Chimerism in health, transplantation and autoimmunity

Koopmans, M.; Kremer Hovinga, I.C.L.

9

PREGNANCY, CHIMERISM AND LUPUS NEPHRITIS: A MULTI-CENTER STUDY

Idske C.L. Kremer Hovinga
Marije Koopmans
Cecile Grootscholten
Annemieke M. van der Wal
Marc Bijl
Ron H.W.M. Derksen
Alexandre E. Voskuyl
Emile de Heer
Jan A. Bruijn
Jo H.M. Berden
Ingeborg M. Bajema

on behalf of the Dutch Working Party on Systemic Lupus Erythematosus

Lupus 2008; 17: 541-547

Abstract

Chimerism occurs twice as often in the kidneys of women with lupus nephritis as in normal kidneys and may be involved in the pathogenesis of systemic lupus erythematosus (SLE). Pregnancy is considered the most important source of chimerism, but the exact relationship between pregnancy, the persistence of chimeric cells, and the development of SLE has not been investigated.

Renal biopsies and clinical data from patients in the First Dutch Lupus Nephritis Study were used. Chimeric cells were identified by in situ hybridization of the Y chromosome. A questionnaire was used to obtain detailed reproductive data including pregnancy history and miscarriages.

Chimerism was found in 12 of 26 (46%) renal biopsies. Of the 12 chimeric women, 5 reported a pregnancy; of 14 women who were not chimeric, 8 reported a pregnancy. Chimeric women who had been pregnant reported significantly more pregnancies than non-chimeric women who had been pregnant (p=0.04). The median age of the youngest child was higher in chimeric women (19 yrs) than in non-chimeric women (6 yrs).

Despite the attention given to pregnancy histories with respect to chimerism, this study shows that in patients with SLE a clear-cut relationship is not apparent. A considerable number of chimeric women did not report a pregnancy: in these women, other sources of chimerism must be considered. Our data support the theory that only certain subsets of chimeric cells persist into the maternal circulation after pregnancy.

Introduction

Systemic lupus erythematosus (SLE) is an immune-mediated disease that affects several organs and has a variety of clinical symptoms.[1] It is characterised by the presence of autoantibodies, especially autoantibodies directed toward nuclear components.[2] Despite extensive research, the aetiology of SLE is still unknown, but is probably multifactorial. One factor that may be involved in the pathogenesis of SLE is chimerism (the presence of cells from one individual in another).

An important finding that points towards a role for chimerism in SLE is an experimental mouse model developed in the 1970s. In the model, the injection of chimeric cells in (DBAxC57BL) F1 mice initiates a graft-versus-host response resulting in manifestations of an SLE-like disease including the production of autoantibodies directed against nuclear antigens (e.g. anti-double-stranded DNA) and the occurrence of proliferative glomerulonephritis.[3,4] Recent studies demonstrating that chimerism is present in humans with SLE underline a possible role for chimerism in the development of SLE.[5-9]

SLE occurs in women and men at a ratio of approximately 10:1. In women, the first symptoms most often occur during their fertile years.[10] These facts are interesting in relation to a possible role of chimerism in SLE, because the most important source for chimeric cells is pregnancy. During pregnancy, foetal cells enter the maternal circulation, making the mother chimeric.[11-13] It should be noted that miscarriages can also result in chimerism of the mother.[14]

Putting pregnancy, chimerism, and SLE together to form a hypothesis about the role of chimerism in the development of SLE, we postulated the following: healthy women become pregnant, which results in chimerism, which under certain circumstances may lead to a chronic immune response initiated by the chimeric cells and result in the initiation of SLE. In this scenario, a clear-cut relationship should be present between pregnancy, the persistence of chimerism, and the development of SLE.

We previously investigated the occurrence of chimerism in kidneys that were affected by SLE (lupus nephritis) and found that chimerism occurs twice as often in kidneys with lupus nephritis as in normal kidneys.[8] However, in the previous study, extensive information on reproductive history was lacking. Therefore, in the present study we investigated the presence of chimerism in a new group of women with proliferative lupus nephritis, of whom extensive information on reproductive history was known, in order to determine whether a relationship between pregnancy, chimerism, and lupus nephritis exists.

