# Male microchimerism in women with systemic sclerosis and healthy women who never gave birth to a son

*Nathalie C Lambert, Jennifer M Pang, Timothy D Erickson, Anne M Stevens, Zhen Yan, Daniel E Furst, and J. Lee Nelson*

# MALE MICROCHIMERISM IN WOMEN WITH SYSTEMIC SCLEROSIS AND HEALTHY WOMEN WHO NEVER GAVE BIRTH TO A SON

Nathalie C. Lambert [a], Jennifer M. Pang [a], Zhen Yan [a], Timothy D. Erickson [a], Anne M. Stevens [a,b], Daniel E. Furst [c] and J. Lee Nelson.[a,d]

[a]Immunogenetics, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA
[b]Pediatrics, Children's Hospital and Regional Medical Center, Seattle, Washington, USA
[c]Rheumatology, University of California, Los Angeles, USA
[d]Rheumatology, University of Washington, Seattle, Washington, USA

**Supported by** the Scleroderma Foundation (Grant # 007/01), NIH grants AI41721 and AR48084.

*Corresponding Author:*
Nathalie Lambert, Ph.D.
INSERM, U639
Faculté de Médecine La Timone
13005 Marseille, France
1 (33) 4 91-32-44-97
nathalie.lambert@medecine.univ-mrs.fr
Key words: microchimerism, pregnancy, systemic sclerosis, Y-chromosome

***Objective.*** Male DNA or cells are often used to measure microchimerism in a woman. In studies of autoimmune diseases male microchimerism is most often attributed to prior birth of a son. The objective of the current studies was to determine the frequency of male microchimerism in women who were healthy or had systemic sclerosis (SSc) who never gave birth to a son.
***Method.*** Real-Time quantitative PCR targeting the Y-chromosome specific sequence DYS14 was employed to test DNA extracted from peripheral blood mononuclear cells of 26 women with SSc and 23 healthy women who never gave birth to a son. Results were expressed as the genome equivalent number of male cells per million host cells (gEq/mil).
***Results.*** Male DNA was found in 15% of women with SSc, range 0 to 23.7 gEq/mil. Thirteen percent of healthy women had male DNA, range 0 to 5.1 gEq/mil. Although two women with male DNA had an induced abortion, most had no history of spontaneous or induced abortion (SSc and healthy).
***Conclusions.*** Microchimerism with male DNA can be found in the circulation of women who no history of birth of a son. These results indicate sources other than a male birth must also be considered when male DNA is used to measure microchimerism. Although other studies are needed, there was no apparent difference in women with SSc and healthy women. Possible sources of the male DNA include from an unrecognized male pregnancy or unrecognized male twin, from an older male sibling transferred via the maternal circulation or from sexual intercourse alone. (252 words)

## INTRODUCTION

During normal human pregnancy some cells traffic between the fetus and mother [1]. Decades later, low levels of exogenous cells, presumed to be fetal in origin, have been described in the maternal circulation and cells presumed to be maternal in origin in her offspring, referred to as fetal and maternal microchimerism (Mc) respectively [2, 3]. Systemic sclerosis (SSc) occurs more often in women than men and has a peak incidence in post-reproductive years. We previously investigated women who were parous (had at least one birth) prior to SSc onset and similar healthy women and found women with SSc who had given birth to a son had higher levels of male DNA than healthy women [4]. However, male DNA in a woman's circulation could potentially derive from sources other than a prior male birth. Persistent Mc from pregnancy can also result from traffic of cells between fraternal twins, initially described in cattle and later confirmed in humans [5]. High levels of male DNA have been described in women undergoing induced abortion [6] and persistent fetal Mc is presumed to occur after induced abortion and miscarriage. Additionally, Mc could potentially derive from an older male sibling since maternal cells pass into the fetal circulation and the mother carries persistent Mc from earlier pregnancies. Because most all studies of fetal Mc employ testing for male DNA as the measure of Mc but the frequency of male Mc in women without a male birth is unknown, we conducted the current study to investigate male DNA in women with no history of a male birth. We employed a quantitative Real Time PCR assay that targets the Y chromosome sequence DYS14 to test DNA extracted from peripheral blood mononuclear cells of women with SSc and healthy women who never gave birth to son.

