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USE OF CGH-ARRAY TO DETECT CHROMOSOME ABNORMALITIES IN FIRST TRIMESTER MISCARRIAGE

by

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in the branch of Obstetrics and Gynaecology

DECLARATION

I, Rachelle Orfanidis, declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Obstetrics and Gynaecology in the University of the Witwatersrand, Johannesburg. It has also been submitted to the Colleges of Medicine of South Africa in partial fulfilment of the requirements for the qualification of Fellowship of the College of Obstetrics and Gynaecology

DEDICATION

I dedicate this work to my late father, Gavin Duffey, for his unending guidance and to my husband, Peter Orfanidis, for his support.

ACKNOWLEDGEMENTS

I would like to thank the following people for their assistance with this report:

1. My supervisors Dr Yossi Unterslak, Dr Marlene Bothma and Dr Jaysen Knezovich.
2. The doctors, nursing and administrative staff at Vitalab Fertility Centre, Johannesburg.

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ABSTRACT

Background and Objectives

Miscarriage affects 10 to 15 % of all pregnancies, with over 80% occurring in the first trimester. Approximately 50% are caused by chromosomal abnormalities. Comparative Genomic Hybridisation-array (CGH-array) is a molecular technology that can be applied to products of conception collected following a miscarriage. Its use in the South African private health sector for investigation of early pregnancy loss is relatively new.

This study aimed to quantify chromosomal abnormalities underlying first trimester miscarriages in the South African setting using CGH-array, as well as to demonstrate that CGH-array has a low test failure rate.

The objectives were to describe the characteristics of the study population, namely patients seeking infertility treatment in South Africa; to describe the results of the CGH-array analysis performed on products of conception; and to determine the rate of test failure.

Methods

This was a retrospective descriptive study conducted at Vitalab Centre for Assisted Conception, which is a private fertility clinic in Johannesburg, South Africa. The study population consisted of patients who had suffered either a sporadic or recurrent first trimester miscarriage from March 2014 to November 2016. Recurrent miscarriage was defined as the loss of three or more consecutive pregnancies. Chorionic villus

samples were obtained under direct vision using a hysteroscope. The samples were analysed by Genesis Genetics, a private laboratory in Midrand, South Africa.

Results

There were 101 samples from 99 patients included in the study. The mean maternal age was 33.9 ± 4.8 years and the patients were predominantly white, employed and in heterosexual partnerships. 79% of the miscarriages were sporadic (the patient had experienced fewer than three consecutive pregnancy losses) and 71% of the pregnancies were conceived using assisted reproductive techniques.

The overall rate of chromosomal abnormalities was 55.4%. Autosomal trisomy accounted for 71.4% of these, followed by structural abnormalities (25%) and monosomy X (7.1%). Chromosomes 16 and 22 were the commonest trisomies, each comprising 19% of the autosomal trisomies. There were three cases of 48 XXY with Trisomy 19 which may represent a test artefact. There were no cases of polyploidy identified. The ratio of female to male euploid results was 2.21:1 and the test failure rate was 0%.

Conclusion

The detection rate of CGH-array in the South African setting is in keeping with international standards. Despite its known limitations and the potential artefacts identified in this study, it is a promising tool to investigate first trimester miscarriages in the local setting.

1. INTRODUCTION

In the setting of infertility, the occurrence of a miscarriage causes a significant emotional and psychological burden for the couple trying to conceive. Most guidelines only recommend investigation into the underlying cause in cases where there has been recurrent pregnancy loss.[1] However, in the context of infertility, investigation may be warranted after a single loss. First trimester miscarriage is defined as spontaneous pregnancy loss before 13 completed weeks.[2,3] Some couples may experience recurrent miscarriages which are defined by The Royal College of Obstetricians and Gynaecologists as “the loss of three or more consecutive pregnancies”.[1]

Chromosomal abnormalities are the commonest cause of first trimester miscarriages and are present in approximately 50% of cases.[4] Considering the high incidence of genetic factors causing miscarriage, there is value in testing the products of conception following an early pregnancy loss. Firstly, the abnormalities are usually sporadic and the risk of a miscarriage as a result of fetal aneuploidy decreases in subsequent pregnancies.[5] Genetic testing can therefore assist with planning of future pregnancies as the prognosis for the next pregnancy is better if a fetal chromosomal abnormality caused the current miscarriage.[5] Secondly, if a fetal genetic cause is excluded, there may be a possible treatable cause such as antiphospholipid syndrome or a uterine abnormality. Investigations can then be aimed at identifying such factors. For couples struggling with infertility, a miscarriage, either sporadic or recurrent, can be especially difficult as significant time, money and effort may have been required to achieve a pregnancy in the first place. As such, every effort should be made to look for reversible causes and establish the outlook for subsequent pregnancies.

Products of conception have been examined by karyotyping since the 1970's.[4] Much of our understanding of the chromosomal abnormalities underlying early pregnancy loss and the frequency with which they occur are derived from these studies. There are, however, shortcomings of cytogenetic testing that have been largely overcome by newer molecular technologies.[4] Comparative Genomic Hybridisation–array (CGH-array) is one of the new methods. The use of this technology is relatively new in South Africa and is limited to the private healthcare setting. As such, its role in the evaluation of first trimester miscarriage in local gynaecology and infertility practices is not well understood and warrants investigation.

2. LITERATURE REVIEW

2.1 GLOBAL INCIDENCE OF FIRST TRIMESTER MISCARRIAGE

The incidence of miscarriages occurring in clinically recognised pregnancies is estimated to be 10 to 15%.[6-8] Of these, approximately 80% occur in the first trimester.[9,10] The criteria for a clinically recognised pregnancy varies between studies, largely due to advances in the detection of human chorionic gonadotropin in urine and serum as well as improvements in ultrasound technology. However, it is thought that there is a high occurrence of embryonic loss prior to the mother becoming aware of the pregnancy, with one study reporting that 91.7% of the first trimester miscarriages studied occurred subclinically.[11]

2.2 SOUTH AFRICAN INCIDENCE OF FIRST TRIMESTER MISCARRIAGE

To the best of the author's knowledge, there have been no large studies examining the incidence of miscarriage in South Africa. The majority of research locally has been aimed at induced and unsafe abortion practices.[12] A large prospective

population-based study conducted in India, which is also a middle-income country, found the incidence of first trimester miscarriage to be 217.5 per 1000 ongoing pregnancies.[13]

2.3 RISK FACTORS FOR FIRST TRIMESTER MISCARRIAGE

Maternal and Paternal Age

Advanced maternal age is an independent risk factor for early pregnancy loss.[4,10] A large population based Danish study showed that the risk of first trimester miscarriage increased from 8.7% at a maternal age of 22 years, to 84.1% at 48 years of age.[14] Another large prospective study of 36 056 women in the United States showed a statistically significant increase in the risk of miscarriage for women aged 35 to 39 years, with an odds ratio of 2.0.[15] The risk was even higher for those older than 40 years, with an odds ratio of 2.4.[15]

The effect of paternal age on the risk of miscarriage has not been studied as extensively as that of maternal age. However, a large multicentre study in Europe determined that there was a significantly higher risk for couples in which the maternal age was greater than 35 years and the paternal age was more than 40 years.[16]

Gravidity

Naylor and Warburton showed that the risk of miscarriage increased substantially with increasing gravidity.[17] Because the study population consisted of young women, with only 10% of participants being over the age of 30 years, this finding could not be explained by a concurrent increase in maternal age.[17]

Previous miscarriage

Naylor and Warburton, in their retrospective study, also found a positive association between the number of previous miscarriages and risk of spontaneous miscarriage.[17] One of the first prospective studies to establish a link between past reproductive performance and risk of miscarriage was the Cambridge early pregnancy loss study.[18] Twelve percent of all the clinically recognised pregnancies that occurred during the study resulted in a miscarriage prior to 20 weeks, of which 96% occurred in the first trimester.[18] There was a significantly higher risk of early pregnancy loss in women with previous miscarriage. The greatest risk (24%) was demonstrated in patients with a history of only having had prior spontaneous miscarriages with no successful pregnancies.[18] Furthermore, only 4% of patients whose previous pregnancies were all successful had a miscarriage during the study.[18]

Lifestyle factors

Alcohol consumption of greater than five units per week has been shown to increase the risk of first trimester miscarriage.[19] Rasch demonstrated an odds ratio for spontaneous miscarriage of 4.84 for alcohol use in early pregnancy and also found an increased risk of spontaneous miscarriage in women who consumed more than 375mg caffeine per day, with an odds ratio of 2.21.[20] However, a large systematic review concluded that the evidence is not sufficient to positively establish a link between caffeine use and miscarriage due to the substantial effect of confounding variables in the majority of studies.[21] Cigarette smoking has also been studied and the evidence does not support an increased risk of miscarriage in women who are exposed to tobacco smoke.[20,22]

2.4 CAUSES OF FIRST TRIMESTER MISCARRIAGE

2.4.1 Non-Chromosomal Causes

The anatomical and medical conditions that are thought to cause early pregnancy loss have been more extensively studied in the context of recurrent miscarriage rather than in sporadic miscarriages. Anatomical causes underlying first trimester miscarriages refer to uterine malformations and abnormalities as opposed to cervical incompetence which mainly affects second trimester pregnancies.[1] Evidence for the causal effect of uterine Mullerian abnormalities includes the severity of uterine distortion in arcuate and subseptate uteri being greater in women with recurrent miscarriage than in low risk women.[23] Medical causes include endocrine, autoimmune and thrombotic conditions.[24]

