrument. DNA from buccal swabs was extracted using Maxwell 16 Buccal Swab LEV DNA Purification Kit on the Maxwell 16 Instrument (Promega, Madison, WI, USA).

HLA-A, -B and -DRB1 genotypes were determined by the Luminex xMAP system LABType SSO, a reverse SSO DNA typing system (One Lambda Inc., Canoga Park, CA, USA) according to the manufacturer's instructions.

Power calculation

According to the Mendelian laws, we would expect the inheritance of the 8.1AH to be 50%, i.e. $P_{exp} = 0.5$.

The power calculation was performed as a two-sided one-sample inference to a known proportion. In our earlier study we found that of the 35 women with secondary RPL, 22 had bequeathed the 8.1AH to their live born child (63%). Therefore, we set the $P_{obs} = 0.65$. With a power of 0.9 and a type I error of 0.05, 113 mother–child pairs were required.

Statistics

To test whether the 8.1AH was significantly more often bequeathed from RPL women to their children than expected, we used the two-sided exact binomial test. The non-parametric median test was used to assess differences in birth weight and the χ^2 test was used to test sex-ratio. All statistical analyses were performed in the Statistical Package for Social Sciences (IBM SPSS, Armonk, NY, USA).

RESULTS

In our cohort of 110 mother–child pairs, we found that 61 (55%) of the live born children had inherited the 8.1AH, which was not significantly higher than the expected 50%, $P = 0.25$. According to the power calculation, we should have included a total of 113 mother-–child pairs. If we assume that all of the remaining three children had inherited the 8.1AH, the proportion would be 57%; $P = 0.19$.

Among the children, there was a trend toward more boys than girls (62 vs 48, $P = 0.34$). There was no trend toward a higher inheritance among boys, 50%, $P = 1.00$. Among girls there was a non-significant trend toward higher inheritance, 63%, $P = 0.11$. In a one-sided test this would be almost significant, $P = 0.055$.

We also analyzed the data for children born by women with primary RPL and secondary RPL separately. We found no statistically significant differences in inheritance, $P = 0.68$ and $P = 0.39$, respectively. Among children born before the series of pregnancy losses, 19 (50%) of the boys and 18 (60%) of the girls had inherited the 8.1AH. In the group of children born after the pregnancy losses, the numbers were not significantly different: 12 (50%) of the boys and 12 (67%) of the girls had inherited the 8.1AH from their heterozygous mother; $P = 1.00$ for boys and $P = 0.64$ for girls.

Furthermore, we ascertained whether the birth weight of children in this cohort was related to their carriage of the 8.1AH. As birth weight is dependent on sex, we stratified the information according to this. However, we found no significant differences in birth weight according to inheritance of the 8.1AH.

The median number of pregnancy losses at referral was 3 (interquartile range 3; 5) and we investigated the inheritance of the 8.1AH stratified for the mothers' number of pregnancy losses prior to referral. We saw no significant differences, nor any trends.

The results are summarized in Table 1.

DISCUSSION

We tested the gestational drive hypothesis for the 8.1AH in a cohort of women with unexplained RPL and their live born children. We did not find a significantly higher degree of inheritance of the 8.1AH among children born by heterozygous women with RPL. It is evident that statistical power is limited, especially in the subgroup analyses.

The study is based on a well-known hypothesis of gestational drive [20]. The studied cohort consists of clinically well-characterized patients with no known risk factors for their pregnancy losses and their live born children. We have included both women with primary and secondary RPL. Women with secondary RPL may be somewhat different from women with primary RPL in their HLA background and especially HLA-DRB1*03 frequency [26]. It would be interesting to include a sufficient number of mother–child pairs to evaluate the groups separately. On the other hand, one could argue that if the theory of gestational drive holds true, there should be no difference between women with secondary and primary RPL as the first fetus is as likely to inherit the 8.1AH from the heterozygous mother as the fourth fetus.

In the present study, we have not investigated the inheritance of the 8.1AH from the children's fathers. However, in future studies of gestational drive and parent–offspring conflict in general, it would be interesting to include all genetic shareholders.

Regrettably, we do not have access to pregnancy loss tissue from the vast majority of our patients, neither before nor after referral. As these women have more failed than successful pregnancies, investigating the miscarried pregnancies for carriage of the 8.1AH, and in addition chromosomal aberrations, would have strengthened the study. This would also have enabled us to include women with exclusively or predominantly euploid pregnancy losses.