## PATIENTS AND METHODS

***Study subjects.*** Twenty-six women with SSc and 23 healthy women were studied, all of whom had no history of a male birth. Women with SSc were classified as having diffuse or limited SSc, 73% (n=19) and 27% (n=7) respectively, according to previous descriptions of SSc subset classification [7]. Among the 26 women with SSc, 58% (n=15) were parous and 42% (n=11) were nulliparous. Among the 23 healthy women 52% (n=12) were parous and 48% (n=11) nulliparous. Six of the nulliparous women with SSc were also nulligravid as were 8 of the healthy women. Some women with SSc and healthy women had a history of spontaneous abortion and a few a history of induced abortion as indicated in Tables 1 and 2.

Study subjects derived primarily from Seattle, Washington, USA and surrounding areas. Eighty-five percent of women with SSc were Caucasian, 4% Asian and 11% of mixed ethnicity. Ninety-five percent of healthy women were Caucasian, 0% Asian, and 4% of mixed ethnicity. Among women with SSc, 24 had no history of blood transfusion (an alternative source of Mc) and for two transfusion history was unknown. Eighteen healthy women never received a blood transfusion, transfusion history was unknown for 4 and one had received a blood transfusion. One healthy woman had a male twin (HW-04). No woman with SSc had a twin. All subjects provided informed consent.

***Isolation of PBMC and DNA extraction.*** Whole blood was drawn into Acid Citrate Dextrose solution A vacutainer tubes and processed by Ficoll Hypaque centrifugation to isolate PBMC (Pharmacia Biotech AB, Uppsula, Sweden). DNA was extracted from PBMC with a Promega Wizard Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and resuspended in water.

***DYS14 Real-Time quantitative PCR.*** A Real-Time quantitative PCR (QPCR) assay that we previously developed and reported was employed that targets the Y-chromosome specific sequence DYS14 [8]. Each DNA sample was tested in 6 to 12

aliquots of 5,000 to 20,000 genome equivalent cells for DYS14 with two similar aliquots tested for *β-globin* to define the total DNA concentration of the sample tested. The conversion factor used was 6.6 pg of DNA per cell. Information was combined across aliquots of PBMC as previously described [8]. For ease of expression the amount of male DNA was expressed as the number of genome equivalent male cells per million maternal cells (gEq/mil). As a conservative estimate of the frequency of male DNA we further required that a sample exhibit a result above threshold in a minimum of two different wells per assay, or be confirmed in a second test.

**Specificity of the DYS14 assay.** As previously reported, the DYS14 sequence was found not to cross-react with any other DNA sequence [8]. In addition, in the current studies, each experimental Q-PCR plate included the equivalent of 10,000 human nulligravid female DNA systematically used as background with increased concentrations of 0, 1, 5 10, 50, 100, 500 equivalent DNA of male cells. Therefore in each plate a triplicate of 0 male DNA in 10,000 female DNA was tested for male DNA amplification with DYS14 PCR assay and was consistently negative. This provided an internal control indicating the DYS14 Q-PCR assay does not cross-react with other DNA sequences.

***Precautions to minimize contamination risk.*** False positive results are always a concern with PCR based methods, and risk of contamination may be of special concern for assays that target male DNA. Multiple aspects of methods used in the current study were designed to minimize any potential contamination risk. The optical detection system of the 7000 Sequence Detector obviates the need to reopen reaction tubes after amplification. A female technician conducted all experiments including DNA extraction and the Q-PCR assays. DNA extractions and Q-PCR preparations were done under a UV equipped safety hood, with UV run 30 minutes between experiments to avoid persistence of any remaining DNA. Filtered tips were used in all pipetting. Additionally, each experiment included controls to monitor for possible contamination and negative controls were always negative. In cases where samples were tested more than once samples negative in a first experiment were also negative in a second test .

