# Clinically recognizable error rate after the transfer of comprehensive chromosomal screened euploid embryos is low

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**Objective:** To determine the clinically recognizable error rate with the use of quantitative polymerase chain reaction (qPCR)–based comprehensive chromosomal screening (CCS).

**Design:** Retrospective study.

**Setting:** Multiple fertility centers.

**Patient(s):** All patients receiving euploid designated embryos.

**Intervention(s):** Trophectoderm biopsy for CCS.

**Main Outcome Measure(s):** Evaluation of the pregnancy outcomes following the transfer of qPCR-designated euploid embryos. Calculation of the clinically recognizable error rate.

**Result(s):** A total of 3,168 transfers led to 2,354 pregnancies (74.3%). Of 4,794 CCS euploid embryos transferred, 2,976 gestational sacs developed, reflecting a clinical implantation rate of 62.1%. In the cases where a miscarriage occurred and products of conception were available for analysis, ten were ultimately found to be aneuploid. Seven were identified in the products of conception following clinical losses and three in ongoing pregnancies. The clinically recognizable error rate per embryo designated as euploid was 0.21% (95% confidence interval [CI] 0.10–0.37). The clinically recognizable error rate per transfer was 0.32% (95% CI 0.16–0.56). The clinically recognizable error rate per ongoing pregnancy was 0.13% (95% CI 0.03–0.37). Three products of conception from aneuploid losses were available to the molecular laboratory for detailed examination, and all of them demonstrated fetal mosaicism.

**Conclusion(s):** The clinically recognizable error rate with qPCR-based CCS is real but quite low. Although evaluated in only a limited number of specimens, mosaicism appears to play a prominent role in misdiagnoses. Mosaic errors present a genuine limit to the effectiveness of aneuploidy screening, because they are not attributable to technical issues in the embryology or analytic laboratories. (Fertil Steril® 2014;102:1613–8. ©2014 by American Society for Reproductive Medicine.)

**Key Words:** Aneuploidy, preimplantation genetic screening, misdiagnosis, trophectoderm biopsy, quantitative PCR

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Embryonic aneuploidy screening has been used with success in assisted reproduction to improve overall pregnancy outcomes. The magnitude of improvement has been demonstrated by class I data that have shown the transfer of chromosomally normal embryos screened by comprehensive chromosomal screening (CCS) to significantly increase implantation

and delivery rates compared with unscreened embryos (1–3). By selecting only euploid embryos for transfer, investigators have also reported a reduced risk of clinical pregnancy loss (4). Perhaps most importantly, elective single-embryo transfer with CCS-screened embryos provides delivery rates per transfer equivalent to multiembryo transfer (5). The dramatic reduction in polyzygotic multiple gestation meaningfully enhances obstetrical and neonatal outcomes for patients who conceive with the use of these technologies (6).

Although clinical results have been excellent, the reality is that no screening paradigm is perfect. Embryonic aneuploidy screening with CCS is subject to both biologic and technical errors (7). Inevitably, that means that some patients will develop aneuploid gestations even after undergoing CCS during their IVF treatment cycle. A biologic error is any misdiagnosis that results from a complexity within the embryo rather than an error of test function. As such, biologic errors are limitations of the test rather than errors of test function. For example, these tests require normalization of each chromosome within a specimen against the other chromosomes within that same specimen (8, 9). This corrects for variation in the number of the cells in the biopsy, the loading volume when the biopsy is placed in the reaction tube, and the variability in the fidelity of the amplification itself. Therefore, haploidy, triploidy, and tetraploidy are not currently predictable.

Perhaps most important is the broader impact of embryonic mosaicism (10, 11). There are two clinically relevant types of mosaicism, that within the embryo and that within the biopsy sample. When mosaicism exists elsewhere within the embryo, an accurately processed and evaluated biopsy may correctly be designated as euploid while some portion of the embryo is aneuploid and may result in an abnormal clinical gestation. However, when mosaicism exists within the biopsy sample it can be detected with the use of single-nucleotide polymorphism (SNP) microarray when $\geq 40\%$ of the cells are mosaic and with the use of array comparative genome hybridization (aCGH) when $\geq 50\%$ are aneuploid (12, 13).

