

## RESEARCH ARTICLE

# Microchimerism in recurrent miscarriage

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Maternal–fetal cell exchange during pregnancy results in acquisition of microchimerism, which can durably persist in both recipients. Naturally acquired microchimerism may impact maternal–fetal interaction in pregnancy. We conducted studies to ask whether microchimerism that a woman acquired from her own mother is detectable before or during pregnancy in women with recurrent miscarriage. Fetal microchimerism was also assayed. Women with primary idiopathic recurrent miscarriage ( $n=23$ ) and controls ( $n=31$ ) were studied. Genotyping was conducted for probands, their mothers and the fetus, a non-shared polymorphism identified and quantitative polymerase chain reaction performed to measure microchimerism in peripheral blood mononuclear cells. Preconception comparisons were made between recurrent miscarriage subjects and controls, using logistic regression and Wilcoxon rank sum. Longitudinal microchimerism in subsequent pregnancies of recurrent miscarriage subjects was described. There was a trend toward lower preconception detection of microchimerism in recurrent miscarriage *versus* controls, 6% *vs.* 19% (1/16 *vs.* 6/31,  $P=0.2$ ). During pregnancy, 3/11 (27%) of recurrent miscarriage subjects who went on to have a birth had detection of microchimerism from their own mother, whereas neither of two subjects who went on to miscarry had detection (0/2). This initial data suggest that microchimerism from a woman's own mother, while detectable in women with recurrent miscarriage, may differ from controls and according to subsequent pregnancy outcome. Further studies are needed to determine the cell types, quantities and any potential functional role of microchimerism in recurrent miscarriage.

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## INTRODUCTION

Of all clinically recognized pregnancies, 15%–20% end in miscarriage, making it the most common pregnancy complication.<sup>1–3</sup> Including preclinical pregnancies, estimated human pregnancy loss rates reach as high as 50%–70%.<sup>1,2,4</sup> Recurrent miscarriage (RM), defined as 3 or more consecutive pregnancy losses, affects approximately 1%–5% of couples.<sup>5</sup> Factors that may influence RM risk include genetic, anatomic, endocrinological, thrombophilic and environmental contributors. Genetic causes are most common, and as genomic technology advances, will likely account for an increasing proportion of RM cases. Currently, however, etiology remains undetermined in about 50% of cases.<sup>5,6</sup> Studies suggest that immune dysfunction underlies a proportion of RM.<sup>7,8</sup> This is evidenced by increased risk in women with some autoimmune diseases, including antiphospholipid syndrome.<sup>9</sup> In addition, maternal–paternal immunogenetic relationships appear to influence

pregnancy outcomes,<sup>10,11</sup> with a beneficial effect of disparity in human leukocyte antigen (HLA) genes in successful pregnancies, and a number of studies showing that excess HLA-sharing in a couple may be a risk factor for RM.<sup>12–15</sup> With this in mind, and extrapolating from the transplant literature in which some data suggest benefit of pre-transplant transfusion,<sup>16</sup> various immunotherapies have been considered for RM, aiming to modulate maternal recognition of the pregnancy. These have included paternal mononuclear cell infusion, third-party mononuclear cell infusion and administration of intravenous immune globulin. Though none of these approaches have shown benefit overall,<sup>17–19</sup> some investigators are still attempting to identify a target, approach or clinical subgroup that may benefit from immunomodulatory therapy.<sup>20,21</sup>

In normal pregnancy, one of the ways in which maternal–fetal interaction occurs is through the exchange of cells. Maternal–fetal exchange results in microchimerism, defined

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as small amounts of foreign cells or DNA detectable in a genetically distinct individual. Microchimerism originally acquired in pregnancy can durably persist for decades, is associated with later-life health benefits and risks, and can be considered in a multigenerational context.<sup>22</sup> An adult woman (or 'proband') acquired microchimerism from her own mother ('mother of the proband', or MP) when she herself was a fetus. This 'graft', acquired during fetal immune system development, contributes to aspects of immune tolerance,<sup>23</sup> can remain in her system into adulthood and represents a preexisting inhabitant as she experiences pregnancy herself.<sup>24</sup> During subsequent pregnancies, new fetal sources of microchimerism are acquired. In the current study, we asked whether MP microchimerism is detectable in women with RM. Additionally, because interaction of a new 'graft' of fetal microchimerism with the preexisting MP microchimerism has been suggested,<sup>25</sup> we also explored whether women with RM who become pregnant have MP microchimerism during pregnancy and whether there is any correlation with pregnancy outcome.