## RESULTS

Microchimerism with male DNA in peripheral blood mononuclear cells was found in some women with SSc and healthy women with no history of prior birth of a son. Among women with SSc 15% (4/26) had male DNA and among healthy women 13% (3/23) [Tables 1 and 2]. Fifty-five percent of all women were parous but had only given birth to daughters, representing 58% of women with SSc (n=15) and 52% of healthy women (n=12). Among parous women with SSc two (8%) had male DNA and among healthy women none; one woman with SSc had no other pregnancies and the other an induced abortion. Forty-five percent of all women were nulliparous, representing 42% of women with SSc (n=11) and 48% of healthy women (n=11). Among nulliparous women with SSc, 2 had male DNA (18%) and among healthy women 3 (27%), with all but one healthy women (who had an induced abortion) also nulligravid. Positive results ranged from 0.4 to 23.7 gEq/mil in women with SSc and 0.7 to 5.1 gEq/mil in healthy women. None of the women with positive results had a history of blood transfusion or a male twin.

For women who had a pregnancy, the time since last pregnancy was somewhat greater among women with SSc than healthy women (mean 18.6, median 14.0 vs. mean 14.5, median 11.5); the number of daughters was similar (mean 1.4 and 1.5 respectively). More women with SSc than healthy women reported a history of spontaneous abortion (44% vs. 14%). Women with SSc were somewhat older when tested than healthy women (mean 47.6, median 49.5 vs. mean 42.6, median 39.0).

To further confirm positive results we conducted additional tests from the same woman from different aliquots of DNA (same blood draw) and from different blood draws. Some women without a history of spontaneous or induced abortion had positive results in multiple wells, from different blood draws (SSc-10, SSc-16, and HW-10) and from additional assays conducted on other dates (SSc-08) (data not shown) confirming results in Tables 1 and 2. The median DNA equivalent total number of cells tested was similar between women with SSc (median: 129,358 gEq) and healthy women (128,430 gEq).

In the overall study population male DNA was sometimes found in women who had given birth to daughters but had no history of prior pregnancy, women who had never given birth but had a prior induced abortion, and also among women with no history of any prior pregnancy. Although we did not find male DNA in any woman who only had a history of prior spontaneous abortion in our study, testing for male DNA would not detect microchimerism from a female fetus. We were able to conduct a quantitative PCR test for a non-shared HLA polymorphism of the husband to test one woman with SSc who had five spontaneous abortions using recently described methods [9]. High levels of microchimerism with the HLA sequence of the husband were found, supporting the likelihood of persistent fetal microchimerism from prior spontaneous abortion in this case (data not shown).

**DISCUSSION**

Recent studies have implicated persistent fetal Mc in parous women with SSc [10]. Most studies investigating fetal Mc in autoimmune diseases have used testing for male DNA (or cells) in women as the measure of persistent fetal Mc, usually testing women known to have previously given birth to a son. This approach is often taken, not because of a proposed biological difference in fetal Mc from sons compared to daughters, but rather for the technical reason that a single assay can be used to test many women. Prior studies, however, have not specifically examined the potential for sources other than a prior male birth to lead to persistent male DNA in a woman. Therefore, in the current study we asked whether male DNA can be found in peripheral blood mononuclear cells of women who had no history of a male birth. We employed a previously developed Real-Time quantitative PCR assay for a Y-chromosome sequence to test women with SSc and healthy women with no prior birth of a son [8].

