DEPARTMENT OF CLINICAL SCIENCE, INTERVENTION
AND TECHNOLOGY
Karolinska Institutet, Stockholm, Sweden

MATERNAL MICROCHIMERISM

Anna Maria Kanold

Karolinska Institutet

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Till minnet av min pappa, Torsten Jonsson.

”Fast själve mig var väl inte så märkvärdigt att göre, men alla di här krumelurerna jag har i öronen, det måste ha vart marigt att få ihop”
Lina, under husförhöret i Katthult (Emil i Lönneberga)

ABSTRACT

Microchimerism refers to one individual harboring cells or DNA at a low level that derive from another individual. The most common source is pregnancy when cells from the fetus and the mother pass the placenta bidirectionally, and give rise to maternal microchimerism (cells from the mother in the fetus) and fetal microchimerism (cells from the fetus in the mother). The cells persist in the individual, at least until middle-age. Several hypotheses have addressed the consequences of harboring semiallogeneic cells. Both maternal and fetal cells within the host are associated with certain autoimmune diseases, as inducers of the disease but also as repairers of already injured tissue. Furthermore, inducing fetal-maternal tolerance during pregnancy seems to be an important purpose of the cell-trafficking. Observations from the transplantation field yield that this effect may be long lasting.

In paper I, we examined the presence of maternal cells within different cellular subsets in tissues from eleven 2nd trimester fetuses. Seven fetuses presented with maternal microchimerism. The cells were widely spread in different tissues and found in both normal fetuses and fetuses with trisomy 21. The cells were of mature immunological and hematopoietic stem cells character.

Paper II aimed to examine maternal microchimerism in healthy children's tonsils and adenoid by a quantitative PCR assay. We found maternal microchimerism in four of 20 children. The children were between four and six years old and harbored maternal cells in the tonsils and/ or adenoid at levels that ranged from 2 x10^{-2} to 7.1 x10^{-5}. The same children were also positive for maternal cells in peripheral blood.

In paper III, we investigated the association between maternal microchimerism in peripheral blood and systemic lupus erythematosus. In the study, 32 nulligravida women with SLE were included. Seventeen brothers of the women and additional 12 unrelated males constituted healthy controls. Two patients and one control appeared with maternal cells in peripheral blood. The result indicates no correlation between maternal microchimerism in blood and SLE (P-value = 0.65). At a follow up, the same individuals tested negative 16 years after the first draw date.

The purpose of paper IV was to evaluate the cellular subset and frequency of maternal cells in umbilical cord blood following vaginal deliveries and caesarian sections, when the time of umbilical cord clamping was known. We included 44 babies from normal term pregnancies who were delivered vaginally (24) and by caesarian section (20). Five of 44 (11%) of the umbilical cord blood samples contained maternal microchimerism. The positive fractions were total DNA, CD34+ and CD56+. Four of the five positive samples were from caesarian sections and one was from a vaginal delivery. The positive samples were from deliveries with a mean clamping time of 37 seconds compared to 54 seconds in the negative group.

Overall, we have shown that maternal cells are common in fetuses, infants and children. Their nature is of mature immunological and hematopoietic stem cell character. There is no correlation between the autoimmune disorder SLE, and maternal microchimerism.

LIST OF PUBLICATIONS

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II. Jonsson AM, Papadogiannakis N, Granath A, Häggström J, Schaffer M, Uzunel M, Westgren M.
Maternal microchimerism in juvenile tonsils and adenoids.
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A research study of the association between maternal microchimerism and systemic lupus erythematousus in adults: a comparison between patients and healthy controls based on single-nucleotide polymorphism using quantitative real-time PCR.
PLoS One. 2013;8:e74534

IV. Kanold AM, Westgren M, Götherström C.
Cellular subsets of maternal microchimerism in umbilical cord blood.
Manuscript

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LIST OF ABBREVIATIONS

Mc	microchimerism
HSCT	hematopoietic stem cell transplantation
MMc	maternal microchimerism
FMc	fetal microchimerism
UCBT	umbilical cord blood transplantation
T regs	regulatory T cells
GvHD	graft-versus-host disease
SSc	systemic sclerosis
MHC	major histocompability complex
HLA	human leukocyte antigen
mHags	minor histocompatibility antigens
HSC	hematopoietic stem cell
GvL	graft-versus-leukemia
APC	antigen-presenting cell
UBC	umbilical cord blood
NK cells	natural killer cells
NIMA	non-inherited maternal antigen
NIPA	non-inherited paternal antigens
uNK cells	uterine NK cells
Th cells	T helper cells
MLR	mixed lymphocyte reaction
FISH	Fluorescent in-situ hybridization
PCR	polymerase chain reaction
SCID	severe combined immunodeficient disease
JDM	juvenile dermatomyocytis
JIIM	juvenile idiopathic inflammatory myopathies
NLS	neonatal lupus syndrome
QRT- PCR	quantitative real time PCR
SLE	systemic lupus erythematosus

1 INTRODUCTION

This thesis is about how your mother may influence you from inside.

"…a thing of immortal make, not human, lion-fronted and snake behind, a goat in the middle and snorting out the breath of the terrible flame of bright fire…" Homer's description in the Illiad is the earliest description of the animal chimera which has its origin in the Greek mythology (1). In medicine, a chimera is defined as an individual that harbors foreign cells or DNA. When the number of cells is small the term microchimerism (Mc) is used.

A microchimeric state may arise after a blood transfusion, an organ transplant or after hematopoietic stem cell transplantation (HSCT) (2,-4). However, the most common source of Mc is not from iatrogenic interventions, but from pregnancy when cells or DNA from the mother and the fetus pass bidirectionally over the placenta and result in maternal (MMc) and fetal Mc (FMc) respectively (5). This observation raises many imperative questions. Cells of fetal or maternal origin will be semiallogeneic in relation to the host (mother and offspring, respectively), and as such would be anticipated to evoke an immune response (6).

The Nobel Prize winner in medicine and physiology in 1960, the immunologist Peter Medawar asked in an article from 1953: "how does the pregnant mother contrive to nourish within itself, for many weeks or months, a fetus that is an antigenically foreign body?" (7). He proposed in his article three theories that could explain this immunological wonder:

1. **An anatomical, impermeable barrier- the placenta- separates the fetus from the mother.**

   This notion had been prevailing for long despite Schmorl reporting already in 1893 about the findings of trophoblasts in the maternal pulmonary circulation (8). However, in the 1907 edition of William's Textbook of Obstetrics it could be read: "The foetal blood in the vessels of the choronic villi at no time gains access to the maternal blood in the intervillous spaces" (9). Later, 1959, trophoblasts were also observed in the maternal blood. (10). Walknowska was first to report, in 1969, about fetal lymphocytes in the maternal circulation of pregnant women (11). Already at that time, the authors

speculated if the findings could be useful for prenatal diagnostics. In the early 1960s, fluorescently or radioisotopically labeled cells were injected into the mothers prior to parturition, and were thereafter detected in cord blood of their infants - the first evidence of maternal microchimerism was displayed (12, 13).

Thus, the first theory of Medawar has been proved wrong and it is now well accepted that there is a bidirectional exchange of cells over the placenta during pregnancy and that the semiallogeneic cells may persist in the offspring and the mother for years (14, 15). This fact make the immunological wonder of pregnancy even more fascinating since it has been proven that the mother and fetus actually "see" each other during the nine months and later in life.

The second theory presented by Medawar went as follow:

2. **The fetus is immunologically immature.**

A growing body of evidence showed that this hypothesis is not sufficient to explain why the fetus does not reject its mother. An early experiment by Billingham et al. showed that fetal chicks and mice who were exposed to foreign antigens in utero tolerated a skin graft from the same donor better, compared to those who were not previously exposed (16). Recent research has shown that the fetus is capable to create tolerance by inducing CD4+ cells to become regulatory T cells, and thereby achieve tolerance to self and to the non-inherited maternal antigens of maternal chimeric cells (17).

The last theory concerned the immune system of the pregnant woman:

3. **The immune system of the mother is inert.**

Today it is well known that fetal antigens elicit both a cellular and humoral response in the mother although she obtains tolerance towards them during pregnancy (18). The tolerance could be long-lived or transitory (19, 20). Recently, indirect evidence that recipients in umbilical cord blood transplantation (UCBT) might benefit from cellular responses from maternal microchimeric cells in the transplant if the recipient and donor shared the same inherited paternal antigen (21).
Regulatory T cells (T regs), actively induced, also seem to contribute to the maternal tolerance (22).

Historically, the consequences of pregnancy related Mc were first focused on FMc due to the fact that many autoimmune diseases have a female dominance and that autoimmune diseases generally have a peak incidence during and after childbearing age. Additionally, the striking resemblance of chronic graft-versus-host disease (GvHD) to certain autoimmune diseases such as systemic sclerosis (SSc) inspired the theory that diseases that had traditionally considered as autoimmune processes might in fact have an alloimmune component. This hypothesis was first formulated in 1996 by Lee Nelson: “Is some autoimmune disease auto-alloimmune or allo-autoimmune?” (23). The microchimeric cells residing in an individual following a pregnancy will be semi allogeneic and as such would be expected to elicit an immune response. The hypothesis has now included MMc and autoimmunity and several diseases in children have been associated with persistent maternal cells. Briefly, three questions are asked in this context: Do maternal cells attack host cells? Are maternal cells the target of an attack? Are maternal cells homing to repair damaged tissue?
However, MMc is common in healthy individuals; in cord blood, peripheral blood of children and adults and in tissues.

In this thesis I will in the first chapters briefly discuss the anatomical and physiological features of the permeable “membrane” between the mother and the fetus i.e. the placenta. Thereafter, I will summarize the concept of transplantation immunology because of its parallels to the circumstances of the”transplanted” fetus. Finally, before the summary of the research addressing naturally acquired microchimerism, I will discuss the immunological events in the placenta, the mother and the fetus during pregnancy.

2 BACKGROUND

2.1 THE PLACENTA

The placenta constitutes the maternal-fetal interface and provides many functions throughout pregnancy, including the transportation of fetal nutrients and metabolic products as oxygen and carbon dioxide. It also produces numerous hormones and enzymes which are released into the maternal blood.

The placenta consists of one fetal part - the umbilical cord, the fetal membranes (amnion and chorion) and the chorionic villi – and one maternal part, i.e. the decidua. Figure 1.

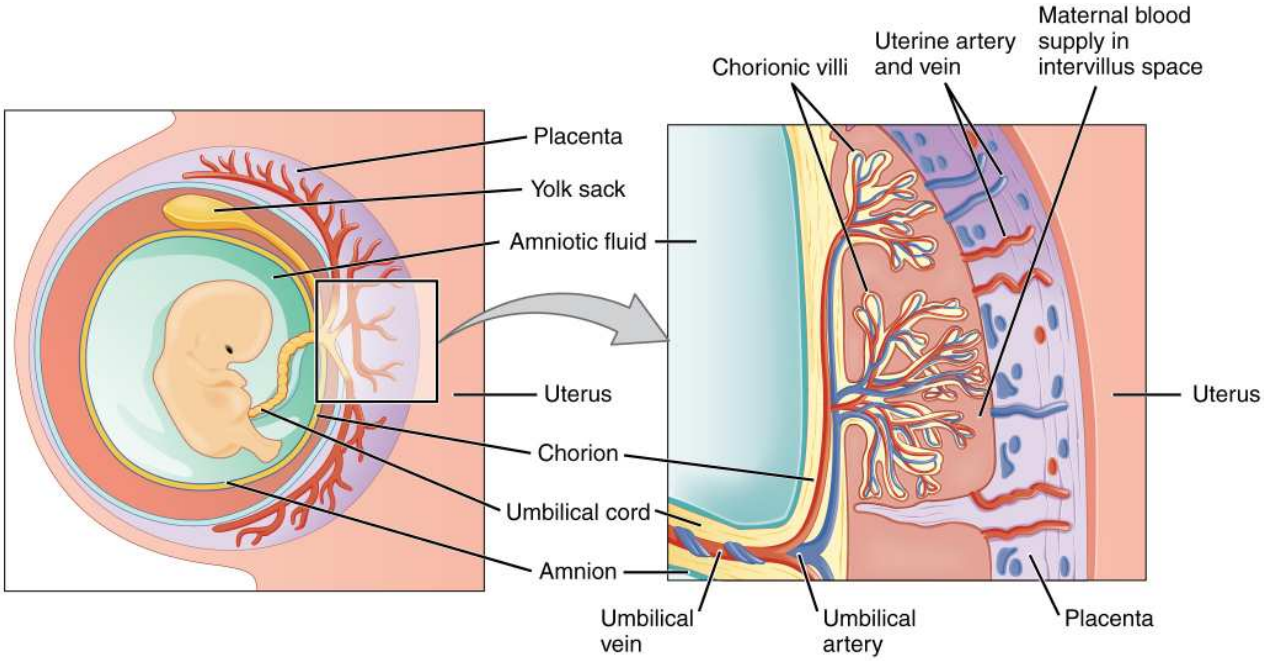

Figure 1. The placenta

During the first gestational week two types of cells develop from the fertilized ovum. The outer layer is covered by one layer of ectodermal cells, the primitive trophoblast, except at one pole where the rapidly dividing cells have formed the inner cell mass, which constitutes the beginning of the embryo. The invasive capacity of trophoblastic cells makes the blastocyst descend into the endometrium around day six or seven post fertilization. After implantation mesodermic cells grow out beneath the primitive trophoblast and form villi. Each villous consists of a mesodermic core covered by two layers of trophoblastic cells (24, 25). The outer cells are called syncytiotrophoblasts whereas the inner cells are termed cytotrophoblasts. Figure 2. The cytotrophoblasts decrease in number as pregnancy progresses.The syncytiotrophoblast is a multinucleated continuous cell layer that covers the surface of the placenta. It forms as

a result of differentiation and fusion of the underlying cytotrophoblast, a process that continues throughout placental development and by that contributes to the barrier function of the placenta. The extravillous trophoblast arises from proliferating cell columns and penetrates deep into the endometrium and the walls of the uterine wall. Invasive extravillous trophoblast plays an active role in the remodeling processes that occur in the uterine spiral arteries (24, 25). Pools of maternal blood, known as lacunae, surround and develop within the trophoblastic syncytium. Throughout pregnancy, the two circulatory systems remain separate. During the third week villi become united with maternal vessels that communicate with the intervillous space. Not until the end of the fourth gestational week, connections are made between the vessels of the chorionic plate and those of the fetus.

