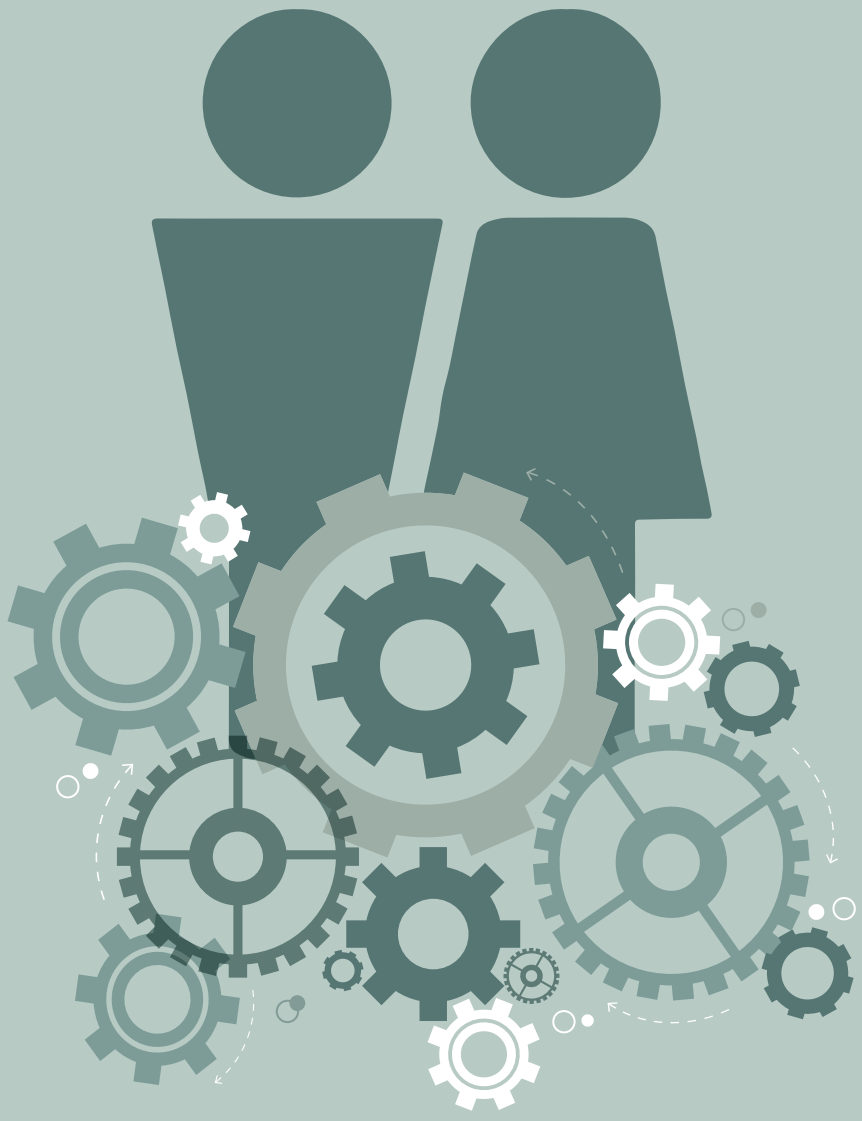
Paternal factors in recurrent miscarriage : a role beyond conception

Meuleman, T.

Paternal factors in recurrent miscarriage

A ROLE BEYOND CONCEPTION



TESS MEULEMAN

Paternal factors in recurrent miscarriage;

A role beyond conception

Tess Meuleman

The studies described in this thesis were performed at the Department of Immunohematology and Blood Transfusion and the Department of Gynaecology and Obstetrics at the Leiden University Medical Center, the Netherlands.

Cover	Wendy Schoneveld | Wenz id
Layout	Renate Siebes | Proefschrift.nu
Printed by	ProefschriftMaken | www.proefschriftmaken.nl
ISBN	978-94-930-1986-7

Financial support for printing this thesis was kindly provided by the department of Gynaecology and Obstetrics Leiden University Medical Center, Nationaal Referentie Centrum voor Histocompatibiliteit, Stichting Oranjekliniek, Stichting NVLE Fonds, Stichting HELLP, Chipsoft, BMA Mosos, VPS Diagnostics, Rabobank.

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 9 oktober 2018
klokke 15.00 uur

door

Tess Meuleman

geboren te Sittard
in 1984

Promotoren	Prof. dr. F.H.J. Claas Prof. dr. J.J.M. van Lith
Leden promotiecommissie	Prof. dr. F. Koning Prof. dr. S. Robertson *Robinson Research Institute, Adelaide, Australia* Prof. dr. I. Joosten *RadboudUMC, Nijmegen* Dr. S. Heidt

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General introduction

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1. Recurrent miscarriage

Recurrent miscarriage is defined as three or more consecutive miscarriages prior to the 20th week of gestation.[1] It is a devastating condition, both from a medical and psychological point of view. Sporadic miscarriages occur frequently (about 10-15% of pregnancies end in a miscarriage), but recurrent miscarriage is only diagnosed after a couple has experienced at least three miscarriages, which is the case in 1 to 2% of all couples trying to conceive.[1] Recurrent miscarriage is etiologically a highly heterogeneous condition. Possible etiologic factors include uterine anomalies, endocrine disorders, maternal inherited and acquired thrombophilia, and parental chromosomal abnormalities.[2,3] Whenever the diagnosis 'recurrent miscarriages' is established, an underlying cause may be identified in 25-50% of the patients.[4] Although the underlying cause remains unclear in a high number of patients, therapeutic options have been widely investigated in the last decade. Unfortunately, medication like low dose of insulin, HCG, immunotherapy, and aspirin alone or in combination with low molecular weight heparin (LMWH) seems not to improve pregnancy rates in the studied populations.[5-7] Also progesterone has not proven to be effective in patients with unexplained miscarriages.[8]

These rather disappointing results leaves 50-75% of couples with the burden of continuous uncertainty and leaves their clinicians without means to treat these women in a next pregnancy in order to prevent further miscarriages.

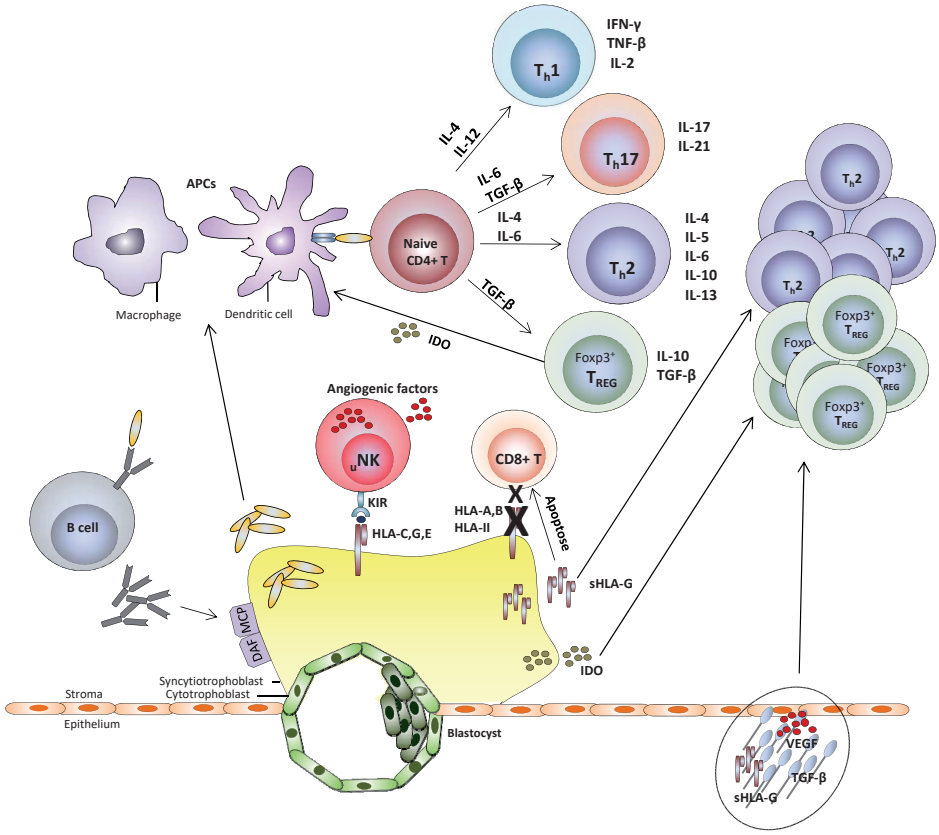
Without any therapy the chance of a live birth after 3 miscarriages is generally assumed to be 75%, while in more recent studies live birth rates range from 57-95%.[5,9-12] The large range found for live birth rate in women with recurrent miscarriages might also be explained by different definitions of recurrent miscarriage. Many studies on recurrent miscarriage include women with ≥2 miscarriages, have no clear classification of explained recurrent miscarriage, and mostly no clear definition of control subjects used.

However, the main problem is that many of the women with unexplained recurrent miscarriage, will never be able to have a live birth. Since the fetus is a semi-allograft, which in normal pregnancy is tolerated by the maternal immune system, it has been suggested that an inadequate maternal allo-immune response to paternal antigens is responsible for a proportion of these unexplained miscarriages.[13,14]

2. Immunological tolerance in early pregnancy

The blastocyst reaches the uterine cavity and penetrates the epithelium of the uterus 8 to 10 days after conception. At that moment fetal antigens make contact with maternal

Figure 1.1 Immunological responses during implantation of the blastocyst leading to normal implantation, normal placentation, and fetal acceptance



peripheral mononuclear blood cells (PBMCs). The trophoblast cells from the outer layer of the placenta are in direct contact with maternal immune cells. Furthermore, entry of fetal material as fetal cells (microchimerism), synctiotrophoblast fragments, and fetal DNA into the maternal circulation occurs.[15] To protect the fetus from immune mediated damage, a state of tolerance must be generated. Several immunological mechanisms at the implantation site contribute to a tolerogenic environment (Figure 1.1), in which HLA molecules play an important role. However, the actual immunological mechanisms by which the maternal immune system accepts the semi-allogeneic fetus is still not completely understood.

2.1 HLA molecules

Major histocompatibility complex (MHC) antigens, in human called the human leukocyte antigen (HLA), are highly polymorphic glycoproteins, of which the main function is peptide

presentation to T cells. The MHC is located on the short arm of chromosome 6 and is divided in three regions; class I, II, and III. The class I region includes the classical genes, HLA-A, HLA-B, HLA-C, and the non-classical genes, HLA-E, HLA-F, and HLA-G. HLA class I molecules present antigens to CD8+ T cells and form ligands for receptors on natural killer (NK) cells. Classical HLA class I molecules are expressed on all nucleated cells and on platelets. HLA class II molecules, which include HLA-DR, HLA-DQ, and HLA-DP, are present on antigen presenting cells (APCs), like macrophages, dendritic cells (DCs), and B cells. The class III region contains a high density of non-HLA genes, like genes coding for complement components (C2,C4), heat shock protein (Hsp70), and cytokines (TNF).[16]

2.1.1 HLA sharing

It is believed that a high degree of HLA sharing between couples could decrease the trigger to develop an immunoregulatory response, as recognition of paternal antigens by the maternal immune system is essential for normal pregnancy. Therefore, the role of HLA sharing has extensively been investigated in recurrent miscarriage.[17] Studies examining HLA sharing showed inconsistent results, probably because they tested a wide range of hypotheses, adopted various classifications for the same disease, used various control groups, and investigated different HLA alleles and loci. In a meta-analysis of selected case control studies a slightly increased and significant risk of recurrent miscarriage among couples who shared at least one allele at the HLA-DR locus was found.[17] However, it remains unclear whether the HLA alleles themselves are the susceptibility factors, or whether other genes linked to HLA are the main causative agents for the onset of recurrent miscarriage.[17]

2.1.2 HLA alleles

One of the mechanisms leading to acceptance of the allogeneic fetus, is that fetal cytotrophoblast cells do not to express HLA class II molecules and the HLA class I molecules, HLA-A and HLA-B. Therefore, direct triggering of CD4+ T cells and cytotoxic lysis of trophoblast cells by CD8+ T cells is prevented (Figure 1.2).

Instead, extravillous cytotrophoblast cells express the non-classical oligomorphic HLA-G and E-molecules while, HLA-C is the only classical HLA I antigen expressed on trophoblast.[18-20] HLA-C is highly polymorphic while both maternal and paternal alleles are expressed on the cell surface.[21] The HLA-C molecules appear to be involved in regulation of the immune response at the fetal-maternal interface on the basis of allorecognition by killer immunoglobulin-like receptor (KIR) on both NK cells and T cells (Figure 1.3).[22-28]

Figure 1.2 Absence of HLA-A and HLA-B and MHC class II molecules on trophoblast prevents direct lysis by CD8+ T cells

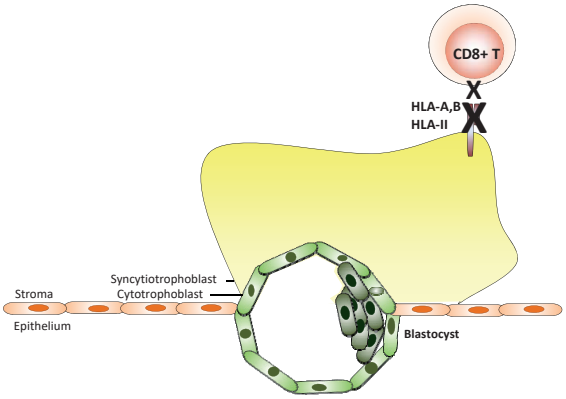
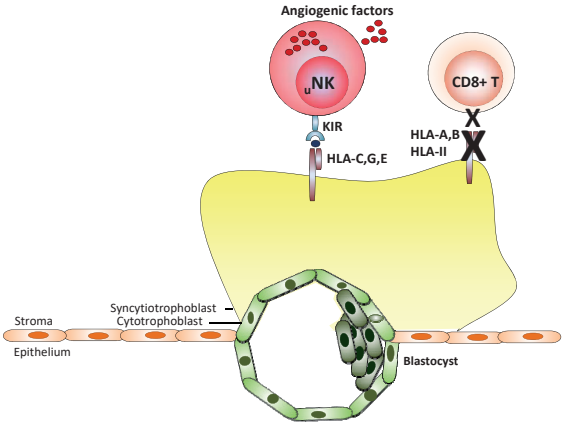


Figure 1.3 Interaction of HLA-G and HLA-C with KIR on NK cells regulates the maternal immune response



HLA-C ligands specifically interact with inhibitory receptors or activating receptors on NK cells, both KIR. Although there are about 350 different KIR genotypes, there are 2 basic KIR haplotypes called the A and B haplotype. The B haplotype consists predominantly of activating receptors whereas the A haplotype mainly consists of inhibiting receptors. The HLA-C ligands are divided into two subtypes, namely HLA-C1 binds inhibitory KIR2DL2/3, while HLA-C2 binds inhibitory KIR2DL1 and activating KIR2DS1.[29] The interaction between these two HLA-C subtypes and KIR receptors, resulting in a balance of inhibiting and activating NK cells, in (un)complicated pregnancy has been studied extensively for the past years.[30-33] In early pregnancy, approximately 70% of decidual leukocytes are uterine NK cells, these

numbers decrease during pregnancy until 3% at term.[34] This suggests that NK cells play a crucial role in the development of the placenta.[35] Uterine NK cells differ from peripheral blood NK cells, by their increased binding abilities to HLA-C and high CD56 levels and the absence of the marker CD16. Their immunological response is mainly based on cytokine supply and not on their cytotoxic potential.[36] NK cells in the decidua play an important role in angiogenesis via the production of angiogenic factors such as TGF-β, angiopoietin 1 and 2, vascular endothelial growth factor (VEGF), and placental growth factor (PlGF), since these factors enhance invasion of extravillous trophoblast (Figure 1.3).[37]

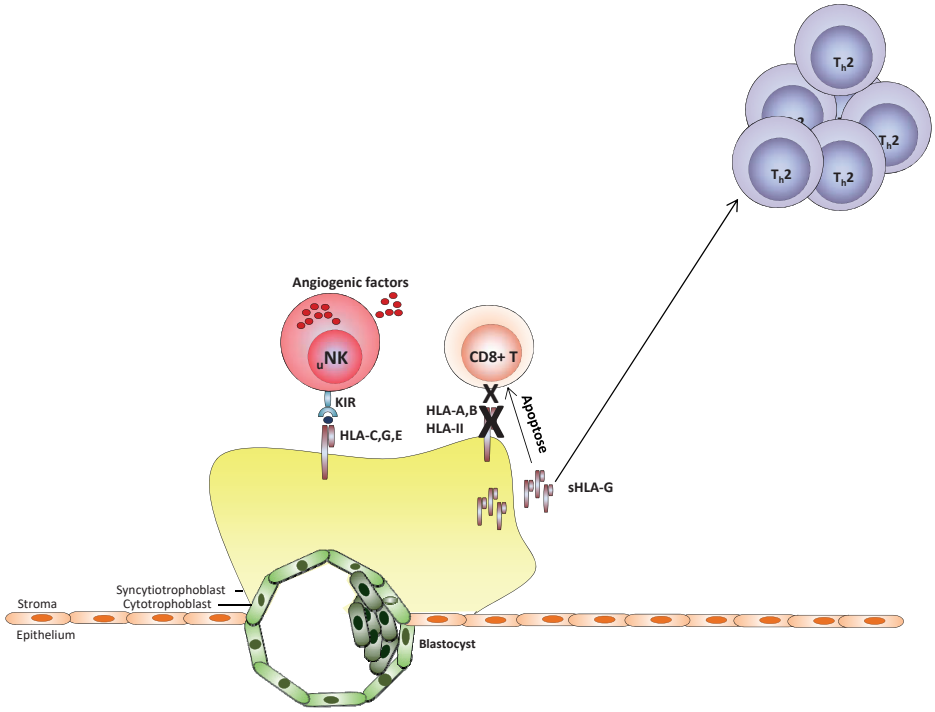
The non-classical class I molecule HLA-G is characterized by its restricted tissue expression, including extravillous trophoblast cells, the thymus, eyes, and various types of tumour and stromal cells,[38] and by its limited polymorphisms.[39] Only 4 membrane bound (HLA-G1, G2, G3, and G4) and 3 soluble isoforms (sHLA-G5, G6, and G7) have been identified.[40,41] It has been postulated that the main function of HLA-G is interacting with NK cells, macrophages, T cells, and possibly B cells by binding with leukocyte immunoglobulin-like receptor (LIR), immunoglobulin-like transcripts (ILT), and KIR.[40,42,43] The interaction of HLA-G on the trophoblast with ILT expressed on DCs promotes regulatory T cells to downregulate the adaptive immune response in the uterus.[44] The ILT2 receptor is also expressed in a small percentage of uterine NK cells and binds HLA-G at low affinity.[45] In addition, binding of HLA-G with KIR2DL4 receptor on uterine NK cells results in the production of various cytokines, chemokines, and angiogenic factors, stimulating trophoblast invasion and blood vessel development associated with normal implantation (Figure 1.3). Nevertheless, no differences in expression of HLA-G on the trophoblast were observed between women with recurrent miscarriage and control subjects.[46-48] HLA-G gene expression is dependent on polymorphism in the promotor region or 5'upstream regulatory region. Insertion of a 14-basepair (bp) segment in the 3'untranslated region (3'UTR) of the HLA-G gene may affect HLA-G mRNA stability,[49] which is associated with lower levels or even absence of soluble HLA-G (sHLA-G) in plasma.[50-52] The HLA-G 3'UTR in exon 8 consists of eight single nucleotide polymorphisms (SNPs), which together generate eight distinct haplotypes.[53,54] Although the level of sHLA-G is dependent of the HLA-G genotype, meta-analyses on the association of the 14-bp insertion with unexplained recurrent miscarriage have led to inconsistent results.[55,56] Most likely, 14-bp insertion is in linkage disequilibrium with other sequence variations that influence the level of soluble isoforms. This is in line with the hypothesis that sHLA-G expression is determined by the combination of multiple SNPs.[53] sHLA-G is highly present in the maternal circulation during pregnancy[57] and the trophoblast is able to produce sHLA-G. sHLA-G, especially sHLA-G5 and sHLA-G6, possess immunosuppressive functions by an apoptotic effect on activated CD8+ T cells[58] and suppression of an alloimmune proliferative

response,[59] which seems to be concentration-dependent.[60] Furthermore, experimental data suggest that the development of a Th2 cytokine response (see paragraph 2.2) is associated with high concentration of sHLA-G and maintenance of pregnancy (Figure 1.4).[60]

On the other hand, low levels of sHLA-G have been associated with spontaneous miscarriage,[61] recurrent miscarriage,[62] and miscarriage in IVF pregnancies.[63]

HLA-E is detected on extravillous trophoblast cells[64-66] and binds to CD94/NKG2A on NK cells. HLA-E is an oligomorphic HLA molecule and only three variants can be distinguished at the protein level. As the E*01:04 allele is very rare, only the two non-synonymous alleles (E*01:01 and E*01:03) are of clinical importance. Affinity of HLA*01:01 for a nonamer peptide derived from HLA-G is lower than for HLA-E*01:03.[67] Recently, co-expression of HLA-E and HLA-G at the surface of preimplantation embryos was detected, suggesting that these antigens, which both have ligands to bind with NK cells, are important in normal pregnancy.[68]

Figure 1.4 The role of sHLA-G in maintenance of pregnancy



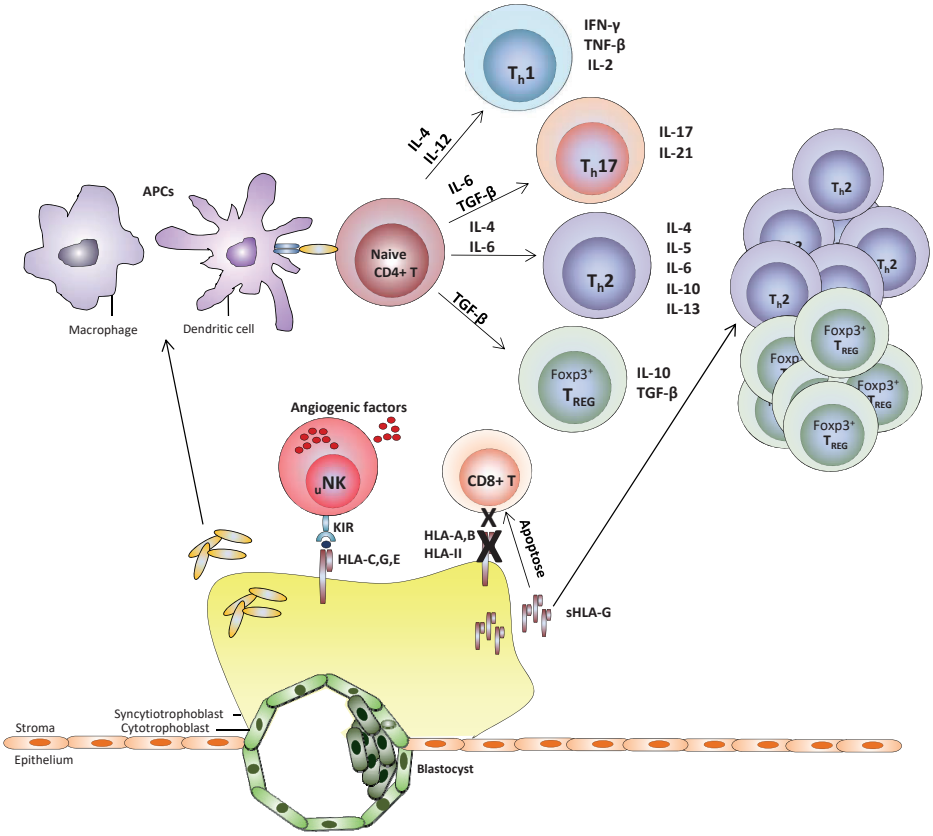
2.2 T cell activation

Besides uterine NK cells (70%), the early decidua is comprised of T cells (10%), and APCs including macrophages (20%), and DCs (2%). T cells are a part of the adaptive immune system. After an initial response to a specific pathogen both CD4+ and CD8+ cells replicate and develop into memory cells that will lead to a more vigorous response to subsequent encounters with that pathogen. The T cell receptor (TCR) binds to short peptides in the binding groove of the MHC on the cell surface. T cell activation requires the interaction of the TCR with the appropriate MHC/peptide complex and interaction of co-stimulatory molecule CD28 with CD80/86 on APCs. Upon activation, T cells express the high affinity interleukin (IL)-2 receptor and produce IL-2, which drives clonal expansion. After this expansion, CD8+ T cells may differentiate into cytotoxic effector cells, whereas naïve CD4+T cells develop into T helper 1 (Th1), T helper 2 (Th2), regulatory T cells or T helper (Th17) cells,[69] depending on the types of cytokines present in the environment where T cell activation occurs (Figure 1.5).

The development of Th1 or Th2 cells is influenced by the presence of pro-inflammatory cytokines such as IL-12 and Il-4. Th1 cells synthesize IL-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-β and thereby induce cellular immunity. Cellular immunity covers the activity by T cells, NK cells, mast cells, basophils, eosinophils, neutrophils, macrophages, and DCs.

Th2 cells synthesize IL-4, IL-5, IL-6, IL-10, and IL-13 which induce humoral immunity, which includes chemokines, cytokines, complement, and B cells and thus stimulate B cells to produce antibodies.[70] Regulatory T cells develop under the influence of cytokines like TGF-β and IL-10, and in the absence of pro-inflammatory cytokines. If inflammatory cytokines IL-6 and/or IL-21 are present along with TGF-β the induction of regulatory cells is inhibited and Th17 cells are generated. The regulatory T cell is a specialized subset of T cells distinguished from the other classes by their role in tolerance. Regulatory T cells can be divided into the naturally occurring CD4+CD25^{bright} regulatory T cell (Treg) derived from the thymus and the peripherally induced type 1 regulatory T cells (Tr1) and Th 3 regulatory cells. Activated CD4+ T cells express intermediate levels of CD25 (CD25^{dim}) and CD4+ T cells expressing high levels of CD25 (CD25^{bright}) have regulatory capacity.[71] CD4+CD25^{bright} regulatory T cells are able to control immune responsiveness to self- and allo-antigens and are able to suppress auto-immunity.[72] FoxP3, the gene encoding the transcription factor Scurfin, is also a marker for Treg cells. Mice deficient for Scurfin, lack regulatory T cells and suffer from autoimmunity, whereas mice with overexpression of Foxp3 display increased immunosuppressive activity compared to wild type mice.[73,74] Other markers to distinguish further between activated and regulatory T cells are cytotoxic T

Figure 1.5 T cell activation and differentiation of CD4+ T helper subsets at the implantation site



lymphocyte associate protein 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), CD95, and CD127, which is inversely correlated with Foxp3 expression.[75,76] However, all these surface markers can be dynamically expressed on other cell populations and functional tests remain necessary to distinguish Treg cells from related cells.

2.2.1 T cell activation in pregnancy

When the blastocyst is penetrating the epithelium of the uterus, the maternal innate immune system comes into action. This first line of defense against pathogens that penetrate the epithelial surface is always available, and does not improve with repeated exposure to the same pathogen. Cells of the innate immune system can interact with cells of the adaptive immunity. Fetal allo-antigens are picked up by invading APCs, which are the second largest subset of leukocytes in the early pregnancy, at the implantation site and transport the antigens to the uterine lymph nodes. There they activate the acquired

immune system. These APCs, especially DCs will interact with naïve T-helper cells and induce differentiation of these cells into T helper cells under the influence of different cytokines as described in paragraph 2.2 and Figure 1.5.

The expansion of Treg cells will actively induce maternal tolerance to paternal antigens during pregnancy. Indeed, in mice Treg cells in the decidua are responsible for maternal tolerance to fetal allo-antigens by preventing rejection and facilitating a successful pregnancy.[77,78] In an abortion prone mouse model, transfer of Treg cells from normal pregnant mice could inhibit proliferation and cytokine production of Th1 cells in the decidua, and prevent abortion.[79,80] Likely Th2 cytokine production as IL-4 and IL-10 was promoted by this transfer of Treg cells.[81] Also in human, Treg cells are increased in the decidua during pregnancy.[82] In women with complicated pregnancies as recurrent miscarriage and preeclampsia, decreased numbers of Treg cells were found in the decidua[83] and placenta,[84] suggesting that Treg cells play a pivotal role in the maintenance of fetal acceptance, normal implantation, and placentation. The possible mechanisms by which Treg cells induce maternal immune tolerance towards fetal antigens are cell-to-cell interaction via membrane-bound TGF-β, LAG-3, Galectin 1, and CTLA-4, secondly by inhibiting T cell activation through production of cytokines such as IL-10 and TGF-β, and thirdly by inducing expression of indomelamine 2,3-dioxygenase (IDO) by APCs through CTLA-4.[69]

IDO, a tryptophan-catabolizing enzyme, is also synthesized by the trophoblast and promotes maternal-fetal tolerance. In mice, the IDO inhibitor, 1-methyl-tryptophan (1-MT), induces fetal rejection by preventing IDO to suppress maternal T cell responses.[85] Moreover, by tryptophan depletion IDO protects the fetus by suppressing T cell-driven local inflammatory responses at the maternal-fetal interface.[86] Under influence of IDO naïve T cells differentiate into cells with a regulatory phenotype, and in turn, these cells create other tolerogenic DCs through the induction of IDO expression by CTLA4 interactions.[87] In summary, IDO can mediate a suppressive effect directly on effector T cells and at the same time activate Tregs cells.[88]

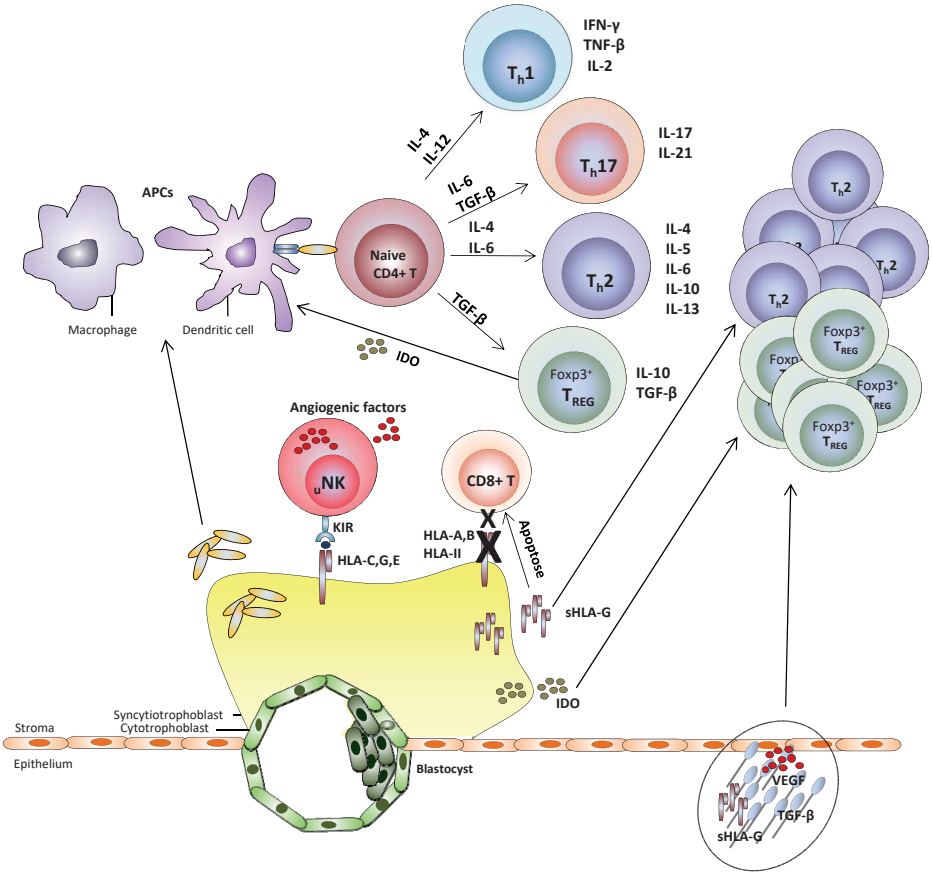
2.2.2 Seminal plasma and T cell activation

Already during copulation, long before implantation, maternal tolerance towards future fetal allo-antigens is induced as was shown in mice studies.[79,89] Normal seminal plasma facilitates spermatozoal transport and survival, and consequently increases fertility.[90] It has been assumed that immunoregulatory factors in seminal plasma protect spermatozoa against a female allo-immune response. In normal sperm, different suppressive mechanisms are active and a range of inhibitors of the immune and inflammatory system has been identified. Also, human seminal plasma contains several types of immunoregulatory

factors such as cytokines, chemokines,[91] and sHLA,[92,93] which may modulate the maternal immune response (Figure 1.6).[94,95] One of those cytokines, TGF-β, is highly present in human seminal plasma. TGF-β is thought to inhibit a type 1 immune response against the semi-allogeneic fetus, by initiating a type 2 or Treg-dominated immune response associated with partner-specific tolerance.[96]

Soluble forms of HLA-G have been found in human seminal plasma and therefore paternal HLA-G may affect the maternal immune system before implantation of the embryo.[97] These allo-antigens present in seminal plasma may also be responsible for the Treg cells expansion as was previously shown in mice.[98] In addition, within two days after insemination, Treg cells with an upregulation of Foxp3 expression can be found in the draining lymph nodes in mice.[77] DCs are partly responsible for this antigen specific Treg expansion (Figure 1.6).[99]

Figure 1.6 Expansion of Treg cells by immunoregulatory factors present in seminal plasma before implantation



Another well-known route to induce immune tolerance is via oral exposure, possible because the gut has the most adequate absorption in the absence of an inflammatory environment.[100,101] In transplantation models of rats, oral administration of MHC molecules prevents the occurrence of allograft rejection.[102] Based on this knowledge, Koelman et al. hypothesized that a potent way of inducing tolerance towards paternal HLA of the fetus in pregnancy, would be exposure of these antigens to the mothers oral mucosa. In support of this theory, they showed that both oral sex and swallowing sperm diminished the prevalence of preeclampsia.[93] Interestingly, in women with two miscarriages the incidence of oral sex practice was similar to a control population, but more women in the control group swallowed sperm compared to women with two miscarriages.[103]

2.3 B cell activation, antibodies, and complement

Besides T cells, B cells are also a part of the adaptive immune system. B cells produce antigen-specific antibodies; after uptake of antigen by the B cell receptor (BCR) and interaction with primed T cells and costimulation through CD40L-CD40 and specific cytokines.[104] Essential for this antibody response is that the interacting T- and B cell recognize epitopes of the same antigen. Antibodies are glycoproteins and there are five classes or isotypes called IgA, IgD, IgE, IgG, and IgM. In addition, there are 4 subclasses of IgG and two subclasses of IgA. Of the five isotypes, IgM is always the first antibody to be secreted in an immune response, as this is the BCR on naïve B cells independent of T cell help. During differentiation so called 'isotype switching' takes place, which means that some B cells start to produce antibodies of a different class that mediate other effector functions at different locations.[105] The main functions of antibodies are neutralization and opsonization. During neutralization, antibodies bind tightly to a site of the pathogen, thereby neutralizing its toxic activity, and preventing interaction with human cells. Opsonization is the process in which IgG antibodies coat the cell surface of a pathogen. The constant region of the antibody binds to receptors on a phagocyte and promotes ingestion and destruction by these phagocytes.

Furthermore, IgM, IgG1, and IgG3 can activate the complement system. The complement system is the so called 'first line of defense' of the human immune system. Complement is activated by 3 mechanisms known as the classical, lectin, and alternative pathways. These three pathways converge by generating enzymes called C3 convertases, which cleave C3 into C3b and C3a. C3b binds to the surface of foreign cells and opsonizes the cells for phagocytosis. A tissue-biomarker for classical complement activation, which is activated by antibodies, is C4d, a non-functional split product of classical complement activation. C4d covalently attaches to cells and tissues, thereby acting as a footprint of

recent antibody-mediated tissue injury. Other biological functions of the complement system are achieved through the production of activation fragments (e.g., C3a, C5a, C5b-9) by forming the membrane attack complex (MAC). This complex can insert into membranes and has the potential to damage cells. Finally, antibodies bound to infected cells, allow NK cells to kill them through antibody-dependent cellular cytotoxicity.

A fraction of B cells do not differentiate into antibody secreting plasma cells but become B memory cells that are able to respond rapidly when the same antigen is encountered again.[106]

2.3.1 B cell activation, antibodies, and complement in pregnancy

When the maternal immune system recognizes the paternal HLA as different, this may lead to the production of allo-antibodies.

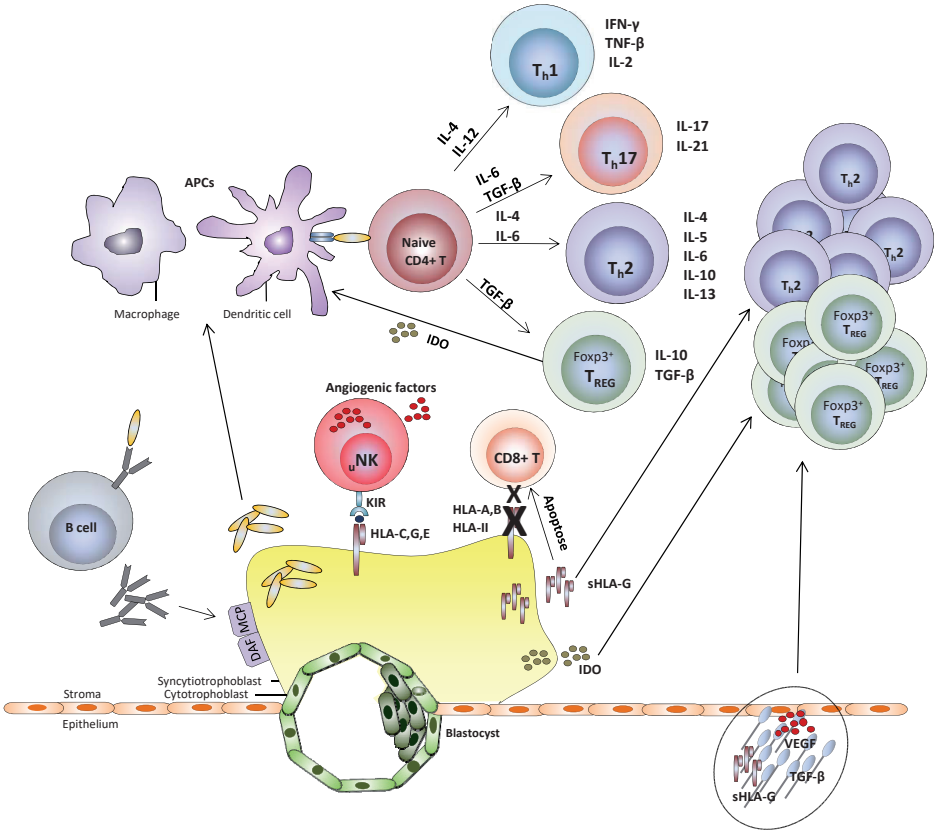
Approximately 30% of healthy women develop anti-HLA antibodies during pregnancy. The presence of these antibodies increases after 28 weeks of pregnancy and antibodies can still be present at time of a new conception.[107,108] Binding of antibodies to paternal HLA antigens of the fetus might lead to complement fixation and antibody-mediated rejection of the fetus. In women with recurrent miscarriages presence of anti-HLA antibodies is associated with a reduced chance of a live birth.[109] In addition, in spontaneous preterm birth C4d deposition on fetal umbilical cord endothelium was associated with circulating maternal anti-HLA class I antibodies.[110]

Also, autoantibodies can initiate local complement activation by the activation of the classical pathway, and recruitment of inflammatory cells which may lead to abnormal placental development in pregnancy. We have recently demonstrated that C4d is abundantly present in placentas of women with autoimmune mediated pregnancy losses caused by SLE and antiphospholipid syndrome.[111] Placental C4d was found at the fetal-maternal interface, and was strongly associated with intrauterine fetal death and severe forms of preeclampsia.[112-116]

During normal pregnancy, uncontrolled complement activation is prevented by the three regulatory proteins: decay accelerating factor (DAF), membrane cofactor protein (MCP) and CD59, highly expressed on the surface of trophoblasts, as well as circulating complement regulatory proteins factor H, factor I, and C4b binding protein (Figure 1.7).[117]

DAF accelerates the destruction of the C3 convertase enzymes that activate C3 and amplifies the classical and alternative complement pathways. MCP is a cofactor for Factor-I mediated degradation and inactivation of C3b and C4b, thereby preventing further activation and amplification of the complement cascade. Finally CD59 prevents the

Figure 1.7 Complement regulatory proteins prevent uncontrolled complement activation in pregnancy



assembly of MAC, thereby blocking the lysis effect of this complex.[118] A recent cohort study of patients with severe preeclampsia demonstrated that 19% of women had mutations in complement regulatory genes, leading to inadequate inhibition of complement activation at the fetal-maternal interface.[119]

4. Outline of this thesis

The general aim of the studies described in this thesis was to further unravel the underlying mechanisms causing recurrent miscarriage of unknown etiology in order to identify these women earlier in their disease course, and to eventually develop more effective and more patient tailored treatment strategies. The focus of these present studies was on the detection of parameters leading to the causative mechanisms, with special emphasis on the role of the partner.

In **chapter 2** we systematically reviewed whether HLA sharing between partners, specific HLA alleles or the 14-bp insertion in the 3'UTR of the HLA-G gene were associated with the occurrence of unexplained recurrent miscarriage. In a second study, **chapter 3**, we compared other polymorphisms in the HLA-G allele in women with recurrent miscarriage with women with uneventful pregnancy.

As HLA-C is the only classical HLA antigen expressed on the trophoblast, we studied whether the immunogenicity of HLA-C plays a role in couples with unexplained recurrent miscarriage (**chapter 4**). The effect of anti-HLA antibodies on pregnancy complications is reviewed in **chapter 5**. The role of HLA-C specific antibodies in pregnancy complications has not been studied, while we know from transplantation settings that a proportion of alloantibodies cause rejection, mostly through their ability to activate complement.[120] Therefore, we investigated the presence of HLA-C antibodies in the first trimester of pregnancy (**chapter 6**), and the presence of C4d in products of conception (**chapter 7**), in women with recurrent miscarriage and compared them to women with uneventful pregnancy.

Seminal plasma contains a variety of immunological factors that can potentially influence the acceptance of the fetus by the maternal immune system. The immunomodulating effect of seminal plasma on human T cells was explored in **chapter 8**. Finally, we determined whether women with recurrent miscarriage had less oral sex with their partner than women with uneventful pregnancy as a possible explanation for the lack of tolerance to the paternal antigens (**chapter 9**).

Chapter 10 provides a general discussion of this thesis. Moreover, implications for clinical practice and future research are discussed.