We found that male DNA was not uncommon in the circulation of women who had no prior history of a son. Male DNA was found in 14% of women overall, without a significant difference in women with SSc and healthy women. The lack of a difference in SSc and healthy women does not argue against prior reports implicating fetal Mc in SSc. Rather the current results indicate that any portion of positive results attributable to male DNA originating from other than a prior male birth would be expected to be similar in SSc and healthy women. It does indicate, however, that the term "exogenous" may be more appropriate than "fetal" when Mc is assessed by testing for male DNA, and that the specific origin of all sources of male DNA in women requires further investigation.

There are at least four potential explanations for male DNA in a woman with no history of a male birth. As a first possibility, male DNA could originate from a pregnancy that was not carried to term. It has been assumed that persistent fetal Mc can result from a spontaneous or induced abortion. High levels of male DNA have been reported in women undergoing elective pregnancy termination [6]. Consistent with this observation, in the current study we found male DNA in two of three women who had a history of induced abortion. A prior spontaneous abortion with a male fetus that was unrecognized could result in male DNA. Disproportionately greater loss of males in *utero* has been proposed as an explanation of the excess male/female ratio at fertilization compared to that at birth [11], however, it is unclear whether loss would occur sufficiently

advanced into pregnancy that fetal cells would have reached the maternal circulation. In a recent review of fetal Mc studies, fetal loss was suggested as potentially important in persistent fetal Mc [12]. We did not find Mc with male DNA in any of the 14 women reporting a prior spontaneous abortion in our study. However, it is reasonable to presume that persistent fetal Mc can occur after a spontaneous abortion and testing for male DNA would not detect fetal microchimerism from a female fetus. Lending support to this possibility, one woman in the current studies who tested negatively for male DNA and who had multiple spontaneous abortions did have microchimerism when tested by targeting a non-shared HLA-specific sequence of her husband.

Although one woman who had a male twin had a negative result, a second possibility that could apply to other women, is that cells from a male twin transferred to a female fetus *in utero* and the male twin was lost early in gestation. A number of studies have described loss of an unrecognized "vanished" twin as not uncommon in healthy pregnancies [13]. Because women harbor persistent fetal Mc, and because maternal cells are known to reach the fetal circulation, a third possibility is transfer of cells from an older brother via the maternal circulation *in utero*. Three women with SSc with positive results with no other history of spontaneous or induced abortion had an older brother. A fourth possibility is that Mc with male DNA might sometimes occur from sexual intercourse alone. Further studies with information concerning sexual history (including type of birth control method etc.) are needed to confirm this last possibility.

Our finding of male DNA in women without sons is consistent with another report that used a kinetic quantitative ELISA to measure male DNA and found positive results in 33% (5/15) of women with SSc and 23% (19/83) of women with other connective tissue diseases [14], although male DNA was not reported among healthy women without sons in this study (0%, 0/8). That results differed for healthy women could be due to small numbers of study subjects, differences in assays, study populations, medication use, or differences in the frequency of prior pregnancy with females since fetal Mc from a female fetus is undetected in assays that test for male DNA.

In conclusion, male Mc was not uncommon in the peripheral blood of women who never gave birth to a son. Results were not significantly different in women with SSc and healthy women. This observation invites further scrutiny as to the origins of male DNA in women without sons. Our results also highlight the need for additional methods to study Mc beyond those measuring male DNA, for example quantitative assays targeting HLA or other genetic polymorphisms [9].

**AKNOWLEDGMENTS**

We would like to thank the Scleroderma Registry and Jennifer Brackensick for their contribution to this study.

## REFERENCES


1.Lo YM, Lo ES, Watson N, Noakes L, Sargent IL, Thilaganathan B, et al. Two-way cell traffic between mother and fetus: biologic and clinical implications. Blood 1996;88(11):4390-5.

2.Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc Natl Acad Sci U S A 1996;93(2):705-8.

3.Maloney S, Smith A, Furst DE, Myerson D, Rupert K, Evans PC, et al. Microchimerism of maternal origin persists into adult life. J Clin Invest 1999;104(1):41-7.

4.Nelson JL, Furst DE, Maloney S, Gooley T, Evans PC, Smith A, et al. Microchimerism and HLA-compatible relationships of pregnancy in scleroderma. Lancet 1998;351(9102):559-62.