Patients and methods

Patients and biopsies

The patients came from a multicenter prospective randomised controlled trial (First Dutch Lupus Nephritis Study). Inclusion and exclusion criteria for this study have been described in detail elsewhere.[15] In short, lupus nephritis patients were included from September 1995 to September 2001 according to the following criteria: the presence of at least 4 American College of Rheumatology criteria for SLE,[1,16] age 18 to 60 years, creatinine clearance (Cockcroft-Gault) > 25 ml/min, and biopsy-proven proliferative lupus nephritis. After randomization patients were treated with either 13 pulses cyclophosphamide (750 mg/m^2) combined with oral prednisone, or with azathioprine (2 mg/kg/day for 2 years) and oral prednisone combined with a total of 9 methylprednisolone pulses (3x3 pulses of 1000 mg) given within the first 6 weeks. In all patients, this therapy was begun after a renal biopsy was taken, and therefore would not influence the results of the present study. Clinical and laboratory parameters, including creatinine clearance, proteinuria, presence of autoantibodies, and SLE disease activity index were recorded.

All women in the First Dutch Lupus Nephritis Study were asked to fill out a questionnaire evaluating reproductive data before, during, and after the study. Questions were formulated regarding the number of pregnancies, number of deliveries, the number of live births, birth dates of children, number of miscarriages and durations of pregnancies, use of oral contraceptives, presence and nature of cycles, and unwilling childlessness.

Paraffin-embedded renal biopsy specimens from 45 women were available for the present study. Unfortunately, tissue blocks from nineteen patients contained material of inferior quality and therefore could not be used for in situ hybridization procedures. Therefore, the biopsy specimens of 26 women with lupus nephritis were included.

In situ hybridization targeting the Y chromosome

In order to detect chimeric cells, in situ hybridization of the Y chromosome was performed as described earlier.[17] In short, paraffin-embedded tissues were cut into 4-µm sections and deposited onto Superfrost plus glass slides (Menzel-Glaser, Braunschweig, Germany). The paraffin was removed, followed by a distilled water rinse. The sections were pretreated with 0.05 M citrate buffer at 80ºC, rinsed in distilled water at 37º, and treated with 0.5% pepsin in 0.01 M HCL at 37ºC.

A Y chromosome-specific DNA probe[18] was labeled with digoxigenin (DIG), precipitated, dried, and dissolved in a hybridization mixture. Slides were covered with a 30 µL hybridization mixture containing 5 ng/µl labeled probe and placed on a metal plate at 80ºC for 10 minutes, followed by incubation at 37ºC overnight. The next day, the sections were washed three times in 2 × SSC/0.1% Tween at 37ºC, and three times in 0.1 × SSC at 60ºC. Sections were incubated consecutively with a mouse anti-DIG monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA), a rabbit anti-mouse immunoglobulin horseradish peroxidase (HRP; Dako, Glostrup, Denmark), and a swine anti-rabbit immunoglobulin-HRP (Dako). Finally, sections were developed with Nova Red Vector. Hematoxylin staining served as a background stain.

Male kidney tissue samples served as positive controls, with red-brown dots confirming a positive signal in 64% of the nuclei. By PCR and sequencing, we confirmed that the probe was specific for the Y chromosome (described in reference [17]). As a negative technical control, a male tissue sample was used on which the complete in situ hybridization protocol was performed, but instead of the hybridization mixture with the Y chromosome probe, only the hybridization mixture was added. This negative control was consistently negative.

Scoring

All specimens were independently scored for the presence of Y chromosome-positive cells by two observers who were blinded to the clinical information of the study subjects. A cell was only scored positive for the Y chromosome if there was a dot inside the nucleus that had a similar size and staining intensity as the dots that were found in the nuclei of the male control samples.

Statistics

Categorical variables were compared with the use of the chi-square test. Continuous variables were compared with the Student's t-test, and non-normally distributed continuous variables were compared with the Mann-Whitney U test. Correlations were determined by calculating point-biserial correlations. A binary logistic regression analysis was used to determine the predictive value of several parameters for the presence of chimerism.