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Chapter 2

HLA associations and HLA sharing in recurrent miscarriage: a systematic review and meta-analysis

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Human Immunology 2015;76 (5):362-373
Supplementary data available on http://dx.doi.org/10.1016/j.humimm.2015.02.004

Abstract

Problem

The aim of this meta-analysis was to evaluate whether specific maternal HLA alleles and HLA sharing of couples are associated with the occurrence of recurrent miscarriage.

Method

A systematic literature search was performed for studies that evaluated the association between HLA alleles, HLA sharing and recurrent miscarriage. Recurrent miscarriage was defined as three or more consecutive unexplained miscarriages and a control group was included of women with at least one live birth and no miscarriages in their history. Meta-analyses were performed and the pooled odds ratio (OR) was calculated.

Results

We included 41 studies. Selection bias was present in 40 studies and information bias in all studies. Meta-analyses showed an increased risk of recurrent miscarriage in mothers carrying a HLA-DRB1*4 (OR 1.41, 95% CI 1.05-1.90), HLA-DRB1*15 (OR 1.57, 95% CI 1.15-2.14), or a HLA-E*01:01 allele (OR 1.47, 95% CI .20-1.81), and a decreased risk with HLA-DRB1*13 (OR 0.63, 95% CI 0.45-0.89) or HLA-DRB1*14 (OR 0.54, 95% CI 0.31-0.94). Pooling results for HLA sharing showed that HLA-B sharing (OR 1.39, 95% CI 1.11-1.75) and HLA-DR sharing (OR 1.57, 95% CI 1.10-1.25) were both associated with the occurrence of recurrent miscarriage.

Conclusion

Although the present systematic review and meta-analysis demonstrates that specific HLA alleles and HLA sharing are associated with recurrent miscarriage, a high degree of bias was present and therefore observed results should be interpreted carefully.

Introduction

Approximately 1% of all couples will be confronted with recurrent miscarriage, which is defined as three or more consecutive miscarriages prior to the 20th week of gestation.[1] Recurrent miscarriage is a highly heterogeneous condition. Possible etiologic factors may include balanced translocations in the maternal or paternal DNA, uterine anomalies, acquired thrombophilia as anti-phospholipid syndrome (APS), and hereditary thrombophilia.[2,3] However, in many couples no causal factor can be identified.[2,4] As the fetus is a semi-allograft, which escapes maternal immune rejection in normal pregnancy, many studies investigated whether the HLA system plays a role in unexplained recurrent miscarriage.

Several authors investigated whether specific maternal HLA class II alleles,[5,6] some of which associated with auto-immune disorders,[7] are also associated with the occurrence of recurrent miscarriage. Other studies investigated the role of HLA-C, the only classical HLA antigen expressed on trophoblast, in recurrent miscarriage,[8,9] with a special focus on the group of HLA-C2 alleles and their interaction with receptors on NK cells suggested to be associated with complicated pregnancies.[10-13] In addition, the association between recurrent miscarriage and the non-classical HLA-E and HLA-G alleles, both present on the trophoblast were investigated.[14,15] Although the invading throphoblast is derived from the fetus, most studies focused on associations of recurrent miscarriage and specific maternal HLA-C, -E and -G alleles likely since collecting and typing of miscarriage material is rather difficult due to logistical problems. Several studies focused on the association of recurrent miscarriage with the insertion of a 14-bp in HLA-G in the mother,[16,17] which has been correlated with reduced mRNA levels of HLA-G[18] and low levels or even absence of sHLA-G in plasma.[19,20] As HLA sharing between couples could decrease the trigger to develop an immunoregulatory response, which may be associated with failure of implantation or fetal loss, the degree of HLA sharing was extensively investigated in recurrent miscarriage.[21]

Studies on the association of unexplained recurrent miscarriage with specific maternal HLA alleles and HLA sharing between couples have led to inconsistent results.[21-24] This inconsistency is not surprising considering the various definitions of recurrent miscarriage and control groups, the analysis of different HLA alleles and loci, and the application of various HLA typing methods.

In order to provide a complete and up-to-date overview on the possible role of the HLA system in recurrent miscarriage, we reviewed the literature on the association between specific maternal HLA alleles and HLA sharing between couples and the occurrence of recurrent miscarriage.

Material and methods

Search strategy

In November 2013 we searched in close collaboration with a trained librarian in the databases PubMed, Embase, Web of Science, and Cochrane for studies that evaluated the association between HLA alleles and HLA sharing with recurrent miscarriage (Supplementary data for the comprehensive search string, Table I). As search limit, only studies published in English and concerning humans were included. In addition, references of other narrative and systematic reviews were checked for relevant articles.

Eligibility criteria

After the literature search, all titles and abstracts were independently assessed by two observers (TM and EL). The following eligibility criteria were applied:

- *Definition of recurrent miscarriage:* Women with three or more consecutive unexplained miscarriages.[1] The classification unexplained recurrent miscarriage was made for women without uterine anomalies and/or, parental chromosomal abnormalities and/or, acquired or hereditary thrombophilia[2] or if the authors stated them as unexplained.
- *Definition of control subject:* Women with at least one live birth and no miscarriages in the medical history.
- *Method of HLA typing:* For the association of specific maternal HLA alleles with recurrent miscarriage only studies were included using molecular HLA typing methods. For the association between HLA sharing and recurrent miscarriage both studies using serological HLA typing methods as well as molecular methods were included. Complement dependent cytotoxicity (CDC) or two color fluorescence (TCF) as HLA typing methods were considered as serological methods. Restriction fragment length polymorphism (RFLP), PCR-RFLP, PCR-sequence specific priming (SSP), PCR-sequence specific oligonucleotides (SSO), and PCR-Luminex, and PCR-sequence based typing (SBT) were considered as molecular methods.
- *Design:* cohort studies, case-control studies, or cross-sectional studies were included in the analysis. Case-reports were not considered.

Risk of bias assessment

Risk of bias was assessed according to an adaptation of the Newcastle-Ottawa scale (http://www.ohri.ca/programs/clinical_epidemiology/nosgen.pdf (downloaded 01-2013)) by two observers (TM and EL):

- *Selection bias:* studies were considered to have a high risk of selection bias if cases were not consecutive or randomly sampled from a defined hospital or clinic over a defined period of time (case-control studies). For the control group high risk of selection bias was considered if control subjects were not from the same population as the cases. Adequate control subjects must have had the chance to become a case (which defines a control in a case-control study). In cohort studies selection bias was considered if the cohort was not representative of the average fertile women in the general population and the non-exposed cohort was not drawn from the same population as the exposed cohort.
- *Information bias:* studies were considered to have a high risk of information bias if the case description (primary recurrent miscarriage (recurrent miscarriage with no history of live births) or secondary recurrent miscarriage (recurrent miscarriage after (a) live birth(s)), gestational age of miscarriages, maternal age at time of diagnosis) was incomplete. As the diagnostic work-up to rule out verifiable causes for recurrent miscarriage (uterine anomalies, parental chromosomal abnormalities, acquired thrombophilia as APS) as recommended by international guidelines[25-27] was inadequate, the risk of bias was considered to be high. For the control subjects high risk of information bias was considered if the description of control subjects (number of live births, course of pregnancies, miscarriages in history) was incomplete.
- *Equal assessment of confounding factors in the case and control subjects:* adequate description of ethnicity in cases and control subjects, as the frequency of HLA alleles varies amongst different populations.[28] Furthermore defining adequately whether auto-immune diseases related with specific HLA types and associated with recurrent miscarriage such as systemic lupus erythematosus (SLE)[29-32] and rheumatoid arthritis (RA)[33-35] were present in cases and control subjects.
- *Description of laboratory procedures for HLA typing in studies:* HLA typing performed by molecular methods is more reliable and sensitive than serological typing.[36,37] Furthermore, molecular typing can be performed with low and high resolution; with the latter method a more specific typing with allele variations at the level of nucleotides is obtained. A result of comparing molecular typing with serological typing and comparing high resolution with low resolution typing is that

the change of finding HLA compatibility is smaller and therefore less HLA sharing is expected to be reported which could lead to heterogeneity between studies.

- *Specification of antigens and alleles used for HLA sharing:* specifying which antigens and alleles are used for calculating HLA sharing in cases and control subjects is important because use of different antigens or alleles could lead to over- or underestimation of HLA sharing and therefore to heterogeneity between studies.

Disagreement about selection of studies and assessing risk of bias was resolved by consensus. If no agreement was obtained, the opinion of a third observer (KB) was asked to gain consensus.

Data extraction

The following data were independently extracted by two observers (TM and EL): design of the study, definition of recurrent miscarriage, definition of control subject, number of case and control subjects, pregnancy with same partner or other partner(s), ethnicity, presence of auto-immune diseases, method of HLA typing, specific HLA allele frequencies, HLA allele phenotypic frequencies, and shared HLA antigens or alleles. We contacted the authors if variables or data was missing. Multiple studies published by the same author(s) were checked for overlap in included case subjects; we used the study with the largest dataset or the study with the best defined case or control group.

Statistical analysis

The primary outcome of the meta-analysis was the pooled odds ratio (OR) and their 95% confidence intervals (CI) for the association between the occurrence of recurrent miscarriage and specific HLA alleles or phenotypes and HLA sharing of the selected studies. HLA-G alleles were typed for the broad, split, and silent mutations in most studies. In order to combine the results of these studies we pooled these data on the broad and split, for example HLA-G*01:01:01 was pooled with HLA-G*01:01:03 and the combined data were used for meta-analysis.

As we expected a between-study heterogeneity a priori, due to different study populations, we used the random effects model by default. For this analysis, a minimum of five studies is generally recognized to be required.[38,39] Meta-analysis with less than five studies was performed in a fixed effects model. In addition, I^2 statistics were calculated, if substantial heterogeneity is present (I^2 >50%), meta-regression was performed to explore heterogeneity. Metaregression was performed between molecular typing used as screening method for HLA typing and serological typing, between studies which include APS screening

in their work-up to rule out women with explained recurrent miscarriage and studies which did not include APS screening, and finally between primary recurrent miscarriage and secondary recurrent miscarriage, as it is still postulated that primary recurrent miscarriage and secondary recurrent miscarriage could be two distinct entities with different underlying pathology.[40] For ethnicity, population stratification was performed.

To assess small study effects in the meta-analyses, funnel plots were generated. We used the Egger's test to explore this potential bias in case more than eight studies were included.[38,39] All analyses were performed with STATA (StataCorp.2011. Stata Statistical Software, Release 10, College Station, TX, USA;StataC).

Results

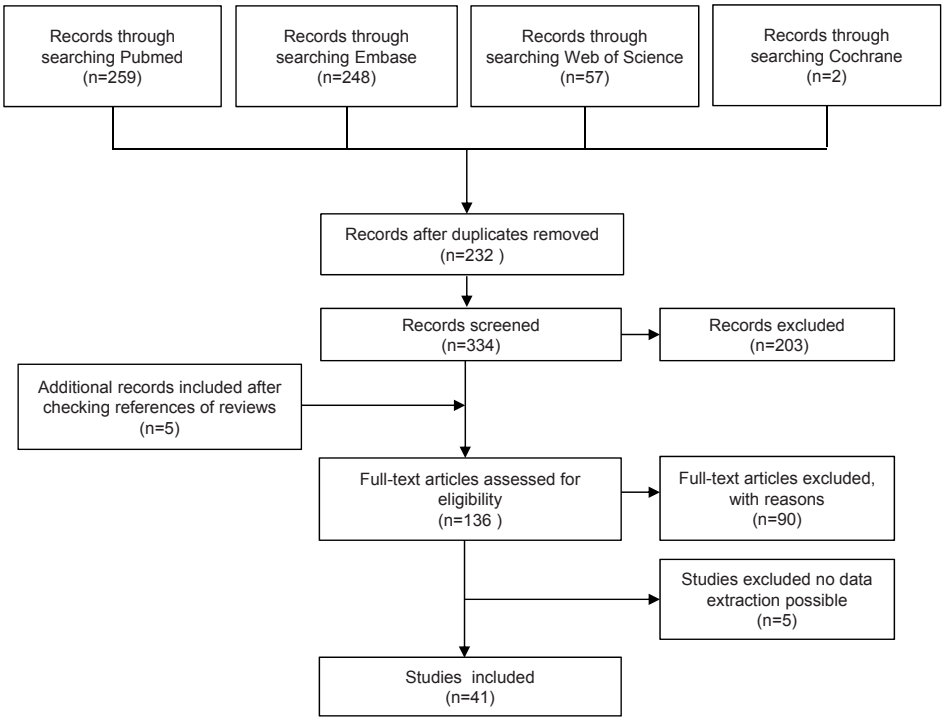
Study selection

The literature search identified 334 records. After review based on title and abstract, 131 records remained. References of other narrative and systematic reviews (n=50) were checked for relevant articles and 5 more studies could be identified. Therefore, a total of 136 were selected for full text review. When reading full text, most studies were excluded because of duplications, studying HLA sharing in women with recurrent miscarriage in the context of treatment evaluation, expressing of HLA antigens on trophoblast, or reporting about linkage disequilibrium in women with recurrent miscarriage (n=43). We furthermore excluded 47 studies that did not meet our eligibility criteria for case and/or control group definitions (for example; a case group with at least 2 miscarriages and no separate data in the study available of women with ≥ 3 miscarriages (see Supplementary data, Table II). Three studies were excluded because data extraction for individual HLA alleles or HLA sharing proved impossible[41-43] and two studies because data was not shown.[12,44] We were not able to retrieve the latter data even after contact with the corresponding authors. Finally, 41 studies were included, all of them case-control studies (Figure 2.1).

Risk of bias assessment

An overview of the risk of bias of the studies included is provided in Supplementary data, Table III. All included studies had a high risk of information bias because none of the selected studies provided a full definition of the cases, and five studies did not mention any of the case definitions (whether women had primary recurrent miscarriage or secondary recurrent miscarriage, the gestational age of the miscarriages and maternal age at diagnosis).[17,45-48] Most studies included in their work-up investigations to rule out uterine

Figure 2.1 Flowchart illustrating how the studies were selected for the meta-analysis



anomalies and parental chromosomal abnormalities (explained recurrent miscarriage). In 15 studies out of 41 studies screening for APS was not part of their work-up. Furthermore none of the 26 studies which included screening for APS described whether they tested for APS on at least two separate occasions 12 weeks apart.[49] Only six out of 41 studies fully described control sampling.[17,50-52]

High risk of selection bias was present as most studies did not indicate whether cases were consecutive or randomly sampled from one hospital. In only two studies it was clear that control subjects were from the same source population as cases.[6,53]

High risk of population stratification bias was present because the ethnicity of the participants was adequately reported in only 15 of the 41 studies and of these only 7 studies matched cases and control subjects or adjusted for ethnicity.[6,8,14,51,54-56] Stratification analysis was not possible because not enough studies were present for each specific ethnic origin.

Only three studies adequately defined whether cases had no auto-immune diseases as SLE and RA.[56-58] In addition, 10 studies measured ANA, anti-dsDNA, or rheumatoid factor

without reporting clinical features. Furthermore, two studies measured anti-phospholipid antibodies in order to detect acquired thrombophilia during the work-up,[45,59] leaving 25 studies which did not define whether auto-immune diseases were present.

All studies clearly defined which method for HLA typing was used. All studies, which investigate HLA sharing and used molecular typing method, mentioned whether they used high or low resolution typing. Three studies used high resolution[59-61] and five used low resolution.[45,53,62-64] A greater risk for bias was that only 14 out of 21 studies which investigated HLA sharing defined adequately which antigens and alleles were used to calculate sharing.

HLA association studies

For associations between specific HLA alleles and recurrent miscarriage, 24 studies were eligible using molecular HLA typing method (Table 2.1).[5,6,8,9,14-17,45-48,51,52,57,60-63,65-69] The following HLA alleles were reported in selected studies:

- Classical HLA I
  - HLA-C2
- Classical HLA II
  - HLA-DRB1*01-04,07-18 (16 alleles)
  - HLA-DQA1*01-06 (6 alleles)
  - HLA-DQB1*01-09 (9 alleles)
  - HLA-DPB*01-06,08-11,13,15-19 (16 alleles)
- Non-classical HLA I
  - HLA-G*01:01,03,04,05N,06 (5 alleles)
  - HLA-E*01:01,03 (2 alleles)
  - HLA-G 14-bp polymorphism

As HLA-DRB1*02,17,18, DQA1*05,06, DQB1*01,07-09, DPB*01-06,08-11,13,15-19 alleles were mentioned in only one study, they could not be considered for meta-analysis. All significant associations on specific alleles and recurrent miscarriage of individual studies can be found in Table 2.1.

The phenotypic frequency of HLA-C2 was investigated in couples with recurrent miscarriage, an overall fixed effects meta-analysis (2 studies) showed an OR of 1.04 with a 95% CI 0.81-1.34 (Figure 2.2a). One study investigated HLA-C2 allelic frequencies in women separately and showed an OR of 1.29 (95% CI 0.91-1.83, p=0.175).[9]

For the HLA class II alleles, a significant association was observed for phenotypic frequencies of HLA-DRB1*4 (OR 1.41, 95% CI 1.05-1.90), HLA-DRB1*13 (OR 0.63, 95% CI

Table 2.1 HLA associations in recurrent miscarriage

	Author	Study design	Cases		Control subjects		Ethnicity	HLA biomarker	Study findings
			N	Definition	N	Definition			Significant
Classical HLA I									
1	Faridi et al. (2011)	Case control	177	≥ 3 PRM	200	≥ 2 uncomplicated live births	Ethnically matched	C1,C2 alleles in couples (allelic)	-
2	Hiby et al. (2008)	Case control	162	≥ 3 PRM, first (92%) and second trimester, same partner	269	1 live birth	NR	C1,C2 alleles in couples (allelic)	C2
3	Christiansen et al. (1997)	Case control	70	≥ 3 RM (20 PRM, 15 SRM), before 28th gestational week	60	≥ 2 live births	Caucasian	C1,C2 alleles in couples (phenotypic)	-
Classical HLA II									
4	Aruna et al. (2011)	Case control	56	143 couples with ≥ 2 RM (130 PRM, 13 SRM) and 56 couples with ≥ 3 RM	140	≥ 1 live birth	Ethnically matched	DR-B1,DQA,DQB (allelic)	DQB1*03:03:02[a] DQB1*03:03:03↓[b]
5	Kruse et al. (2004) (study II)	Case control	354	≥ 3 RM (212 PRM, 142 SRM), 20-45 years	202	≥ 1 live birth	Caucasian	DRB1,DQA1, DQB1 (phenotypic)	DRB1*04[b] DRB1*13[b] DRB1*14[b] DQA1*01:03[b] DQB1*03:02[b] DQB1*06:03/06:04[b] (study II)
6	Takakuwa et al. (2003)	Case control	93	≥ 3 RM (79 PRM, 14 SRM) first trimester, same partner	115	≥ 2 term deliveries	Japanese	DRB1 (phenotypic)	DRB1 *15:02
7	Sasaki et al. (1997)	Case control	27	≥ 3 RM, first trimester	22	≥ 2 term deliveries	NR	DRB1 (phenotypic)	DRB1*04

8	Takakuwa et al. (1999)	Case control	30	≥ 3 PRM, first trimester, same partner	30	≥ 2 term deliveries		DPB (phenotypic)	DPB*04[b] DPB*04:02[b]
9	Bellingard et al. (1995)	Case control	10	≥ 3 PRM, mean age 33.9 years	21	≥ 2 live births	NR	DRB1 (allelic & phenotypic)	-
10	Dizon-Townson et al. (1995)	Case control	51	≥ 3 RM, consecutive	43	≥ 7 live births	Caucasian	DQA1 (allelic)	-
11	Takakuwa et al. (1992)	Case control	22	≥ 3 RM, same partner, first trimester	20	≥ 2 term deliveries	NR	DQB1 (allelic & phenotypic)	-
Non-classical HLA II									
12	Christiansen et al. (2012)	Case control	339	≥ 3 RM (154 PRM, 185 SRM), median age at referral 32-33 years	125	≥ 2 uncomplicated live births	NR	HLA-G (exon 8)	G14bp ins/ins
13	Vargas et al. (2011)	Case control (matched age, socio-economic)	60	≥ 3 PRM (clinically verified), before 20th gestational week, same partner, mean age at miscarriage 26.4 years	68	≥ 2 live births	Ethno-geographically matched	HLA-G (exon 2,3,8) (allelic)	HLA-G 01:01A ↓
14	Zhu et al. (2010)	Case control	51	≥ 3 RM	251	≥ 1 live birth	NR	HLA-G (exon 8)	-
15	Suryanarayana et al. (2008)	Case control	169	≥ 3 PRM, first trimester	92	≥ 1 uncomplicated pregnancy and birth	Ethnically matched	HLA-G (exon 2 and 8) (allelic)	-
16	Xue et al. (2007)	Case control	24	≥ 3 RM	88	≥ 1 uncomplicated pregnancy and birth	NR	HLA-G (exon 8)	G14 bp ins/del
17	Yan et al. (2006)	Case control	79	≥ 3 RM	107	≥ 2 uncomplicated pregnancy and births	NR	HLA-G (exon 8)	G14 bp ins

Table 2.1 continues on next page

Table 2.1 *Continued*

	Author	Study design	Cases		Control subjects		Ethnicity	HLA biomarker	Study findings
			N	Definition	N	Definition			Significant
18	Yan et al. (2006)	Case control	69	≥ 3 RM	146	≥ 2 uncomplicated pregnancy and births	NR	HLA-G (exon 2,3,4) (allelic)	-
19	Abbas et al. (2004)	Case control	120	≥ 3 PRM	120	≥ 3 live births	NR	HLA-G (exon 2,3) (allelic)	HLA-G 01:01:03
20	Tripathi et al. (2004)	Case control	120	≥ 3 PRM	120	≥ 3 live births	NR	HLA-G (exon 8)	G14 bp ins/del
21	Pfeiffer et al. (2001)	Case control	78	≥ 3 RM (56 PRM, 22 SRM), same partner, 22-42 years	52	≥ 1 successful pregnancy	Caucasian	HLA-G (exon 2,3) (allelic) HLA-E (codon 107) (allelic)	-
22	Mosaad et al. (2011)	Case control	108	≥ 3 PRM, 19-38 years	120	Parous	Same	HLA-E (codon 107) (allelic)	HLA-E 01:01 HLA-E 01:03↓ HLA-E 01:01/ HLA-E 01:01
23	Tripathi et al. (2006)	Case control	120	≥ 3 PRM, 22-40 years	120	≥ 3 live births	NR	HLA-E (allelic)	HLA-E 01:01 HLA-E 01:01/ HLA-E 01:01
24	Kanai et al. (2001)	Case control	30	≥ 3 PRM, first trimester	38	≥ 1 uncomplicated pregnancy and live birth	Japanese	HLA-E (allelic)	-

[a]Not included in meta-analysis, significant after correction for multiple testing, [b]Not included in meta-analysis, not significant after correction for multiple testing, RM; recurrent miscarriage, PRM; primary recurrent miscarriage, SRM; secondary recurrent miscarriage, NR; not reported, ns; not significant.

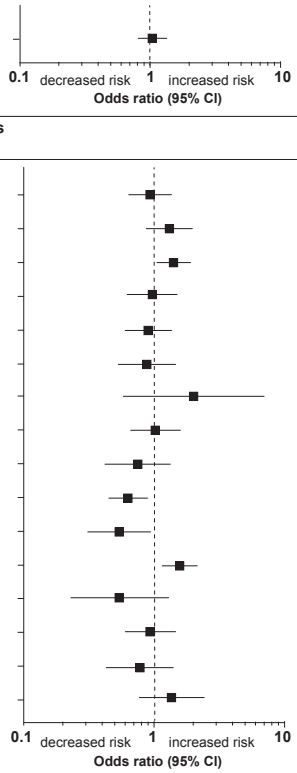
Figure 2.2 Associations of classical HLA I (a) and classical HLA II (b) in recurrent miscarriage

a

Classical HLA I	studies (#)	cases (n/N)	control subjects (n/N)	*p*-value	I^2 (%)
HLA-C2	2	231/494	245/520	0.748	31.1

b

Classical HLA II	studies (#)	cases (n/N)	control subjects (n/N)	*p*-value	I^2 (%)
HLA-DRB1*01	4	83/484	58/360	0.710	31.9
HLA-DRB1*03	2	95/364	47/223	0.193	22.8
HLA-DRB1*04	4	186/484	109/360	0.021	29.8
HLA-DRB1*07	3	64/391	43/245	0.881	0.0
HLA-DRB1*08	4	59/484	56/360	0.609	0.0
HLA-DRB1*09	4	36/484	42/360	0.616	71.8
HLA-DRB1*10	4	8/484	3/360	0.272	28.6
HLA-DRB1*11	4	60/484	39/360	0.916	0.0
HLA-DRB1*12	4	25/484	28/360	0.319	0.0
HLA-DRB1*13	3	92/457	91/338	0.008	0.0
HLA-DRB1*14	3	22/457	36/338	0.029	0.0
HLA-DRB1*15	3	170/474	92/339	0.004	0.0
HLA-DRB1*16	3	9/474	13/339	0.167	0.0
HLA-DQA1*01	4	136/214	280/364	0.758	0.0
HLA-DQA1*02	4	22/214	38/364	0.398	0.0
HLA-DQA1*03	4	31/214	26/364	0.287	74.4



Odds ratio with 95% CI are shown of a meta-analysis in a fixed effects model. For individual meta-analysis see supplementary data, figures 1-3.

0.45-0.89), HLA-DRB1*14 (OR 0.54, 95% CI 0.31-0.94), HLA-DRB1*15 (OR 1.57, 95% CI 1.15-2.14) and recurrent miscarriage in a fixed effects meta-analysis (Figure 2.2b).

Of all HLA II alleles which were only reported once in literature, only HLA-DQB1*03:03:02 was associated with recurrent miscarriage after correction for multiple testing (Table 2.1).[5] Observed heterogeneity for HLA-DRB1*09 (71.8%) could not be explained in a meta-regression by APS included in the work-up (p=0.552). As only one study reported separate data for women with primary and secondary recurrent miscarriage,[65] it was not possible to perform a meta-regression by primary recurrent miscarriage and secondary recurrent miscarriage.

For the presence of allelic frequencies of HLA-G*01:01, HLA-G*01:03, HLA-G*01:04, and the null allele G*01:05N in the mother no association was found with recurrent miscarriage

in a fixed effects model (respectively OR 0.90, 95% CI 0.71-1.14, OR 0.97, 95% CI 0.68-1.140, OR 1.32, 95% CI 1.00-1.76, OR 0.88, 95% CI 0.56-1.39) (Figure 2.3a). Pooled analysis of studies on the carrier ship of HLA-E*01:01 in the mother (4 studies) showed an association with recurrent miscarriage in a fixed effects meta-analysis (OR 1.50, 95% CI 1.20-1.88). Because HLA-E has only two non-synonymous alleles, for HLA-E*01:03 a fixed-effects meta-analysis showed an OR of 0.66 (95% CI 0.53-0.83) (Figure 2.3a).

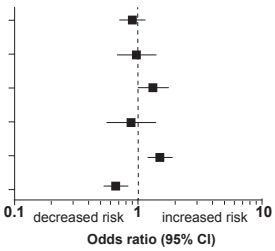
For HLA-G*01:04 high heterogeneity was observed (69.2%). Only two studies showed data on HLA-G*01:04 in women with primary recurrent miscarriage[14,66] and none of the studies in women with secondary recurrent miscarriage. We could therefore not explore the heterogeneity by meta-regression. In addition, all studies in this meta-analysis included screening for APS in their work-up.

In total 7 studies investigated the HLA-G 14-bp polymorphism in women with recurrent miscarriage. Pooled analysis in a random effects model of 14-bp insertion alleles and recurrent miscarriage showed an OR of 1.20 (95% CI 0.96-1.50). Studies on 14-bp genotype showed for 14-bp insertion/insertion an OR of 1.38 (95% CI 0.85-2.26), for 14-bp insertion/

Figure 2.3 Associations of non-classical HLA I alleles (a) and 14-bp polymorphism (b) in recurrent miscarriage

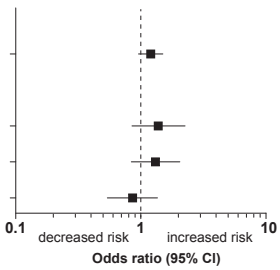
a

Non-classical HLA I	studies (#)	cases (n/N)	control subjects (n/N)	*p*-value	I^2 (%)
HLA-G*01:01	4	389/654	481/772	0.371	43.9
HLA-G*01:03	4	70/654	73/772	0.886	0.0
HLA-G*01:04	4	122/654	130/772	0.051	69.2
HLA-G*01:05N	4	40/654	46/772	0.588	0.0
HLA-E*01:01	4	423/668	352/656	<0.001	0.0
HLA-E*01:03	4	245/668	304/656	<0.001	0.0



b

HLA-G 14-bp polymorphism	studies (#)	cases (n/N)	control subjects (n/N)	*p*-value	I^2 (%)
HLA-G 14-bp allele					
Ins	6	603/1202	627/1460	0.104	44.0
HLA-G 14-bp genotype					
Ins/ins	7	213/940	148/953	0.196	69.8
Ins/del	7	477/940	427/953	0.232	79.9
Del/del	7	250/940	278/953	0.525	74.4



Except for 14-bp polymorphism, odds ratio with 95% CI are shown of a meta-analysis in a fixed effects model. For 14-bp polymorphism odds ratio with 95% CI are shown of a meta-analysis in a random effects model. For individual meta-analysis see supplementary data, figures 4-7.

deletion an OR of 1.31 (95% CI 0.84-2.05), and for 14-bp deletion/deletion an OR of 0.86 (95% CI 0.54-1.36) with recurrent miscarriage (Figure 2.3b).

Meta-analyses on 14-bp genotype showed high heterogeneity, which could not be explained by meta-regression for primary recurrent miscarriage and secondary recurrent miscarriage for 14-bp insertion homozygosity, for 14-bp heterozygosity and for 14-bp deletion homozygosity (respectively p=0.315, p=0.621, p=0.570). All studies included for meta-analyses on 14-bp genotype included screening for APS and therefore this could not explain the heterogeneity observed.

HLA sharing studies

In total 21 eligible studies reported on classical HLA I sharing (HLA-A, HLA-B, HLA-C sharing) and classical HLA II sharing (HLA-DR, HLA-DQ, HLA-DP sharing) in couples with recurrent miscarriage.[45,50,53-56,58-64,70-77]

For HLA-A, B, C sharing most studies used serological typing method, except one study[61] which used molecular method to define HLA-C alleles. For HLA-DR and HLA-DQ sharing both molecular and serological typing methods were used in the included studies and for HLA-DP sharing only molecular typing method was used (Table 2.2).

Sharing of HLA-B and HLA-DR were both associated with recurrent miscarriage in a random effects meta-analysis (respectively OR 1.39, 95% CI 1.11-1.75, OR 1.57, 95% CI 1.10-1.25). Pooled analysis for HLA-A and HLA-C in a random effects model showed no association with recurrent miscarriage (respectively OR 1.11, 95% CI 0.71-1.74, OR 0.99, 95% CI 0.73-1.35) (Figure 2.4). Egger's test indicating that these meta-analyses were not biased because of small studies reporting large effects (see Supplementary data, figures13-15).

A fixed effects model showed an OR of 1.62 (95% CI 0.99-2.63) and an OR of 1.60 (95% CI 1.00-2.56) for HLA-DQB1 and HLA-DQ sharing and not for HLA-DQA1 (OR 0.99, 95% CI 0.56-1.75) with recurrent miscarriage (Figure 2.4). Only one study investigated DPB1 sharing in women with recurrent miscarriage, this study reported an OR of 1.14.[60]

For HLA-A sharing, APS included in the work-up, meta-regression for primary recurrent miscarriage and secondary recurrent miscarriage could not explain heterogeneity (respectively p=0.746, p=0.989). In addition, observed heterogeneity for HLA-DR was not explained in a meta-regression by method of HLA typing, APS included in the work-up, or primary recurrent miscarriage and secondary recurrent miscarriage (respectively p=0.664, p=0.354, p=0.960). For HLA-DQ and DQA1-sharing only two studies were eligible for meta-analysis and meta-regression could not be performed. Considering primary recurrent

Table 2.2 HLA sharing (HLA-A, B, C, DR, DQ, DP) in couples with recurrent miscarriage

	Author	Study design	Cases		Control subjects		Ethnicity	HLA biomarker	HLA typing method	Study findings	
			N	Definition	N	Definition				Significant	ND
1	Takakuwa et al. (2006)	Case control	91	≥ 3 PRM, first trimester, same partner	72	≥ 2 normal term deliveries	Japanese	DR, DQB1	DNA (high resolution)	-	DR, DQB1
2	Laurentaci et al. (1999)	Case control	86	≥ 3 RM (65 PRM, 21 SRM), first or second trimester	100	≥ 2 live births	NR	A, B, DR, DQ	Serological	-	A, B, DR, DQ
3	Takakuwa et al. (1999)	Case control	30	≥ 3 PRM, first trimester, same partner	30	≥ 2 term deliveries	NR	DPB1	DNA (high resolution)	-	DPB1
4	Christiansen et al. (1997)	Case control	35	≥ 3 RM (20 PRM, 15 SRM) before 28th gestational week	30	≥ 2 live births	Caucasian	Bw4, Bw6, C	DNA (high resolution)	-	Bw4, Bw6, C
5	Kishore et al. (1996)	Case control	100	≥ 3 PRM, mean age 23.7 years	100	≥ 3 live born	Ethnically matched	A, B, C, DR	Serological	A, Class 1, DR ≥ 1	B, C
6	Sbracia et al. (1996)	Case control	75	≥ 3 RM, mean age 34.1 years	30	≥ 2 successful pregnancies	NR	A, B, DR	Serological	-	A, B, DR
7	Dizon-Townson et al. (1995)	Case control	51	≥ 3 RM, consecutive	43	≥ 7 live births	Caucasian	DQA1	DNA (low resolution)	-	DQA1
8	Bellingard et al. (1995)	Case control	7	≥ 3 PRM, mean age 33.9 years	21	≥ 2 live births	NR	A, B, DRB1	Serological, DNA (low resolution)	-	A, B, DR

9	Ober et al. (1993)	Case control	68 (37 DQB1)	≥ 3 RM, age 23-43 years	36	≥ 1 live birth	NR	DQA1, DQB1	DNA (low resolution)	DQA1 ≥ 2	DQB1
10	Eroglu et al. (1992)	Case control (age matched)	60	≥ 3 PRM	60	≥ 2 live births	Race matched	A, B, DR	Serological	-	A, B, DR
11	Takakuwa et al. (1992)	Case control	22	≥ 3 RM, same partner, first trimester	20	≥ 2 term deliveries	NR	DQB1	DNA (low resolution)	-	DQB1
12	Chang et al. (1991)	Case control	36	≥ 3 RM (25 PRM, 11 SRM), first trimester, age 23-42 years	269	≥ 2 uncompli-cated preg-nancies	NR	A, B, C, DR	Serological	-	A, B, C, DR
13	Ho et al. (1990)	Case control (age matched)	123	≥ 3 RM (91 PRM, 32 SRM)	51	≥ 2 live births	Chinese	A, B, DR, DQ	Serological	A,DQ (PRM) A,B,DR,DQ ≥ 3 (Both)	-
14	Christiansen et al. (1989)	Case control	39	≥ 3 RM, age 20-42 years	33	≥ 2 live births	NR	A, B, DR	Serological/ DNA (low resolution)	-	A, B, DR
15	Johnson et al. (1988)	Case control	113	≥ 3 RM (80 PRM, 33 SRM), same partner, first trimester, age 23-42 years	51	Childbearing couples	NR	A, B, DR	Serological	B (PRM)	A, C, DR
16	Takakuwa et al. (1986)	Case control	18	≥ 3 RM, first trimester	13	Normal pregnancy history	NR	A, B, C, DR	Serological	DR	A, B, C

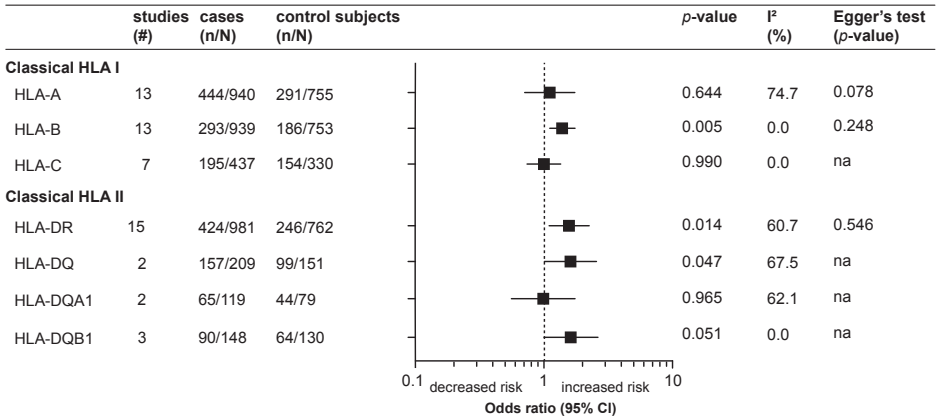
Table 2.2 continues on next page

Table 2.2 *Continued*

	Author	Study design	Cases		Control subjects		Ethnicity	HLA biomarker	HLA typing method	Study findings	
			N	Definition	N	Definition				Significant	ND
17	Vanoli et al. (1985)	Case control	47	≥ 3 RM first trimester, age 25-45 years	65	≥ 2 live births	NR	A, B, C, DR	Serological	-	A, B, C, DR
18	Taylor et al. (1985)	Case control	139	≥ 3 PRM, same partner	103	≥ 2 children	NR	A, B, C, DR	Serological	All ≥ 2 antigen	
19	Reznikoff-Etievant et al. (1984)	Case control	20	≥ 3 RM, first trimester	32	Several successful pregnancies	NR	A, B, C, DR	Serological	DR	A, B, C, all
20	Schacter et al. (1984)	Case control	60	≥ 3 RM, first trimester	30	≥3 live births without congenital abnormalities	NR	A, B, DR	Serological	A	B, DR
21	Oksenberg et al. (1984)	Case control (age matched)	60 (A,B) 30 (DR)	≥ 3 PRM, first trimester	30	≥ 2 live births	Ethnically matched	A, B, DR	Serological	-	A, B, DR

RM; recurrent miscarriage, PRM; primary recurrent miscarriage, SRM; secondary recurrent miscarriage, NR; not reported.

Figure 2.4 HLA-A, B, C, DR, DQ, DQA1, DQB1 sharing in recurrent miscarriage



For HLA-A, B, C, DR sharing odds ratio with 95% CI are shown of a meta-analysis in a random effects model and for HLA-DQ, DQA1, DQB1 sharing in a fixed effects model. For individual meta-analysis see supplementary data, figures 8-12.

miscarriage and secondary recurrent miscarriage, none of the studies reported separate data for DQA1 sharing and for HLA-DQ sharing only one study reported separate data,[78] therefore heterogeneity could not be explored by meta-regression.

Discussion

In this meta-analysis we investigated the association of HLA alleles with the prevalence of recurrent miscarriage in a pre-specified population. Although associations between specific HLA alleles and HLA sharing with recurrent miscarriage were found, no consistent conclusions can be drawn since observed ORs were relatively small and high risk of selection and information bias was present in selected studies.

In comparison to previous reviews and meta-analyses,[21-24] the current meta-analysis gives a complete overview of all possible associations between HLA alleles in the mother and HLA sharing between couples with the occurrence of recurrent miscarriage. To obtain a more homogenous group of women with unexplained recurrent miscarriage, only women with at least three previous miscarriages should be taken into account and women with possible explanatory factors should be excluded.[79] However, previous meta-analyses also included studies with women with ≥ 2 or more miscarriages in the case group and/or did not define whether these miscarriages were unexplained or not.[22-24] In a previous meta-analysis by Beydoun et al.[21] on HLA sharing and recurrent miscarriage it was indicated that difficulties arise when comparing studies with different definitions for the same

disease, with various control groups, and different techniques for HLA typing. Therefore, we corrected for these disturbing factors by applying strict inclusion criteria both for the case and the control group. Moreover, as current HLA typing performed by molecular methods is more reliable and sensitive compared to serological methods used in the past,[36,37] we only included studies which used molecular HLA typing for our analysis on the possible association between HLA alleles and the occurrence of recurrent miscarriage.

All studies included in the present meta-analysis were case-control studies with small sample sizes, which implies a high risk of selection and information bias (Supplementary Table III). Furthermore different methods were used to diagnose uterine anomalies and only a few studies screened for the presence of APS in a correct way.[49] None of the studies mentioned whether control subjects were screened for uterine anomalies, parental chromosomal abnormalities, and APS. Though this information was lacking, it is unlikely that these conditions have affected the outcome because of the low prevalence in general population.

By including only data from published papers, there is a risk of publication bias. Although funnel plots showed no publication bias in this review, only 3 out of the 34 meta-analyses were performed with more than eight studies and funnel plots could be generated.

Although meta-regression could not explain the observed heterogeneity, not always sufficient data was available to perform meta-regression and therefore definite conclusions cannot be drawn. Subgroup analysis for classifying antigens or alleles for sharing and for high or low resolution molecular typing could not be performed as too little data were available in selected studies.

Differences found in HLA specificity could be purely dependent on genetic differences between populations. However, population stratification in the meta-analysis was not possible since only few studies mentioned ethnicity.

Most studies focused on couple sharing and only few studies have investigated whether homozygous fetuses are preferentially miscarried. Still, HLA sharing between couples is the nearest approach to identify sharing of HLA antigens at the fetal-maternal interface since typing of miscarriage material is rather difficult due to logistical problems. In future research typing of living children from women with recurrent miscarriage could help unravel whether incompatible fetuses have a higher survival rate in women with recurrent miscarriage.

It would be clinically more relevant to study specific HLA-C,E,G alleles expressed on the trophoblast as these alleles are important for maternal immune recognition of the fetus.

However, most studies focused on the maternal alleles probably because it is difficult to collect and type trophoblast tissue.