Endocrine Disorders

There is sufficient evidence to establish a link between uncontrolled diabetes and first trimester loss.[25,26] Well controlled patients with diabetes are not at increased risk of miscarriage.[26] There is contradictory evidence for a causal relationship between thyroid dysfunction and pregnancy loss.[24] The incidence of abnormal thyroid function has not been found to be higher in the recurrent miscarriage population.[1,27] Furthermore, adequately treated patients with hypothyroidism do not have an increase in risk.[28] However, inadequately treated patients with hypothyroidism do have a significantly higher risk of spontaneous miscarriage.[28] The role of thyroid antibodies in the aetiology of miscarriage remains unclear.[1,24] Lastly, Polycystic Ovarian Syndrome (PCOS) has been linked with recurrent miscarriage as shown by a study that found a prevalence of 40.7% among women with recurrent miscarriage

compared to 23% in the general population.[29] However, the mechanism and exact hormone abnormality responsible remains poorly understood.[29, 30]

Autoimmune Disorders

Antiphospholipid Syndrome (APLS) is known to be an important cause of recurrent miscarriage.[1] In fact, a loss of three or more consecutive pregnancies before 10 weeks gestation is one of the clinical criteria for diagnosis of the condition.[31] Untreated APLS has been associated with a first trimester miscarriage rate of up to 90%.[32]

Thrombophilic Disorders

Inherited thrombophilias have been implicated in recurrent miscarriage.[1,24] There is sufficient evidence to establish a causative role for Factor V Leiden, activated protein C resistance, prothrombin G20210A mutation and protein S deficiency.[33,34] Literature does not support an association between protein C and antithrombin deficiencies and first trimester miscarriage.[33]

The conditions discussed above are by no means a comprehensive list of all possible aetiologies that have been suggested as causes of early miscarriage. However, they do represent those that have the most evidence supporting their role in spontaneous miscarriages. Research is still ongoing into immunological, infective and other causes.[1,32] Even with a detailed assessment for all the conditions above, as well as genetic testing of the products of conception, almost half of cases remain

unexplained with the frequency of unexplained miscarriages being reported from 33% to 45%.[35-37]

2.4.2 Chromosomal Causes

Chromosomal abnormalities are widely accepted to be the commonest cause of early pregnancy loss.[10] The rate of genetic abnormalities is thought to be higher in sporadic than recurrent miscarriages.[38] Most studies included in large review articles and meta-analyses do not examine sporadic miscarriages exclusively. As such, the overall rate of chromosome abnormalities in all miscarriages is approximately 50% with rates as high as 61% being reported.[4, 38,39] The reported rate of genetic aberrations in studies that investigated recurrent miscarriage samples only ranged from 29 to 46%.[5,38, 40]

2.4.2.1 Background and Definitions

The normal human cell has 23 pairs of chromosomes. 22 of these are autosomes and the last pair are the sex chromosomes, X and Y. An organism with the correct complement of chromosomes is said to be euploid. Aneuploidy refers to an abnormal number of chromosomes, which may be either greater or fewer than normal. Trisomy occurs when there is an extra copy of a chromosome, monosomy refers to a missing chromosome from one of the pairs and polyploidy is a condition in which there is an additional haploid set (or multiple additional sets) of chromosomes in the cell.[41]

Chromosomal abnormalities can also occur in which only part of a chromosome is aberrant, with either additional or missing segments from the long or short arm of the

chromosome.[42] These are referred to as duplications and deletions respectively and because they result in a net gain or loss of genetic material, are further described as unbalanced rearrangements.[42] Balanced rearrangements occur where there is a structural rearrangement of genetic material between one or two chromosomes, but which does not result in a net gain or loss of material.[42] With all rearrangements, the phenotype may be normal, or there may be phenotypic effects depending on the amount and location of DNA affected.[42] In several studies, the terms “numerical” and “structural” abnormalities are used to describe aneuploidy and balanced or unbalanced rearrangements respectively.

2.4.2.2 Frequency of Chromosomal Abnormalities that cause Miscarriage

Miscarriage samples have been examined by karyotyping, also known as cytogenetic testing, since the 1970’s.[4] Much of our understanding of the chromosomal abnormalities outlined above and the frequency with which they occur are derived from these studies. Hassold, et al published one of the largest studies in 1980.[43] One thousand miscarriage samples were karyotyped and the authors included a comparison of their findings with seven large studies that also performed cytogenetic assessment on human miscarriage samples. All eight studies included second trimester miscarriages, although the majority of cases karyotyped were first trimester losses. The rate of chromosomal anomalies as well as the proportion of each type of abnormality found by Hassold, et al were consistent with the previous studies.[43]

Autosomal trisomy was the commonest type of abnormality with a frequency of 44.5%.[43] Trisomy 16 accounted for 24.7% of the autosomal trisomies.[43] An aggregate of the data from the other seven studies showed a frequency of 29.8% for trisomy 16,

confirming it to be the commonest occurring autosomal trisomy.[43] Thereafter, the commonest chromosomes involved in autosomal trisomy across all the studies were 22, 21, 15, 13, 7, 4 and 18.[43] There were no reported cases of Trisomy 1 and only one reported case of Trisomy 19.[43] Polyploidy was found to be the next most common abnormality with 15.1% of the abnormal fetuses having triploidy and 7.1% having tetraploidy; the total polyploidy rate was therefore 22.2%.[43] The previous studies showed a rate of polyploidy ranging from 8.3% to 17.4%.[43] Monosomy X was the anomaly that occurred most frequently overall, accounting for 24.2% of all the abnormal cases. This was comparable to the previous studies.[43] The early studies found a relatively low incidence of structural chromosomal abnormalities with Hassold, et al finding a balanced or unbalanced chromosomal rearrangement in only 4.3% of the anomalous karyotypes.[43] The aggregate analysis of the data from the previous studies found a similar frequency of 6.9% for structural abnormalities.[43]

More recent studies have been performed using various combinations of cytogenetic testing and newer molecular techniques. The results are largely consistent with the original studies. In all studies autosomal trisomy is the commonest abnormality accounting for 61% to 76% of abnormal cases.[40, 44-46] Of these, the five chromosomes most frequently involved are 16, 15, 22, 21 and 14.[40, 44-46] Polyploidy has been found to be the second most common type of abnormality in all studies where karyotyping was used, occurring in approximately 19% of the aneuploidy cases.[40,44.45] The rate of Monosomy X ranges from 9% to 22%.[40, 44-46] In all studies, structural abnormalities account for the least number of abnormal miscarriage samples and are reported to have a frequency of 3%-5%.[40, 44-46]

2.5 GENETIC TESTING

2.5.1 Traditional Cytogenetic Testing

Cytogenetic testing, otherwise referred to as karyotyping, is performed by obtaining a sample of the products of conception and culturing the tissue in culture media.[5,44] Thereafter, the chromosomes are prepared on slides and examined under a microscope.[5,44] Various stains and banding techniques have been developed and applied over the years, the most well-known being Giemsa-banding.[5,44] There are two main disadvantages to karyotyping, both of which are related to the need for viable cultured tissue.

Firstly, there is a significant risk of culture failure which leads to no result being provided for the physician and patient. The rate of failed tissue culture varies in the literature from 10% to 30%.[5,40,43,47,48] Secondly, maternal contamination is a common problem with subsequent overestimation of the 46 XX genotype.[49] There is an inherent risk of maternal tissue being sampled regardless of which genetic testing technique is being used. This is sometimes unavoidable due to the nature of miscarriages, particularly where the products of conception have been partially or completely passed prior to collection of the specimen. It is a greater problem, however, for cytogenetic testing because there can be selective overgrowth of maternal cells during tissue culture.[43,49] Evidence for this phenomenon includes an increase in the female to male ratio from samples that remained in culture media for longer than a month, compared to those that were examined after less than two weeks.[43]

2.5.2 Overview of New Techniques

The new molecular technologies improve on both these flaws as they do not require tissue culture.[4,38] The modalities are categorised as either site-specific or comprehensive for all the chromosomes. The site-specific methods include Fluorescence in situ Hybridisation (FISH), Multiplex ligation-dependent probe amplification (MLPA) and Quantitative fluorescent polymerase chain reaction (QF-PCR).[4] FISH is used most commonly in clinical practice. The major drawback for all these methods is that they only screen a subset of chromosomes. Typically, these would be chromosomes 13, 18, 21, X and Y as these are commonly included in panels for prenatal testing.[50] Whole genome techniques include Chromosomal Comparative Genomic Hybridisation (CGH), CGH-array and Next Generation Sequencing (NGS).[4,38]

2.5.3 Comparative Genomic Hybridisation-Array (CGH-array)

2.5.3.1 Basic Concepts

CGH-array is a comprehensive chromosome screening technique that examines chromosomes 1 to 22, X and Y. It is a molecular technique in which a reference DNA sample as well as the test sample are fluorescently labelled with different colour fluorophores, usually green and red respectively. The two are then co-hybridised into an array of genomic clones. Each sample (the reference and test DNA) binds proportionately to complementary regions of the genome. The fluorophores are excited by a laser causing them to emit a light signal. The signal is captured by a high-speed camera. If there is an equal amount of DNA in both samples at a particular region on the genome, the image will be an even mixture of the two colours, and in combination, produce an orange light signal. If the test sample is

missing a chromosome, the image will appear skewed to the colour of the reference sample (green). If there is an additional chromosome at a particular location, the image captured will appear closer to the colour of the test sample (red). In cases of unbalanced rearrangements, the differential signal will still detect the gain or loss of genetic material in the same manner, but the effect may be less pronounced depending on the amount of material gained or lost.[51]