We compared detection rates of MP microchimerism prior to conception in probands with a history of unexplained RM to healthy women with no reproductive complications. In addition, we evaluated MP microchimerism in women with unexplained RM who had subsequent pregnancies which were successful or ended in demise. Fetal microchimerism was also measured in some pregnancies.

## MATERIALS AND METHODS

### Study population

We conducted a prospective cohort study of women with idiopathic primary RM, defined as three or more documented consecutive unexplained miscarriages of less than 20 weeks gestation, of which one had an informative euploid karyotype result (46,XX or 46,XY). Primary was defined as no prior ongoing pregnancy of at least 20 weeks' gestation. All of the pregnancies had to be conceived with the same reproductive partner. Idiopathic was defined as a negative comprehensive clinical evaluation.<sup>26,27</sup> Exclusion criteria included the woman being a twin, had received a prior blood transfusion, had a history of autoimmune disease, prior elective pregnancy termination and inability or refusal to give written informed consent.

RM subjects who met all the preceding criteria were enrolled following clinical evaluation in the University of Chicago Recurrent Pregnancy Loss Program (Chicago, IL, USA) affirming idiopathic RM. All study subjects (probands) who became pregnant were then also followed longitudinally throughout subsequent pregnancies during the study period (2007–2011). One of the authors (MDS) was the clinician for all subjects. Comprehensive acquisition and abstracting of demographic information, partner-specific and maternal age-specific past pregnancies, medical, surgical, social and family history information, was performed. The data were thoroughly checked by the investigators with any omissions or discrepancies resolved. For subsequent pregnancies in RM subjects, gestational age was determined by the last menstrual period and adjusted by a first or second trimester ultrasound.

Control participants without RM were derived from a population of healthy, reproductive age women followed longitudinally at regional hospitals and medical centers in Seattle, WA, USA between 1991 and 2011. Medical records and a self-reported questionnaire were collected and reviewed for clinical and demographic information. Information obtained included age, ethnicity, medical history, reproductive history and maternal and fetal outcomes. Women with pregnancy complications or a history of transfusion were excluded.

### Sample collection

For probands with RM, peripheral blood samples were drawn prior to the proband's next pregnancy, and if she became pregnant again, at 5 and 10 weeks of gestation and/or at the time of pregnancy demise, second trimester (24–26 weeks), third trimester (36–38 weeks), as well as 6 weeks and 6 months after pregnancy loss or delivery. For MP microchimerism studies, the proband's mother was asked to provide a buccal swab sample. The proband's partner was also asked to provide a buccal swab and cord blood and miscarriage tissue were collected when possible.

Control participants underwent peripheral blood draws prior to conception, each trimester and postpartum. Samples drawn prior to conception, or between pregnancies and outside of the postpartum window, were included in this analysis.

For RM subjects who had subsequent miscarriage(s), chromosome testing was offered with conventional cytogenetic analysis and/or comparative genomic hybridization, and excess tissue was used for research. For RM subjects with subsequent delivery(ies), fetal cord blood or a segment of cord tissue was obtained immediately for research use. For both RM subjects and controls, all maternal and cord blood samples were drawn into acid citrate dextrose solution A-vacutainer tubes. Maternal peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll Histopaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation at a density of 1.077 g/ml, cryopreserved in dimethylsulfoxide and stored in liquid nitrogen. Genomic DNA was extracted from PBMCs or whole blood using Wizard Genomic DNA Purification Kits (Promega, Madison, WI, USA) according to manufacturer's instructions. DNA was extracted from buccal swabs, fetal tissue or cord tissue using 'QIAmp DNA Mini Kit' (Qiagen, Hilden, Germany).

This study was approved by the Institutional Review Committees of the University of Chicago and the Fred Hutchinson Cancer Research Center. All individual participants gave written informed consent prior to participation in this study.

