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# ReproGenQ

Volume 01, (September 2020)



## APPLIED GENETICS SPECIAL INTEREST GROUP

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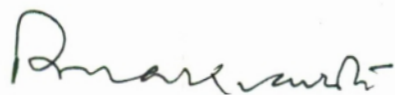
### FOREWORD

I wish to congratulate the team of SIG Applied Genetics of IFS at the release of the first of their series of newsletter.

Integration of the of the field of genetics as an integral part of today's reproductive practice has become imperative due to increase in the burden of reproductive disorders.

Advances in molecular diagnostic techniques relevant to infertility and assisted reproductive techniques (next generation sequencing and microarray), advent of NIPS/NIPT, preimplantation genetic screening/diagnosis (PGD/PGS) to PGT-a for aneuploidy and non-invasive PGT-a, epigenetic studies to genome editing etc. has further increased this drive and expectations of public.

This introduction to the field of Applied Genetics to our clinicians can try to bridge the interface between clinics and diagnostics in the field of Reproductive Sciences.



(Dr B N CHAKRAVARTY)

Director





I am privileged to acknowledge the first newsletter of SIG team of Applied Genetics.

At its core, reproductive medicine attempts to explain how human life is created and how it develops throughout pregnancy. The role of genetic testing to guide medical decision making in this regard is sizable and will likely continue to grow in the future.

Genetic techniques are used in medicine to diagnose and treat inherited human disorders. Knowledge of a family history of various disorders may indicate a hereditary tendency to develop these afflictions. Cells from embryonic tissues can reveal certain genetic abnormalities. Intended for readers with a background in fertility medicine as well as those less familiar with IVF, this comprehensive newsletter presents an update on role of cytogenetics and recurrent pregnancy loss.



Based on this understanding, therapies are developed and used to maximize outcomes. Specifically, increased pregnancy rates, decreased incidence of obstetric complications and miscarriage, and the avoidance of fetuses affected by birth defects or other deficiencies are the stated goal of much of the current research in reproductive medicine.

Knowledge about genetic disease among gynecologists, obstetricians and the general public will help to create appropriate awareness and address personal benefits of screening in a non-directive manner.

I would like to congratulate Applied Genetics SIG Team for the future endeavor.

**Long live IFS!**

**Prof. Sudha Prasad**

President

Indian Fertility Society

It gives me great pleasure to introduce the first of the series of newsletters named "ReproGenQ" from the recent special interest group (SIG) of Applied Genetics.

Over the last decade there has been an exponential development in the field of genomics, including novel diagnostics and therapies relevant to infertility and assisted reproduction techniques. These have expanded beyond male and female infertility, pre-implantation genetic testing for aneuploidy (PGT-a) to non-invasive PGT-a, issues related to mitochondrial replacement in human oocytes and to cross-generational epigenetic inheritance or germline genome editing (GGE) technologies. All this has brought a paradigm shift in our understanding relevant to the practice of ART.

The interface of Genetics and Assisted Reproductive Techniques is well recognised and the SIG of Applied genetics is making all efforts to bring academics to our members through newsletters and CMEs.

The enthusiastic team of this young SIG of Applied Genetics is out with its first news-letter with many more in the offing. I wish their endeavours success promising hoping to have many such newsletters in their academic tenure.

Good wishes

**Dr Neena Malhotra**

General Secretary

Indian Fertility Society





It is my pleasure and privilege to present to you the first issue of newsletter "ReproGenQ" of the Special Interest Group on Applied Genetics.

This SIG is an initiative which aims to promote a forum for discussion and exchange of information related to different aspects of genetics and epigenetics of human reproduction, be it natural or assisted.

The field of reproductive genetics is gaining importance with the identification of genetic factors responsible for infertility, abortion, stillbirth, malformation and cancer. Its study plays an important role in predicting and preventing a disorder thus, trying to decrease the burden of disease right from the initial stages of family planning. The use of molecular analysis in this field has advanced exponentially and is still fragmented and cumbersome.

The last few decades have witnessed these striking advancements in reproductive and laboratory medicine that has caused these two fields to become intricately connected. This newsletter is an endeavour to focus on the role of reproductive genetics and to bridge the interface between clinical and applied sciences and to enhance the insights on when, what and how of diagnostics.

We are blessed to have a team of distinguished experts in the field of genetics and their experience and enthusiasm drives us to address a broad spectrum of topics in reproductive genetics.

#### **Dr Sarabpreet Singh**

Convenor

Special Interest Group - Applied Genetics



I am immensely happy to learn that SIG, Indian Fertility Society is releasing the newsletter "ReproGenQ".

This is an important newsletter, which will address the issues related to reproductive health of women and men with focus on infertility and assisted reproduction. I hope that the newsletter will provide a platform for dissemination of knowledge amongst various scientists and clinicians. The commentaries and perspectives of the experts from various disciplines will help in understanding the problems related to reproductive health so that scientific societies in future will come out with a strategic plan to resolve these problems.

My wishes to the editorial team for the release.

#### **Dr Rakesh Kumar**

Co-Convenor

Special Interest Group - Applied Genetics

On behalf of **Special Interest Group: Applied Genetics**, it gives me immense pleasure and pride in presenting to you the first issue of newsletter "**ReproGenQ**". Similar to IQ, **ReproGenQ** signifies extent of our applied knowledge in the field of reproductive genetics. The need of hour is to get current updates of cutting edge research and to transfer the gained knowledge into customized clinical care in the form of comprehensive genetic counseling and testing. Genomics and genomic based technologies will keep evolving and influencing the pre and post-conception outcomes. The focus of this newsletter is to highlight clinical application of genomics in the field of infertility, preimplantation and prenatal diagnosis. To begin with, this very first part pen down mix of notes touching the basic and molecular concepts of some fertility issues.