Results

Y chromosome-positive, chimeric cells were found in 12 of 26 (46%) renal biopsy specimens with lupus nephritis. Four renal tissue specimens contained 1 chimeric cell, 3 specimens contained 2 chimeric cells, 3 specimens contained 4 chimeric cells, 1 specimen contained 5 chimeric cells, and in 1 specimen 6 chimeric cells were found. The mean surface area of all biopsies was 6.25 mm^2; a logistic regression analysis showed that surface area (OR =1.09 , CI 0.89-1.33, p=0.40) did not predict for chimerism.

Clinical data and results are given in Table 1. Age at biopsy was not related to the presence of chimerism (OR = 1.02, p=0.62). There was no relationship between the presence of chimerism and age at diagnosis (OR = 1.01, p=0.89), or time since diagnosis (OR = 1.03, p=0.65). Clinical parameters comprising serum creatinine (OR = 1.00, p=0.75), proteinuria (OR= 1.09, p=0.61), the presence of anti-double stranded DNA autoantibodies (OR= 0.85, p=0.91), the titer of C3 (OR= 3.10, p=0.49) or C4 complement (OR= 243.18, p=0.42), and the SLE disease activity index (OR= 0.99, p=0.83) did not predict significantly for the presence of chimerism.

Table 1. Clinical data and results of women with lupus nephritis

		Total group	Chimerism present	Chimerism absent
Number of women		26	12	14
Caucasian (%)		19/26 (73)	9/12 (75)	10/14 (71)
Lupus nephritis presenting symptom (%)		6/26 (23)	1/12 (8)	5/14 (36)
Age at biopsy, yrs				
	mean	35.4	36.5	34.4
	range	17 - 54	19 - 52	17 - 54
Time since diagnosis, yrs				
	median	5.0	6.8	2.8
	range	0 - 34	0 - 15	0 - 34
Serum creatinine, µmoles/liter				
	median	94.5	101.5	86.0
	range	64 - 236	69 - 175	64 - 236
Proteinuria, g/24 h				
	median	4.3	4.7	3.7
	range	0.5 - 8.9	0.5 - 7.9	1.0 - 8.9
Anti-dsDNA antibodies positive/total (%)		24/26 (92)	11/12 (92)	13/14 (93)
Complement C3, g/liter				
	median	0.42	0.44	0.4
	range	0.20 - 1.12	0.32 - 1.03	0.20 - 1.12
Complement C4, g/liter				
	median	0.1	0.11	0,1
	range	0.04 - 0.26	0.04 - 0.26	0.06 - 0.21
SLE disease activity index				
	median	17	14	17
	range	6 - 38	8 - 26	6 - 38

There were no statistically significant differences; to convert values of creatinine to milligrams per deciliter, divide by 88.4

Of the 26 women, 13 (50%) reported at least one pregnancy. Three of these 13 women had no live births, but only spontaneous abortions of which the gender of the fetus was unknown. Of the 10 women with live birth children, 7 had at least one son and 3 had only daughters. Reproductive data and results for chimerism are given in Table 2. Of the 10 women with children and SLE, 7 had their first pregnancy before SLE was diagnosed. One was pregnant at diagnosis. Two women had their first child after SLE was diagnosed.

Table 2. Reproductive data and results of women with lupus nephritis at biopsy

	Total group	Chimerism present	Chimerism absent
Number of women	26	12	14
Pregnancy before biopsy			
yes	13	5	8
No	13	7	6
Number of live birth children			
0	16	8	8
1	3	0	3
2	4	3	1
3	3	1	2
Abortions			
yes	4*	2	2
No	22	10	12
Time since last pregnancy			
Mean (yrs)	12.1	16.4	8.7
Range (yrs)	0.2 - 21.5	5.8 - 21.5	0.2 - 18.6
Time since first pregnancy			
Mean (yrs)	14.9	20.4	10.5
Range (yrs)	0.2 - 27.3	8.3 - 27.3	0.2 - 24.1
Age at first pregnancy			
Mean (yrs)	24.0	22.5	25.2
Range (yrs)	20-29	20-24	21-29
Menses before study			
Regular with oral contraceptive use	11	4	7
Regular without oral contraceptive use	4	1	3
Irregular	2	2	0
No menses	8	5	3
Unknown	1	0	1
Problems getting pregnant			
Yes	1	0	1
No	22	11	11
Unknown	3	1	2