### Identification and quantification of microchimerism

Because of the extensive polymorphism in HLA genes, HLA genotyping of two individuals, even if related usually results in identification of a unique HLA polymorphism that can then be targeted to identify and quantify microchimerism. HLA genotyping was conducted using a Luminex-based (One Lambda) polymerase chain reaction (PCR) sequence-specific oligonucleotide

probe technique. All samples were HLA-genotyped for the class II loci DRB1, DQA1 and DQB1 because most of our quantitative PCR assays target polymorphisms at these loci. The HLA genotypes of a proband and her mother were then examined to identify a non-shared HLA polymorphism that could be used to identify MP microchimerism. For women with subsequent pregnancies, HLA genotyping was done for the child (from cord blood) or miscarried tissue, and for partners when they were willing to participate. These studies provided confirmation (or not) that the assay for MP microchimerism detected only MP microchimerism and would not also detect fetal microchimerism. Because an HLA polymorphism unique to a proband's mother was not always present, genotyping for several other polymorphic, non-HLA genes (antithrombin III, thyroglobulin and glutathione *S*-transferase theta 1) was also performed in order to identify other non-shared polymorphisms. Genotyping for these non-HLA loci utilized a conventional PCR system described previously.<sup>28</sup>

After identifying a polymorphism that was unique to the MP, we employed the appropriate assay from a panel of polymorphism-specific quantitative PCR assays that we developed for this purpose<sup>29,30</sup> to test DNA extracted from the proband's PBMCs for MP microchimerism. For each sample, approximately 100 000 genome equivalents (GEq) of total extracted DNA were tested. Samples were run in sextuplicate with total reaction volumes of 50 µl per well. The maximum amount of DNA tested per well was 25 000 GEq, as higher concentrations of DNA may inhibit the PCR reaction. A calibration curve for the polymorphism-specific assay was included to quantify the amount of microchimerism and validate the assay for each experiment. Every sample was also tested for a non-polymorphic gene, beta globin (BGLOB). A BGLOB calibration curve (obtained from commercially prepared human genomic DNA (Promega)) was concurrently evaluated on each plate to quantify the total number of GEq of DNA tested in each reaction. DNA quantities were reported as the DNA GEq number of microchimeric cells per 100 000 total GEq by using a conversion factor of 6.6 pg of DNA per cell.<sup>31</sup> For some pregnancies fetal microchimerism was also assayed, identifying a polymorphism unique to the fetus and assaying using a similar approach.

Multiple safeguards were taken to avoid the risk of contamination. The optical detection system of the 7000 Sequence Detector obviates the need to reopen reaction tubes after amplification. DNA extractions and quantitative PCR preparations were performed under an ultraviolet light equipped safety hood, with ultraviolet run for 30 min between experiments. Filtered tips were used during pipetting. Each experiment included multiple control wells to ensure the absence of contamination.

### Statistical considerations

The current studies were conducted to determine whether MP microchimerism is detectable in women with RM prior to and/or during subsequent pregnancies, supporting (or refuting) rationale for further investigation in this area. The trend observed in preconception results for RM compared to controls was analyzed by Fisher's exact, and Wilcoxon rank sum was used to compare clinical and demographic information across the two groups. The primary outcome for analysis of preconception samples was subject status (RM case *vs.* control) and the principal predictor of interest was the presence of MP microchimerism. Logistic regression models were used to estimate the association between presence of MP microchimerism and case status while adjusting for the confounding factor of total number of cells tested. Preconception MP microchimerism concentrations were compared using Wilcoxon rank sum. Analyses were performed using Stata software version 12.1 (Statacorp, Inc., College Station, TX, USA). Among the RM probands, detection of MP and fetal microchimerism were also described according to subsequent pregnancy outcome (successful pregnancy *versus* subsequent miscarriage).

### RESULTS

Overall, 107 samples from 23 RM subjects were studied for microchimerism. Clinical and demographic information is shown in Table 1. Overall, maternal age and race were similar among women with RM and controls. As expected, women with RM had higher gravidity and lower parity than the uncomplicated controls.

Of the 23 RM probands, 16 had preconception samples that were then tested for MP microchimerism. Results were compared with preconception samples from 31 control subjects.