I wish the readers an enlightened journey through ReproGenQ!

**Dr. Mona Sharma**

Editor

ReproGenQ



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- He is trained in reproductive biology and human molecular genetics
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- She did her graduation and post graduation (MD) from GMCH, Chandigarh
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- Her research is based on assessing the role of sperm RNA as well as genomic integrity and overwhelming oxidative stress as a critical determinant of embryo viability in recurrent pregnancy loss and recurrent implantation failure patients
- She has received CMC In-Training Travel Award from ASRM (2019), Lalor Foundation Award from ASA in 2019 and 2016 and travel awards from ICMR and CSIR



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- In her department, she is coordinating Andrology Lab Services and Animal IVF Research Laboratory
- Her ongoing research activities are related to preeclampsia, premature ovarian failure, polycystic ovarian syndrome, sperm and oocyte contributing factors in fertilization
- She has authored numerous research publications and has co-edited an Andrology textbook as well
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- Received PhD in the area of Reproductive Genetics from All India Institute of Medical Sciences (AIIMS), New Delhi while working on topic "Chromosome Aneuploidies & Mosaicism in Preimplantation Embryos."
- Professional Experience of more than 12 years with special interest in the area of Prenatal Diagnosis & Male Infertility, , Recurrent Pregnancy Loss (RPL), Preimplantation Genetics Screening (PGS), Clinical Embryology Cytogenetics & Molecular Cytogeneomics
- With more than 25 publications in indexed National & International journals, chapters, scientific presentations as both oral, poster in conferences & demonstrator at national level workshops
- He has experience and worked in various capacity leading teams at hospitals like AIIMS, Indraprastha Apollo Hospitals, Quest Diagnostics, ESIC Hospitals, New Delhi

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- Dr. Sam has a PhD in Human Genetics and a Post-Doctoral Fellowship from NIMHANS, Bangalore. He has authored 5 papers in well-known international journals as well as co-authored chapter in International book
- At ECGI, Dr. Balu is coordinating clinical reporting operations as well as overseeing the prenatal clinical genetic tests such as qNIPS, NIPS, PGS and microarray testing and reporting
- He has more than 9 years of experience in the field of human genetics
- He was a part of the PGS/ PGD domain right from its inception. He played a key role in developing and validating the PGS test using NGS technology
- He has also helped develop custom PGD testing for various single gene disorders



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- Field of Interest - Clinical application of PGT & PNGT



## Recurrent Pregnancy Loss (RPL): A Clinical Geneticist Perspective

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### ABSTRACT

Spontaneous loss of pregnancy is one of the most common complications of pregnancy. Random numeric chromosome abnormalities are the single most common cause of pregnancy loss of sporadic losses before 10 weeks of gestation. There are recommendations from different international professional bodies based on evidence from the published studies regarding evaluation and management of recurrent pregnancy loss (RPL). The present diagnosis and management of RPL can be done only in 50% of patient while remaining patients had to categorize under false idiopathic category with no diagnosis. Most of professional societies suggest chromosomal evaluation of product of conception (POC) samples as part of the clinical management of couples with pregnancy loss. POC Microarray along with ASRM RPL work-up can identify causes of miscarriage in majority of cases and it's a better technology as compared to POC Karyotype in terms of diagnostic yield. The genetic evaluation of RPL work-up can be started by offering couple karyotype to rule out balanced translocation with two or more failed pregnancies in first trimester followed by in-vitro fertilization (IVF) and preimplantation genetic screening (PGS).

### INTRODUCTION

Spontaneous loss of pregnancy is one of the most common complications of pregnancy. It's been estimated that approximately 70% of all human conceptions fails to achieve viability and approximately 15-20% of clinically recognized pregnancies ends up as pregnancy loss with approximately 5% of them experiences two consecutive miscarriages.<sup>1,2,3</sup>

The exact prevalence of RPL is difficult to estimate but few studies show 1-2% of women affected with RPL.<sup>4</sup> The burden of recurrent miscarriages in India is higher as compared to reported literature worldwide i.e. around 7.4%.<sup>5</sup>

Clinically Early Pregnancy Loss is defined as a non-viable, intrauterine pregnancy with either an empty gestational sac or a gestational sac

containing an embryo or fetus without fetal heart activity within the first 13 weeks of gestation. Whereas Pregnancy Loss (miscarriage) is the spontaneous demise of a pregnancy before the fetus reaches viability from time of conception until 24 weeks of gestation. RPL is assigned to a state when there is loss of two or more failed pregnancies in first trimester.<sup>6,7</sup>

### ETIOLOGY OF RPL

At present there are many studies that have reported various etiologies in RPL patients including genetic abnormalities, uterine anatomic abnormalities, antiphospholipid syndrome, endocrine abnormalities, infections, hormonal or metabolic disorders, sperm quality and life style related disorders (Figure 1). There are also recommendations from different international professional bodies (ASRM, ESHRE, ACOG) based on evidence from the published studies regarding evaluation and management of RPL (Table 1).<sup>6,7</sup> At present diagnosis and management of RPL can be done only in 50% of patient while remaining patients had to be categorized under false idiopathic category with no diagnosis.