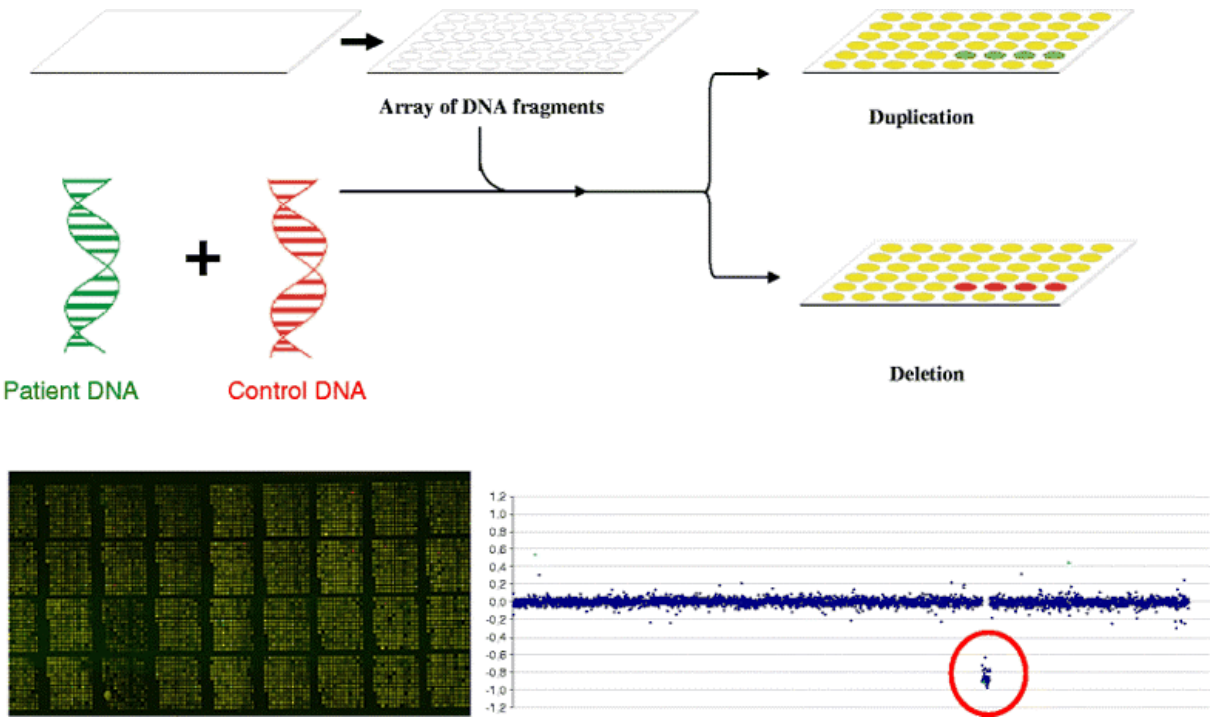


Figure 1. Schematic representation of the CGH-array technique[52]

2.5.3.2 Advantages

Sample collection

One of the most important advantages of CGH-array is the fact that it does not require any tissue culture and that the test can be reliably performed on very small amounts of viable tissue or fluid.[51, 53] Therefore the problems associated with tissue

culture, namely culture failure and selective overgrowth of maternal cells, are largely eliminated by this approach. Multiple studies have applied CGH-array to samples that failed to produce a culture for karyotyping, and the rate of failure to obtain a microarray result on these samples was reported to be between 5% and 8%.[48,54,55] The other advantage of tissue culture not being required is that the time to obtain a result is reduced from upward of two weeks to a few days depending on the capacity of the laboratory performing the test.[42,51,56]

Resolution

Cytogenetic analysis cannot detect abnormalities less than 10Mb, whereas CGH-array has a much higher resolution and can therefore diagnose submicroscopic rearrangements.[51, 57]

Improved Detection Rates

There have been several studies that have tested samples from spontaneous abortions using both CGH-array and karyotyping. Schaeffer, et al demonstrated that CGH-array detected all the abnormalities found on cytogenetic analysis and picked up additional abnormalities, including deletions and duplications, in 9.8% of cases.[58] Dhillon, et al conducted a systematic review and meta-analysis of similar studies that yielded nine papers and 314 samples.[56] They found that CGH-array detected 13% additional abnormalities compared to karyotyping, whereas karyotyping only picked up 3% additional abnormalities that were missed by CGH-array.[56] The additional abnormalities detected by CGH-array included mosaicism, autosomal trisomies as well as deletions and duplications.[56] The few cases of abnormalities detected by karyotyping that were missed by CGH-array were all balanced translocations and

polyploidies.[56] The reasons underlying the improved detection demonstrated by these studies have not been explored in detail in the articles, but it is possible the combination of the higher resolution and the superior performance of the test on smaller amounts of poorer quality tissue may be contributing factors.

2.5.3.3 Limitations

Difficult to detect certain abnormalities

CGH-array is not able to detect polyploidy and balanced rearrangements in the majority of cases.[38] Two studies have shown that polyploidy was missed by CGH-array, but when combined with flow cytometry, those cases were detected.[48,55]

Cost

Because of the advanced technology required, the test is significantly more expensive than conventional karyotyping.[57]

Abnormalities of uncertain significance

CGH-array can be applied to the whole genome at very high resolutions. As such it has detected submicroscopic rearrangements, also known as copy number variants (CNV's) that have uncertain clinical significance.[59,60] Because these CNV's are not well understood, such results create difficulty in the counselling of patients and in clinical decision making. There are two types of arrays available, one that is targeted to comprehensively screen for all the chromosomes and known pathological submicroscopic rearrangements, and a genome-wide array.[51,57] The targeted array is

preferred for clinical practice as this eliminates the chance of CNV's of uncertain clinical significance being detected, however, new pathologies and mechanisms that could explain the large proportion of unexplained miscarriages would be missed by such arrays.[51,57]

3. PROBLEM STATEMENT

Chromosomal abnormalities are the commonest cause of first trimester miscarriage and should be identified in these cases to exclude potentially reversible causes and prognosticate future pregnancies. Traditional cytogenetic testing of the products of conception has limitations which are largely overcome by molecular techniques. CGH-array is one such technique that has recently been used in the private health sector in South Africa to analyse samples from first trimester miscarriages.

The aim of this study is to quantify chromosomal abnormalities present in first trimester miscarriages using CGH-array in the South African setting, particularly in the context of a population seeking treatment for infertility. Furthermore, this study aims to demonstrate that the test overcomes the disadvantage of culture failure seen with cytogenetic testing, and therefore results in reduced test failure rates.

4. OBJECTIVES

To describe the demographic features and pregnancy-related clinical information of the study sample.

To describe the chromosomal abnormalities detected by CGH-array testing carried out on tissue obtained from sporadic and recurrent first trimester miscarriages.

To determine the rate of indeterminate results or failure to obtain a result using CGH-array due to culture failure, maternal contamination or any other cause.

5. METHODS

5.1 STUDY SETTING

The study was performed at Vitalab Centre for Assisted Conception in Johannesburg, South Africa, which is a private healthcare facility. The scope of practise at the clinic includes assessment, investigation and management of couples experiencing infertility and recurrent pregnancy loss. Once they conceive, patients are managed at the centre until ten weeks gestation. Thereafter they are referred to an obstetrician for the remainder of their pregnancy and delivery. The patients and obstetricians are routinely advised to inform Vitalab if a miscarriage occurs between ten and thirteen weeks gestation so that they can be offered genetic testing. There is therefore minimal loss to follow up of patients that suffer a miscarriage in the first trimester.

The samples were analysed by Genesis Genetics in Midrand, South Africa, which is a private laboratory service.

5.2 STUDY DESIGN

A descriptive study was performed by way of a retrospective review of patient records.

5.3 STUDY POPULATION

All patients who had a first trimester miscarriage, either sporadic or recurrent, who had CGH-array testing on the chorionic villus sample obtained by hysteroscopy during the period 1 March 2014 to 2 November 2016 were identified. This group is not representative of the general South African population as the cost of fertility investigation and treatment is prohibitively expensive for the majority of patients. However, the genetic test that was studied is only available in the private health sector and the demographic profile of patients treated at Vitalab is consistent with the population that utilises private health care in South Africa.

5.4 STUDY SAMPLE

5.4.1 Inclusion Criteria

All patients above the age of 18 who had a first trimester miscarriage after conceiving naturally or by assisted reproduction were included. Multiple pregnancies, both dichorionic and monochorionic, that met the criteria for sample collection detailed below were included.

5.4.2 Exclusion Criteria

Tissue samples from ectopic pregnancies that were tested using CGH-array were excluded. Cases where the result of a CGH-array test was obtained from Genesis Genetics, but the patient file at Vitalab was missing, were excluded as the clinical information of the patient could not be obtained.

5.5 SAMPLING AND SAMPLE SIZE

The potential cases were identified by a comprehensive list provided by Genesis Genetics of every CGH-array test conducted on miscarriage samples from patients of Vitalab. The clinical records for each patient were then obtained from Vitalab and reviewed. Cases that met the inclusion criteria were included in the study.

The sample size was determined using a specified time period from the inception of the use of CGH-array at Vitalab and Genesis Genetics until the time of the ethics application for the study. The time period was therefore from 1 March 2014 to 2 November 2016. All cases of CGH-array performed on first trimester miscarriage samples during that period were evaluated against the inclusion and exclusion criteria.

5.6 SAMPLE COLLECTION

Patients who suffered a miscarriage at Vitalab were offered a hysteroscopic procedure in which a chorionic villus sample was taken under direct vision to minimise accidental sampling of maternal endometrium. Should patients have declined for reasons including cost or personal preference, they were offered other

procedures to evacuate the uterus and no specimen was sent for genetic testing. The rate of uptake was estimated to be 90%. The study sample was therefore representative of the study population.