**Table 1 Clinical/demographic information**

	RM (n=23)	Controls (n=31)	P value
Maternal age in years, median (IQR)	33.0 (29.5–35.5)	33.9 (25.1–38.5)	0.85
Race, n (%)			0.69
White	22 (95.7)	26 (83.9)	
Black	0 (0)	1 (3.2)	
Asian	1 (4.4)	2 (6.5)	
Other/mixed	0 (0)	2 (6.5)	
Gravidity, median (IQR)	4 (3–5)	1 (0–2)	<0.0001
Parity, median (IQR)	0 (0–0)	1 (0–2)	<0.0001

Abbreviations: IQR, interquartile range; RM, recurrent miscarriage.

Factors reported at the time of initial blood draw unless otherwise indicated.

**Table 2 Preconception MP microchimerism in women with recurrent miscarriage and controls**

	RM (n=16)	Controls (n=31)	OR for detection (95% CI)	aOR* for detection (95% CI)
MP microchimerism detection, n (%)	1 (6.3)	6 (19.4)	0.28 (0.03–2.54)	0.30 (0.03–3.02)
MP microchimerism concentration in GEq of microchimerism per total 100 000 GEq tested, median (range)	0 (0–714.4)	0 (0–0.13)**		

Abbreviations: CI, confidence interval; GEq, genome equivalents; RM, recurrent miscarriage.

\* adjusted for total number of maternal genome equivalents tested.

\*\*  $P=0.2$  by Wilcoxon rank sum.

Only one RM proband tested positive, and there was a trend to lower MP microchimerism in RM probands compared to controls. However, the difference between the groups was not significant for detection or concentration of MP microchimerism in preconception samples in this study (Table 2). The average total number of GEq tested was similar in samples from RM subjects and controls (101 333±32 140 GEq and 94 651±28 208 GEq, respectively).

Among the 23 RM probands, microchimerism testing was completed for 15 subsequent pregnancies of 14 women (one woman had two pregnancies). Of the 15 pregnancies, 11 resulted in a birth and 4 in another miscarriage. Results of MP microchimerism testing are provided (Table 3) for 11 pregnancies: nine that resulted in a birth and two in a miscarriage. Whether the assay was exclusive to MP microchimerism or detected both MP and fetal microchimerism is indicated. For some pregnancies, more than one assay was run. The limited number of pregnancies precludes statistical analysis; however, 27% of RM probands who went on to have a birth were positive (3/11) for MP microchimerism whereas neither that went on to miscarry was positive (0/2). In the two pregnancies that ended in miscarriage, only the subjects' post-pregnancy samples showed detectable MP microchimerism (postpartum MP microchimerism was also observed in some RM probands who had a birth). Table 3 also includes testing specific for only fetal microchimerism for six of the pregnancies.

For comparison, in a previous published study, we evaluated MP microchimerism in a cohort of uncomplicated pregnancies. We found that MP microchimerism increased with increasing gestational age, with frequency of detection 0% (0/9) in the first trimester, 16% (3/19) in the second trimester, 29% (7/24) in the third trimester and 14% (4/28) postpartum.<sup>24</sup>

## DISCUSSION

In this initial study of women with RM, analysis suggested a trend toward diminished MP microchimerism in RM compared to control women with no history of miscarriage but was not significant. During pregnancy, MP microchimerism was more common, detectable in more than a quarter of RM probands who went on to have a birth, although not in two who went on to have another miscarriage.

These data add to an evolving literature addressing the role of maternal–fetal exchange during pregnancy and its impact on health.<sup>22,32,33</sup> Mold *et al.*<sup>23</sup> have shown that

maternal cells transfer to fetal tissues, including lymph nodes, and influence the development of fetal regulatory T cells to non-inherited maternal HLA antigens. These cells impart durable tolerance to non-inherited maternal antigens, an effect which can impact maternal health and transplant outcomes later in life.<sup>34,35</sup> We previously found that in normal pregnancy, cells from a woman's own mother (MP) are detectable in circulation, particularly during the third trimester. In the same study, MP microchimerism was not detected in preeclampsia, a pregnancy disorder thought by some to reflect maternal immune dysfunction.<sup>24</sup> It is unknown whether MP microchimerism plays an active role in, or reflects, the capacity for maternal adaptation to pregnancy, but further investigation of these questions has the potential to identify biomarkers and/or targets for immune modulation in women with RM.