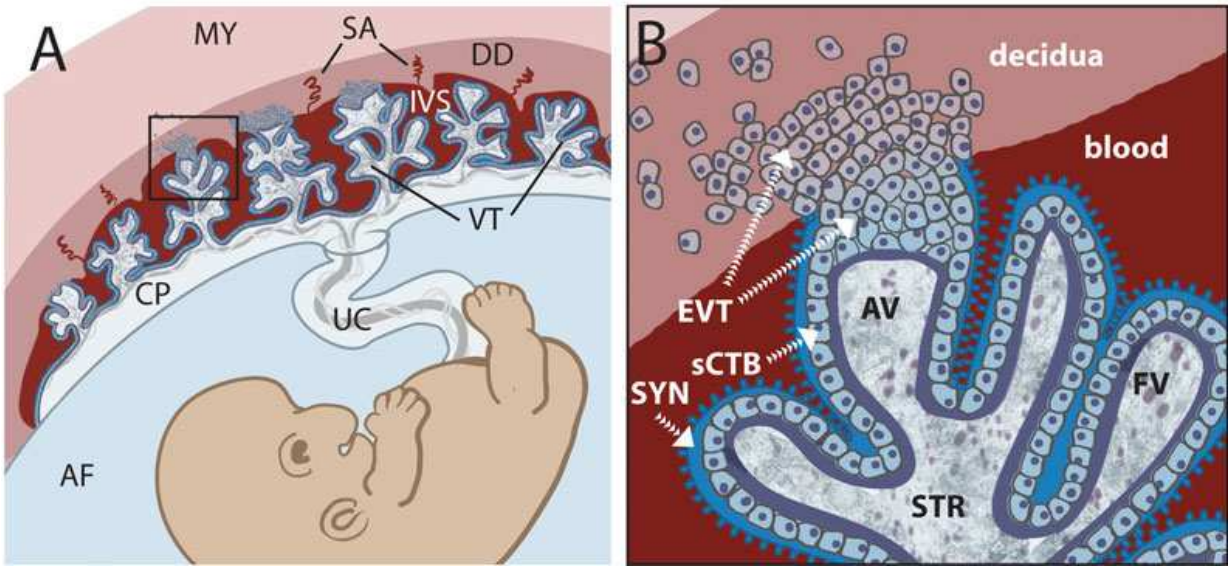

Figure 2. Details of the placenta.
AF= amniotic fluid
CP= chorion plate
DD=deciduas
IVS=intervillous space
MY=myometrium
SA= spiral arteries
UC=umbilical cord
VT=villi tree
AV= anchoring villi
CTB= subsyncytial cytotrophoblasts
EVT= extravillous cytotrophoblast
FV=floating villi
STR=stroma
SYN= syncytiotrophoblasts

2.1.1 Cellular traffic across the placenta

Maternal or fetal cells passing across the placental barrier must migrate both through the fetal capillary endothelial cell layer and the trophoblast cell layers. The mechanism by which this happens is uncertain. Dawe et al. hypothesized in a review about possible

explanations which include 1) microtraumatic hemorrhage of the placental blood channels 2) specific cell types being capable of adhesion to the trophoblasts and of the walls of the fetal blood channels and migrate through the placental barrier created by the trophoblasts 3) deportation of trophoblast lining the maternal vessels and intervillous space into the maternal circulation (FMc) (26).

Previous research that supports the first theory include the discovery of intervillous thrombi containing mixed maternal and fetal cells in the human and rodent placenta, and the finding of histological defects in the continuity of the trophoblast lining (27, 28, 29). The incidence of transplacental bleeding and if there is a certain time this happens during pregnancy it is not known (30, 31). Massive fetomaternal transplacental hemorrhage may have subsequent adverse outcomes for the neonate. It is not uncommon and it confirms the occurrence of events with defect trophoblast lining (32). The appearance of trophoblasts in maternal blood may also be explained by "the microtraumatic theory".

The second theory, presented by Dawe et al., includes active adhesion and transmigration of lymphocytes with help from special cell adhesion molecules including PECAM-1 and ICAM-1, which are expressed in the fetal capillary endothelial cell layer (33, 34). Trophoblasts also express ICAM-1 and other adhesion molecules (35). The authors hypothesized that the transfer of microchimeric cells might resemble the migration of cells that occurs across high endothelial venule endothelium in peripheral lymph nodes and at the blood-brain barrier (26).
By term most cytotrophoblast and syncytiotrophoblast are replaced by a very thin (1-2 µm) vasculosyncytial membranethat contains transtrophoblastic channels or pores that may also explain Mc exchange between mother and fetus (36).

2.2 TRANSPLANTATION IMMUNOLOGY

2.2.1 Major histocompability complex (MHC)

MHC is a cell surface molecule group encoded by a large polymorphic gene family located on chromosome six and consisting mainly of two types, MHC I and II (37). Their main function is to mediate interactions of leukocytes, for example to present antigens to the T cells. In humans they are called human leukocyte antigen (HLA) class I and II. All nucleated cells carry HLA class I (HLA-A, -B, -C) which present antigen

of intracellular origin (synthesized from self proteins or viral antigens) to CD8+ cells (cytotoxic T cells) (37). Class II molecules (HLA-DR, -DP, -DQ) are expressed primarily on antigen presenting cells (APC), including dendritic cells and macrophages, and also on activated T cells and present antigen of extracellular origin to CD4+ cells (T helper cells) (38). A specific T cell recognizes only a few HLA class-peptide combinations but is dependent on them to be activated, a phenomenon known as MHC restriction. Certain HLA class I and class II genes are highly polymorphic; some have several hundred alleles. Furthermore, polymorphism of HLA class I heavy-chain genes and HLA class II α- and β- chain genes increases the variety and strengthen T cell immunity. Both the paternal and maternal inherited HLA genes are expressed simultaneously, contributing to the diversity of possible antigen recognition. At a population level, the diversity of HLA molecules is important to survive epidemic diseases but it also creates the main immunological barrier to clinical transplantation. Some HLA class II molecules particularly DR and DQ are associated with autoimmunity.

In general, HLA antibodies are not naturally occurring but may develop as a consequence of encountered non-self HLA for example in blood transfusion, pregnancy (paternally inherited antigens), or organ or tissue transplant.

2.2.2 Minor histocomability antigens (mHags)

mHags are polymorphic self-proteins presented as breakdown products in the context of HLA class I or class II molecules. They do not evoke immune reactions as strong as MHCs but they elicit an immune response due to allelic differences between humans and have been shown to play a role in transplantation and pregnancy. In HLA- matched HSCT mHags may provoke a GvHD or modulate graft rejection in organ transplantations. (40). Parous female donors of hematopoietic stem cells (HSCs) are more likely to elicit a GvHD in recipients compared to non-parous or male donors, explained by previous sensitization to Y chromosome mHag peptides (HY) with subsequent initiation of an HY-specific CD8+ T cell response (41). Approximately 50 different mHags have been identified and they are encoded on the Y chromosome, and on many autosomes (42). The large number of mHags indicates that histo-incompatibility between donors-recipients or mother-fetuses is not a rare event. In transplantation not only unfavorable immune responses to mHags are elicited; mHag-specific donor T cells may mediate a graft-versus-leukemia effect (GvL) or graft-

versus-tumor effect (43). This function can be of advantage by using donor T-cells specific for a certain mHag, whose expression is restricted to the GvL effect by isolating them from the recipient's blood, expand them and then re-infuse them into the patient (44, 45, 46). The role of mHags in pregnancy will be further discussed in the chapter of Reproductive Immunology.

2.2.3 Transplantation

The genetic differences in the highly polymorphic HLA molecules are the dominant underlying cause of immune responses against transplanted tissue or organs. After organ transplantation, alloreactions developed by the recipient's immune system are directed at the cells of the graft and may cause a transplant rejection or a host-versus-graft reaction. In contrast, in HSCT, the recipient's immune system has been destroyed and the major type of alloreaction arises from mature T cells in the graft and is directed towards cells in the host. A graft-versus-host reaction starts and leads, with varying degrees of severity, to GvHD in almost all patients who undergo a HSCT. The degree of disparity of HLA molecules between donor and recipient correlate to the risk of GvHD and host-versus-graft reactions (47). T cells recognize alloantigens via two different pathways; the direct and the indirect (48). The direct pathway of allorecognition implicates T cells recognizing alloantigens as intact molecules presented by donor antigen-presenting cells (APCs). In the indirect pathway, T cells recognize alloantigens presented by host APCs by direct interaction of their receptors with the donor HLA molecules. Figure 3.

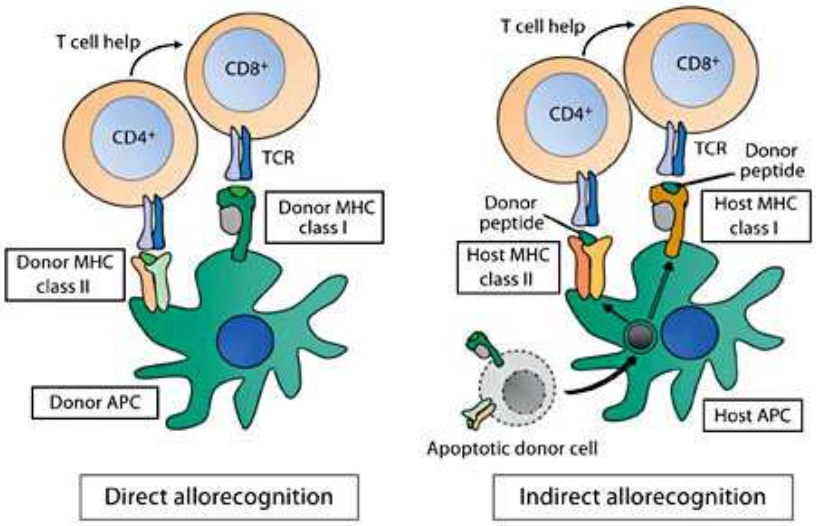

Figure 3. Direct and indirect allorecognition demonstrated. With kind permission from dr Tomo Saric.

HSC are used in both autologous (a patient is given back his own stem cells) and allogeneic (a patient receives another individuals stem cells) transplantations with the aim to reconstitute the hematopoietic cell lineages in patients with both malignant and non-malignant disorders (49). The sources of HSC may be different; bone marrow, mobilized stem cells from peripheral blood or umbilical cord blood (UCB). When considering an unrelated donor, matching of HLA class I A, B and C and HLA class II DR and DQ is of most importance (50). If the match of these genes is perfect, the over-all survival rate is comparable to matched related donor HSCT (51). However, achieving this perfect match is rare. A prevailing ranking of what genes are more important is not established. Prior to the HSCT, the recipient is subjected to destruction of his or her immune system (conditioning) in order to create space for donor cells and to suppress the ability to react against the allograft. This can be accomplished by chemotherapeutic agents or by radiation. Depletion of donor T cells by neutralizing antibodies in the graft is sometimes performed in order to prevent GvHD. The disadvantages however will be increased risks of graft failure and infections and less GvL effect (52). After the HSCT the patient's immune system must be restored and different cells reconstituted with different velocity (53). Neutrophil granulocytes, used as a marker for engraftment, are recovered after 2-3 weeks, UCBT after 4 weeks, whereas T cells are not recovered until 3-6 months post transplantation (53).

2.2.4 Umbilical cord blood transplantation (UCBT)

In 1988, the first transplantation with cord blood was performed. The patient was a boy with Fanconi anemia who underwent UCBT with his sister as the donor (54). Since then, collection and storing of UCB in international cord blood banks has increased the availability of UCB as a source of stem cells for the treatment of both malignant and non-malignant disorders. One of several advantages with cord blood transplantations is the accessibility, through cord blood banks, when no HLA-matched donor is available. The risk for the donor is absent. However, recently clamping time (the time passing before cutting the cord after delivery) prolonged to three minutes has been shown to result in higher hematocrite and higher ferritin concentration up to four months of age, which may be beneficial to the child, compared to children with immediate clamping (55). This regime cannot be attained with clinical collection of UCB since the amount

of blood is too limited to enable retrieval of sufficient numbers of cells for transplantation. At the two centers for UCB banking in Sweden, Stockholm and Gothenburg, a compromise has been introduced; clamping time of one minute in vaginal deliveries and 30 seconds in caesarian sections. Furthermore, the risk of developing GvHD is reduced due to the naivety character of UCB (56). One mayor disadvantage of using UCB as a source of HSC is the limited number of stem cells, leading to slower engraftment and reconstitution of the immune system with increased infections as a consequence (57). Still, a beneficial feature of UCB is the content of CD34+ cells with high capacity to self-renewal and proliferation (58). The use of double cord blood units i.e. the use of two unrelated UCB units has partly counteracted the problem of limited number of cells (59). The GvL effect after UCBT does not seem to be decreased, despite the lower incidence of GvHD (58).

2.2.5 Graft-versus-host disease (GvHD)

Clinical manifestations of GvHD depend on the degree of donor-host histocompatibility. The immune system of the recipient is usually not destroyed completely but co-exists transiently with the donors. Bidirectional alloreactions result in GvHD, host-versus-graft and GvL effects. These effects are mainly carried out by CD8+T cells (60). Billingham formulated in 1966, three requirements necessary for GvHD in general: 1.The graft must contain immunologically competent cells 2. The recipient must express tissue antigens that are not present in the transplant donor. 3. The host must be incapable of rejecting the transplanted cells (61). Acute GvHD arises within 100 days and is characterized by a systemic, progressive attack on the cells of skin, liver and intestines. The reaction derives from mature T cells from the donor that interact with the recipient's dendritic cells (direct pathway of recognition). Alloreactive T cells will be stimulated to divide and differentiate into effector cells under the influence of inflammatory cytokines (60). Acute GvHD affects up to 85% of patients after HSCT and depend on donor type, HLA disparity and prophylaxis of GvHD (62). Chronic GvHD usually develops after 100 days following HSCT and affects approximately 30-50% of long-time survivors; it resembles an autoimmune disease, including the development of antibodies directed to the allogeneic HLA molecules and it involves the indirect pathway of recognition (63, 64). Chronic GvHD affects almost any organ although liver, skin and mouth are the most common sites (65).

In spite of HLA matching, GvHD may arise. This can be exemplified by male patients who receive HSC from their HLA identical sister. The sister may be previously sensitized to HY (mHags encoded on the Y chromosome) that could influence the outcome of HSCT (66).
Both acute and chronic GvHD are treated with different immunosuppressive drugs.

2.2.6 Graft-versus-leukemia effect (GvL effect)

Alloreactive donor T cells are not only exerting negative reactions since they also have the capacity to react against tumor cells. The development of GvHD is thought to mirror the benefits of a GvL effect. Weiden et al. reported that patients who developed GvHD also had significantly lower relapse rates (67). By giving patients with leukemia donor lymphocytes infusion induction of remission can be achieved (68). Additionally, patients receiving T cell depleted grafts in order to prevent GvHD have been shown to have significantly higher relapse rates (69). Natural killer (NK) cells may also be effectors in GvL reactions. They are among the first to be reconstituted after HSCT and mediate cytotoxic effects without prior sensitization. (70).

2.2.7 Non-inherited maternal antigen effect (NIMA effect)

The theory that the natural acquisition of maternal cells by the fetus may lead to durable, maternal-specific tolerance is called the NIMA effect. The theory has been based on results from transplantation studies. Patients who receive many blood transfusions become highly sensitized and develop antibodies against almost all HLA-alloantigens making it hard to find a cross-matched negative organ donor if needed. In a study by Claas et al. 50 % of 26 such highly sensitized renal-transplant patients failed to produce antibodies against mismatched NIMA but were capable of producing antibodies against non-inherited paternal antigens (NIPA) indicating that exposure to NIMA induce partial B cell tolerance in the offspring (71). When mice and chicks were exposed to allogeneic tissue suspensions in utero and later, as adults transplanted with skin from the donor strains, acceptance of the graft tissue was achieved in contrast to the animals who received the skin graft from another strain than that they had been exposed to in utero (16). The NIMA effect has clinical relevance; a human solid organ transplantation study reported that the survival of a haplotype- mismatched kidney allograft from a sibling donor was significantly better if the mismatched HLA

haplotype was maternal (NIMA), as compared with paternal (NIPA)-mismatched donor-recipient pairs. (72). A similar effect of NIMA has been seen in HSCT. A better transplantation outcome and less GvHD is achieved when patients receive HLA haploidentical stem cell transplants from mothers or NIMA-mismatched siblings compared to those from fathers or siblings without NIMA mismatch (73- 76).