5.De Moor G, De Bock G, Noens L, De Bie S. A new case of human chimerism detected after pregnancy: 46,XY karyotype in the lymphocytes of a woman. Acta Clin Belg 1988;43(3):231-5.

6.Bianchi DW, Farina A, Weber W, Delli-Bovi LC, Deriso M, Williams JM, et al. Significant fetal-maternal hemorrhage after termination of pregnancy: implications for development of fetal cell microchimerism. Am J Obstet Gynecol 2001;184(4):703-6.

7.LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr., et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988;15(2):202-5.

8.Lambert NC, Lo YM, Erickson TD, Tylee TS, Guthrie KA, Furst DE, et al. Male microchimerism in healthy women and women with scleroderma: cells or circulating DNA? A quantitative answer. Blood 2002;100(8):2845-51.

9.Lambert NC, Erickson TD, Yan Z, Pang JM, Guthrie KA, Furst DE, et al. Quantification of maternal microchimerism by HLA-specific real-time polymerase chain reaction: studies of healthy women and women with scleroderma. Arthritis Rheum 2004;50(3):906-14.

10.Lambert N, Nelson JL. Microchimerism in autoimmune disease: more questions than answers? Autoimmun Rev 2003;2(3):133-9.

11.Diamond JM. Causes of death before birth. Nature 1987;329(6139):487-8.

12.Khosrotehrani K, Johnson KL, Lau J, Dupuy A, Cha DH, Bianchi DW. The influence of fetal loss on the presence of fetal cell microchimerism: a systematic review. Arthritis Rheum 2003;48(11):3237-41.

13.Robinson HP, Caines JS. Sonar evidence of early pregnancy failure in patients with twin conceptions. Br J Obstet Gynaecol 1977;84(1):22-5.

14.Gannage M, Amoura Z, Lantz O, Piette JC, Caillat-Zucman S. Feto-maternal microchimerism in connective tissue diseases. Eur J Immunol 2002;32(12):3405-13.

**Table 1. Male DNA in women with SSc who never gave birth to a son.**

| ID | Age | Disease | Age at disease onset | # of daughters | # of fetal losses IB[1] | SAB[2] | Years from pregnancy[6] | Mc / million |
|---|---|---|---|---|---|---|---|---|
| **SSc 01** | 52 | DIFF[3] | 50 | 2 | 0 | 1 | 28.3 | 0 |
| **SSc 02** | 46 | DIFF | 44 | 2 | 0 | 0 | 13.4 | 0 |
| **SSc 03** | 49 | DIFF | 43 | 1 | uk[5] | uk | 26.0 | 0 |
| **SSc 04** | 50 | DIFF | 39 | 1 | 0 | 1 | 10.2 | 0 |
| **SSc 05** | 37 | LIM[4] | 26 | 1 | 0 | 2 | 14.0 | 0 |
| **SSc 06** | 31 | DIFF | 28 | 1 | 0 | 1 | 1.2 | 0 |
| **SSc 07** | 40 | DIFF | 34 | 1 | 1 | 0 | 8.9 | **3.9** |
| **SSc 08** | 19 | DIFF | 19 | 0 | 0 | 0 | na[7] | **23.7** |
| **SSc 09** | 24 | DIFF | 7 | 0 | 0 | 0 | na | 0 |
| **SSc 10** | 34 | DIFF | 33 | 0 | 0 | 0 | na | **2.0** |
| **SSc 11** | 36 | DIFF | 29 | 0 | 0 | 1 | 7.4 | 0 |
| **SSc 12** | 70 | DIFF | 45 | 2 | 0 | 1 | 40.9 | 0 |
| **SSc 13** | 40 | DIFF | 36 | 1 | 0 | 1 | 3.0 | 0 |
| **SSc 14** | 51 | LIM | 49 | 0 | 0 | 1 | 31.0 | 0 |
| **SSc 15** | 47 | LIM | 38 | 0 | 0 | 0 | na | 0 |
| **SSc 16** | 47 | DIFF | 22 | 1 | 0 | 0 | 17.8 | **0.4** |
| **SSc 17** | 58 | LIM | 54 | 1 | 0 | 0 | 33.9 | 0 |
| **SSc 18** | 40 | DIFF | 38 | 2 | 0 | 0 | 14.0 | 0 |
| **SSc 19** | 51 | LIM | 44 | 1 | 0 | 2 | 8.7 | 0 |
| **SSc 20** | 49 | DIFF | 43 | 3 | 0 | 0 | 12.3 | 0 |
| **SSc 21** | 22 | DIFF | 20 | 0 | 0 | 0 | na | 0 |
| **SSc 22** | 51 | LIM | 49 | 0 | 1 | 0 | 31.0 | 0 |
| **SSc 23** | 46 | DIFF | 42 | 0 | 0 | 5 | uk | 0 |
| **SSc 24** | 53 | DIFF | 50 | 1 | 0 | 0 | 34.0 | 0 |
| **SSc 25** | 53 | LIM | 45 | 0 | 0 | 3 | 18.0 | 0 |
| **SSc 26** | 37 | DIFF | 35 | 0 | 0 | 0 | na | 0 |