Technical errors might be attributed to specimen processing and handling, amplification fidelity, and a variety of factors affecting the informatics used to calculate the final result. Any one or more of these factors, alone or in combination with the biologic factors, may compromise the predictive value of the test and lead to the transfer of an embryo that results in an aneuploid gestation.

Data evaluating the predictive value of a normal result from quantitative polymerase chain reaction (qPCR) and SNP microarray are available, and within those studies no embryo that screened as euploid implanted and progressed into a clinical aneuploid pregnancy (1, 2, 4). Similarly, data are available evaluating the predictive value of a normal result from 204 day-3 aCGH cycles; 13 miscarriages were observed, three with evaluable products of conception, and no misdiagnoses were identified although maternal contamination was not excluded (14). The published misdiagnosis rate with aCGH is 1.9% when comparing outcomes to fluorescence in situ hybridization (FISH) results, which is problematic given the substantial error rate associated with FISH (15). In a comparative study, SNP microarray reanalysis indicated a significantly higher error rate with aCGH (7%) compared with qPCR (0%) (16). Although this is reassuring that the clinically recognizable error rate is low with all techniques, the reality is that these studies were not powered to provide a comprehensive evaluation of the false-normal rate of embryonic aneuploidy screening.

Unfortunately, clinical experience has shown that clinical misdiagnoses do occur and that aneuploid gestations have rarely developed after transferring embryos that were screened as euploid (7). These pregnancies represent adverse outcomes for patients. In those cases where the pregnancies arrest in early development and miscarry, the patients suffer the physical and emotional consequences of pregnancy loss and lose valuable time from their efforts to conceive and deliver a healthy gestation. Development into an ongoing aneuploid gestation has even more complex and potentially longstanding consequences for these patients.

Only very large clinical experiences would be sufficiently powered to estimate how often these potentially serious adverse outcomes result. The present study sought to review a large multicentered clinical experience to determine how often clinically detectable aneuploid gestations develop after transferring embryos designated as euploid with the use of CCS.

## MATERIALS AND METHODS
### Experimental Design

All centers using CCS in conjunction with Reproductive Medicine Associates Genetics were queried regarding the outcomes of the cycles in which screened embryos were transferred. All transfer cycles from the participating centers were reviewed to determine the following: 1) the total number of transfers; 2) the total number of embryos transferred; 3) the number of transfers where a clinical pregnancy was established; 4) the number of transfers where no pregnancies occurred; and 5) the number of transfers where evidence of aneuploid gestation was found. In the event where an aneuploid gestation was identified, the pregnancy was further categorized as having resulted in either a clinical loss or an ongoing/delivered gestation.

Owing to the multicenter and retrospective nature of the study design, not all pregnancies that resulted in a loss had fetal cellular material obtained for examination: ~50% of patients with a clinically recognized loss had an evaluation with tissue for diagnosis, and ~90% of those underwent cytogenetic analysis; therefore, no conclusion can be drawn from cases that did not undergo this procedure. In the case of a misdiagnosis, the study center was alerted and all other tested specimens were assumed to be of normal karyotype. Clearly, this methodology is not comprehensive, because some clinical pregnancies were lost but did not undergo dilation and curettage and thus had no tissue available for cytogenetic analysis. It is unknown if these losses were euploid or aneuploid. Regarding pregnancies that delivered, it seems very unlikely that an aneuploid gestation would remain unrecognized by the couple or the clinicians caring for the baby.