The procedure was done under general anaesthesia within 48 hours after the miscarriage was diagnosed. A hysteroscope was passed into the uterus. The majority of cases were missed miscarriages with an intact embryo which was visualised (Figure 2) and an embryoscopy was done where the hysteroscope was guided into the gestational sac (Figure 3). In the few cases where there was an incomplete miscarriage, the hysteroscope was passed into the uterus and if there was good quality fetal or trophoblastic tissue, a sample was also taken under direct vision. In the case of a multiple gestation, both embryos were visualised, and the samples taken under direct vision were labelled immediately to ensure accurate results were obtained for each embryo. For multiple gestations, only missed miscarriages with intact embryos that were able to be clearly visualised and sampled were included in the study. The description of the procedure and the findings of the hysteroscopy and embryoscopy were available in the clinical records for each case at Vitalab.



Figure 2. Hysteroscopic image of an intact embryo enclosed in the amniotic sac

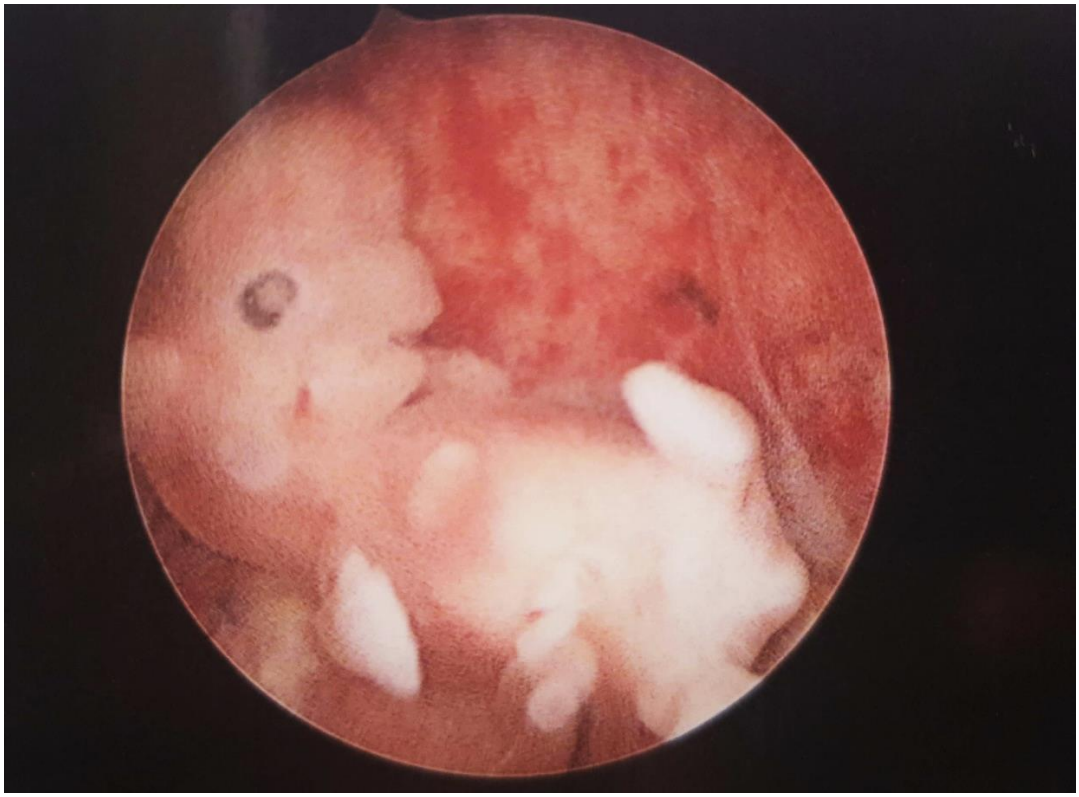


Figure 3. Hysteroscopic image of an embryo after entry through the amnion

The tissue was placed in normal saline in a specimen container which was shaken and then examined. A typical coral-like appearance of the tissue confirmed that the sample contained chorionic villi. Once confirmed, the chorionic villus sample was sent to the Genesis Genetics laboratory for the CGH-array. One of the advantages of CGH-array is that it requires no specific conditions for transport. The temperature of the sample does not need to be regulated and there is no time limit for the sample to arrive at the laboratory. However, the samples were sent on the day of collection and prepared for analysis immediately upon arrival at Genesis Genetics. The results were available within five to seven days and were sent electronically to Vitalab. They were then accessible using the patient's file number.

Genesis Genetics used the Qiagen FlexiGene DNA extraction kit to extract DNA from the sample, followed by DNA amplification using the Illumina SurePlex kit. Lastly the Illumina 24sure array was used to screen the chromosomes. This array is the targeted array preferred for clinical practise that comprehensively screens all of the chromosomes.

5.7 DATA ANALYSIS

Data was analysed using Microsoft Excel. The descriptive data was described using frequencies and percentages as well as medians with ranges and means with standard deviations.

5.8 ETHICS

Patient confidentiality was protected by the allocation of a study number to each case. Only the study number was recorded on the data collection forms and therefore no personal information was captured. There is a detailed consent form signed by all patients undergoing treatment at Vitalab Centre for Assisted Conception in which permission is obtained to use information from clinical records anonymously for research purposes. An extract from this form is attached as Appendix B. Ethics clearance was obtained from the Human Research Ethics Committee for the University of the Witwatersrand (Appendix C).

6. RESULTS

102 potential cases were identified from the comprehensive list of CGH-array results obtained from Genesis Genetics. Of these, one was excluded as the test was done on an ectopic pregnancy sample. Another two cases were excluded because the clinical records could not be obtained from Vitalab Centre for Assisted Conception. In total, 99 patients were included in the study. Two of these patients had dichorionic twin pregnancies for which two separate samples were taken. There was one set of monochorionic, monoamniotic twins as well as one case of identical quadruplets in a single sac. For both cases a single sample and genetic test was performed. Therefore the total number of samples included in the study was 101.

The mean maternal age was 33.9 years and the mean age of the partner was 36.1 years (Table 1). The majority of patients were in heterosexual relationships with two (2.0%) same sex couples and three (3.0%) single females. The ethnicity of both

patients and partners was mainly white. All of the partners and most of the patients were employed.

Table 1. Demographic characteristics of patients and their partners

Demographic variable		Maternal (n=99) N (%)	Paternal / Partner (n=96) N (%)
Age in years (mean ± SD)		33.9 ± 4.8	36.1 ± 6.5
Ethnicity	White	70 (70.7)	69 (71.9)
	African	15 (15.2)	15 (15.6)
	Indian	10 (10.1)	10 (10.4)
	Arabic	3 (3.0)	2 (2.1)
	Coloured	1 (1.0)	0 (0.0)
Occupation	Employed	89 (89.9)	96 (100)
	Unemployed	10 (10.1)	0 (0)

The majority of patients were nulliparous with a median gravidity of 2 (Table 2). Most of the women were attending the clinic for primary or secondary infertility. Sixty women had never had a miscarriage before, while two women had experienced more than three miscarriages previously.

Table 2. Reproductive history of patients (n=99)

Reproductive variable		N (%)
Gravidity	1	45 (45.5)
	2	25 (25.3)
	3	12 (12.1)
	Greater than 3	17 (17.2)
Parity	0	76 (76.7)
	1	17 (17.2)
	2	6 (6.1)
Reason for consultation	Primary infertility	42 (42.2)
	Secondary infertility	33 (33.3)
	Recurrent miscarriage	18 (18.2)
	Same sex	5 (5.1)
	Genetic condition	1 (1.0)
Previous ectopic		6 (6.1)
Previous termination of pregnancy		3 (3.0)
Number of previous miscarriages	0	60 (60.6)
	1	18 (18.2)
	2	14 (14.1)
	3	5 (5.0)
	Greater than 3	2 (2.0)

There were only three patients with HIV in the study sample (Table 3). Fifteen of the women were smokers. Forty patients had gynaecological disorders, while 34 had

uterine abnormalities and 12 had tubal factors. Of the 94 male partners, 19.1% had male factor infertility.

Table 3. Clinical conditions and risk factors in study participants (n=99)

Clinical variable		N (%)
Medical disorders	HIV seropositive	3 (3.0)
	Hyperthyroidism	2 (2.0)
	Hypothyroidism	8 (8.1)
Smoking		15 (15.2)
Gynaecological disorders	Endometriosis	26 (26.3)
	Pelvic adhesions	4 (4.0)
	Polycystic ovarian syndrome	10 (10.1)
Uterine abnormalities	Leiomyomas	11 (11.1)
	Septate uterus	12 (12.1)
	Polyps	5 (5.1)
	Intrauterine synechiae	6 (6.1)
Tubal pathology	Hydrosalpinx	6 (6.1)
	Salpingectomy	4 (4.0)
	Occlusion	2 (2.0)
Male factor infertility (n=94)		18 (19.1)

The mean gestational age was 7.3 weeks based on early ultrasound findings (Table 4). The majority of the pregnancies that resulted in the current miscarriage were singleton gestations with only four cases of multiple gestations. There were 78 sporadic miscarriages as defined by the Royal College of Obstetricians and Gynaecologists[1] and most of the cases were missed miscarriages diagnosed on

ultrasound. Sixty-two of the pregnancies were conceived using In-Vitro Fertilisation (IVF) or Intra-Cytoplasmic Sperm Injection (ICSI), twenty were spontaneously conceived, fourteen were as a result of ovulation induction and three pregnancies occurred after Zygote Intra-Fallopian Transfer (ZIFT) or Gamete Intra-Fallopian Transfer (GIFT). There were only nine cases of donor gametes being used.