2.3 REPRODUCTIVE IMMUNOLOGY

A goal of both the mother and the fetus during pregnancy is to achieve a tolerogenic state to the semi-allogeneic counterpart. Mainly, this is carried out in three areas; the immunological features at the maternal-fetal interface i.e. the placenta and the decidua and the adaptive mechanisms of the maternal and developing fetal immune systems. All three parts may be of interest to understand the trafficking and the maintenance of MMc and FMc.

2.3.1 The maternal-fetal interface

The maternal-fetal interface is a dynamic site that comprehends multiple cellular interactions in an environment rich in cytokines and hormones (77). The fetus itself does not come into direct contact with maternal tissue. It is the trophoblast that forms the interface between the maternal and fetal compartments. During the first trimester, in the hemochorial human pregnancy, fetal extravillous trophoblast cells penetrate deeply into the decidua that will elicit recruitment of maternal immune cells and the production of pro-inflammatory cytokines (78). All fetal trophoblasts lack Class I HLA-A and HLA-B as well as Class II molecules, instead the extravillous trophoblasts bear HLA-C, -E and -G (79). Through this unique HLA expression, extravillous trophoblasts are capable of interacting with receptors expressed by uterine NK cells (uNK), T cells subsets and macrophages, leading to inhibition or activation with the purpose to protect the trophoblasts from attacks by maternal cells, and thereby maintaining normal pregnancy (80, 81). HLA-G molecules seem to be of high importance for successful embryo implantation in pregnancy and allograft acceptance in transplantation (82, 83).

The decidua is populated by a variety of maternal immune cells. The purpose of those cells is to support placenta development, inhibit attacks of maternal effector cells and to combat infections. In decidualized endometrium, in both non-pregnant and pregnant women, the primary leukocyte subpopulations are uNK cells, macrophages and T cells.

The specific role of uNK cells is not clear but it has been suggested that they regulate fetal trophoblast invasion and the uterine vascular changes for maximizing maternal blood flow through placenta (84). Macrophages constitute 20-30% of the leukocytes in the decidua and are believed to, beside their function as protectors and effectors against infections, be involved in implantation, placental growth and the development of maternal-fetal tolerance (85). Approximately 10–20% of leukocytes in the first-trimester human decidua are T cells and within this population a very small proportion constitutes T regs (86). The function of decidual T cells is so far undefined but they seem to exhibit their function by cell–cell contact and suppression of T cell responses (87, 88). The secretion of human chorionic gonadotropin from the trophoblast appear to attract T regs to the fetal-maternal interface and to modulate their regulating function (89).

An association between increased T cells and decreased T regs levels has been noted in spontaneous abortion (90, 91). A decreased proportion of decidual T regs in preeclampsia has also been reported (92).

2.3.2 The maternal immune system

Proliferating T helper cells that develop into mature CD4+ cells differentiate into two major subtypes of cells known as T helper (Th) 1 and Th2 cells that are defined by their function, cytokine production profiles and surface chemokine receptors.

For long the alteration of cytokine profile termed, “the Th1/ Th2 shift”, seen in pregnant women was considered the main mechanism for achieving a tolerogenic state during pregnancy. It refers to the fact that the maternal immune system downregulates Th1-induced cellular immunity and enhances Th2-induced humoral responsiveness in pregnancy, with a subsequent increased levels of anti-inflammatory cytokines such as IL-4, IL-5, IL-10 compared to IFN-γ, TNF-α and IL-2 that are considered inflammatory (93, 94). Pregnancy related hormones such as progesterone, estradiol, leukaemic inhibitory factor and prostaglandins promote the Th2 profile (95-97). Trophoblasts also contribute to the Th2-cytokine dominance in pregnancy (98).

More recent research suggests that the maternal immune system is aware of the semi-allogeneic fetus and develops strategies to tolerate it. During pregnancy large amounts of apoptotic trophoblast are shed into the maternal circulation that can be captured by APCs that present fetal antigen to T cells but maternal tolerance is generally not compromised by these T cells, whose activity seem to be restricted by clonal deletion

or suppression (99). T regs are considered to mediate a large part of active immune tolerance that inhibits maternal lymphocytes from attacking the fetus (22). Recent data shows that T regs specific to fetal antigens expand more than 100-fold (100). In a mouse model depletion of T regs generated a higher rate of spontaneous abortions (22). The mechanism of how T regs contribute to the tolerogenic state of pregnancy has been proposed to be attributed to their secretion of IL-10 and PD-1 (programmed cell death protein 1) and by the up-regulation of IDO (indoleamin-pyrrole 2,3-dioxygenase) expression on dendritic cells and monocytes in peripheral blood and deciduas, leading to a local tolerant microenvironment and suppression of T effector cells (101-103). Interestingly, it seems that already the encounter with seminal fluid will stimulate the expansion of T regs as a preparation for pregnancy (104).

T cells specific for at least three different mHags (HA1, HA2, HY) have been identified in women following pregnancy and may be detected decades afterwards (105-107). The mHag specific T cells are elicited during pregnancy and appear to be able to lyse target cells and produce IFN-γ (108). Three hypotheses by Linsheid et al. may explain why theses T cells do not cause rejection of the fetus; firstly, T cells are not fully activated during pregnancy and consequently are deficient in their effector function *in vivo,* secondly T regs at the maternal-fetal interface prevent rejection and thirdly, fetal-specific T cells are unable to traffic to the maternal-fetal interface and thus cannot mediate their attack. (109). Both placenta and microchimeric fetal cells are likely sources of fetal mHags during pregnancy.

2.3.3 The fetal immune system

Less is known about how the immune system of a fetus deals with the challenges of developing in a semi-allogeneic host. In the past, the fetal immune system was considered inert and functionally impaired due to limited antigen experience, but recent research has confirmed that the human fetal immune system is decidedly active. The fetus must learn to tolerate benign antigens, including self-antigens as well as maternal, food and environmental antigens that cross the placenta. Given that the fetus is relatively protected against microbial infections inside the uterus and that the fetus, the premature and newborn has an enhanced susceptibility to infections, it makes sense that the developing fetal immune system mainly is tuned to generate tolerance.

Heamatopoesis originates in the early yolk sac, but from the 11^{th}-12^{th} gestational week the liver is the primary organ of the fetal hematopoesis, whereas from the 20^{th}

gestational week heamatopoesis mainly resides in the bone marrow (110). With increasing gestational age the innate immune system of the fetus is formed. However, neonatal innate cells display fewer functions and produce less cytokines compared to adult (111). NK cells are also a part of the fetal developing innate immune system and have the capacity to detect and destroy virus infected cells through a cytotoxic mechanism. The levels increase through pregnancy and reach the maximum level at birth (approximately 10% of lymphocytes in cord blood). The levels then decrease to reach adult levels around the age of 5 years (112). Complement factors, contributing to the innate defense, are produced in the fetal liver, but in neonates they are reduced to 10-70% of adult levels (l13).

The adaptive immune defense, which is characterized by the elimination of specific pathogens and by development of an immunological memory, includes T cells and B cells. The levels of those cells increase with gestational age, but the first week after birth they expand immensely (114). T cells migrate from the fetal liver or bone marrow to mature in the thymus. The fetal thymus develops around the 9^{th}-10^{th} gestational week but is not mature until the14^{th}-16^{th} gestational week (115). In the thymus the genes of the T cell receptor rearrange, resulting in a large diversity of potential T cell antigen specifities. The T cells bearing receptors which recognize self-MHC on the epithelial cells are positively selected for differentiation to CD4+ or CD8+ single positive cells (116). The T cells leave the thymus as naïve CD4+ or CD8+ cells. In peripheral lymphoid tissues they get activated and start to mature by binding to their corresponding antigen/ HLA. Yet, this activation is dependent on co-stimulation of APCs. By 16^{th}-20^{th} gestational week T cell development is well under way and T cells migrate to the periphery (117). At the same gestational age the peripheral lymph nodes are colonized by lymphocytes (118).

The fetal immune system needs not only to develop in order to be armed against infections after birth, it must also learn to tolerate benign and/or necessary antigens (including maternal). In the thymus, parallel to the positive selection of CD4+ cells and CD8+, the T cells whose receptor bind to self-antigen/ MHC and by that have a potential to be auto reactive, undergo apoptosis in a process known as clonal deletion (119). By that, central tolerance is achieved. However, mature auto reactive T cells may escape from the negative selection in the thymus (120). To deal with those escaping T cells that may exert potentially harmful autoimmune reactions in the individual, a secondary line of tolerance promoting exists in the periphery, imposed by

a population of regulatory T cells. The best characterized are the CD4+ CD25+FoxP3+ T regs. They are identified by their expression of transcription factor FoxP3 and the IL-receptor α-chain (CD25) (121). They suppress immune responses through production of IL-10 and TGF-β (transforming growth factor β). Mouse studies have shown that depletion of the CD4+CD25+ T cells result in severe autoimmunity (121). An X-linked syndrome characterized by early onset of autoimmune disease in multiple organs was defined in humans in 1982. The syndrome, named IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) was found to emerge from disruption of FoxP3 leading to the absence of T regs (123). The mechanism used by T regs includes binding to the same APC as the autoreactive T cell followed by secretion of non-inflammatory cytokines, direct target-cell cytotoxicity and modulation of antigen presentation, and thereby inhibiting proliferation and activation of the T cell (124). The development of T regs starts approximately in the 13^{th}gestational week, in the fetal thymus (125). Fetal lymph nodes in the second trimester contain a high amount of T regs (on average 15-20% of CD4+ cells) compared to adult lymph nodes, full-term cord blood and adult peripheral blood (less than 5% of CD4+ cells)(118, 126) Michaelsson et al. showed that depletion of T regs in the fetal lymph node generated a spontaneous proliferation of conventional T cells and increased production of IFN-γ upon stimulation, but not in the assays of T regs depletion in adult lymphnodes (118). This finding made the authors suggest that 1) fetal T cells are, in the absence of T regs, highly responsive and that T regs have a suppressive influence in the fetal immune system to achieve peripheral tolerance 2) adult and fetal adaptive immune systems exhibit significant differences with respect to the requirements for maintenance of peripheral tolerance (118).

Based on these observations and the previous findings of MMc in fetuses, the same research group tested the hypothesis that fetal T cells could react on maternal antigen and the fetal immune response could be inhibited by fetal T regs (17). First of all, MMc was found at a rather high level (up to 0.8%) in fetal lymph nodes in 2^{nd} trimester fetuses. Then fetal immune responses against maternal APC bearing NIMA and against unrelated, third party APC were tested in vitro by mixed lymphocyte reaction (MLR). Fetal responses against NIMAs were less prominent compared to unrelated antigens, indicating tolerance. When fetal cells were depleted of T regs prior to MLR, the immune response increased against maternal and self APC but not against unrelated donor alloantigen. Subsequent experiments were conducted on peripheral blood of

healthy children with the aim to investigate if T regs could suppress T cell activity against maternal or paternal APC. A small but significant suppression against maternal APCs compared to paternal was noted. It is not known if theses T regs orginated from fetal life or if they developed more recently due to ongoing MMc (17).
In conclusion; it appears that fetal T regs are involved in the induction and maintenance of the NIMA effect.

2.4 METHODS TO STUDY MICROCHIMERISM

The technological challenges to study Mc are related to the limited number of cells, and therefore the identification requires sensitive methods. One strategy to identify fetal cells in a woman is the utilization of the Y chromosome as a marker to detect male cells in a female host (5,15). Fluorescent in-situ hybridization (FISH) with Y chromosome-specific probes can be used to microscopically identify male cells in blood or tissues. The practicality is however restricted by the time-consuming work of screening a very large amount of cells. FISH analysis can be combined with concomitant cellular phenotyping by immunohistochemistry (127). MMc may also be studied with FISH assays, by identification of female cells in male tissue or blood (127). Figure 4.

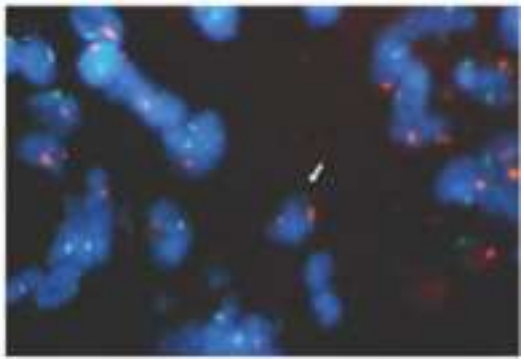

Figure 4. Maternal epithelial cell in the lung identified by cytokeratin expression. X-probe signal = red. Y-probe signal=green. With kind permission from dr Anne Stevens.

DNA amplification using the polymerase chain reaction (PCR) provides a sensitive method to detect and quantify microchimeric cells. In certain assays the sensitivity can reach one donor cell per million host cells (128). DNA is extracted from tissues or peripheral blood and detected by targeting nonshared, maternal-specific HLA

polymorphism (129). Other variants of genetic polymorphism, including the use of markers for the Y chromosome in FMc assays, may also be used (130).

2.5 FETAL MICROCHIMERISM

2.5.1 Fetal microchimerism in blood and tissue

In humans, fetal DNA has been detected in maternal blood as early as at four to five weeks of gestational age, and both fetal cells and DNA are consistently detected from seven weeks (131,132). In 2nd trimester pregnancies the number of circulating fetal cells in maternal blood is estimated to 2-6 per milliliter and distinctly increase until parturition with a rapid decline post partum (133,134). Free fetal DNA has been demonstrated to be much more frequent than fetal nucleated cells (100% vs. 26%) in the circulation of pregnant women and with a more rapid clearance (5). The discrepancy made Lo et al. speculate if the cell populations predominantly involved in cellular traffic and plasma DNA liberation are different, suggesting that the trophoblasts are the main source of liberated plasma fetal DNA and that persistent FMc would originate from intact fetal cells (5).

Male hematopoietic stem cells, presumably fetal, have been demonstrated in the circulation of women decades after they had given birth to sons (15). The fact that the fetal cells persist may be explained by their ability to engraft in bone marrow as progenitor cells with the capacity to differentiate, proliferate and re-infiltrate blood and tissue later (135).

Conditions associated with placental dysfunction appear to result in an increased passage of fetal cells into the mother. For example, in pregnancies complicated by abnormal fetal karyotype, Bianchi and others have shown that the levels of FMc was several times higher in pregnancies associated with trisomy 21compared to normal pregnancies (136, 137). Increased amounts of free fetal DNA in the maternal circulation have also been reported in preeclampsia and HELLP (hemolysis, elevated liver enzymes, and low platelets) as well as in preterm labour and fetal growth restriction(138-141). Whether similarly high levels are present years later is currently unknown.

The cellular subsets in FMc are various, with both mesenchymal and hematopoietic origin (142). Hematopoetic lineage markers have been demonstrated in subsets of CD3+, CD4+, CD8+, CD19+, CD14+,CD56/16, CD66b+ and CD34+(5, 143,144).

