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ORIGINAL ARTICLE ***Early pregnancy***

Recurrent pregnancy loss evaluation combined with 24-chromosome microarray of miscarriage tissue provides a probable or definite cause of pregnancy loss in over 90% of patients

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STUDY QUESTION: Will the addition of 24-chromosome microarray analysis on miscarriage tissue combined with the standard American Society for Reproductive Medicine (ASRM) evaluation for recurrent miscarriage explain most losses?

SUMMARY ANSWER: Over 90% of patients with recurrent pregnancy loss (RPL) will have a probable or definitive cause identified when combining genetic testing on miscarriage tissue with the standard ASRM evaluation for recurrent miscarriage.

WHAT IS KNOWN ALREADY: RPL is estimated to occur in 2–4% of reproductive age couples. A probable cause can be identified in approximately 50% of patients after an ASRM recommended workup including an evaluation for parental chromosomal abnormalities, congenital and acquired uterine anomalies, endocrine imbalances and autoimmune factors including antiphospholipid syndrome.

STUDY DESIGN, SIZE, DURATION: Single-center, prospective cohort study that included 100 patients seen in a private RPL clinic from 2014 to 2017. All 100 women had two or more pregnancy losses, a complete evaluation for RPL as defined by the ASRM, and miscarriage tissue evaluated by 24-chromosome microarray analysis after their second or subsequent miscarriage.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Frequencies of abnormal results for evidence-based diagnostic tests considered definite or probable causes of RPL (karyotyping for parental chromosomal abnormalities, and 24-chromosome microarray evaluation for products of conception (POC); pelvic sonohysterography, hysterosalpingogram, or hysteroscopy for uterine anomalies; immunological tests for lupus anticoagulant and anticardiolipin antibodies; and blood tests for thyroid stimulating hormone (TSH), prolactin and hemoglobin A1c) were evaluated. We excluded cases where there was maternal cell contamination of the miscarriage tissue or if the ASRM evaluation was incomplete. A cost analysis for the evaluation of RPL was conducted to determine whether a proposed procedure of 24-chromome microarray evaluation followed by an ASRM RPL workup (for those RPL patients who had a normal 24-chromosome microarray evaluation) was more cost-efficient than conducting ASRM RPL workups on RPL patients followed by 24-chromosome microarray analysis (for those RPL patients who had a normal RPL workup).

MAIN RESULTS AND THE ROLE OF CHANCE: A definite or probable cause of pregnancy loss was identified in the vast majority (95/100; 95%) of RPL patients when a 24-chromosome pair microarray evaluation of POC testing is combined with the standard ASRM RPL workup evaluation at the time of the second or subsequent loss. The ASRM RPL workup identified an abnormality and a probable explanation for pregnancy loss in only 45/100 or 45% of all patients. A definite abnormality was identified in 67/100 patients or 67% when initial testing was performed using 24-chromosome microarray analyses on the miscarriage tissue. Only 5/100 (5%) patients, who had a euploid loss and a normal ASRM RPL workup, had a pregnancy loss without a probable or definitive cause identified. All other losses were explained by an abnormal 24-chromosome microarray analysis of the miscarriage tissue, an abnormal finding of the RPL workup, or a combination of both.

Results from the cost analysis indicated that an initial approach of using a 24-chromosome microarray analysis on miscarriage tissue resulted in a 50% savings in cost to the health care system and to the patient.

LIMITATIONS, REASONS FOR CAUTION: This is a single-center study on a small group of well-characterized women with RPL. There was an incomplete follow-up on subsequent pregnancy outcomes after evaluation, however this should not affect our principal results. The maternal age of patients varied from 26 to 45 years old. More aneuploid pregnancy losses would be expected in older women, particularly over the age of 35 years old.

WIDER IMPLICATIONS OF THE FINDINGS: Evaluation of POC using 24-chromosome microarray analysis adds significantly to the ASRM recommended evaluation of RPL. Genetic evaluation on miscarriage tissue obtained at the time of the second and subsequent pregnancy losses should be offered to all couples with two or more consecutive pregnancy losses. The combination of a genetic evaluation on miscarriage tissue with an evidence-based evaluation for RPL will identify a probable or definitive cause in over 90% of miscarriages.

STUDY FUNDING/COMPETING INTEREST(S): No funding was received for this study and there are no conflicts of interest to declare.

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Key words: recurrent pregnancy loss / miscarriage / products of conception testing / karyotype / 24-chromosome microarray analysis

Introduction

For parents seeking a successful pregnancy, recurrent pregnancy loss (RPL) is a greatly distressing problem that occurs in up to 4% of reproductive age couples (Stephenson and Kutteh, 2007). It also poses a frustrating challenge to clinicians seeking to diagnose and treat patients who have suffered multiple losses. The etiologies most often thought to contribute to RPL include parental chromosomal translocations, congenital and acquired uterine abnormalities, endocrine imbalances, autoimmune factors including antiphospholipid syndrome, as well as infectious and thrombophilic causes (Stephenson, 1996; Donders *et al.* 2000; Martinelli *et al.*, 2000; George *et al.*, 2002; Hogge *et al.*, 2003; Anselmo *et al.*, 2004; Christiansen *et al.*, 2006; Jauniaux *et al.*, 2006; Stephenson and Kutteh, 2007; Jaslow *et al.*, 2010; Brezina and Kutteh *et al.*, 2013). Of the various diagnostic tests available for evaluation of RPL, many require considerable time and expense, leading to questions about when testing is warranted and limiting the tests to those recommended as a result of randomized controlled trials and meta-analyses (Stephenson, 1996; Jauniaux *et al.*, 2006).