Table 4. Characteristics of index pregnancy that resulted in miscarriage (n=99)

Pregnancy variable		N (%)
Number of fetuses	Singleton	95 (96.0)
	DCDA twins	2 (2.0)
	MCDA twins	1 (1.0)
	Quadruplets	1 (1.0)
Category of miscarriage	Sporadic	78 (79.0)
	Recurrent	21 (21.0)
Type of miscarriage	Missed	95 (96.0)
	Incomplete	4 (4.0)
Method of conception	IVF	32 (32.3)
	ICSI	30 (30.3)
	Spontaneous	20 (20.2)
	Ovulation induction with timed intercourse	8 (8.1)
	Ovulation induction with insemination	6 (6.1)
	ZIFT / GIFT	3 (3.0)

Use of donor gametes	Donor oocytes	2 (2.0)
	Donor spermatocytes	7 (7.1)

CGH-array was performed successfully on 100% of the samples (Table 5). Chromosomal abnormalities were detected in 55.4% of the 101 samples included in the study. Of the euploid samples, 31 (68.9%) were female and 14 (31.1%) were male. The rate of chromosomal abnormalities among the cases of sporadic miscarriage was 55.0%, while 52.4% of the cases of recurrent miscarriage had an abnormal result.

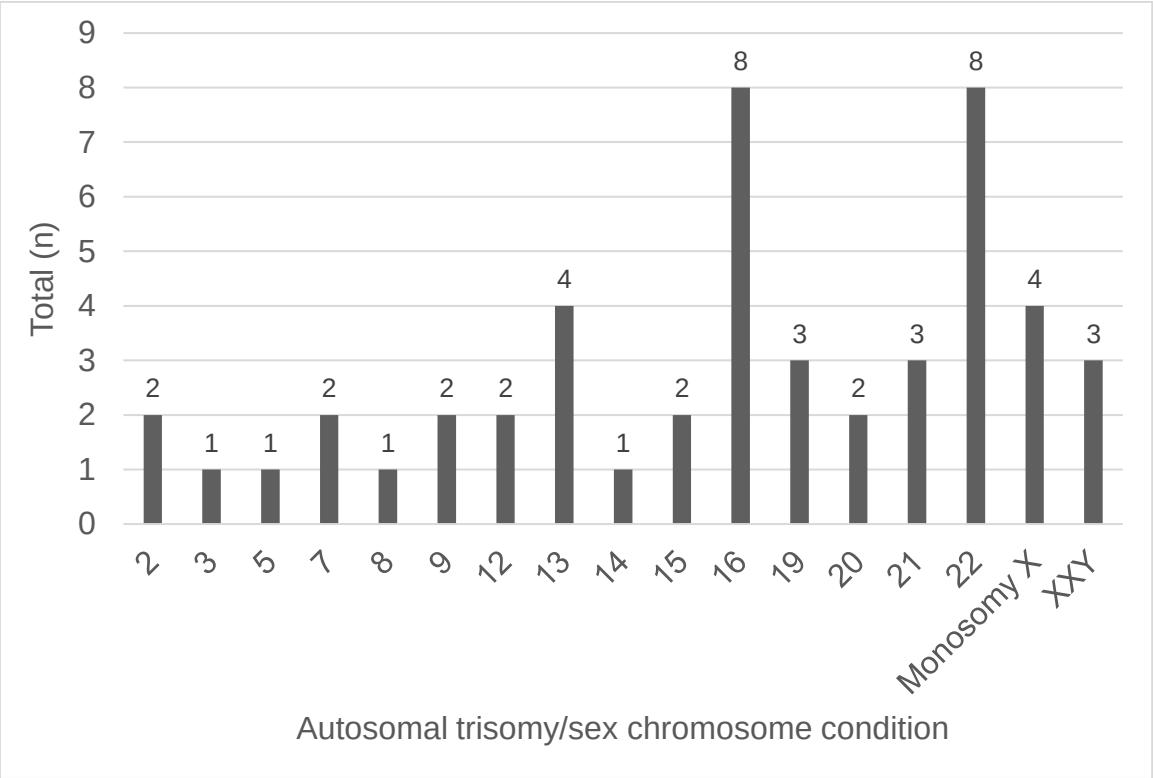
Of the 56 abnormal cases, 49 had a single abnormality and seven had two or more abnormalities. There were 38 cases in which an autosomal trisomy was identified and four cases of monosomy X. Three cases of XXY were identified, one occurring in isolation, and two were found in addition to an autosomal trisomy. Structural abnormalities were found in 13 of the samples. Ten of these occurred as a single duplication or deletion of one chromosome. There were two cases in which the structural abnormality was found in addition to a numerical abnormality, and a single case in which three structural abnormalities were detected in the same sample. Polyploidy was not detected in any of the cases.

Table 5: CGH-array results (n=101)

CGH-array result		N (%)
Result obtained		101 (100.0)
Euploid		45 (44.6)
	Female (XX)	31 (68.9)
	Male (XY)	14 (31.1)
Aneuploid		56 (55.4)
Numerical	Autosomal Trisomy	40 (71.4)
	Monosomy X	4 (7.1)
	XXY	3 (5.3)
Structural	Duplications	9 (16.1)
	Deletions	5 (8.9)

There were 49 numerical abnormalities detected in the study (Figure 4). While there were 40 cases in which an autosomal trisomy was found, two of the samples were found to have two autosomal trisomies. As such, there were 42 autosomal trisomies in total. There were no cases of autosomal monosomy. The seven cases of sex chromosome abnormalities occurred mostly in isolation, except for the three cases of XXY that were found in conjunction with trisomy 19. Trisomy 16 and 22 were the commonest autosomal trisomies, with eight cases of each identified. These were followed by trisomy of chromosomes 13, 19 and 21. There were no cases of trisomy 1, 4, 6, 10, 11, 17 or 18 identified in the study.

Figure 4: Frequency of numerical chromosomal abnormalities detected by CGH-array (n=49)



7. DISCUSSION

7.1 DEMOGRAPHIC AND CLINICAL CHARACTERISTICS

The relatively high maternal age is expected for a group of patients attending an infertility clinic. The risk of miscarriage and chromosomal abnormalities for this age group of mothers is increased compared to mothers younger than 30 years.[14,43] However, the rate of chromosomal abnormalities detected in this study was in keeping with the literature.

The prevalence of HIV seropositive individuals in South Africa is estimated to be 12.6%.[61] The rate is higher among adults between 15 and 49 years with 18.0% of

people of reproductive age believed to be infected.[61] The rate of HIV seropositive women in this study was found to be significantly lower than the national average at 3%. This is a reflection of the study population differing from the general South African population with regards to socio-economic status. This is unlikely to impact the applicability of the study to the private health sector, where the test is available. However, this discrepancy highlights the need for further research at public health facilities were the test to be considered for broader use in South Africa.

There were few cases of medical conditions identified in the study sample with only ten patients having a thyroid disorder. No other significant general medical conditions were detected in the clinical records of any of the patients. However, there was a much higher rate of gynaecological conditions and disorders identified. This is expected as patients are assessed and intensively investigated for these gynaecological conditions during the evaluation of infertility or recurrent miscarriage. It is possible that there was a higher prevalence of general medical conditions that were not detected due to the focus of the clinical assessment of these patients. However, it is not expected that a relatively young, healthy population seeking medical help for reproductive issues would have a high burden of comorbid diseases.

7.2 PREGNANCY FACTORS

As expected, the majority of pregnancies were conceived using assisted reproductive techniques (ART). The question of whether there is a higher occurrence of chromosomal abnormalities in pregnancies conceived with ART has not been adequately answered in the literature to date. Multiple studies have assessed products of conception after first trimester miscarriage in patients that underwent ART, however, they have relatively small sample sizes ranging from 18 to 273 specimens.[62-65] The rate of aneuploidy found in these studies was between 45% and 83%.[62-65] The authors of some of these studies concluded that there was a higher rate of chromosomal abnormalities in this particular population, however, the aneuploidy rate for miscarriage samples overall has been shown to be approximately between 29% and 61%.[4,5,38-40] As such it is not possible to draw any conclusions from the high rate of aneuploidy in these descriptive studies alone. A comparative study with 560 cases of first trimester spontaneous miscarriages did not find a statistically significant difference between the aneuploidy rate of miscarriages occurring in pregnancies conceived naturally, compared to those achieved using IVF, ICSI or IUI.[66] The high number of pregnancies following ART in this study therefore is unlikely to represent a confounding variable in the results.

Most of the miscarriages were missed miscarriages with only four incomplete miscarriages in the study sample. This is expected in a setting where pregnancies are monitored from early gestations with ultrasonography. Similarly, the mean gestational age of the pregnancies at the time of the miscarriage being diagnosed was 7.3 weeks, which is the typical gestational age for the first antenatal scan. Due to the fact that there were mostly missed miscarriages with intact embryos at the time of hysteroscopy and chorionic villus sampling, it would be expected that the rate of

successful sampling of fetal tissues would be high. However, the female to male ratio of the euploid specimens indicates that there may have been maternal contamination in the specimens obtained.