The presence of fetal HLA-C2 in combination with maternal KIRAA is suggested to be associated with complicated pregnancies.[13] In our meta-analysis maternal HLA-C2 was not associated with recurrent miscarriage, but the frequency of the male HLA-C2 is also a determinative factor for the chance that a fetus will be HLA-C2 positive. Only one selected study investigated HLA-C2 frequencies in males and reported an increased frequency of C2 alleles in males of couples with recurrent miscarriage.[9]

In line with studies that found no differences in expression of HLA-G on the trophoblast[80-82] between women with recurrent miscarriage and control subjects, our meta-analysis revealed no association of specific HLA-G alleles with recurrent miscarriage. HLA-E*01:01 surface expression on transfected cells and peptide affinity of HLA*01:01 for a nonamer sequence with HLA-G is lower than for HLA-E*01:03.[83] This may explain the increased presence of the HLA- E*01:01 allele in women with recurrent miscarriage although the actual expression of HLA-E was not decreased in women with recurrent miscarriage.[80-82,84] Pooled analysis for HLA-G 14bp insertion allele showed no significant association with recurrent miscarriage in our meta-analysis. This is in line with the hypothesis that the expression of HLA-G depends on the combination of several polymorphisms.[85] Future research in recurrent miscarriage should focus on these haplotypes rather than only on 14-bp polymorphism.

HLA class II alleles are known to be associated with auto-immune disorders.[7] The occurrence of recurrent miscarriage is strongly associated with APS[2] and possible with RA.[34] Only one study showing an association between HLA-DRB1*04 and recurrent miscarriage, excluded cases with APS and other auto-immune disorders.[57] Therefore it is not clear whether the association found in our meta-analysis for specific HLA-DRB1 alleles and recurrent miscarriage can be explained by underlying auto-immune disorder(s) in women with recurrent miscarriage or by another mechanism.

The association between HLA-B sharing and recurrent miscarriage observed in this meta-analysis is in line with observations by Ober et al. who found that fetal loss rates were increased among couples matched for HLA-B.[86] However, classical HLA class I and II, with exception of HLA-C, are not expressed on trophoblast tissues and therefore unlikely to be directly involved in the key mechanism that leads to a detrimental maternal immune response to the fetus. The gene for HLA-B is closely linked with that coding for HLA-C, the products of which play an important role in the interaction between uterine NK cells and trophoblast HLA-C, which is relevant for a proper placentation.

Incompatibility of HLA-DR between couples could facilitate the occurrence of a normal pregnancy. The immunogenetic conditions for a successful pregnancy seem to have similarities with those associated with the beneficial effect of a pre-transplant blood transfusion in transplantation. One HLA-DR antigen has to be shared between blood transfusion donor and recipient in order to induce a beneficial (tolerating) effect on the course of a subsequent renal transplantation[87] while incompatibility for the other HLA-DR antigen enhances a stable, rejection-free, allograft function.[88] CD4+ regulatory T cells are suggested to play a pivotal role in this beneficial effect of blood transfusions on graft survival.[89] In accordance with this blood transfusion effect, the pregnant mother has to accept the semi-allogeneic fetus and CD4+ regulatory T cells are needed for the maintenance of early pregnancy.[90] Indeed in this meta-analysis HLA-DR couple sharing was associated with recurrent miscarriage, which is in line with the results of a previous meta-analysis.[21]

The questions remains whether HLA sharing and HLA specificity itself is related with recurrent miscarriage or whether these genes are linked with susceptibility genes that influence reproductive outcome. In an inbred population of European descent matching for the entire 16-locus haplotype was clearly associated with the occurrence of recurrent miscarriage.[86]

Although many studies were conducted on HLA associations and recurrent miscarriage, no consistent conclusions can be drawn. Although strict inclusion criteria were applied in this meta-analysis, a high risk of information and selection bias was still present in the selected studies. Future studies on the association between HLA alleles and maternal immune recognition of the fetus should use strict inclusion criteria, including ethnicity, and should focus on the combination of HLA alleles expressed on the trophoblast and the maternal HLA alleles.

Acknowledgements

The authors would like to thank J.W. Schoones for his help with the literature search.

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Chapter 3

Lower frequency of the HLA-G UTR-4 haplotype in women with unexplained recurrent miscarriage

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Journal of Reproductive Immunology 2018;126:46-52
Supplementary data available on https://doi.org/10.1016/j.jri.2018.02.002

Abstract

Problem

HLA-G expressed by trophoblasts at the fetal-maternal interface and its soluble form have immunomodulatory effects. HLA-G expression depends on the combination of DNA polymorphisms. We hypothesized that combinations of specific single nucleotide polymorphisms (SNPs) in the 3'untranslated region (3'UTR) of HLA-G play a role in unexplained recurrent miscarriage.

Method

In a case control design, 100 cases with at least three unexplained consecutive miscarriages prior to the 20th week of gestation were included. Cases were at time of the third miscarriage younger than 36 years, and they conceived all their pregnancies from the same partner. The control group included 89 women with an uneventful pregnancy. The association of HLA-G 3'UTR SNPs and specific HLA-G haplotype with recurrent miscarriage was studied with logistic regression. Odds ratios (OR) and 95% confidence intervals (95% CI) were reported.

Results

Individual SNPs were not significantly associated with recurrent miscarriage after correction for multiple comparisons. However, the presence of the UTR-4 haplotype, which included +3003C, was significantly lower in women with recurrent miscarriage (OR 0.4, 95% CI 0.2-0.8, p=0.015).

Conclusion

In conclusion, this is the first study to perform a comprehensive analysis of HLA-G SNPs and HLA-G haplotypes in a well-defined group of women with recurrent miscarriage and women with uneventful pregnancy. The UTR-4 haplotype was less frequently observed in women with recurrent miscarriage, suggesting an immunoregulatory role of this haplotype for continuation of the pregnancy without complications. Thus, association of HLA-G with recurrent miscarriage is not related to single polymorphisms in the 3'UTR, but is rather dependent on haplotypes.

Introduction

About 1% of all couples trying to conceive, are confronted with recurrent miscarriage, which is defined as three or more consecutive pregnancies prior to the 20th week of gestation.[1] Possible etiologic factors include uterine anomalies, endocrine disorders, maternal inherited and acquired thrombophilia, and parental chromosomal abnormalities.[2,3] However, in only 25-50% of the couples an underlying cause for recurrent miscarriage can actually be identified.[4,5]

Since the fetus is a semi-allograft, which normally escapes maternal immune rejection, it has been suggested for many years that unexplained recurrent miscarriage is associated with specific maternal human leukocyte antigens (HLA) alleles or with the degree of HLA mismatching between mother and child. Since the role of classical HLA alleles remains controversial,[6] more attention has been drawn to non-classical HLA antigens expressed on the trophoblast, which interact directly with maternal immune cells.

The non-classical class I molecule HLA-G is characterized by its restricted expression on human extravillous trophoblast cells and by limited polymorphism of the HLA-G DNA sequence.[7] The HLA-G 3' untranslated region (3'UTR) in exon 8 consists of eight single nucleotide polymorphisms (SNPs), which together generate eight distinct haplotypes.[8,9] Insertion of 14 bp at the 3'UTR may affect HLA-G mRNA stability,[10] which is associated with lower levels or even absence of soluble HLA-G (sHLA-G) in plasma.[11-13] sHLA-G is highly present in the maternal circulation during pregnancy.[14] sHLA-G possess immunosuppressive functions,[15] which seem to be concentration-dependent.[16] Low levels of sHLA-G have been associated with spontaneous miscarriage,[17] recurrent miscarriage,[18] and miscarriage in IVF pregnancies.[19] Although the level of sHLA-G likely results from the HLA-G genotype, meta-analyses on the association of the 14-bp insertion with unexplained recurrent miscarriage have led to inconsistent results.[6,20,21] Most likely 14-bp insertion is in linkage disequilibrium with other sequence variations that influence the level of soluble isoforms. This is in line with the hypothesis that HLA-G expression is determined by the combination of multiple SNPs.[8]

For this reason we studied the HLA-G 3' UTR in exon 8, thereby including the combination of SNPs to compose UTR haplotypes, in women with recurrent miscarriage and in women with uneventful pregnancy.

Material and methods

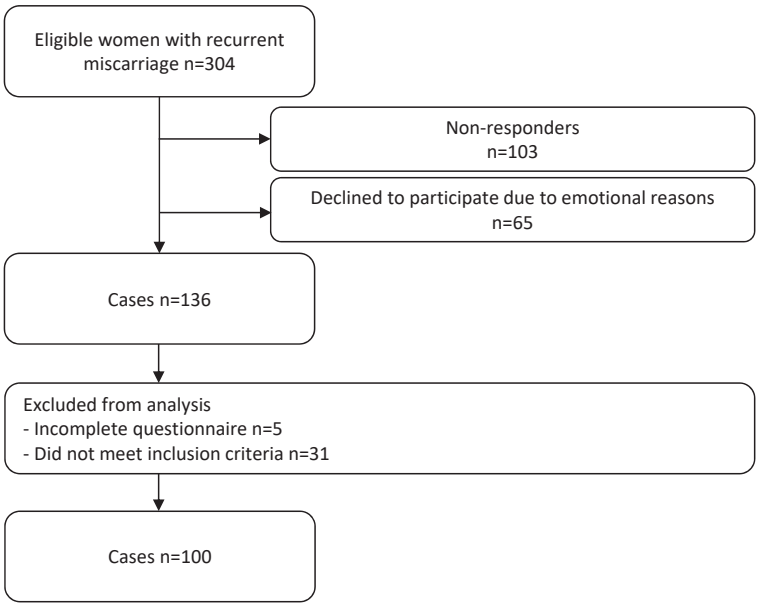
Case group

In this case control study, eligible cases were couples with recurrent miscarriage, who visited the recurrent miscarriage clinic of the department of Obstetrics and Reproductive Medicine at the Leiden University Medical Centre (LUMC), a tertiary referral centre in the Netherlands, from 2000 onwards. The clinical work-up includes documentation of a standardised history from the couple, karyotyping, an ultrasound or hysteroscopy, and thrombophilia screening. Hereditary thrombophilia was defined by the presence of a factor V Leiden mutation, factor II mutation (prothrombin gene mutation), protein C or S deficiency, high factor VIII, or antithrombin deficiency. Acquired thrombophilia (anti-phospholipid syndrome) was defined by the presence of IgG or IgM anticardiolipin antibodies or lupus anticoagulant in repeated samples taken 3 months apart and at least 6 weeks after pregnancy.[22] After revision of the classification criteria the presence of IgG or IgM β2-glycoprotein I antibodies was added to the work-up.[23] Most of the women who visited the clinic were counselled to participate in randomized controlled trial such as the Habenox (NCT0095962)[22] and Promise (ISRCTN92644181)[24]. In the Habenox trial, women were randomly allocated to one of three intervention groups (enoxaparin 40 mg, or enoxaparin 40 mg and aspirin 100 mg, or aspirin 100 mg). In the Promise trial, women were allocated to a group receiving vaginal progesterone or a placebo.

To obtain a homogenous case group, we only included women who had had three or more consecutive miscarriages prior to the 20th week of gestation with the same partner, and who were younger than 36 years at the time of their third consecutive miscarriage. Cases with known causes for miscarriage such as uterine anomalies, parental chromosomal abnormalities, and anti-phospholipid syndrome were not eligible. Women with hereditary thrombophilia were not excluded, because the evidence that hereditary thrombophilia is associated with recurrent miscarriage is less clear.[3,25] Both women with primary recurrent miscarriage (no history of live birth) and secondary recurrent miscarriage (history of live birth) were eligible.

From the 433 couples who visited the clinic, 304 women met the inclusion criteria and were asked to participate by filling in one digital or paper questionnaire. The questionnaire was made using ProMISe, an internet based application for the design, maintenance, and use of data management projects. Data were entered and stored in a clinical database (ProMISe Database, https://www.msbi.nl/promise/). From the 304 women, a total of 100 women were eligible, and blood samples were taken after inclusion (Figure 3.1).

Figure 3.1 Flowchart of cases



Control group

Control subjects were women with an uneventful pregnancy, who gave birth at the department of Obstetrics at the LUMC between 2004 and 2007. Women were included when they had one or more uneventful pregnancies, i.e., not suffering from pregnancy complications such as recurrent miscarriage, pregnancy induced hypertension, preeclampsia, Hemolysis Elevated Liver enzymes and Low Platelets (HELLP) syndrome, preterm birth, fetal growth restriction, and perinatal death in their history. Pregnancy induced hypertension was defined as systolic blood pressure above 140 mmHg and/or diastolic pressure above 90 mmHg combined with proteinuria (≥ 300mg/day or a spot urine protein/creatinine ratio ≥ 30 mg protein/mmol) as preeclampsia.[26] Preterm birth was defined as a delivery between 24 and 37 weeks gestation, fetal growth restriction as birth weight below the 2.3th percentile for gestational age and sex,[27] and perinatal death as fetal loss after 22 weeks of gestation till 7 days after birth. A total of 89 control subjects donated blood for genotyping.

Variables and definitions

Data were collected from the obstetrical records and ProMISe Database (questionnaires). Information about medical history, use of medication, intoxications, and pregnancy outcome was cross-checked in obstetrical records to overcome recall bias. The data of

the obstetrical records were used in case of discrepancies between the ProMISe Database and obstetrical records. The following data were collected: personal characteristics, intoxications (smoking, alcohol, drugs), use of medication, general disease history, outcome and complications of all pregnancies, and neonatal characteristics. Maternal age in the case group was defined as the age at time of the third consecutive miscarriage, and in the control group as the age at time of the delivery of the first uncomplicated pregnancy.

Ethnicity was divided into four groups according to the definitions of the Central Bureau of Statistics of the Netherlands (CBS). All persons of whom the mother was born in Europe (excluding Turkey), Indonesia, Japan, North-America, and Oceania were defined as native or Caucasian ethnic origin. Persons of whom the mother was born in Morocco or Turkey were from Moroccan or Turkish ethnic origin, and for Surinamese and Antillean it was Surinamese or Antillean ethnic origin. All persons of whom the mother was born in Africa, Asia (exclusive Indonesia and Japan) and South-America were defined as other non-Caucasian ethnic origin.

Detection of HLA-G 3'UTR polymorphisms and composition of haplotypes

DNA was isolated from EDTA blood to sequence a 590-bp fragment covering the 3'UTR of exon 8, starting at the 14-bp insertion/deletion and ending 585 bp further downstream. To sequence and determine the haplotype on each of the two alleles, amplification reactions were performed in two steps. First, the 14-bp insertion/deletion was determined in a SybrGreen-based qPCR reaction using a specific 5'-primer that detected either the insertion or deletion, in combination with a generic 3'-primer. Second, in case of Ins-Del heterozygosity (47.6%), two sequencing reactions were performed using the 5'-primers in combination with the generic 3'-primer that was tailed with a M13 sequence to cover the 3'UTR region of HLA-G, as described previously.[28] In case of homozygosity (52.4%), one sequencing reaction was performed using either the insertion- or deletion-specific 5'-primer together with the M13-tailed 3'-primer.

The following SNPs were identified: the 14-bp insertion/deletion (rs371194629), +3003C/T (rs1707), +3010C/G (rs1710), +3027A/C (rs17179101), +3035C/T (rs17179108), +3142C/G (rs1063320), +3187A/G (rs9380142), and +3196C/G (rs1610696. Eight distinct UTR haplotypes were defined as described previously.[8] Interpretation of the sequencing data, alignment, and conversion to UTR-haplotypes was carried out using a specialized software tool (SBT Engine, GenDX, Utrecht, The Netherlands).

As an additional Dutch reference group, the genotype frequencies of rs371194629 and rs1707 and the haplotype frequencies of the HLA-G 3' UTR were obtained from the

Genome of the Netherlands (GoNL) reference panel (http://www.nlgenome.nl/). In total, 499 persons of Dutch ancestry have been whole-genome sequenced for this reference panel.[29] The samples were collected without phenotypic ascertainment, but due to the trio-based design of the study, all women in the GoNL had at least one successful live birth.

Statistical analyses

For comparison of baseline characteristics between cases and control subjects the Mann Whitney U test was performed. For categorical variables the Chi-squared test was used, and if expected counts were less than five the Fisher's exact test was used.

To exclude the possibility of a selection of a certain genotype within our groups, the Hardy-Weinberg equilibrium[30,31] was assessed for genotypes and haplotypes of the HLA-G 3'UTR in cases and control subjects using Pypop Software 0.7.0.

The association between the presence of different HLA-G genotypes and haplotypes and recurrent miscarriage was studied with univariate logistic regression. Per HLA-G genotype the highest prevalence was defined as the reference group. If percentages in a group were below 5%, no calculations were performed. For the calculations on the HLA-G genotypes Bonferroni adjustment was used to correct for multiple comparisons.

For the HLA-G haplotypes two specific calculations were performed, the presence of the haplotype in percentage was calculated and the frequency per haplotype was calculated in the case and control group. Furthermore, subgroup analyses were performed to evaluate the presence of different HLA-G genotypes and haplotypes in Caucasian women in the case group compared to control subjects. As it is still postulated, that primary recurrent miscarriage and secondary recurrent miscarriage could be two distinct entities with different underlying pathology,[32] subgroup analysis of primary recurrent miscarriage and secondary recurrent miscarriage was performed.

Statistical analysis was performed using SPSS Statistics (version 24.0, IL, USA). Descriptive statistical analysis was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, CA, USA, www.graphpad.com).

Ethical approval

The protocol was approved by the Ethics committee of the LUMC (P12.099) and all participants gave informed consent before inclusion in this study. The study was registered with the Dutch trial registry NTR 3402.

Results

Baseline characteristics of subjects

Baseline characteristics of women with recurrent miscarriage and women with an uneventful pregnancy are depicted in Table 3.1.

Table 3.1 Baseline characteristics of subjects

	Recurrent miscarriage (N=100)	Uneventful pregnancy (N=89)	*P*-value
Maternal age at time of 3rd miscarriage or 1st uneventful delivery (years;median[IQR])¬	31.0 (28.0-33.0)	30.0 (26.7-33.0)	0.368
BMI (Kg/m^2;median[IQR]) ±	23.3 (21.0-27.1)	24.1 (21.3-28.2)	0.415
Ethnic origin (n(%))§			0.916
Native/Caucasian	89 (89.0)	79 (90.8)	
Turkish/Moroccan	2 (2.0)	2 (2.3)	
Antillean/Surinamese	2 (2.0)	2 (2.3)	
Other non-Caucasian	7 (7.0)	4 (4.6)	
Gravidity (median[IQR])	6 (5-8)	3 (2-4)	<0.001
Gravidity at time of inclusion	5 (4-6.7)	3 (2-3)	<0.001
Parity (median[IQR])	1.5 (1-2)	2 (2-3)	<0.001
Parity at time of inclusion	0 (0-0)	1 (0-2)	<0.001

All χ^2 tests or Mann-Whitney U-test. IQR; interquartile range, BMI; Body mass index. ¬2.1% missing values (1 of 100 cases and 3 of 89 control subjects), ±4.7% missing values (2 of 100 cases and 7 of 89 control subjects), §1% missing values (0 of 100 cases and 2 of 89 control subjects).

In the case group, 69 women (69.0%) had primary recurrent miscarriage and 31 (31.0%) women had secondary recurrent miscarriage. Most women had 4 or more consecutive miscarriages (71.0%), and 41 (41.0%) had 5 or more miscarriages. A total of 11 (11.0%) cases had thrombophilia, i.e., factor V Leiden (n=4), prothrombin gene mutation (n=4) or high factor VIII (n=5). None had protein C or S deficiency. Out of 100 cases, 75 had at least one live birth after the consecutive miscarriages.

In the case and control subjects all genotypes and haplotypes for HLA-G 3'UTR were in Hardy-Weinberg equilibrium (Supplementary data, Table I).

HLA-G polymorphisms

As far as individual SNPs in the HLA-G 3'UTR exon 8 region are concerned no significant differences were seen after correction for multiple comparisons between women with recurrent miscarriage and women with uneventful pregnancy (Table 3.2). In the subgroup analyses similar results were seen (supplementary data, Table II, III, and IV).

Table 3.2 HLA-G 3'UTR genotypic polymorphisms in women with recurrent miscarriage and uneventful pregnancy

SNP		Recurrent miscarriage (N=100)	Uneventful pregnancy (N=89)	OR	95% CI	*P*	*Pc*
14-bp	InsIns	21 (21%)	12 (13.5%)	1.2	0.5-2.9	0.558	1.000
	InsDel	52 (52%)	38 (42.7%)	ref			
	DelDel	27 (27%)	39 (43.8%)	0.5	0.2-0.9	0.038	0.304
+3003	CC	3 (3%)	3 (3.4%)	nc			
	CT	17 (17%)	29 (32.6%)	0.4	0.2-0.8	0.013	0.104
	TT	80 (80%)	57 (64%)	ref			
+3010	CC	30 (30%)	20 (22.5%)	1.1	0.5-2.3	0.662	1.000
	CG	50 (50%)	39 (43.8%)	ref			
	GG	20 (20%)	30 (33.7%)	0.5	0.2-1.0	0.069	0.552
+3027	AA	0 (0%)	0 (0%)	nc			
	AC	16 (16%)	13 (14.6%)	1.1	0.5-2.4	0.791	1.000
	CC	84 (84%)	76 (85.4%)	ref			
+3035	CC	76 (76%)	73 (82%)	ref			
	CT	24 (24%)	15 (16.9%)	1.5	0.7-3.1	0.243	1.000
	TT	0 (0%)	1 (1.1%)	nc			
+3142	CC	20 (20%)	30 (33.7%)	0.5	0.2-1.0	0.069	0.552
	CG	50 (50%)	39 (43.8%)	ref			
	GG	30 (30%)	20 (22.5%)	1.1	0.5-2.3	0.662	1.000
+3187	AA	49 (49%)	43 (48.3%)	ref			
	AG	41 (41%)	34 (38.2%)	1.0	0.5-1.9	0.856	1.000
	GG	10 (10%)	12 (13.5%)	0.7	0.2-1.8	0.511	1.000
+3196	CC	43 (43%)	50 (56.2%)	ref			
	CG	44 (44%)	31 (34.8%)	1.6	0.8-3.0	0.110	0.880
	GG	13 (13%)	8 (9.0%)	1.8	0.7-4.9	0.199	1.000

Data are all n (%). All univariate logistic regression analysis. Per HLA-G genotype the highest prevalence was defined as the reference group. If percentages in a group were below 5%, no calculations were performed. P; *p*-value, Pc; *p*-value corrected for multiple comparisons, OR; odds ratio, 95% CI; 95% confidence interval, nc; not calculated, ref; reference group.

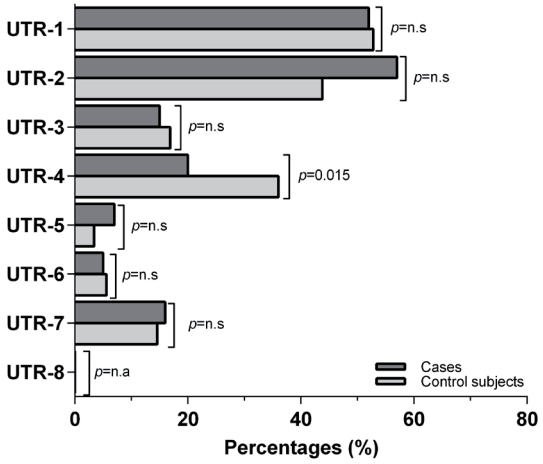
The presence of the UTR-4 haplotype was significantly lower in women with recurrent miscarriage compared to women with an uneventful pregnancy (20% vs. 36%, OR 0.4, 95% CI 0.2-0.8, *p*=0.015) (Figure 3.2).

These differences were also observed in haplotype frequencies (0.116 vs. 0.198, OR 0.5, 95% CI 0.3-0.9, *p*=0.036) (Table 3.3). The UTR-4 haplotype frequency in the Dutch genomic reference population is 0.157.

Differences in the UTR-4 haplotype frequency between cases and control subjects were similarly observed in the subgroup of Caucasian women (supplementary data, Table V). Data of haplotypes in women with primary recurrent miscarriage compared to control subjects are depicted in supplementary data, Table VI and VII.

Besides the observation of a significantly lower haplotype frequency of UTR-4 haplotype in women with secondary recurrent miscarriage compared to control subjects, the frequency of UTR-2 haplotype was significantly higher (0.403 vs. 0.280, OR 1.9, 95% CI 1.0-3.6, *p*=0.037) (supplementary data, Table VIII). Similar results were observed in the presence of the UTR-2 haplotype in women with secondary recurrent miscarriage compared to women with an uneventful pregnancy (supplementary data, Table VIIII).

Figure 3.2 The presence of HLA-G 3'UTR haplotypes in women with recurrent miscarriage compared to women with uneventful pregnancy



For cases and control subjects UTR-1 51% vs. 52.8% (OR 0.9, 95% CL 0.5-1.6, *p*=0.804), for UTR-2 57% vs. 43.8% (OR 1.6, 95% CL 0.9-3.0, *p*=0.071), for UTR-3 15% vs. 16.9% (OR 0.8, 95% CL 0.3-1.9, *p*=0.728), for UTR-4 20% vs. 36% (OR 0.4, 95% CL 0.2-0.8, *p*=0.015), for UTR-5 7% vs. 3.4% (OR 2.1, 95% CL 0.5-8.6, *p*=0.276), for UTR-6 5% vs. 5.6% (OR 0.8, 95% CL 0.2-3.1, *p*=0.850), for UTR-7 16% vs. 14.6% (OR 1.1, 95% CL 0.5-2.4, *p*=0.791), for UTR-8 not applicable. All univariate logistic regression analysis. n.s; not significant, n.a; not applicable. In the sensitivity analysis for ethnicity similar results were seen for the presence of HLA-G 3'UTR haplotypes as showed in Figure 3.1 between Caucasian cases and control subjects (data not shown).

Table 3.3 3'UTR haplotype frequencies of the HLA-G locus in women with recurrent miscarriage compared to women with uneventful pregnancy

HLA-G 3'UTR haplotypes									Haplotype frequencies				
	14-bp	+3003	+3010	+3027	+3035	+3142	+3187	+3196	Recurrent miscarriage 2n=198*	Uneventful pregnancy 2n=177*	OR	95% CI	*P*
UTR-1	Del	T	G	C	C	C	G	C	0.308	0.333	0.8	0.5-1.3	0.594
UTR-2	Ins	T	C	C	C	G	A	G	0.354	0.260	1.5	0.9-2.3	0.063
UTR-3	Del	T	C	C	C	G	A	C	0.081	0.085	0.9	0.4-1.9	0.879
UTR-4	Del	C	G	C	C	C	A	C	0.116	0.198	0.5	0.3-0.9	0.036
UTR-5	Ins	T	C	C	T	G	A	C	0.035	0.023	1.4	0.4-4.7	0.506
UTR-6	Del	T	G	C	C	C	A	C	0.025	0.028	0.8	0.2-3.1	0.850
UTR-7	Ins	T	C	A	T	G	A	C	0.081	0.073	1.1	0.5-2.4	0.791
UTR-8	Ins	T	G	C	C	G	A	C	0.000	0.000	na	na	na

All univariate logistic regression analysis. *P*; *p*-value, OR; odds ratio, 95% C; 95% confidence interval, na; not applicable. *In 2 cases and 1 control subject one of the UTR haplotype could not be defined (1.5%). The 3'UTR haplotype nomenclature is consistent with publication by Castelli et al.

Discussion

The HLA-G UTR-4 haplotype was less frequently observed in women with recurrent miscarriage, whereas single polymorphisms in the 3'UTR region of HLA-G were not significantly associated with outcome.

The strength of the study is that a large homogenous well-defined case group of women with at least three consecutive unexplained recurrent miscarriages within 20 weeks of gestation with the same partner was included. Such a clear definition was not used in previous studies.[6,33]

Furthermore, in contrast to many other studies in women with recurrent miscarriage investigating exclusively the 14-bp insertion polymorphism,[6] we analyzed all individual SNPs to distinguish eight different haplotypes of the 3'UTR in exon 8. This is crucial, since the expression of soluble and membrane-bound forms of HLA-G most likely depends on the combination of several polymorphisms at the 3'UTR.[8]

A limitation of the study may be that the link between genetic polymorphisms and levels of soluble or membrane-bound forms of HLA-G in the case and control group could not be studied. Peripheral blood levels of sHLA-G vary substantially between non-pregnant and pregnant individuals, and they decline in pregnant individuals during the third trimester.[34] As blood sampling by most control subjects was performed directly after delivery and most cases were not pregnant in our study, it was not justified to assess sHLA-G levels and correlate these to the polymorphisms studied. Therefore, the exact mechanism, by which these polymorphisms may affect the occurrence of recurrent miscarriage remains to be established. Nevertheless, most polymorphisms in the 3'UTR determined in this study were previously found to be related to the extent of HLA-G expression.[10,35,36] Both HLA-G expressed at the fetal-maternal interface and sHLA-G possess immunosuppressive functions.[15,37] It is therefore not surprising that low levels of sHLA-G have been recently associated with recurrent miscarriage.[18] However, no differences in expression of HLA-G on the trophoblast were observed between women with recurrent miscarriage and control subjects.[38-40]

At the moment, the role of HLA-G in complicated pregnancy is not easily comprehensible, and it is not clear whether all variations in the HLA-G gene, which affect HLA-G expression, have been identified. For the HLA-G coding region a lower degree of polymorphisms has been observed than at the 3'UTR. Although some HLA-G alleles in the coding region, including HLA-G*01013, HLA-G*0105N, and HLA-G*01041, are associated with expression of soluble HLA-G,[41] these associations could also be explained by the fact that these HLA-G alleles are in linkage disequilibrium with 3'UTR haplotypes.[8] Recently

Di Cristofaro et al. showed that the HLA-G*0104 allele was in linkage disequilibrium with the presence of the UTR-3 haplotype.[42]

For the HLA-G 3'UTR, Castelli et al. characterized the variability and its haplotype structure in one of the most heterogeneous populations of the world.[8] In their population only less than 1% of haplotypes was undefined, which is in line with our data, and most likely not clinically relevant. The allelic and haplotype frequencies of our control group were similar to those described for European populations.[43]

Among the eight haplotypes, UTR-4 was less frequently seen in recurrent miscarriage, and more frequently in uneventful pregnancy, suggesting a protective effect of this haplotype in pregnancy. In contrast to pregnancy where a high HLA-G expression seems to be beneficial, HLA-G expression in tumour cells contributes to the escape of tumour cells from a tumour specific immune response.[44] In line with our data, in prostate cancer UTR-4 was associated with an increased risk in disease development and supports the idea that the presence of UTR-4 haplotype influences HLA-G expression.[45] Conversely, UTR-4 was associated with a lower risk of colorectal carcinoma, although after adjustment for possible confounders no significance was reached.[46]

The UTR-4 haplotype is the only haplotype bearing the +3003C allele, which was also increased in women with uneventful pregnancy. The +3003C allele is in linkage disequilibrium with the allele -725G at the promotor region.[43] Although this allele was previously associated with increased risk for miscarriage in couples,[47] it was also found to be related with a higher HLA-G expression, as compared to the -725C or T allele.[48] The affinity of microRNAs binding to this region is probably dependent on the type of nucleotide present at this +3003 position.[49] In our study, women with recurrent miscarriage had more often the genotypic variants +3010CC and +3003TT. The presence of +3003T, in combination with +3010C, shows low affinity for miR-513a compared to the +3003C/+3010G haplotype.[49] The combination of the polymorphisms +3010C and +3003T are part of the UTR-2, UTR-3, UTR-5, and UTR-7 haplotypes, of which only the UTR-3 haplotype contains the 14-bp insertion. The UTR-2, UTR-5 and UTR-7 were found to be associated with lower expression of sHLA-G.[13] Interestingly, in the subgroup analysis in the current study the frequency of UTR-2 was significantly higher in women with secondary recurrent miscarriage compared to control subjects. In addition, UTR-4 was only significantly associated with uneventful pregnancy compared to women with secondary recurrent miscarriage, and not with women with primary recurrent miscarriage. Although the size of this group was limited, this finding may support the idea that secondary recurrent miscarriage has different underlying pathology than primary recurrent miscarriage.[32] It could be that in women with secondary recurrent miscarriage a previous

live birth increases immunity to fetal antigens in the next pregnancy by for example the presence of HLA antibodies or H-Y antibodies.[50,51] Hypothetically, women with secondary recurrent miscarriage and an UTR-2 haplotype could have less immunomodulatory capacity during implantation and therefore possibly an increased risk of miscarriage due to increased immunity by the presence of antibodies from a previous live birth. Although the presence of HLA antibodies increases after 28 weeks of pregnancy and antibodies can still be present at time of a new conception,[52,53] no significant differences were observed in paternal-specific HLA antibodies between women with primary recurrent miscarriage and women with secondary recurrent miscarriage in a recent study.[54] These differences between primary recurrent miscarriage and secondary miscarriage should be subject for further studies.

Furthermore, the UTR-2, UTR-5, and UTR-7 haplotypes share three genotypic variants which have previously been associated with decreased HLA-G levels: 1.) the 14-bp insertion influencing mRNA stability,[10] resulting in lower levels or even absence of sHLA-G in plasma[11-13]; 2.) the +3142G allele, which increases the affinity of several microRNAs, including miR-148a, miR148b, and miR152, leading to downregulation of HLA-G expression[36]; and 3.) the +3187A allele, which is related to decreased HLA-G expression due to increased number of adenines in AU-rich motif mediating mRNA degradation.[35] In line with these data we observed a decreased frequency of the 14-bp deletion/deletion in women with recurrent miscarriage (27%) compared to the control group (43.8%), the latter being comparable to the Dutch genome reference group (37.1%) (data not shown).

The current study shows that the 3'UTR HLA-G haplotype, rather than single polymorphisms in the 3'UTR region of HLA-G, is most significantly associated with recurrent miscarriage. On the basis of these data we hypothesize that a combination of polymorphisms in the 3'UTR region of the HLA-G gene affects the degree of microRNA binding to these regions, which influences HLA-G expression. If this is the case, microRNAs may be used in the future as biomarkers and possible targets for therapeutic strategies, aiming at the induction of a higher HLA-G expression and improved pregnancy outcome.

Acknowledgements

The authors would like to thank Clara Kolster, Marjolein Bourgonje-Verhart, and Marise Wagner for their help obtaining blood samples from all the cases. The authors would like to thank Geert Haasnoot for his help to assess the Hardy-Weinberg equilibrium for genotypes and haplotypes of the HLA-G studied.

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Chapter 4

Paternal HLA-C is a risk factor in unexplained recurrent miscarriage

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American Journal of Reproductive Immunology 2017;79(2):e12797
Supplementary data available at the end of the chapter

Abstract

Problem

HLA-C is the only classical HLA I antigen expressed on trophoblast. We hypothesized that the alloimmune response to paternal HLA-C plays a role in unexplained recurrent miscarriage.

Method

In a case control design, we included 100 women with at least three unexplained consecutive miscarriages along with their partners and children. For the first control group we included 90 women with an uneventful singleton pregnancy without pregnancy complications in their history along with their children. The second control group consisted of 425 families. HLA-C*07 and HLA-C*17 frequencies, which are the most immunogenic HLA-C antigens, along with HLA-C mismatches, and the presence of specific HLA antibodies in the mother were determined.

Results

HLA-C and HLA-C*07 mismatches were significantly increased in couples with recurrent miscarriage compared to control subjects (p=0.016, p=0.008, respectively). The incidence of child-specific HLA-C*07/HLA-C*17 antibodies was increased in women with recurrent miscarriage (p=0.007).

Conclusion

The results show that HLA-C incompatibility between couples is significantly associated with unexplained recurrent miscarriage.

Introduction

About 1% of all couples are confronted with recurrent miscarriage, which is defined as three or more consecutive pregnancies prior to the 20th week of gestation.[1] Possible etiologic factors include uterine anomalies, endocrine disorders, maternal inherited and acquired thrombophilia, and parental chromosomal abnormalities.[2] In only 50% of the couples an underlying cause for recurrent miscarriage can be identified.[3]

As the fetus is a semi-allograft, which escapes maternal immune rejection in normal pregnancy, it has been proposed that unexplained recurrent miscarriage is associated with the presence of specific maternal HLA alleles and with the degree of HLA mismatching between mother and child. As the role of classical HLA alleles remains controversial,[4] more attention has been drawn to antigens expressed on the trophoblast, which can interact directly with maternal immune cells. The only classical HLA antigen expressed on the trophoblast is HLA-C, and both the maternal and paternal HLA-C allele are expressed by the trophoblast.[5]

HLA-C*07 and C*17 are the most immunogenic antigens of the HLA-C alleles as they contain the highest number of epitope mismatches compared to other HLA-C alleles.[6] Women with recurrent miscarriage develop significantly more often HLA-C antibodies in the first trimester,[6] suggestive for a higher incidence of paternal HLA-C mismatches in couples with recurrent miscarriage.

Here, we investigated the role of paternal HLA-C in couples with unexplained recurrent miscarriage and with uneventful pregnancy. Thereby, we determined frequencies of HLA-C*07 and C*17 alleles, paternal HLA-C mismatches, and the presence of specific maternal HLA-C antibodies.

Material and methods

Case group

Cases were couples with recurrent miscarriage, who visited the recurrent miscarriage clinic of the department of Obstetrics and Reproductive Medicine at the Leiden University Medical Center (LUMC) in the Netherlands from 2000 onwards. The clinical work-up includes documentation of a standardised history from the couple, karyotyping, an ultrasound or hysteroscopy, and thrombophilia screening. Hereditary thrombophilia was defined by the presence of a factor V Leiden mutation, factor II mutation (prothrombin gene mutation), protein C or S deficiency, high factor VIII, or antithrombin deficiency. Acquired thrombophilia (anti-phospholipid syndrome) was defined by the presence of

IgG or IgM anticardiolipin antibodies or lupus anticoagulant in repeated samples taken 3 months apart and at least 6 weeks after pregnancy.[7] After revision of the classification criteria the presence of IgG or IgM β2-glycoprotein I antibodies was added to the work-up.[8]

Most of the women who visited the clinic were counselled to participate in randomized controlled trials such as the Habenox (NCT0095962)[7] and Promise (ISRCTN92644181).[9] In the Habenox trial, women were randomly allocated to one of three intervention groups (enoxaparin 40 mg, or enoxaparin 40 mg and aspirin 100 mg, or aspirin 100 mg). In the Promise trial, women were allocated to a group receiving vaginal progesterone or a placebo.

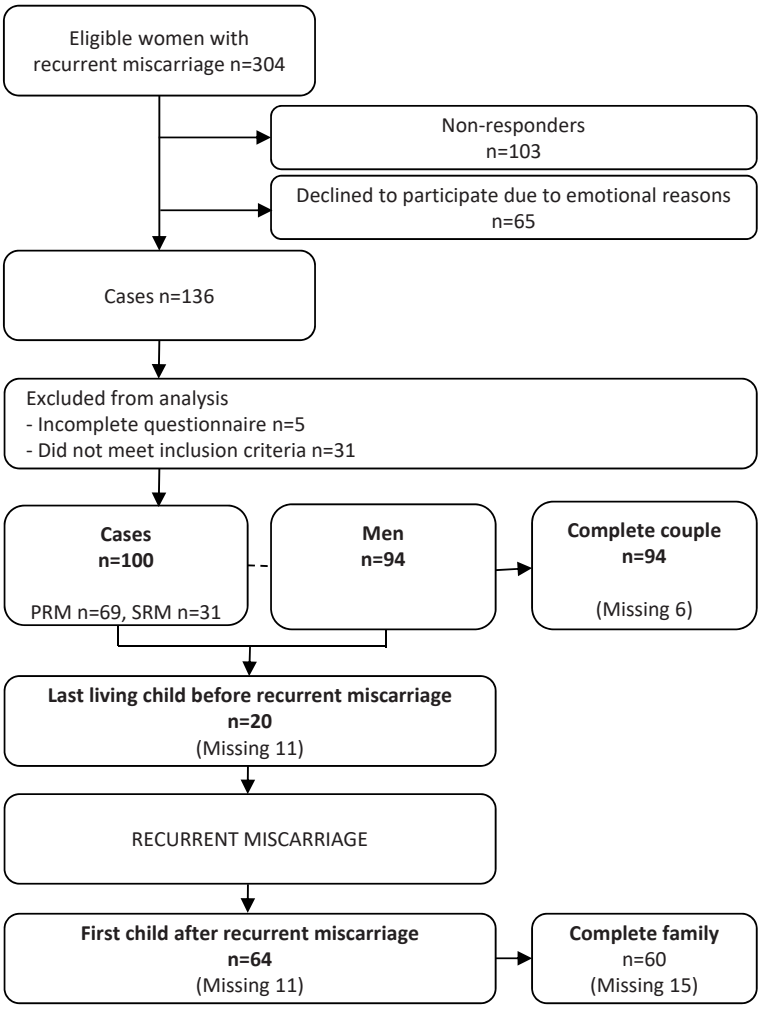
To obtain a homogenous study group, we only included women with three or more consecutive miscarriages prior to the 20th week of gestation with the same partner. The women had to be younger than 36 years of age at the time of their third consecutive miscarriage. Cases with known causes for miscarriage such as uterine anomalies, parental chromosomal abnormalities, and anti-phospholipid syndrome were not eligible. Women with hereditary thrombophilia were not excluded because the evidence that hereditary thrombophilia is associated with recurrent miscarriage is less clear.[10,11] Both women with primary recurrent miscarriage (no history of live birth) and secondary recurrent miscarriage (history of live birth) were eligible.

From the 433 couples who visited the clinic, 304 couples met the inclusion criteria and they were asked to participate by filling in one digital or paper questionnaire. The questionnaire was made using ProMISe, an Internet-based application for the design, maintenance, and use of data management projects. Data of the women were stored in a clinical database (ProMISe Database, https://www.msbi.nl/promise/).

From the 304 couples, 31 couples did not meet the inclusion criteria. Finally, 100 eligible women were included (Figure 4.1). Analysis with regard to baseline characteristics between the 100 included women and 173 eligible women, who did not participate, is depicted in Supplementary data, Table I.

Of the 100 women included, 69 had primary recurrent miscarriage and 31 women had secondary recurrent miscarriage. From the 31 women with secondary recurrent miscarriage, only the last living child before the consecutive miscarriages was asked to participate, in total 20 children could be included. From the 100 women, 94 partners could be included. Out of 100 cases, 75 had at least one live birth after the consecutive miscarriages. Only the living child directly born after the consecutive miscarriages was included (n=64, 11 children were missing) (Figure 4.1).

Figure 4.1 Flowchart of cases



PRM; primary recurrent miscarriage, SRM; secondary recurrent miscarriage.

After inclusion, blood samples from women and their partner were obtained and the children were asked to donate buccal mucous membrane for HLA genotyping (Oragene Discover, DNA Genotek, Ontario, Canada).