Table 1. Inheritance of the 8.1 AH from RPL women to their live born children

	Inherited *N* (%)	Did not inherit *N* (%)	*P* value
All live born children (*N* = 110)	61 (55%)	49 (45%)	0.29[a]
Type of RPL			
Secondary RPL (*n* = 87)	48 (55%)	39 (45%)	0.39[a]
Primary RPL (*n* = 23)	13 (57%)	10 (43%)	0.68[a]
Median birth weight (range)[b]			
Boys (*n* = 53)	3320 (1992; 4270)	3481 (1000; 5300)	0.90[c]
Girls (*n* = 37)	3300 (1240; 4800)	3100 (2440; 3675)	0.37[c]
Sex of live born			
Boys (*n* = 62)	31 (50%)	31 (50%)	1.0[a]
Girls (*n* = 48)	30 (63%)	18 (37%)	0.11[a]
Pregnancy losses before referral (number of women)			
3 (n = 43)	25 (58%)	18 (39%)	0.36[a]
4 (n = 25)	13 (52%)	12 (48%)	1[a]
5 or more (n = 42)	23 (55%)	19 (45%)	0.64[a]

[a]Exact binomial test.
[b]We did not have information on birth weight for all live born children.
[c]Non-parametric median test.

We found a trend toward higher inheritance of the 8.1AH among live born girls, $P = 0.11$. As we tested a hypothesis with a specified directionality (i.e. the 8.1AH being inherited more often than expected by Mendelian inheritance), it could be argued that a one-sided test would be appropriate. This would have yielded a *P*-value of 0.055 in the subgroup analysis of the girls. However, we could not *a priori* exclude the possibility that the 8.1AH was bequeathed less often than expected, and therefore we chose the two-sided binomial test.

We have previously shown that firstborn children born by women with secondary RPL are significantly more often boys. Children born after the series of pregnancy losses are significantly more often girls than boys compared with the expected 1.06 sex ratio [30]. Furthermore, women with secondary RPL have a higher chance of live birth in the first pregnancy after referral if the firstborn is a girl [27]. Immunity to male-specific minor histocompatibility (HY) antigens and maternal carriage of the HY restricting HLA class II alleles HLA-DRB1*15, -DQB1*05:01/02 play a significant role in secondary RPL following the birth of a boy, but not a girl [22, 27]. Therefore, *a priori*, we would not necessarily expect that the 8.1AH is associated with secondary RPL following the birth of a boy. Both among RPL women and on a population basis, we have also found that a firstborn boy leads to a lower birth weight of later born sibs, especially if the later born child is a boy [31, 32]. Therefore, gestational drive in RPL may be more prominent in secondary RPL after a firstborn girl as HY immunity does not seem to play a large role in this subset of patients. Our finding of a trend toward increased inheritance of the 8.1AH among live born girls ($P = 0.11$) is also interesting taking into consideration that others have reported sex-specific effects of the 8.1AH [9–12]. The gestational drive hypothesis does not specify sex-specific effects, although sibling rivalry may be more intense within sexes rather than between them (D. Haig, personal communication).

CONCLUSIONS AND IMPLICATIONS

To our knowledge, this is the first attempt to identify the highly conserved 8.1AH as a 'selfish gene' [33] in a human cohort. The lack of a significantly higher inheritance of the 8.1AH by live born children of women with RPL does not prove the hypothesis wrong. As outlined above, there are several other plausible explanations. We found a near-significant trend toward an increased inheritance in the order of 13% among live born girls. For the etiology of RPL, the impact of gestational drive by the 8.1AH seems negligible from a clinical point of view. However, even seemingly weak effects could theoretically explain the prevalence of the 8.1AH in Caucasian

populations. As such this type of study is important for empirical testing of evolutionary hypotheses of mother–offspring conflict in human reproduction.