* three women reported only spontaneous abortions, no live births; one woman reported one spontaneous abortion and has two daughters; there were no significant differences

There were three women without children who had miscarriages of which one occurred before SLE was diagnosed and of the other two it is unknown. In the biopsies of the 13 women that reported a pregnancy, Y chromosome-positive cells were present in 5 (38%). Of the 13 women that did not report a pregnancy, 7 (54%) showed Y chromosome-positive cells in their biopsies (Figure 1). In one of the 3 women who experienced only spontaneous abortions and had no live birth children, chimerism was present. One woman experienced a spontaneous abortion once and also had two live birth daughters, and chimerism was found in her renal biopsy. There was no significant difference between the occurrence of chimerism in the biopsies of women who had been pregnant compared with women who had not been pregnant ($p = 0.43$). However, when focussing on the number of pregnancies experienced by the women who had reported a pregnancy, chimeric women who had been pregnant reported significantly more pregnancies (median 2, mean 2.4, range 2-3) than non-chimeric women (median 1, mean 1.5, range 1-3, $p=0.04$). In the logistic regression analysis number of pregnancies did not significantly predict for chimerism (OR= 6.70, p= 0.08), but a significant correlation was found (correlation coefficient = 0.59, $p=0.04$). A relationship between the presence of Y chromosome-positive cells and gender of the children was not observed (Figure 2.).

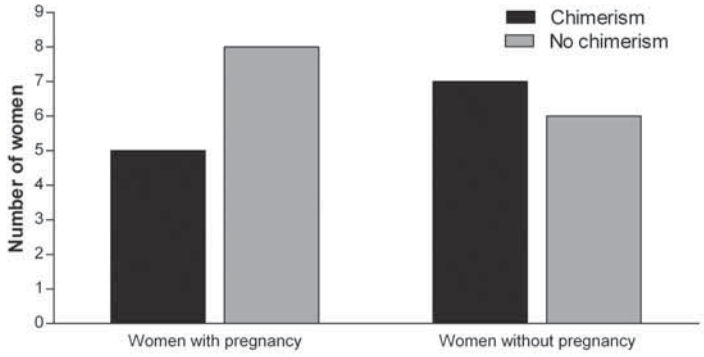

Figure 1. The presence of chimerism in women with lupus nephritis who had or did not have at least one pregnancy. No significant differences were found between the groups with chimerism present (black bars) and chimerism absent (grey bars).

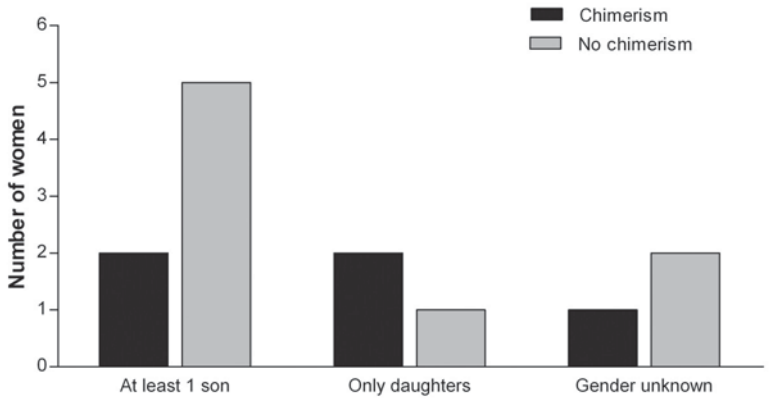

Figure 2. The presence of chimerism in parous women with lupus nephritis related to the gender of their children. Of three women that experienced only spontaneous abortions, the gender of the lost fetus was unknown. No significant differences were found between the groups with chimerism present (black bars) and chimerism absent (grey bars).

The median age of the youngest child was higher in chimeric women (16 years) than in non-chimeric women (9 years), although this difference was not statistically significant, possibly due to the small sizes of both groups. A similar tendency was observed between the age of the oldest child and the presence of chimerism (median age of the oldest child of chimeric women was 23 years and of non-chimeric women was 9 years). We did not observe a significant relationship between other reproductive data such as the number of miscarriages, duration of pregnancy, use of oral contraceptives, or nature of cycles and the presence of chimerism. Also there was no relationship between parity of the patient and the number of chimeric cells. In none of the women who became pregnant after SLE was diagnosed, chimerism was found.