Y chromosome bearing cells have been identified in diverse female tissues, including thyroid, skin, lung, lymph nodes , kidney, liver, hearts, spleen, cervix, intestine and bone marrow (135, 145-151). Thus, it seems that fetal cells are widely spread within the body and become incorporated into a range of various cell types. Additionally, differentiated fetal cells have been demonstrated for example among hepatocytes, cardiomyocytes and thyrocytes (152-154).

2.5.2 Fetal microchimerism in autoimmunity

A maternal cellular immune response directed against fetal antigens can be detected in humans, suggesting that persisting FMc could be recognized by maternal T cells mediating an allo-autoimmune reaction (19,105, 106). So, the association between FMc and several disorders that traditionally have been referred to as autoimmune have been investigated, the first being SSc. SSc has clinical resemblance to chronic GvHD, including development of auto antibodies and has its peak incidence in women after reproductive years (155). Women with SS had significantly higher levels of male DNA in peripheral blood, detected by Y chromosome specific PCR, compared to healthy controls (156, 157). Subsequent studies have reported comparable results, i.e. FMc is more prevailing in the peripheral blood mononuclear cells, skin lesions and other affected organs of women with systemic sclerosis (158, 159). However, healthy controls also harbor FMc, and other studies have not clearly identified an association between SSc and FMc (160,161).

Hypotheses regarding how FMc contributes to the disease include both a fetal vs. maternal type reaction (graft vs. host) and a maternal vs. fetal reaction (host vs. graft) (162). The maternal vs. fetal reaction is suggested to involve immune recognition of fetal cells by maternal T cells, either in a direct or an indirect way. In the direct way fetal antigens are presented directly by persistent fetal APC to maternal T cells, whereas in the indirect way, fetal antigens are presented to the maternal effectors by maternal APCs.

Various other autoimmune diseases have been investigated for their association with FMc. Fanning et al found male cells, presumably fetal, in liver biopsies from patients with primary biliary cirrhosis, but not in controls, whereas other studies did not found

any clear association.(163-165). Fetal chimeric cells have been identified in affected salivary glands in patients with Sjogren's syndrome, but not in peripheral blood (166,167). The study of autoimmune thyroid disease is of particular interest because of its frequent onset or relapse in the postpartum period, but, once again, study results have been controversial. Klintschar et.al found an increase in FMc in affected thyroid tissue of women with Hashimoto's disease, but in the study by Srivitsa et al. similar frequency of FMc was detected in Hashimoto's disease and thyroid neoplasms (145, 168). In conclusion, the association between FMc and systemic sclerosis appears to be the most clearly established. The role of the fetal cells remains controversial- are they an essential part of the pathogenesis or is the finding of fetal cells in affected tissues and the increased frequency in blood a manifestation of recruitment of fetal cells to damaged tissue, where they may take part in repair processes?

The fact that FMc is present in a wide range of tissues, not only those affected by autoimmune processes, formulated the alternative hypothesis that the occurrence of fetal cells in these tissues is a result of the fetal cells homing to areas of damaged tissue participating in repair processes. Descriptions from the literature include: in a woman with hepatitis C, invading fetal cells with a mature liver phenotype were observed in the liver; the fetal cells were assumed to originate from a pregnancy 17 years earlier (169). Well-differentiated liver cells of male origin were demonstrated in 36% of liver biopsies from women with different hepatic diseases (149). Fully differentiated male thyroid have been observed in thyroid of a woman with autoimmune thyroid disease (145). Furthermore, synovial tissue of patients with rheumatoid arthritis contained fetal mesenchymal stem cells with possible ability to participate to bone and cartilage repair (170). The fact that FMc were more likely to be found in patients with a severe SLE compared to those with a mild variety formulated the theory that the fetal cells were not causing the disease but rather home to the damaged maternal tissue once the injury reaches a certain level (171).

2.5.3 Fetal microchimerism in cancer

Recent reports suggest an association between pregnancy, Mc and cancer. It is less likely that parous women who harbors FMc develop cancer than parous women without FMc suggesting a immunosurveillance and/ or tumor suppressor function, especially in breast cancer (172-174). Gadi proposed three mechanisms how the fetal microchimeric cell could contribute to alloreactivity against cancer antigens in the

mother: 1) fetal T cells effectors attack maternal, malignant cells expressing cancer antigens 2) fetal APCs present maternal cancer antigen to maternal effector cells 3) fetal NK-cells initiate a cytotoxic reaction towards the maternal malignant cells (175). Additionally, fetal cells in cervical, lung and thyroid cancer tissues have been demonstrated (176-178). However, it is hard to evaluate those findings; are the fetal cells effectors, an accompanying marker of concomitant process or providers of tissue repair?

2.6 MATERNAL MICROCHIMERISM

In the beginning of the eighties the idea that allogeneic maternal cells might be involved in triggering disease in children was born. Children affected with severe combined immunodeficient disease (SCID) were shown to harbor maternal cells in the circulation and the maternal cells were also engrafted in tissues (179). SCID is a genetic disorder characterized by the absence of functional T-lymphocytes. The defect immune system manifests by impaired antibody response due to either direct involvement with B lymphocytes or through improper B lymphocyte activation due to non-functional CD4+ cells. In a large cohort by Muller et al., as many as 40% of infants with SCID harbored engrafted maternal T cells and GvHD was developed in 76% of them (180). The phenotypes of the engrafted maternal chimeric cells in SCID infants have been confirmed to be T and B cells but without the ability to functionally replace the immune system of the infant (179-181). Since then, additional pediatric diseases have been proposed to be associated with MMc; juvenile dermatomyocytis (JDM), juvenile idiopathic inflammatory myopathies (JIIM), neonatal lupus syndrome (NLS), type 1 diabetes, pityriasis lichenoides, biliary atresia (182-190).So far, one autoimmune disorder affecting adults has been shown to harbor MMc to a higher frequency; SSc (129).

2.6.1 Maternal microchimerism in blood and tissue

The fetal immune system has been shown to be exposed to maternal microchimeric cells from the 13th gestational week (in blood samples taken prior to induced abortions) (191). Studies in the 60ties detected MMc in cord blood by labeling maternal blood cells with a fluorescent dye or Chromium-51 and injecting them back into the mothers before delivery (12,13). More modern studies investigating MMc in cord blood have

shown very diverging results depending on the method (5,192-200). The frequency has varied greatly; 0% to 100% with a mean frequency of 14%.
Maternal cells are also common in infants and healthy individuals and have been identified in the peripheral blood up to the age of 49 (14). Maloney et al. developed a PCR assay which could detect one maternal cell in the background of 100.000 host cells. In healthy offspring, DNA sequences, presumed to be maternal, were identified in 17 (55%) of 31 individuals from the age of 9 to 49 years (14). Quantitative methods as quantitative real time PCR (QRT-PCR), have estimated the levels for example in cord blood to 2.6 x 10^{-4}(5). The subsets of maternal cells in peripheral blood of healthy individuals were investigated by flow cytometry followed by QRT-PCR and were found to be CD3+ (T cells), CD19+ (B cells), CD 14+ (macrophages, monocytes), CD56+ (natural killer cells) (201). The study revealed a frequency of MMc of 39% in healthy individuals. Granulocyte populations also contain maternal cells (144).
Several studies have explored the tissue engraftment capacity of the maternal cells. In a 2nd trimester fetus, MMc was found in a variety of tissues including liver, lung, heart, thymus, spleen, adrenal gland and kidney (202). The cells were of hematopoietic and stem cells origin. In infants with inflammatory and non-inflammatory diseases maternal cells were detected by FISH analysis for X- and Y-chromosomes in liver, pancreas, kidney, heart, lung, skin, thyroid and thymus (127, 203). Thus, MMc in tissues could conceivably be targets of an immunological attack.

2.6.2 Maternal microchimerism in autoimmunity

Maternal cells appear to have multi-linage plasticity since female (presumed to be maternal) cells expressed sacromeric α-actin, a marker for myocardial cells, in the heart tissue of male infants who died from NLS (187). The development of NLS starts in utero in fetuses of mothers with anti-SSA antibodies leading to congenital heart block, rash, hepatic and hematologic abnormatilities (204). Not all babies born of mothers with those antibodies develop the disease suggesting other pathological mechanisms. Using a FISH assay, MMc was demonstrated in 15 of 15 sections of NLS heart tissue, with a frequency range of 0,025% to 2,2% of total cells (187). In controls, only 2 of 8 sections showed maternal cells (0-0,1% of total cells). Furthermore, 86% of the maternal cells expressed a marker for cardiac myocytes- sacromeric α-actin, indicating differentiating capacity.

Pityriasis lichenoides

Pityriasis lichenoides is a skin disease that predominately affects children and young adults with unknown cause although some researchers have suggested that the initiation of the lesions could be viral (205). The clinical characteristics are flares of inflammatory skin lesions. Pathological findings include lymphocyte infiltration deep into the dermis with lymphocytes within the subsets of CD3+ and CD8+ (205). A study by Khosrotherani et al. demonstrated increased levels of maternal cells in skin tissue of male children with pityriasis lichenoides compared to healthy children, although the difference in frequency was not significant (189). Numbers of maternal cell were 99/ million in patients compared to 5/ million in controls ($p=0.005$). Combined FISH with immunostaining using anti-CD45 antibody was used on nine sections from patients and five from controls. None of the 19 chimeric cells stained positive with CD45+ but in seven of 10 patients the maternal cells stained positive for cytokeratin indicating that they had differentiated into keratinocytes.

Type 1 Diabetes

Type 1 Diabetes is characterized by autoimmune destruction of insulin secreting islet beta cells in pancreas leading to insulin deficiency and lymphocyte and macrophage infiltration. In children with diabetes, maternal cells were more frequent in peripheral blood compared to unaffected siblings and unrelated controls (188). By in situ analys, 4 male pancreases were examined in regard to MMc. Few female, hematopoetic cells were found but female, insulin- positive cells, presumably β cells were detected at slightly higher levels in diabetic tissue compared to controls indicating ability to differentiate into a tissue specific phenotype. Since MMc not was found in the lymphocyte subset, the authors suggested that it was unlikely that the maternal cells act as effectors but the finding of insulin- positive chimeric cells could rather indicate a consequence of tissue repair (188). A similar study was conducted by Vanzyl et al. (206). They found maternal cells in pancreas, both in patients and controls but the frequency of cells was significant higher in patients. The maternal cells ranged between 0.31 and 0.80%,mean 0.58% compared to controls; 0.24-0.50%, mean 0.38%) ($p = 0.05$). The maternal cells were both insulin negative and insulin positive but CD 45+ was not expressed, indicating absence of effector function of microchimeric cells. The findings of differentiated maternal cells in tissues could be interpreted that they home to the area of damaged tissue and participate in a tissue repair process.

Juvenile dermatomyosytis

JDM is a multi-organ autoimmune disease that causes inflammation in small vessels of muscles, skin and the gastrointestinal tract and has been associated with the HLA Class II allele DQA1*0501 (207). The pathogenesis remains unclear but muscle biopsies have demonstrated lymphocyte and phagocyte infiltration (208). Maternally derived cells have been shown to be more frequent both in muscle biopsies and in whole blood of children with JDM compared to controls (182, 183, 185).

In a recent study by Ye et.al muscle biopsies from 7 male children with JDM and 4 healthy male controls were investigated with FISH combined with confocal imaging (184). Concomitant immunofluoroscence for CD45+ was used to determine whether MMc in muscle were lymphocytes. The study confirmed previous results: the frequency of MMc was significantly higher in JDM muscle (0.42-1.14%) compared to controls (0.08-0.42%). Among the maternal cells no CD45+ cells were observed. Thus, the findings of differentiated maternal cells in tissues and with absence of maternal lymphocytes could be interpreted that they home to the area of damaged tissue and participate in a tissue repair process.

Additionally, in the study of Reed et al. the presence of MMc in healthy subjects was shown to be associated with the HLA genotype of the mother, in particular HLA DQA1*0501 (184).

Systemic sclerosis

SSc is a chronic immunological disease characterized by increased accumulation of collagen in the skin and in various internal organs. Cellular infiltration constitutes above all of T cells. Like in FMc, there has been an association between MMc and the disease. MMc occurred more frequently among women with SSc (72%) compared to healthy controls (22%) but levels of maternal cells were not significantly different (129). However, in a patient negative for MMc in blood who then died from the disease, high levels of MMc was found in both affected and non-affected internal organs and bone marrow (129).

Biliary atresia

Several studies have exposed an association between biliary atresia (190, 209-211). It is an autoimmune, progressive disease of the biliary tree in infants and it is the most common cause of pediatric liver transplantation. Maternal CD8+ cells were more

frequently found in livers of patients with biliary atresia compared to controls and some maternal cells were cytokeratin-positive (211).

In conclusion, studies of MMc and autoimmunity have its origin in the same reasoning as FMc and autoimmunity i.e. the similarities between chronic GvHD and some autoimmune diseases where maternal cells could act as effector cells in the progeny, for example as in JDM (183,185). Examples of conditions with a possible beneficial effect of MMc are NLS and type 1 diabetes where maternal cells appeared with tissue specific character in the absence of maternal inflammatory cells. (187, 188, 206). A theory that maternal cells may influence a fetal response towards self antigens has been proposed by Leveque et al. (212).

The observation made in the transplantation field that incompatibility between HLA genes between patient and donor highly affects the risk to a patient to develop chronic GvHD have led to the hypothesis that naturally acquired microchimerism and developing of autoimmunity are dependent of the HLA combination of host and donor (156, 183).

2.6.3 HLA and naturally acquired microchimerism

HLA may affect the presence of Mc in different ways. First of all, a specific HLA allele in the mother or the child may influence the occurrence of FMc or MMc (156, 183). Secondly, a compatibility of HLA alleles from the perspective of the mother (regarding FMc) or the fetus (regarding MMc) may influence the ability of host cells to pass the placenta undetected and/or engraft. For example, increased levels of FMc have been described in SSc (156). Women with SSc had significantly more often given birth to a child compatible to the mother for HLA-DRB1, prior to disease onset (156). Berry et al. investigated the presence of MMc in cord blood, collected for a variety of medical reasons between gestational week 18 and 38 (200). MMc was detectable in 16 of 30 samples. The frequency and concentration of MMc was higher when there was maternal compatibility for HLA-DQB1 from the fetal perspective, that is, the mother was DQB1 homozygous or HLA identical.

Mother	DQB1*	DQB1*
Child	DQB1*	DQB1#

The mother is DQB1 homozygous

Mother	DQB1*	DQB1¤
Child	DQB1*	DQB1¤

The mother is DQB1 identical

Figure 5. Compatibility of the mother from the child's perspective

Thirdly, a transfer of an autoimmune HLA risk allele through Mc may be possible: a study by Rak et al. showed that FMc may have an adverse effect in RA patients who lack the most common RA risk-associated HLA alleles, HLA-DRB1*04 and HLA-DRB1*01 (213). An increased frequency and higher levels of FMc with those alleles were observed in patients with RA compared to controls. Related to MMc, on the other hand, several studies have demonstrated that patient with RA who lack HLA-DRB1*04, have mothers with the risk allele (NIMA) to a higher frequency rather than fathers (NIPA) carrying this allele (214, 215). However, it was not investigated if patients with RA harbored MMc.