Control groups

Two control groups were selected. The first control group consisted of 90 nulliparous and multiparous women with an uneventful pregnancy, who gave birth at the department of

Obstetrics at the LUMC between 2004 and 2007. Women were included in case they had one or more uneventful pregnancies, that is, not suffering from pregnancy complications such as recurrent miscarriage, pregnancy-induced hypertension, preeclampsia, hemolysis elevated liver enzymes and low platelets (HELLP) syndrome, preterm birth, fetal growth restriction, and perinatal death in their history. Pregnancy-induced hypertension was defined as systolic blood pressure above 140 mmHg and/or diastolic pressure above 90 mmHg combined with proteinuria (≥300 mg/day or a spot urine protein/creatinine ratio ≥30 mg protein/mmol) as preeclampsia.[12] Preterm birth was defined as a delivery between 24 and 37 weeks gestation, fetal growth restriction as birth weight below the 2.3th percentile for gestational age and sex,[13] and perinatal death as fetal loss after 22 weeks of gestation till 7 days after birth. All control subjects donated blood within 48 hours after labor.

The second control group consisted of 425 families who had been typed for HLA-A, HLA-B, HLA-C, HLA-DQB1, and HLA-DRB1, and who had a screening and work-up for stem cell transplantation for a child with leukemia. We selected these families as the occurrence of leukemia is not linked to specific HLA types. From all the living children who had been screened in these families, the child that was screened for stem cell transplantation was used in the analyses.

Variables and definitions

Data were collected from the obstetrical records and the ProMISe database (questionnaires). Information about medical history, use of medication, intoxications, and pregnancy outcome was cross-checked in obstetrical records to overcome recall bias. The data of the obstetrical records were used in case of discrepancies between the ProMISe database and obstetrical records. The following data were collected: personal characteristics, intoxications (smoking, alcohol, drugs), use of medication, general disease history, outcome and complications of all pregnancies, and neonatal characteristics. Maternal age in the cases was defined as the age at time of the third consecutive miscarriage, and in the first control group as the age at time of the delivery of the first uncomplicated pregnancy.

Ethnicity was divided in 4 groups according to the rules of the Central Bureau of Statistics of the Netherlands (CBS). All persons of whom the mother was born in Europe (excluding Turkey), Indonesia, Japan, North-America, and Oceania were defined as native or Caucasian ethnic origin. Persons of whom the mother was born in Morocco or Turkey were from Moroccan or Turkish ethnic origin, and for Surinamese and Antillean it was Surinamese or Antillean ethnic origin. All persons of whom the mother was born in Africa, Asia (exclusive Indonesia and Japan), and South-America were defined as other non-Caucasian ethnic origin.

HLA typing

The samples from the case group, the first control group, and the second control group (only the maternal and paternal samples from this last group) were typed at intermediate resolution level for the loci HLA-A, HLA-B, and HLA-C with a PCR-based reversed sequence specific bead hybridization assay (Lifecodes HLA-SSO Typing Immucor Norcross, Georgia), which involves PCR amplification of targeted regions within the MHC class I regions with group specific primers, followed by a process of probing the amplicon with Luminex beads, each coated with sequence-specific oligonucleotide probes to identify the presence or absence of specific alleles. The assignment of HLA type is then based on the reaction pattern observed, compared to patterns associated with published sequences (Lifecodes HLA-SSO Typing Immucor Norcross, Georgia USA).

The samples from the children from the second control group taken before 2004 were typed at high resolution level for HLA-A, HLA-B, and HLA-C loci using the PCR-SSP technique[14] with commercially available HLA-primer sets (Dynal, Oslo, Norway). Those taken after 2004 were typed by the sequence-based Typing (PCR-SBT) technique.

HLA antibody screening

HLA antibody screening was performed with maternal sera obtained from the case group and the first control group. The detection of HLA class I antibodies in maternal sera was performed by an enzyme-linked immunosorbent assay (ELISA) (LAT TM, One Lambda, CA, USA) with readouts at 630 nm, detecting both complement fixing and non-complement fixing antibodies, or by Luminex-based method (Luminex Corporation, Texas, USA). Positive sera (ratio patient/control >2.0) by ELISA or Luminex were further tested for HLA antibody class I specificity with single-antigen beads (SAB) by the Luminex method (Gen Probe, Stamford, CT, USA), following the manufacturer's instructions. Purified anti-human C1q was used in the IgG SAB assay to detect complement fixing antibodies. An MFI (median fluorescence intensity) >1000 was considered positive, as reported elsewhere.[15,16]

Statistical analyses

For descriptive analysis of baseline characteristics between cases and control subjects the Mann-Whitney U test was performed. For categorical variables, the chi-squared test was used, and if expected counts were less than five, the Fisher's exact test was used.

To exclude the possibility of a selection of a certain genotype within our groups, the Hardy-Weinberg equilibrium[17,18] was assessed for all genotypes of the HLA-C allele using Pypop Software 0.7.0. Statistical analysis was performed using SPSS Statistics (version 24.0, IL,

USA). Descriptive statistical analysis was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, CA, USA, www.graphpad.com).

The following comparisons between cases and control subjects were performed: frequencies of HLA-C*07 and HLA-C*17 alleles in mother, father, and children, the incidence of paternal HLA-C mismatches, and the incidence of (child-specific or paternal-specific) HLA class I, HLA-C, and HLA-C*07/17 antibodies

As previously described using the HLAMatchmaker program developed by Duquesnoy,[19] HLA-C*07 and C*17 showed the highest mean of epitope mismatches with other alleles within the HLA-C locus.[6] Therefore frequencies of HLA-C*07 and HLA-C*17 in cases and control subjects were determined. Odds ratio (OR) and confidence interval (CI) were calculated with the Woolf-Haldane method,[20,21] *p*-values were calculated with the Fisher's exact test. In cases, expected number of HLA-C*07 and HLA-C*17 alleles of the child was calculated on basis of the actual data from mother and father. For example, in case of maternal C*07C*x and paternal C*xC*x typing, the chance of a C*07 child is 0.5. All these chances were added and divided by the number of children.

The number of paternal HLA-C mismatches between mother and father or child was determined on basis of two-digit HLA typing. For the analysis of HLA-C*07 or C*17 mismatches, only mothers were selected who were negative for HLA-C*07 or C*17.

Furthermore, child-specific or paternal-specific HLA class I antibodies, HLA-C antibodies, and HLA-C*07/17 antibodies were assigned by comparing the HLA antigen of the child or father with the specificity of the maternal HLA-C antibody and class I antibody.

Ethical approval

The protocol was approved by the Ethics committee of the LUMC (P12.099) and all participants gave informed consent before inclusion in this study. The study was registered with the Dutch trial registry NTR 3402. All participants included in the control groups gave informed consent that samples, which were obtained, could be used in future studies.

Results

Baseline characteristics of subjects

Baseline characteristics of women with recurrent miscarriage and women with an uneventful pregnancy are depicted in Table 4.1. Out of 100 cases, 75 had at least one live birth after the consecutive miscarriages (Figure 4.1). From the 75 children directly born

after recurrent miscarriage, 29 were boys (38.7%) and 46 were girls (61.3%). From the 31 children born before the consecutive miscarriages, 18 were boys (58.1%) and 13 were girls (41.9%).

Table 4.1 Baseline characteristics of subjects

	Recurrent miscarriage (N=100)	Uneventful pregnancy (Control group 1) (N=90)	*P*-value
Maternal age at time of 3rd miscarriage or 1st uneventful delivery (years;median[IQR])[a]	31 (28-33)	30 (26-33)	0.368
BMI (Kg/m^2;median[IQR])[b]	23.3 (21.0-27.1)	24.1 (21.2-28.2)	0.415
Ethnic origin (n(%))[c]			0.916
Caucasian	89 (89)	79 (89.8)	
Turkish/Moroccan	2 (2)	2 (2.3)	
Antillean/Surinamese	2 (2)	2 (2.3)	
Other non-Caucasian	7 (7)	5 (5.7)	
Gravidity (median[IQR])	6 (5-8)	3 (2-4)	<0.001
Parity (median[IQR])	1.5 (1-2)	2 (2-3)	<0.001
Miscarriages (median[IQR])	4 (3-6)	0 (0-1)	<0.001
Children for whom DNA was available (n(%))	64 (64)	89 (98.8)	
Gravidity (median[IQR])	5 (4-6.7)	3 (2-3.2)	<0.001
Parity (median[IQR])	0 (0-0)	1 (0-2)	<0.001
Nulliparous (n(%))	51 (79.7)	25 (27.8)	<0.001
Gestational age (days;median[IQR])	273 (266-280)	275 (270-283)	0.836
Birthweight (gram;median[IQR])	3495 (3039.5-3775.0)	3555 (3111.2-3870.0)	0.298
Gender (n(%))[d]			0.496
Boy	24 (37.5)	37 (42.5)	
Girl	40 (62.5)	49 (57.0)	
Complications (n(%))	11 (17.2)	0	na
Preterm birth	8 (8)		
Fetal growth restriction	5 (5)		
Preeclampsia	2 (2)		
Perinatal death	0		

All chi-squared tests or Mann-Whitney *U* test. IQR; interquartile range, na; not applicable. BMI; Body mass index.
[a]2.1% missing values (1 of 100 cases and 3 of 90 control subjects).
[b]4.7% missing values (2 of 100 cases and 7 of 90 control subjects).
[c]1% missing values (0 of 100 cases and 2 of 90 control subjects).
[d]1.9% missing values (3 out of 89 control subjects).

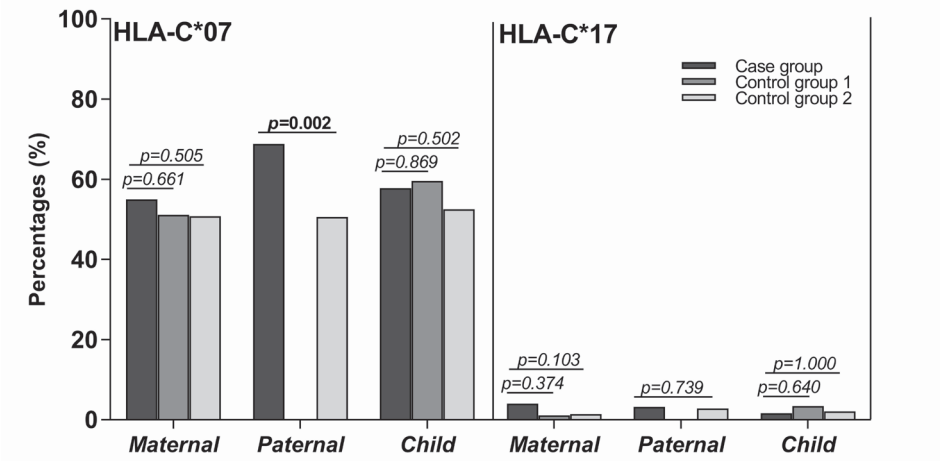
Most women had 4 or more consecutive miscarriages (71%), and 41 (41%) 5 or more miscarriages. A total of 11 cases had inherited thrombophilia, that is, factor V Leiden (n=4), prothrombin gene mutation (n=4), or high factor VIII (n=5). None had protein C, or S deficiency.

In the case group and the first control group, numbers were too small to assess Hardy-Weinberg equilibrium for all genotypes of HLA-C alleles. In the case and both control groups all loci for homozygotes and heterozygotes genotypes and in the second control group, all genotypes of the HLA-C allele were in Hardy-Weinberg equilibrium (Supplementary data, Table II).

Phenotype frequencies of HLA-C*07 and HLA-C*17

The incidence of HLA-C*07 was significantly increased in paternal cases compared to paternal control subjects (OR 2.1, CI 1.3-3.4, *p*=0.002). No significant differences were present with regard to the frequencies of HLA-C*07 and HLA-C*17 in mother and child between cases and control subjects (Figure 4.2).

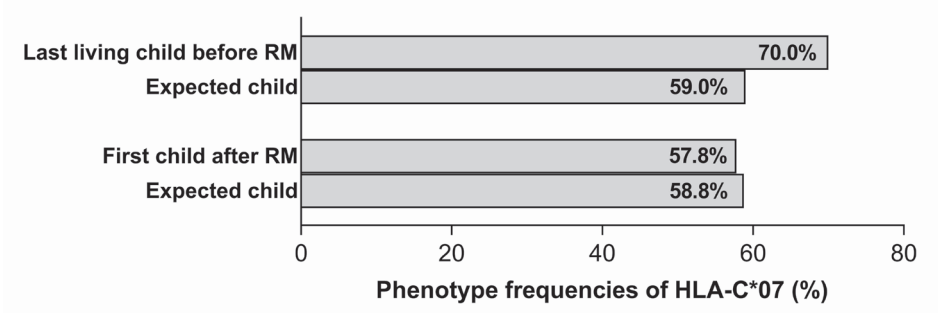
Figure 4.2 Phenotype frequencies of HLA-C*07 and C*17 in women with recurrent miscarriage compared to control groups



Frequencies for HLA-C*07 in maternal case group and control group 1: 55% vs. 51.1% (OR 1.1, 95% CI 0.6-2.0, p=0.661) and control group 2: 55% vs. 50.8% (OR 1.1, 95% CI 0.7-1.8, *p*=0.505). For paternal case group and control group 2: 68.8% vs. 50.6% (OR 2.1, 95% CI 1.3-3.4, *p*=0.002). For children in case group and control group 1: 57.8% vs. 59.6% (OR 0.9, 95% CI 0.4-1.7, *p*=0.869) and control group 2: 57.8% vs. 52.5% (OR 1.2, 95% CI 0.7-2.0, *p*=0.502).
Frequencies for HLA-C*17 in maternal case group and control group 1: 4% vs. 1.1% (OR 2.7, 95% CI 0.4-17.6, *p*=0.374) and control group 2: 4% vs. 1.4% (OR 3.0, 95% CI 0.8-10.2, *p*=0.103). For paternal case group and control group 2: 3.2% vs. 2.8% (OR 1.2, 95% CI 0.3-4.2, *p*=0.739). For children in case group and control group 1: 1.6% vs. 3.4% (OR 0.5, 95% CI 0.0-4.0, *p*=0.640) and control group 2: 1.6% vs. 2.1% (OR 1.0, 95% CI 0.1-5.9, *p*=1.000). Odds ratio (OR) and confidence interval (CI) were calculated with the Woolf-Haldane method, *p*-values were calculated with the Fisher's exact test.

HLA-C*07 phenotype frequency of the last living child born before the occurrence of recurrent miscarriage was 70% (14 out of 20 children). The expected frequency of HLA-C*07 in this group was 59%, and the frequency of HLA-C*07 of children born after recurrent miscarriage was 57.8% (37 out of 64 children) (Figure 4.3).

Figure 4.3 Phenotype frequencies of HLA-C*07 in children from women with recurrent miscarriage



Expected percentages were calculated from parental haplotypes of HLA C*07. RM; recurrent miscarriage.

HLA–C mismatches

The number of paternal HLA-C mismatches was significantly different between cases and control subjects (Table 4.2). Significantly more paternal C*07 mismatches were observed in women with recurrent miscarriage (67.5%) compared to control subjects (44.5%) (p=0.008). Accordingly, mismatches for HLA-C*07 between mother and child were increased in the case group compared to the second control group (p=0.040).

HLA antibody formation

Information on time of blood sampling is depicted in Table 4.3. Three cases were pregnant at time of blood sampling, of which two were in their first trimester and one woman in her third trimester. As all control subjects donated blood within 48 hours after labor, they had significantly more previous live births than cases at the time of blood sampling. For that reason, significantly more nulliparous women were included in the case group. Nevertheless, the incidence of HLA class I antibodies was significantly higher in women with recurrent miscarriage (p=0.008) (Table 4.3).

HLA class I antibodies against the first child born after recurrent miscarriage were more often detected in women with recurrent miscarriage compared to women with uneventful pregnancies (p=0.022). Especially the incidence of child-specific HLA-C*07 and HLA-C*17 antibodies was significantly increased in cases compared to control subjects (p=0.007)

Table 4.2 HLA-C mismatches of maternal-paternal and maternal-child in women with recurrent miscarriage and control subjects

Mismatch		Maternal-paternal					Maternal-child								
		Case group N=94		Control group 2 N=425			Case group N=64		Expected	Control group 1 N=87[a]		Control group 2 N=425			
		n	%	n	%	*P*	n	%	%	n	%	n	%	P_1	P_2
C locus	0	8	8.5	52	12.2	0.016[b]	15	23.4	30.8	27	31	136	32	0.303	0.167
	1	58	61.7	195	45.9		49	76.6	69.2	60	69	289	68		
	2	28	29.8	178	41.9										
C*07[c]	0	13	32.5	116	55.5	0.008	16	53.3	55.6	25	59.5	150	71.8	0.601	0.040
	1	27	67.5	93	44.5		14	46.7	44.4	17	40.5	59	28.2		
C*17[c]	0	86	96.6	408	97.4	0.720	61	100.0	100.0	84	97.7	414	98.8	0.511	1.000
	1	3	3.4	11	2.6		0	0.0	0.0	2	2.3	5	1.2		

[a]For 3 out of 90 children of control group 1, HLA typing was incomplete and therefore not included.
Chi-squared tests and [b]overall chi-squared comparison.
[c]For this analysis, only mothers which were C*07 or C*17 negative were included both for the case group and control groups.
Expected percentages were calculated upon parental haplotypes of HLA-C. P; *p*-value. P_1 comparison between women with recurrent miscarriage and control group 1. P_2 comparison between women with recurrent miscarriage and control group 2.

Table 4.3 Presence of HLA antibodies

	Recurrent miscarriage N=100	Uneventful pregnancy (Control group 1) N=82[b]	*P*-value
At the time of blood sampling:			
Gravidity (median[IQR])	6 (5-8)	3 (2-3.2)	<0.001
Parity (median[IQR])	1 (1-2)	2 (1-3)	<0.001
Nulliparous	14 (14)	0 (0)	<0.001[a]
Miscarriages (median[IQR])	4 (3-6)	0 (0-1)	<0.001
Pregnant	3 (3)	0	0.253[a]
Blood transfusion[c]	5 (5)	11 (12.6)	0.069
Presence of HLA antibodies			
Presence of HLA-I antibodies	33 (33)	13 (15.9)	0.008
Presence of HLA-C antibodies	12 (12)	10 (12.2)	0.968
Presence of HLA-C*07/17 antibodies	7 (7)	5 (6.1)	0.807
Presence of specific HLA antibodies (n)	**64**	**79[d]**	
Presence of child-specific HLA-I antibodies	20 (31.3)	12 (15.2)	0.022
Presence of child-specific HLA-C antibodies	8 (12.5)	5 (6.3)	0.202
Presence of child-specific HLA-C*07/17 antibodies	6 (9.4)	0 (0.0)	0.007[a]
Presence of specific HLA antibodies (n)	**94**		
Presence of paternal-specific HLA-I antibodies	32 (34)	-	-
Presence of paternal-specific HLA-C antibodies	9 (9.6)	-	-
Presence of paternal-specific HLA-C*07/17 antibodies	6 (6.4)	-	-

Data are n(%) unless otherwise indicated. All Mann-Whitney *U* test or chi-squared test except for [a]Fisher's exact test.
[b]In 8 control subjects, no serum was available to detect HLA antibodies.
[c]2.6% missing values (2 of 100 cases and 3 of 90 control subjects).
[d]For 3 of 90 children of the control subjects, HLA typing was incomplete and therefore not included.

(Table 4.3). HLA-C antibodies were C1q fixing antibodies in 3 of 8 women with recurrent miscarriage compared to 4 of 5 women with uneventful pregnancy (p=0.266).

In the pregnancies of the first living child born after recurrent miscarriage, where antibodies against HLA class I were present, 60.0% of the women used anticoagulants as aspirin (n=4) or LMWH (n=7). In women with child-specific HLA-C antibodies, 62.5% used anticoagulants as aspirin (n=2) or LMWH (n=3). In women with child-specific HLA-C*07 or HLA-C*17 antibodies 50.0 % used anticoagulants (aspirin (n=1) or LMWH (n=2)).

No significant differences were observed in paternal-specific HLA class I, HLA-C or HLA-C*07/17 antibodies between women with primary recurrent miscarriage and women

with secondary recurrent miscarriage (p=0.257, p=0.471, and p=0.392, respectively). Also, no significant differences were seen in paternal-specific HLA class I, HLA-C or HLA-C*07/17 antibodies between couples who had at least one living child born after recurrent miscarriage and couples who had no living child after recurrent miscarriage (p=0.279, p=0.106, p=0.332, respectively).

Discussion

The frequency of HLA-C*07, one of the most immunogenic HLA-C alleles, was significantly increased in partners of women with recurrent miscarriage compared to control subjects. Accordingly, an increased number of mismatches for paternal HLA-C*07 was found. The incidence of HLA-C*07 mismatches was significantly higher between mother and father compared to mother and child, which is not surprising as in only half of the cases the mismatched HLA-C*07 was inherited by the child. This is in accordance with Mendelian segregation. However, the consequence of the high incidence of HLA-C*07 in the father is that in all previous pregnancies the incidence of HLA-C*07 in the child has been higher than in the control populations. The presence of HLA antibodies, including antibodies against the high immunogenic HLA-C*07/17, was increased in women with recurrent miscarriage compared to women with uneventful pregnancy.

The strength of the study is that a large homogenous group of women with a history of recurrent miscarriage was selected and compared to women with uneventful pregnancy. Furthermore, analyses of cases could be compared with a large cohort of complete families which were found to be in Hardy-Weinberg equilibrium, indicating that there was no selection of a certain genotype within these families. Furthermore, in comparison with recent studies,[6,22] by including complete families in combination with antibody screening, specificity of these antibodies could be determined.

A possible limitation is that this study is subject to selection bias, as couples, that did not participate in this study had overall significantly fewer children and fewer live births after they had recurrent miscarriages. However, this suggests that the observed effects are rather an underestimation due to the fact that the group with worse outcome amongst the recurrent miscarriage cases did not participate.

Furthermore, most women with recurrent miscarriage included in this study took part in the Habenox trial and were therefore randomly assigned to use anticoagulants as LMWH, aspirin, or a combination of both during pregnancy. In case of anti-phospholipid antibodies treatment with heparin is beneficial, and protects mice from pregnancy complications,

because it inhibits complement activation.[23] More C4d deposition was found at the maternal-fetal interface in women with unexplained recurrent miscarriage, which may reflect antibody-mediated rejection in these women.[24,25] In spontaneous preterm birth the presence of C4d in fetal cord endothelium was associated with circulating maternal anti-HLA-I antibodies.[26] In addition, HLA antibodies in early pregnancy are associated with lower chance of a live birth in women with recurrent miscarriage.[27] Although recent studies show no significant effect of LMWH in women with unexplained recurrent miscarriage,[28-30] the effect of LMWH in a homogenous group of women with unexplained recurrent miscarriage has not been studied yet. Therefore, the use of anticoagulants as LMWH, aspirin, or a combination of both could have influenced outcome in our study. In addition, in women with recurrent miscarriage having anti-phospholipid antibodies, prophylactic use of heparin and low-dose aspirin may reduce pregnancy loss by 50%.[31]

Activation of the complement cascade by anti-phospholipid antibodies is a specific trigger provoking a thrombotic event.[32,33] Whether HLA antibodies are capable of precipitating the coagulation pathway by the same mechanism as anti-phospholipid antibodies needs to be further investigated. In addition, it remains to be established in future studies whether aspirin induces similar anticoagulant effect and thereby reduces pregnancy loss in women with recurrent miscarriage and presence of HLA antibodies.

Although in a recent study HLA-C-specific antibodies were found more often in women with recurrent miscarriage,[6] the present study showed that HLA class I antibodies, but not particularly HLA-C antibodies, were significantly increased in women with recurrent miscarriage. In the present study, blood sampling in control subjects was performed directly after delivery and control subjects were more often multiparous. Therefore the presence of HLA antibodies in control subjects could have been overestimated with respect to cases, as HLA antibodies against the paternal antigens are only demonstrable after the pregnancy has reached a gestational age of 28 weeks[6,34] and the highest incidence of HLA antibodies is after delivery.[34] Moreover, Adeyi et al. showed that donor-specific antibodies could be undetectable, since the graft had absorbed them. After removal of the graft, these antibodies became more readily detectable.[35] In the same line, child-specific antibodies detected postpartum could be higher as a result of absorption by the placenta during pregnancy. Despite the fact that control subjects were more often multiparous, significantly more child-specific HLA class I and HLA-C*07/17 antibodies were present in women with recurrent miscarriage.

Not all HLA-C antibodies in cases were complement fixing, and we know from trans-plantation settings that only a proportion of allo-antibodies cause rejection, especially those with the ability to activate complement and a high avidity for the antigenic target.[36]

Possibly, the presence of HLA antibodies in women with recurrent miscarriage can be considered as a marker for a broader immune response, as was previously shown in HLA identical family transplantations.[37] In that study the presence of HLA antibodies was a risk factor for worse outcome, although HLA antibodies themselves could not have caused any harm. Alternatively, preformed HLA antibodies, which can be present at time of new conception,[34,38] may induce platelet activation by placental thromboxane production causing pregnancy complications.[39]

In conclusion, this study demonstrates that in a proportion of women with recurrent miscarriage an immune response to the paternal HLA-C allele may play a role in the occurrence of so far unexplained recurrent miscarriage. Our study confirms recent findings,[6,24] that humoral rejection of the fetal allograft may indeed be present in a subgroup of women with recurrent miscarriage, thereby shedding new light on current theories about the pathophysiology of this devastating condition. A possible next step might be the use of complement inhibitors or aspirin in the subsequent pregnancy in this specific subgroup of women with recurrent miscarriage.

Acknowledgements

The authors would like to thank Clara Kolster, Marjolein Bourgonje-Verhart, and Marise Wagner for their help obtaining blood and saliva samples from all the cases. The authors would like to thank Simone Brand-Schaaf and Sophia Stein for their help with antibody screening. The authors would like to thank Michael Eikmans for his critical reading and valuable feedback on the manuscript.

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Supplementary data

Table I Characteristics of included and excluded cases

	Included cases (N=100)	Excluded cases (N=173)	*P*-value
Gravidity (median[IQR])	6 (5-8)	5 (4-6)	<0.001
Parity (median[IQR])	1.5 (1-2)	1 (0-1)	<0.001
Miscarriages (median[IQR])	4 (3-6)	3 (3-4)	<0.001
Consecutive miscarriages	4 (3-5)	3 (3-4)	<0.001
Primary recurrent miscarriage Secondary recurrent miscarriage	69 (69%) 31 (31%)	100 (57.8%) 73 (42.2%)	0.066
Live birth after miscarriages	75 (75%)	23 (13.3%)	<0.001

Table II Hardy Weinberg analyses for homozygotes and heterozygotes C allele in the case and control groups

		Maternal			Paternal			Child		
C allele		Obs	Exp	*P*-value	Obs	Exp	*P*-value	Obs	Exp	*P*-value
Case group	hom	17	16.5	0.903	18	20.9	0.525	5	9.9	0.122
	het	83	83.5	0.957	75	72.1	0.732	59	54.1	0.509
Control group 1	hom	21	15.8	0.187	-	-	-	18	18.5	0.905
	het	67	72.2	0.538	-	-	-	71	70.5	0.951
Control group 2	hom	58	62.0	0.609	72	64.6	0.358	73	68.2	0.558
	het	367	363.0	0.832	353	360.4	0.697	352	356.8	0.798
	all			0.155			0.302			0.416

Hom; homozygote, het; heterozygite, obs; observed, exp; expected.

Chapter 5

Beneficial or harmful effect of anti-paternal human leukocyte antibodies on pregnancy outcome? A systematic review and meta-analysis

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American Journal of Reproductive Immunology 2013;70(2):87-103
Appendices available on http://doi:10.1111/aji.12109

Abstract

Problem

During pregnancy antibodies are induced that target the paternal human leukocyte antigens of the semi-allogeneic fetus. The level and presence of these antibodies have been reported elevated as well as decreased for a variety of pregnancy complications; the clinical relevance and consequences of these antibodies is not very clear. Therefore, the objective of this review is to determine whether the presence of anti-paternal antibodies influences pregnancy outcome.

Method

We performed a systematic search of studies that described the effect of anti-paternal antibodies on pregnancy complications. The primary outcome was the risk ratio for HLA Class I and Class II antibodies on pregnancy complications. Furthermore, we calculated the risk for first and third trimester complications.

Results

The seventeen studies which were selected for meta-analysis showed high level of statistical and clinical heterogeneity. In the meta-analysis we found no significant effect of HLA Class I or Class II antibodies on pregnancy outcome.

Conclusions

No consistent conclusions can be drawn from the meta-analysis. Discrepancies in the meta-analysis are the result of different screening techniques, varying time points of screening and use of incorrect control groups. Furthermore, more detailed analyses of the characteristics and specificity of the antibodies involved are essential.

Introduction

During pregnancy the maternal immune system has to tolerate the persistence of semi-allogeneic fetal cells in maternal tissue, both locally at the fetal-maternal interface, as well as systematically. The entry of fetal material into the maternal circulation exists as microparticles are released from the syncytiotrophoblasts and shed into the maternal peripheral blood.[1,2] Furthermore, fetal cells (microchimerism),[3] fetal DNA, and debris from apoptotic cells flow into the circulation. The presence of these fetal antigens, as well as processing and presenting of major histo-compatibility complex (MHC) alloantigens by macrophages, enables fetus-specific antigen recognition by the maternal adaptive immune system. Indeed, primed T cells to paternal HLA antigens and fetus-specific minor histocompatibility complexes, like HY, have been demonstrated in the peripheral blood of pregnant women.[4-6] In addition, studies by our group show that the CD4+CD25dim (activated) T-cell population increases in maternal peripheral blood during pregnancy.[7]

B cell activation occurs after uptake of antigen by the B cell receptor, followed by interaction with the primed T cells and costimulation through CD40L-CD40, producing anti-paternal antibodies.[8] Anti-paternal antibodies have been first described by van Rood et al.[9] and Payne et al.[10] in 1958. These antibodies are developed in 10-30% of healthy women during pregnancy[11] and their incidence is increased after 28th week of pregnancy.[12] The number of pregnancies enhances the prevalence of HLA antibodies, although the result of the immunizing effect appears to be the strongest in the first two pregnancies.[13]

The immunogenicity of the paternal HLA antigens during pregnancy is affected by the HLA antigens of the mother[14] and more recent studies showed that the induction of antibodies to paternal HLA is directly correlated with the number of foreign epitopes, triplets of amino acid sequences accessible for antibodies on the paternal HLA antigens.[15,16] There is a loss of detectable antibodies months to years after immunization,[5] though primed cytotoxic T lymphocytes specific for these antigens can persist for more than 10 years, even if the antibodies have disappeared.[17]

A number of important clinical effects of HLA antibodies have been clearly established in transplantation and transfusion settings, including acute and chronic graft failure,[18] platelet transfusion refractoriness[19] and transfusion-related acute lung injury (TRALI) syndrome.[20] For pregnancy however the clinical relevance of these antibodies is controversial; both the presence and absence of HLA- or paternal antibodies have been described in the context of pregnancy complications. Moreover, increasing evidence suggests a role of anti-paternal antibodies in the pathogenic mechanism of pregnancy

complications such as preeclampsia[21] and recurrent miscarriage.[22] We therefore reviewed the literature to determine the effect of presence of anti-paternal antibodies on pregnancy outcome.

Method

Criteria for eligibility

A search in the National Centre for Biotechnology Information Pubmed database was performed in June 2012 using the Medical Subject Headings (MeSH) terms 'HLA antigens', 'antibodies' and 'major histo-compatibility complex' in combination with 'pregnancy complications' and 'pregnancy outcome'. As a search limit English-language publications and human research was used. Additional articles were identified from the Embase and Web of Science databases. Detailed search strategy is displayed in Appendix 1. We selected all reports with data of HLA antibodies in respect to pregnancy complications. The pregnancy complications studied were preeclampsia, recurrent miscarriage, placental abruption, gestational diabetes, growth restriction and preterm labor. These various complications are defined in Appendix 2. The definition of presence of HLA antibodies was based on the criteria used by the various authors. Only studies with a comparative, uncomplicated pregnant control group were included for the meta-analysis. Exclusion criteria were: case reports, letters, comments and articles focusing on anti-paternal antibodies after paternal leukocyte immunization.

Quality assessment

Two authors (E.L. and T.M.) independently assessed for inclusion all potential studies of the search strategy. These authors also assessed quality and risk of bias, according to the Newcastle-Ottawa scale (http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp maximum score 9 points), and carried out data extraction (Appendix 3). Disagreement was resolved by consensus. Low methodological quality was not an exclusion criterion.

Data analysis

The following data were extracted from the reports for the meta-analysis: design of the study, definition of the pregnancy complication, total numbers of pregnancies, births and miscarriage, number of patients and healthy controls in the study, number of patients and controls with HLA antibodies, the technique and timing of screening for anti-paternal antibodies, the ethnicity and the specificity of the antibodies. We contacted the authors if

the variables were missing. When available, history of blood transfusion or transplantation was also noted.

The primary outcome of the meta-analysis was the pooled risk ratio for HLA Class I and Class II antibodies on pregnancy complications. In further analyses we calculated the pooled risk for first and third trimester complications.

Furthermore, we expected statistical heterogeneity, so we used the random effects model for the meta-analysis; the heterogeneity was calculated with the I^2 statistics. This, with all of the other analyses was performed with Review Manager (RevMan) Version 5.1. Copenhagen: The Nordic Cochrane Centre, the Cochrane Collaboration, 2011.

Results

Literature search

The main search identified 332 potentially relevant studies. Figure 5.1 shows the flow chart, leading to the final 17 references included in this review. A total of 316 studies were not relevant for the research question and were excluded. One study was identified in the reference list of an excluded article and was added to the final number.

In one study[23] two complications, placental abruption and preterm labor, were compared with uncomplicated pregnancies. All other studies focused on one pregnancy complication. The design of the seventeen studies included[23-39] was a case control study. Only three groups mentioned the ethnicity of participants which was Hispanic[24,25] and Italian respectively[32]; one study only stated that the participants and controls came from the same geographic area.[30] Different techniques for detecting HLA antibodies were used; the standard NIH complement dependent cytotoxicity (CDC) assay[40] detected antibodies in twelve studies,[26,29-39] the enzyme-linked immunosorbent assay (ELISA) was the technique used in three studies.[23,26,28] In one study both the CDC assay and the ELISA was applied,[26] Coulam et al.[33] made use of the standard CDC and a complement dependent cytotoxicity assay with ^{51}Cr release. Finally, flowcytometry was used in three studies[24,25,27]; Bartel et al.[27] used both flowcytometry and Luminex.

For antibody detection and specificity determination a panel with HLA typed individuals was used for five studies though the size of these panels differed (Appendix 3). The CDC assay was tested against only paternal lymphocytes in five studies,[29,33,35,37,39] both paternal lymphocytes as a panel of donors were used in the study of Jenkins et al.[31] and no information was present in the study of Sargent et al.[36] and Shankarkumar et al.[38] HLA

Figure 5.1 Flow chart depicting selection of articles for systematic review

* Major causes for exclusion were: no determination of HLA antibodies, articles focusing on paternal leukocyte immunization, articles focusing on techniques of screening, articles focusing on solid organ transplantation and case reports, letters or comments.
** Major causes for exclusion were: non-comparative studies and no well-defined complication group.

positivity was differently defined (Appendix 3) within all the study groups, though the same definition (and technique) was used for both patients as controls.

Nine studies not only detected HLA antibodies, but also determined the specificity of these antibodies.[24-28,30,32,36] Finally the timing of measurement differed among the studies (Appendix 3); for four studies the moment of detection was not described,[29,33,38,39] for two studies this was only described for the control group.[32,37]

Earlier blood or tissue transplant was mentioned only in the study of Nielsen et al.[26] and Sargent et al.[36] as an exclusion criterion.

In Appendix 3 the assessment of methodological quality according to the Newcastle-Ottawa scale is summarized. Five studies showed high quality scoring seven or six points out of nine.[24-26,29,33] Mediate quality was scored by three studies[27,28,39] and nine studies[23,30-32,34-38] scored four points or less, indicating low quality.

Meta-analysis

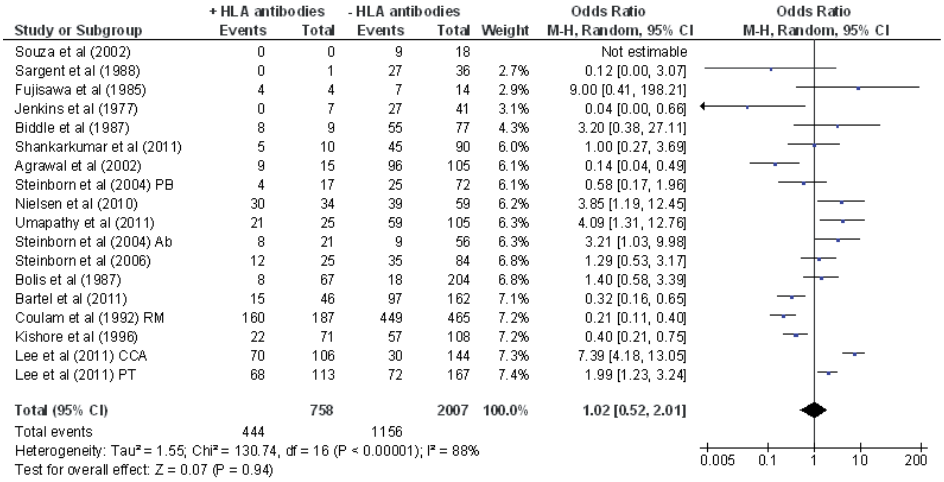
To check for the existence of publication bias a funnel plot was drawn, which showed a symmetrical shape, indicating the absence of publication bias (data not shown). From all included studies we calculated the Odds Ratio (OR) with its accompanying standard error (Table 5.1). The OR from the seventeen studies with class I antibodies ranged from 9.00 to 0.04; this was plotted in Figure 5.2. In the study of Souza et al.[39] no HLA antibodies were detected in patients or controls; the OR could therefore not be determined.

The pooled OR was 1.02 (95% confidence interval 0.52-2.01), showing no significant effect of HLA antibodies Class I on pregnancy complications in general. The I^2 showed high

Table 5.1 Characteristics and outcome of the 17 included studies

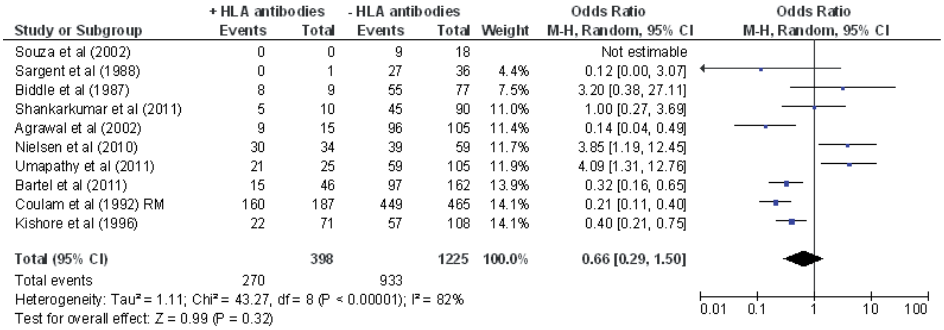
Study	Year of publi-cation	Complication	Participants (n)	Odds Ratio (95% CI) HLA Class I antibodies	Odds Ratio (95% CI) HLA Class II antibodies
Lee et al.[25]	2011	CCA	Patients 100 Controls 150	7.39 [4.18-13.05]	2.81 [1.56-5.09]
Lee et al.[24]	2011	Preterm labor	Patients 140 Controls 140	1.99 [1.23-3.24]	1.10 [0.61-1.99]
Nielsen et al.[26]	2010	RM	Patients 69 Controls 24	3.85 [1.19-12.45]	0.8 [0.25-2.57]
Bartel et al.[27]	2011	RM	Patients 112 Controls 96	0.32 [0.16-0.65]	0.20 [0.08-0.46]
Steinborn et al.[28]	2006	Gestational diabetes	Patients 47 Controls 62	1.29 [0.53-3.17]	3.48 [1.12-10.85]
Steinborn et al.[23]	2004	Placental abruption	Patients 17 Controls 60	3.21 [1.03-9.98]	
		Preterm labor	Patients 29 Controls 60	0.58 [0.17-1.96]	
Kishore et al.[29]	1996	RM	Patients 79 Controls 100	0.40 [0.21-0.75]	
Fujisawa et al.[30]	1985	Preeclampsia	Patients 11 Controls 7	9.00 [0.41-198.21]	0.43 [0.06-2.97]
Jenkins et al.[31]	1977	Preeclampsia	Patients 27 Controls 21	0.04 [0.002-0.66]	
Bolis et al.[32]	1987	Preeclampsia	Patients 26 Controls 245	1.40 [0.58-3.39]	0.85 [0.11-6.87]
Coulam et al.[33]	1992	RM	Patients 609 Controls 43	0.21 [0.11-0.40]	

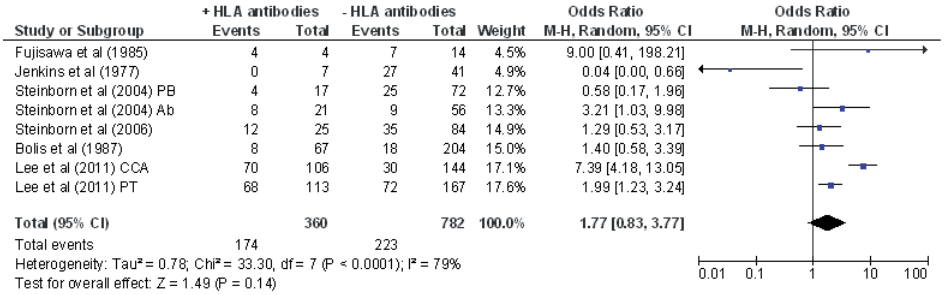
Figure 5.2 Meta-analysis of studies on the risk of HLA Class I antibodies with pregnancy complications



Events = number of patients with complication, Total = number of patients and controls.

Figure 5.3 Meta-analysis of studies on the risk of HLA Class I antibodies with first (a) and third (b) trimester complications



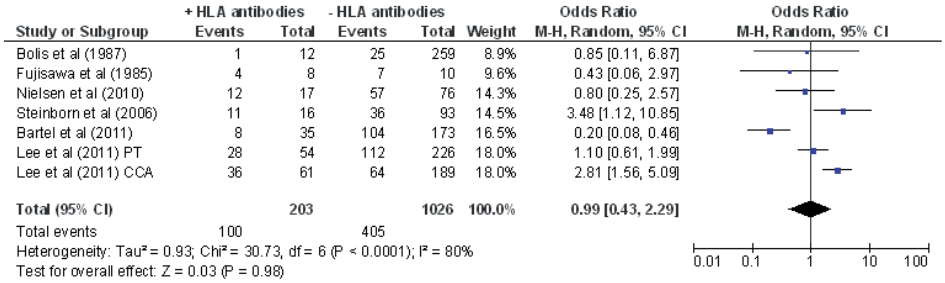


Events = number of patients with complication, Total = number of patients and controls.

heterogeneity (88% $p<0.00001$) among the studies. Pooling all pregnancy complications with different gestational ages this heterogeneity was expected. Therefore we also calculated the risk of HLA Class I antibodies for first and third trimester complications (Figure 5.3). The pooled OR for first trimester complications was 0.66 (95% confidence interval 0.29-1.50) with high heterogeneity (I^2=82% $p<0.00001$). For third trimester complications a pooled odds ratio of 1.77 (95% confidence interval 0.83-3.77, I^2=79% $p<0.0001$) was calculated, showing no effect of HLA Class I antibodies on late pregnancy complications and high heterogeneity.