In 2012, the American Society for Reproductive Medicine (ASRM) issued a committee opinion on the evaluation and treatment of RPL (American Society for Reproductive Medicine, 2012). This guideline recommended genetic karyotypes on both partners, evaluation of the uterine cavity (sonohysterography, hysterosalpingogram, or hysteroscopy), immunologic tests for lupus anticoagulant, anticardiolipin antibodies, and β2-glycoprotein I antibodies along with blood tests for thyroid stimulating hormone (TSH), prolactin and hemoglobin A1c. Our group and others have proposed a new algorithm for the evaluation and treatment of RPL based on the results of genetic testing obtained from products of conception (POC) at the time of the second or subsequent pregnancy loss (Fig. 1) (Bernardi *et al.*, 2012; Brezina and Kutteh, 2013). Several studies have indicated that the risk of recurrent miscarriage after two successive losses is only slightly lower (24–29%) than that of women with three or more spontaneous miscarriages (31–33%) (Jaslow *et al.*, 2010). Thus, evaluation and treatment can reasonably be started after two consecutive miscarriages, especially when the woman is older than 35 years of age, or when the couple has had difficulty conceiving (Sullivan *et al.*, 2004; ASRM, 2012).

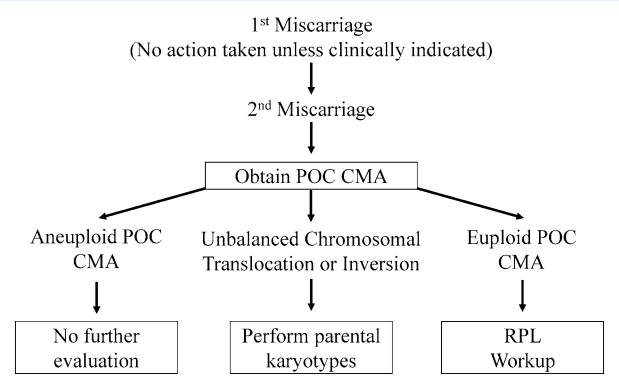

Figure 1 The proposed testing algorithm for recurrent pregnancy loss (RPL) evaluation and treatment based on the chromosome microarray analysis (CMA) diagnosis obtained from products of conception (POC) at the time of the second or subsequent pregnancy loss.

Following this algorithm, if the genetic test results from the POC are aneuploid, no further evaluation or treatment is recommended. If an unbalanced chromosomal translocation or inversion are identified in the miscarriage tissue, then parental karyotypes should be performed as well as genetic counseling, and potentially preimplantation genetic diagnosis may be warranted (Dahdouh *et al.*, 2015). If the miscarriage tissue is found to be chromosomally normal (euploid) and maternal cell contamination has been ruled out, then a full ASRM RPL workup is advised because the cause for the pregnancy loss is unknown.

In this study, we prospectively tested this RPL algorithm to evaluate the cause of miscarriage in a group of well-characterized women with RPL. This is the first study to have prospectively tested this algorithm to assess its usefulness in the evaluation and treatment of RPL. RPL patients who had a full RPL workup based on ASRM guidelines and had a CMA on POC following their second or subsequent pregnancy

loss were included. We then used this RPL algorithm to evaluate the cause of miscarriage in our patient population. We excluded cases in which maternal cell contamination was present in CMA and/or if the patient did not receive both a POC CMA and an ASRM RPL workup evaluation.

Our primary objective was to validate that the proposed testing algorithm based on the results of genetic testing obtained from POC acquired at the time of the second or subsequent pregnancy loss was more efficient than the workup for RPL currently recommended by the ASRM. We determined the frequency of abnormal results on miscarriage tissue by CMA analysis and compared it to the frequency of abnormal results for a full RPL workup evaluation as determined by the ASRM. Additionally, we prospectively calculated the cost savings to the health care system that would result from initially evaluating miscarriage tissue at the time of the second or subsequent loss followed by a reflexive full ASRM RPL workup in those patients with euploid miscarriages.

Materials and Methods

Ethical approval

The Institutional Review Board at Rhodes College approved this prospective cohort study for exempt status. The research involved the collection and study of existing data recorded by the investigators in such a manner that the subjects could not be identified directly or indirectly though identifiers linked to the subjects. The study was conducted at Fertility Associates of Memphis between January 2014 and March 2017.

Study population and participants

Subjects eligible for inclusion were patients with RPL referred to Fertility Associates of Memphis from 2014 to 2017. All women aged 20–45 years old were eligible, regardless of socioeconomic status or race. This age range is similar to that of previous studies (Stephenson, 1996; Clifford *et al.*, 1997; Salim *et al.*, 2003; Sullivan *et al.*, 2004; Christiansen *et al.*, 2006; Jaslow *et al.*, 2010). For each RPL patient, a thorough medical and obstetric history was obtained and a physical examination was performed. As part of an ongoing investigation of the causes of RPL, all patients were encouraged to have a full evaluation as recommended by ASRM (American Society for Reproductive Medicine, 2012). All testing was performed when women were non-pregnant and at least 6 weeks remote from a miscarriage.