In cases where a misdiagnosis was confirmed and fetal cellular material was available, a sample was obtained to allow investigation of possible mosaicism. Individual cells were isolated from each product of conception sample, and several of these cells were tested. Microarray copy number analysis and genotyping were performed on each cell tested so that ploidy status could be determined in addition to allowing DNA fingerprinting and definitive designation of the cells as being of maternal or fetal origin. Once these criteria were satisfied, the chromosomal analysis of fetal cells was compared with the original biopsy results. If mosaicism was identified, then the error was considered to be biologic in origin. If no mosaicism was identified, then it was not possible to designate the error as being either biologic or technical because it remained possible that mosaicism existed in some part of the developing conceptus which was not sampled and tested during the analysis.

### Participating Centers

The ten Reproductive Medicine Associates (RMA) centers that participated included RMA of New Jersey, RMA of Connecticut, Colorado Center for Reproductive Medicine (CCRM), RMA Michigan, RMA of New York, Spokane Center for Reproductive Health, RMA of Texas, Houston IVF, RMA of Philadelphia, and RMA of Pennsylvania–Allentown.

Every center was responsible for clinical care of their patients and all related embryologic procedures. Each center cultured embryos to the blastocyst stage and performed trophectoderm biopsies that were subsequently loaded into PCR tubes containing lysis buffer for analysis. The molecular genetics department at RMA Genetics in Basking Ridge, New Jersey, performed qPCR amplification steps which included multiplex preamplification, qPCR, and bioinformatics analysis for copy number determination. The one exception was CCRM, which performed the preamplification and qPCR internally before bioinformatics analysis at RMA Genetics. The reagents and test plates for the analyses at CCRM were provided by RMA Genetics and were identical to those used for the other assays. Final reports were generated and forwarded to the program caring for each patient.

### Outcomes and Data Analysis

The purpose of this retrospective observational study was to determine the clinically recognizable error rate after the transfer of embryos whose screening had indicated that they were euploid. In this study, embryos with normal screening results (either 46,XX or 46,XY) are described as being "designated euploid." All references to the ploidy status of embryos before transfer refer to their screening result and thus their designated status. Although this is not a routinely used term, it seeks to emphasize that the true status of the embryo is unknown. The present study specifically sought to determine how often that designation is incorrect and leads to a clinically recognizable error due to the establishment of an aneuploid implantation.

The first step in the analysis is to determine the number of aneuploid implantations that occurred after transfer of embryos that had been screened and designated as euploid. Where products of conception are available, this is determined by analysis of products of conception after a clinical loss, amniocentesis, or chorionic villi sampling of ongoing implantations or cytogenetic analysis of a newborn suspected to have an abnormality.

Given those data, the clinically recognizable error rate per euploid embryo transferred can be calculated. The total number of implantations found to be aneuploid is divided by the total number of embryos transferred and expressed as a percentage. It is also possible to calculate the proportion of clinically evident implantations that were aneuploid. That rate is determined by dividing the number of aneuploid implantations by the total number of gestational sacs identified. Finally, the subset of aneuploid implantations which were ongoing (i.e., did not miscarry) may be used to calculate the risk for an ongoing aneuploid implantation per CCS-screened embryo transferred.

Clearly, these types of data cannot provide a comprehensive evaluation of the overall misdiagnosis rate among embryos designated as euploid. If the embryo either failed to implant or arrested before developing a clinically evident gestational sac, then there are no products of conception to evaluate. Similarly, if the embryo implanted and a miscarriage occurred, only cases in which tissue was collected could be evaluated. It is not possible to determine if the designation of euploid for such embryos was correct or incorrect. It does not mean that the screening results for those embryos were incorrect, it only means that any misdiagnoses would be undetectable. The only analyses possible with clinical data are of the clinically recognizable error rate.

Evaluating the outcomes per transfer follows similar logic. First, the overall clinically recognizable error rate per transfer is calculated by dividing the number of pregnancies that had an aneuploid implantation by the total number of transfers. The risk of an ongoing aneuploid gestation is determined by dividing the number of pregnancies with an ongoing aneuploid implantation by the number of transfers.

The final analysis was based on the evaluation of the products of conception from those aneuploid implantations where cellular material was made available to the analytic laboratory doing the CCS testing. Comparison of SNP microarray analysis profiles were compared to determine the presence or absence of embryonic mosaicism. The mosaicism rate among the aneuploid embryos was reported as a percentage of the samples that were available for analyses. This analysis was approved by the Western Institutional Review Board, protocol no. 20021333.