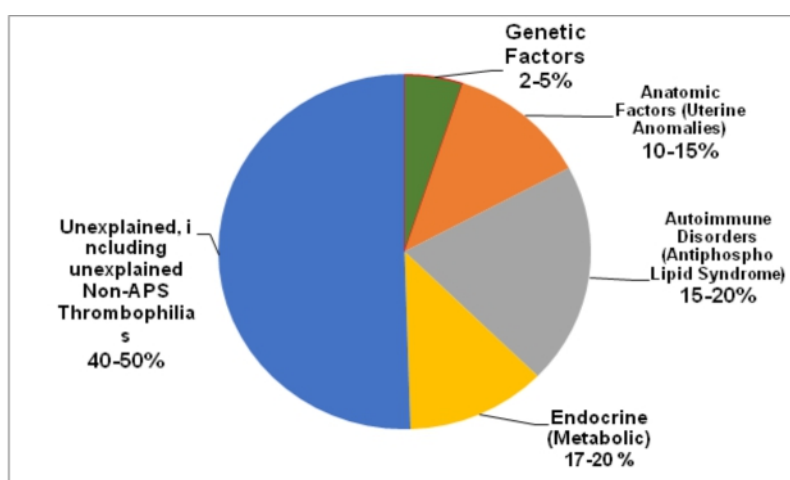


Figure 1: Pie-chart showing various etiologies observed in RPL patients<sup>13</sup>

### CHALLENGES IN MANAGEMENT OF RPL

- Differentiation between sporadic miscarriages from RPL.
- Accuracy of self-reported loss by patient may not be accurate.
- Current investigations and interventions recommended by guidelines are with evidences of low, very low & moderate quality.
- Treatment interventions in idiopathic (50%) cases.
- Counseling is difficult in patient with no definitive diagnosis and with no definitive cause of pregnancy loss.

### COMMON GENETIC ABNORMALITIES IN RPL

Random chromosome abnormalities are the single most common cause of pregnancy loss of sporadic losses before 10 weeks of gestation and are due to numeric chromosome abnormalities (>60%) including whole chromosome aneuploidies specifically trisomy, monosomy and polyploidy.<sup>6,8-10</sup> It can be further summarized that pregnancy loss can be due to (a) Numerical chromosomal abnormalities i.e. trisomy, monosomy (60-80%) (b) Structural chromosomal abnormalities, translocations or inversions (2-5%), (c) Polyploidy including triploidy or tetraploidy (20%) arising due to aberrant fertilization. The most common structural chromosome abnormality are balanced



translocation that could be either robertsonian translocation (within same chromosome) or reciprocal translocation (involving two different chromosome) and is observed in 2-5% of couples with recurrent miscarriage. The most common hypothesis proposed for aneuploidy is random error in segregation or non-disjunction of chromosome in

meiosis I or II during embryogenesis or germ cell development. In recent years due to advancement of genetic technologies there are various methodologies utilized for examination of embryonic/fetal material (POC) and advantages and disadvantages of all technologies are been summarized in Table 2.

**Table 1: Summary of current recommendations from different professional bodies for genetic evaluation of RPL**

Professional	Current Recommendations	Evidence
<b>ESHRE<sup>7</sup></b>	Array based comparative genomic hybridization (array-CGH) or microarray is recommended based on a reduction in maternal cell contamination.	<b>Strong</b>
	Genetic analysis of pregnancy tissue though is not routinely recommended in RPL but it could be performed for explanatory purposes.	<b>Conditional</b>
	Parental Karyotyping at present not routinely recommended in couple with RPL, but it could be carried out after individuals risk assessment.	<b>Conditional</b>
<b>ASRM<sup>6</sup></b>	Peripheral karyotyping is preferred for parents for detection of balanced structural chromosome abnormalities.	<b>Strong</b>
	Karyotype analysis of POC may be useful in the setting of ongoing therapy of RPL but there is possibility of maternal tissue contamination in the specimen	<b>Conditional</b>
<b>ACOG/SMF M<sup>12</sup></b>	In cases of intra uterine fetal demise or still birth further cytogenetic analysis is desired, Chromosomal microarray analysis on the fetal tissue (i.e. amniotic fluid, placenta, or products of conception) is recommended in the evaluation with increased likelihood of obtaining results and improved detection of causative abnormalities	<b>Recommended</b>

<sup>6</sup>ESHRE: European Society of Human Reproduction & Embryology; <sup>7</sup>ASRM: American Society of Reproductive Medicine; <sup>12</sup>ACOG: The American College of Obstetricians and Gynecologists, SMFM: Society of Maternal Fetal Medicine.

**Table 2: Comparison between some of the common technologies used for examination of embryonic/fetal material (product of conception)**

Method Characteristics (Technique)	Microarray (Comparative Genomic Hybridization)	Karyotype (Conventional Culture technique)	FISH (Fluorescence in-situ Hybridization)	QF-PCR (Quantitative Fluorescent-Polymerase chain Reaction)	NGS (Next Generation Sequencing)
<b>Detect</b>	Chromosome abnormalities (aneuploidies, triploidy), Unbalanced structural changes (duplication, deletion, amplification)	Changes in chromosome number (aneuploidies, polyploidy) Structural Abnormalities (balanced & unbalanced translocation)	Chromosome aneuploidies Diagnosis of sub-microscopic chromosome aberration, Structural translocation	Detect aneuploidies for chromosome 13, 18, 21, X, Y, 15, 16 and 22	Sequencing of large genomic regions, high number of genes with high throughput
<b>Samples Type</b>	Fresh Tissue, FFPE Block	Fresh Tissue, culture cells	Fresh Tissue, uncultured (interphase) cells	Fresh Tissue/ DNA	Fresh/ FFPE
<b>Limitation</b>	Cannot detect unbalanced translocation & low level of mosaicism (<10%),	The requirement of culture of cells, (high culture failure rate 10-20%)	Diagnosis specific and limited to probes utilized in kit	Diagnosis within intended use of kit only	Very high sensitivity, excess of information, uninterpretable for diagnosis the genetic cause
<b>Culture Failures</b>	No	Yes	No	No	No
<b>Diagnostic Yield</b>	High (100-400 kb)	Low (5- 10 MB)	Moderate (100-200 Kb)	Moderate	Very High (<50Kb)
<b>Maternal Cell Contamination (MCC)</b>	No	Yes	Yes	No	No
<b>Turn Around Time (TAT) for getting results</b>	10-12 days	14-21 days	24-48 hours	< 24 hours	21-28 days
<b>Recommended by Guidelines</b>	Yes	Yes	No	No	No
<b>Cost (INR)</b>	15000-18000	7000-8000	5000-7000	4000-6000	20000-25000