Given the common incidence of MMc in healthy individuals it is logical to assume that the presence of MMc, or naturally acquired Mc as a whole, not always contribute to disease. So, the possible consequences of MMc range from tolerance to sensitization but what process might disrupt the balance it is not known. Maternal microchimeric cells, normally present at a low level might be expanded by an environmental stimulus and proliferate in blood and tissues. At higher levels MMc could reach the threshold for activation of the host's immune system or become more prone to be effectors in an "alloimmune" process. Alternatively, the occurrence of the semi-allogeneic cells may reflect induction of host tolerance

Very few studies have provided tests of MMc and its functions in humans. In the study by Reed et al. maternal T lymphocytes were isolated from children with JDM and were shown to react to the children's cells in vitro by producing IFN-γ (183). In contrast, the maternal cells did not react to the healthy sibling. A case report describes a 21-year old male who was exposed to volatile chemicals in a tyre factory. He developed a condition with resemblance to GvHD including lymphocytic infiltrates and fibrosis in lung, skin and intestinal mucosa. He was compatible with his mother at all HLA class II loci and by a mixed leukocyte reaction his CD4+ and CD8+ cells responded by a two-fold increase when activated to his mother's cells (216).

2.7 OTHER SOURCES OF MICROCHIMERISM

Additional sources of microchimerism than the actual known pregnancy and a woman's born children could make the interpretation of results hard. Male microchimerism has been detected in women who have never given born to a son (217-219). Since it is known that FMc may follow spontaneous or induced abortion it is

feasible that male microchimerism in a woman could stem from an early, unnoticed miscarriage (220). However, the findings of male cells in female children and fetuses cannot be explained by this (221). Furthermore, female UCB samples compromised HY-specific cytotoxic T cells from neonates both with older brothers and without which made the authors speculate in that male microchimerism from the mothers' older brothers or a miscarriage of the mother could induce an anti HY-response (222). Thus, the findings may be a result of a "trans-maternal" flow of male cells. Microchimerism in an individual has also been proposed to derive from a vanished twin (223). Iatrogenic interventions as blood transfusion organ- and HSC transplantation may also give rise to Mc (2-4).
Whether sexual intercourse will be followed by male Mc is currently unknown. However, the findings that expansion of T regs is stimulated at the encounter with seminal fluid might indicate that (104).

The findings that maternal cells can be transferred from the mother to the offspring by nursing stem from mouse studies (224, 225). Consequently it seem to lead to additionally induced tolerance to NIMAs in mice (226). Interestingly enough, breast milk in humans contains stem cells with multi-lineage properties (227). Whether these cells give rise to MMc in the baby is currently not known.

2.8 ANIMAL MODELS

Rodents are the most frequently used animal model for Mc research. For detection of the Y chromosome in PCR, FISH and immunofluorescence assays green fluorescent protein (GFP) or bioluminescence-positive male animals are often used (228). Congenic or allogenic matings between wild-type females and homozygous GFP $^{+/+}$ males result in the possibility to identify maternal and fetal cells. Maternal cells will be GFP^{-} and fetal cells GFP^{+}. When studying MMc in offspring it is required to use GFP$^{+/-}$ female mice mated to GFP$^{-/-}$ males.

MMc has appeared to be common both in healthy and in immunodeficient mice (224, 229). In mice with immunodeficiency they appear earlier in gestation compared to healthy mice and are more widely spread (229). Like in humans, the MHC compability of mother and pup influences the levels of MMc (230). Although the placenta is hemochorial in both rodents and humans, major differences exist between their pregnancies. The mouse placenta contains two layers of trophoblasts and one

layer of syncytiotrophoblasts whereas the human contains only one layer of trophoblasts and has villi resulting in a thicker placenta (231). In mice, trophoblast invasion into maternal vasculature is minor, and the exchange of nutrients occurs in the labyrinth area. Decidualization occurs only at the site of implantation. Pregnancy differs between the two species also in immunological and endocrinological manners (231). Taken together these differences, cautions must be made when comparing MMc and its consequences in humans and in rodents.

3 AIMS

The overall aim of this thesis was to study the phenomenon of MMc and explore its physiogical role and kinetics in normal pregnancy as well as investigate the possible persistence of MMc during childhood and adult life, especially in connection with development of autoimmune disease, like SLE.

The specific aims were:

1. To investigate the occurrence of maternal cells of different cellular subsets in tissues of 2nd trimester fetuses. (Paper I)
2. To investigate the occurrence of maternal cells in healthy children's tonsils, adenoid and blood. (Paper II)
3. To investigate the association between SLE in adults and MMc in peripheral blood. (Paper III)
4. To investigate the occurrence maternal cells of different cellular subsets in cord blood of term infants. (Paper IV)

4 SUBJECTS AND METHODS

4.1 SUBJECTS

Paper I

Tissues were sampled from five 2nd trimester fetuses that were aborted on request (gestational weeks 14-17). An additional five cases of termination of pregnancy, aborted due to trisomy 21 and/ or malformations, were included (gestational weeks 15-18). Finally, a stillbirth from the 27th gestational week was investigated. Seven fetuses were male and four were female.

Gestational age was determined by measurements of the biparietal diameter of the fetus by abdominal ultrasound prior to the abortion. Gestational age was expressed in completed weeks. The termination of the pregnancies was performed by oral administration of mifeprostone 600 mg on day 1 and misoprostol 800 mg vaginally followed by 400 mg misoprostol orally every third hour until the abortion was completed on day 3. The intrauterine fetal death was induced with 600 mg mifeprostone followed by vaginal administration of gemeprost the following day.

The bodies of the fetuses were accurately washed and the tissues were collected at autopsy and placed in sterile NaCl. Peripheral blood was drawn from the mothers.

Paper II

Tissue samples from the tonsils and/ or the adenoid of 20 children undergoing tonsillectomy and/or adenoidectomy due to recurrent tonsillitis and/ or respiratory obstruction were collected prospectively. A questionnaire about the health conditions of the mother and the child was answered by the mother. Details regarding the questionnaire are provided in Discussion.

The children were between two and 15 years of age (mean age 5,2) and consisted of 14 boys and six girls.

Peripheral blood was collected from the mothers and the children. The tonsils and adenoids were removed by curettage under general anesthesia and dissected immediately after the surgery under sterile conditions. Four central parts of the tonsil

and two central parts from the adenoid, each measuring approximately 5 × 5 × 5 mm, were collected for analysis in order to avoid contamination of blood, epithelial cells and inflammatory exudates on the surface. No histopathological examination of the tissues was performed.

Paper III

The study subjects originated from a large genetic study at the Department of Rheumatology, Karolinska University Hospital that was conducted during the period 1995-1998. In the original study, 208 patients and their first-degree relatives were included. All patients fulfilled at least four of the 1982 revised American College of Rheumatology Criteria for classification of SLE. Information about the patients was collected from medical records, by review of medical history and examination of the patients according to a structured protocol by a rheumatologist. Systemic Lupus Activity Measure (SLAM) and Systemic Lupus International Collaborating Clinics (SLICC) damage index were used to measure SLE disease activity and organ damage respectively. First degree relatives filled in a questionnaire about their health. Blood samples were drawn for genetic and other analysis from patients and their relatives and stored at -70˚ C.

Thirty-two patients of the originally 208 were recruited to our study (30 women and two men), with a mean age of 32 (range 18-52). The female patients were selected due to the fact that they had reported themselves as nulligravida in the questionnaire. The healthy control group was consisted of 17 brothers of the patients. Even those men whose sisters were excluded from the study because of past pregnancy were included. Additionally, 12 healthy, unrelated males were recruited to the control group. The mean age of the 29 controls was 31 (range 10-54). No difference in age between the patients and controls appeared (p-value=0.8).

Paper IV

UCB samples, included in the study originated from 44 normal, term pregnancies. They derived from 24 vaginal deliveries, 18 elective and two emergency caesarian sections. The donors, 23 girls and 21 boys, were all born healthy. The characteristics of patients and UCB samples are shown in Table I.

Table I

Patient and umbilical cord blood characteristics.

	Whole group	Vaginal	C/S
Age mothers (years)	30 (±4)	28 (±4)	31 (±4)
Gestational age (weeks+days)	39+1 (±1w+1d)	39+3 (±1w+2 d)	38+6 (±1 w)
Weight baby (grams)	3394 (±302)	3427 (±324)	3355 (±276)
Clamping time (seconds)	52 (±25)	71 (±20)	30 (±4)
WCB (10^8/L)	78 (±19)	81 (±22)	75 (±16)
Total amount WCB (10^8)	6.7(±1,7)	6.9 (±1.5)	6.7 (±1.8)

C/S= caesarian section WCB= white cell blood count

The UCB samples were collected with the purpose to be altruistically donated to Sweden's national UCB bank but the units used in the study included a too low total white cell count for banking and were therefore donated to research.

The sampling of UCB is standardized and is performed as sterile as possibly; the umbilical cord is washed at least three times with chlorhexidine, the umbilical cord vein punctured and the UCB collected into a UCB collection bag by gravitation before the placenta is delivered. In vaginal deliveries the cord is clamped and cut after approximately one minute and in caesarian sections after 30 seconds. Specific clamping time is recorded with a timer for each collection.

Peripheral blood was collected from the mothers at the time of delivery.

4.2 METHODS

4.2.1 Disintegration of tissues. Paper I and II

The fetal, tonsil and adenoid tissues were disintegrated by passage through a 100-µm nylon mesh to form a single cell suspension and diluted in NaCl.

4.2.2 Cell separation. Paper I and IV

To evaluate lineage-specific chimerism in fetal tissues (paper I) and in UBC (paper IV) separations of cells were made by means of immunomagnetic beads according to the manufacturer's instructions (Dynal Biotech, Oslo, Norway). In fetal tissues separations

of CD3+, CD19+, CD34+ and CD45+ were derived from various organs. In UCB samples the separation included CD3+, CD19+, CD33+, CD34+ and CD56+.

4.2.3 DNA extraction. Paper I, II, III and IV

In paper I and II, DNA was extracted from tissues and whole blood using Quiagen Mini Kit (Quiagen, Hilden, Germany). The method includes enzymatic lysation of the cells by proteinase K. The tissue samples were digested at 37˚C overnight on a shaker whereas the blood was digested at 60˚C for 10 minutes. To purify and to concentrate the DNA, a silica-membrane-based nucleic acid purification, procedure was used. The same method was also used to extract and purify the samples from the unrelated controls in paper III. In paper IV, the extraction of DNA was automatized in an EZ1 machine using magnetic silica particles (Quiagen, Hilden, Germany). Genomic DNA concentrations were measured using NanoDrop (Thermo Scientific, Delaware, USA) and diluted to required concentrations.

4.2.4 HLA typing. Paper I and II

In paper I, mothers and fetuses were HLA typed with PCR-SSP (OlerupSSP, Stockholm, Sweden) with the intention to identify a NIMA in the fetus that could be targeted in the PCR assays in order to detect MMc in the fetal tissues. In paper II, the purpose was to examine if any correlation between the presence of MMc and the various combinations of mother/ child HLA classes was found. The PCR assays in this paper were based on polymorphism in biallelic systems (see 6.1).
In paper I, HLA typing was performed by PCR amplification with HLA-DR. If the sequence difference was not large enough between mother and fetus, typing for HLA-A and HLA-B was performed. Primers specific for maternal HLA sequences were designed for each fetus- mother pair and tested for specificity and sensitivity (see 4.2.5).
In paper II, HLA-A,-B and DRB1 polymorphism were determined using standard PCR-based methods (LabType, One Lambda, Inc, CA) including sequence-specific oligonucleotide probes (PCR-SSOP) using xMAP technology (Luminex System, Luminex Corporation, Austin, TX).

4.2.5 Semi-quantitative PCR. Paper I

PCR amplifications were performed with maternal-specific primers in a volume of 50 L containing 1g genomic DNA, 0.5M of each primer, 200M of each dNTP (Perkin-Elmer, Branchburg, CA), 1 PCR buffer (1.5 mM MgCl, 50 mM Mg, 10 mM KCl, 0.001% gelatin), 5% glycerol, 5g cresol red, and 1.5 U AmpliTaq polymerase (Perkin-Elmer). After an initial 3 minute hot-start/ denaturation step at 94°C, 40 PCR amplification cycles were carried out in a PTC-200 thermal cycler (MJ Research, Watertown, CA). The positive control contained maternal DNA and the negative control distilled H2O. The PCR products were separated on a ready-to-use polyacrylamide gel electrophoresis gel for 1 hour and visualized by an automated silver staining method. The band patterns were then analyzed under visible light. We considered the analyzed organs positive for maternal cells when we were able to identify a band pattern of maternal alleles on the PCR gel either in whole tissue or cell-separated fractions. Figure 6.

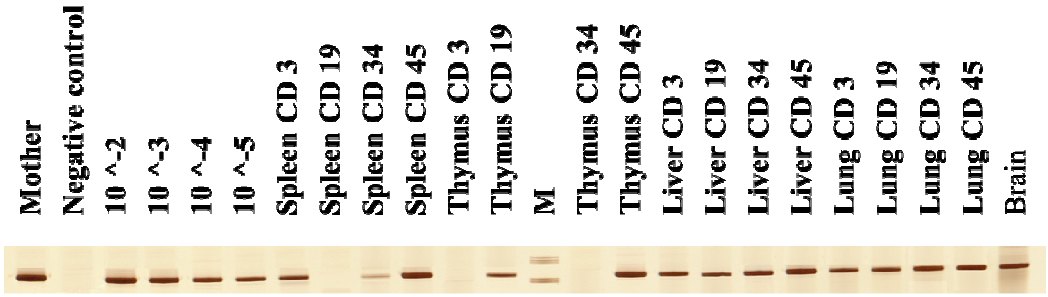

Figure 6. Example of PCR gel from a semi-quantitative assay.

Serial dilution experiments were performed to establish the sensitivity of the PCR amplification. Decreasing amounts of maternal DNA were mixed with DNA from a third individual, with the same HLA type as the fetus to give fixed amounts of 1g DNA with final concentrations of maternal DNA of 1%, 0.1%, 0.01%, and 0.001%. The resulting DNA mixture was subsequently amplified using the maternal-specific primers and analyzed as described in previous text. A semiquantitative analysis was performed in which the intensity of the sample bands on the gel was compared with the 10-fold dilution series. In conclusion; the sensitivity was estimated to 1/ 100.000.

4.2.6 Quantitative Real-Time PCR (QRT-PCR). Paper II, III and IV

In paper II, III and IV, QRT- PCR was used based on single-nucleotide polymorphism (SNP) and insertion/ deletion polymorphism (INDEL) referred to as biallelic genetic systems. Initially, screening of the mother and the offspring was performed by using a small amount of DNA (10 ng) in a PCR assay. The allelic markers used were: S01a, S02, S03, S04a, S05b, S07a, S07b, S08b, ID1, ID2, ID4 and ID7. If no differences were found the pair was screened for additional 10 markers (S01b, S04b, S06, S09b, S010a, S011b, ID9, ID10, ID11 and ID12). For each biallelic system, one of the primers was from the polymorphic region to specifically amplify each allele, whereas the second primer and the probe were common to both alleles. An allele was considered informative when it was positive for maternal DNA and negative for the child's DNA. Detection and quantification with one to three markers were performed with the 7500 Sequence Detection System (Applied Biosystems, Foster City, CA), using TaqMan technology. The amount of amplifiable DNA in each sample was assessed by parallel amplification of the reference gene glyceraldehyde phosphate dehydrogenase (GAPDH). All the samples were run in duplicate, and both maternal and patient/ control DNA samples were included in each run. Relative quantification of recipient DNA was calculated according to the ΔΔCt method (Applied Biosystems, user bulletin 2), using GAPDH as a reference gene and the maternal DNA sample as a calibrator. The amount of DNA used in the studies varied between 150– 500 ng. The specificity and sensitivity of the QRT-PCR method was determined for all markers included in this study using artificial DNA mixtures and varying DNA amounts. With 150-450 ng of DNA, a sensitivity of 0.01% was reached for most markers and no false positive results was found using 40 cycles of PCR amplification. Figure 7.