## RESULTS

The outcomes of cycles using qPCR-based aneuploidy screening from January 2010 to June 2013 were available for analysis. A total of 3,168 transfers involving 4,974 blastocysts that had been designated as being euploid by CCS were completed.

### Analyses per Embryo

Of the 4,974 blastocysts transferred, 2,976 gestational sacs developed, providing a clinical implantation rate of 62.1%. Of those, 2,738 (92%) progressed to delivery and 238 (8%) either miscarried or underwent spontaneous reduction. Ultimately, 57.1% of transferred embryos implanted and progressed to delivery.

Ten of the implantations where products of conception were available for analysis were found to be aneuploid. These included one tetraploid, two monosomic, and seven trisomic gestations. Seven of these aneuploid gestations were identified in first-trimester clinical pregnancy losses, and the remaining three were identified in the early second trimester of ongoing gestations.

The clinically recognizable error rate per embryo designated as euploid was 10 out of 4,974, or 0.21% (95% confidence interval [CI] 0.10%–0.37%). Three of these aneuploid gestations were found in ongoing gestations, so the proportion of screened embryos that ultimately resulted in an ongoing aneuploid gestation was 3 out of 4,974, or 0.06%.

The clinically recognizable error rate may also be expressed as a proportion of total gestational sacs. A total of 10 out of 2,976 (0.3%) gestational sacs were discovered to be aneuploid. The clinically recognizable error rate among the ongoing gestational sacs was 3 out of 2,976, or 0.1%.

### Analyses per Transfer

There were a total of 3,168 transfers using embryos that had been designated as euploid with the use of CCS. A mean of 1.5 embryos were included per transfer. A total of 2,354 (74.3%) of the transfers progressed to the point of having one or more gestational sacs. Of these, 2,189 developed into ongoing pregnancies, providing a delivery rate per transfer of 69.1%.

The 10 errors occurred in 10 separate pregnancies. The clinically recognizable error rate per transfer was 10 out of 3,168, or 0.32% (95% CI 0.16%–0.56%). The clinically recognizable error rate per clinical pregnancy was 10 out of 2,354, or 0.4%. Finally, ongoing aneuploid gestations occurred in 3 out of 2,184 ongoing pregnancies, or 0.13%. Specific numbers for each are presented in Tables 1 and 2.

### Evaluation for Possible Mosaicism

There were four cases in which products of conception were available for comparison and all underwent detailed analysis to confirm the origin of misdiagnosis. In all four cases the products of conception showed evidence of mosaicism. For example, one case followed the transfer of an embryo with a predicted karyotype of 46,XY. The products of conception were analyzed with the use of routine g-banded karyotype analysis and determined to be 47,XY+15, yielding an abnormal male karyotype that contradicted the predicted result. SNP microarray analysis with copy number analysis and genotyping demonstrated clear evidence of mosaicism with some fetal cells exhibiting the predicted euploid karyotype (46,XY) and the remainder of the cells exhibiting the aneuploid karyotype (47,XY+15).

### TABLE 1

**Clinically recognizable error rate per designated euploid embryo (N = 4,794).**

| Parameter Evaluated | | N (% of total) |
|---|---|---|
| Embryos developed into clinically evident gestational sacs, n (%) | | 2,976 (62.1) |
| Embryos progressed to delivery, n (%) | | 2,738 (57.1) |
| Clinically evident gestational sacs that arrested in development and did not deliver, n (%) | | 238 (8.0)[a] |

| Clinically recognizable errors per embryo | Clinically recognizable error rate per embryo (%) | 95% CI |
|---|---|---|
| Total (n = 10) | 0.21 | 0.10–0.37 |
| Ongoing (n = 3) | 0.10 | 0.03–0.27 |

*Note:* CI = confidence interval.
[a] Percent of clinically evident gestational sacs.