Kilobases (Kb), MB (Megabase), FFPE (Fresh Frozen Paraffin Embedded Tissue)

## MICROARRAY

Chromosomal microarray analysis (CMA) is method of choice for measuring gains and losses of DNA throughout the human genome. Microarray can also identify chromosomal aneuploidy, submicroscopic abnormalities, and large structural abnormalities of chromosomes. Results obtained are expressed as Copy Number Variants (CNVs) that is defined as duplicated or deleted segments of DNA of at least 1000 base pairs (1 Kb) in size with difference from reference genome. CNVs

obtained are expressed as:

- **Pathogenic:** CNV of clinical significance detected; 15% genetic disease burden
- **Nonpathogenic:** CNV of no clinical significance
- **Variants of uncertain significance (VOUS):** CNV of uncertain significance

One of the latest study identified probable cause of pregnancy loss in majority of RPL patients by evaluation of POC through 24-chromosome pair microarray along with standard RPL workup by ASRM guidelines<sup>11</sup>. It was concluded that evaluation of POC using 24-chromosome microarray analysis adds significantly to the existing ASRM guidelines recommended for RPL evaluation. It was also concluded that all couples with RPL should be offered genetic evaluation on miscarriage tissue obtained at the time of the second and subsequent pregnancy losses. There was a testing algorithm proposed in combination of a genetic evaluation that will identify a probable or definitive cause of RPL in over 90% of miscarriages (Figure 2).

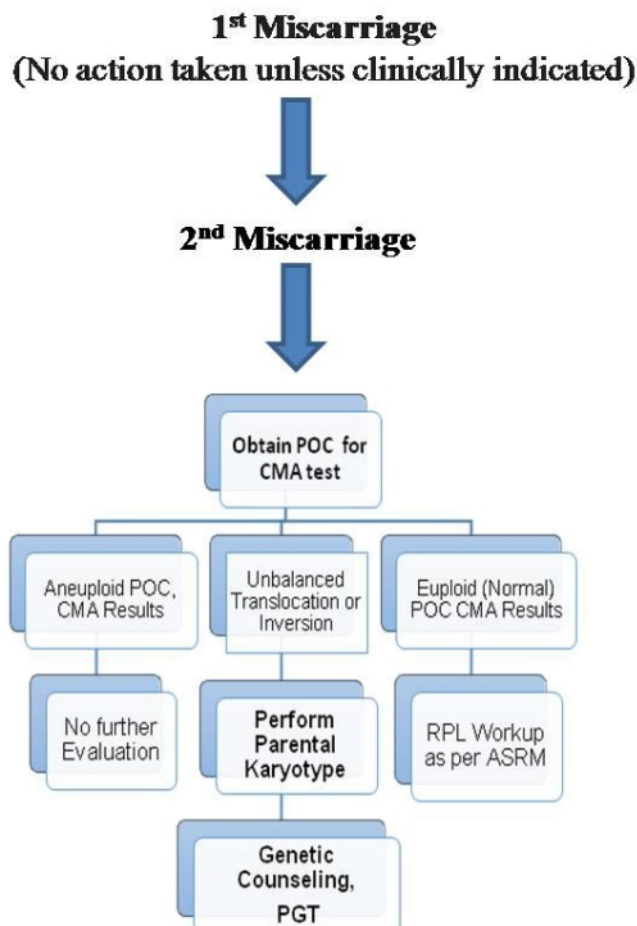


Figure 2: Algorithm for recurrent pregnancy loss (RPL) evaluation and treatment based on chromosome microarray analysis (CMA) <sup>11</sup>.

## CONCLUSION

Most of the professional bodies including ACOG, ASRM, RCOG and ESHRE advocate chromosomal evaluation of POC samples as part of the clinical management of couples with RPL. This helps in identifying 50-60% of women having pregnancy loss due to gross chromosomal abnormality. CMA prevents patients from undergoing unnecessary costly investigations whereas negative results should be followed with routine RPL work as suggested by ASRM guidelines. POC Microarray along with ASRM RPL work-up can identify causes of miscarriage in majority of cases and it is a better technology as compared to POC Karyotype in terms of diagnostic yield. The genetic evaluation of RPL work-up can be started by offering couple karyotype to rule out balanced translocation after two or more failed pregnancies in first trimester followed by in-vitro fertilization and preimplantation genetic screening. Genetic counseling should be offered to couples along with informed consent explaining the advantages and limitations for any genetic investigation. Routine genetic analysis for inherited thrombophilia is not recommended in women with RPL unless indicated or if family history exists.