Pregnancy was determined by multiple positive urine β-hCGs and ultrasound documentation. Pregnancy loss was defined in accordance with ASRM guidelines as the loss of any pregnancy documented with ultrasound and/or histopathological evaluation. The mean gestational age of the pregnancy losses for the women included in this study was 7.5 weeks (range 5.5–11.5 weeks). Molar pregnancies, ectopic pregnancies, biochemical pregnancy losses and pregnancy terminations were excluded from analysis.

In this study, 114 patients were enrolled because they had experienced at least two pregnancy losses, had undergone a full RPL workup as recommended by the ASRM, and had a CMA analysis on POC following their second or subsequent pregnancy loss. However, 14 of the 114 (12.3%) women had either maternal cell contamination of the POC CMA (12/14; 85.7%) or had a POC CMA that was unable to be read (2/14; 14.3%). Therefore, these women were excluded from analysis. Data recording the incidence of aneuploidy and euploidy in POC samples as well as results from ASRM RPL diagnostic testing were evaluated.

Evidence-based diagnostic tests performed and criteria for abnormal test results

Testing was based on current ASRM recommendations that identified definite or probable causes of RPL (American Society for Reproductive Medicine, 2012). For each diagnostic test performed, patients were categorized as normal or abnormal according to the criteria described below.

Parental genetics

A geneticist reviewed parental karyotypes, and significant rearrangements (e.g. balanced translocations and mosaics) were considered abnormal. Normal variants were considered normal. This category was ranked as abnormal if either a patient or her partner had an abnormal karyotype.

Uterine anatomy

Uterine anatomic defects were identified by hysterosalpingogram (HSG), hysteroscopy, or sonohysterography (SHG). Considered abnormal were congenital uterine anomalies (such as unicornuate and bicornuate uteri), and significant fundal filling defects or intrauterine abnormalities in the upper two-thirds of the uterine cavity (Jaslow and Kutteh, 2013). These included fibroids and polyps greater than 1.0 cm that were in the cavity, septa greater than 1.0 cm wide and 1.0 cm deep, or Asherman's syndrome adhesions.

Lupus anticoagulant

Serum levels of lupus anticoagulant were evaluated using the dilute Russell viper venom test and PTT-LA. Results greater than 42 s that were not corrected with a 1:1 mix with normal serum were considered abnormal if confirmed by a hexagonal phase phospholipid test.

Anticardiolipin and anti-β2 glycoprotein antibodies

Serum levels of anticardiolipin (aCL) IgG and IgM were measured by enzyme-linked immunoassay, ELISA, with abnormal levels exceeding 20 phospholipid units. All positive tests were confirmed by repeat testing at least 6 weeks later.

Thyroid function

Serum levels of thyroid stimulating hormone less than 0.45 μIU/mL or greater than 4.0 μIU/mL were considered abnormal.

Hemoglobin A1c

A patient's serum hemoglobin A1c level was considered abnormal if it was greater than 6.4%.

Prolactin

Serum levels of prolactin that were greater than 23.3 ng/mL were considered abnormal.

24-chromosome microarray analysis

All miscarriage tissue was genotyped using Illumina Cyto Single Nucleotide Polymorphism (SNP)-12b microarrays, which contains more than 300 000 probes covering all 24 chromosomes with median distance between probes of 6500 base pairs. SNP-microarray testing identifies DNA copy number, uniparental disomy, parental origin of chromosome abnormalities and maternal cell contamination using the previously described proprietary Parental Support TM algorithm (Johnson *et al.*, 2010) (Anora, Natera, San Carlos, California, USA). Comparison of the SNP identities between the maternal and POC data is used to identify maternal cell contamination, the parental origin of aneuploidy and unbalanced chromosome segments. Overall, results were obtained on 100/114 (87.7%) of samples submitted.

Cost analysis of evaluations for RPL

We performed a cost analysis of the evaluations for RPL comparing the costs of the standard ASRM evaluation with the costs of the POC CMA. We used the self-pay cost of each test as currently used in our RPL clinic as of March 2017 to determine the total cost of $3288.50. Costs of all tests were expressed in United States Dollar (USD) amounts. The self-pay costs for each of the etiologic categories that we evaluated in the ASRM RPL workup are listed in Table I. Although these prices vary depending on each RPL center, the total cost is a fairly standardized price. We excluded the expenses related to office visits, ultrasound examinations, pregnancy tests and prenatal lab test because these were similar in all patients and not related to the evaluation of RPL. We did not include the cost of obtaining the miscarriage tissue as it was passed spontaneously in some cases, obtained after medical induction, or surgical evacuation that would add to the estimated cost. The self-pay cost for the CMA on POC was $700.00. We evaluated the cost-effectiveness of the new proposed testing algorithm by comparing it to the recommended ASRM RPL workup evaluation.

Table I Cost analysis of recurrent pregnancy loss (RPL) evaluation methods (all in US dollars).