Discussion

There are experimental data suggesting that chimeric cells may play a role in the development of SLE, and that these cells are derived mainly from pregnancies. Our group previously demonstrated that chimerism is present more often in kidney specimens from women with SLE compared with healthy control kidneys.[8] In the present study, we

investigated the relationship between pregnancy history and the presence of chimerism in the kidneys of women with proliferative lupus nephritis.

We found that chimeric Y chromosome-positive cells were present in 46% of the kidneys of women with proliferative lupus nephritis. This finding is comparable to our previous results in kidneys from women with SLE in a different patient group, in which chimerism was found in 51% of the kidneys of women with SLE compared with only 25% of healthy control kidneys.[8]

We did not observe a clear-cut relationship between the number of live birth boys and the presence of Y chromosome-positive chimeric cells in renal biopsy specimens with lupus nephritis, for which there are several possible reasons. First, not every former pregnancy may lead to the persistence of chimeric cells, and if foetal-derived cells do persist, their number may differ per pregnancy. Ariga et al.[12] demonstrated that at delivery fetal-derived cells are present in all mothers, but one month after delivery only 10% of women still had fetal-derived cells present in their blood. This finding may indicate that only particular subsets of fetal-derived chimeric cells can survive for longer periods of time and therefore a linear relationship between pregnancy and chimerism would not be expected. This phenomenon has previously been described by Johnson et al.[19] who demonstrated that the chimeric cells found in the liver of a woman who had 5 known pregnancies were derived from one fetus. In a mouse study, Khosrotehrani et al.[20] investigated the number of foetal-derived chimeric cells in several tissues during allogenic pregnancies and congenic pregnancies. They found that the median number of foetal chimeric cells was lower in allogenic matings compared with congenic matings, possibly indicating that foeto-maternal histocompatibility influences the persistence of chimeric cells. Our finding that there is a tendency towards a higher occurrence of chimeric cells in parous mothers who experienced more pregnancies also supports this theory, since they have a higher chance of having at least one child that meets the criteria for tolerance.

Our data may also indicate that the assumption that pregnancy is the main source of chimerism is incorrect. An important indicator that there may be other sources of Y

chromosome-positive chimeric cells besides pregnancy is that, in this study, two women with only daughters were found to be positive for Y chromosome-positive cells. In the literature, other studies also observed that Y chromosome-positive cells can be present in women without sons [6,14,21]. For example, Gannagé et al.[6] previously found male cells in the blood of 15 women with SLE without sons. Although it cannot be excluded that these chimeric cells are derived from unrecognised pregnancies (which we will discuss later), other options should be considered as well. Yan et al.[14] investigated the presence of Y chromosome-positive chimeric cells in different groups of women with rheumatoid arthritis without sons, and found that 10% of 48 nulligravid women had Y chromosome-positive cells in their blood. They concluded that there must be other sources of Y chromosome-positive cells besides known pregnancies and miscarriages, including cells from an older brother that were transferred by the maternal circulation, or sexual intercourse. Other than these, sources of chimerism that have been described in the literature are bone marrow or stem cell transplantations, blood transfusions, and twinning.[22-24] In the present study, no patient had a bone marrow or stem cell transplantation. We did not have information regarding blood transfusions for the women in this study, but we knew from a previous study that a clear-cut relationship between blood transfusions and the presence of chimerism is absent.[8,17] Twinning may play a role as a source for chimerism in the present study, since we do not know whether our patients were (originally) part of a twin. However, the incidence of twins is quite low (17.6/1000 twin deliveries in 2006 in the Netherlands[25]), making it unlikely that most of the chimerism found in the women with SLE comes from twinning. Sexual intercourse as a source of Y chromosome-positive cells in women with SLE is another interesting option. Unfortunately, investigations addressing this subject are simply lacking.