7.3 FEMALE TO MALE EUPLOID RATIO

The ratio of 46 XX to 46 XY results was 2.21:1. Statistically speaking, it would be expected that the ratio should be approximately 1:1[61]. There is a possibility that the ratio in this cohort was skewed towards 46 XX, however, it is also possible that maternal cell contamination (MCC) of the samples occurred. CGH-array does not require cell culture which is known to result in MCC due to overgrowth of maternal cells and therefore the incidence of contamination was expected to be low in this study. Two previous studies analysed products of conception with both karyotyping and CGH-array. Menten, et al demonstrated a female to male euploid ratio of 4.00:1 after karyotyping, compared to 1.14:1 after CGH-array, which supports the theory that eliminating overgrowth of maternal cells during culture will reduce MCC.[55] However, Schaeffer, et al found a similar discrepancy in the sex ratio of the euploid specimens using CGH-array to that which was present in this study.[58] The likely conclusion is that regardless of the genetic testing technique used, there remains a risk of obtaining maternal cells during the collection and preparation of the specimen. Besides the inherent risk of sampling maternal tissue instead of fetal products of conception, one theory is that maternal blood cells may contaminate fetal or trophoblastic tissues due to rupture of uterine vessels during separation of the chorion from the decidua.[67] The apparent occurrence of MCC in this study represents a limitation and must be considered when interpreting the results of the frequency of chromosomal abnormalities detected.

7.4 RATE OF TEST FAILURE

A result was reported in 100% of the cases in this study. This is in keeping with the literature that demonstrated CGH-array is associated with significantly less test failure than karyotyping by eliminating the need for tissue culture.[48,54,55]

However, while it can be concluded that the test was successfully applied to every specimen obtained, it cannot be stated that 100% of the results were accurate due to the possibility of maternal cell contamination.

7.5 CGH-ARRAY RESULTS

The overall rate of chromosomal abnormalities was 55.4%. This is in keeping with the literature which reports the frequency of chromosomal abnormalities in early pregnancy loss to be approximately 50%.[4, 38,39] The incidence of aneuploidy in this study may be under-reported due to the potential maternal cell contamination discussed previously, which may have resulted in abnormalities not being detected. However, the overall aneuploidy rate was in keeping with expectations based on previous studies, and so the possibility that this cohort was simply skewed towards more euploid females cannot be excluded. There may also have been over-reporting of aneuploidy cases due to the fact that many patients undergoing IVF and ICSI opt for preimplantation genetic screening of their embryos. Such couples would not have been offered genetic testing on the products of conception if they had suffered a miscarriage after transferring euploid embryos.

There were fewer chromosomal abnormalities present in the specimens obtained from patients who had suffered a recurrent miscarriage compared to those from patients with sporadic miscarriages. The aneuploidy rates for the two groups were 52.4% and 55.0%, respectively. This is consistent with previous studies that have demonstrated a lower incidence of chromosomal causes in recurrent miscarriages.[5, 40]

A chromosomal abnormality was present in all of the cases of multiple pregnancy. The two cases of non-identical twins showed an abnormality in both fetuses. The abnormalities differed between the fetuses in each case, implying that the sampling was correct and 2 separate specimens were in fact obtained.

A double trisomy was detected in the sample analysed from the identical quadruplet pregnancy as well as from the identical twin pregnancy. In both cases, the result was 48 XXY and trisomy 19. This is a highly unusual finding because trisomy 19 is exceedingly rare, with one systematic review finding no direct evidence of its occurrence in the literature.[4] Hassold, et al detected only one case of trisomy 19 from 1000 miscarriage samples.[43] Furthermore, the sex chromosome abnormality of XXY has been shown to occur as a double trisomy with multiple chromosomes including 4, 8, 9, 13, 16, 18 and 22.[68, 69] However, to the best of the author's knowledge there are no reported cases of 48 XXY with trisomy 19 in the literature. It is therefore possible that the unexpectedly high rate of trisomy 19 in this cohort might suggest a CGH-array artefact.

The most common autosomal trisomies detected in this study were of chromosomes 16 and 22, followed by chromosomes 13 and 21. This is consistent with both the older cytogenetic studies and the newer studies that utilised various new techniques.[40,43-46] The results from this study differ from the literature in that trisomy 18 was found to be one of the most frequently reported chromosomal abnormalities in the cytogenetic studies, but there were no cases present among the miscarriage samples analysed.[44-46] Also, chromosome 15 has been reported to occur with greater frequency than was seen in this study.[40,43-46]

As expected due to the known limitations of CGH-array, there were no cases of polyploidy detected. Monosomy X was also found less frequently than reported in the literature, which has cited it as being the commonest aneuploidy overall.[23] Structural abnormalities were present in 23.21% of the abnormal cases. This is significantly higher than the rates of 3 % to 6.9% reported in the literature from both older cytogenetic studies and newer studies that included various molecular techniques.[23,40,43-46] This may suggest another artefact of the CGH-array with cases of partial aneuploidies being falsely identified.

7.6 LIMITATIONS

The sample size of the study was limited by the time that CGH-array was introduced to analyse miscarriage samples at Vitalab Centre for Assisted Conception.

As discussed previously, preimplantation genetic screening may have introduced a possible source of selection bias. Couples who opted to screen their embryos prior to

transfer during IVF or ICSI would not have been included in this study if they subsequently suffered a miscarriage of a euploid pregnancy.

The unexpectedly high incidence of suspected maternal cell contamination represents another limitation to the study. The absence of polyploidy present in the results could be considered a limitation as well, considering that CGH-array was not used in conjunction with flow cytometry during analysis of the specimens. However, the results do provide further evidence that CGH-array alone does not detect polyploidy. As such, a recommendation can be reiterated from this study that it should be used with flow cytometry in order to improve detection of chromosomal abnormalities in first trimester miscarriage.

Applicability of this study is limited to the private health sector and the population it serves. CGH-array is an expensive test and not available in all sectors of society. The need for a hysteroscope and theatre facilities to collect the specimen in the same manner as the study further adds to the prohibitive cost in the broader health services. However, the sample may be collected with an ordinary evacuation procedure and the test is being applied in other gynaecological practices without hysteroscopic sampling. It would be a useful area of research to ascertain the accuracy of results of specimens obtained with less expensive sampling methods.

8. RECOMMENDATIONS FOR RESEARCH

To the best of the author's knowledge, this is the first study that has described CGH-array results for products of conception in the South African setting. Further research is recommended to directly compare the effectiveness of the test to karyotyping in

the local setting, considering that karyotyping is less expensive and more readily available in the public health sector.

Also, additional studies are recommended to investigate the potential artefacts of CGH-array, namely the unexpectedly high rates of structural abnormalities and trisomy 19, found in this study. Prospective studies that combine CGH-array with other molecular techniques, such as FISH or QF-PCR to confirm unusual results would achieve this objective.

9. CONCLUSION

Early pregnancy loss is a common clinical problem and can cause significant distress for couples trying to conceive, particularly in the context of infertility. This study determined that the rate of chromosomal abnormalities underlying first trimester miscarriages in the South African context is 55.4%. Despite the subfertile population examined in this study, this rate is in keeping with the incidence reported previously in the literature.

The use of CGH-array to test products of conception is relatively new in South Africa. This study demonstrates that the detection rate of chromosomal abnormalities using this technology is comparable with international standards and that the rate of test failure was 0%. Furthermore, this array detected numerical and structural abnormalities on a wide range of chromosomes, which would have been missed had site-specific methods, such as FISH, been used with their limited panels. However, the study also confirmed a known limitation of the test, namely that it is unable to detect polyploidy, and identified potential artefacts present in the array. Overall it is a promising tool to investigate first trimester miscarriages in the local setting.

10. REFERENCES

1. Royal College of Obstetricians and Gynaecologists. Green-top guideline number 17: the investigation and treatment of couples with recurrent first-trimester and second-trimester miscarriage. 2011 [cited 2016 June 4]. Available from https://www.rcog.org.uk/en/guidelines-research-services/guidelines/gtg17/
2. Pattinson RC, Snyman LC. Miscarriage. In: Kruger TF, Botha MH, editors. Clinical Gynaecology. 4th ed. Cape Town: Juta, 2011; p.196-209.
3. National Institute for Health and Care Excellence. NICE clinical guideline number 154: ectopic pregnancy and miscarriage: diagnosis and initial management. 2012 [cited 2018 August 21]. Available from https://www.nice.org.uk/guidance/cg154/resources/ectopic-pregnancy-and-miscarriage-diagnosis-and-initial-management-pdf-35109631301317
4. Hardy K, Hardy PJ. 1st trimester miscarriage: four decades of study. Trans Pediatr. 2015;4(2):189-200. doi:10.3978/j.issn.2224-4336.2015.03.05
5. Carp H, Toder V, Aviram A, Daniely M, Mashiach S, Barkai G. Karyotype of the abortus in recurrent miscarriage. Fertil Steril. 2001;74(4):678-682. doi:10.1016/S0015-0282(00)01801-X
6. Zinaman MJ, Clegg ED, Brown CC, O'Connor J, Selevan SG. Estimates of human fertility and pregnancy loss. Fertil Steril. 1996;65(3):503-509. doi:10.1016/S0015-0282(16)58144-8
7. Wilcox AJ, Weinberg CR, O'Connor JF, et al. Incidence of early loss of pregnancy. N Engl J Med. 1988;319(4):189-194. doi:10.1056/NEJM198807283190401
8. Wang X, Chen C, Wang L, Chen D, Guang W, French J. Conception, early pregnancy loss, and time to clinical pregnancy: a population-based prospective study. Fertil Steril. 2003;79(3):577-584. doi:10.1016/S0015-0282(02)04694-0