Test performed	Self-pay cost in USD
Maternal karyotype	$899.00
Paternal karyotype	$899.00
Lupus anticoagulant	$180.50
Antiphospholipid antibodies	$230.00
Sonohysterogram	$795.00
Thyroid stimulating hormone	$70.00
Prolactin	$80.00
Hemoglobin A1c	$45.00
Total per patient for ASRM workup	$3288.50

All costs were based on self-pay charges in our RPL clinic as of August 2017. American Society of Reproductive Medicine (ASRM) RPL workup based on ASRM (2012). Costs for office visits, ultrasounds and pregnancy tests would be similar using either evaluation strategy so these costs were not included in the cost evaluation

Statistical analyses

Statistical analysis was conducted using GraphPad Instat and Microsoft Excel with Statplus software. In order to determine whether maternal age correlated with frequency of abnormal results obtained by CMA or ASRM RPL workup, a Fisher's exact test was performed to determine statistical significance between maternal age groups. A 95% confidence interval was set for all calculations and $P < 0.05$ was considered to be statistically significant for all statistical tests.

Results

Abnormal findings of ASRM RPL workup

In those women who underwent a comprehensive RPL workup as defined by ASRM, 45/100 (45%) had an abnormality identified that could be a potential contributing factor for RPL (Fig. 2). In total, 14/100 (14%) women had two or more abnormal factors identified by the ASRM RPL workup. The frequencies of specific abnormalities identified by the ASRM RPL workup are detailed in Fig. 2. These findings are consistent with our previous study conducted at the same clinic that evaluated abnormalities in a larger sample population of 1020 women with RPL (Jaslow *et al.*, 2010).

Abnormal findings of POC chromosomal microarray analysis

All women in this study had a CMA of the miscarriage tissue at the time of their second or subsequent miscarriage. The majority of losses (67/100; 67%) had an abnormal POC CMA (Fig. 3, right section). In those women with an aneuploid loss, the majority 50/67 (74.6%) had a normal RPL workup. Interestingly, of the 33/100 (33%) women who had a normal POC CMA result (euploid loss) the majority 28/33

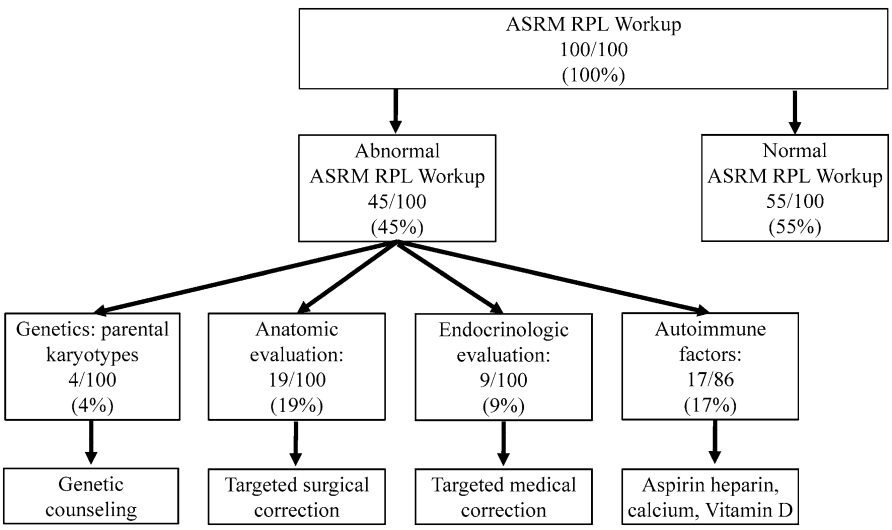

Figure 2 Frequency of American Society of Reproductive Medicine—recurrent pregnancy loss (ASRM RPL) workup abnormalities among all 100 RPL patients evaluated and the recommended treatment for each abnormality result. See 'Materials and Methods' for details of evaluation.

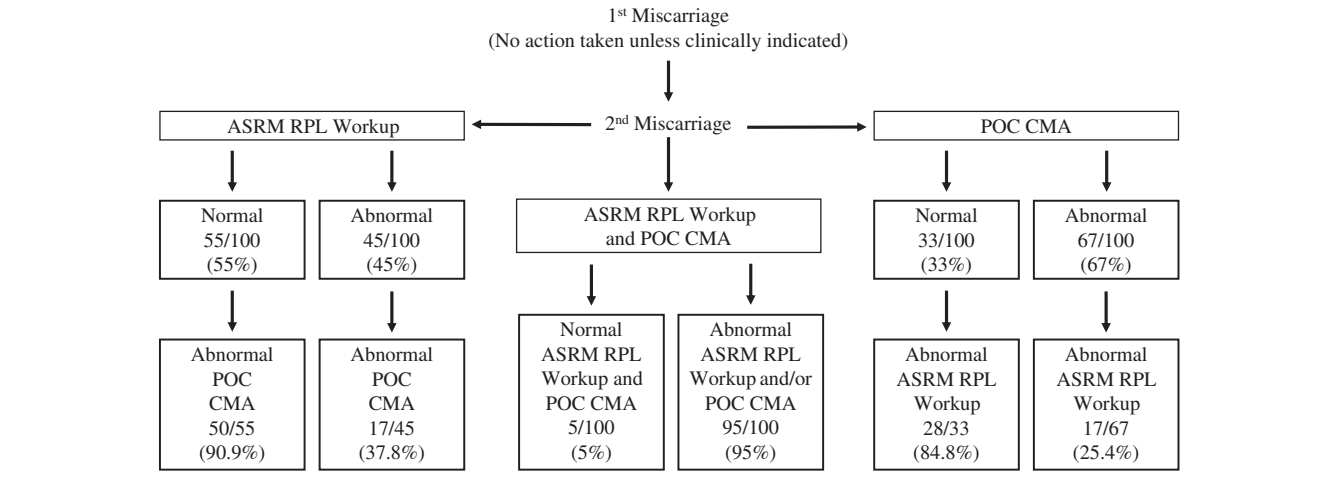

Figure 3 Three strategies for identifying cause of RPL were evaluated: ASRM RPL workup, products of conception chromosome microarray analysis (POC CMA) and a combination of both. Results suggest that a combination of both can identify the cause of RPL in 95% of cases.