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## Role of Cytogenetics in Infertility

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### ABSTRACT

Chromosome analysis is a progressively evolving and an essential technique in the field of medicine. The altered chromosomal arrangement is considered as genetic disorder but not very often the recognition of this altered arrangement is clearly made. Unbiased identification of the target chromosome or finding of the composite karyotypes is imperative in clinical diagnosis. Fluorescence in situ hybridization (FISH) is an appropriate cytogenetic technique as it provides a crucial opportunity in the instant evaluation of chromosomal aneuploidies in stagnant cells. Here we review the universal beliefs of the chromosomal malformations and the cytogenetic procedures which assist in the detection of chromosomal abnormalities in infertile patients.

### INTRODUCTION

Presently, cytogenetics is an emerging molecular technique for interpreting the numerical and structural arrangement of chromosomes. Traditional cytogenetics applying normal banded chromosomal analysis stays as an easy and widespread method to get an outline of the human genome.<sup>1</sup> For identification of genetic disorders in infants the regular banded karyotype evaluation can currently be merged with multiplex FISH (M-FISH) and a variety of other molecular procedures. The permutation of comparative genomic hybridization (CGH) with iridescent FISH was found to be a powerful blend for portraying complicated karyotypes. Chromosomal deformities is still a mystery of human syndromes.<sup>2</sup> Thus, molecular cytogenetics consisting of karyotyping and FISH is a vital approach for identification of genetic alterations and suggesting potential therapy.<sup>3</sup>

Childlessness is a considerable dilemma. It has been reported that 15% of couples bear infertility during their reproductive years. It was an earlier belief that reproductive troubles were mostly due to female factors but investigations in past years have proved that 50% of infertility is affected by male factors.<sup>4</sup> The advancing latest cytogenetic procedures permitted the initial assessment of human chromosomes. Karyotyping helps in the determination of chromosomal deformities. However, the procedure is tedious with few laboratories providing information.

Additionally the information produced, still of great importance, is minimal.<sup>5</sup>

The area of human cytogenetics was introduced in 1956, when the amount of genetic material in a diploid cell was precisely revealed to be 46.<sup>2</sup> In the field of infertility, the cytogenetics began to create approaches to envision chromosome structure. It has been shown that chromosomes vary in size and position of centromere also differs. Cytogenetic findings have revealed that an additional, misplaced or lost copy of particular human chromosome have syndromic association. For ex. additional copy of chromosome 21 was seen with Down syndrome (also called trisomy 21), the presence a single X chromosome and no Y chromosome (45,XO) associated with Turner's syndrome, whereas the presence of two copies of the X chromosome and one copy of the Y chromosome (47,XXY) was shown in Klinefelter's syndrome.<sup>6</sup> Chromosomal cytogenetic anomalies are the most critical causes of male infertility. Karyotyping in infertile males is considered the best method for optical analysis of genetic material to find structural rearrangements. Cytogenetics has also been used for antenatal diagnosis for aneuploidies, investigation for recurrent abortions, delay in mental growth, learning disabilities etc.<sup>7</sup> In karyotyping, the cells are cultivated from blood. The separated cells in the metaphase stage contain chromosomes in their distinct shape.<sup>8</sup> The cells are fixed, stained with dyes (Giemsa, Leishman's or variant) and analysed under microscope for the exact identification of any deformities in chromosomes.

G-banding karyotyping cannot identify small structural malformations or that exists inside G-negative bands or entails translocations among regions that have parallel banding patterns. Resolution of cells during and after G-banding also depends upon the origin of cells.

FISH offers assessment of chromosomes with their DNA probes allowing a quicker, not expensive, simpler substitute for identifying aneuploidies in human sperm. Additionally, sperm hindered by anomalies in movements or other facets of reproduction can be evaluated utilizing FISH.<sup>9</sup>

By using FISH the detection of microdeletions as well as translocations is feasible. In this method the short sequences of DNA from infertile male samples has been attached with probes (fluorescent tags) of complementary sequence. This method of detection is "locus specific" where altered multiplication of chromosomes is detected easily. The mixture of chromosomes with their specific probes allows the chromosome to get painted.<sup>10</sup> Numerous other expansions of the procedure are under progress with this technique for the exact diagnosis of infertility i.e. spectral banding, which produces a colourful banding pattern.

The possibility of FISH to identify minute chromosomal deformities that cannot be identified with karyotyping has previously been stated. FISH does not require any cells to be arrested in the metaphase stage; it depends on the appearance or non-appearance of a fluorescent signal to identify chromosomes. Progressively, this method has shifted from the research laboratory into regular use, especially when samples are in restricted number or give poor-outcomes (polyps) or negative reporting (as in microdeletions) on karyotyping.

**CONCLUSION**

For infertile couples the advancement in the field of cytogenetics with their positive effects is really a boon. It is crucial to carry out cytogenetic assessment to evaluate the exact cause of infertility. The appropriate treatment as well as diagnosis can be made by an aberrant karyotype or FISH and will substantially raise the chances of a successful pregnancy in future.

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## PATERNAL CONTRIBUTIONS IN RECURRENT PREGNANCY LOSS

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### ABSTRACT

Recurrent pregnancy loss (RPL), is loss of two or more pregnancies, occurring before 20 weeks of gestation. With an already complex etiology involved in miscarriages, idiopathic/unexplained RPL (iRPL) account for up to 40-50% of RPLs. With substantial literature focusing on the causes and consequences of RPL, the current focus is shifting towards analyzing the paternal contribution in not only providing the haploid genome but also in regulating early embryogenesis. The defects in sperm chromatin integrity and overwhelming seminal oxidative stress are a potential cause of pregnancy loss as it is essential for sperm function, fertilization, implantation and subsequent embryonic development. Recent studies have also highlighted the role of sperm factors in early embryonic development as the transcriptionally inert spermatozoa transcribe genes critical for early embryonic development.