9. French FE, Bierman JM. Probabilities of fetal mortality. Public Health Rep. 1962;77(10):835-848. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1915016/pdf/pubhealthreporig00094-0013.pdf
10. American College of Obstetricians and Gynaecologists. Practice bulletin number 150: early pregnancy loss. 2015 [cited 2018 January 6]. Available from https://m.acog.org/Clinical-Guidance-and-Publications/Practice-Bulletins/Committee-on-Practice-Bulletins-Gynecology/Early-Pregnancy-Loss
11. Edmonds DK, Lindsay KS, Miller JF, Williamson E, Wood PJ. Early embryonic mortality in women. Fertil Steril. 1982;38(4):447-453. doi:10.1016/S0015-0282(16)46579-9
12. Rees H, Katzenellenbogen J, Shabodien R, et al. The epidemiology of incomplete abortion in South Africa. S Afr Med J. 1997;87(4):432-437. Available from https://www.ajol.info/index.php/samj/article/view/157185/146796
13. Dhaded SM, Somannavar MS, Jacob JP, et al. Early pregnancy loss in Belagavi, Karnataka, India 2014-2017: a prospective population-based observational study in a low-resource setting. Reprod Health. 2018;15(suppl1):15-22. doi:10.1186/s12978-018-0525-4
14. Nybo Anderson A, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal age and fetal loss: population based register linkage study. BMJ. 2000;320:1708-1712. doi:10.1136/bmj.320.7251.1708
15. Cleary-Goldman J, Fergal M, Vidaver J, et al. Impact of maternal age on obstetric outcome. Obstet Gynecol. 2005;105(5):983-990. doi:10.1097/01.AOG.0000158118.75532.51

16. de La Rochebrochard E, Thonneau P. Paternal age and maternal age are risk factors for miscarriage; results from a multicentre European study. Hum Reprod. 2002;17(6):1649-1656. doi:10.1093/humrep/17.6.1649
17. Naylor AF, Warburton D. Sequential analysis of spontaneous abortion. II. Collaborative study data show that gravidity determines a very substantial rise in risk. Fert Ster. 1979;31(3):282-286. doi:10.1016/S0015-0282(16)43875-6
18. Regan L, Braude PR, Trembath PL. Influence of past reproductive performance on risk of spontaneous abortion. BMJ. 1989;299:541-545. doi:10.1136/bmj.299.6698.541
19. Kesmodel U, Wisborg K, Olsen SF, Henriksen TB, Secher NJ. Moderate alcohol intake in pregnancy and the risk of spontaneous abortion. Alcohol Alcohol. 2002;37(1):87-92. doi:10.1093/alcalc/37.1.87
20. Rasch V. Cigarette, alcohol, and caffeine consumption: risk factors for spontaneous abortion. Acta Obstet Gynecol Scand. 2003;82(2):182-188. doi:10.1080/j.1600-0412.2003.00078.x
21. Peck JD, Leviton A, Cowan LD. A review of the epidemiological evidence concerning the reproductive health effects of caffeine consumption: a 2000-2009 update. Food Chem Toxicol. 2010;48(10):2549-2576. doi:10.1016/j.fct.2010.06.019
22. Lindbohm M, Sallmen M, Taskinen H. Effects of exposure to environmental tobacco smoke on reproductive health. Scand J Work Environ Health. 2002;28(suppl2):84-96. Available from https://www.jstor.org/stable/40967257
23. Salim R, Regan L, Woelfer B, Backos M, Jurkovic D. A comparative study of the morphology of congenital uterine anomalies in women with and without a history of recurrent first trimester miscarriage. Hum Reprod. 2003;18(1):162-166. doi:10.1093/humrep/deg030

24. Regan L, Rai R. Epidemiology and the medical causes of miscarriage. Baillieres Clin Obstet Gynaecol. 2000;14(5):839-854. doi:10.1053/beog.2000.0123
25. Hanson U, Persson B, Thunell S. Relationship between haemoglobin A_{1c} in early type 1 (insulin-dependent) diabetic pregnancy and the occurrence of spontaneous abortion and fetal malformation in Sweden. Diabetologia. 1990;33(2):100-104. doi:10.1007/BF00401047
26. Mills JL, Simpson JL, Driscoll SG, et al. Incidence of spontaneous abortion among normal women and insulin-dependent diabetic women whose pregnancies were identified within 21 days of conception. N Engl J Med. 1988;319(25):1617-1623. doi:10.1056/NEJM198812223192501
27. Bussen S, Sutterlin M, Steck T. Endocrine abnormalities during the follicular phase in women with recurrent spontaneous abortion. Hum Reprod. 1999;14(1):18-20. doi:10.1.93/humrep/14.1.18
28. Abalovich M, Gutierrez S, Alcaraz G, Maccallini G, Garcia A, Levalle O. Overt and subclinical hypothyroidism complicating pregnancy. Thyroid. 2004;12(1). doi:10.1089/105072502753451986
29. Rai R, Backos M, Rushworth F, Regan L. Polycystic ovaries and recurrent miscarriage-a reappraisal. Hum Reprod. 2000;15(3):612-615. doi10.1093/humrep/15.3.612
30. Clifford K, Rai R, Watson H, Franks S, Regan L. Does suppressing luteinising hormone secretion reduce the miscarriage rate? Results of a randomised control trial. BMJ. 1996;312:1508-1511. doi:10.1136/bmj.312.7045.1508
31. Wilson WA, Gharavi AE, Koike T, et al. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome. Arthritis Rheum. 1999;42(7):1309-1311. doi:10.1002/1529-0131(199907)42:7<1309::AID-ANR1>3.0CO:2-F

32. Rai RS, Clifford K, Cohen H, Regan L. High prospective fetal loss rate in untreated pregnancies of women with recurrent miscarriage and antiphospholipid antibodies. Hum Reprod. 1995;10(12):3301-3304. doi:10.1093/oxfordjournals.humrep.a135907
33. Rey E, Kahn SR, David M, Shrier I. Thrombophilic disorders and fetal loss: a meta-analysis. Lancet. 2003;361(9361):901-908. doi:10.1016/S0140-6736(03)12771-7
34. Kovalevsky G, Gracia CR, Berlin JA, Sammel MD, Barnhart KT. Evaluation of the association between hereditary thrombophilias and recurrent pregnancy loss: a meta-analysis. Arch Intern Med. 2004;164(5):558-563. doi:10.1001/archinte.164.5.558
35. Ford HB, Schust DJ. Recurrent pregnancy loss: etiology, diagnosis and therapy. Rev Obstet Gynecol. 2009;2(2):76-83. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2709325/
36. Clifford K, Rai R, Watson H, Regan L. Pregnancy: an informative protocol for the investigation of recurrent miscarriage: preliminary experience of 500 consecutive cases. Hum Reprod. 1994;9(7):1328-1332. doi:10.1093/oxfordjournals.humrep.a138703
37. Salim D, Stephenson MD. Frequency of factors associated with habitual abortion in 197 couples. Fertil Steril. 1996;66(1):24-29. doi:10.1016/S0015-0282(16)58382-4
38. van den Berg MM, van Maarle MC, van Wely M, Goddijn M. Genetics of early miscarriage. Biochim Biophys Acta Mol Basis Dis. 2012;1822:1951-1959. doi: 10.1016/j.bbadis.2012.07.001.
39. Zhang YX, Zhang YP, Gu Y, et al. Genetic analysis of first-trimester miscarriages with a combination of cytogenetic karyotyping, microsatellite genotyping and

arrayCGH. Clin Genet. 2009;75(2):133-140. doi:10.1111/j.1399-0004.2008.01131.x

40. Stephenson MD, Awartani KA, Robinson WP. Cytogenetic analysis of miscarriages from couples with recurrent miscarriage: a case-control study. Hum Reprod. 2002;17(2):446-451. doi:10.1093/humrep/17.2.446
41. de Jong G, Gebhardt GS. Genetics. In: Kruger TF, Botha MH, editors. Clinical Gynaecology. 4th ed. Cape Town: Juta, 2011; p.78-90.
42. Griffiths AJ, Gelbart WM, Miller JH, et al. Chromosomal rearrangements. In: Modern Genetic Analysis. New York: WH Freeman, 1999. Available from https://www.ncbi.nlm.nih.gov/books/NBK21367/
43. Hassold T, Chen N, Funkhouser J, et al. A cytogenetic study of 1000 spontaneous abortions. Ann Hum Genet. 1980;44:151-78. doi: 10.1111/j.1469-1809.1980.tb00955.x
44. Philip T, Philip K, Reiner A, Beer F, Kalousek DK. Embryoscopic and cytogenetic analysis of 233 missed abortions: factors involved in the pathogenesis of developmental defects of early failed pregnancies. Hum Reprod. 2003;18(8):1724-1732. doi:10.1093/humrep/deg309
45. Sullivan AE, Silver RM, LaCoursiere DY, Porter TF, Branch DW. Recurrent fetal aneuploidy and recurrent miscarriage. Obstet Gynecol. 2004;104(4):784-788. doi:10.1097/01AOG.0000131832.68727.e2
46. Shen J, Wu W, Gao C, et al. Chromosomal copy number analysis on chorionic villus samples from early spontaneous miscarriages by high throughput genetic technology. Mol Cytogenet. 2016;9(1):1-7. doi:10.1186/s13039-015-0210-z
47. Kajii T, Ohama K, Niikawa N, Ferrier A, Avirachan S. Banding analysis of abnormal karyotypes in spontaneous abortion. Am J Hum Genet. 1973;25(5):539-547. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1762642/
48. Lomax B, Tang S, Separovic E, et al. Comparative genomic hybridisation in combination with flow cytometry improves results of cytogenetic analysis of