(84.8%) had an abnormal result in the ASRM RPL workup. Thus, the majority of women with two or more pregnancy losses who had a normal POC CMA had an abnormal ASRM RPL workup in this study. Conversely, the majority of women who had a POC CMA that was abnormal (aneuploid) had a normal ASRM evaluation (50/67; 74.6%).

Combined results of ASRM RPL workup and POC CMA

Overall, 95/100 (95%) had an abnormal POC CMA and/or an abnormal ASRM RPL workup (Fig. 3, center section). If the proposed strategy of performing a POC CMA first and only analyzing patients with normal POC CMA results by ASRM RPL workup had been conducted, then 95/100 (95%) women would have had a probable or definite cause of RPL identified, with 67/100 having had an abnormal POC CMA and 28/100 having had an abnormal evidence-based test result identified by the ASRM RPL workup. Thus, only 5 out of 100 women who were evaluated in this study had a pregnancy loss without a potential explanation. All other pregnancy losses (95/100; 95%) had a definite or probable cause of the loss explained by an abnormal POC CMA or an abnormal finding on an evidence-based test during the ASRM RPL workup or a combination of both.

Identification of genetic anomalies for abnormal POC CMA

Of the 100 POC evaluated, 67 (67%) had an abnormality determined by CMA. Table II depicts the frequency of abnormal results of the POC CMA performed. As expected, the most common POC CMA abnormality was trisomy, accounting for 53/67 (79.1%) of all POC CMA abnormalities. The one case of multiple aneuploidy that occurred had a POC CMA result of 48 XY + 20, +22.

Maternal age assessment

The mean age of women who were included in this study was 35.7 ± 4.4 (mean ± SD) years old with a range of 26–45 years old. To determine the possible effect of maternal age on the frequency of aneuploidy in the POC, the women were stratified into two groups: less than 35 years old (44/100) or greater than 35 years (56/100). The age of 35 years old was chosen as the division point between the two groups due to the results of previous studies that have found that abnormal POC CMA results are more likely to occur with increased age, especially above the age of 35 years old (Sullivan *et al.*, 2004). The average age of the 44 women included in the younger maternal age group was 31.7 ± 2.8 years old. The average age of the 56 women included in the advanced maternal age group was 38.8 ± 2.4 years old.

Figure 4 shows a stratified percentage of abnormal POC CMA and RPL workup results by these two maternal age groups. Age had no significant effect on the frequency of abnormal ASRM RPL workups in the women <35 years old compared to those who were ≥35 years (45.5% versus 44.6%, $P = 1.00$). As expected, the frequency of chromosomal abnormalities identified by CMA in the POC from women ≥ 35 years old was greater that in those women <35 years old, however these differences were small and not statistically significant (69.6% versus 63.6%, $P = 0.67$). Therefore, the ASRM RPL workup and POC CMA results between the two maternal age groups were analyzed together.

Cost analysis

In this study, if all 100 patients following their second or subsequent pregnancy loss had initially undergone an ASRM RPL workup (approximately $3288.50 USD self-pay cost per patient), 45/100 (45%) patients would have had an abnormal RPL workup result (Fig. 5, left section). The expenses related to the evaluation of these 45 women would be limited to the ASRM workup. However, 55/100 (55%) women would have had a normal RPL workup result and would follow-up with CMA ($700.00 USD per patient). Thus, 55/100 (55%) of women would have had to pay for both the RPL workup and CMA for a total of $3988.50 USD per patient for these 55 patients. The total self-pay cost for all tests performed (RPL workup and CMA) on all 100 patients evaluated would be approximately $367 350.00 USD.

Table II Chromosomal abnormalities in POC from RPL patients.

Anomaly	Cases	Frequency (%)
Monosomy X	7	10.4
Trisomy 2	3	4.5
Trisomy 4	2	3.0
Trisomy 7	1	1.5
Trisomy 8	2	3.0
Trisomy 9	2	3.0
Trisomy 10	3	4.5
Trisomy 13	1	1.5
Trisomy 15	6	9.0
Trisomy 16	13	19.4
Trisomy 20	1	1.5
Trisomy 21	9	13.4
Trisomy 22	10	14.9
Triploidy	6	9.0
Multiple aneuploidy	1	1.5
Total	67	100

Abnormal products of conception (POC) chromosome microarray analysis results were found in 67/100 patients. See text for details.