In addition to a role for MP microchimerism in pregnancy adaptation, the acquisition of fetal cells by the proband during prior pregnancies may impact her subsequent pregnancies. It has been hypothesized that evolutionary pressures suggest competition between fetal microchimeric 'grafts' in the mother, perhaps influencing the interbirth interval to facilitate maximal delivery and minimal sharing of maternal resources for each offspring.<sup>36,37</sup> Whether fetal microchimerism from a prior pregnancy negatively or positively impacts subsequent pregnancies in women with RM is unknown and warrants further study.

The major drawback of this study is the small size of the study population, which limits the conclusions that can be drawn. Strengths include the careful characterization of RM subjects as well as controls. The RM subjects were strictly defined and had a negative comprehensive clinical evaluation. We were able to follow RM probands longitudinally, including time points from prior to conception throughout their pregnancies and postpregnancy. Also, the sensitive techniques used to detect and quantify microchimerism are well established.<sup>29</sup>

In conclusion, this initial study of MP microchimerism in RM demonstrates that MP can be detected in a proband's peripheral blood mononuclear cells, sometimes during pregnancy and rarely prior to conception. Other studies are needed to extend these observations, characterize cell types, determine any functional role they may play in pregnancy outcomes and evaluate the potential for 'graft' vs. 'graft' interactions among the different sources of microchimerism that can be harbored by women.

**Table 3 MP and fetal microchimerism<sup>1</sup> in women with recurrent miscarriage and subsequent pregnancy**

Subject #	Gravidity/parity	Outcome	Clinical notes	Source of microchimerism	Preconception	First trimester <sup>5</sup>	Second trimester	Third trimester	6 weeks post	6 months post
1	4/0	39 WGA <sup>3</sup> live birth	Vaginal delivery, female, 3725 g	MP <sup>3</sup>	0.13	0, 0	6.77	0	0	0
2	3/0	39 WGA live birth	Vaginal delivery, male, 3155 g	MP	0	0	0			
3	4/0	34 WGA live birth	Cesarean section, female, 1956 g preeclampsia, neonatal demise	MP	0	0, 1.43	0.69		0	
4	6/0	40 WGA live birth	Vaginal delivery, male, 3941 g	MP	0	0		0		
5	5/0	39 WGA live birth	Vaginal delivery, male, 3147 g	MP		0.16, 0	0		0	
6	4/0	39 WGA live birth	Cesarean section, female, 3630 g	MP		0, 0		15.32		
7	6/0	39 WGA live birth	Cesarean section, female, 4438 g	MP or fetal	0	0	0	0		
8	4/0	40 WGA live birth	Cesarean section, female, 2665 g, gestational hypertension	MP	0	0, 0	0	0	1.55	0
9	4/0	36 WGA live birth	Twins, Cesarean section, male/ female, 2438 g/2750 g	MP or fetal Fetal MP or fetal	0 0	0 0, 0	0 0.97 0	0 0	0 0	0 0
10	4/0	6 WGA <sup>2</sup> miscarriage		Fetal		0, 3.39	0	0	0	0
11	12/0	6 WGA miscarriage		MP <sup>3</sup>		0, 0, 0	0	0	0	0
12	4/0	41 WGA live birth	Cesarean section, female, 3799 g	MP or fetal	0	0, 0	0	0	2.56	0
13	3/0	39 WGA live birth	Vaginal delivery, female, 3657 g	Fetal <sup>4</sup>	0	0, 0	0	0	0	0
14a <sup>6</sup>	5/0	8 WGA miscarriage		Fetal	0	0, 0	0	0	0	0
14b <sup>6</sup>	6/0	6 WGA miscarriage		Fetal		0, 0			0, 0.35	0.89

<sup>1</sup> In GEq of microchimerism per total 100 000 GEq tested.

<sup>2</sup> Weeks gestational age (WGA).

<sup>3</sup> For these subjects, partner or child HLA typing not available to definitively rule out fetal source.

<sup>4</sup> For these subjects, MP HLA typing not available to definitively rule out MP source.

<sup>5</sup> Some subjects had more than one sample tested during the first trimester; these are all listed and delimited by commas.

<sup>6</sup> For subject #14, two pregnancies were followed, and results are listed.

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