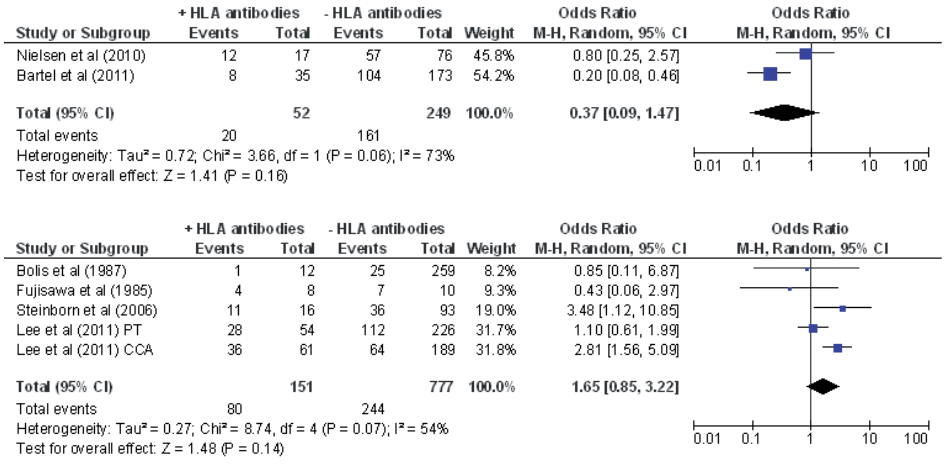
Moreover, we calculated the pooled risk for HLA Class II antibodies on pregnancy complications. Seven studies[24-28,30,32] screened and detected Class II antibodies, by

Figure 5.4 Meta-analysis of studies on the risk of HLA Class II antibodies with pregnancy complications



Events = number of patients with complication, Total = number of patients and controls.

Figure 5.5 Meta-analysis of studies on the risk of HLA Class II antibodies with first (a) and third (b) trimester complications



Events = number of patients with complication, Total = number of patients and controls.

flowcytometry, ELISA, Luminex or CDC (B lymphocytes). The odds ratio of Class II antibodies ranged from 3.48 to 0.2 with high heterogeneity (I^2=80% p<0.0001); plotted in Figure 5.4. No significant effect was found of HLA Class II antibodies on pregnancy complications. As described above, we also calculated the OR for first trimester and third trimester complications (Figure 5.5). The OR for first and third trimester complications was 0.37 (95% confidence interval 0.09-1.47) respectively 1.65 (95% confidence interval 0.85-3.22), showing no effect of HLA Class II antibodies on first or third trimester complications. The I^2 was 73% p=0.06 and 54% p=0.07; high to medium heterogeneity.

Discussion

In human pregnancy the presence of antibodies against red blood cell or platelet antigen are associated with serious complications, resulting in hemolytic disease of the fetus and fetal/ neonatal allo-immune thrombocytopenia (FNAIT) respectively. In contrast with these fetal blood cell antigens, the role of HLA antibodies is not that clear as both harmful and beneficial effects have been described. In this systematic review we found no significant effect of HLA antibodies on pregnancy outcome (Figure 5.2 and 5.4). The seventeen studies included however showed high statistical and clinical heterogeneity.

As we pooled all different pregnancy complications we expected a certain level of heterogeneity. Therefore, we separated the complications based on the time of onset, which resulted in a first trimester complication group and a late pregnancy complication group. The latter included chronic chorioamnionitis, preterm labor, preeclampsia, placental abruption and gestational diabetes. Though an immunological patho-mechanism is described for all of these different complications, pooling them could contribute to the reported heterogeneity. Unfortunately, too little data exist to execute a meta-analysis for each complication separately. On the other hand, the first trimester group consisted of the same pregnancy complication (recurrent miscarriage) and still showed high level of heterogeneity.

The methodological quality ranged from 3 points until 7 points maximally, with a median of 4 points which implies a low quality of the majority of studies. The largest weight in the meta-analysis was provided by a high quality study with a large patient group included. For the meta-analysis we included data from published papers only. The funnel plot actually showed a symmetric scatter plot, which indicated no publication bias (data not shown).

The ethnicity of the participants was mentioned in only two studies. Knowledge of the race or ethnicity is of importance though as the haplotype frequencies of HLA

vary amongst different populations[41] and certain combinations of fetal-maternal HLA generate significantly more antibodies.[14] Therefore it is not surprising that there is also a wide variation in incidence of anti-HLA antibodies in the sera of healthy women among different populations.[37]

The timing of screening was different amongst the included studies, ranging from a gestational age of 4 weeks until 12 months postpartum. Regan et al.[12] was the first to document the incidence and development of HLA antibodies measured throughout the pregnancy in a healthy population. They detected HLA antibodies only after the pregnancy has reached a gestational age of 28 weeks, probably since the influx of fetal material into the circulation peaks in the last trimester. One study included in our meta-analysis showed indeed higher IgG HLA class I and II positivity in samples obtained at time of delivery than before a gestational age of 16 weeks both for uncomplicated as complicated pregnancies.[25] Regan et al. further showed that the cumulative frequency of positive HLA antibody sera increased even more after delivery, at 4 weeks postpartum.[12] This means that the highest incidence of HLA antibodies is after delivery, when fetal material entry into the maternal circulation is at maximum, in coincidence with Rhesus antibodies. Moreover, during graft rejection donor-specific antibodies may be undetectable by routine serum screening because the graft has absorbed them. After removal of the antigen source these antibodies become more readily detectable.[42] Therefore, the level of child specific antibodies detected postpartum could be higher as a result of absorption by the placenta during pregnancy. Six studies in our meta-analysis did not mention the timing of screening for their patient and control group. In two other studies non-pregnant women were used as control group, within 2 years after their last pregnancy, while their patients were screened during pregnancy.[26,35] These differences may have impact on their results.

HLA specific antibodies formed during a previous pregnancy can be present at time of new conception[12] and studies that report a beneficial effect of HLA antibodies suggest these antibodies enhance the development of maternal-fetal immunologic tolerance. This is in line with epidemiological studies that a prior birth confers a protective effect against pre-eclampsia and IUGR.[43] Furthermore, exposure to new or different paternal antigens as a result of changed paternity is associated with an altered risk for pre-eclampsia.[44] However, it is not clear whether this beneficial effect can be attributed to the HLA antibody itself, or, for example to the period of semen exposure.[45] It is assumed that semen contains a variety of immune factors which can support the induction of maternal immunomodulation[46] and an association was found between short duration of sexual intercourse and preeclampsia.[45,47] Still, stratification according to the presence or absence of previous pregnancies is required to rule out the effect of earlier formed HLA

antibodies; this was only mentioned in the study of Lee et al.[25] They observed similar results in nulliparous and multiparous women though.

For primary recurrent miscarriage, almost all the included studies used healthy multiparous women as control group. However, since the incidence of HLA antibodies in multiparous is per definition higher than in first trimester pregnancies, only nulliparous women with recurrent (≥3) elective abortions, as Biddle et al.[34] stated, would reflect the appropriate control group. Interestingly, in the study of Nielsen et al.[26] a subgroup of secondary recurrent miscarriages with a boy prior to the miscarriage showed a significant higher prevalence of HLA antibodies compared to healthy multiparous controls. The authors explain this higher prevalence of HLA antibodies as the result of a higher degree of microchimerism. Microchimerism occurs within 50-70% of pregnant women[3] and can persist until 27 years after delivery.[48] The traffic of fetal cells into the maternal circulation microchimerism may lead to its activation. Indeed, a reproductive history including elective termination of pregnancy[49] or early fetal loss[50] is associated with a higher incidence of microchimerism in maternal tissues. Also for preeclampsia a significant increase of fetal erythroblasts was seen compared to normotensive pregnant women,[51,52] as with intra-uterine growth restriction.[53,54] This enhanced cell trafficking however may be a result of the pathological condition of the placenta rather than as a cause for these complications. A higher level of HLA antibodies may thus be just a result of higher level of microchimerism, without a causal link and without any clinical relevance. Furthermore, regarding the study of Nielsen et al., no data exists about the kinetics of appearance of HLA antibodies around abortion or at 2 years post delivery, the time points of screening in their study.

To detect HLA antibodies five different techniques were used: the standard NIH CDC assay, the ELISA, complement dependent cytotoxicity assay with ^{51}Cr release, Luminex and flowcytometry. The CDC assay is based on complement activation and requires immunoglobulins that bind to complement. Immunoglobulins that are not bound will thus not be detected.[55] The indirect immunofluorescence method (flowcytometry) is independent of complement fixation and can detect all antibodies. This technique is used in the studies of Lee et al. and Bartel et al.[24,25,27] Furthermore, the CDC assay uses lymphocytes as target for HLA antibodies, which implies that also other irrelevant cell membrane structures may be targets for antibody reactivity as well,[56] possibly overestimating the actual level of HLA antibodies. To increase the sensitivity and specificity of HLA antibody detection, Luminex and flowcytometric assays are introduced that use isolated HLA molecules on beads. Indeed, Lee et al. and Bartel et al.[25,27,57] made use of these single-antigen beads. With the binding of HLA molecules to microspheres however a conformational change in the HLA molecule may occur, unmasking 'hidden' epitopes,

which may lead to the detection of unexpected antibody reactivity.[58,59] Furthermore, whether the detected antibodies are directed against the paternal HLA of the fetus was not mentioned,[24,27] or only for certain cases.[25] This child-specificity was also not mentioned in studies that used the CDC assay serum with lymphocytes from a panel of donors,[30,32,34] with the exception of the study by Nielsen et al. Using paternal lymphocytes as donor to determine the extent of immunization will generate only child-specific antibodies.

The question remains nevertheless, what the clinical relevance of these (non) child-specific antibodies is. Extravillous trophoblasts, interacting with maternal cells at the decidua basalis, express only HLA-C and the non-classical HLA-E, -F and -G. Antibodies directed against these human leukocyte antigens in theory may have a detrimental effect. However, HLA-C mismatches are low in immunogenicity and the detection of HLA-C antibodies with CDC assay is not reliable; the expression of HLA-C on nucleated cells is lower than HLA-A or -B and there is linkage disequilibrium with HLA-B antigens.[60] In fact, all IgG type HLA antibodies are able to cross the placenta; HLA antibodies were demonstrated in the fetal circulation[61] and neonatal thrombocytopenia by maternal HLA antibodies was reported as side effect following leukocyte immunization in a woman with recurrent miscarriages.[62]

The clinical relevance of HLA antibodies is also a matter of debate in transplantation setting.[55] Preceding organ transplantation, screening for HLA antibodies is performed as routine. The detection of donor specific HLA antibodies with CDC was considered a contraindication for transplantation, as these antibodies were associated with graft failure after transplantation,[18] With the introduction of newer screening techniques the sensitivity is increased, however to determine the individual risk factor for a patient before transplantation appears impossible; the debate concerning interpretation still continues.[55]

Conclusion

In this systematic review and meta-analysis we found no significant effect of HLA antibodies class I and/or II on pregnancy complications. Notably, the included studies showed high heterogeneity, both clinical as statistical. The discrepancies in the meta-analysis are the result of different screening techniques, varying time points of screening and use of incorrect control groups. Furthermore, analyses of the characteristics of the antibodies involved are essential; these include their (child) specificity, capacity to fix complement, their titer and the HLA epitopes[16] recognized. Large, observational studies, considering these characteristics are necessary to determine whether there is a beneficial or harmful effect of HLA antibodies, or that it is simply an epiphenomenon of any pregnancy.

Acknowledgements

The authors would like to thank Claire la Chapelle for her epidemiological advice, Geert Haasnoot for his help with the statistical analysis and Prof. Dr. T. Stijnen for advice related to the meta-analysis.

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Chapter 6

HLA-C antibodies in women with recurrent miscarriage suggests that antibody mediated rejection is one of the mechanisms leading to recurrent miscarriage

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Journal of Reproductive Immunology 2016;116:28-34

Abstract

Problem

HLA-C is the only polymorphic classical HLA I antigen expressed on trophoblast cells. It is known that higher incidence of C4d deposition on trophoblast cells is present in women with recurrent miscarriages. C4d is a footprint of antibody-mediated classical complement activation. Therefore, this study hypothesize that antibodies against HLA-C may play a role in the occurrence of unexplained consecutive recurrent miscarriage.

Method

Present case control study compared the incidence of HLA-C specific antibodies in 95 women with at least three consecutive miscarriages and 105 women with uneventful pregnancy. In the first trimester of the next pregnancy, presence and specificity of HLA antibodies were determined and their complement fixing ability. The incidence of HLA antibodies was compared with uni- and multivariate logistic regression models adjusting for possible confounders.

Results

Although in general a higher incidence of HLA antibodies was found in women with recurrent miscarriage 31.6% versus in control subjects 9.5% (adjusted OR 4.3, 95% CI 2.0-9.5), the contribution of antibodies against HLA-C was significantly higher in women with recurrent miscarriage (9.5%) compared to women with uneventful pregnancy (1%) (adjusted OR 11.0, 95% CI 1.3-89.0). In contrast to the control group, HLA-C antibodies in the recurrent miscarriage group were more often able to bind complement.

Conclusion

The higher incidence of antibodies specific for HLA-C in women with recurrent miscarriages suggests that HLA-C antibodies may be involved in the aetiology of unexplained consecutive recurrent miscarriage.

Introduction

About 1-3% of all couples are confronted with recurrent miscarriage, which is internationally defined as ≥3 consecutive miscarriages before 20 weeks of gestation.[1] Recurrent miscarriage is a heterogeneous disorder. Possible etiologic factors include uterine anomalies, endocrine disorders, acquired thrombophilia (anti-phospholipid syndrome), hereditary thrombophilia, or balanced translocations in the maternal or paternal DNA.[2,3] However, in many couples no causal factor can be identified.[2,4]

During pregnancy the maternal immune system has to tolerate the persistence of the semi-allogeneic fetal cells. The extravillous trophoblasts, which is in direct contact with maternal cells, expresses only HLA-C and the non-classical HLA-E, -F, and -G. Chimeric fetal cells in the peripheral circulation express all classical HLA class I and II antigens. Approximately 30% of healthy women develop HLA antibodies during pregnancy, the presence of these antibodies increases after 28 weeks of pregnancy and antibodies can still be present at time of a new conception.[5,6] Therefore, the incidence of HLA antibodies in the first trimester is higher in multiparous women than in nulliparous women.[6]

Binding of antibodies to paternal HLA antigens of the fetus might lead to complement fixation and antibody-mediated rejection of the fetus. In women with recurrent miscarriage an increased deposition of C4d, a marker of classical complement activation, was found at the maternal-fetal interface.[7] In spontaneous preterm birth C4d in fetal umbilical cord endothelium was associated with circulating maternal anti-HLA class I antibodies.[8] A recent meta-analysis showed no significant association between HLA antibodies and the occurrence of recurrent miscarriage,[9] however the included studies showed a high level of clinical and statistical heterogeneity. Interestingly, the role of HLA-C specific antibodies has not been studied yet, while from transplantation settings we know that a proportion of allo-antibodies cause rejection, amongst others depending on their ability to activate complement.[10]

We hypothesize that antibody-mediated reactivity plays a role in unexplained recurrent miscarriage. Therefore, the incidence of HLA antibodies, especially those directed against the only polymorphic classical HLA I antigen expressed on trophoblast (HLA-C), is compared between women with recurrent miscarriage and women with uneventful pregnancy.

Material and methods

Subjects

We performed a case control study. The case group was selected from the Habenox trial (NCT0095962),[11] a multicentre multinational trial which investigated thrombophylaxis

(enoxaparin (low molecular weight heparin (LMWH)), aspirin, or combination of both) for women with recurrent miscarriage with and without thrombophilia. The clinical work-up included documentation of a standardised history from the couple, karyotyping of the couple, an ultrasound or hysteroscopy, and thrombophilia screening. Exclusion criteria for the Habenox trial were a history of thromboembolism or bleeding disorders, allergy to aspirin or enoxaparin, uterine anomalies, cervical insufficiency, untreated thyroid disease, poorly regulated diabetes mellitus, parental chromosomal abnormalities, and pregnancies achieved by assisted reproductive techniques. Hereditary thrombophilia was defined by the presence of a factor V Leiden mutation, increased factor II (prothrombin gene mutation), protein C or S deficiency, increased factor VIII, or antithrombin deficiency. Acquired thrombophilia (anti-phospholipid syndrome) as the presence of IgG anticardiolipin antibodies or lupus anticoagulant or IgG β2-glycoprotein I antibodies in repeated samples taken 3 months apart and at least 6 weeks after pregnancy.[12]

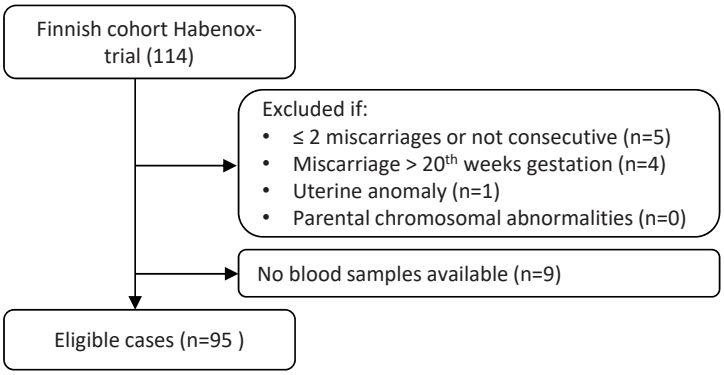
To obtain a more homogenous case group in this case control study, only women with three or more, consecutive miscarriages prior to the 20th week of gestation without uterine anomalies and parental chromosomal abnormalities were selected. Women with hereditary thrombophilia were not excluded, as the evidence that hereditary thrombophilia is associated with recurrent miscarriage is less clear.[3,13]

The presence of HLA antibodies can be considered as a marker for a broader immune response.[14] Although the presence of anti-phospholipid antibodies is highly associated with recurrent miscarriage,[3,15] women with acquired thrombophilia were not excluded since anti-phospholipid antibodies are potential candidates for this broader antibody response. Sensitivity analyses were performed to evaluate the presence of HLA antibodies in women with and without acquired thrombophilia in the case group compared to control subjects.

Both women with primary recurrent miscarriage (no history of live birth) and secondary recurrent miscarriage (history of (a) live birth(s)) were included. The Finnish women were selected, since blood samples were available from this group. Samples were taken at the first antenatal visit before enrolment in the Habenox trial, and every 4 weeks, till the pregnancy ended. This pregnancy was indicated as the index pregnancy. In the Habenox trial, 114 Finnish women were consecutive enrolled from January 2002 until December 2007 and 95 cases were eligible for this case control study (Figure 6.1).

The control group consisted of women who had an uncomplicated index singleton pregnancy, ending in an uncomplicated live birth without congenital abnormalities, delivering at the department of Obstetrics at the Leiden University Medical Centre

Figure 6.1 Flow chart of the case group



(LUMC) in the Netherlands between June 2003 and June 2012. In total 105 women were eligible and consisted of both nulliparous and multiparous women. All these women had no recurrent miscarriage or complicated pregnancy (preeclampsia, Hemolysis Elevated Liver enzymes and Low Platelets syndrome, preterm birth, preterm rupture of membranes, fetal growth restriction, perinatal death, or maternal death defined as described below) in history and current pregnancy. Women were at the time of inclusion healthy individuals with an uneventful medical history. Samples were taken in the first trimester during pregnancy, which was indicated as the index pregnancy.

Variables and definitions

From the cases and control subjects data was collected from medical files. The following data was collected: personal characteristics, intoxications, use of medication, general medical history, information on previous blood transfusions and transplantations, outcome and complications of all previous pregnancies before the index pregnancy, gender of previous children, treatment for previous miscarriages, gestational age at blood sampling, outcome and complications of index pregnancy, medication use during index pregnancy, and neonatal characteristics.

Pregnancy induced hypertension was defined as systolic blood pressure above 140 mmHg and/or diastolic pressure above 90 mmHg, combined with proteinuria as preeclampsia, preterm birth as a delivery between 24 and 37 weeks gestation, fetal growth restriction as birth weight below the 2.3th percentile for gestational age and sex,[16] perinatal death as fetal loss after 22 weeks of gestation till 7 days after birth, and postpartum haemorrhage as blood loss above 1000 mL in the 24hrs postpartum.

Antibody screening

The presence of HLA antibodies was determined in the first trimester (till 13 weeks gestation) of the index pregnancy, both in cases and control subjects. The detection of HLA class I and II antibodies in maternal sera was performed by an enzyme-linked immunosorbent assay (LAT TM, One Lambda, CA) with readouts at 630 nm, detecting both complement fixing and non-complement fixing antibodies. Positive sera (ratio patient/control >2.0) was further tested for HLA antibody specificity by the standard NIH complement dependent cytotoxicity assay[17] against a panel of 54 HLA-typed individuals and for their class I and II specificity with Luminex single antigen beads (Gen Probe, Stamford, CT) following the manufacturer's instructions. Purified anti-human C1q was used in the IgG SAB assay to detect complement fixing antibodies. An median fluorescence intensity >1000 was considered positive as reported elsewhere.[18,19]

Statistical analyses

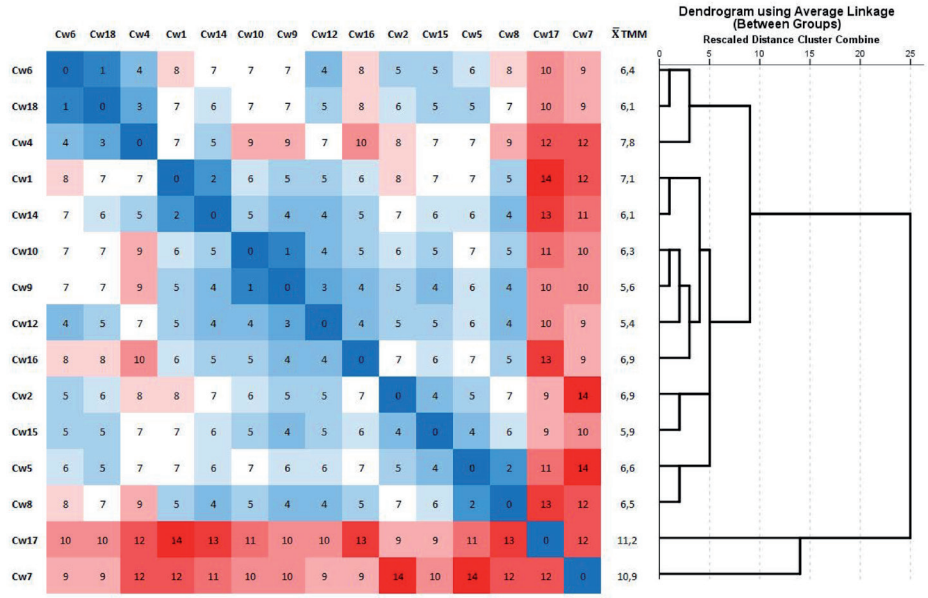
For descriptive analysis of baseline characteristics between cases and control subjects the Mann Whitney U test was performed and for categorical variables the Chi-squared test, if expected counts were less than five Fisher's exact test was used.

HLA antibody presence and specificity (HLA-A, HLA-B, HLA-C, HLA class I and II antibodies) were compared between cases and control subjects.

Bias can arise due to genetic differences between the Finnish case group and the Dutch control group. As we had special interest in HLA antibodies against the most polymorphic antigen on trophoblast the HLA-C antigen, we determined the relative immunogenicity of the different HLA-C antigens using the HLAMatchmaker program developed by Duquesnoy[20] as previously described for HLA-A and HLA-B.[21] The number of epitope (triplet) mismatches was calculated on basis of polymorphic amino acid configurations that represent defined areas of HLA epitopes on protein sequences of HLA-A, HLA-B, and HLA-C chains accessible to allo-antibodies and depicted in a cross-analysis. HLA-C*07 and C*17 showed the highest mean of triplet mismatches within the HLA-C allele (Figure 6.2), suggesting that these are the most immunogenic antigens. The frequencies of the HLA-C alleles in the Finnish and Dutch population were compared using www.allelefrequencies.net and appeared to be comparable i.e. HLA-C*07 (0.30 vs. 0.33) and C*17 (0.022 vs. 0.004) between the Finnish and Dutch population.

Furthermore, potential confounding factors for the presence of HLA antibodies and the occurrence of recurrent miscarriage include an older age, acquired thrombophilia, thyroid autoimmunity, previous pregnancy complications, and having a boy prior to the

Figure 6.2 Cross-analysis of single HLA-C antigen for triplet mismatches shown in a heatmap



The median number of triplet mismatches is colored white, increasing number of triplets are colored red and decreasing are colored blue. Mean triplet mismatches ($\bar{x}$ TMM) of specific HLA-C alleles towards all the other HLA-C alleles and clustering between group using average linking are given.

index pregnancy. More pregnancy complications are observed prior and subsequent to the miscarriages in women with recurrent miscarriage.[22,23] In a recent meta-analysis no beneficial or harmful effect of HLA allo-antibodies was observed in pregnancy, however more studies are necessary to determine definite effect of HLA antibodies on pregnancy outcome,[9] and therefore pregnancy complications could still be a potential confounder for the presence of HLA antibodies. Furthermore, previous studies showed that recurrent miscarriage is more often preceded by a firstborn boy than girl.[24,25] A boy prior to the index pregnancy is related with presence of HLA antibodies,[26] due to the higher incidence of pregnancy complications, if the child preceding the secondary recurrent miscarriage is a boy compared to a girl.[23] Acquired thrombophilia was not measured in the control group, none of the cases and control subjects had thyroid disease, and control subjects were selected on basis of uncomplicated previous pregnancies. All these possible confounding factors, including having a boy prior to the index pregnancy, could not be included in the multivariate analysis. Therefore, the association between the presence of HLA antibodies and recurrent miscarriage was studied with uni- and multivariate logistic regression, adjusting for age as continuous variable in a multivariate logistic regression analysis using the enter method.

In the sensitivity analysis for parity the presence of HLA antibodies was compared between nulliparous, multiparous cases and control subjects. Gravidity was not included in a sensitivity analysis because antibodies are rarely demonstrable before 28 weeks gestation.[5] Of the 50 pregnancies ending in a spontaneous miscarriage, only two women developed antibodies during the index pregnancy.[5] As previous curettages and the gestational age at the time of blood sampling can hypothetically affect the incidence of HLA antibodies, these parameters were included in sensitivity analyses.

Statistical analysis was performed using SPSS Statistics (version 20.0, IL, USA). Descriptive statistical analysis was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, CA, USA, www.graphpad.com).

Ethic statements

Approval to use blood samples for research from the Finnish women in the case group was given (NCT0095962). The determination of HLA antibodies in serum of women with uneventful pregnancies was approved by the Ethic Committee of the LUMC (P13.048).

Results

Baseline characteristics of subjects

No differences between the cases and control subjects were observed with respect to maternal age, BMI, smoking habits, and parity (Table 6.1). In both groups the proportion of nulliparous women was equal. None of the cases and control subjects had blood transfusions or transplantation in their medical history.

In the case group the median consecutive miscarriage rate was 3, while 33 patients (34.4%) had 4 or more consecutive miscarriages. Of the 105 control subjects, 23 women (21.9%) had at least one miscarriage and 12 women (11.5%) had at least one termination of pregnancy before the index pregnancy. Overall no differences were observed in pregnancy complications between cases and control subjects (Table 6.2).

The incidence of HLA antibodies is higher in women with recurrent miscarriage

The incidence of HLA antibodies in the first trimester was significantly higher in women with recurrent miscarriage 31.6% versus in control subjects 9.5% (OR 4.3, 95% CI 2.0-9.5) (Figure 6.3a). After adjustment for age, in multivariate analysis, the estimator of interest did not change (adjusted OR 4.3, 95% CI 2.0-9.5). The contribution of HLA-C

Table 6.1 Baseline characteristics of subjects

	Recurrent miscarriage (N=95)	Uneventful pregnancy (N=105)
Maternal age (years; median[IQR])	32 (29-35)	32 (27.5-35)
BMI (Kg/m^2; median[IQR])$^{\pm}$	22.6 (20.7-25.3)	23.2 (20.7-26.0)
Maternal smoking§	10 (11.2)	9 (8.6)
Blood transfusions	0	0
Transplantation	0	0
Thrombophilia V Leiden mutation Prothrombin gene mutation Protein C or S deficiency High factor VIII Anti-thrombin deficiency Anti-phospholipid syndrome	24 8 2 1 0 0 17	nt
Gravidity (median[IQR])	5 (4-6)[a]	2 (1-3)
Parity (median[IQR])	0 (0-1)	1 (0-1)
Nulliparous	55 (57.9)	51 (48.6)
Previous miscarriages (range)	95 (100) 3-8	23 (21.9) 0-2
Previous termination of pregnancy (range)	6 (6.2) 0-2	12 (11.5) 0-2
Curettage	73 (76.8)[a]	14 (13.3)
Gestation blood sampling (days; median[IQR])	67.5 (44.5-70)[a]	77 (71-84.5)
Having a boy prior to the index pregnancy	26 (65.0)	34 (63)

Data are n (%) unless otherwise indicated, IQR; interquartile range, nt; not tested, BMI; body mass index. All chi-squared tests or Mann-Whitney U-test, $^{\pm}$11% missing values (4 of 95 cases and 18 of 105 controls), §4% missing values (6 of 95 cases and 2 of 105 controls).
[a]compared with women with uneventful pregnancy $p<0.001$.

antibodies to the humoral sensitization was significantly higher in women with recurrent miscarriage i.e. 9 out of 95 cases compared to only one out of 105 control subjects (OR 10.8, 95% CI 1.3-87.6 and adjusted OR 11.0, 95% CI 1.3-89.0) (Figure 6.3b). In 5 out of 9 women with recurrent miscarriage these HLA-C antibodies were C1q fixing antibodies, the HLA-C antibodies found in the women with an uneventful pregnancy were non-C1q fixing antibodies.

As anti-phospholipid antibodies had not been measured in the control subjects, we could not correct in multivariate logistic regression for the presence of anti-phospholipid

Table 6.2 Characteristics of the ongoing index pregnancy in subjects

	Recurrent miscarriage (N=95)	Uneventful pregnancy (N=105)
Live births	60 (63.2)	105 (100)
Gestational age (days;median[IQR])	281 (271-288)	281 (276-287)
Birthweight (gram;median[IQR])	3372.5 (3137.7-3957.5)	3460 (3147.5-3802.5)
Blood pressure (mmHg;median[IQR])¬		
Systolic	120 (115-130)	123 (120-130)
Diastolic	78 (73-90)	78 (71.2-80)
Complications	19 (31.7)	17 (16.2)
Preterm birth	4 (6.8) [a]	0 (0.0)
Fetal growth restriction	0	0
Fluxus	8 (14.3) [b]	1 (1.0)
Pregnancy induced hypertension	10 (16.7)	16 (15.2)
Preeclampsia	1 (1.7)	0
HELPP syndrome	0	0
Perinatal death	0	0
Maternal death	0	0

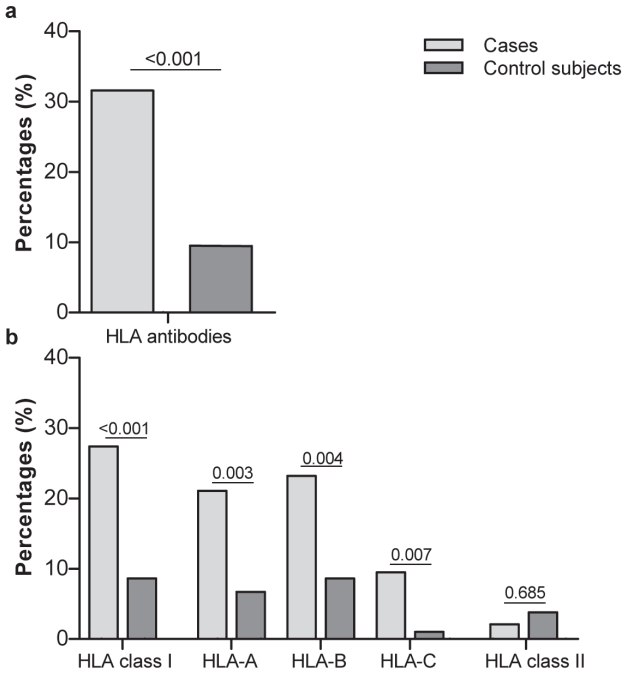
Data are n (%) unless otherwise indicated, IQR; interquartile range, HELLP; Hemolysis Elevated Liver enzymes and Low Platelets. All chi-squared tests or Mann-Whitney U-test, ¬6.0% missing values (9 of 60 cases and 1 of 105 controls).
[a]compared with women with uneventful pregnancy $p<0.05$.
[b]compared with women with uneventful pregnancy $p<0.001$.

antibodies, but even after exclusion of the 17 cases with anti-phospholipid syndrome, the incidence of HLA antibodies was still higher in the recurrent miscarriage group (OR 3.0, 95% CI 1.3-7.0).

Sensitivity analysis for parity showed that HLA antibodies were more often present in nulliparous cases than in nulliparous control subjects (29.1% vs. 0%, $p<0.001$). In nulliparous cases four women had HLA-C antibodies compared to none of the control subjects ($p=0.050$). Furthermore, multiparous cases had significantly more often HLA-C antibodies than control subjects (12.5% vs. 1.9%, $p=0.037$).

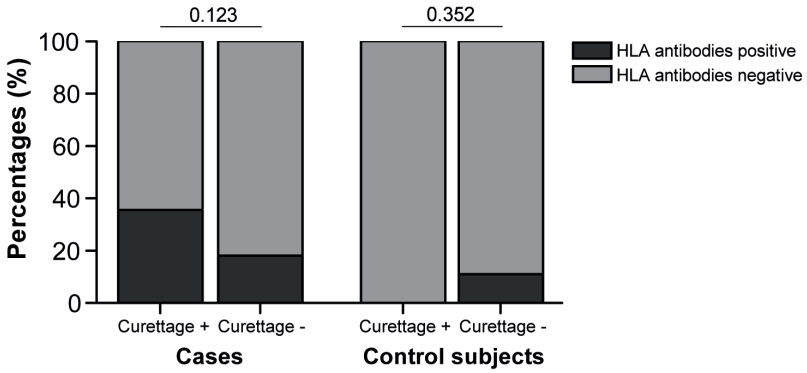
Previous curettage was not associated with an increase of HLA antibodies, neither in cases nor in control subjects (Figure 6.4). In addition, no HLA antibodies were detected in nulliparous control subjects, although 7 control subjects had a termination of pregnancy before the index pregnancy and one control subject had two terminations of pregnancy. In the case group the mean gestational age at the time of blood sampling was comparable in women with and without HLA antibodies (61.4 vs. 58.0 days, $p=0.391$). In the control group gestational age at time of blood sampling was significantly later in women with HLA antibodies (81.8 vs. 76.6 days, $p=0.035$).

Figure 6.3 Presence of HLA antibodies (a) and specific HLA antibodies (b) in cases and control subjects



HLA antibodies in the first trimester were significantly higher in women with recurrent miscarriage 31.6% versus in control subjects 9.5% (a). For specific HLA antibodies, especially those directed against the only polymorphic classical HLA I antigen expressed on trophoblast (HLA-C) were increased. In 9 out of 95 cases HLA-C antibodies were detected compared to only one out of 105 control subjects (b).

Figure 6.4 Presence of HLA antibodies in cases and control subjects with and without previous curettages



No association between the presence of HLA antibodies in the cases or in the control subjects (p=0.123 and p=0.352 respectively).

Discussion

The incidence of HLA-antibodies in the first trimester was found to be significantly increased in women with recurrent miscarriage. Particular relevant for the local situation in the placenta, is the observation that significantly more often HLA antibodies specific for HLA-C are found in women with recurrent miscarriage.

The strength of the study is that, in comparison to other studies,[9] a large homogenous case group of women with a history of recurrent miscarriage were selected. In addition sensitivity analysis for parity was performed. Finally, the detection of the HLA-C specificity of the HLA antibodies is important, considering that HLA-C is the only classical HLA antigen expressed on the trophoblast. Furthermore, by analysing the ability of the HLA-C antibodies to fix complement, a possible link can be made with the previously observed increased C4d deposition on the trophoblast in recurrent miscarriages.[7]

HLA-antibodies in early pregnancy are associated with lower chance of a live birth in women with recurrent miscarriage.[26] However, in our study we could not evaluate presence of HLA antibodies related to pregnancy outcome because the use of medication. In our case group, all women used medication as part of the randomized control trial, i.e. enoxaparin, aspirin, or a combination of these two, during blood sampling for this study, which could influence pregnancy outcome. In vivo studies in mice showed that treatment with heparin prevents complement activation and protects mice from pregnancy complications induced by anti-phospholipid antibodies.[27] Recently, a similar effect of LMWH in women with at least two consecutive miscarriages or one late miscarriage has not been observed,[28,29] however the effect of LMWH in a homogenous group of women with recurrent miscarriage has not been studied yet.

Furthermore, the HLA typing of the fetus or products of conception from the index pregnancy was unknown, which made it impossible to determine whether the HLA antibodies were specific for the index pregnancy. Future research in a different group of women with recurrent miscarriage, without using medication, should focus on the presence of HLA antibodies in first trimester, whether these antibodies are child specific, and relate this to pregnancy outcome.

The fact that the gestational age at blood sampling was significantly higher in control subjects than in cases, could have affected the results. However, a later gestational age is supposed to be associated with a higher production of HLA antibodies,[5] whereas less HLA antibody production was seen in the controls subjects, suggesting that the observed difference might even be an underestimation.

The presence of HLA antibodies can be considered as a marker for a broader immune response, as was previously shown in HLA identical family transplantations, where the presence of HLA antibodies was a risk factor for worse outcome, although HLA antibodies themselves could not have caused any harm.[14] Anti-phospholipid antibodies, which are highly associated with recurrent miscarriage,[3] are potential candidates for this broader antibody response. No correction could be made in multivariate logistic regression for the presence of anti-phospholipid antibodies since these antibodies were not measured in the control subjects. However, even after exclusion of cases with anti-phospholipid syndrome, a significant association between the presence of HLA antibodies and occurrence of recurrent miscarriage was present.

Some studies suggest that the presence of specific antibodies directed to the paternal HLA could be beneficial for a pregnancy by enhancing the development of maternal-fetal immunologic tolerance,[30-34] others demonstrate that HLA antibodies against the paternal antigens are rarely demonstrable before 28 weeks gestation in uneventful pregnancy,[5] which is in line with our finding that none of the nulliparous control subjects had HLA antibodies. In contrast, almost one third of the women with primary recurrent miscarriage produced HLA antibodies in the first trimester. The presence of HLA-C antibodies in women with primary recurrent miscarriage may partly explain the high incidence of C4d found in women with primary recurrent miscarriage shown previously.[7] However, from transplantation settings it is known that not all allo-antibodies cause rejection and that their ability to activate complement might play a determinative role.[10] The majority of the HLA-C antibodies in the case group were able to fix complement, whereas no complement fixing HLA-C antibodies were found in the control group.

In conclusion, in this study which included a homogenous well-defined group of women with recurrent miscarriage, a higher incidence of HLA antibodies was observed compared to women with an uneventful pregnancy. Especially, the presence of complement fixing HLA-C antibodies in relation to the selective expression of HLA-C on trophoblast tissue might explain the aetiology in a proportion of the women with recurrent miscarriage.

Acknowledgements

The authors would like to thank Sophia Stein and Simone Brand-Schaaf for her help with antibody screening and Robert-Jan Meerman for his help with data collecting of the control subjects.

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Chapter 7

Increased complement C4d deposition at the maternal-fetal interface in unexplained recurrent miscarriage

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Journal of Reproductive Immunology 2015;113:54-60

Abstract

Problem

C4d is a footprint of antibody-mediated classical complement activation, and has evolved as a useful diagnostic marker of antibody-mediated rejection. It is unknown if complement activation, as reflected by C4d deposition plays a role in unexplained recurrent miscarriage.

Method

In a case-control study products of conception of 35 women with three or more unexplained consecutive miscarriages within 20 weeks of gestation with the same partner (case group), 22 women with one spontaneous sporadic miscarriage and no history of complicated pregnancy(ies) (control group 1), and 40 women who underwent an elective abortion for psychosocial reasons (control group 2) were included. Immunohistochemical staining for C4d was performed on products of conception. Positivity for C4d was scored semi-quantitatively.

Results

C4d deposition was present in products of conception of 14 out of 35 women with unexplained recurrent miscarriage (40.0%), compared to 6 out of 22 women with a sporadic miscarriage (27.3%), and 4 out of 40 women with an elective abortion (10.0%) (p=0.020).

Conclusion

C4d is increased at the maternal-fetal interface in women with unexplained recurrent miscarriage, which may reflect an aberrant anti-fetal immunity in these women. Further knowledge of the specific pathogenic mechanism may lead to the development of new treatment strategies for this group of women.

Introduction

About 1-3% of all couples are confronted with recurrent miscarriage, which is defined as ≥3 consecutive miscarriages before 20 weeks of gestation.[1] Recurrent miscarriage is a heterogeneous condition. Possible etiologic factors for recurrent miscarriage include uterine anomalies, endocrine disorders, acquired thrombophilia (antiphospholipid syndrome), hereditary thrombophilia, or balanced translocations in the maternal or paternal DNA.[2,3] However, in many couples no causal factor can be identified.[2,4] This uncertainty has a major impact on women and their partners.[5] For clinicians, the lack of etiological insight and nonexistent evidence based therapeutic interventions makes the management of these women complex and sometimes frustrating.

In analogy with solid organ transplantation it has been hypothesized that unexplained recurrent miscarriage is a form of maternal anti-fetal allograft rejection.[6] In transplantation, antibody mediated rejection has gained much attention since the discovery of C4d. C4d is a non-functional split product of classical complement activation that covalently attaches to cells and tissues, thereby acting as a footprint of recent antibody mediated tissue injury.[7-9]

In unexplained recurrent miscarriage complement activation could be caused by excessive allo- or auto-antibody reactivity. Auto-antibodies could for instance be antiphospholipid-like antibodies that are not picked up by current assays. Allo-antibodies could be anti-HLA antibodies directed to paternal HLA antigens of the fetus. We hypothesize that if an ongoing antibody-mediated process is responsible for miscarriage, C4d should be present at the maternal-fetal interface. A recent study showed more C4d deposition in products of conception of women with two or more previous miscarriages compared to sporadic miscarriages.[10] In this study it was not clear whether the miscarriages of the small study group were unexplained, consecutive and the same partner was involved.