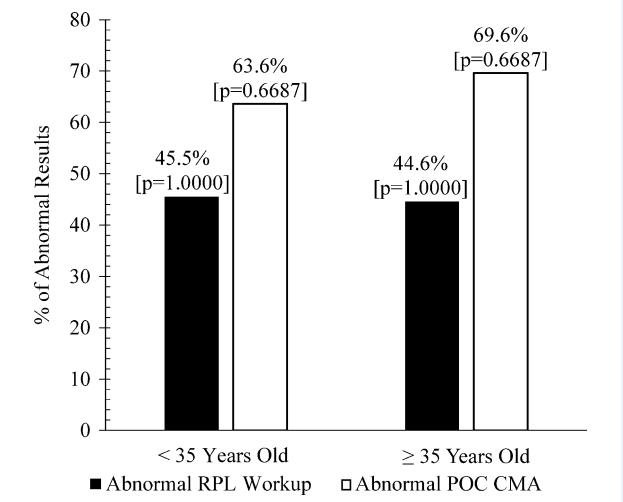

Figure 4 Comparison of abnormal ASRM RPL evaluation and abnormal POC CMA results based on maternal age. Numbers above columns show the percentage of the abnormal results to total number of women in that maternal age group. Mean maternal age was 35.7 years (see text). There were no statistically significant differences in the frequency of abnormal results identified by the ASRM RPL workup result ($P = 1.0000$) and 24-chromosome microarray ($P = 0.6687$) between the two age groups.

If the initial strategy were to obtain a POC CMA following the second or subsequent miscarriage, 67/100 (67%) patients would have an abnormal result and thus would only have expenses of the microarray of $700.00 USD per patient (Fig. 5, right section). Thus, 33/100 (33%) patients who had a normal POC CMA result would have needed to complete an ASRM RPL workup ($3288.50 USD per patient). Consequently, these 33/100 (33%) patients would have expenses for both the RPL workup and the CMA for a total of $3988.50 USD per patient. The total self-pay cost for all tests performed (RPL workup and CMA) on all 100 patients using this proposed algorithm (Fig. 5) would be approximately $178 520.50 USD. Thus, this proposed evaluation strategy of initially obtaining a CMA on the POC results in a cost savings to the health care system of over 50%.

Discussion

In this study of women with RPL, a definitive or probable cause could be identified for the vast majority of cases (95/100 women; 95%) when combining genetic testing of the miscarriage tissue with the ASRM evaluation for recurrent miscarriage. Although the presence of a particular abnormal test result does not prove that it caused the pregnancy losses in these patients, abnormalities in parental genetics, uterine anatomy, lupus anticoagulant, anticardiolipin antibodies, TSH and hemoglobin A1c are considered either probable or definite causes of RPL (Christiansen *et al.*, 2006; Jauniaux *et al.*, 2006; ASRM, 2012). In addition, the occurrence of an embryonic aneuploidy does not exclude, for example, the possibility the mother also has a uterine septum or elevated TSH. Only 5 out of 100 women (5%) had a euploid loss and normal evidence-based test results identified by the ASRM RPL workup. Possibly, these losses are linked to factors other than those assessed by the recommended evidence-based tests. For example, the density and the functions of T helper and T regulatory cells have been found to differ in women with recurrent pregnancy loss compared to women with normal pregnancies (Yang *et al.* 2008; Wang *et al.*, 2010, 2013). Exposure to environmental toxins ranging from solvents to tobacco have also been speculated to play a role in RPL (Gardella and Hill, 2000), although the effects of such factors are extremely difficult to quantify and evaluate, especially when they require self-reporting. It is also possible that some of the patients who had a euploid loss and a normal ASRM workup (those 5/100 women whose pregnancy losses were completely unexplained) have a subtler chromosome abnormality that was below the resolution of the SNP-CMA.

Although our study included 100 patients, the frequencies of abnormal diagnostic test results are comparable to those documented in several other studies. Abnormal parental karyotypes were detected among 4/100 (4%) of our patients; which is within the range of 2.5–8% reported by others for women with RPL (Harger *et al.*, 1983; Stray-Pedersen and Stray-Pedersen, 1984; Clifford *et al.*, 1994; Stephenson, 1996; Yetman and Kutteh, 1996; Rey *et al.*, 2003; Stephenson and Kutteh, 2007; Jaslow *et al.*, 2010). Additionally, uterine anatomic defects occurred in 19/100 patients (19%); a rate also within the cited range (15–20%) and comparable to the percentages reported by Stephenson (1996), Jaslow *et al.* (2010), and Stray-Pederson and Stray-Pedersen (1984). We detected abnormal lupus anticoagulant and anticardiolipin antibodies test results among 17/100 patients (17%); other researchers have reported frequencies from 14% (Clifford *et al.*, 1994) to 20.3% (Stephenson, 1996). These similar results support our findings and are indicative that our sample was appropriately representative.