### INTRODUCTION

Miscarriage or spontaneous pregnancy loss is one of the devastating complications of pregnancy and is defined as pregnancy loss before 20 completed weeks of gestation. The condition affects 10-15% of clinical pregnancies. 30% of human conceptions are lost both before and after implantation.<sup>1</sup> Recurrent pregnancy loss (RPL), affecting 0.4-1% of couples is defined as having at least three consecutive embryo miscarriages within the first or early second trimester of pregnancy before the fetus reaches viability.<sup>2</sup> The American Society for Reproductive Medicine defines RPL as two or more failed pregnancies, which have been documented by either ultrasound or histopathological examination.<sup>3</sup>

Unlike sporadic miscarriages, recurrent miscarriages tend to occur even if the fetus has normal chromosomal component.<sup>4</sup> The risk of RPL is higher at a lesser gestational age and is also seen to be affected by the number of miscarriages if happened previously, i.e the risk increases

with number of consecutive pregnancy losses which happened in the past.<sup>5,7</sup> Parental age at conception especially the maternal age is a strong independent causal factor for RPL and the incidence has been seen to increase from 10% to 51% when the maternal age at conception advances from 20-24 yrs to 40-44 years.<sup>1</sup>

### ETIOLOGY

Nearly 40-50% cases of RPL remain idiopathic RPL (iRPL) despite of multiple etiologies suggested for its occurrence. Among the already accepted etiologies ascribed to RPL, the major causes are i) genetic (parental chromosomal anomalies, aberrations in products of conception), ii) uterine anatomic anomalies, iii) endocrine and autoimmune, iv) thrombophilias, v) infections and environmental.

Parental chromosomal abnormalities account for nearly 2-5% of all cases of RPL. The most common chromosomal aberration witnessed in the patients is Robertsonian and reciprocal chromosomal translocations. Antiphospholipid syndrome (APS) also an important contributor is seen in 3-5% of general population. The other non-APS heritable thrombophilias associated with RPL include hyperhomocysteinemia resulting from MTHFR (methylene tetrahydrofolate reductase) gene mutations, protein C and protein S deficiencies, factor V Leiden mutations, antithrombin and prothrombin promoter mutations.<sup>8,9</sup> However, some environmental and lifestyle factors have also been seen to affect pregnancy outcome.

### PATERNAL CONTRIBUTIONS

Parental genetic and epigenetic contributions have been seen to navigate embryonic development. The paternal contributions to early embryonic development as a potential causal factor for adverse pregnancy outcomes have remained underexplored as they have mainly relied on basic semen analysis. But the role has been recently realized in couples experiencing sporadic ART failures even after ICSI. While the effect of advanced maternal age on fertilization and reproduction is well known, much less has been explained about possible effects of parental ageing. Male reproductive functions show a gradual decline with advanced age due in part to the fact that spermatogenesis is a continuous process. With limited germ cell divisions seen in the female, the male gametes witness an increase in germline mutation rate.<sup>10</sup> Advanced paternal age at conception has been associated with impaired fertilization rates, implantation failures, pregnancy loss, newborn birth defects and infant mortality.<sup>11-14</sup>

### SPERM GENOME

Spermatogenesis is characterized by ordered histone replacement. The compaction of the sperm DNA where the nucleohistone structure is replaced by the nucleo-protamine structure is characterised by nucleosomal disassembly. The sperm DNA is extensively complexed with transition proteins (TNPs) which are then finally replaced by protamines to form a highly compact nucleoprotamine complex.<sup>15</sup> But only 85% of the total DNA in sperm nucleus is coiled into toroids by protamines and 5-15% remains associated with histones.<sup>15-17</sup> Along with this transition, some of the DNA remains attached to the nuclear matrix at the matrix attachment regions (MARs). This is seen at approximate 50kb intermediate intervals throughout the genome. These proteins are responsible for a highly stable chromatin



condensation leading to a shutdown of the transcriptional machinery of the sperm genome.<sup>18</sup>

### SPERM TRANSCRIPTS

The contributions of sperm to the oocyte are more than just a mere vector for providing the haploid genome. The epigenetically marked, terminally differentiated spermatozoa are transcriptionally inactive cells due to compaction of sperm DNA, but are capable of post-meiotic production of functionally viable transcripts.<sup>16-20</sup> This non-genomic paternal delivery of RNA to the oocyte at fertilization is retained by the newly formed embryo and can translate proteins which are involved in crucial processes related to stress response, implantation and early embryonic development. Hence, paternal genome contributes to the transcript signature of the embryo prior to the activation of embryonic genome.<sup>16,21,22</sup> The dysregulation of mRNA transcripts from transcriptionally silent spermatozoa have been associated with increased incidence of pregnancy loss.<sup>16</sup> Various studies have been conducted to analyse the expression of the genes critical for early embryonic development. In an attempt to elucidate the role of these transcripts in pregnancy loss, the relative expression of some of the genes critical for embryonic development such as FOXG1, SOX3, RPS6, RBM9, RPS17, RPL29 and those which are responsible for the DNA damage detection and repair in the sperm i.e OGG1 and PARP1 was assessed in male partners of couples with RPL and implantation failure in the studies conducted in our laboratory.<sup>16,17,22,23</sup> The presence of RNA in sperm thus might be a useful method for the early detection of affected sperm and perhaps provide the means to prevent transmission to future generations.<sup>16,17,23</sup>