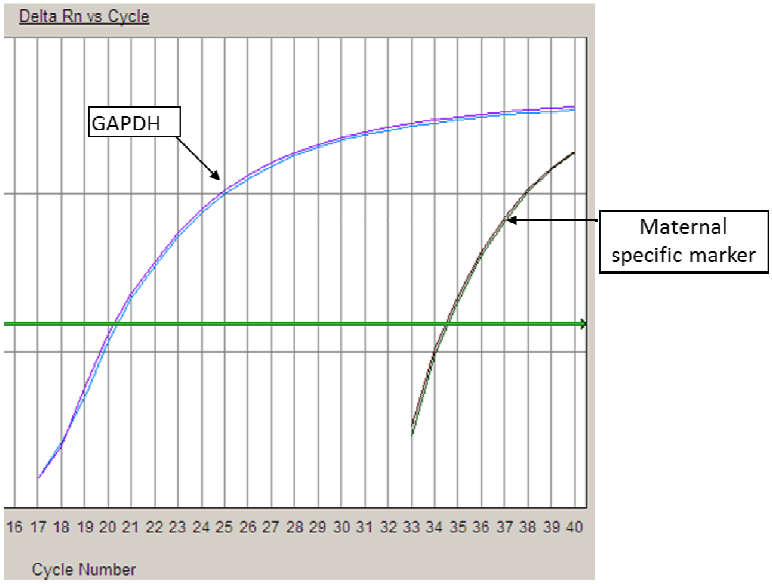

Figure 7. Example of a QRT-PCR assay

4.3 STATISTICAL METHODS

Paper III. Statistical analyses for age differences between patients and controls were conducted with student's t-test. Chi-square test was performed to analyze differences in MMc frequency between the two groups. Statistical analysis was performed using Statistica 10, StatSoft, Tulsa, USA.

4.4 ETHICAL CONSIDERATIONS

Paper I: The study was approved by the Etics Committee at Huddinge University Hospital (Dnr 129/01)

Paper II: This study was approved by the Ethics Committee at Karolinska University Hospital (Dnr 2008/3:3). Institutional Review Board consent was obtained from all patients

Paper III: The Regional Ethics Committee, Stockholm, Sweden (2012/203–31/2) approved the study. The Local Ethics Committee at Karolinska University Hospital approved the original study in 1995 (Dnr 95–178 (950616)). Written informed consent was obtained from all participants. The caretakers of minors gave written consent on their behalf. The signed consent forms are stored with the research file.
Paper IV: The study was approved by the Regional Ethics Committee, Stockholm, Sweden (Dnr 2012/480-31/3). Informed written consent was obtained from all participants.

5 RESULTS

Paper I. Maternal cells are widely distributed in 2nd trimester fetal tissues.

Maternal cells were found in seven of 11 fetuses. The fetuses were between gestational week 14 and 18 and consisted of four males and three females. MMc was detected in all organs investigated (lung, thymus, kidney, heart, liver, spleen, brain, gonads). In four fetuses, no MMc was detected in any tissue. No differences were detected between abnormal or normal fetuses; three of the five abnormal and four of the five normal fetuses harbored MMc and. The stillborn fetus was negative for MMc.
No specific organ appeared more positive for maternal cells than others but there was a tendency that cases with stronger positivity showed uniformly strong positivity in most fetal tissues. Levels of maternal cells ranged from 1/ 100- 1/100.000 (semiquantitatively estimated). Five of the seven cases subjected to analysis of different cellular subsets of maternal cells in fetal tissues presented with maternal mature immunological cells (CD3+, CD19+, CD45+) as well as cells of progenitor type (CD34+). The results are shown in Table II.

Table II

Distribution of maternal alleles in fetal tissues after cell separation.

Tissue	Antigen	Case 1	Case 3	Case 4	Case 5	Case 8	Case 9	Case 10
Lung	CD3	+	++	-	NT	-	NT	-
Lung	CD19	-	++	-	NT	-	-	-
Lung	CD34	-	++	-	++++	-	-	-
Lung	CD45	-	+++	-	++++	-	-	-
Thymus	CD3	NT	-	+	++++	-	-	-
Thymus	CD19	NT	+	-	++++	-	-	-
Thymus	CD34	-	-	-	++++	-	NT	-
Thymus	CD45	NT	+++	+	++++	-	NT	-
Liver	CD3	-	++	-	+++	-	NT	-
Liver	CD19	-	++	-	+++	-	-	-
Liver	CD34	-	+++	-	+++	-	NT	-
Liver	CD45	-	+++	-	++++	-	-	-
Spleen	CD3	NT	++	+	++++	-	NT	+
Spleen	CD19	NT	-	-	++++	-	-	-
Spleen	CD34	+	+	-	++++	-	NT	-
Spleen	CD45	NT	+++	+	++++	-	-	-
Kidney	CD34	-	-	-	++++	NT	-	-
Heart	CD34	-	-	-	++++	NT	-	-
Adrenal gland	CD34	-	+	-	NT	NT	-	-
Pancreas	CD34	NT	NT	+	NT	NT	NT	-
Ovary	CD34	NT	NT	NT	NT	NT	NT	NT
Testis	CD34	NT	-	-	++++	NT	-	+
Brain	CD34	NT	++	NT	NT	NT	NT	+

"-": no maternal DNA detected, "+": maternal cells detected at a level of 10^{-4}-10^{-5}, "++": 10^{-3}-10^{-4}, "+++": 10^{-2}-10^{-3}, "++++":>10^{-2}. "NT" = not tested

Paper II. Maternal cells are common in children's tonsils and/ or adenoid.

MMc was present in four of 20 (20%) children's tonsils or adenoids. The age of the children (two girls and two boys) ranged from four to six years. Two of them were positive in all fractions of tonsils (four fractions) and adenoid (two fractions) tested respectively, one was positive in three of the four tonsil fractions and one child was only positive in one of the four tonsil fractions. All children positive in tissues were also positive in blood. Levels of maternal cells ranged from 2 x10^{-2} to 7.1 x10^{-5} in tissues and from 1 x10^{-3} to 7 x10^{-3} in peripheral blood. No noticeable correlation

between the presence of MMc in the tissues and the various combinations of HLA relationship of mother and child was found.

Paper III. There is no correlation between MMc and SLE in adults.

MMc was detected in peripheral blood of two (patient 153 and patient 156) of 32 patients with SLE (6%) and in one of 29 healthy controls (3%) (P-value = 0.65). The phenotype of the patients did not differ from the negative ones regarding phenotype or age. The characteristics of the two positive patients in relation to all study subjects are shown in Table III.

Table III.
Patient characteristics.

			Pat 153	Pat 156
Age at inclusion	31,7 ± 10,4	Range 18,3-51,3	43	38
Gender, female	30/32 (94%)		+	+
Age at SLE onset	23.4 ± 7.5		23	36
Discoid lupus	3/32 (9%)		0	0
Butterfly erythema*	19/32 (59%)		1	1
Photosensitivity*	18/32 (56%)		1	1
Oral ulcers*	9/32 (28%)		0	0
Arthritis*	26 /32 (81%)		1	1
Serositis*	5/32 (16%)		0	0
Nephritis*	17/32 (53%)		0	0
Haematology*	26/32 (81%)		0	0
Neurology*	3/32 (9%)		0	0
Anti nuclear antibodies*	31/32 (97%)		1	1
Immunology*	25/32 (78%)		1	1
Number of ACR criteria*	Median 6	Range 4-8	6	6
SLAM	Median 6	Range 1-15	5	7
SLICC Damage index	Median 0	Range 0-5	0	0
APS syndrom	3/32 (9%)		0	0

*Manifestations are defined according to American College of Rheumatology´s 1982 revised criteria for SLE (232), SLAM = Systemic Lupus Activity Measure , a validated measure of disease activity (233), SLICC = Systemic Lupus International Collaborating Clinics damage index, a validated cumulative organ damage (234), APS =antiphospholid syndrome was diagnosed according to the Sydney criteria(235)

The concentration of maternal cells was 0,006% and 0,002% in the two patients, respectively. In the healthy male control, the maternal cells constituted 0.004%. At the follow up in present time, all three individuals tested negative for MMc, 16 years after the first draw date.

Paper IV. Maternal CD 34+ and CD 56+ cells are present in UCB. The frequency of MMc in cord blood may depend on clamping time and/ or mode of delivery.

Five out of 44 (11%) UCB samples were positive for MMc (Table IV).

Table IV
Summary of MMc-positive UCB samples.

Patient no	Mode of delivery	Gestational age *(weeks+days)*	Parity	Previous fetal loss	Clamping time *(seconds)*	Gender child	WCB (10^8/L)	% MMc in CD34	% MMc in CD56	% MMc in total DNA
5	El C/S	38+1	0	0	30	B	84	0	0	0.01
6	El C/S	38+6	1	1	30	B	100	1.5	0.3	0.2
8	El C/S	39+0	0	1	30	B	61	0.5	0	0
17	El C/S	38+4	2	1	35	B	45	0	0	0.1
37	vaginal	38+5	1	0	60	G	95	0	0.07	0

El C/S = Elective caesarian section B = boy G = girl WBC = white cell blood count

Two were positive only in the total DNA fraction (case 5 and 17), one in the CD34+ fraction (case 8), one in the CD56+ fraction (case 37) and one in the fractions of total DNA, CD34+ and CD56+ (case 6). The estimated concentration of maternal DNA ranged from 0.01% to 1.5%. No sample was positive for maternal cells in the CD3+, CD19+ or CD45+ fractions. All five positive samples were from babies with healthy mothers of whom four were delivered by elective caesarian section (80%) and one vaginally. In the group of negative samples 16 of 39 (41%) mothers were delivered by caesarian. The mean clamping time in the positive group was 37 ±13 seconds compared to 54 ±26 seconds in the negative group. Four of the five (80%) mothers of the positive babies had been pregnant before (54% in the negative group). Two of them (40%) had had a previous delivery (38% in the negative group), two (20%) had experienced at least one fetal loss (miscarriage or elective abortion) but no previous delivery (negative group 38%) and one (20%) had experienced at least one fetal loss and had given birth before (20% in the negative group). The mean white cell blood count per liter in the UCB samples of the positive group was 77 ±23 and in the negative group, 80 ±21.

6 DISCUSSION

6.1 METHODOLOGICAL CONSIDERATIONS

Studying MMc requires exceptionally sensitive methods due to the limited number of foreign (maternal) cells in the sample of the offspring. With too high sensitivity, the risk of false positivity, i.e. less specificity, follows. In our work on MMc we have used two different kinds of PCR; conventional PCR with subsequent separation of the PCR products with electrophoresis followed by visualization by an automated silver staining method (paper I) and Quantitative Real-Time PCR (QRT-PCR) (paper II, III and IV). One of the advantages with QRT-PCR compared to conventional PCR is the lower risk of contamination; the amplified sample is not taken out from the tube for further analysis. It is also less time consuming because the detection step is included in the PCR reaction and post PCR analysis with electrophoresis or blotting is not necessary. The detection of the amplified PCR product is rendered by means of fluorescent molecules, in our assays a TaqMan-probe. The fluorescent signal that is emitted is proportional to the amount of amplified DNA in the PCR reaction, resulting in quantification of the amount of DNA in the original sample. The conventional PCR method is only semiquantitative. The intensity of the sample bands was in paper I compared with a 10-fold dilution series with fixed concentrations of maternal DNA of 1%, 0.1%, 0.01%, and 0.001%

The sensitivity and specificity of the PCR method is, among other things, dependent on the choice of primers. In paper one, we used primers specific for a non inherited maternal HLA allele which are more specific compared to the system of biallelic primers (markers) used in paper two, three and four. The biallelic system we used was both based on single-nucleotide polymorphism (SNP) and insertion/ deletion polymorphism (INDEL). Due to short polymorphic sequences, only one primer is specific in the PCR reaction while the second primer and the TaqMan-probe can bind to both maternal and off-spring DNA. Furthermore, in the SNP case, specificity lies in only one base in the specific primer. This low specificity increases the risk of false positive amplification of negative alleles. The false positive signal can arise during late PCR cycles and thereby also limit the sensitivity of detecting the minor allele.

In the case of HLA, several SNP mismatches are available in the DNA sequence and primers can be designed so that both primers are specific in the PCR reaction. This increases specificity and sensitivity of the primers. However, the utilization of HLA

primers are burdened with much more time consuming work; the mother and offspring needs to be HLA typed followed by design of specific primers for the non inherited maternal allele and this is not always feasible with a large number of samples.
The amount of DNA in the sample is also essential to achieve proper sensitivity and specificity. The use of HLA specific primers allows higher concentrations due to higher specificity whereas biallelic markers work satisfactorily at DNA amounts up to 250 ng. Too high DNA amounts may increase the risk of false positive results, due to factors explained above. Considering that each cell contains 6 pg of DNA, the use of 250 ng DNA will render a sample of 42.000 cells with, theoretically, the ability to detect one maternal cell therein. Thus, the sensitivity of our QRT-PCR assays may exceed 0.01. In addition, cell separation like performed in paper I and paper VI, also increases the sensitivity of the method by removing the inappropriate cell types in the background (236).
The specificity and sensitivity of the QRT-PCR method was determined for markers included in our assays using artificial DNA mixtures and varying DNA amounts. The PCR assay was validated by mixing DNA from one individual (positive for the three markers) in different concentration in a second individual (negative for the markers). We detected no false positive results using 500 ng DNA and 40 cycles of PCR amplification.
To strengthen the specificity in the QRT-PCR we used as many different informative alleles as possible, usually two, and the samples were always run in duplicates, i.e. four wells in total using two markers. We did not consider it positive if not at least two wells showed evidence of MMc. This requirement is not always claimed in other studies.
The use of FISH is very common in MMc research but caution is recommended when interpreting results due to low specificity (237). The approach is to use probes directed to X and Y chromosomes which limits the detection to female cells in male tissue. The greatest advantage lies within the in situ method itself. It enables the visualization of maternal cells in a tissue and combined with phenotypic markers that are tested simultaneously with the XX/ XY probes make possible the characterization of maternal cells within the tissue. However, the risk of false positive results in determination of those rare cells is high due to misinterpretation of cells going through mitosis and overlapping or fusion with host cells. Verification by the microchimeric cells in a FISH assay could be performed by laser microdissection and subsequent laser-catapulting followed by PCR (238).

6.2 FINDINGS AND INTERPRETATIONS

6.2.1 Ontogeny of MMc. Paper I, II and IV

In paper one, two and four we investigated the presence of MMc in fetuses, children's tissues and in cord blood. Still, the ontogeny of MMc is ambiguous. Elucidation of questions as "when does the establishment of MMc occur during fetal development and in what individuals?", "where and in what cellular subsets does it persist?" would not only contribute to the understanding of how potential allo-autoimmune diseases may arise but would also shed new light on mechanisms underlying naturally acquired fetal-maternal tolerance. This might in turn contribute to the development of alternative strategies in HLA- mismatched HSCT, for instance using NIMA- and/ or IPA-matched recipient and donor-pairs in UCBT (21, 76).