*Werner. Error rate with comprehensive chromosomal screening. Fertil Steril 2014.*

## DISCUSSION

This study details a large multicenter experience with qPCR-based aneuploidy screening which allowed characterization of the clinically recognizable error rate. This information should be helpful when counseling patients about the clinical results that may be anticipated following the transfer of embryos that have undergone CCS-based aneuploidy screening. It also provides some insights into the mechanisms that lead to these clinical failures.

The clinical utility of 24-chromosome aneuploidy screening has been validated in several studies providing class I data (1, 2, 4, 9, 17). Increased implantation rates, lower loss rates, and higher delivery rates have all been demonstrated (2, 5, 6). Although enthusiasm for these enhanced outcomes is reasonable, it should be tempered by acknowledgement of the inevitable misdiagnoses that accompany the use of any laboratory diagnostic test.

### TABLE 2

**Clinically recognizable error rate per transfer (N = 3,168).**

| Parameter Evaluated | | N (% of total) |
|---|---|---|
| Clinical pregnancies (≥ 1 gestational sacs), n (%) | | 2,354 (74.3) |
| Pregnancies progressed to delivery, n (%) | | 2,189 (69.1) |
| Clinically evident gestational sacs that arrested in development and did not deliver, n (%) | | 170 (7.2)[a] |

| Clinically recognizable errors per transfer | Clinically recognizable error rate per transfer (%) | 95% CI |
|---|---|---|
| Total (n = 10) | 0.32 | 0.16–0.56 |
| Ongoing (n = 3) | 0.13 | 0.03–0.37 |

*Note:* CI = confidence interval.
[a] Percent of pregnancies.

*Werner. Error rate with comprehensive chromosomal screening. Fertil Steril 2014.*

Misdiagnoses are known to occur with all forms of preimplantation genetic diagnosis. The European Society for Human Reproduction and Embryology Preimplantation Genetic Diagnosis (PGD) Consortium reported a 0.27% rate per transfer of misdiagnoses for qPCR-based PGD for single gene disorders. There was also a relatively high error rate of 3.6% when performing sex determination for X-linked diseases (18). Preimplantation genetic screening has evolved with introduction of routine trophectoderm biopsy and different analytic testing platforms. The new paradigms may provide greater speed and accuracy as well as potentially reduced costs. However, no technique is perfect and it is important to measure both the actual and the clinically recognizable error rates.

The initial study defining the limits to the predictive value of 24-chromosome aneuploidy screening was a prospective blinded nonselection study (1). That study sought to determine the false-positive (embryo predicted to be abnormal but a healthy child resulted) and the false-negative rate (embryo predicted to be euploid but an aneuploid gestation developed after transfer) by biopsying embryos and then transferring them to the patients without waiting for any analysis of that biopsy. The results of the analysis were eventually correlated with the ultimate outcomes for those embryos. That nonselection study calculated the clinically recognizable error rate of a euploid screening result. Specifically, it found that the clinically recognizable error rate was zero because there were no misdiagnoses following the transfer of embryos that were designated as euploid after CCS.

Those data were certainly reassuring, but the small sample size significantly limited their power. Implantation and progression of an aneuploid embryo to a clinical stage of development occurs relatively infrequently (1). That means that the chance of an error being detected in a clinical implantation is low even if the embryo is mislabeled as being euploid when in fact it is aneuploid. Further complexity is added by the fact that embryonic mosaicism may allow euploid cells to be biopsied and correctly labeled as euploid when the embryo contains a substantial amount of aneuploid cells and may subsequently develop into an abnormal gestation. The clinically recognizable error rate of a euploid CCS result is a function of many factors. These include a combination of the laboratory error rate, the prevalence of embryonic mosaicism, and the relatively low efficiency with which aneuploid embryos implant and progress to clinically evaluable stages of development.