### ROLE OF OXIDATIVE STRESS

Though the adverse effects of semen quality and sperm have been characterized, various biological markers as well as protective measures for this cell in crisis need to be elucidated. The vulnerable sperm cells exists in a state of oxygen paradox and requires oxygen for ATP production and hence are exposed to high levels of reactive oxygen species (ROS) thus generated. These free radicals are the byproducts of sperm metabolism have been shown to exert beneficial roles in the sperm such as hyperactivation, moderation of tyrosine phosphorylase required for sperm capacitation, acrosome reaction and sperm-oocyte fusion at physiological levels but are considered detrimental at pathological levels.<sup>24</sup> Apart from this, the excessive production of ROS have been seen to affect key functions involved in inducing cholesterol oxidation during capacitation, thus further facilitating efflux of sterols from plasma membrane of sperm thereby increasing sperm plasma membrane fluidity.<sup>17,25,26</sup> Oxidative stress occurring when the proportion of oxidants in the body increase manifold and antioxidants lack in adequate proportion to scavenge the free radicals.<sup>27-30</sup> This state of homeostatic imbalance is shown to compromise human sperm function and further leads to the activation of the intrinsic apoptotic cascade in these cells.

Sperm is most vulnerable to oxidative damage by various endogenous and exogenous factors due to being transcriptionally inert, lack of cytosolic antioxidants, having a high content of polyunsaturated fatty acid in the sperm plasma membrane and being deficient in DNA damage detection and repair mechanisms.<sup>31,32</sup>

Modern lifestyle of many people is unhealthy succumbing the vulnerable sperm to increasing oxidative stress. This includes various modifiable life-style factors including high levels of psychological stress, physical

inactivity, high calorie intake, obesity, mobile phone radiations, smoking, consumption of alcohol, cocaine etc. owing to the increasing corporate culture in the society. Metabolites in the cigarette smoke containing cotinine, cadmium and lead cause DNA strand breaks. Cigarette smoke also decreases the antioxidant capacity in body. Cocaine use and even moderate consumption of alcohol (3-5 units per week) seen to have adverse effects on fertility and fetal development thereby increasing the risk of pregnancy failures.<sup>33,34</sup> The advent of the holistic science of yoga and meditation has emerged out to be the method for prevention as well as an aid in the management of such disorders as it has shown to decrease oxidative stress and improve genomic integrity.<sup>35-37</sup>

Excessive production of ROS in mammalian sperm leads to the induction of lipid peroxidation cascades due to the high content of PUFA which culminates in the formation of electrophilic lipid aldehydes such as malondialdehyde (MDA), 4-hydroxynonenal (4HNE), and acrolein (ACR).<sup>38</sup> These are seen to further bind to proteins in mitochondrial electron transport chain culminating in a sustained formation of ROS.<sup>31,38</sup> Both genomic and mitochondrial DNA of sperm is highly vulnerable to oxidative stress-induced damage.

The ROS thus generated attack the poorly compacted sperm DNA with a subsequent generation of mutagenic oxidized DNA base adducts such as 8-hydroxy 2'deoxyguanosine (8-OHdG). The inert nature of sperm chromatin leaves them with a very limited capacity for DNA repair and with the insufficient repair mechanisms; the sperm is thus dependant on the oocyte repair mechanisms.<sup>37-39</sup> Sperm cell is characterized by a truncated DNA damage detection and repair mechanism whereby it only possesses the first enzyme in the base excision repair pathway i.e OGG1, while it lacks the downstream enzymes APE1 and XRCC. This DNA damage in the sperm being dependent upon the oocyte repair mechanism needs to be corrected before the S1 phase of the first mitotic division. Deficit in the oocyte repair capacity will thus predispose the damage to be carried over to the next generation and will adversely affect the early embryonic development, and may be a causal factor for spontaneous pregnancy loss, implantation failure and risk of birth defects.<sup>31,32</sup> DNA damage induced by oxidative stress causes pro-mutagenic changes affecting spermatogenesis and even impairs fertilization.

### SPERM DNA DAMAGE

Sperm DNA integrity has been recognized as one of the key determinants of fertilization and early embryonic development. The vulnerable sperm cell with compromised genomic integrity may still be able to fertilize the oocyte, but can on the contrary may result in implantation failure, poor cleavage, blastocyst formation, implantation failure, increased chances of spontaneous pregnancy loss, birth defects and paediatric carcinomas.<sup>16,17,30,40-43</sup>

The increased propensity of the sperm for oxidative attack and predisposition to derangements in genomic integrity is due to its vulnerability during spermatogenesis, sperm function and transport in reproductive tract. It is well known from various studies that abnormal protamination leading to decreased protamine content and altered histone:protamine ratio is linked with decreased DNA integrity leading to higher levels of DNA damage and poor fertility outcomes.<sup>15-17,29,44-46</sup>

The increase in sperm DNA damage is one of the main paternal causes of implantation failure even after ICSI as the sperm bypasses the

natural barriers to fertilize the oocyte with ART.<sup>18</sup> Sperm DNA integrity is critical for accurate transmission of paternal genetic information and normal embryonic development. Oxidative DNA damage in the male germ line has been strongly associated with an increase in de novo germ line mutations and hypomethylation in the sperm epigenome. This further predisposes to an alarming increase in the risk of genetic and epigenetic disorders in the offspring. The accumulation of highly mutagenic oxidative DNA base adduct 8-OHdG has the propensity to induce global hypomethylation by impairing the function of de novo DNA methyltransferases, causing genomic instability and predisposing to mutations and epimutations impairing the embryonic development. Normal sperm chromatin structure is thus important for sperm fertilizing potential and birth of healthy offspring.