(1) induced abortion, (2) spontaneous abortion, (3) diffuse SSc, (4) limited SSc, (5) uk = unknown, (6) years from outcome of most recent pregnancy, (7) na = not applicable

**Table 2. Male DNA in healthy women who never gave birth to a son**

| ID | Age | # of daughters | # of fetal losses IB[1] | SAB[2] | Years from pregnancy[5] | Mc / million |
|---|---|---|---|---|---|---|
| **HW-01** | 28 | 0 | 0 | 0 | na[6] | 0 |
| **HW-02** | 29 | 1 | 0 | 0 | 1.0 | 0 |
| **HW-03** | 38 | 1 | 0 | 0 | 18.3 | 0 |
| **HW-04**[3] | 41 | 2 | 0 | 1 | 2.5 | 0 |
| **HW-05** | 26 | 0 | 0 | 0 | na | 0 |
| **HW-06** | 35 | 1 | 0 | 0 | 2.5 | 0 |
| **HW-07** | 29 | 0 | 0 | 0 | na | **0.7** |
| **HW-08** | 50 | 1 | 0 | 0 | 30.2 | 0 |
| **HW-09** | 39 | 2 | 0 | 0 | 4.0 | 0 |
| **HW-10** | 30 | 0 | 0 | 0 | na | **5.1** |
| **HW-11** | 17 | 0 | uk[4] | uk | na | 0 |
| **HW-12** | 16 | 0 | uk | uk | na | 0 |
| **HW-13** | 22 | 0 | 0 | 0 | na | 0 |
| **HW-14** | 38 | 1 | 0 | 0 | 5.8 | 0 |
| **HW-15** | 32 | 2 | 0 | 0 | 11.5 | 0 |
| **HW-16** | 31 | 0 | 0 | 0 | na | 0 |
| **HW-17** | 62 | 2 | 0 | 0 | 39.1 | 0 |
| **HW-18** | 53 | 0 | 0 | 0 | na | 0 |
| **HW-19** | 59 | 1 | 0 | 0 | 30.2 | 0 |
| **HW-20** | 46 | 2 | 0 | 1 | 14.2 | 0 |
| **HW-21** | 37 | 0 | 0 | 0 | na | 0 |
| **HW-22** | 47 | 0 | 1 | 0 | uk | **1.8** |
| **HW-23** | 58 | 2 | 0 | 1 | uk | 0 |

(1) Induced abortion, (2) spontaneous abortion, (3) subject with twin brother, (4) uk = unknown, (5) years from outcome of most recent pregnancy, (6) na = not applicable.