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REFERENCES

1. Aly TA, Eller E, Ide A *et al*. Multi-SNP analysis of MHC region: remarkable conservation of HLA-A1-B8-DR3 haplotype. *Diabetes* 2006; **55**:1265–9.
2. Corvol H, Beucher J, Boelle PY *et al*. Ancestral haplotype 8.1 and lung disease severity in European cystic fibrosis patients. *J Cyst Fibros* 2012; **11**:63–7.
3. Laki J, Laki I, Nemeth K *et al*. The 8.1 ancestral MHC haplotype is associated with delayed onset of colonization in cystic fibrosis. *Int Immunol* 2006; **18**:1585–90.
4. Aladzsity I, Madach K, Szilagyi A *et al*. Analysis of the 8.1 ancestral MHC haplotype in severe, pneumonia-related sepsis. *Clin Immunol* 2011; **139**:282–9.
5. Cameron PU, Mallal SA, French MA *et al*. Major histocompatibility complex genes influence the outcome of HIV infection. Ancestral haplotypes with C4 null alleles explain diverse HLA associations. *Hum Immunol* 1990; **29**: 282–95.
6. Fialho RN, Martins L, Pinheiro JP *et al*. Role of human leukocyte antigen, killer-cell immunoglobulin-like receptors, and cytokine gene polymorphisms in leptospirosis. *Hum Immunol* 2009; **70**:915–20.
7. Candore G, Lio D, Colonna Romano G *et al*. Pathogenesis of autoimmune diseases associated with 8.1 ancestral haplotype: effect of multiple gene interactions. *Autoimmun Rev* 2002; **1**:29–35.
8. Price P, Witt C, Allcock R *et al*. The genetic basis for the association of the 8.1 ancestral haplotype (A1, B8, DR3) with multiple immunopathological diseases. *Immunol Rev* 1999; **167**:257–74.
9. Toth EK, Kocsis J, Madaras B *et al*. The 8.1 ancestral MHC haplotype is strongly associated with colorectal cancer risk. *Int J Cancer* 2007; **121**:1744–8.
10. Franciotta D, Cuccia M, Dondi E *et al*. Polymorphic markers in MHC class II/III region: a study on Italian patients with myasthenia gravis. *J Neurol Sci* 2001; **190**:11–6.
11. Rojana-udomsart A, Needham M, Luo YB *et al*. The association of sporadic inclusion body myositis and Sjogren's syndrome in carriers of HLA-DR3 and the 8.1 MHC ancestral haplotype. *Clin Neurol Neurosurg* 2011; **113**:559–63.
12. Caruso C, Candore G, Colonna Romano G *et al*. HLA, aging, and longevity: a critical reappraisal. *Hum Immunol* 2000; **61**:942–9.
13. Capittini C, Pasi A, Bergamaschi P *et al*. HLA haplotypes and birth weight variation: is your future going to be light or heavy? *Tissue Antigens* 2009; **74**:156–63
14. Williams GC. Pleiotropy, natural selection, and the evolution of senescence. *Evolution* 1957; **11**:14.
15. D'Alfonso S, Cappello N, Borelli I *et al*. HLA supratypes in an Italian population. *Immunogenetics* 1994; **39**:114–20.
16. Degli-Esposti MA, Leaver AL, Christiansen FT *et al*. Ancestral haplotypes: conserved population MHC haplotypes. *Hum Immunol* 1992; **34**:242–52.
17. Haig D. Evolutionary conflicts in pregnancy and calcium metabolism—a review. *Placenta* 2004; **25**:S10–5.
18. Haig D. Gestational drive and the green-bearded placenta. *Proc Natl Acad Sci U S A* 1996; **93**:6547–51.
19. Haig D. Altercation of generations: genetic conflicts of pregnancy. *Am J Reprod Immunol* 1996; **35**:226–32.
20. Haig D. Maternal-fetal interactions and MHC polymorphism. *J Reprod Immunol* 1997; **35**:101–9.
21. Larsen EC, Christiansen OB, Kolte AM *et al*. New insights into mechanisms behind miscarriage. *BMC Med* 2013; **11**:154.
22. Nielsen HS. Secondary recurrent miscarriage and H-Y immunity. *Hum Reprod Update* 2011; **17**:558–74.
23. Stephenson MD, Awartani KA, Robinson WP. Cytogenetic analysis of miscarriages from couples with recurrent miscarriage: a case-control study. *Hum Reprod* 2002; **17**: 446–51.
24. Ogasawara M, Aoki K, Okada S *et al*. Embryonic karyotype of abortuses in relation to the number of previous miscarriages. *Fertil Steril* 2000; **73**:300–4.
25. Christiansen OB, Kolte AM, Dahl M *et al*. Maternal homozygocity for a 14 base pair insertion in exon 8 of the HLA-G gene and carriage of HLA class II alleles restricting HY immunity predispose to unexplained secondary recurrent miscarriage and low birth weight in children born to these patients. *Hum Immunol* 2012; **73**:699–705.
26. Kruse C, Steffensen R, Varming K *et al*. A study of HLA-DR and -DQ alleles in 588 patients and 562 controls confirms that HLA-DRB1*03 is associated with recurrent miscarriage. *Hum Reprod* 2004; **19**:1215–21.
27. Nielsen HS, Steffensen R, Varming K *et al*. Association of HY-restricting HLA class II alleles with pregnancy outcome in patients with recurrent miscarriage subsequent to a firstborn boy. *Hum Mol Genet* 2009; **18**: 1684–91.
28. Kolte AM, Bernardi LA, Christiansen OB *et al*. Terminology for pregnancy loss prior to viability: a consensus statement from the ESHRE early pregnancy special interest group. *Hum Reprod* 2015; **30**:495–8.
29. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**:1215.

30. Christiansen OB, Steffensen R, Nielsen HS *et al*. Multifactorial etiology of recurrent miscarriage and its scientific and clinical implications. *Gynecol Obstet Invest* 2008; **66**:257–67.
31. Nielsen HS, Mortensen L, Nygaard U *et al*. Brothers and reduction of the birth weight of later-born siblings. *Am J Epidemiol* 2008; **167**:480–4.
32. Nielsen HS, Steffensen R, Lund M *et al*. Frequency and impact of obstetric complications prior and subsequent to unexplained secondary recurrent miscarriage. *Hum Reprod* 2010; **25**:1543–52.
33. Wade MJ, Beeman RW. The population dynamics of maternal-effect selfish genes. *Genetics* 1994; **138**:1309–14.

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A. refers to:	*Has contributed to the co-operation*	*0-33 %*
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