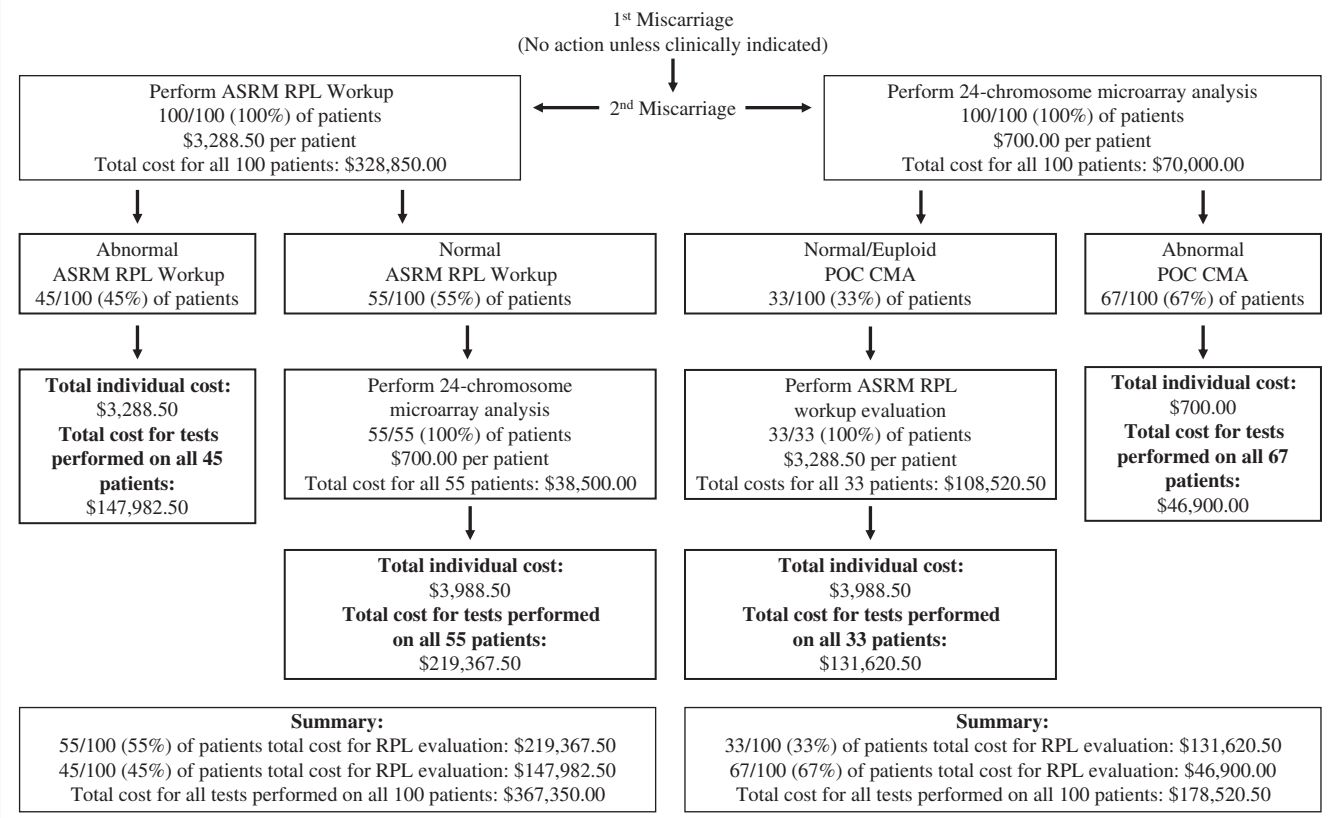

Figure 5 Cost analysis and comparison between using American Society for Reproductive Medicine (ASRM) recurrent pregnancy loss (RPL) evaluation first then follow-up with 24-chromosome microarray analysis (CMA) of products of conception (POC) (outlined on the left side of figure) versus using the proposed algorithm of initially using the 24-chromosome microarray test on POC followed by ASRM RPL workup (outlined on the right side of the figure). Results show that the new algorithm was a more cost-effective approach.

Overall, only 45/100 women (45%) had at least one abnormal evidence-based diagnostic test that was identified by the ASRM RPL workup evaluation. This result is similar to that of larger studies such as Jaslow *et al.* (2010) that reported an abnormal evidence-based diagnostic test in 403 out of 1020 (40%) RPL patients examined. In contrast, 67/100 women (67%) had an abnormal POC CMA. This result is similar to that of larger studies such as Menasha *et al.* (2005) in which a genetically abnormal POC was found in 65.8% of 1273 cases examined. It is also comparable to the results of Boue *et al.* (1975) in which an abnormality was discovered in miscarriage karyotypes for over 60% of 1500 samples evaluated. The apparent increase in abnormalities detected over time parallels the advancement of technologies and techniques for evaluating miscarriage tissue as diagnostic capabilities improve. Moreover, studies on endometrial stromal cells from women with recurrent miscarriage suggest a delay in early discrimination between high- and low-quality embryos (Weimer *et al.*, 2012). Thus, some women with RPL may be 'super fertile' by allowing genetically abnormal embryos to implant before ultimately being recognized as abnormal. Another reason for the high abnormality rate in our study may be related to the slightly higher maternal age of the cohort studied (Mean = 35.7 y). Although aneuploidy in miscarriage tissue is more likely to occur with increased maternal age, especially above age 35 (Sullivan *et al.*, 2004), there were no statistically significant differences between the number of abnormal POC CMA results ($P = 0.67$) or abnormal ASRM RPL workup results ($P = 1.00$) among our cohort based on maternal age. This higher maternal age may be a representation of the fact that more women are choosing to become pregnant later on in their lives.