6.2.1.1 Paper I. MMc in 2nd trimester fetuses.

In paper one, seven of eleven 2nd trimester fetuses showed evidence of MMc in all tissues investigated and included all cellular subsets. It would be closest at hand to compare our results with MMc in tissues of newborns and infants. Stevens et al. found, in contrast to us, few maternal hematopoietic cells when they investigated seven male infants who had died from lupus, infections and fetal hydrops with congenital anomalies (127). Similar to our study, maternal cells were widely spread and found in all tissues examined, including liver, pancreas, lung, bladder, skin and spleen. Interestingly enough, in contrast to the rare CD45+ and CD3+ cellular subsets, the frequency of differentiated tissue-specific maternal cells was high; in liver up to 42% of the maternal cells stained positive for cytokeratin and was found in six of seven infants, in kidney all maternal cells expressed cytokeratin and had the morphology of renal tubular cells and in pancreas, among islet β cells, up to 1.9% were maternal. MMc was not found in inflammatory areas but in normal organs. This is in line with Nelson et al who detected islet β cells of maternal origin in children with TD1 but infrequent CD 45+ cells (188). Since we did not (and no others either) examine differentiated tissue-specific maternal cells in the fetuses, it is not possible to say if the differentiation takes place already in fetal life or after birth. Speculations could be made that the maternal cells may develop towards different directions as the immune system develops in the host. MMc in tissues of older children is common, both in healthy and diseased, but few studies have evaluated if the cells are tissue-specific.

All fetuses in our study that were positive of MMc and then subjected to analysis of cellular subsets presented with CD 34+, a marker for HSC, in different organs. HSC are multipotent cells that differentiate into all kinds of blood cells and simultaneously replicate themselves to maintain self-renewal (239). Furthermore, recent reports have demonstrated that HSC may be even more plastic. The findings that HSCs are capable of differentiating into cell types of unrelated tissues originate from both mouse and human clinical studies where donor HSC cells have been found to give rise to endothelial precursors, neuronal brain cells, hepatic cells, skeletal muscle and cardiac muscle cells (240-244). In humans, donor specific marrow cell-derived epithelial and endothelial cells have also been found in the skin of the recipient and in endovascular epithelium (245, 246). These reports suggest that HSCs are able to transdifferentiate across the embryonic germ layer barrier, for example differentiation of HSCs into neural cells (mesoderm to ectoderm). A transfer of HSCs into muscle, however, would be within the same germ layer, the mesoderm. If the microchimeric, maternal CD34+ is comparable in its feature with the transplanted adult HSC, it could be interpreted that maternal CD 34+ cells in the fetus may give rise to tissue-specific cells after birth. However, other types of maternal stem cells could also be the origin. It is unknown whether MMc could be considered as benign and part of normal tissue generation in the fetus and infant but be recognized by the host as foreign later in life.
In paper I we did not conduct an in situ analysis. This is a limitation due to the fact that we cannot say for certain that the maternal cells were incorporated in the fetal tissues or if they were circulating freely.

Where do the residing transferred maternal cells hide? Do they home to bone-marrow as other stem cells? No previous study has investigated MMc in bone marrow. An interesting study by Gammill et al. investigated the presence of MMc in pregnant women longitudinally, that is if the soon-to-be grandmother's DNA was detectable in the soon-to-be mother (247). Twenty-seven women with normal pregnancies and 20 women who developed preeclampsia were included. In healthy pregnancies, the prevalence was 0% early in gestation but increased with every trimester, to be detectable in 16% in 2^{nd} trimester and 29% in 3^{rd} trimester. Post partum the prevalence was 14%. The concentration of MMc also increased through pregnancy. In the pregnancies complicated by preeclampsia, none had detectable MMc at any time. This finding may reflect that different immunological conditions may have an effect on the detectability of MMc. The authors speculated that the increase in MMc in peripheral

blood may be a consequence of a generalized stem cell mobilization that occurs in healthy pregnancy which in turn could be triggered by the load of fetal antigenic material seen in the third trimester and that function could be impaired in preeclamptic patients. A future study could be to analyze MMc in bone marrow of prospect HSCT donors or to evaluate if maternal cells can be mobilized to peripheral blood by G-CSF (granulocyte colony-stimulating factor) given the donor prior a peripheral HSCT.
We sought to investigate pregnancies complicated with trisomies due to earlier findings of increased fetal-maternal trafficking in pregnancies complicated by aneuploidy (136). In paper I, three of four fetuses with trisomy 21 had detectable MMc compared to four of seven fetuses without trisomy 21. Of course, the number of subjects is too small to draw any definite conclusions from this finding. But the fact that individuals with Down's syndrome has a failing immune system and more often suffer from autoimmune disorders, infections and malignancies, makes it interesting to study the frequency and levels of MMc in those individuals. It is not only known that T and B cells have impaired function in individuals with Down's syndrome- the thymus is smaller and has an abnormal structure, even in newborns. (248). Speculations could be made that the semi-allogenic maternal cells pass as not that foreign due to impaired immunological function and that the maternal cells later in life could serve as effectors or targets in autoimmunity. A small study by Srivitsa et al. examining MMc in different tissues of four neonates who had died within the first week after birth, showed the highest number of MMc in a neonate with Down's syndrome (203).
We did an attempt to analyze MMc in the liver of 1st trimester fetuses that were sampled for isolation of different tissue progenitors but we realized that the risk of contamination with maternal blood was too high due to the findings of very high levels of MMc, to go further with that study. The fetuses were delivered after vacuum extraction and though we chose those fetuses that were intact, we could not be sure that the maternal blood was properly washed away.
In conclusion, the cellular subsets in the 2nd trimester fetuses had a mature immunological feature that may induce feto maternal tolerance but they could also serve as functional cells within the host.

6.2.1.2 Paper II. MMc in children's tonsils and/ or adenoid

The choice of investigating tonsils and adenoid in children originated in our wish to explore MMc in tissues of healthy individuals. In studies examining MMc in different

disorders of autoimmunity, usually other patients' biopsies serve as controls. It can be questioned whether such controls reflect the actual MMc status in healthy individuals.
A critical remark to the use of tonsils and adenoids could be that they were not normal, due to the fact that they needed to be ablated and that children with respiratory obstruction and/ or recurrent tonsillitis not can be considered as "healthy". However, tissues of healthy individuals are available to a very limited extent. The finding of MMc in these specimens could also reflect homing of maternal cells to an immunological site. MMc was found in four of 20 children which correlate well to the frequency in controls of other studies. The percentage of maternal cells varied between 0,00075 to 0,02. In our study the children who were found positive in tonsils, were also positive in blood. It might be true that tissue and blood MMc frequency correlate well in healthy individuals where the maternal cells might appear as "innocent bystanders", but that may not be necessarily true in a diseased individual. An example of this could be the report of a patient who died from scleroderma (129). She had no MMc in peripheral blood at several sample-takings but was found to harbor high levels in a number of organs when she died.
In paper II we did not make cell separations, so whether the maternal cells are of hematopoietic nature or not, we cannot say. Recent research shows evidence that at least tonsils might be an extrathymic lymphoid tissue, due to the findings of T cell lineage precursors in it (249). But again, the homing of maternal cells in the host is not known. Parallels to the findings of differentiated maternal cells in pancreas and heart tissue could lead to speculations that maternal cells in the inflamed tonsils and/or adenoid were part of a tissue repair process and had migrated there secondarily. This would have been possible to confirm or exclude using XX/ XY based FISH simultaneously with tissue specific markers for cytokeratin for instance. However, that would have limited the study to boys.

The questionnaire that was answered by the mothers reflects what confounders must be considered when analyzing MMc. It also included questions about conditions during pregnancy and breast feeding that also could influence MMc. We asked if the children had received a blood transfusion, if the child was a twin, if the mother was healthy and had had a normal pregnancy and for how long the child was breast-fed. First, an earlier blood transfusion could be a confounder even though irradiation of the blood in order to eliminate leukocytes routinely is performed, but Mc following blood transfusion has been described (2). Mc from a twin could also be interpreted as

maternal (223). It has not been investigated if MMc is increased after preeclamptic preganancies as it is in FMc but it is not unlikely that MMc is also facilitated due to placental dysfunction (138). Brest feeding as an additional source of MMc is interesting. In mice it has been shown that maternal cells can be transferred to the pup by nursing (224). However, MMc did not persist into adulthood in the absence of neonatal exposure through breast feeding (250). In humans, the influence of breast feeding on MMc is not investigated.

6.2.1.3 Paper IV. Cellular subsets of maternal cells in umbilical cord blood

The information on cellular subsets of MMc in cord blood is scarce. Only two previous studies have explored this; Wernet et al. found that as much as 10% of all nucleated cells in whole blood to be maternal in the one of 16 UCB samples positive for maternal cells (193). Five % of the CD3+ cell fraction and 15% of the myelomonocytic cells from mononuclear cells were maternal. Of picked BFU-E (erythroid burst-forming units) and CFU-GM (colony forming units-granulocyte-macrophage), 15% were maternal. That should not be interpreted as microchimerism but rather chimerism and consequently questioning of contamination or misinterpretation of maternal cells would be proper. Also by FISH, Hall et al. found 7 of 49 UCB samples to be positive for MMc. Female CD8+ cells were found in 5 of 39 samples and CD 34+ only in one of 27 (196) The levels ranged from 0.04% to 1.0%.
In our study, the frequency of MMc was detected to 11%, somewhat unexpectedly low as will be discussed below.

The maternal cellular subsets in cord blood are of importance in two aspects; first of all it is of interest in order to understand the ontogeny of MMc. Are maternal cells actually present at birth and do they exhibit a character of progenitor lineages with potential to engraft and differentiate? The other aspect is what subsets of maternal cells are actually co-transmitted as “third party” in UCBT. This is specifically of interest since van Rood et al. in two studies showed that the mother of the donor of cord blood seems to influence the outcome of the transplantation. The first study showed that when donors and recipients were mismatched for NIMAs, the recipient was more likely to have an earlier engraftment, less GvHD and decreased rate of relapse of acute myeloid leukemia (76). This might be explained by the effect of donor’s T cells (both effectors and T regs) with anti-NIMA activity, developed during fetal life. Later, the same group showed that if the donor and recipient shared the same IPA, the relapse risk was

significantly reduced (hazard ratio=0.35, P <0.001) with a non-significant slight increase in GvHD (21). An interpretation could be that maternal T cells might mediate this effect. Thus, the microchimeric maternal cells could be the heroes (or heroines) in this case, but the real evidence would of course be to demonstrate maternal cells exhibiting this effect in the recipient. A consequence of the findings by Van Rood would be to HLA-type the mothers of the UCB donors, a procedure that is not yet a routine in UCB banking.

The frequency of MMc in this study (11%) appeared to be somewhat lower than the frequency of MMc in children's tonsils and/ or adenoid (20%). We used the same method as in paper II but the samples derived from different ages (and tissues) and the kinetics of MMc and natural course is not known. Since the presence of MMc seems to depend on HLA compatibility between fetus and mother, the subjects in paper IV might have not been compatible by chance.

Results from previous studies of MMc in cord blood vary greatly (0-100%, mean 14%) but they include several different detection methods.

We were interested in two further aspects; whether MMc in UCB is dependent on clamping time or mode of delivery. Most of previous studies on MMc in UCB refer to "immediate" clamping of the cord, but the practice in Sweden is about to change to longer clamping time since infants may benefit from increased hematocrite and higher ferritin concentration after birth following clamping time after three minutes. Since this will not yield adequate numbers of cells needed in UCBT, a compromise implying clamping time of one minute in vaginally deliveries and 30 minutes in caesarian sections at the two UCB bank centers (Stockholm and Gothenburg) in Sweden has been introduced.

Actually, we speculated in the planning stage of the study if contractions during labour could facilitate the transfer of MMc. The number of positive cases was low but four of the five were from deliveries by caesarian sections indicating that this assumption might be wrong. However, among the positive cases the clamping time was considerably shorter (37 ±13 seconds) compared to the negative group (54 ±26 seconds) leading to speculations if the clamping time itself affects the transfer of maternal cells. If the indirect findings by van Rood that maternal cells in a UCB graft could contribute to an improved outcome of UCBT could be directly shown and larger studies could confirm that MMc in UCB is enhanced by short clamping time, there

would be two advantages with immediate clamping; more cells to the transplant and more maternal cells to exhibit the GVL effect.
The mean concentration of maternal CD34+ and CD56+ cells in paper IV was 1% and 0.185% respectively. Considering that a typical UCB transplant harbors $3x10^7$nucleated cells/ kg recipient body weight and assume that 1% are CD34+ and 20% are CD56+ implying that $3x10^3$ maternal CD34+cells and 1.1 $x10^4$ CD56+cells /kg are transferred with the UCB graft. This amount of cells may be large enough to engraft if co-transmitted in UCBT (251).

6.2.2 MMc in autoimmunity. Paper III

Indications that MMc could be involved in autoimmune disorders stem from studies of SCID that showed that maternal cells persist at high levels and engraft in children with this immunodeficiency. Additionally, SCID children develop GvHD to a great extent. However, almost all disorders that have been proposed to be associated with MMc (and FMc) are basically describing the presence of MMc in peripheral blood or target tissues of patients with autoimmune disorders and healthy controls and do not provide evidence for the possible function(s) of MMc.
There are difficulties comparing autoimmunity and GvHD. Autoimmune diseases are a heterogeneous group of over 80 disorders that are characterized by pathologic immune responses directed at self-tissue and, like in GvHD, the spectra of pathophysiology and clinical presentation are broad (252, 253). A further aspect when comparing chimerism and consequent GvHD after HSCT and naturally acquired chimerism regards the differences in the size of cell populations. After HSCT, the hematopoietic cells of the recipient are almost replaced by the donors in contrast to MMc or FMc where they constitute less than 0.1%. MMc has been associated with several autoimmune diseases, especially in children; JDM, JIIM, neonatal lupus, type 1 diabetes, pityriasis lichenoides, biliary atresia (182-190).
In adults, patients with SSc were found to harbor MMc more often than controls (129). All of the above mentioned autoimmune disorders display similarities to GVHD.

6.2.2.1 Paper III. MMc in SLE

The seed to our study arose from Stevens et al.'s findings that bidirectional compatibility for DRB1 was associated with SLE when they compared mother/ son

compatibility in 30 male SLE patients with 76 healthy males (254). They showed that the frequency and concentration of MMc was higher when there was maternal compatibility for HLA-DQB1 from the fetal perspective, that is, the mother was DQB1 homozygous or HLA identical. This, together with the fact that a SLE like disease could be evoked in mice by transfusion of homozygous parental lymphocytes into heterozygous progeny made Stevens et al. suggest that MMc in SLE patients might be elevated. A collaboration with the Rheumatology Department at Karolinska Institutet led to our access to stored DNA from SLE patients and their first degree relatives (which originated from a large genetic study from the 90s) and gave us the opportunity to see if the assumptions of Stevens et al. were true.
However, we did not find any association; two patients (6%) and one control (3%) harbored maternal cells in peripheral blood.