The most challenging of the sources of clinically recognizable errors may ultimately be mosaicism. Given the reality of embryonic mosaicism, even at the blastocyst stage, it is unlikely that any testing paradigm will ever attain perfect precision. Interestingly, all four cases where a portion of the products of conception were provided to the CCS laboratory showed clear evidence of mosaicism. In these cases, the biopsy may very well have been normal despite sufficient aneuploid cells being present in the embryo to create an abnormal pregnancy. These errors may be the result of a biologic error (mosaicism), an analytic error, or both in the event that the biopsy itself was mosaic but went undetected in the analysis.

Given the unavailability of cellular material, it is unknown if any of the other aneuploid pregnancies were mosaic or if they represented analytic errors. An analytic error occurred in at least one case. The normalization and statistical smoothing required for qPCR-based CCS means the technique is currently incapable of detecting tetraploidy. In the end, it seems likely that the errors reflected both biologic and laboratory-related issues.

In the future, it may be possible to determine if mosaicism is present in the biopsy. Next-generation sequencing (NGS) has great potential to detect mosaicism in that setting. Still, that would require that mosaicism exist in the small amount of trophectoderm removed at the time of the biopsy, typically 3–5 cells. It is certainly possible that a great deal of mosaicism, although significantly affecting the embryo, would remain undetected because it may not be represented in the biopsy itself. Some NGS protocols may also be able to identify polyploid samples. Additionally, it may become possible to integrate these data with those of other types of evaluations, such as time-lapse imaging or assessment of the transcriptome of the cumulus, to further enhance selection and improve clinical outcomes. Studies evaluating all of these approaches are underway.

In the end, the greatest value of the data from the present study may be in empowering accurate patient counseling for those couples considering embryonic aneuploidy screening. Our group has elected to focus on three specific pieces of information when using these data to counsel patients. They are the clinically recognizable error rate per embryo tested, which is 0.21%, the clinically recognizable error rate per transfer, which is 0.32%, and the risk of an ongoing aneuploid gestation per transfer, which is 0.13%. We also emphasize that these are the clinically recognizable error rates found only in evaluable pregnancies and does not account for the error rate in embryos that do not implant or progress to the clinical stage. It should be emphasized that not every miscarriage had products available for conception, and so the clinically recognizable error rate can not account for those pregnancies that miscarried and no subsequent analysis was performed. Assuming 45% of all miscarriages underwent cytogenetic analysis, in the worst case scenario this would mean that 6.5% of all miscarriages were aneuploid, and a hypothetic misdiagnosis would occur in 0.40% of embryos. This would be an overestimate of the misdiagnosis rate, but it is interesting to postulate a worst-case scenario given the limitation of this study design.

It would be most helpful if it were possible to determine if the misdiagnosis risk varied for different subpopulations of patients. Clinicians and patients may choose CCS testing for specific risk factors, such as increasing age, recurrent implantation failure, diminished ovarian reserve, and other factors traditionally associated with an increased risk of aneuploidy. Differences in mosaicism and other unknown factors may influence the validity of the diagnostic result. In this way, the present analysis may not be directly applicable to all cases and would require a large sample size, given the very low prevalence of clinically recognizable errors.

These data may also be useful in discussions with obstetricians, perinatologists, and geneticists who counsel and care

for these patients once the patient becomes pregnant. Clearly, they and the patients need to remain vigilant to the fact that aneuploid gestations may occur even after embryonic aneuploidy screening. Indications for first- and second-trimester antenatal screening should remain unchanged. The chance of finding an abnormality is reduced but clearly not eliminated. At the present time, there is no validated way to adjust the risk calculations used during antenatal screening. In the future, as more data accrue, it may be possible to incorporate the fact that CCS was done when considering calculation of aneuploidy risk following serum or ultrasound screening paradigms.

In conclusion, the use of qPCR-based CCS results in a very low risk for a clinically recognizable aneuploid pregnancy. New technologies, adjunctive screening of the embryo, and enhanced clinical management strategies offer opportunities for further improvements in the precision of the screening result and possibly clinical outcomes. Studies validating the use of more advanced technologies and screening paradigms are already ongoing.

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