This study has several limitations. Firstly, sampling error, i.e. the underestimation of chimeric cells actually present, may have influenced the data. Secondly, relationships between the presence of chimerism and certain (reproductive factors) may exist that are not recognised in the present study due to the small number of patients in the several subgroups involved. For instance, there is a tendency for chimeric parous women to have a longer period of time between their last baby and their biopsy than non-chimeric parous women. This finding is interesting in the light of a publication

by Abbud Filho et al.[5] in which it was found that in the peripheral blood of women with SLE the number of male DNA copies increased with the increasing age of the youngest son. These observations could indicate that, once they are tolerated by the maternal immune system, chimeric cells can proliferate over time. Thirdly, although a detailed reproductive history of the women with SLE was available in this study, the number of reported pregnancies is always an underestimation of the real number because pregnancies can occur unnoticed. Macklon et al.[26] discussed the so-called 'Pregnancy Loss Iceberg' showing that 60% of pregnancies are lost before they are clinically noticed, of which 50% have reached the implantation phase. From studies investigating the clinical usefulness of detecting chimerism for prenatal screening, it is known that foetal DNA can be detected in the mother as early as 4 weeks and 5 days of gestation and foetal cells as early as 6 weeks of gestation during normal pregnancy.[27,28] Miscarriages and induced abortions have been described to lead to an increased number of chimeric cells in the mother compared with normal pregnancies of the same gestational age.[14,29] Therefore, it is plausible that clinically unrecognised pregnancies could lead to chimerism as well. Also, in the study by Yan et al. [14] that we mentioned earlier, unrecognised spontaneous abortions and vanished male twins have been proposed as possible sources of Y chromosome-positive cells. It was impossible to take this into account in the design of the present study.

We investigated the presence of chimerism in kidney biopsies of women with lupus nephritis and searched for a relationship between the presence of chimerism and pregnancy history. We hypothesised that, since the main source of chimerism is assumed to be pregnancy, a clear-cut relationship between pregnancy and the presence of chimerism would be present. However, we could not deduce a relationship between pregnancy history and the presence of chimerism in this study. This finding has several interesting implications. Factors influencing the persistence of chimerism after pregnancy may result in a predilection for certain chimeric cells to persist long term, resulting in a non-linear relationship between pregnancy and chimerism. Another possibility is that sources of chimerism other than completed pregnancies are more important than was initially anticipated. Our data give rise to intriguing questions about the source of chimerism in women with SLE, and a demand for new hypotheses and investigations.

Acknowledgments

We thank the members of the Dutch Working Party on Systemic Lupus Erythematosus for their cooperation and the patients that participated in the First Dutch Lupus Nephritis Study. The first Dutch Lupus Nephritis Study was supported by grants from the Dutch Kidney Foundation (C02.2023 and C94.1363) and the Dutch Arthritis Association (No.735). Cecile Grootscholten is a recipient of a Netherlands Organisation for Scientific Research Fellowship for Clinical Investigators (NWO No. 920-03-115). We kindly acknowledge the Gratama Foundation for supporting Marije Koopmans and Idske Kremer Hovinga financially.

Reference List

1. Tan EM, Cohen AS, Fries JF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982;25:1271-1277.
2. Sherer Y, Gorstein A, Fritzler MJ, et al. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. *Semin Arthritis Rheum* 2004;34:501-537.
3. Via CS, Shearer GM. T-cell interactions in autoimmunity: insights from a murine model of graft-versus-host disease. *Immunol Today* 1988;9:207-213.
4. Bruijn JA, Van Elven EH, Hogendoorn PC, et al. Murine chronic graft-versus-host disease as a model for lupus nephritis. *Am J Pathol* 1988;130:639-641.
5. Abbud FM, Pavarino-Bertelli EC, Alvarenga MP, et al. Systemic lupus erythematosus and microchimerism in autoimmunity. *Transplant Proc* 2002;34:2951-2952.
6. Gannage M, Amoura Z, Lantz O, et al. Feto-maternal microchimerism in connective tissue diseases. *Eur J Immunol* 2002;32:3405-3413.
7. Johnson KL, McAlindon TE, Mulcahy E, et al. Microchimerism in a female patient with systemic lupus erythematosus. *Arthritis Rheum* 2001;44:2107-2111.
8. Kremer Hovinga IC, Koopmans M, Baelde HJ, et al. Chimerism occurs twice as often in lupus nephritis as in normal kidneys. *Arthritis Rheum* 2006;54:2944-2950.
9. Mosca M, Curcio M, Lapi S, et al. Correlations of Y chromosome microchimerism with disease activity in patients with SLE: analysis of preliminary data. *Ann Rheum Dis* 2003;62:651-654.
10. Cervera R, Abarca-Costalago M, Abramovicz D, et al. Systemic lupus erythematosus in Europe at the change of the millennium: lessons from the "Euro-Lupus Project". *Autoimmun Rev* 2006;5:180-186.
11. Bianchi DW, Zickwolf GK, Weil GJ, et al. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci U S A* 1996;93:705-708.
12. Ariga H, Ohto H, Busch MP, et al. Kinetics of fetal cellular and cell-free DNA in the maternal circulation during and after pregnancy: implications for noninvasive prenatal diagnosis. *Transfusion* 2001;41:1524-1530.
13. Evans PC, Lambert N, Maloney S, et al. Long-term fetal microchimerism in peripheral blood mononuclear cell subsets in healthy women and women with scleroderma. *Blood* 1999;93:2033-2037.
14. Yan Z, Lambert NC, Guthrie KA, et al. Male microchimerism in women without sons: quantitative assessment and correlation with pregnancy history. *Am J Med* 2005;118:899-906.