One limitation of our study is that we only investigated MMc in blood and not in tissues. This might not reflect a true picture because affected tissues may harbor MMc while peripheral blood does not. One patient with SSc in the study by Lambert et al., who was negative for MMc in peripheral blood in four occasions, was found to harbor MMc in bone marrow prior to her death. After she had died, evaluation of MMc in different organs was conducted and it was found in several affected organs. In a study of FMc in SLE the presence of chimeric cells in kidney was related to injury (255). A patient with SLE was at autopsy found to harbor FMc in every injured organ, but not in unaffected tissue (256). This information suggest that future studies of the association between microchimeric cells and inflammatory disease should include evaluation of Mc in affected organs where microchimeric cells are assumed to be active. In our study of MMc and SLE, additional examinations of the occurrence of MMc in for example skin, kidneys and synovial fluid would have provided additional information.
In paper III, the levels of MMc in the two patients (mean 0.004%) and the control (0.004%) did not differ. In the literature of Mc in autoimmunity, the association of Mc and the disease is sometimes based on an increased frequency of Mc, as in MMc and SSc, when other correlation is founded on increased levels of Mc as in FMc and SSc.(156,157).
An advantage with our study was that almost 60% of the control group constituted of healthy brothers to the patients. Using siblings as controls reduces genetic and environmental influences.

When evaluating the presence of maternal cells in an individual, the possibility of detecting confounding microchimeric cells of other origin should not be neglected, for example from a previous pregnancy i.e. FMc. Not only live births result in FMc. Of 120 women without sons, 21% tested positive for male Mc (218). Male Mc prevalence was significantly greater in the group of induced abortions (57%) compared to those with only daughters (8%), those with spontaneous abortions (22%) or those who were nulligravida (10%). Interestingly, the levels of MMc were also higher in the group of induced abortions indicating that residing FMc could theoretically be misinterpreted as MMc if the fetuses of the abortions share the same allele that is considered as the NIMA in a woman. The surprising finding of 10% male Mc among the nulligravida might be explained by other possible sources of male microchimerism including unrecognized spontaneous abortion or vanished male twin.

In order to minimize the risk of amplifying a polymorphic region of fetal origin in the PCR procedure we strictly selected SLE patients who had reported themselves as nulligravida. Since this information of the healthy sisters of the patients was not available we chose the patients' brothers and unrelated men as healthy controls. If the hypothesis that FMc could be a confounder in detecting MMc in women is true, the prevalence and levels of (false) MMc would be higher in parous compared to nulliparous women and would consequently increase with number of children. Studies that evaluated this specifically are not available but in the report of different cellular subsets of MMc in womens peripheral blood, MMc was more prevalent in the parous group compared with the nulliparous, 45% (10/22) and 22% (2/9) respectively (201). However, the two groups were of limited size. Still, this study had the advantage that they had determined the phenotype of the womens children in order to be able to exclude them as confounding MMc. Additionally; parous women may have experienced fetal losses to a greater extent than nulliparous women (and as a consequence have acquired more FMc).

If residing FMc or MMc in a women could pass over to her current pregnancy in a transgenerational manner is not known. Interestingly enough, Dierselhuis et al. found male Mc in six of nine UCB samples from girls with older brothers, but none in the three samples from firstborn girls (222).

FMc acquired by the mother from an earlier pregnancy that pass into her current could theoretically be misinterpreted as MMc in the current child if the same NIMA is shared by the older sibling and the mother. If that hypothesis would be true, the consequence

would be that the later birth order a child has, the greater likelihood of MMc (false) would be recognized. No studies have addressed this specifically but in the report of MMc in cord blood by Scaradavou there were no differences in the frequency of MMc in cord blood from babies whose mothers were multigravida (70%) compared to primigravida (72%) (198). But in our work, both in paper I and paper IV, there was a tendency that the mothers in the positive cases had experienced more pregnancies than mothers in the negative cases. A recent study of the association between asthma and MMc did not find any correlation of birth order and MMc (257). Accordingly, conflicting indirect data of possibly confounding transmaternal flow of FMc in women investigated for MMc exists.

The low prevalence of MMc in paper III could lead to speculations that the characteristics of the subjects included (nulligravida women and healthy men) may influence the result towards lowe frequency. In adults, men specifically have not been subjected to MMc analysis before, and neither have nulligravida women. What if previous studies of MMc in women are confounded with FMc? Most studies of autoimmune disorders and MMc are conducted in children. Could MMc be more prevalent in children, and thus more important as part of certain autoimmunity? Studies that investigate the natural course of MMc in healthy individuals could contribute to that answer.

Thus, MMc could be misinterpreted in women with previous pregnancies. The most proper way would be to address MMc in males or children with no history of blood transfusion. When investigating the incidence of MMc in women, an accurate obstetric history and the exclusion of her children carrying the same targeted polymorphism used in the analysis, is to be recommended. However, Mc from a vanishing twin is hard to rule out as a confounder for MMc.

In our study of SLE and MMc, we were fortunate to get new blood test from the three subjects (two patients and one healthy male) with MMc in the middle of the 90s. All three tested negative for MMc 16 years later. The natural course of MMc over time has never been investigated. The reason for MMc absence in our follow up could be that maternal microchimeric cells age and disappear as other cells even though MMc has been detected up to middle age (14). The fact that hematopoietic progenitors are among detectable MMc cells make them likely to persist for a long time but to what extent they are able to self-renewal is unknown. One can also speculate if immune modulatory treatment affects their prescence. For example, MMc disappeared in two patients with SSc under cyclophosphamide treatment (258). JDM patients treated with methotrexate

harbored MMc less frequently than patients treated with prednisone (259). MMc present in autoimmune diseases may also differ during amelioration or flares. The study by Gammill et.al. referred to above (6.2.2) showed that the prevalence of MMc in healthy pregnant women increased from 0% in the first trimester to 29% during the third, suggesting that factors (immunological or due to the increasing maternal blood volume) dependent on the pregnancy lead to the mobilization of all sorts of stem cells from the bone marrow (247).

As in many autoimmune diseases, specific HLA alleles are well documented to increase susceptibility to SLE. The MHC class II alleles HLA-DRB1*03 and HLA-DRB1*15 are associated with the disease but just over half of the patients carry the risk alleles suggesting other factors to be essential to the development of the disease. In the view of HSCT and the importance of histocompatibility in regulating Mc in the recipient and subsequent GvHD, the suggestion has been proposed that the HLA relationship of host and non-host cells in naturally acquired Mc may be important. This matter is already discussed in Introduction.

In our study of MMc and SLE we did not conduct any HLA analysis of the mother/ child pairs. Retrospectively, in the light of the negative association this would however not have added any further conclusions. In two of our other studies we conducted HLA typing. In the study of 2^{nd} trimester fetuses the typing was done with the purpose of finding a non-inherited maternal allele to use in the PCR assay. The typing did not result in any particular pattern of compatibility between fetuses and mothers and the presence of MMc. In our report of MMc in tonsils and/or adenoids the 20 mother/ child pairs were typed for HLA-A, B and DR (results not published) with this intention but there were no findings of compatibility from the perspective of the fetus and the presence of MMc in this study either. However, the numbers of subjects were presumably too small to expect this.

7 CONCLUSION AND FURTHER DIRECTIONS

- Maternal cells of lymphoid and myeloid lineages and hematopoietic progenitors are widely distributed in 2nd trimester fetuses, including healthy fetuses and fetuses with Down's syndrome.

  - Where do maternal cells home? Investigating bone marrow for the presence of MMc compared to peripheral blood in donors of HSC. Do MMc increase in peripheral blood in donors treated with G-CSF in order to mobilize stem cells before HSCT?

  - Do children and adults with Down's syndrome harbor MMc to a greater extent than healthy individuals? Investigating MMc in peripheral blood within different cellular subsets in individuals with Down's syndrome.

  - Do infants, children and adults with preeclamptic mothers harbor MMc at higher levels and frequency compared to individuals born of healthy mothers?

- MMc is common in children's tonsils and/ or adenoids and corresponds to levels and frequency in peripheral blood.

- MMc in cord blood is present and includes CD34+ and CD56+ cellular subsets. The transfer of maternal cells may depend on clamping time and/ or mode of delivery.

  - Larger studies of cellular subsets of MMc in UCB are needed with defined, different clamping times, for example: immediate, after 30 seconds, after one minute and after three minutes.

  - Evaluate the presence of MMc and functionality in a recipient after UCBT.

- MMc is not associated with SLE in adults and exhibits low frequency in adult nulligravid women with SLE and healthy men.

- Future studies of MMc should preferably include children or men exclusively to exclude the probability of confounding FMc. If women of reproductive age are included, an accurate obstetric history is essential.
- Analyze the natural course and time kinetics of MMc over time in healthy individuals.

8 SVENSK SAMMANFATTNING

Begreppet chimär härrör från den grekiska mytologin där det syftar till ett vidunder bestående av kroppsdelar från ett lejon, en get och en orm. Inom medicin uppstår chimerism som följd av blodtransfusion, benmärgstransplantation eller organtransplantation. Chimerism kan beskrivas som närvaron av en annan persons DNA eller celler i en kropp. När denna andel av celler är liten (mindre än 1 %) benämns tillståndet som mikrochimerism. Den absolut vanligaste orsaken till mikrochimerism är graviditet. Länge uppfattades moderkakan som en helt tät barriär mellan mamma och foster, men nu är det ett etablerat faktum att det sker en överföring av celler i båda riktningarna under en graviditet. Dessa celler kan kvarstå i blod och vävnad upp i vuxen ålder. Celler från barnet i mamman benämns fetal mikrochimerism och celler från mamman i barnet maternell mikrochimerism. Eftersom kvinnan och barnet bara till hälften är lika varandra genetiskt (barnets halva genuppsättning kommer från pappan) finner man det anmärkningsvärt att dessa främmande celler inte stöts bort eller gör skada. Ett parallellt exempel från medicinen är situationen som kan uppkomma efter en benmärgstransplantation där en patients immunförsvar nästan helt ersätts av en donators immunceller. De flesta sådana patienter utvecklar en sk graft-versus-host reaktion , där donatorns celler uppfattar patientens celler som främmande och angriper dem i olika vävnader. Angreppen kan ske i många olika organ men vanliga exempel är angrepp på hud, slemhinnor och tarm vilket ofta leder till utslag i huden och diarré.

Eftersom många autoimmuna sjukdomar (sjukdomar där kroppens immunförsvar uppfattar de egna cellerna som främmande) liknar en graft-versus-host-sjukdom har det spekulerats i om mikrochimära celler efter en graviditet kan vara en bidagande faktor?. Man har upptäckt en association mellan kvarvarande fetala celler hos kvinnor med tex skleroderma som är en typisk autoimmun sjukdom vilken debuterar framförallt i kvinnor efter barnafödande ålder. Hos dessa kvinnor har man funnit en högre andel av fetala celler i blod och i sjuk vävnad jämfört med friska individer. Celler från mamman som återfinns hos hennes barn har sammankopplats med framför allt ganska ovanliga autoimmuna barnsjukdomar, men också med en så pass vanlig sjukdom som diabetes. Hur förklarar man fyndet av en högre andel mikrochimära celler i samband med vissa sjukdomar?

Teorier som framkastas som förklaring lyder:

1. De främmande cellerna verkar som attackceller i mammans eller avkommans kropp.

2. De främmande cellerna är själva målet för mammans eller avkommans egna immunologiska celler.

3. De främmande cellerna förflyttar sig till en redan skadad vävnad och bidrar till reparation.

Således finns det spekulationer om att cellerna är av både ondo och godo.
Celler från mamman i avkomman, som denna avhandling handlar om, finns i både friska och sjuka individer och kan finnas där under lång tid. I en studie hittades celler i blod hos en 49- åring som man antog var mammans. En nytta som fostret kan dra av att härbärgera en liten del mammaceller är att de hjälper fostret att tolerera sin mamma under pågående graviditet. Egentligen borde båda mamma och barn stöta bort varandra pga. olika genetiska uppsättningar. Mammaceller i ett foster verkar kunna påverka fostret till att producera celler som hämmar andra celler från att attackera mamman. Två relativt nya studier som handlar om transplantation av navelsträngsblod till patienter med leukemi påvisar att kvarvarande celler från mamman i navelsträngsblod kan ha en positiv effekt på utfallet.

Denna avhandling består av fyra arbeten som innefattat följande:

Arbete 1: Förekomsten av immunologiska mammaceller i olika organ i foster från 14:e till 18:e graviditetsveckan. Fem foster var friska och fem hade Down's syndrom och/ eller missbildningar. Vi undersökte också ett foster som hade dött i livmodern i anslutning till 27:e graviditetsveckan.
Resultat: Vi fann immunologiskt mogna celler från mamman i sju av 11 foster och vissa av cellerna var av stamcellskaraktär. Av de friska fostren hade fyra stycken mammaceller och bland de sjuka hade tre av fem. Det döda fostret från 27: e veckan hade inga mammaceller. Cellerna fanns spridda i samtliga organ vi undersökte, t.ex. i lever, brässen, hjärna och lungor.
Slutsats: Immunologiskt mogna celler i foster är ett vanligt fenomen.

Arbete 2: Förekomsten av mammaceller i friska barns halsmandlar och näspolyper samt i blod.
Resultat: Fyra av 20 barn hade mammaceller i sina halsmandlar och/ eller polyper. Samma barn hade mammaceller i blod också.

Slutsats: Mammaceller i friska barns vävnader och blod är vanligt förekommande. Detta kan vara viktig information när man undersöker förekomsten av mammaceller hos sjuka barn. Oftast jämför man med vävnad från en individ som har en annan sjukdom än den aktuella och då kan det bli svårt att tolka resultaten.

Arbete 3: Finns det någon association mellan sjukdomen systemisk lupus erythematousus och mammaceller? Vi undersökte förekomsten av maternell mikrochimerism i 32 patienters blod och jämförde dessa med 29 stycken friska män. Proverna var tagna i mitten av 90-talet. Vi upprepade analysen bland de som var positiva för mammaceller nu 16 år senare.

Resultat: Bland patienterna hade två stycken mammaceller i blodet och av kontrollpersonerna hade en mammaceller. Samtliga tre personer var negativa vid uppföljningen.

Slutsats: Det föreligger ingen association mellan systemisk lupus eryhematousus och maternell mikrochimerism i blod. Förekomsten av mammaceller förefaller variera med tid.

Arbete 4: Förekomsten av olika typer av mammaceller i navelsträngsblod hos 44 stycken fullgångna, friska nyfödda. Vi analyserade om andelen mammaceller kunde påverkas av förlossningssätt - kejsarsnitt eller vaginal förlossning - eller av avnavlingstiden (tiden mellan barnets födelse och när man stänger av flödet i navelsträngen och klipper av den).

Resultat: Vi fann mammaceller i navelsträngsblodet hos fem av 44 nyfödda. Fyra av dessa var förlösta med kejsarsnitt. Mammacellerna var av stamcellskaraktär och ”NK celler” (en typ av mördarceller som är viktiga för immunförsvaret). Avnavlingstiden var kortare i de fall där man fann mammaceller.

Slutsats: Mammaceller förekommer i navelsträngsblod hos friska nyfödda. Det är möjligt att förlossningssätt och/ eller avnavlingstid påverkar andelen. Fynden kan ha relevans vid navelsträngstransplantationer.

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