We therefore investigated the incidence of C4d deposition on trophoblast tissue in a well-defined homogenous large case group of women with at least three consecutive unexplained recurrent miscarriages within 20 weeks of gestation with the same partner compared to control subjects.

Material and methods

Subjects

We studied products of conception of 97 women, which were available in the archives of the pathology department of the Leiden University Medical Center (LUMC), the

Netherlands. They were divided into three groups: a case group of 35 women with recurrent miscarriage, a first control group of 22 women with only one spontaneous miscarriage (sporadic miscarriage group), and a second control group of 40 women who underwent elective abortion for psychosocial reasons (elective abortion group). In 12 of the latter control subjects, the tissue samples were derived from an abortion clinic.

The case group consisted of women with recurrent miscarriage who visited the recurrent miscarriage clinic of the department of Obstetrics and Reproductive Medicine at the LUMC in the Netherlands, from 2000 onwards. The clinical work-up includes documentation of a standardised history from the couple, karyotyping of the couple, a standard blood check including glucose, and thyroid function and antibodies, thrombophilia screening, and an ultrasound or hysteroscopy. Some of these women participated in the Habenox trial (NCT0095962).[11] In the Habenox trial women were randomly allocated to three intervention groups. The first group received enoxaparin 40mg (low molecular weight heparin (LMWH)), the second group enoxaparin 40mg and aspirin 100mg, and the third group received aspirin 100mg.

To obtain a homogenous case group, we only included women with at least three consecutive miscarriages within 20 weeks of gestation with the same partner. Both women with primary recurrent miscarriage (no history of live births) and secondary recurrent miscarriage (history of (a) live birth(s)) were included. As parental chromosomal abnormalities, acquired thrombophilia, uterine anomalies, and thyroid autoimmunity are associated with recurrent miscarriage,[3,12,13] women with at least one of these factors were excluded. Acquired thrombophilia was defined as the presence of IgG anticardiolipin antibodies or lupus anticoagulant or IgG β2-glycoprotein I antibodies in repeated samples taken 3 months apart and at least 6 weeks after a delivery or miscarriage.[14] Women with hereditary thrombophilia or hyperhomocysteinemia were not excluded, as the evidence that hereditary thrombophilia or hyperhomocysteinemia is associated with recurrent miscarriage is less clear.[3,15] Hereditary thrombophilia was defined by the presence of a factor V Leiden mutation, factor II (prothrombin) gene mutation, protein C or S deficiency, or antithrombin deficiency.

The first control group included 22 healthy women who experienced a spontaneous miscarriage and had no history of recurrent miscarriage or complicated pregnancies as preeclampsia, Hemolysis Elevated Liver enzymes and Low Platelets (HELLP) syndrome, preterm birth, intra-uterine growth restriction (IUGR), or perinatal death. Preeclampsia was defined as systolic blood pressure above 140 mmHg and/or diastolic pressure above 90 mmHg combined with proteinuria,[16] preterm birth as a delivery between 24-37 weeks gestation, intra-uterine growth restriction as birth weight below the 10th percentile for

gestational age and sex according to the Netherlands Perinatal Registry birth weight percentiles,[17] perinatal death as fetal loss after 22 weeks of gestation till 7 days after birth.

The second control group included 40 women with elective abortion due to psychosocial indications.

Tissue samples of products of conception

All products of conception, except for 12 tissue samples received from the abortion clinic in Leiden, were collected in the LUMC. In 52 out of 57 women with recurrent miscarriage and women with a sporadic miscarriage an ultrasound was performed (91.3%) to detect a fetal demise (68.5%) or blighted ovum (12.3%), and in 10.5% ultrasound findings were unknown. In 95 out of 97 women (97.9%) a curettage was performed by a gynecologist either at the LUMC (83 cases) or at the abortion clinic (12 cases). Two women used vaginal misoprostol and collected products of conception at home. In 93% of the cases products of conception were fixed in 4% buffered formalin and embedded in paraffin within 48 hours at the department of Pathology in the LUMC, in two cases products of conception were fixed within three days, one case within four days, and one case within five days.

Sequential serial sections (4µm-thick) were cut on adhesive coated glasses and dried overnight at 37°C. Paraffin sections were routinely stained with HE. To study classical complement activation, immunohistochemically staining was performed for C4d (BI-RC4d, Biomedica Gruppe, Austria). In short, tissue sections were deparaffinized and hydrated by xylene and decreasing alcohol concentration to demi-H_2O. Optimal antibody dilutions and incubation times for the different antibody were pre-determined by means of titration on positive control sections. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 minutes. After a wash step with demi-H_2O, antigen retrieval was performed by boiling the sections for 10 minutes in 10mM/1mM TRIS/EDTA (pH9.0). A polyclonal rabbit anti-human C4d antibody (Biomedica Gruppe, Austria), was applied at a dilution of 1:80 in 1% BSA/PBS, and slides were incubated for one hour at room temperature. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) as a chromogen and Demi-H_2O was used to stop the reaction. Subsequently tissue sections were counterstained with Haematoxylin (SIGMA, Switzerland, Steinheim). The slides were mounted in mounting medium (Surgipath Medical Ind., Inc. Richmond) and covered. A tissue sample from a placenta of a woman with preeclampsia with C4d-positive staining used in a previous study served as a positive control.[18] As negative control Rabbit immunoglobulin fraction

(Solid-Phase Absorption, X0936, Dako) was used. Four batches were needed to stain all slides, and in every single batch positive and negative controls were included.

Quantification of morphology and immunohistopathology

Sections were independently evaluated by two experienced observers (TM and GS), who scored the sections blinded to the clinical data of the women. Differences in scorings were resolved by re-reviewing the sections with a third observer to obtain consensus (KV). In three cases the third observer (KV) was involved in the evaluation in order to obtain consensus. From most products of conception only one section was available, if more sections were available, we scored every single section and combined the results of these sections. C4d stains brown and was defined as positive when present at the maternal-fetal interface, on the maternal side of the syncytiotrophoblast suggesting a maternal origin of complement activation. The degree of C4d deposition of the section(s) was scored semi-quantitatively. The different staining patterns are shown in Figure 7.1.[19]

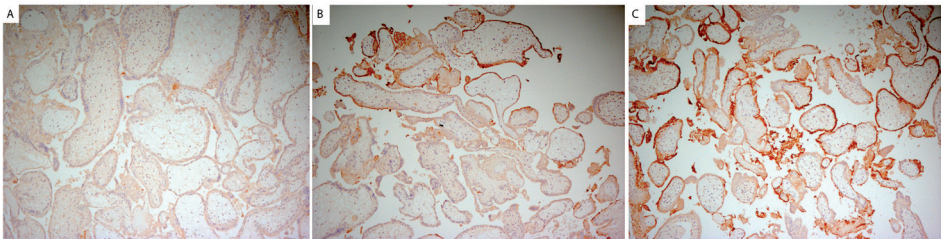
Because direct binding of C1q to apoptotic cells can cause C4d deposition,[20] we performed a subgroup analysis of C4d presence in non-apoptotic trophoblast cells. All trophoblast cells were counted and the percentages of non-apoptotic and apoptotic trophoblast cells were determined (Figure 7.2). Only sections with at least 10 non-apoptotic trophoblast cells were included. From 97 women, who were eligible, 8 women (1 from case group, 1 from first control group, 6 from second control group) had to be excluded for this subgroup analysis, because sections did not contain sufficient non-apoptotic trophoblast cells. For this subgroup analysis C4d deposition exclusively on non-apoptotic trophoblast cells was defined per women.

Statistical analysis

For descriptive analysis of baseline characteristics the Mann Whitney U test was performed and for categorical variables the Chi-square test, if expected counts were less than five Fisher's exact test was used.

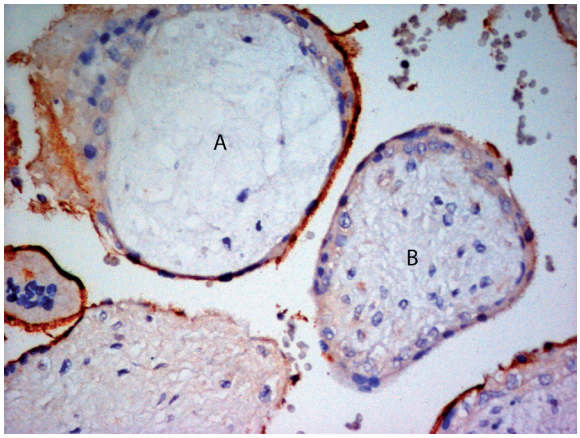
C4d deposition was compared between cases and control subjects using the Chi-square test and its trend version (linear-by-linear analysis) was used in analyzing C4d staining divided in absent, focal, or diffuse C4d staining. All analyses were performed using SPSS statistical software package (version 20.0; IL, USA). A *p*-value less than 0.05 was considered statistically significant. Descriptive statistical analysis was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, California, USA, www.graphpad.com).

Figure 7.1 Examples of immunohistochemical staining patterns of C4d



A. Absent C4d staining, 0-10% of vital trophoblast cells showing linear C4d staining per section.
B. Focal C4d staining, 10-50% of vital trophoblast cells showing linear C4d staining per section.
C. Diffuse C4d staining, >50% of vital trophoblast cells showing linear C4d staining per section.

Figure 7.2 Typical example of an apoptotic trophoblast cell



An apoptotic trophoblast cell (A) and a non-apoptotic trophoblast cell (B). Trophoblast cells containing few nuclei, which were hydropic, and had ruptures in their cell membrane were defined as apoptopic trophoblast cells.

Ethics statement

All tissue samples were handled in a coded and anonymized fashion, according to the Dutch National Ethical guidelines (Code for Proper Secondary Use of Human Tissue).[21] This national guideline enables scientists to perform research with human material that came available within the framework of patient care. Consequently, when properly coded and anonymized, human material can be used for research purposes without patient's informed consent and without additional approval by an ethics committee.

Table 7.1 Baseline characteristics

	Recurrent miscarriage (N=35)	Sporadic miscarriage (N=22)	Elective abortion (N=40)^
Maternal age (years;median[IQR])	31 (28-36)[b]	31.5 (24.7-36.2)	26 (20-31)
Thrombophilia (n)	4	na	na
Factor V Leiden mutation	1		
Factor II (prothrombin) gene mutation	2		
C or S protein	1		
Antithrombin	0		
Hyperhomocysteinemia	5		
Gravidity (median[IQR])~	7 (5-8)[a, c]	3 (2-3.2)	2 (1-3)
At time of index pregnancy (median[IQR])~	4 (3-6)[a, c]	2 (1-3)	2 (1-3)
Parity (median[IQR])~	2 (1-2)[b]	1.5 (1-2.2)	1 (0-1.5)
Live births before index pregnancy			
(n(%))~	18 (51.4)	12 (54.5)	9 (42.8)
(median[IQR])	0.5 (0-1)	1 (0-2)	0 (0-1.5)
Miscarriages (median[IQR])	5 (4-6)[a, c]	1 (1-1)	0
Miscarriages before index pregnancy			
yes/no (n(%))	32 (91.4)	0	1 (3.6)
(median[IQR])	2 (1-4)		
Gestational age at miscarriage or abortion (days; median[IQR])	64 (59-73)[b]	72.5 (66-79)	56 (43.7-70)
Time between determination with ultrasound of fetal demise and actual fetal loss (days; median[IQR])*	6 (4-12)	5 (2-12)	na
Fixation within 24 hours after curettage or abortion			
(n(%))	25 (71.4)	14 (63.6)	23 (82.1)
(days; median[IQR])	0 (0-1)	0 (0-1)	0 (0-0)
Non-apoptotic trophoblast cells			
(%; median[IQR])	50 (30-75)[b]	52.5 (27.5-71.2)	37.5 (18.7-60.0)
(number; median[IQR])	142.5 (33-247)	183.7 (33.7-434.2)	67.5 (17-158.7)
Fetal loss (n(%))			
Spontaneous	0	0	0
Curettage	30 (85.7)	22 (100)	40 (100)
Vaginal misoprostol	2 (5.7)	0	0
Curettage because unsuccessful vaginal misoprostol	3 (8.6)	0	0

^In all variables, except for gestational age, clinical data of 12 control subjects of which tissue samples were received from the abortion clinic is missing, ~25.0% missing values (7 out of 28 control subjects with elective abortion in the Leiden University Medical Centre), *22.8% missing values (4 out of 35 patients with recurrent miscarriage and 7 out of 22 control subjects with sporadic miscarriage), IQR; interquartile range, na; not available, na; not applicable. [a]compared with elective abortion $p<0.001$. [b]compared with elective abortion $p<0.05$. [c]compared with sporadic miscarriage $p<0.001$.

Results

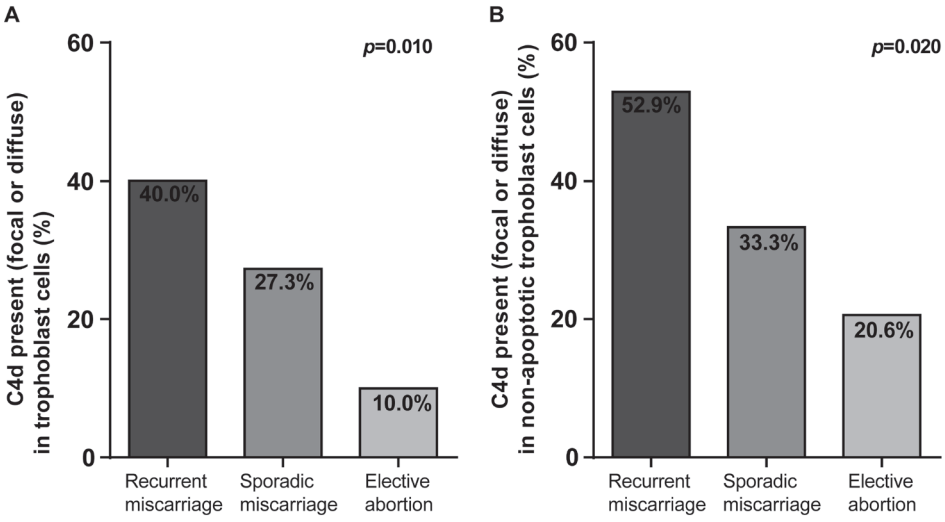
Clinical characteristics

Table 7.1 shows the clinical characteristics between the case and the control groups. In the case group median maternal age at the time of the third consecutive miscarriage was 31 years. Five women with recurrent miscarriage, participating in the Habenox trial, were treated with anticoagulant therapy; a prophylactic dose of LMWH in one case (2.8%), aspirin in two cases (5.7%), a combination of both in two cases (5.7%). LMWH was administered to one woman with a prothrombin gene mutation and to one woman experimentally. None of the women with sporadic miscarriage used any medication during pregnancy. In the elective abortion group this information was not available, but it is very unlikely that these women used anticoagulants.

C4d deposition

C4d deposition was present in 14 out of 35 women with recurrent miscarriage (40.0%), compared to 6 out of 22 women with a sporadic miscarriage (27.3%), and 4 out of 40 women with an elective abortion (10.0%) (p=0.010) (Figure 7.3A). In addition, C4d staining

Figure 7.3 Presence of C4d in women with unexplained recurrent miscarriage, women with sporadic miscarriage and women with elective abortion



Presence of C4, including focal and diffuse C4d staining, on trophoblast cells (A) and non-apoptotic trophoblast cells (B).

patterns differed significantly among the three groups in a chi-square linear-by-linear analysis (p=0.006) (Table 7.2).

In 47.1% of women with primary recurrent miscarriage C4d deposition was found compared to 33.3% women with secondary recurrent miscarriage (p=0.407). C4d was present in 47.1% of women with primary recurrent miscarriage compared to 13.6% of women without a prior live birth in the control groups (p=0.033).

Analysis of the case group showed that in 44.4% of the women with thrombophilia or hyperhomocysteinemia C4d was present compared to 38.5% of women without thrombophilia or hyperhomocysteinemia (p=1.000). In 57.1% of women using anticoagulants (LMWH, aspirin, or a combination) C4d was found compared to 35.7% of women using no anticoagulants. Significantly more women with thrombophilia or hyperhomocysteinemia used anticoagulants compared to women without thrombophilia or hyperhomocysteinemia (44.4% vs. 11.5%, p=0.033). After exclusion of women with thrombophilia or hyperhomocysteinemia (9 women), or women using medication in the case group (7 women), C4d deposition in women with recurrent miscarriage compared to control subjects remained significantly different (respectively p=0.022, p=0.035).

The subgroup analysis on C4d deposition and C4d staining patterns exclusively on non-apoptotic trophoblast cells also showed significant differences between women with recurrent miscarriage and control subjects (Figure 7.3B) (Table 7.3).

Table 7.2 Recurrent miscarriage and C4d staining patterns in trophoblast cells

	Absent C4d deposition	Focal C4d deposition	Diffuse C4d deposition	Total
Recurrent miscarriage n(%)	21 (60.0)	10 (28.6)	4 (11.4)	35
Sporadic miscarriage n(%)	16 (72.7)	2 (9.1)	4 (18.2)	22
Elective abortion n(%)	36 (90.0)	3 (7.5)	1 (2.5)	40

p=0.006 in a chi-square linear-by-linear association analysis.

Table 7.3 Recurrent miscarriage and C4d staining patterns in non-apoptotic trophoblast cells

	Absent C4d deposition	Focal C4d deposition	Diffuse C4d deposition	Total
Recurrent miscarriage n(%)	16 (47.1)	12 (35.3)	6 (17.6)	34
Sporadic miscarriage n(%)	14 (66.7)	3 (14.3)	4 (19.0)	21
Elective abortion n(%)	27 (79.4)	6 (17.6)	1 (2.9)	34

p=0.006 in a chi-square linear-by-linear association analysis.

Discussion

Recurrent miscarriage is a devastating complication of pregnancy. In many couples no underlying cause can be found.[2,4] For many years it has been questioned whether the fetus can indeed be considered as an 'allograft' and, as a consequence, miscarriage as 'rejection'.[2,22] The present study shows that C4d deposition at the maternal side of the syncytiotrophoblast was significantly increased in women with unexplained consecutive recurrent miscarriages compared to women with a sporadic miscarriage and with an elective abortion. This confirms and extends earlier observations that antibody-mediated rejection of the fetal allograft may be present in pregnancy complications.[10,23]

Strengths of this study are that C4d deposition was examined in products of conception of a well-defined homogenous case group of women with at least three consecutive unexplained recurrent miscarriages with the same partner within 20 weeks of gestation and compared to control subjects with sporadic miscarriage without complicated pregnancy history and women with elective abortion for psychosocial indication. Such a clear definition was not used in a previous study by Lee et al.[10]

As C1q can cause C4d deposition on apoptotic cells,[20] a subgroup analysis was performed on C4d deposition on non-apoptotic trophoblast cells in order to test for additional pathways leading to C4d deposition.

It is suggested that recurrent miscarriage is caused by failure of endometrial selectivity of impaired embryos,[24] possibly leading to more aneuploidy embryos in women with idiopathic recurrent miscarriage.[25] In a recent study it was suggested that complement activation is a common mechanism of placental and fetal injuries regardless of chromosomal integrity.[10] Indeed C4d deposition along the trophoblast was found to be similar in chromosomally normal and abnormal miscarriages,[10] indicating that increased complement activation in recurrent miscarriage is not dependent on chromosomal aneuploidies and demonstrates a different underlying pathophysiology.

Complement deposition can be either interpreted as a sign of local dysregulation of the placental complement system or as excessive complement activation for example caused by allo- or auto-antibody deposition.[23,26]

Auto-antibodies like antiphospholipid antibodies are possible candidates, as we have recently demonstrated that C4d is abundantly present in placentas of women with autoimmune mediated pregnancy losses caused by systemic lupus erythematosus (SLE) and antiphospholipid syndrome.[23] Therefore, all women in our recurrent miscarriage population were tested for IgG anticardiolipin antibodies and lupus anticoagulant and if these were present the women were excluded from the study.

On the other hand, allo-antibodies, directed against inherited paternal HLA antigens expressed on trophoblast cells, could play a role. Lee et al. showed that in spontaneous preterm birth the presence of C4d in fetal cord endothelium was associated with circulating maternal anti-HLA I antibodies.[27] After a live birth around 30% of healthy women have circulating anti-HLA antibodies and these antibodies can still be present at time of conception.[28,29] The incidence of C4d in products of conception i.e. 27.3% in the combined control group with a prior live birth and in 33.3% of women with secondary recurrent miscarriage is almost similar to the incidence of HLA antibodies after pregnancy. We found, interestingly, the highest incidence of increased C4d staining in women with primary recurrent miscarriage (47.1%), where we would expect the lowest circulating maternal HLA antibodies. It is still not clear whether HLA antibodies predispose to a higher risk of adverse pregnancy outcome.[30] Therefore, it remains to be established whether the increased C4d deposition especially in women with primary recurrent miscarriage is due to an increased incidence of HLA antibodies. From transplantation settings, we know that only a proportion of allo-antibodies cause rejection, amongst others depending on their ability to activate complement and their avidity for the antigenic target.[31] The role of auto- or allo-antibodies in the C4d deposition in recurrent miscarriage should be subject for further studies.

Under physiological conditions the placenta is strongly protected from spontaneous complement activation by regulatory mechanism such as Decay Accelerating Factor (DAF), Membrane Cofactor Protein (MCP), and CD59.[32-34] Therefore, increased complement deposition can also be interpreted as a sign of local dysregulation of the complement system.[23,26] In a recent cohort study up to 19% of women with SLE and antiphospholipid syndrome, who developed severe preeclampsia, had mutations in complement regulatory genes, leading to inadequate inhibition of complement activation at the maternal-fetal interface.[35] The excessive C4d deposition in placental tissue of some of our patients is in line with the concept, that genetic defects in complement regulation may cause recurrent miscarriage. However, in a recent study mutations in the Membrane Cofactor Protein (MCP) gene were found not to be associated with recurrent miscarriage.[36]

At present there is no evidence based treatment for women with unexplained recurrent miscarriage. A proportion of women in the present study were using LMWH, aspirin, or a combination of both at time of miscarriage, but we did not find any relation between use of anticoagulants and presence of C4d. These results should be interpreted carefully as significantly more women with thrombophilia were using medication and therefore it seems that the results on medication are confounded. Defects in thrombophilia genes could lead to disruption of endothelium or inflammation resulting in activation of the complement cascade.[37,38]

Unexplained recurrent miscarriage is probably not due to a single cause. Further unraveling possible pathophysiological mechanisms for unexplained recurrent miscarriage in order to define patient tailored treatment strategies is essential. Studies by Girardi et al., show that heparin is beneficial in case of antiphospholipid antibodies because it inhibits complement activation, and not because of its effects on the coagulation cascade.[39] If complement activation at the maternal-fetal interface indeed plays a role in a subgroup of women with recurrent miscarriage mediated by impaired placentation, treatment with inhibitors of the complement cascade such as LMWH, statins,[40] or biologicals would be advocated.[41] The presence of C4d in products of conception may potentially serve as a diagnostic indicator for a next pregnancy, making this subgroup of women eligible for treatment with complement inhibitors in the following pregnancy.

Acknowledgements

The authors would like to thank Prof. Jan van Lith for his critical reading and valuable feedback on earlier versions of the manuscript.

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Chapter 8

The immunomodulating effect of seminal plasma on T cells

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Journal of Reproductive Immunology 2015;110:109-116

Abstract

Problem

Seminal plasma (SP) contains immunomodulatory factors, which may contribute to the formation of a tolerogenic environment at the embryo implantation site. The main focus of this study was to investigate the influence of SP on female T cells in the presence and absence of antigen presenting cells (APCs) in an in vitro model.

Method

Female PBMCs and T cells were incubated with SP from seminal fluid samples of known and variable sperm quality. The immediate effect of SP on the mRNA expression of CD25, IL-10, IFN-γ, and Foxp3 was measured. Furthermore proliferative responses, cytokine production, and CD25 expression was determined.

Results

Exposure to SP leads to an increased mRNA expression of CD25, IL-10, and Foxp3 in T cells. Induction of mRNA for IL-10 and CD25 was dependent on the presence of APCs. Both PBMCs and T cells exposed to SP, showed a proliferative response and produced several cytokines. The observed proliferative effects of SP on T cells were independent of sperm quality parameters, cytokines, or soluble HLA molecules in SP. Furthermore, presence of SP induced a higher expression of CD25 on the membrane of CD4+ T cells.

Conclusion

SP has a direct immunomodulatory effect on T cells as reflected in a proliferative response and upregulation of Foxp3. The presence of APCs is needed to induce IL-10 and CD25 upregulation in T cells exposed to SP. In conclusion, SP has both a direct and an indirect effect mediated through APCs on T cells.

Introduction

During pregnancy the maternal immune system has to tolerate the presence of the semi-allogeneic fetus. Allorecognition takes place at the site of embryo implantation, where trophoblast cells invade and are confronted with maternal PBMCs.

In mice regulatory T cells (Tregs) usually implicated in maintenance of tolerance to self-antigens,[1] are present in the decidua and responsible for maternal tolerance to fetal allo-antigens.[2,3] Also in human, Tregs are increased in the decidua during pregnancy.[4] In women with complicated pregnancies, decreased numbers of Tregs were found in the decidua[5] and placenta[6] suggesting that these Tregs play a pivotal role in uncomplicated pregnancies.

Other studies in mice have shown that already during copulation, long before implantation, maternal tolerance towards fetal allo-antigens is induced.[7,8] In fact allo-antigens are present in human SP[9,10] and may be responsible for the Treg expansion as was previously shown in mice.[11] In addition, within two days after insemination, Tregs with an upregulation of Foxp3 expression can be found in the draining lymph nodes in mice.[2]

Dendritic cells (DCs), highly present in the decidua are partly responsible for this antigen specific Treg expansion.[12] SP contains also a large variety of cytokines, which may modulate the maternal immune response.[13,14] TGF-β is highly present in human SP and is thought to inhibit a type 1 immune response against the semi-allogeneic fetus by initiating a type 2 or Treg-dominated immune response associated with partner-specific tolerance.[15] In addition, TGF-β elicits expression of pro-inflammatory cytokines as IL-6 and GM-CSF in human cervical epithelial cells,[14] which may also contribute to improved antigen presentation by DCs.[12]

In humans most of SP is deposited at the cervix,[16] where it may affect the function of multiple cell types, including immune cells and the endometrium.[17] Balandya et al. showed that exposure of human PBMCs to SP resulted in an increased intracellular expression of markers of Tregs and TGF-β.[18]

To aim of the present study was to study the immunomodulating effect of SP on human T cells. To investigate the direct effect of SP on T cells, T cells were enriched and isolated from female PBMCs (fPBMCs) and the effect of SP on mRNA expression of CD25, IL-10, IFN-γ, Foxp3 was measured. Furthermore the proliferative response and cytokine production was measured. As a T cell response is often the result of the interaction between T cells and APCs, the possible role of APCs was studied as well.

Subjects and methods

SP samples

In all couples with infertility for at least one year visiting the reproductive medicine clinic at the Leiden University Medical Center (LUMC), an exploratory study of fertility is performed, which includes determination of sperm quality (volume, concentration, motility, morphology, and viscosity). In this study sperm quality was defined as VCM: (volume x concentration x motility)x10^6.[19] Low quality was defined as a VCM below 10x10^6 and high quality as a VCM above 100x10^6. SP samples were collected by masturbation, and sperm quality was assessed the same day. All males were HIV-negative and asymptomatic for Chlamydia trachomatis and Neisseria gonorrhoeae. In addition, SP samples containing leukocytes, as a marker for infection, were excluded from this study.

Within two hours after determination of the quality, samples were centrifuged at 2,000rpm for 10 minutes, the sperm was discarded and only SP was stored at -20° C. For the cultures, samples were thawed at room temperature and centrifuged at 14,000rpm for 4 minutes.

Messenger RNA (mRNA) transcript analysis

Cultures were performed to demonstrate the effect of SP on fPBMCs or T cells (CD3+ fraction) either enriched by depletion of non-T cells from fPBMCs according to magnetic cell sorting (Pan T Cell isolation Kit II, no. 130-091-156, MACS) or isolated. In short the procedure to isolate T cells, APCs were depleted from PBMCs. PBMCs were stained for CD14-FITC, CD19-FITC, CD40-FITC, CD56-FITC, CD36-FITC (Beckton Dickinson, New Jersey, USA). FACS (FACS-Aria II with FACS-Diva software, Beckton Dickinson) sorted into a viable CD45+ population depleted for all FITC stained cells and washed with culture medium containing RPMI 1640 with 10% human serum and L-glutamine. In order to confirm that this procedure indeed leads to the depletion of APCs fractions were stained with CD14-FITC, CD19-PE, CD3-PercCP, CD45-APC (Beckton Dickinson). We added the autologous APCs fractions to the isolated T cells.

fPBMCs were selected from a panel of healthy HLA typed volunteers, who, after informed consent, donate blood for transplantation and pregnancy related research. SP used in these cultures was from unrelated men.

fPBMCs, enriched T cells, isolated T cells, or isolated T cells with addition of autologous APCs (500 µl of 2x10^6 per ml) were separately cultured with 500 µl of SP (1:100) or with culture medium (negative control) in round-bottom 24-well plates (Greiner Bio-one) for 1 day, and stored in 50 µl of RNA*later* (RNA stabilization Buffer, Qiagen, Venlo,

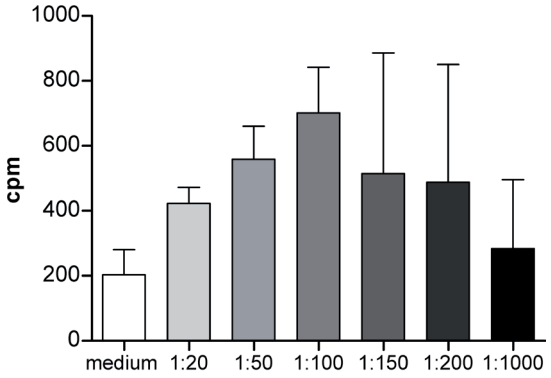
the Netherlands) at -20°C. RNA extraction was performed using NucleoSpin columns (Macherey-Nagel, Düren, Germany). To synthesize cDNA, RNA was combined with oligo dT (Invitrogen; 0.25mg) and random nucleotide hexamers (Invitrogen; 0.25mg), and incubated at 65°C for 5 min.[20] SuperScript III RT (Invitrogen; 200 U), 0.5mM dNTP, 40U of RNAse OUT rRNAse inhibitor, and 5mM DTT were added on ice. Reactions were allowed to proceed at 25°C for 5 min and at 50°C for 1 hour. Reactions were terminated at 70°C for 5 min. PCR assays were carried out using iQ™ SYBR® Green Supermix and a MyiQ Real-Time PCR detection system (Bio-Rad). The PCR program consisted of 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Primers pairs were selected in the coding sequence of the mRNA transcripts using Primer 3 (v. 0.4.0; SourceForge). Primers spanned at least one intron with a size of 800 bp or more. To ensure high specificity, primer sets were tested on control cDNA (from Human Reference Total RNA; Clontech, Mountain View CA, US) and genomic DNA. The expected size of the amplicons was checked on agarose gels. Efficiency of each PCR assay was 90110%. A final melting curve analysis during the PCR runs was performed to check assay specificity.

Levels of mRNA transcripts for CD25, Foxp3, IL-10, IFN-γ were normalized to the geometric mean signal of reference genes GAPDH and β-actin. In five different qPCR experiments, Cq values of the reference genes in the samples highly correlated with each other (r=0.91±0.06). Signals of individual targets were standardized using the ΔΔCq method and the formula $2^{-(Cq\,[transcript]-AVG\,Cq\,[references])}$. Sequences for the transcripts investigated have been previously described.[20,21]

Functional analysis

Cultures were set up with 50µl of $1x10^6$ concentration per ml enriched T cells or fPBMCs in culture medium added in triplicate wells in round-bottom 96-well plate (Costar) to 50µl SP at a final dilution of 1:100. A final dilution of 1:100 induced the highest response in a preliminary study (Figure 8.1). Cells were cultured and incubated for 6 days. On day 5, supernatant was taken from each well for cytokine analysis and ^{3}H-thymidine was added to measure ^{3}H-thymidine incorporation on day 6. The results are expressed as the median counts per minute (cpm) for each triplicate culture. The degree of proliferative response was measured by the difference in ^{3}H-thymidine incorporation between experiments with and without the addition of SP.

Figure 8.1 Proliferation of PBMCs with different dilutions of seminal plasma



Proliferation of 10 PBMCs incubated with different dilutions of 3 SP with different quality of sperm (median with IQR).

Cytokine and sHLA analysis

The levels of cytokines released into the culture supernatants and already present in the SP were assessed by the Bio-Plex Luminex™ system assay (Bio-Rad, Veenendaal, The Netherlands) following the manufacturer's instructions. The following cytokines IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, IFN-γ, and TGF-β1, TGF-β2, TGF-β3 in latent and active form were tested, IDO was analyzed in SP with ELISA method (Uscn Life Science Inc Us). Cytokine concentration was expressed as picogram per ml (pg/ml).

For sHLA class I in SP monoclonal antibody to HLA-class I purified antibody W6/32 (Department of IHB, LUMC, the Netherlands) and for sHLA-G in SP purified antibody Mem-G/9 (20µg,11-292-C100, Exbio Praha a.s., Vestec, Czech Republic) were coupled via carboxyl groups on the surface of polystyrene beads (COOH bead, Bio-Rad) according procedure of the Bio-Plex Amine Coupling kit (Bio-Rad). Luminex system (Bio-Plex, Bio-Rad) was used for read outs. The results for sHLA class I and sHLA-G were expressed in Median Fluorescence Intensity (MFI).

Flow cytometry

Differentiation of fPBMCs with and without SP was measured every 24 hours for three days. The following directly conjugated mouse-anti-human mAb were used for four-color immunofluorescence surface staining: CD45-APC, CD14-FITC, CD25-PE, CD3-PerCP, CD4-APC (Becton Dickinson) and used in concentrations according to manufactures instructions. Flowcytometry was performed on a FACS Calibur using Cellquest-pro

Software (Becton Dickinson) as described previously.[22] To investigate the presence of Tregs, MFI of CD4+CD25dim, CD4+CD25bright were calculated within the CD3+CD4 fraction.

FACS analysis of all fPBMCs was done using Flowjo-V10 Cytometry Analysis, the fluorescence intensity to distinguish between CD25dim and bright was determined on each individual sample.

Data analysis

In total 21 fPBMCs were tested. In 4 cases the experiments were performed with enriched and isolated T cells. The purity of the enriched CD3+ fraction by depletion of non-T cells from fPBMCs was variable in these 4 samples: 99.4%, 86.4%, 80.1%, 79.5%. The purity of T cell fraction isolated with FACS sorting was 96.8%, 97.7%, 97.9%, 99.3%. In order to determine the role of APCs, increasing number of APCs were added to these highly purified T cell suspension.

In total 61 different SP samples were tested. The number of fPBMCs and SP samples used in the different experiments is indicated in the result part for each experiment.

To analyse the relationship of quality of sperm and proliferative response of PBMCs, relative difference of the proliferative response with and without SP was calculated per SP sample (proliferation in cpm with seminal plasma minus proliferation in cpm without seminal plasma divided through the proliferation in cpm without seminal plasma) and the median value of the relative difference in proliferation of these responders per SP sample was compared between SP samples with a low and high sperm quality.

Only the results of SP samples, which were used at least two times on different fPBMCs, were analyzed for correlation and association between seminal cytokine and sHLA concentration and proliferative response of the cells. The relative difference of the proliferative response with and without SP of the different fPBMCs per SP sample was calculated as described above and the median relative difference of the proliferative response per SP sample was correlated with seminal cytokine and sHLA concentration.

For the measurements of cytokine expression at day 5 in the cultures in the presence of SP specific cytokine concentration of SP alone was subtracted to correct for cytokine concentration present in SP.

Statistical analysis

Descriptive statistical analysis was performed using SPSS Statistics 20 (IBM SPSS Software). Comparisons between groups were made using the Wilcoxon signed-rank test for paired analysis or the Mann-Whitney U test for unpaired analysis. Correlations were calculated using Spearman's rank resulting in a correlation coefficient (ρ) and a $p<0.05$ was considered significant.

Results

mRNA expression by T cells and fPBMCs after incubation with SP

Contact with SP induced an increased mRNA expression of Foxp3 in purified T cells. fPBMCs incubated with SP showed an increased mRNA expression of IL-10 and CD25 (both $p<0.001$), whereas mRNA expression of Foxp3 was slightly increased (Table 8.1).

As a T cell response often depends on the interaction with APCs, we determined whether addition of autologous APCs to the isolated T cells and SP, affected mRNA expression of IL-10, CD25, and Foxp3 in these isolated T cells. Addition of increasing numbers of autologous APCs led to an increased expression of IL-10 and CD25 (Figure 8.2a,b), whereas for mRNA expression of Foxp3 a decreased expression was observed (Figure 8.2c). Correlation analysis between mRNA expression of IL-10, CD25, and Foxp3 in T cells and remaining APCs in the T cell fraction after enrichment (respectively 0.5%, 13.6%, 19.9%, 20.5%) confirmed these results for IL-10 (Figure 8.2d). mRNA expression of Foxp3 was negatively correlated with the presence of remaining APCs (Figure 8.2f).

Proliferative effect of SP on T cells and fPBMCs

SP showed a direct effect on enriched T cells as reflected in a significant increase in proliferative response when SP was added ($p=0.002$) (Figure 8.3a) A median of 4.34 fold increase in cpm was observed in the presence of SP (Interquartile range (IQR) 3.98-5.15). The degree of the proliferative response was not correlated with the percentage of APCs in the CD3 fraction ($\rho=0.007$, $p=0.983$).

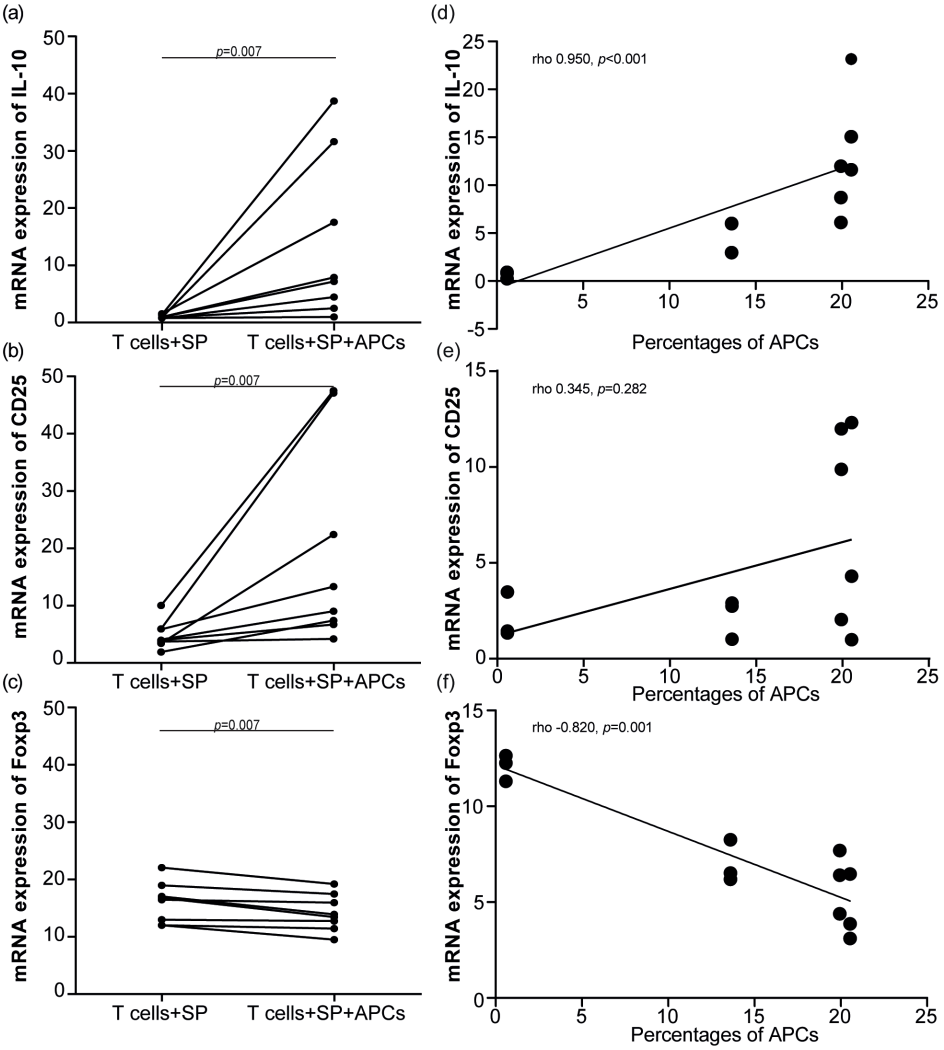
Exposure to SP resulted also in a significantly increased proliferation of the fPBMCs ($p<0.001$) (Figure 8.3b), with a median of 2.99 fold increase in cpm (IQR 1.79-5.37) in all fPBMCs and a median of 1.35 fold increase in cpm (IQR 1.35-2.49) in the fPBMCs which were used for T cell enrichment. Exposure of seminal plasma to enriched T cells from PBMCs led to a significantly higher increase in cpm compared to whole fPBMCs exposed to seminal plasma ($p<0.001$).

Table 8.1 mRNA expression of T cells and fPBMCs in the absence and presence of SP

	mRNA expression of T cells without SP	mRNA expression of T cells with SP	*P*-value	mRNA expression of fPBMCs without SP	mRNA expression of fPBMCs with SP	*P*-value
CD25	3.66 (1.15-8.58)	3.60 (1.93-5.90)	0.247	3.10 (1.06-5.55)	21.66 (11.72-39.34)	<0.001
IL-10	1.39 (0.54-4.38)	0.93 (0.31-8.06)	0.526	6.76 (3.68-11.76)	39.02 (24.17-52.09)	<0.001
Foxp3	5.73 (3.57-14.50)	10.40 (6.42-13.67)	0.002	4.17 (0.90-6.90)	4.93 (2.33-10.41)	0.014
IFN-y	1.34 (0.91-2.60)	0.60 (0.38-0.92)	0.001	0.62 (0.18-1.10)	0.97 (0.31-1.95)	0.161

Data are median (IQR). In total 6 different fPBMCs and 4 different enriched and isolated T cells were used all incubated with at least 4 different seminal plasma samples (for fPBMCs in total 33 combinations and for T cells in total 12 combinations). mRNA; messenger RNA, SP; seminal plasma.