spontaneous abortions. Am J Hum Genet. 2000;66(5):1516-1521. doi:10.1086/302878

49. Bell KA, Van Deerlin PG, Haddad BR, Feinberg RF. Cytogenetic diagnosis of "normal 46,XX" karyotypes in spontaneous abortions frequently may be misleading. Fertil Steril. 1999;71(2):334-341. doi:10.1016/S0015-0282(98)00445-2
50. Tepperberg J, Pettenati MJ, Rao PN, et al. Prenatal diagnosis using interphase fluorescence *in situ* hybridization (FISH): 2-year multi-centre retrospective study and review of the literature. Prenat Diagn. 2001;21(4):293-301. doi:10.1002/pd.57
51. Shinawi M, Cheun SW. The array CGH and its clinical applications. Drug Discov Today. 2008;13(17):760-770. doi: 10.1016/j.drudis.2008.06.007
52. de Ravel TJ, Devriendt K, Fryns JP, Vermeesch JR. What's new in karyotyping? The move towards array comparative genomic hybridisation (CGH). Eur J Pediatr. 2007;166(7):637-643. doi:10.1007/s00431-007-0463-6
53. Rickman L, Fiegler H, Shaw-Smith C, et al. Prenatal detection of unbalanced chromosome rearrangements by array CGH. J Med Genet. 2006;43(4):353-361. doi:10.1136/jmg.2005.037648
54. Fritz B, Hallermann C, Olert J, et al. Cytogenetic analyses of culture failures by comparative genomic hybridisation (CGH) – Re-evaluation of chromosome aberration rates in early spontaneous abortions. Eur J Human Genet. 2001;9:539-547. doi:10.1038/sj.ejhg.5200669
55. Menten B, Swerts K, Delle Chiaie B, et al. Array comparative genomic hybridization and flow cytometry analysis of spontaneous abortions and mors *in utero* samples. BMC Med Genet. 2009;10:89-93. doi:10.1186/1471-2350-10-89
56. Dhillon RK, Hillman SC, Morris RK, et al. Additional information from chromosomal microarray analysis (CMA) over conventional karyotyping when

diagnosing chromosomal abnormalities in miscarriage: a systematic review and meta-analysis. Br J Obstet Gyneacol. 2013;121(1):11-21. doi:10.1111/1471-0528.12382

57. American College of Obstetricians and Gynaecologists. Committee opinion number 446: array comparative genomic hybridization in prenatal diagnosis. Obstet Gynecol. 2009;114(5):1161-1163. doi:10.1097/AOG.0b013e3181c33cad
58. Schaeffer AJ, Chung J, Heretis K, Wong A, Ledbetter DH, Martin CL. Comparative genomic hybridization-array analysis enhances the detection of aneuploidies and submicroscopic imbalances in spontaneous miscarriages. Am J Hum Genet. 2004;74(6):1168-1174. doi:10.1086/421250
59. Rajcan-Separovic E, Diego-Alvarez D, Robinson WP, et al. Identification of copy number variants in miscarriages from couples with idiopathic recurrent pregnancy loss. Hum Reprod. 2010;25(11):2913-2922. doi:10.1093/humrep/deq202
60. Viaggi CD, Cavani S, Malacarne M, et al. First-trimester euploid miscarriages analysed by array-CGH. J Appl Genet. 2013;54(3):353-359. doi:10.1007/s13353-013-0157-x
61. Statistics South Africa. Mid-year population estimates 2017. July 2018 [cited 2018 October 8]. Available from https://www.statssa.gov.za/publications/P0302/P03022017.pdf.
62. Nayak S, Pavone ME, Milad M, Kazer R. Aneuploidy rates in failed pregnancies following assisted reproductive technology. J. Womens Health. 2011;20(8):1239-1243. doi:10.1089/jwh.2010.2648
63. Shields LE, Serafini PC, Schenken RS, Moore CM. Chromosomal analysis of pregnancy losses in patients undergoing assisted reproduction. J Assist Reprod Genet. 1992;9(1):57-60. doi:10.1007/BF01204116

64. Causio F, Fischetto R, Sarcina E, Geusa S, Tartagni M. Chromosome analysis of spontaneous abortions after in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI). Eur J Obstet Gynaecol Reprod Biol. 2002;105(1):44-48. doi:10.1016/S0301-2115(02)00151-3
65. Lathi RB, Westphal LM, Milki, A.A. Aneuploidy in the miscarriages of infertile women and the potential benefit of preimplantation genetic diagnosis. Fertil Steril. 2008;89(2):353-357. doi:10.1016/j.fertnstert.2007.02.040
66. Wu T, Yin B, Zhu Y, et al. Molecular cytogenetic analysis of early spontaneous abortions conceived from varying assisted reproductive technology procedures. Mol Cytogenet. 2016;9:79. doi:10.1186/s13039-016-0284-2
67. Massalska D, Zimowski JG, Bijok J, et al. First trimester pregnancy loss: clinical implications of genetic testing. J Obstet Gynaecol Res. 2017;43(1):23-29. doi:10.1111/jog.13179
68. Jenderny J. Chromosome aberrations in a large series of spontaneous miscarriages in the German population and review of the literature. Mol Cytogenet. 2014;7:38. doi:10.1186/1755-8166-7-38.
69. Reddy KS. Double trisomy in spontaneous abortions. Hum Genet. 1997;101(3):339-345. doi:10.1007/s004390050638.

11. APPENDICES

APPENDIX A: DATA SHEET

DATA CAPTURE SHEET

Use of CGH-array to detect chromosome abnormalities in first trimester miscarriage

Dr R Orfanidis

Study Number	

Demographic Information			
Maternal	Age		
	Race		
	Occupation		
Paternal	Age		
	Race		
	Occupation		

Clinical Information		
Parity		
Gravidity		
Gestation		
No. of Previous Miscarriages		
Tick one	Sporadic	
	Recurrent	

Method of Conception		
Tick one	Spontaneous	
	Ovulation induction timed intercourse	

Ovulation induction IUI	
IVF	
ICSI	
GIFT/ZIFT	

Risk Factors		
Tick all that apply	Uterine or tubal pathology	
	Smoking	
	Infections	
	HIV	
	Hepatitis B and C	
	Rubella	
	Endocrine disorders	
	Diabetes	
	Thyroid disorder	
	Autoimmune disorders	
	Antiphosp-holipid syndrome	
	Other	
	Specify	

Reproductive History		
Tick one	Primary infertility	
	Secondary infertility	

Multiple gestation?		
Tick one	Singleton	
	Twin gestation	
	Higher order multiples	
	Specify	

Results of CGH-Array

Result obtained		Yes	No
Indeterminate result		Yes	No
	Reason		
Test failure		Yes	No
	Reason		

No abnormality detected		Yes	No
Tick one		46 XX	
		46 XY	

Abnormality/ies detected	Yes	No
Specify		

APPENDIX B: EXTRACT FROM VITALAB CONSENT FORM



The Inner Circle, 159 Rivonia Road, Morningside, 2196 | P.O. Box 652837, Benmore, 2010
Tel: (011) 911 4700 or 0861 882522 | Fax: 0865100951
www.vitalab.com | info@vitalab.com

19. We understand that it may be necessary to produce a semen (sperm) sample to be frozen. This will be used for back-up purposes and will be thawed on the day of oocyte retrieval. If it is not required, it will be discarded after the procedure.

20. We understand that medical aid coverage for any or all of the above procedures might not be available and that we are personally responsible for the expenses incurred during treatment. These expenses have been discussed with us by the competent persons.

21. We understand that we can choose to discontinue participation in the programme at any time, provided we have notified the authorised institution of our written consent thereto. We also understand that, should we decide to discontinue participation in the programme, we will be responsible for all expenses during the period prior to discontinuation and the relevant treatments incurred.

22. We understand that this consent extends from the start of the treatment programme until the treatment is completed, or until we decide to discontinue participation. This consent is binding, and required for participation in subsequent treatment programmes.

23. We understand that, should the results of our treatment and/or any aspect of it be published in medical or scientific journals, all possible precautions will be taken to protect our anonymity. We grant permission to the medical team to publish in professional journals relating to our case, provided our names are withheld.

24. In light of having selected either one or more of the processes listed at A – C on page 1 of this consent, we wish to commence with treatment:

    24.1. We consent that all information supplied by us in connection with the removal and withdrawal of our gamete(s) be updated in the central data bank;

    24.2. We have previously made a donation of our sperm / eggs. Yes ☐ No ☐

    Initials: _____ _____

    24.2.1. If yes, please fill in the details below.

    In the event that we have previously made a donation of our gamete(s), such donation took place on the _____ (date) at _____ (place).

    Initials: _____ _____

    24.3. We consent to a competent person of the authorised institution to perform:

    24.3.1. A physical examination and a medical history questionnaire;

APPENDIX C: ETHICS CLEARANCE CERTIFICATE

R14/49 Dr Rachelle Orfanidis

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M161145

NAME: (Principal Investigator)	Dr Rachelle Orfanidis
DEPARTMENT:	Obstetrics and Gynaecology Vitalab Fertility Centre
PROJECT TITLE:	Use of CGH-array to Detect Chromosome Abnormalities in First Trimester Miscarriage
DATE CONSIDERED:	25/11/2016
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr M. Bothma, Dr Y. Unterslak and Dr Y. Knezovich
APPROVED BY:	Professor P. Cleaton-Jones,Chairperson, HREC (Medical)
DATE OF APPROVAL:	29/03/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floo Senate House/2nd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand th the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake t ensure compliance with these conditions. Should any departure be contemplated,from the research protocol a approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date fc annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. i this case, the study was initially review November and will therefore be due in the month of November each yea Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX D: TURN IT IN REPORT

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