As reported previously, the majority of chromosomal abnormalities from POC in this present study were trisomy (Boue *et al.*, 1975; McFadden and Friedman, 1997; Menasha *et al.* 2005). The most common abnormalities were Trisomy 16, 21 and 22, which is consistent with the findings of previous studies (Hassold *et al.*, 1980, 1996; Kajii *et al.*, 1980; Eiben *et al.*, 1990; Subramaniyam *et al.*, 2014). Furthermore, our study found that Trisomy 15 occurred in 6/67 (9.0%) of miscarriage tissue, a result similar to that of Moraes *et al.* (2005) (9%).

We utilized 24-chromosome microarray analysis in this study, which has some benefits when compared to standard karyotype analysis. Unlike a standard karyotype, 24-chromosome microarray analyses does not require viable tissue and does not require cell culture, thus reducing test failure rates often seen with traditional cytogenetic methods. A standard karyotype analysis results in double the number of female to male fetuses, an abnormal ratio (Kajii *et al.*, 1980; Eiben *et al.*, 1990; Strom *et al.*, 1992; Fritz *et al.*, 2001; Fabro *et al.*, 2011). This high frequency rate of a normal female karyotype (46, XX) in

miscarriage tissue is due to the inability of a standard karyotype to detect maternal cell contamination. In our study, maternal blood was submitted along with a miscarriage tissue sample allowing confirmation of all 46, XX results as fetal tissue or as maternal cell contamination (Lathi et al., 2014). Previous investigators have demonstrated that the detection of genetic abnormalities in miscarriage tissue is significantly improved by CMA analysis compared to standard karyotype (Reddy et al., 2012; Wapner et al., 2012; Lo et al., 2014). Currently the self-pay cost of 24-chromosome microarray analysis ($700.00 USD) is comparable to a standard karyotype of miscarriage tissue ($899.00 USD), and the cost of microarray analysis is expected to continue to decrease (Strassberg et al., 2011).

The initial strategy of performing a CMA analysis allowed us to identify a definite or probable cause of RPL in more patients than the ASRM RPL workup. This increased diagnostic rate was accomplished at a significantly lower cost than the ASRM RPL workup for all 100 patients, providing benefits to both the patients and the health care system. Similar results were reported in a retrospective study by previous investigators, such as Bernardi et al. (2012), who estimated that the proposed algorithm would be more cost-efficient. Bernardi et al. (2012) found that the cost of selective RPL evaluation based on chromosomal testing after the second miscarriage was $3352 and that the cost for a full RPL evaluation was $4507, resulting in a cost savings of $1155. The present study is the first prospective study to evaluate the effectiveness and cost efficiency of the new testing algorithm for RPL.

For the proposed algorithm, with the initial evaluation being the CMA on miscarriage tissue, the total cost to make a diagnosis per patient would be $1879.16 USD ($178,520.50 USD for the cost of all evaluations/95 patients diagnosed). If the ASRM RPL evaluation were initially used, the total cost to make a diagnosis per patient would be $3866.84 USD ($367 350.00 USD for the cost of all evaluations/95 patients diagnosed). Thus, if the proposed algorithm were to be adopted, it would be more effective than the ASRM RPL strategy in identifying a definite or probable cause for RPL at less than half the overall cost to the health care system.

Limitations of this study include an incomplete follow-up on all patients for subsequent pregnancy outcome and a small sample size that was studied. However, results from the cohort studied were found to be consistent with previous and/or larger findings, indicating that the sample was adequately and appropriately representative. Likewise, although follow-up on subsequent pregnancy outcome was incomplete, this should not affect our principal results. Strengths of this prospective study are that the cohort studied consists of a group of well-characterized women with RPL. In addition, all RPL women included had a complete ASRM RPL workup and CMA of POC. Detailed evaluations of all patients were maintained throughout the study. Moreover, all labs, evaluations and tests were conducted at the same facility, ensuring consistency of results. Furthermore, criteria for the determination of abnormal/normal results of ASRM RPL workup and POC CMA were strictly defined prior to the investigation based on objective measurements recommended by the ASRM and previous studies.

For some patients, knowing the cause for a loss may provide comfort. The surveillance mechanisms of the human body are both sensitive and specific in their ability to detect these anomalies at an early gestational age; however, these mechanisms remain to be discovered. The loss of an abnormal pregnancy through natural mechanisms, which is called as a miscarriage, is one of the last opportunities to prevent abnormal babies from being born. Knowing that their body recognized their pregnancy as abnormal and allowed the pregnancy to fail provides assurance to the patient that their body is working properly and fittingly rejected the fetus. As demonstrated by our study, the CMA of miscarriage tissue is a cost-efficient and time-efficient tool that provides answers.

In conclusion, these data support the proposed new algorithm for the evaluation of RPL. The performance of a CMA of miscarriage tissue after the second or subsequent pregnancy loss, followed by the ASRM RPL workup when POC results are normal, is an effective means for determining the cause of RPL. The combination of a genetic evaluation on miscarriage tissue with an evidence-based evaluation for RPL will provide a probable or definitive cause in over 90% of all miscarriages.

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Authors' role

Study conception and design: W.H.K.; Interpretation and synthesis of data: F.P. and W.H.K.; Analysis: F.P. and W.H.K.; Manuscript—Original draft: F.P.; Manuscript—Review and Editing: F.P., W.H.K. and C.R.J.; Critical discussion: F.P., W.H.K. and C.R.J.

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Conflict of interest

None declared.

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