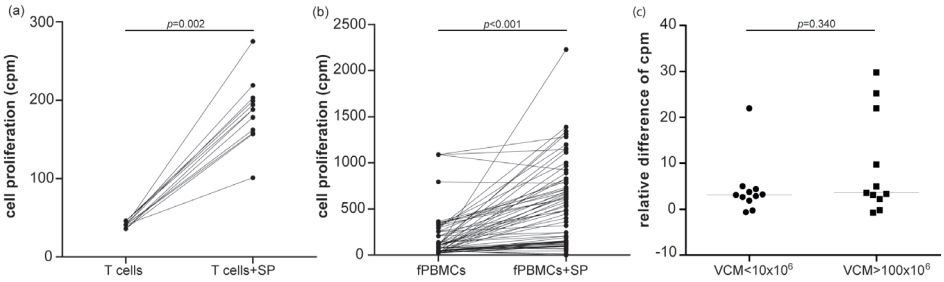
Figure 8.2 mRNA expression of IL-10, CD25, and Foxp3



mRNA expression of IL-10 (a), CD25 (b), and Foxp3 (c) in isolated T cells with addition of autologous APCs. Correlation between T cells enriched from fPBMCs and the percentages of remaining APCs after enrichment and mRNA expression of IL-10 (d), CD25 (e), and Foxp3 (f) at day 1 in the presence of SP. In total four responders were used for these experiments incubated with at least two different SP samples.

The quality of sperm expressed in VCM had no direct relationship with the degree of proliferation of PBMCs (ρ=-0.059, *p*=0.557). SP with a VCM below $10x10^6$ and above $100x10^6$ induced a similar proliferation of fPBMCs (Figure 8.3c).

Figure 8.3 Proliferative response of T cells and fPBMCs in the absence and presence of seminal plasma



a) Proliferative response of enriched T cells in cpm in the absence and presence of SP. In total four different fPBMCs were included for enrichment of T cells and for every responder three different SP samples were used. (b) Proliferative response of 19 fPBMCs in cpm in the absence and presence of SP. Every responder was incubated with at least three different seminal plasma samples of different sperm quality. We used a total of 72 combinations using 45 different SP samples. (c) Of 11 different fPBMCs the relative difference of the proliferative response with and without SP was calculated per SP plasma sample (proliferation in cpm with seminal plasma minus proliferation in cpm without seminal plasma divided through the proliferation in cpm without seminal plasma) and the median value of the relative difference in proliferation of these responders per SP sample was compared between SP samples with a low (VCM<$10x10^6$) and high sperm quality (VCM>$100x10^6$). In total 22 different SP samples were used, 11 with a VCM lower than $10x10^6$ and 11 with a VCM higher than $100x10^6$. Of the SP samples which were used at least twice on different responders, a total of 9 samples, median proliferative response was calculated. Line indicates median.

Several factors in SP in relation to the proliferative response of fPBMCs

As the presence of cytokines and sHLA may affect the proliferative response of fPBMCs, the concentration of different cytokines and sHLA class I and sHLA-G were determined in 11 SP samples added at least twice to 14 different fPBMCs (in total 50 combinations). Latent and active isoforms of TGF-β, IDO, and sHLA class I were present in high concentrations in SP. However, no correlation between the concentration of any of these factors and the degree of cell proliferation in the cultures was observed (Table 8.2).

SP also affects the cytokine production by T cells and fPBMCs

IL-10 and IFN-γ were found to be significantly increased in the supernatant of enriched T cells incubated with SP (Table 8.3). Similarly to the mRNA expression of IL-10, production of IL-10 was highly correlated with percentages of APCs in the CD3 fraction (ρ=0.769, p=0.003). Several cytokines were induced when SP was added to fPBMCs (Table 8.3).

Table 8.2 Relationship between several cytokines, sHLA class I, and sHLA-G in seminal plasma and proliferative response in fPBMCs

	Concentration in semen (pg/ml)			Correlation of the median relative difference and cytokine concentration*
	Min	Median	Max	(ρ, *p*)
TGF-β1l	42601.7	554980.5	12031743.6	0.118, 0.729
TGF-β2l	6378.2	9130.5	27208.0	0.200, 0.555
TGF-β3l	13528.0	54506.0	334687.3	0.073, 0.832
TGF-β1a	62.7	1118.7.9	3423.1	0.027, 0.937
TGF-β2a	55.1	238.1	1408.6	-0.036, 0.915
TGF-β3a	8.1	587.6	1880.1	0.255, 0.450
IDO	56985.9	122161.0	313255.8	0.200, 0.555
IL-10	22.8	33.2	49.0	-0.132, 0.699
TNF-α	3.5	18.9	64.5	0.318, 0.340
IFN-y	263.0	292.1	425.8	0.236, 0.484
IL-2	6.5	8.8	13.9	0.082, 0.811
IL-6	10.1	22.0	605.1	0.055, 0.873
sHLA class I	1134	2322.5	4524	0.155, 0.650
sHLA-G	50	83.5	196	0.269, 0.424

TGF-β1 latent form (TGF-β1), TGF-β1 active form (TGF-β1a). *Per responder (fPBMCs) the relative difference in proliferation (proliferation in cpm with seminal plasma minus proliferation in cpm without seminal plasma divided through the proliferation in cpm without seminal plasma) was calculated and the median value of the relative difference in proliferation of these responders per seminal plasma sample (median relative difference) was correlated with the concentration of several factors in seminal plasma. ρ; correlation coefficient, *p*; *p*-value

CD25 expression is induced on fPBMCs by exposure to SP

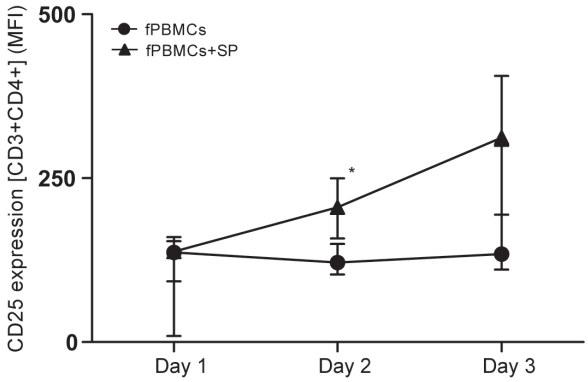
Expression of CD25 on fPBMCs that were exposed to semen was investigated for three subsequent days. Exposure to SP led to a significantly higher expression of CD25 on CD4+ T cells (p=0.006) (Figure 8.4).

Table 8.3 Concentration of cytokines in supernatants of cultures with enriched T cells and fPBMCs in the absence and presence of SP

	Cytokine expression (pg/ml) of T cells without SP	Cytokine expression (pg/ml) of T cells with SP*	*P*-value	Cytokine expression (pg/ml) of fPBMCs without SP	Cytokine expression (pg/ml) of fPBMCs with SP*	*P*-value
IL-1β	0.82 (0.46-1.10)	0.73 (0.27-7.72)	0.814	1.66 (1.39-45.39)	195.41 (42.92-728.97)	<0.001
IL-2	2.39 (0.59-4.89)	2.14 (1.11-7.45)	0.308	3.48 (1.87-5.35)	21.06 (9.66-29.54)	<0.001
IL-4	0.09 (0.07-0.37)	0.04 (0.00-0.90)	0.723	0.76 (0.50-3.23)	3.42 (1.03-7.81)	<0.001
IL-10	2.24 (0.67-3.25)	2.99 (1.79-7.67)	0.028	3.67 (0.98-5.18)	21.50 (8.27-77.57)	<0.001
IL-17	1.08 (0.82-6.60)	1.57 (0.11-7.23)	0.754	5.63 (2.65-32.67)	27.96 (16.17-100.54)	<0.001
TNF-α	1.99 (0.72-3.93)	2.21 (0.41-6.90)	0.433	3.58 (2.84-56.63)	55.38 (17.67-229.64)	<0.001
IFN-y	9.24 (1.20-63.99)	38.40 (16.92-94.09)	0.019	29.10 (19.60-121.79)	154.04 (65.01-231.46)	<0.001

Data are median (IQR). In total 9 different fPBMCs and 4 different enriched T cells were used all incubated with at least 3 different seminal plasma samples (for fPBMCs in total 39 combinations and for T cells in total 12 combinations). SP; seminal plasma. *cytokine concentration of seminal plasma alone was subtracted to correct for cytokine concentration present in seminal plasma.

Figure 8.4 CD25 expression of fPBMCs stimulated with seminal plasma



Four different fPBMCs were stimulated with semen for three subsequent days, at least three SP samples for every responder were used, resulting in 14 different combinations. Expression of CD25 (MFI) within CD3+CD4+ population were given in the absence and presence of SP (median (IQR)). *p<0.05. At day 1 p=0.595, at day 2 p=0.006, at day 3 p=0.056.

Discussion

It has been postulated that SP is important for the immune response at the implantation site to assure embryo implantation and placentation. However, the exact influence of SP on maternal immune cells is still unclear. We investigated the direct effect and the indirect APC-mediated effect of SP on T cells. The present data shows that SP contains active immunoregulatory factors that influence T cells. Some of these effects were due to a direct effect of SP on T cells, others require the presence of APCs. Upregulation of IL-10 and CD25 in T cells seems to be an APCs-mediated effect as mRNA expression of both IL-10 and CD25 were highly increased when autologous APCs were added to isolated T cells and SP. The induction of an increased IL-10 and CD25 mRNA expression and production of IL-10 in the presence of APCs in combination with the increased CD4+CD25 expression suggests that SP is important in driving differentiation of T lymphocytes to a more regulatory phenotype. The early expansion of a more regulatory phenotype is in line with the necessity for a quick induction of tolerance to protect the fetus in the initial stages of contact and are consistent with the observation that a T regulatory response is already operational when the fetus is encountered.[8]

Optimal proliferation was seen when SP was added in a final dilution of 1:100. This dilution seems to mimic the relevant amount of SP that enters the uterus after sperm loss from the vagina following coitus (flowback) and dilution by reproductive tract secretions. SP is deposited at the cervix[16] and in vitro observations in human suggest that the effects of SP extend to the endometrium.[17] After flowback, less than 1% of the sperm cells

retain in the female.[23] The few sperm cells entering the cervix and uterus remain for days following deposition.[24] In vivo observations show that seminal components are carried together with sperm into the higher tract.[25] Furthermore, vascular connections between cervix and endometrium further facilitate vagina-to-uterus transport of progesterone and other mediators.[26] As SP is transported after ejaculation through the female reproductive tract, seminal components could possible come in direct contact with local leukocytes as T cells and epithelial cells in the cervix and endometrium.

Our study shows that SP has a direct effect on T cells as reflected by a proliferative response, mRNA expression of Foxp3, and cytokine production of IFN-γ. It seems that APCs are needed to induce a more regulatory response in T cells in the presence of SP as both mRNA expression of CD25 and IL-10 in isolated T cells were highly increased when APCs were added to the culture with SP. In addition, a positive correlation was found between the percentages of remaining APCs in the enriched T cell fraction and IL-10 mRNA expression and production in the presence of SP. Furthermore, fPBMCs exposed to SP showed highly increased IL-10 expression and IL-10 cytokine levels in supernatants, confirming that APCs are needed to induce IL-10 in T cells under influence of SP. Besides upregulation of IL-10 also the increased CD25 expression on the CD4+ T cell population suggests triggering of a CD4+CD25+ Tregs subset, which is supposed to be necessary for the acceptance of the allogeneic fetus[2,13,27] and the maintenance of a normal pregnancy.[5,6]

In contrast, Foxp3, one of the key proteins responsible for Tregs function,[28] was highly increased in isolated T cells and not as high in fPBMCs in the presence of SP. In addition, a negative correlation was found between the number of APCs and mRNA expression of Foxp3 in the T cell fraction. In the assays with fPBMCs, including APCs, extreme high levels of IL-1β were measured in the presence of SP. IL-1β is a pro-inflammatory cytokine facilitating immune responses, not leading to Foxp3+ Treg activation, which corresponds with data by Sharkey et al. who showed that numbers of Foxp3+ Tregs in the human cervix remained the same after sexual intercourse.[29] Furthermore, SP was also found to induce mRNA expression of IL-1β in human endometrial epithelial cells.[17] The expression of IL-1β is suppressed in patients with recurrent miscarriages,[30] indicative for an important role of IL-1β in regulation of endometrial function and implantation.

Although paternal antigens present in SP in the form of sHLA class I molecules and sHLA-G[10,31] could be involved in the induction of a specific T cell response, no association between the concentration of sHLA class I and sHLA-G present in SP and the proliferative response was observed in the present study. Non-specific mitogens in SP may be responsible for the proliferative immune response as was described for porcine SP.[32]

Of course, our in-vitro model cannot totally capture the rich interplay of multiple cell types found in the endometrium and cervix. Exposure of women to SP after copulation elicits expression of pro-inflammatory cytokines, chemokines, recruitment of macrophages, and dendritic cells (DCs) in the cervical epithelia.[29] In vivo studies in mice show that SP causes upregulation of granulocyte-macrophage-colony-stimulating factor, IL-6, and TNF-α in uterine epithelial cells, which induces infiltration of uterine tissue by macrophages, DCs, and granulocytes.[33,34]

The data is consistent with previous studies in mice showing that seminal vesicle-derived components of the ejaculate have an important role in the immune regulation of the proliferative response of maternal T cells.[11,33,35] Indeed in men, it seems likely that prostate as well as seminal vesicle is important source of seminal plasma factors. Corresponding with previous data, SP samples in our study contained high levels of active and latent isoforms of TGF-β.[17] In addition, we found that IDO is present in high concentration in SP. IDO can mediate a suppressive effect directly on effector T cells and activate Tregs.[36] Both TGF-β and IDO are likely key factors in the immunomodulation at implantation. However, in our study no direct correlation between the amount of TGF-β and IDO in SP and the proliferative response by fPBMCs was observed. Further research should emphasize on the cytokine network and its role in immunomodulation, rather than focusing exclusively on the role of single cytokines.

In summary, our data show that SP has both a direct and indirect immunological effect on T cells, where APCs seem to be essential for the induction of IL-10 in T cells. However, the relevance of these findings for the occurrence of normal and aberrant pregnancy remains to be established.

Acknowledgements

The authors would like to thank Carin van der Keur, Vera Schenk, and Rowena Schaap for their help with the experiments.

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Chapter 9

Oral sex is associated with reduced incidence of recurrent miscarriage

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Submitted to *Journal of Reproductive Immunology*
Supplementary data available at the end of the chapter

Abstract

Problem

A possible way of inducing tolerance towards paternal HLA antigens of the fetus in pregnancy would be exposure of these antigens via seminal fluid to oral mucosa. We hypothesized that women with recurrent miscarriage have had less oral sex compared to women with uneventful pregnancy.

Method

In a matched case control study, 97 women with at least three unexplained consecutive miscarriages prior to the 20th week of gestation with the same partner were included. Cases were younger than 36 years at time of the third miscarriage. The control group included 137 matched women with an uneventful pregnancy. The association between oral sex and recurrent miscarriage was assessed with conditional logistic regression, odds ratios (ORs) were estimated. Missing data were imputed using Imputation by Chained Equations.

Results

In the matched analysis, 41 out of 72 women with recurrent miscarriage had have oral sex, whereas 70 out of 96 matched controls answered positive to this question (56.9% vs. 72.9%, OR 0.50 95% CI 0.25-0.97, p=0.04). After imputation of missing exposure data (51.7%), the association became weaker (OR 0.67, 95% CI 0.36-1.24, p=0.21).

Conclusion

In conclusion, this study suggests a possible protective role of oral sex in the occurrence of recurrent miscarriage. This should however be confirmed in an independent study.

Introduction

About 1% of all couples trying to conceive, are confronted with recurrent miscarriage, which is often defined as three or more consecutive pregnancies prior to the 20th week of gestation.[1] Possible etiologic factors include uterine anomalies, endocrine disorders, maternal inherited and acquired thrombophilia, and parental chromosomal abnormalities.[2,3] However, in only 25-50% of the couples an underlying cause for recurrent miscarriage can actually be identified.[2,4]

A disturbance of maternal immunologic tolerance to the semi-allogeneic fetus has been proposed as one of the possible mechanisms.[4,5] Most research into the immunology of recurrent miscarriage focused on the maternal immune system, leaving paternal factors aside. However, males seems to be capable to affect the female immune system prior to conception.[6] Studies in mice have shown that during copulation, thus before implantation, fetus specific maternal tolerance toward paternal antigens is induced.[7]

A well-known route to induce immune tolerance is via oral exposure, possibly because the gut has the most adequate absorption in the absence of an inflammatory environment.[8,9] In transplantation models of rats, oral administration of MHC molecules diminishes the occurrence of allograft rejection.[10] Based on this knowledge, Koelman et al hypothesized that a potent way of inducing tolerance towards paternal HLA antigens of the fetus in pregnancy would be exposure of these antigens to oral mucosa.[11] To support this theory, they showed that both oral sex and swallowing sperm reduced the incidence of preeclampsia.[11] Another study showed that the pattern of oral sex practice was similar in 66 women with two miscarriages and a control population (N=110), but more women in the control group swallowed sperm than was expected.[12] Here we describe the outcome of a matched case control study to assess the effect of oral sex on the occurrence of recurrent miscarriage in a well-characterized population.

Material and methods

Case group

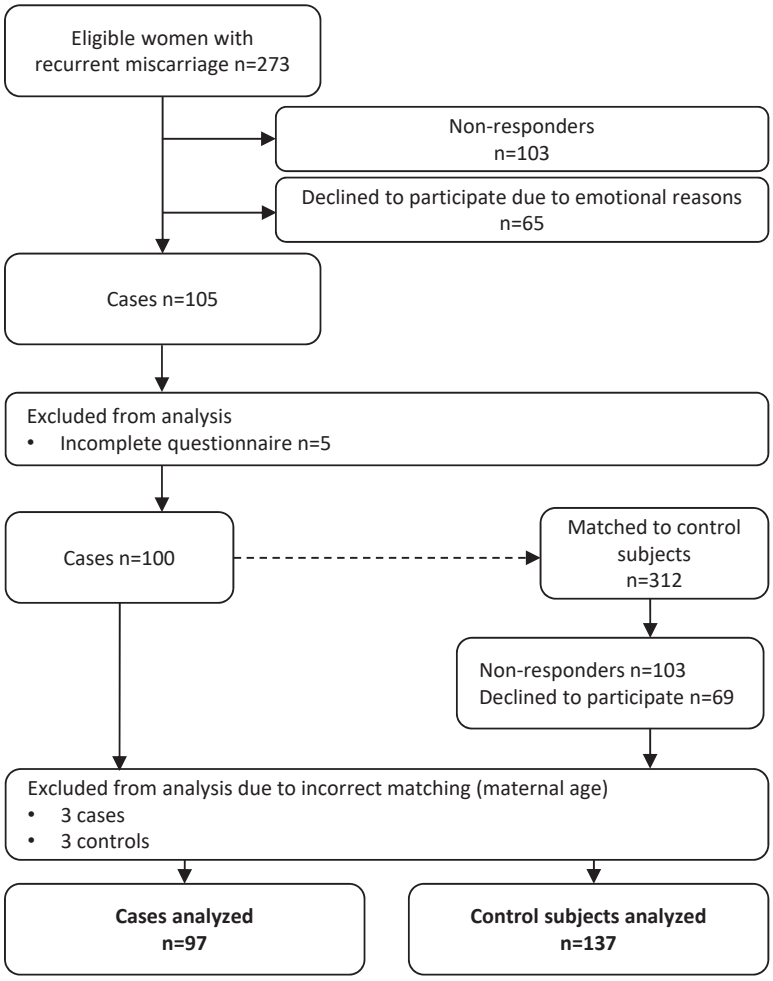
From 433 women who visited the recurrent miscarriage clinic of the department of Obstetrics and Reproductive Medicine at the Leiden University Medical Center (LUMC), a tertiary referral center in the Netherlands, between 2000 and 2014, 273 women were eligible and invited to participate in this study.

Eligible cases were women who had three or more consecutive miscarriages prior to the 20th week of gestation with the same partner, and who were younger than 36 years at

time of their third consecutive miscarriage. Women with known causes for miscarriage such as uterine anomalies, parental chromosomal abnormalities, and anti-phospholipid syndrome were not eligible. The clinical work-up and definition for known causes is previously described.[13] Women with hereditary thrombophilia were not excluded because the evidence that hereditary thrombophilia is associated with recurrent miscarriage is only weak.[3,14] Both women with primary recurrent miscarriage (no history of live birth) and secondary recurrent miscarriage (history of live birth) were eligible.

From the 273 eligible women, 100 eligible women were included (Figure 9.1). Baseline characteristics from the 100 included women and 173 eligible, but not included women of which most women were non-responders, is depicted in Supplementary Table I.

Figure 9.1 Flowchart of subjects



Control group

As it is postulated that the primary pathogenesis of various pregnancy complications is the same within individuals,[15] controls were women with no miscarriage and only uncomplicated pregnancy(ies), i.e. no history of pregnancy complications such as pregnancy-induced hypertension, preeclampsia, Hemolysis Elevated Liver enzymes and Low Platelets (HELLP) syndrome (all defined according to the criteria of the International Society for the study of Hypertension in Pregnancy (ISHHP)), preterm birth (24-37 weeks), fetal growth restriction (birth weight below the 2.3th percentile for gestational age and sex),[16] and perinatal death (fetal loss after 20 weeks of gestation till 7 days after birth).

In the Netherlands it is common practice that community midwives are taking care of low-risk women (with no medical or obstetrical history) during pregnancy and child birth. The zip code of each woman with recurrent miscarriage was used to contact the nearest midwifery practice to control for the impact of socio-economic status (SES) and urbanity in the current analyses. Women with the same zip code, the same age (difference in birth date maximally 1 year), and of which the time of first delivery was close to the time of the third miscarriage of the matched exposed woman (maximum 6 months before or 6 months after) were asked to participate. We contacted at least 3 controls per case. Enrolment took place between 2012 and 2014 (Figure 9.1).

Ethical approval

The protocol was approved by the Ethics committee of the LUMC (P12-099) and all participants gave informed consent. The study was registered with the Dutch trial registry NTR3402 and is part of the REMI (REcurrent MIscarriages) studies, which investigate causes and consequences of recurrent miscarriages.

Dutch reference group

In order to make the study more robust we obtained another control group, i.e., Dutch reference group in which participants were asked to fill in a digital questionnaire about relationships and sexual behavior,[17] for specific details about selection of participants: Wijsen and de Haas.[18] In total 14,892 persons, including men and women, were approached of which 4170 (28%) filled in the questionnaire completely. For our reference group we selected, from a total of 2075 women, 1259 women in the fertile age (between 16 and 50 years) with a heterosexual relationship.

Variables and definitions

All cases and controls were asked to participate by filling in a digital questionnaire or on paper in case women had no access to internet between 2012 and 2014. The questionnaire was made using ProMISe, an internet based, application for the design, maintenance, and use of data management projects. Data were entered and stored in a good clinical practice approved database (ProMISe Database, https://www.msbi.nl/promise/).

The questionnaire contained questions about personal characteristics, general disease history, intoxications (smoking, alcohol, drugs), use of medication at different time points, outcome and complications of all pregnancies, neonatal characteristics, family disease history, partner's characteristics, and questions about their recent sexual behaviour. Information about medical history, use of medication, intoxications, and pregnancy outcome was cross-checked in obstetrical records to overcome recall bias. The data of the obstetrical records were used in case of discrepancies between the questionnaire and obstetrical records. For the questions about recent sexual behaviour additional informed consent was requested. The sexual behaviour part entailed questions about recent (frequency of) oral sex, swallowing the ejaculate, length of the relationship, and monthly sexual frequency. To investigate whether vaginal exposure of sperm was different between cases and controls, recent contraception methods including use of condom were asked for. (Supplementary data, Appendix 1).

Maternal age was defined as age at third consecutive miscarriage for cases or age at first pregnancy for controls. Socioeconomic status was categorized into high, middle or low by using mean household income levels of a neighborhood, which was determined with the first four digits of the zip code, using data from the Netherlands Institute for Social Research.[19] Education was defined as whether or not university level (college and university education together). Ethnicity was based on country of birth of the woman and divided in 4 groups according to the rules of the Central Bureau of Statistics of the Netherlands.[20]

Sample size considerations

Sample size calculation was performed assuming that 40-50% of the cases and 60-80% of the controls would have oral sex,[11,21] leading to the following more precise assumptions adapted for the matched design:

Combination 1: (18%): Cases and controls both don't have oral sex
Combination 2: (15%): Cases have oral sex, controls don't have
Combination 3: (35%): Cases don't have oral sex, controls have
Combination 4 (32%): Cases and controls both have oral sex

This implies an odds-ratio on the event of 0.43 for oral sex vs. no oral sex. A sample size of 186 women (93 exposed, 93 non-exposed) was expected to provide sufficient power (two-sided alpha .05. power 80%), taking a 10% drop-out in consideration. We planned 1:1 case:control ratio, that is, one woman who had recurrent miscarriage matched to one control. On forehand we expected a lot of non-responders and therefore we contacted at least 3 controls per case. PASS 2008, Power Analysis and Sample Size Software (Hintze J., NCSS Kaysville USA) was used for the sample size calculation.

Statistical analysis

The association between oral sex and recurrent miscarriage was studied with conditional logistic regression using a stratified Cox regression and odds ratios (ORs) were estimated. Statistics were performed using SPSS (Version 24.0, Inc., Chicago, IL, USA). A *p*-value <0.05 was considered statistically significant.

Of the 97 cases and 137 controls, 51.7% did not complete all the questions about sexual behaviour, including questions about oral sex. We compared the cases and controls who did complete questionnaires to cases and controls who did not using chi-square tests or Fisher's exact tests or Mann Whitney U tests, whichever were appropriate. We repeated the analyses with missing exposure data imputed using Imputation by Chained Equations. In the imputation models the case/controls status oral sex, swallowing the ejaculate, relationship duration at time of index pregnancy, sexual frequency, and condom use as contraception, and the variables used for matching cases and controls (SES, urbanity, maternal age at time of index pregnancy) were included. In addition, variables that were significantly different between cases who completed all questions on sexual behaviour and cases who did not complete these questions were also included in the imputation model. Ten imputed datasets were created.

Results

Baseline characteristics

In total, 97 women with recurrent miscarriage were included and 137 matched controls (Figure 9.1). Table 9.1 shows the baseline characteristics of the study population.

In the case group, 63 women (64.9%) had primary recurrent miscarriage and 34 (35.1%) secondary recurrent miscarriage. A total of 65 women had 4 or more consecutive miscarriages (67.0%), and 39 women (40.2%) had 5 or more miscarriages. A total of 6

Table 9.1 Baseline characteristics

	Cases with recurrent miscarriage (N=97)	Controls without miscarriage (N=137)
Maternal age at index pregnancy (years;median[IQR])	30.0 (27.0-32.0)	30.0 (27.0-32.0)
Maternal age at time of questionnaire (years;median[IQR])	35.0 (32.0-38.0)	36.0 (33.0-39.0)
BMI (median[IQR])[a]	23.3 (21.5-26.6)	23.1 (21.0-25.8)
Smoking at time of questionnaire	14 (14.6)	18 (13.2)
Use of alcohol at time of questionnaire	51 (52.6)	87 (64.4)
Ethnic origin		
Native/Caucasian	86 (88.7)	130 (94.9)
Turkish/Moroccan	3 (3.1)	2 (1.5)
Antillean/Surinamese	2 (2.1)	1 (0.7)
Other non-Caucasian immigrants	6 (6.2)	4 (2.9)
University level education	47 (48.5)	82 (59.9)
Urbanity		
Few	14 (14.4)	15 (10.9)
Strong to moderate	53 (54.6)	78 (56.9)
Very strong	30 (30.9)	44 (32.1)
SES		
Lowest 25% (<25%)	10 (10.3)	19 (13.9)
Median 50% (25-75%)	51 (52.6)	64 (46.7)
Highest 25% (>75%)	36 (37.1)	54 (39.4)
Gravidity (median[IQR])	6 (5-8)	2 (1-2)
Parity (median[IQR])	1 (1-2)	2 (1-2)

Data are n (%) unless otherwise indicated, BMI; Body mass index, SES; Socioeconomic status, IQR; interquartile range.
[a]1.7% missing values (1 of 97 cases and 3 of 137 controls).

(6.2%) cases had hereditary thrombophilia, i.e., factor V Leiden (n=4), prothrombin gene mutation (n=3), or antithrombin deficiency (n=1). None had protein C or S deficiencies. Out of 97 cases, 70 had at least one live birth after the consecutive miscarriages (72.2%).

Sexual behaviour

Of the 97 cases, 46 cases (47.4%) and of the 137 controls, 75 controls (55.9%) did not complete all the questions about sexual behaviour. In Table 9.2 characteristics are shown of women with completed and women with not-completed questionnaire. Cases who

Table 9.2 Baseline characteristics of cases and controls with complete and incomplete information on sexual behaviour

	Cases (N=97)			Controls (N=137)		
	Incomplete questions about sexual behavior (n=46)	Complete question about sexual behavior (n=51)	*P*-value	Incomplete questions about sexual behavior (n=75)	Complete question about sexual behavior (n=62)	*P*-value
Maternal age at index pregnancy (years;median[IQR])	30 (27-32.2)	30 (27-32)	0.82[a]	30 (27-32)	30 (27.7-31)	0.77
Maternal age at time of questionnaire (years;median[IQR])	35 (33-39)	34 (31-37)	0.12[a]	36 (33-39)	35 (32-39)	0.35
BMI (median[IQR])[b]	23.0 (21.5-25.7)	24 (21.0-27.1)	0.31[a]	23.1 (20.9-26.0)	23.1 (21.0-25.8)	1.00
Smoking at time of questionnaire	11 (23.9)	3 (6.0)	0.02	11 (14.9)	7 (11.3)	0.54
Use of alcohol at time of questionnaire	25 (54.3)	26 (51.0)	0.74	51 (68.9)	36 (59.0)	0.23
Ethnic origin			0.19			0.73
Native/Caucasian	42 (91.3)	44 (86.3)		72 (96.0)	58 (93.5)	
Turkish/Moroccan	1 (2.2)	2 (3.9)		1 (1.3)	1 (1.6)	
Antillean/Surinamese	2 (4.3)	0 (0.0)		0 (0.0)	1 (1.6)	
Other non-Caucasian immigrants	1 (2.2)	5 (9.8)		2 (2.7)	2 (3.2)	
University level education	22 (47.8)	25 (49.0)	0.91	45 (60.0)	37 (59.7)	0.97

Table 9.2 continues on next page

Table 9.2 *Continued*

	Cases (N=97)			Controls (N=137)		
	Incomplete questions about sexual behavior (n=46)	Complete question about sexual behavior (n=51)	*P*-value	Incomplete questions about sexual behavior (n=75)	Complete question about sexual behavior (n=62)	*P*-value
Urbanity			0.62			0.68
Few	8 (17.4)	6 (11.8)		7 (9.3)	8 (12.9)	
Strong to moderate	23 (50.0)	30 (58.8)		42 (56.0)	36 (58.1)	
Very strong	15 (32.6)	15 (29.4)		26 (34.7)	18 (29.0)	
SES			0.13			0.73
Lowest 25% (<25%)	7 (15.2)	3 (5.9)		12 (16.0)	7 (11.3)	
Median 50% (25-75%)	26 (56.5)	25 (49.0)		34 (45.3)	30 (48.4)	
Highest 25% (>75%)	13 (28.3)	23 (45.1)		29 (38.7)	25 (40.3)	
Gravidity (median[min-max])	6 (5-8.2)	7 (5-8)	0.79[a]	2 (1-2)	2 (1-2)	0.81
Parity (median[min-max])	1 (1-2)	2 (1-2)	0.36[a]	2 (1-2)	2 (1-2)	0.61

Data are n (%) unless otherwise indicated, BMI; Body mass index, SES; Socioeconomic status, IQR; interquartile range.
All χ^2 tests or Fisher's exact tests except [a]Mann Whitney U test.
[b]1.7% missing values (1 of 97 cases with incomplete questions and 3 of 137 controls with incomplete questions).

did not complete the questions about sexual behaviour were significantly more often smokers (p=0.02). No other statistical differences were observed. Due to incomplete questionnaires on sexual behaviour matched analysis on oral sex was performed with 72 cases matched with 96 controls.

In the matched analysis, 41 out of 72 women with recurrent miscarriage reported to have oral sex, compared to 70 out of 96 matched controls (56.9% vs. 72.9%, OR 0.50 95% CI 0.25-0.97, p=0.04) (Table 9.3). From the 41 women with recurrent miscarriage who indicated to have oral sex, 39 women filled in the question on swallowing the sperm and 9 indicated to swallow sperm (23.1%). In controls 68/70 matched controls who indicated to have oral sex filled in this question and 10 controls (14.7%) swallowed sperm. No significant differences were observed in the incidence of oral sex in women with primary recurrent miscarriage and secondary recurrent miscarriage (63.3% vs. 46.4%, p=0.15).

Table 9.3 also shows results after missing values being imputed. The association became weaker with a crude OR of 0.67 (95% CI 0.36-1.24, p=0.21).

Out of the 1259 women selected as Dutch reference group, 1206 women filled in the question on oral sex, of which 1076 stated to have oral sex (89.2%). Clearly more than 44 out of 77 women with recurrent miscarriage who filled in this question (57.1%) (OR 0.16, 95% CI 0.09-0.26, p<0.001).

Discussion

This matched case control study suggests that women with recurrent miscarriage had less oral sex compared to women with uneventful pregnancy. This is in line with the hypothesis that the gut has the most adequate absorption in the absence of an inflammatory environment,[8,9] and seminal fluid contains soluble HLA antigens which can already induce maternal immune tolerance towards inherited paternal antigens of the fetus before implantation.

The strength of this study is that a large homogenous well-characterized case group of women with at least three consecutive unexplained recurrent miscarriages less than 20 weeks of gestation with the same partner was included. Furthermore, we compared our data in women with recurrent miscarriage to a representative group of Dutch women in the fertile age. In this reference group 89.2% of the women stated to have oral sex, this percentage is comparable to research on heterosexual behaviour in the USA, that showed that 83.5% of the women between age 35 years and 44 years ever had oral sex.[22] In addition, in a recent study on oral and vaginal exposure to the father's seminal fluid in

Table 9.3 Oral sex in women with recurrent miscarriage

	Recurrent miscarriage (N=97)	No miscarriage (N=137)	OR (95% CI)	*P*	OR (95% CI)	*P*
			Missing values not imputed		With missing values imputed	
Oral sex[a]	41/72 (56.9)	70/96 (72.9)	0.50 (0.25-0.97)	0.04	0.67 (0.36-1.24)	0.21
Relationship duration at time of index pregnancy (median [IQR])[b]	7 (4-9)	7 (4-9.2)	0.94 (0.84-1.05)	0.30	0.97 (0.88-1.07)	0.56
Sex frequency (median [IQR])[c]	8 (4-8)	4 (4-8)	1.07 (0.96-1.19)	0.18	1.03 (0.95-1.12)	0.41
Condom use as contraception[d]	7/56 (12.5)	15/69 (21.7)	0.59 (0.23-1.49)	0.27	0.82 (0.39-1.70)	0.60

Data are n (%), OR; odds ratio, CI; confidence interval, *P*; *p*-value.
[a]17.5% missing values (20 of 97 cases and 21 of 137 controls), 10.6% lost by matching (5 of 97 cases and 20 of 137 controls).
[b]26.0% missing values (21 of 97 cases and 40 of 137 controls), 13.6% lost by matching (13 of 97 cases and 19 of 137 controls).
[c]32.4% missing values (29 of 97 cases and 47 of 137 controls), 18.3% lost by matching (16 of 97 cases and 27 of 137 controls).
[d]30.3% missing values (26 of 97 cases and 45 of 137 controls), 16.2% lost by matching (15 of 97 cases and 23 of 137 controls).

preeclampsia, 78.6% of controls subjects had oral sex.[21] In contrast, in our study this was only 56.9% of the women with recurrent miscarriage, suggesting indeed that having less oral sex might be associated with pregnancy complications such as recurrent miscarriage.

Although it is suggested that particularly the vaginal route of exposure to paternal antigens is critical to successful pregnancy,[21] earlier findings suggest that oral exposure to paternal antigens reduced the incidence of preeclampsia,[11] which is in line with our findings in recurrent miscarriage. Seminal fluid contains all types of immunoregulatory factors such as cytokines, hormones and soluble HLA (sHLA) antigens[23] including sHLA-G. sHLA-G appears to have an important role in creating tolerance during pregnancy,[24-26] and sHLA-G in seminal fluid may affect the maternal immune system before implantation of the embryo.[27] The gut has the most adequate absorption in the absence of an inflammatory environment,[8,9] and therefore having oral sex before implantation of the semi-allogeneic fetus could be a potent way of inducing immune tolerance to the paternal HLA antigens.

Previous epidemiological studies indeed suggest that vaginal exposure and the length of sexual relationship are relevant to induce maternal immune tolerance to paternal antigens and decrease the occurrence of pregnancy complications. In these studies a short sexual relationships, limited seminal exposure, and barrier methods as contraception are associated with an increased risk of preeclampsia.[28-31] Furthermore, exposure to seminal fluid, either by application of vaginal capsules or by natural intercourse prior to embryo transfer in IVF procedures improves implantation success.[32,33] Most likely a combination of oral and vaginal exposure is needed to induce maternal immune tolerance to paternal antigens and an inadequate immunomodulation as early as during mating might be responsible for the development of a variety of pregnancy complications. However, the exact mechanism remains unclear and should be subject for further studies.

We were confronted with incomplete data from questionnaires, especially missing data on sexual behaviour, which was our exposure of interest. We tried to overcome this problem by imputation, a standard statistical approach to deal with missing data.[34] However, valid imputation assumes missing at random, meaning that other variables with complete information are completely accountable for the missing data. However this missingness at random is an untestable assumption, but may be valid in our study as comparing responders to non-responders showed no significant difference, except for smoking. However, missingness at random could still be dependent on variables not included in this study. The observed negative association between oral sex and recurrent miscarriage became smaller after imputation of the missing data, and the confidence interval included the null effect. When performing an unmatched analysis between cases and controls using only complete matched pairs, results were similar to the matched

analysis, when performing an unmatched analysis using all cases and controls, results were similar to the results of the imputed analysis (data not shown). This shows that our results should be interpreted with caution. Potential information bias should also be taken into account, as misclassification may have occurred due to the use of questionnaires and self-reported data, which is impossible to overcome. Importantly, seminal fluid exposure is not commonly recognized as a potential factor that could influence the occurrence of recurrent miscarriage, this will likely not have influenced the way women filled in the questionnaire. For this reason, information bias is not likely explanation for the observed association.

Our study is limited by the fact that the questions about sexual behaviour and contraception did not concern the period before the index pregnancy. This might explain the discrepancy in frequency of sexual intercourse between our study and others showing that limited seminal exposure or the use of barrier methods before conception play a role in the occurrence of pregnancy complications such as preeclampsia.[28,29,31] In our study the frequency of sexual intercourse was similar for women with recurrent miscarriage and controls. It is unknown how sexual behaviour changes during the years in individuals and therefore the questions about sexual behaviour might not reflect sexual behaviour before the index pregnancy especially in the women with recurrent miscarriage. By questioning sexual behaviour after the occurrence of recurrent miscarriage, the question remains whether having recurrent miscarriage affects sexual behaviour or sexual behaviour influence the occurrence of recurrent miscarriage.

Another possible limitation is that couples with recurrent miscarriage who did not participate in this study had overall significantly fewer children and fewer live births after they had recurrent miscarriages. However, this suggests that the observed effects are rather an underestimation due to the fact that the group with worse outcome amongst the recurrent miscarriage cases did not participate.

Despite the limitations of this study and the issues addressed, orally exposure to seminal fluid seems to induce maternal tolerance to paternal antigens and therefore influence pregnancy outcome in a positive way. Our results suggest an association between less oral sex and the occurrence of recurrent miscarriage; this however needs confirmation given the limitations of the present study.

Acknowledgements

The authors would like to thank R. Wolterbeek for his help with the power analysis.

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Supplementary data

Table I Characteristics of included and excluded cases

	Included cases (N=100)	Excluded cases (N=173)	*P*-value
Gravidity (median[IQR])	6 (5-8)	5 (4-6)	<0.001
Parity (median[IQR])	1.5 (1-2)	1 (0-1)	<0.001
Miscarriages (median[IQR])	4 (3-6)	3 (3-4)	<0.001
Consecutive miscarriages	4 (3-5)	3 (3-4)	<0.001
Primary recurrent miscarriage Secondary recurrent miscarriage	69 (69%) 31 (31%)	100 (57.8%) 73 (42.2%)	0.066
Live birth after miscarriages	75 (75%)	23 (13.3%)	<0.001

Appendix 1 Questionnaire on sexual behaviour

We hypothesize that contact with seminal fluid (both vaginally and orally) could influence the immune system of the mother. Too few or too many contact to seminal fluid could possible play a role in accepting the unborn child. Therefore, this questionnaire contains questions about sexual behaviour. We totally understand if these questions are too private for you, if so, please indicate whether you want to skip these questions.

1. I skip the questions on sexual behaviour.
2. I fill in the questions on sexual behaviour.

Question 1
Do you have oral sex with your partner? Yes or No or Unknown

Question 2
If yes, how many times a month?

Question 3
If yes, do you swallow the sperm? Yes or No or Unknown

Question 4
How long is your relationship in years?

Question 5
How often do you have sexual intercourse a month?

Question 6
Do you use contraceptives? No, condoms, IUD, contraceptive pill, implanon, injectable contraceptive, sterilization, interrupted intercourse, inapplicable, unknown.

Chapter 10

Summary and general discussion

Contents

1. Summary

In normal pregnancy the fetus, although a semi-allograft, is tolerated by the maternal immune system. During implantation, both the innate and the adaptive immune system are activated and several immunological mechanisms are playing a role in the acceptance of the semi-allogeneic fetus. It has been suggested that an inadequate maternal allo-immune response to the paternal antigens of the fetus is responsible for a proportion of the unexplained recurrent miscarriage.

Previous studies on the association of unexplained recurrent miscarriage with specific maternal HLA alleles and HLA sharing between couples have led to inconsistent results,[1-4] due to various definitions of recurrent miscarriage and control groups, analysis of different HLA alleles and loci, and application of different HLA typing methods. In **chapter 2** we provide a complete and up-to-date overview on the possible role of the HLA system in recurrent miscarriage, including only studies with strict eligibility criteria and molecular HLA typing methods. Although associations between specific HLA alleles and HLA sharing with recurrent miscarriage were found, no consistent conclusions can be drawn since the observed odd ratios were relatively small and the risk of selection and information bias in the selected studies was high.