15. Grootscholten C, Ligtenberg G, Hagen EC, et al. Azathioprine/methylprednisolone versus cyclophosphamide in proliferative lupus nephritis. A randomized controlled trial. *Kidney Int* 2006;70:732-742.
16. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997;40:1725.
17. Koopmans M, Kremer Hovinga IC, Baelde HJ, et al. Chimerism in kidneys, livers and hearts of normal women: implications for transplantation studies. *Am J Transplant* 2005;5:1495-1502.
18. Lau YF. Detection of Y-specific repeat sequences in normal and variant human chromosomes using in situ hybridization with biotinylated probes. *Cytogenet Cell Genet* 1985;39:184-187.
19. Johnson KL, Samura O, Nelson JL, et al. Significant fetal cell microchimerism in a nontransfused woman with hepatitis C: Evidence of long-term survival and expansion. *Hepatology* 2002;36:1295-1297.
20. Khosrotehrani K, Johnson KL, Guegan S, et al. Natural history of fetal cell microchimerism during and following murine pregnancy. *J Reprod Immunol* 2005;66:1-12.
21. Lambert NC, Pang JM, Yan Z, et al. Male microchimerism in women with systemic sclerosis and healthy women who have never given birth to a son. *Ann Rheum Dis* 2005;64:845-848.
22. Lee TH, Paglieroni T, Ohto H, et al. Survival of donor leukocyte subpopulations in immunocompetent transfusion recipients: frequent long-term microchimerism in severe trauma patients. *Blood* 1999;93:3127-3139.
23. van Dijk BA, Boomsma DI, de Man AJ. Blood group chimerism in human multiple births is not rare. *Am J Med Genet* 1996;61:264-268.
24. Woywodt A, Schroeder M, Gwinner W, et al. Elevated numbers of circulating endothelial cells in renal transplant recipients. *Transplantation* 2003;76:1-4.
25. Central Bureau of Statistics of The Netherlands. Births according to diverse characteristics. Available at: http://statline.cbs.nl/StatWeb/table.asp?PA=37422ned&D1=28-32&D2=(l-11)-l&DM=SLNL&LA=nl&TT=2; Voorburg/Heerlen. 25-5-2007.
26. Macklon NS, Geraedts JP, Fauser BC. Conception to ongoing pregnancy: the 'black box' of early pregnancy loss. *Hum Reprod Update* 2002;8:333-343.
27. Ganshirt D, Smeets FW, Dohr A, et al. Enrichment of fetal nucleated red blood cells from the maternal circulation for prenatal diagnosis: experiences with triple density gradient and MACS based on more than 600 cases. *Fetal Diagn Ther* 1998;13:276-286.
28. Thomas MR, Williamson R, Craft I, et al. Y chromosome sequence DNA amplified from peripheral blood of women in early pregnancy. *Lancet* 1994;343:413-414.

29. Bianchi DW, Farina A, Weber W, et al. Significant fetal-maternal hemorrhage after termination of pregnancy: implications for development of fetal cell microchimerism. *Am J Obstet Gynecol* 2001;184:703-706.