A diminished allorecognition of fetal trophoblast by the maternal immune system, is accomplished by the fact that trophoblast only expresses the non-classical oligomorphic HLA-G and E-molecules and HLA-C, the only classical HLA I antigen.[5-7] As a consequence, maternal immune cells are only in contact with these HLA alleles at the implantation site. For this reason the studies described of this thesis were mainly focussed on different aspects of these specific HLA alleles. In **chapter 3** we compared the genetic polymorphisms of HLA-G in women with recurrent miscarriage with those of women with uneventful pregnancy. As the expression of soluble and membrane-bound forms of HLA-G depends on the combination of several polymorphisms at the 3'UTR,[8] we included these polymorphisms in our study. We found no association between single polymorphism and recurrent miscarriage. However, the HLA-G UTR-4 haplotype was less frequently observed in women with recurrent miscarriage, suggesting an immunoregulatory role of this haplotype facilitating an uncomplicated continuation of pregnancy.

As HLA-C is the only classical HLA antigen expressed on the trophoblast, we questioned in **chapter 4**, whether a maternal allo-immune response to paternal HLA-C plays a role in unexplained recurrent miscarriage. We observed an increased frequency of HLA-C*07, one of the most immunogenic HLA-C alleles, in partners of women with recurrent miscarriage. As a consequence, more mismatches for HLA-C*07 between mother and father were

observed compared to couples with uneventful pregnancy. The incidence of anti-HLA antibodies was also increased in women with recurrent miscarriage compared to women with uneventful pregnancy. As the clinical relevance of these antibodies in pregnancy is not clear, we reviewed in **chapter 5** the effect of anti-paternal antibodies on pregnancy complications including recurrent miscarriage. The selected studies showed a high level of statistical and clinical heterogeneity due to using different screening techniques, varying time points of screening, and use of incorrect control groups. In addition, detailed analyses of the characteristics and specificity of these antibodies were missing in most studies. Information on specificity, capacity to fix complement, titer, and the HLA epitopes recognized is essential, because from transplantation settings we know that a proportion of allo-antibodies cause rejection, for example depending on their ability to activate complement.[9] Surprisingly, the role of HLA-C specific antibodies in recurrent miscarriage has not been studied yet, while HLA-C is the only polymorphic classical HLA I antigen expressed on trophoblast. In **chapter 6**, we hypothesized that antibodies against HLA-C may play a role in the occurrence of unexplained consecutive recurrent miscarriage. The presence, specificity of anti-HLA antibodies, and their complement fixing ability was determined in women with recurrent miscarriage and compared to women with uneventful pregnancy in the first trimester of the next pregnancy. Significantly more often anti-HLA antibodies specific for HLA-C were found in women with recurrent miscarriage, suggesting that these antibodies may play a role in a subpopulation of women with recurrent miscarriage. As not all of these anti-HLA-C antibodies were complement fixing, it remains to be established which effector mechanism is involved in the etiology, complement fixation, antibody-dependent cellular cytotoxicity or both. In **chapter 7**, we observed a higher incidence of C4d deposition in products of conception in women with unexplained recurrent miscarriage compared to women with a sporadic miscarriage and women with an elective abortion. The combined results from **chapter 4**, **chapter 6** and **chapter 7** suggest that in a portion of women with unexplained recurrent miscarriage antibody-mediated rejection of the fetal allograft may play a role.

Studies in mice have shown that already during copulation, long before implantation, maternal tolerance towards fetal allo-antigens is induced.[10,11] In addition, human seminal plasma contains different types of immunoregulatory factors such as cytokines, chemokines,[12] and sHLA,[13,14] which may modulate the maternal immune response.[15,16] We showed in **chapter 8** that human seminal plasma contains all kinds of immunoregulatory factors including high concentrations of TGF-β, IDO, and sHLA class I. Furthermore, we observed that seminal plasma has an immunomodulatory effect on T cells as reflected in an increased proliferative response and upregulation of Foxp3. The presence of APCs is needed to induce IL-10 and CD25 upregulation in T cells exposed to seminal plasma. In accordance to Koelman et al.,[14] we hypothesized that a potent way of inducing tolerance

towards paternal HLA antigens of the fetus in pregnancy would be exposure of these antigens to oral mucosa. In **chapter 9** we tested the hypothesis, whether women with recurrent miscarriage have less oral sex compared to matched control subjects with uneventful pregnancy. In this matched case control study practicing oral sex was negatively associated with the occurrence of recurrent miscarriage. However, many issues have to be overcome before a final conclusion can be drawn.

2. Clinical implications and future perspectives

In couples with unexplained recurrent miscarriage inadequate immune reactions may play a role in the etiology. In this thesis we focussed mainly on reasons why insufficient tolerance towards paternal and fetal antigens may occur and parameters possibly related to occurrence of antibody-mediated rejection of the fetus with paternal HLA-C as the proposed target.

2.1 Background

Most studies published on recurrent miscarriage lack consistency, mainly due to the various definitions of recurrent miscarriage and control groups. The majority of women classified as women with recurrent miscarriage may even have suffered of these miscarriages due to chance rather than on basis of an underlying pathology. Furthermore, recurrent miscarriage is a highly heterogeneous condition with different possible underlying etiological factors. In order to identify only women with unexplained recurrent miscarriage, a high number of previous miscarriages, maternal age at the time of the diagnosis, karyotype of the conception, and a complete diagnostic work-up to rule out explained recurrent miscarriage should be taken into account.[17,18] In most studies no distinction is made between primary recurrent miscarriage and secondary recurrent miscarriage although it is postulated that primary recurrent miscarriage and secondary recurrent miscarriage could be two distinct entities with different underlying pathology.[19] Often ethnicity is not taken into account while, differences in HLA allele frequencies between women with recurrent miscarriage and control subjects could be purely dependent on genetic differences between populations. To overcome these issues, we tried to obtain a homogenous group of women with unexplained recurrent miscarriage, made distinction between primary and secondary recurrent miscarriage, and took ethnicity into account in all our studies. As it is postulated that the primary pathogenesis of various pregnancy complications and recurrent miscarriage is shared,[20,21] we only included in our studies control subjects with uncomplicated pregnancy and no recurrent miscarriage in medical history.

2.2 Possible reasons for a poor immune regulation

2.2.1 The influence of HLA alleles not expressed on trophoblast

As mentioned, classical HLA class I and II, with the exception of HLA-C, are not expressed on embryonic and trophoblast tissues and therefore unlikely to play a key role in the immune mechanism that leads to maternal-fetal tolerance. Nevertheless, a lot of research has been focussed on the role of HLA alleles of the mother related with recurrent miscarriage. Despite the high level of heterogeneity between the studies included in the meta-analysis in **chapter 2**, the presence of maternal HLA-DRB1*4 was associated with recurrent miscarriage.[22] The question remains whether these genes are self-related with recurrent miscarriage or linked with susceptibility genes that influence reproductive outcome. For example HLA DRB1*4 might play a role in an autoimmune response in the mother, which may also lead to pregnancy complications.[23-25]

2.2.2 The role of (s)HLA-G

The presence of sHLA-G in maternal circulation appears to have an important role in creating tolerance during pregnancy.[26-28] HLA-G polymorphisms in the 3'UTR may affect HLA-G mRNA stability,[29] which is associated with lower levels or even absence of sHLA-G in plasma.[30-32] In line with these data, in **chapter 3** we showed that in women with recurrent miscarriage HLA-G UTR-4 haplotype was less frequently present, suggesting an immunoregulatory role of this haplotype leading to an uncomplicated continuation of pregnancy. The question remains whether these maternal HLA-G polymorphisms found in recurrent miscarriage only influence sHLA-G levels in maternal circulation – and therefore pregnancy outcome – or that they also reflect fetal levels of sHLA-G and HLA-G levels on trophoblast due to shared genetic factors. Dahl et al.[33] found that sHLA-G is not freely transferred over the placental barrier, although sHLA-G levels are correlated in maternal and umbilical cord blood during uncomplicated pregnancy. These results may indicate that sharing genes is important for production of sHLA-G in mother and child. The 3'UTR HLA-G haplotype is most significantly associated with recurrent miscarriage (**chapter 3**), rather than single polymorphisms in the 3'UTR region of HLA-G as was previously believed.[4] In addition, in heterozygous 14DelC/14InsG mothers (14 bp ins/del and +3142C/G polymorphism) increasing numbers of 14InsG haplotypes in mother-child genotype combinations were associated with higher levels of sHLA-G at term.[34] The exact factors determining sHLA-G levels in pregnancy and the exact role of different isoforms of sHLA-G in pregnancy are difficult to establish also because sHLA-G in pregnant women is of mixed origin consisting of both fetal sHLA-G molecules from cytophoblast cells and maternal derived sHLA-G molecules.

Furthermore of interest, is the role of seminal sHLA-G as an immunomodulatory factor in the female reproductive tract before, and at the time of conception. A recent study indicates that the level of sHLA-G in seminal plasma may even be associated with the chance of pregnancy in couples.[35] A pilot study in male partners in women with recurrent miscarriage showed that concentration of sHLA-G in seminal plasma is associated with HLA-G genotypes (data not shown in this thesis). Further studies are needed to verify these preliminary findings and to test whether these levels are lower in couples with recurrent miscarriage compared to couples with uneventful pregnancy. As was previously mentioned, studies in mice have shown that already during copulation, long before implantation, maternal tolerance towards fetal allo-antigens is induced.[10,11] A well-known route to induce immune tolerance is via oral exposure, possible because the gut has the most adequate absorption in the absence of an inflammatory environment.[36,37] Although, in accordance to a previous study,[14] our data in **chapter 9** suggest that practicing oral sex is associated with a lower occurrence of recurrent miscarriage, Saftlas et al. suggest that particularly the vaginal route of exposure to paternal antigens is critical to successful pregnancy.[38] Indeed, short sexual relationships, limited vaginal seminal exposure, or usage of barrier methods for contraception are associated with an increased risk of preeclampsia[39-42] and exposure to seminal fluid, either by application of vaginal capsules or by natural intercourse prior to embryo transfer improves implantation success.[43,44] Therefore, the induction of maternal immune tolerance to paternal antigens is believed to be essential for the acceptance of the semi-allogeneic fetus. An inadequate immunomodulation as early as during mating might be responsible for the development of a variety of pregnancy complications.

2.2.3 The role of various signalling molecules in seminal plasma

Besides allo-antigens in the form of sHLA, and in particular sHLA-G, seminal plasma contains various signalling molecules including IL-8, TGF-β, and IFN-γ[45] and several inhibitors of complement and the prostaglandin PGE2. Exposure to seminal fluid induces lymphocyte proliferation (**chapter 8**)[46], NK cell activity, and modified cytokine release from APCs,[12] resulting in tolerance towards paternal allo-antigens. In human, it seems that seminal plasma increases T cell proliferation, triggering a CD4+CD25+ Tregs subset (**chapter 8**),[46] which is supposed to be necessary for the acceptance of the allogeneic fetus[15,47,48] and the maintenance of a normal pregnancy.[49,50] Taken together, above described studies suggest that exposure of the maternal immune system to seminal fluid, decrease the occurrence of a variety of pregnancy complications. Further research should focus on the exact role of seminal plasma in pregnancy and complicated pregnancy. It remains to be established whether variations in level of immunomodulatory factors and interactions between these factors in seminal plasma influences pregnancy outcome

and, if so, whether both orally and vaginally exogenous administration of these factors deceases the risk on pregnancy complications.

2.3 HLA-C as a possible target for immune reactivity

Part of our research was focussed on HLA-C, the only classical HLA allele present on the trophoblast and therefore likely to be directly involved in the detrimental maternal immune response to the fetus. Indeed, in two separate case groups of women with recurrent miscarriage in **chapter 4** and **chapter 6** we found that HLA-C antibodies were significantly increased compared with women with uneventful pregnancy.[51] These antibodies were mostly child-specific and directed against the most immunogenic HLA-C alleles.[52]

Although other studies showed that antibodies are rarely demonstrable before 28 weeks gestation[53] and spontaneous miscarriage is almost never causing formation of antibodies,[53] in our studies the incidence of anti-HLA-I and anti-HLA-C antibodies in nulliparous was similar to that of multiparous women with recurrent miscarriage, and significantly higher than nulliparous control subjects. The increased incidence of anti-HLA antibodies in nulliparous cases could not be explained by previous miscarriages or curettages.[51] These antibodies present in nulliparous women could be formed during the index pregnancy – and causing direct harm – or the presence of these antibodies can be considered as a marker for a broader immune response, as was previously shown in HLA identical family transplantations.[54] The presence of anti-HLA antibodies was a risk factor for worse outcome, although anti-HLA antibodies themselves could not have caused any harm.[54] Anti-phospholipid antibodies, which are highly associated with recurrent miscarriage,[55] are potential candidates for this broader antibody response. However, the presence of these antibodies could not explain the high incidence of anti-HLA antibodies in women with unexplained recurrent miscarriage in our studies.

The production of allo-antibodies is also related to the degree of HLA-DR compatibility. Lashley et al. showed that a higher percentage of women with oocyte donation pregnancies produce HLA class I antibodies in case of HLA-DR incompatibility, independent of the number of HLA class I mismatches.[56] In addition, a higher incidence of donor specific antibodies in patients transplanted with an HLA-DR incompatible graft compared to HLA-DR compatible transplants was seen.[57] This is in contrast with previous meta-analyses, which showed a significantly higher incidence of HLA-DR compatibility instead of incompatibility in women with recurrent miscarriage (**chapter 2**).[3,22] Furthermore, we could not observe more HLA-DR incompatibility of couples in recurrent miscarriage compared with control subjects (**chapter 4**) (Table 10.1, data not earlier shown).

Table 10.1 HLA-DR incompatibility in couples with recurrent miscarriage

Mismatch	Couples	Cases N=97		Controls group 2 N=425		*P*-value
DR-locus	0	10	10.3%	34	8.0%	ns
	1	42	43.3%	183	43.1%	
	2	45	46.4%	208	48.9%	

The increased presence of anti-HLA antibodies in combination with the increased presence of C4d in products of conception in women with unexplained recurrent miscarriage observed in **chapter 7** actually emphasizes that antibody-mediated rejection may play a role in unexplained recurrent miscarriage.[58] However, C4d deposition can be interpreted as a sign of local dysregulation of the placental complement system, as excessive complement activation caused by antibody deposition,[59,60] or interpreted as a non-specific reaction, triggered by damaged tissue and apoptotic cells.[61] A subgroup analysis in our study on C4d deposition on non-apoptotic trophoblast cells in order to test for additional pathways leading to C4d deposition showed similar differences between women with recurrent miscarriage and control subjects suggesting that apoptosis is not the main pathway of the excessive complement deposition seen in unexplained recurrent miscarriage. Not all allo-antibodies cause rejection and their ability to activate complement might play a determinative role.[9] In our studies most anti-HLA-C antibodies were non-C1q fixing antibodies. From transplantation settings we know that assessment of complement-fixing capability with C1q method is more closely correlated with worse outcome after transplantation, as antibody-mediated rejection and graft failure, than antibodies that are only detected by the traditional IgG method.[62] It remains to be established which effector mechanism is involved in the etiology, complement fixation, antibody-dependent cellular cytotoxicity or both.

The clinical relevance of these (child-specific) anti-HLA-C antibodies and C4d in products of conception could not be answered in our studies because in all our case groups medication was used as part of a randomized control trial, i.e. enoxaparin, aspirin, or a combination of these two, which could have influenced pregnancy outcome.[63]

Although it is tempting to assume that antibody-mediated rejection plays a role in unexplained recurrent miscarriage, we need prospective cohort studies, focussing on the presence of anti-HLA antibodies in the first trimester, investigating whether these antibodies are child-specific and complement fixing, and relate this to C4d presence and finally pregnancy outcome. Only then, we will able to answer above mentioned issues and to confirm and extend earlier observations.

2.4 Primary and secondary recurrent miscarriage, two distinct entities?

Previous research postulated that primary recurrent miscarriage and secondary recurrent miscarriage could have different underlying pathologies.[19] In all our research we performed subgroup analyses between primary recurrent miscarriage and secondary recurrent miscarriage. Interestingly, in **chapter 3** the frequency of UTR-2 was significantly higher in women with secondary recurrent miscarriage compared to women with uneventful pregnancy. The UTR-2 haplotype, is one of the UTR haplotypes, found to be associated with lower expression of sHLA-G.[32] Hypothetically, women with secondary recurrent miscarriage and an UTR-2 haplotype could have less immunomodulatory capacity during implantation and therefore possibly an increased risk of miscarriage due to increased immunity to fetal antigens in the next pregnancy by for example the presence of anti-HLA antibodies or H-Y antibodies from a previous live birth.[51,64] The increased frequency of HLA-C*07, one of the most immunogenic HLA-C alleles, of the last living child born before the occurrence of recurrent miscarriage in women with secondary recurrent miscarriage (**chapter 4**) could support the idea of increased immunity. However, as expected with higher occurrence of HLA-C*07 in the previous child and the fact that the presence of anti-HLA antibodies increases after 28 weeks of pregnancy and antibodies can still be present at time of a new conception,[53,65] no significant differences were observed in paternal-specific anti-HLA class I, anti-HLA-C, or anti-HLA-C*07/17 antibodies between women with primary recurrent miscarriage and women with secondary recurrent miscarriage. Whether primary recurrent miscarriage and secondary recurrent miscarriage are two distinct entities, should be subject for further studies.

2.5 Increased endometrium receptivity

Nowadays, new ideas are rising about increased endometrium receptivity in recurrent miscarriage. Normal endometrium does not allow low-quality embryos to implant,[66] but the endometrium of women with recurrent miscarriage may be less selective for the embryo quality,[67-69] possibly leading to more aneuploidy embryos in women with idiopathic recurrent miscarriage.[70] These embryos may be allowed to implant and as the embryo fails to develop further, a miscarriage will follow. As a result, women with recurrent miscarriage should have more karyotypically abnormal miscarriage. However to our knowledge most studies show no difference in the distribution of cytogenetically abnormal miscarriages in couples with recurrent miscarriage compared with controls.[71,72] Studies showing differences included mostly patients with two miscarriages, and observed that the incidence of chromosomal anomalies may decrease as the number of miscarriages increases.[73] Regardless of the chromosome pattern, C4d deposition along the trophoblast

was found to be similar in chromosomally normal and abnormal miscarriages,[74] indicating that increased complement activation in recurrent miscarriage is not dependent on chromosomal aneuploidies and demonstrates a different underlying pathophysiology. In future studies products of conception of women with recurrent miscarriage should always be tested with karyotyping, even though contamination often occurs and collecting and typing of miscarriage material is difficult, to differentiate between underlying immunological pathology and endometrium receptivity problems.

2.6 Treatment options

Despite the conflicting findings and various limitations in studying immunological aspects of recurrent miscarriage several immunotherapeutic strategies have been introduced. There is an ongoing interest in corticosteroid drugs to treat women with recurrent miscarriage. It has been proposed that corticosteroids could improve the intrauterine environment by reducing NK cell count, normalisation of the cytokine expression profile in the endometrium, and by suppression of endometrial inflammation.[75-77] However, there is clear evidence that controlled inflammation and activation of the immune response is essential for embryo implantation.[78] So administration of corticosteroids may be harmful as it potentially elevates the risk of altered fetal growth and developmental programming, congenital anomalies and preterm birth.[78] Fortunately, treatment is not licensed for use in reproductive medicine.

Furthermore, in a systematic review by Wong et al. no significant beneficial effect of immunotherapy (as paternal leukocyte immunization, or intravenous immunoglobulin (IVIG)) over placebo was observed in improving live birth rate in women with recurrent miscarriage.[79] In recent years the use of IVIG – a fractioned blood product which modulates the maternal immune system in recurrent miscarriage – has grown. Two different effects of IVIG has been described. Firstly, downregulation of systematic NK cells, abrogation of NK cell activity at the implantation site, and improvement of regulatory T cells. Secondly, the level of anti-HLA antibodies and auto-antibodies may be reduced by the antibodies in IVIG. Still, the use of IVIG is only approved by the Food and Drugs Administration (FDA) for autoimmune thrombocytopenia.

In women with unexplained recurrent miscarriage administration of exogenous progesterone to improve implantation is widely used. However, a recent RCT showed no evidence that first-trimester progesterone therapy improves outcomes in women with a history of recurrent miscarriage.[80]

As hypercoagulability might result in recurrent miscarriage, the use of anticoagulants as LMWH and aspirin, which both have anti-clotting properties, may increase live birth in women with recurrent miscarriage. In addition, the use of heparin could potentially reduce complement activation in women with recurrent miscarriage. Treatment with heparin is beneficial, and protects mice from pregnancy complications in case of anti-phospholipid antibodies through inhibition of complement activation.[63] Prophylactic use of heparin and low-dose aspirin may reduce pregnancy loss by 50% in women with recurrent miscarriage having anti-phospholipid antibodies.[81] At present there is no evidence of a beneficial effect of anticoagulants – LWMH or aspirin or a combination of both – in women with unexplained recurrent miscarriage.[82] However, the effect of anticoagulants in a homogenous group of women with unexplained recurrent miscarriage has not been studied yet. It is of interest, whether anti-HLA antibodies are capable of precipitating the coagulation pathway by the same mechanism as anti-phospholipid antibodies. And if so, whether coagulants can increase live birth rate after recurrent miscarriage in women having anti-HLA antibodies. As complement activation at the maternal-fetal interface indeed plays a role in a subgroup of women with recurrent miscarriage, treatment with inhibitors of the complement cascade could potentially have effect on live birth rate, such as LMWH, statins,[83] or biological,[84] such as anti-TNF (infliximab).[85] The presence of anti-HLA antibodies and/or C4d in products of conception may potentially serve as a diagnostic indicator for a next pregnancy, making this subgroup of women eligible for treatment with complement inhibitors or anticoagulants.

3. Conclusion

For many years research on recurrent miscarriage was mainly focussed on finding the underlying pathophysiological pathway in the woman. In this thesis, we showed that the interaction with the partner, and fetal antigens inherited from the partner, may play an important role in unexplained recurrent miscarriage. Future research on possible causes for unexplained recurrent miscarriage should therefore focus more on couples and their immunological interactions.

Furthermore, as also illustrated in this thesis, it is of great importance to use an adequate definition of recurrent miscarriage and control subjects in order to make it possible to compare the results of the different studies.

Regardless of the cause, the long-term prognosis of couples with recurrent miscarriage is good; in all our studies we observed a live birth rate around 70% after recurrent miscarriage comparable with previous observations.[86,87] However, one should realise that

multiple pregnancy losses can have a significant psychological toll on affected couples, and therefore efforts need to be made to improve treatment and decrease the time needed to achieve a successful pregnancy. In this thesis we identified several possible parameters related to the immunological pathways leading to recurrent miscarriage. The challenges for future studies is unravelling these possible immunological pathways even further, in order to identify diagnostic markers that can serve as a tool for patient tailored therapy.

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Chapter 11

Nederlandse samenvatting

Het gunstige verloop van een zwangerschap is vanuit immunologisch perspectief gezien heel interessant. De foetus, die zowel maternale genen als paternale genen heeft en dus gedeeltelijk lichaamsvreemd is voor de moeder, wordt in een ongecompliceerde zwangerschap door het immuunsysteem van de moeder geaccepteerd. Dit terwijl een getransplanteerde donornier met dezelfde genetische verschillen zou worden afgestoten.

Bij het onderscheid tussen lichaamseigen en lichaamsvreemd spelen de Humaan Leukocyten Antigenen (HLA) een belangrijke rol. HLA-antigenen zijn moleculen die op het membraam van alle cellen voorkomen en waarvan er heel veel varianten zijn. Dit wordt ook wel polymorfisme genoemd. Tijdens de innesteling van de foetale cellen in de baarmoeder wordt het maternale immuunsysteem geactiveerd, maar het maternale immuunsysteem moet ook worden onderdrukt zodat de foetale cellen zich kunnen innestelen en niet afgestoten worden. Verschillende immunologische mechanismes spelen een rol bij het ontstaan van maternale tolerantie ten opzichte van haar ongeboren kind. De gedachte is dat een inadequate maternale immuunreactie verantwoordelijk is voor een gedeelte van de tot nu toe nog onverklaarde herhaalde miskramen.[1,2]

Men spreekt van herhaalde miskramen indien tenminste 3 opeenvolgende miskramen zijn opgetreden. In 1 tot 2% van de stellen met kinderwens is dit het geval.[3] Tot nu toe bekende oorzaken van herhaalde miskramen zijn uterus anomalieën, endocriene factoren, cytogenetische factoren, erfelijke trombofiliefactoren en antifosfolipiden syndroom.[4,5] Bij 50-75% van deze stellen wordt geen oorzaak gevonden en blijven de herhaalde miskramen onverklaard.[6] Bij stellen met onverklaarde herhaalde miskramen zijn in de afgelopen jaren verschillende behandelingen uitgeprobeerd zoals immunotherapie, progesteron, HCG, aspirine, laag moleculair gewicht heparine en lage dosering insuline. Al deze behandelingen geven echter geen verbetering wat betreft zwangerschapsuitkomst.[7-10] Zonder behandeling is de kans op een levend geboren kind bij stellen met onverklaarde herhaalde miskramen ongeveer 75%, al laten recente studies wisselende percentages zien tussen 57-95%.[7,11-14] Ondanks dat de kans op een levend geboren kind hoog is, is de emotionele belasting en onzekerheid bij stellen met onverklaarde herhaalde miskramen groot. Een gedeelte van deze stellen zal ook nooit een levend geboren kind krijgen.

Het doel van dit proefschrift is om bij stellen met onverklaarde herhaalde miskramen de mogelijk onderliggende immunologische mechanismes te identificeren, waarbij we met name geïnteresseerd zijn in de interactie tussen het maternale immuunsysteem en de lichaamsvreemde vaderlijke HLA-antigenen. Door deze mechanismes beter te begrijpen hopen we in de toekomst effectieve en patiëntgerichte behandelingen te kunnen geven bij stellen met onverklaarde herhaalde miskramen.

In **hoofdstuk 2** geven we een systematisch overzicht van de mogelijke rol die het HLA systeem speelt bij herhaalde miskramen. Eerdere studies tonen associaties aan tussen onverklaarde herhaalde miskramen en specifieke maternale HLA-allelen en tussen onverklaarde herhaalde miskramen en het delen van dezelfde HLA-allelen tussen stellen. Deze studies geven geen eenduidige resultaten onder andere door het gebruik van verschillende definities voor geïncludeerde cases en controles, analyses van verschillende HLA allelen, en het gebruik van verschillende technieken om HLA te typeren. In ons review includeerden we alleen studies die strikte inclusiecriteria gebruikten en waarbij de HLA-antigenen bepaald waren met moleculaire typering technieken. Ondanks deze strikte inclusiecriteria, is nog steeds sprake van grote selectie en informatie bias in de geselecteerde studies en zijn de gevonden associaties klein, zodat we geen definitieve conclusies kunnen trekken.

Op het foetale trofoblast komen maar een deel van de HLA-antigenen tot expressie zodat een verminderde allo-immuunreactie van het maternale immuunsysteem optreedt. Alleen het niet-klassieke oligomorfe HLA-G en HLA-E en maar een enkel klassiek HLA-gen, namelijk HLA-C, komen op het foetale trofoblast tot expressie. Aangezien het maternale immuunsysteem tijdens innesteling alleen in contact komt met deze antigenen, zijn wij in dit proefschrift geïnteresseerd in verschillende aspecten van deze antigenen bij stellen met herhaalde miskramen.

In **hoofdstuk 3** beschrijven we genetische polymorfismen van HLA-G in vrouwen met herhaalde miskramen en vrouwen met een ongecompliceerde zwangerschap. We waren met name geïnteresseerd in een combinatie van verschillende polymorfismen omdat juist die combinatie zorgt voor de expressie van oplosbaar (sHLA) en membraangebonden vormen van HLA-G. We zagen inderdaad dat een combinatie van polymorfismen, leidend tot het HLA-G UTR-4 haplotype, significant minder vaak werd gevonden bij vrouwen met herhaalde miskramen. Dit suggereert dat HLA-G UTR-4 haplotype een belangrijke immunoregulatoire rol speelt bij een ongecompliceerde doorgaande zwangerschap.

Omdat HLA-C het enige klassieke HLA-gen is dat op de trofoblast tot expressie wordt gebracht, hebben we in **hoofdstuk 4** gekeken of een maternale allo-immuunreactie tegen het HLA-C allel van de vader een rol speelt in onverklaarde herhaalde miskramen. We zagen een verhoogde frequentie van HLA-C *07, een van de meest immunogene HLA-C allelen, bij partners van vrouwen met herhaalde miskramen. Als gevolg hiervan werden meer mismatches gevonden voor HLA-C *07 tussen moeder en vader in vergelijking met paren met een ongecompliceerde zwangerschap. De incidentie van HLA antilichamen was ook verhoogd bij vrouwen met herhaalde miskramen in vergelijking met vrouwen met een ongecompliceerde zwangerschap.

Omdat de klinische relevantie van deze antilichamen tijdens de zwangerschap niet duidelijk is, hebben we in **hoofdstuk 5** het effect van antilichamen op zwangerschapscomplicaties, waaronder herhaalde miskramen, onderzocht. De geselecteerde studies tonen een hoog niveau van statistische en klinische heterogeniteit als gevolg van het gebruik van verschillende screeningtechnieken, variërende tijdstippen van screening en gebruik van onjuiste controlegroepen. Bovendien ontbrak in de meeste geïncludeerde studies gedetailleerde analyse van de karakteristieken en specificiteit van deze antilichamen. Zoals we van onderzoek bij transplantatie weten zal maar een deel van de antilichamen afstoting veroorzaken, daarom is informatie over specificiteit, de potentie om complement te fixeren, titer van de antilichamen, en welke HLA-epitopen antilichamen herkennen essentieel. De rol van specifieke HLA-C antilichamen was nog niet onderzocht bij vrouwen met herhaalde miskramen, terwijl HLA-C toch het enige polymorfe klassieke HLA-antigen is dat op trofoblast tot expressie wordt gebracht. Daarom hebben we in **hoofdstuk 6** gekeken naar de rol van specifieke HLA-C antilichamen bij vrouwen met herhaalde miskramen. De aanwezigheid, de specificiteit van HLA-antilichamen en de mogelijkheid om complement te binden werd bepaald in het eerste trimester van de volgende zwangerschap bij vrouwen met herhaalde miskramen en vergeleken met vrouwen met een ongecompliceerde zwangerschap. HLA-C antilichamen werden significant vaker gevonden bij vrouwen met herhaalde miskramen, wat suggereert dat deze antilichamen een rol kunnen spelen in een deel van de vrouwen met herhaalde miskramen. Omdat niet al deze HLA-C antilichamen complement konden binden, moet nog vastgesteld worden welk mechanisme precies betrokken is bij de etiologie, het binden van complement of antilichaam afhankelijke cellulaire cytotoxiciteit.

In **hoofdstuk 7** zagen we vaker C4d depositie in miskraamweefsel bij vrouwen met herhaalde miskramen in vergelijking met vrouwen met een sporadische miskraam en vrouwen met een electieve abortus. C4d is een biomarker voor klassieke complementactivatie, die in de regel plaats vindt door binding van antilichamen. C4d hecht aan cellen en weefsel en werkt daardoor als een marker van recente door antilichaam aangebrachte weefselbeschadiging.

Concluderend wijzen de gecombineerde resultaten van **hoofdstuk 4**, **hoofdstuk 6** en **hoofdstuk 7** erop dat bij een deel van de vrouwen met onverklaarde herhaalde miskramen, antilichaam-gemedieerde afstoting van het foetale allo-transplantaat een rol kan spelen.

Eerdere studies in muizen laten zien dat ten tijde van de geslachtsgemeenschap, al lang voordat de implantatie plaatsvindt, maternale tolerantie tegen foetale allo-antigenen optreedt.[15,16] Humaan semen bevat ook verschillende factoren zoals cytokinen, chemokinen,[17] en sHLA,[18,19] die de maternale immuunreactie kunnen moduleren.[20,21] We laten in **hoofdstuk 8** zien dat humaan semen inderdaad allerlei immuun regulerende

factoren bevat, waaronder hoge concentraties TGF-β, IDO en sHLA klasse I. Tevens heeft humaan semen een immuun modulerend effect op T-cellen. Contact met semen leidde tot een verhoogde proliferatieve respons van T-cellen en de expressie van Foxp3, een marker voor regulerende T-cellen. Verder zagen we dat antigeen presenterende cellen nodig zijn om IL-10- en CD25 expressie te induceren op T-cellen na blootstelling aan humaan semen.

Zoals Koelman en anderen[19] eerder beschreven, is contact met HLA-antigenen in semen via de orale mucosa een manier om tolerantie voor de vaderlijke HLA-antigenen van de foetus tijdens de zwangerschap te creëren. In **hoofdstuk 9** hebben we daarom gekeken of vrouwen met onverklaarde herhaalde miskramen minder vaak orale seks hebben in vergelijking met gematchte vrouwen met een ongecompliceerde zwangerschap. In deze gematchte case-controle studie is het hebben van minder orale seks geassocieerd met het optreden van herhaalde miskramen. Gezien de methodologische en statische problemen in dit hoofdstuk, kan echter nog geen definitieve conclusie getrokken worden.

Concluderend laten we in dit proefschrift zien dat verschillende immunologische interacties tussen de vrouw en de partner, en de foetale antigenen afkomstig van de partner, een rol kunnen spelen in onverklaarde herhaalde miskramen. De uitdaging voor de toekomst is om deze immunologische interacties nader te onderzoeken om diagnostische markers te identificeren die kunnen dienen als hulpmiddel in de keuze van therapie, toegespitst op de individuele patiënt.

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Chapter 12

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List of abbreviations

3'UTR	3'untranslated region
ACTB	β-actin
APCs	Antigen presenting cells
APS	Anti-phospholipid syndrome
BCR	B cell receptor
Bp	Basepair
BMI	Body mass index
CDC	Complement dependent cytotoxicity
CI	Confidence interval
Cpm	Counts per minute
CTLA-4	Cytotoxic T lymphocyte associate protein 4
DAF	Decay accelerating factor
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
FNAIT	Fetal/ neonatal allo-immune thrombocytopenia
fPBMCs	Female PBMCs
HELLP	Hemolysis Elevated Liver enzymes and Low Platelets
HLA	Human leukocyte antigen
IFN	Interferon
IDO	Indomelamine 2,3-dioxygenase
IL	Interleukin
ILT	Immunoglobulin-like transcripts
IUGR	Intra-uterine growth restriction
IQR	Interquartile range
ISHHP	International Society for the study of Hypertension in Pregnancy
GITR	Glucocorticoid-induced tumor necrosis factor receptor
KIR	Killer immunoglobulin-like receptor
LIR	Leukocyte immunoglobulin-like receptor
LMWH	Low molecular weight heparin
LUMC	Leiden University Medical Center
MAC	Membrane attack complex
MCP	Membrane cofactor protein
MESH	Medical subject headings
MFI	Median Fluorescence Intensity
MHC	Major histocompatibility complex

mRNA	Messenger RNA
NK	Natural killer
OR	Odds ratio
PBMCs	Peripheral mononuclear blood cells
Pg/ml	Picogram per ml
PIGF	Placental growth factor
RA	Rheumatoid arthritis
REMI	REcurrent MIscarriage studies
RFLP	Restriction fragment length polymorphism
SBT	Sequence based typing
SES	Socio-economic status
sHLA-G	Soluble HLA-G
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphims
SP	Seminal plasma
SSO	Sequence specific oligonucleotides
SSP	Sequence specific priming
TCF	Two color fluorescence
TCR	T cell receptor
Th	T helper
TNF	Tumor necrosis factor
TRALI	Transfusion-related acute lung injury
Treg	Regulatory T cell
Tr1	Type 1 regulatory
VCM	volume x concentration x motility
VEGF	Vascular endothelial growth factor
1-MT	1-methyl-tryptophan

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Curriculum vitae

Tess Meuleman was born on May 13th 1984 in Sittard and grew up in Nijmegen. In 2002 she graduated from the secondary school at the Stedelijk Gymnasium in Nijmegen. In 2003 she got a field hockey scholarship at the Kent State University in Ohio and studied there for one year following mainly premedical school courses. In 2003 she started medical school at the Leiden University. During her medical education she played field hockey at the highest Dutch competition level and in the Dutch national team under 21. As player of the Dutch national team she became European Champion in 2004. In 2009 she attained her medical degree and started working as a physician (ANIOS) in Obstetrics and Gynaecology at the Westeinde Hospital, The Hague. In February 2011 she started as prenatal doctor at the Department of Obstetrics at the Leiden University Medical Center (LUMC) and combined her work with research on paternal factors in recurrent miscarriage. The studies described in this thesis were performed at two departments in the LUMC under supervision of Prof. K.W.M. Bloemenkamp and Prof. J.M.M. van Lith (Dept. of Obstetrics) and Prof. F.H.J. Claas (Dept. of Immunohematology and Blood Transfusion). In 2012 she worked for 3 months as a medical officer in Minga Mission Hospital, Zambia, as her future husband was employed there as a tropical medicine doctor. Together they started there the See&Treat project via the Female Cancer Foundation. In July 2014 she began her residency in Obstetrics and Gynaecology at the Hagaziekenhuis, The Hague (Dr. B. Hellebrekers). After this year she worked for 3 months at the Department of Obstetrics and Gynaecology of the Moi Teaching Referral Hospital in Eldoret, Kenia, where she performed many obstetric interventions and trained residents in performing obstetric ultrasound. In January 2016 she continued her residency training at the Department of Obstetrics and Gynaecology of the LUMC (Prof. J.M.M. van Lith).

In 2010 she met Bas Vestjens at the Westeinde Hospital, The Hague, while supervising him with a labour. They married in 2013 in Leiden and have two sons, Teun and Guus, both born in February 2017.

Dankwoord

Allereerst wil ik de patiënten bedanken die bereid waren om mee te doen aan dit onderzoek en speciaal terugkwamen op de "terugkomdagen" en gelukkig de meesten vol trots hun kind(eren) konden laten zien.

Frans Claas, een man van weinig woorden maar wel de juiste woorden, die mij inspireerde en uitdaagde om dieper de materie in te duiken. Bedankt dat jij vertrouwen in mij bleef houden door alle jaren heen.

Jan van Lith, heel erg dank voor je persoonlijke begeleiding in het terugkomen na mijn verlof waardoor de afronding van dit proefschrift mogelijk was. Dit heeft heel erg veel betekend voor me.

Kitty Bloemenkamp, bij jou begon het allemaal. Jouw fascinatie voor vrouwen met herhaalde miskramen en de onbekende wereld van de immunologie was aanstekelijk.

Researchverpleegkundigen, eerst Clara en Marjolein, die ontzettend veel hulp boden bij het opzetten van dit onderzoek en vol enthousiasme meededen aan de "terugkomdagen". Later Birgit en Marianne die het vervolgonderzoek bij vrouwen met herhaalde miskramen naar een professioneel niveau hebben getild.

Ivanka en Gladys, dank voor alle kleine klusjes oplossen, die samen heel groot waren.

De Polidames Verlos, wat is het altijd een feest bij jullie en daarmee zorgden jullie onbewust dat ik vol overgave kliniek en onderzoek kon blijven combineren.

Marise en Jantien, samen Kitty's meisjes, met jullie sparren gaf altijd de juiste motivatie.

Afdeling Immunohematologie en Bloedbank, dank aan iedereen. In het bijzonder Geert Haasnoot, die mij bijstond met koppelen van enorme databases en onmogelijke analyses en Jos Drabbels, Jacqy Anholts, Sophia Stein, Simone Brand-Schaaf, Nelleke Korfage en Willem Verduyn, die mij hielpen met alle bepalingen die verricht moesten worden voor dit onderzoek.

Afdeling Reproductieve Immunologie, PhD studenten, studenten, en analisten, in het bijzonder Els, Godelieve en Carin, jullie stonden mij altijd bij en deden velen experimenten alleen als ik weer snel de kliniek in moest voor echo's.

Michael Eikmans, in jouw vond ik een fijne begeleider bij de laatste loodjes van mijn proefschrift, waarvoor dank. Houd het enthousiasme over je nieuwe hobby, de reproductieve immunologie, vast.

Marie-Louise en Lisa, mijn voorgangers. Jullie hebben mij geïnspireerd en geholpen waar nodig, mooi om te zien dat het onderzoek bij stellen met herhaalde miskramen door blijft gaan onder jullie bezielende leiding.

Afdeling Pathologie, met name dank aan Danielle Cohen, haar bevlogenheid over complement was de start van dit proefschrift en Kimberley Veraar, die hielp met C4d kleuringen.

Olaf Dekker en Saskia le Cessie, jullie stonden mij bij met de lastige epidemiologische vraagstukken van dit proefschrift waarvoor dank.

Alle mede-onderzoekers door alle jaren heen, Fenna, Sanne, Aletta, Peggy, Claire, Mathijs, Sara, Evelien, Lukas, Dacia, Linda, Ada, Jolijn, Inge, Ellen, Vivian, Marlies, Joost, Suus, Femke, dank voor alle gezamenlijke kroketten en discussies die beide ontzettend veel motivatie gaven. En natuurlijk Jeroen en Kim voor Mar10 op vrijdag, de lach die nodig was voor de afronding.

Lieve ouders, door jullie enorme onvermoeibare inzet en door jullie aan mij geleerde doorzettingsvermogen is dit proefschrift af, dank hiervoor en voor alles. Lieve broers, kleine zusjes worden groot dankzij grote broers. Renee, mijn kleine zusje maar oh zo groot in steun naar mij.

Lieve schoonouders, jullie altijd luisterende oor en meedenken waren heel erg verhelderend.

Paranimf, Frederike, lieve Freddie, jouw steun bij lief en leed door de jaren heen is altijd onvoorwaardelijk geweest, onze vriendschap is uitgegroeid tot iets heel moois.

Paranimf, lieve Marlies, onze vriendschap zo kort maar zo krachtig, ontstaan tijdens het onderzoek, ik had het niet willen missen, je bent een topper.

Lieve Edith, op voor mij cruciale momenten onmisbaar, zowel in daad als woord.

Lieve vriendinnen, Lot, Stef, Do, Hannah, Bregje, ook al hebben we afgelopen jaren minder tijd voor elkaar, momenten met jullie zijn altijd weer geweldig.

Allerliefste Bassie(man), van alle teams waar ik in heb gezeten, ben jij absoluut de beste, leukste, grappigste, meest zorgzame, en gezelligste teamgenoot ooit. Als team samen de hoofdprijs gewonnen, ons goud, onze jongens.

Allerliefste jongens, lieve Teun en Guus, jullie aanwezigheid hebben mij de rust en het relativeringsvermogen gegeven om dit proefschrift af te ronden en nog veel meer. Jullie lachende aanwezigheid maakt elke dag tot een feest.