**Various theories of sperm DNA damage have been cited before:**<sup>16,27,31,45,46</sup>

- 1) Abortive apoptosis: Sperm with DNA damage is derived from germ cells whose apoptotic process could not be completed during spermatogenesis.<sup>41</sup>
- 2) DNA strand breaks during chromatin remodeling during spermiogenesis.
- 3) DNA damage induced by ROS during transit through the male reproductive tract.
- 4) DNA damage induced by endogenous endonucleases, radiotherapy and chemotherapy.
- 5) DNA damage induced by various environmental factors such as air pollution, cigarette smoking, electromagnetic radiation, high testicular temperature and exposure to insecticides and pesticides.

Numerous causal factors have been cited for sperm DNA damage such as advanced age, obesity, poor social habits such as smoking, alcohol and drug intake, intake of processed food, exposure to xenobiotics, chemotherapy, radiotherapy, occupational exposure to endocrine disrupting chemicals. Different assays have been developed for the analysis of sperm DNA damage. These include a) the tests which can directly measure the extent of DNA damage using different probes and dyes and b) the tests which measure the susceptibility of the DNA to denature.

The tests which use different probes and dyes are acridine orange test, toluidine blue staining, and Chromomycin A3 staining, while the tests which measure the susceptibility of DNA to denature are TUNEL (terminal deoxynucleotidyltransferase UTP nick end labeling) assay, Comet assay, SCSA (Sperm chromatin structure assay), SCD (Sperm chromatin dispersion) test.

There is indeed a need for new markers that better outline the influence of paternal factors on the outcome of both spontaneous and assisted conceptions and pregnancy outcomes. Conventional semen parameters fall short. With evidences to suggest the potential of sperm DNA integrity markers for male infertility, larger studies are needed to define the clinical implications of assessing sperm DNA integrity for unexplained RPL. DNA damage in the sperm is a major contributor of infertility, miscarriage and birth defects in the offspring.<sup>16,17,27,28,30,42,43</sup>

Oxidative stress is the major cause of sperm DNA damage but may be modifiable in many cases as already discussed. A healthy diet undoubtedly plays a role in being a major modifiable factor in management of the sperm cell crisis. An adequate dietary intake of fruits

and vegetables rich in antioxidants such as vitamin C, vitamin E, zinc and selenium has been associated with a significantly lower DNA damage. With higher evidenced impact of vitamin C in reduction of seminal ROS levels, a mixed supplementation of both vitamin C and vitamin E has been seen to act synergistically in ROS reduction and an increase in 8-OHdG levels.<sup>47</sup> Spermatozoal development and DNA synthesis is facilitated by zinc, the action of which is also aided by the co-supplementation with selenium.<sup>48</sup> Significant increase in fertilization, embryo quality and pregnancy success has been observed with mixed supplementation including vitamin C and E, zinc and selenium.<sup>49</sup> Another set of powerful antioxidants are L-Carnitine and coenzyme Q10 which help in sperm maturation and metabolism, prevent lipid peroxidation. It calls for a more thorough evaluation as limited studies have been conducted.

## YOGA AND RPL

The stresses of modern life seem to be accelerating, however, to a greater extent for younger men. The advent of various complementary and alternative medicine therapies, is being widely adopted and many studies have been conducted which have emphasized the potential of these therapies to be effective in enhancing the reproductive health in men to produce a successful pregnancy.<sup>35-37,50,51</sup> Yoga and meditation-based lifestyle interventions have been observed to decrease oxidative stress, DNA fragmentation and mutagenic load in sperm DNA, telomere length regulation, normalization of gene expression and thus prove to be therapeutic.<sup>35-37,50,51</sup> Previous studies from our laboratory have assessed the impact of the intervention on sperm parameters not only in infertile males but also in male partners of couples with RPL and implantation failure in IVF cycles. Yoga has been beneficial in decreasing the oxidative stress in our patients as early as 10 days of practice, improvement of sperm genomic integrity, decreasing the mutagenic load by decrease in 8-OHdG levels and maintenance of telomere length with longer practice of yoga.<sup>35,36,51</sup> Yoga intervention has been found to be instrumental in normalizing the gene expression levels within a brief period of 21 days in RPL and implantation failure.<sup>35,36</sup> Gene expression in patients with primary open angle glaucoma showed an upregulation of genes involved in cellular repair while a downregulation was observed in pro-inflammatory and pro-apoptotic genes.<sup>52</sup> An increase in expression of transcripts that maintain mitochondrial integrity in infertile men with rheumatoid arthritis was observed in another study with yoga intervention for 12 weeks.<sup>53</sup>

The distressing issue of infertility and pregnancy loss is associated with a load of psychological stress faced by the patients. The profound science of yoga has been found to decrease clinical severity and an increase in neuroplasticity in another randomized control trial in major depressive disorder patients from our laboratory. This was associated with a significant improvement in the levels of BDNF, DHEAS, sirtuin 1, and telomerase activity and a decrease in the levels of cortisol and IL-6 with the intervention.<sup>54</sup>

The impact of the intervention on sperm epigenome in a pilot study from our laboratory witnessed differentially methylated regions on the sperm genome where 147 genes were found to be hypomethylated, while 229 genes showed hypermethylation with the intervention. The improvement in the biomarkers of sperm biology, gene regulation and sperm epigenome may prove to be beneficial in the sperm functions.<sup>55</sup> This may have lifelong implications on health trajectory of offspring, decrease incidence of pre and post implantation losses and congenital malformations.

## CONCLUSION

With most of the focus centered on the evaluation of the females, the current paradigm has shifted towards evaluation of the male partner. Oxidative stress and derangements in sperm chromatin integrity are one of the major causative factors of defective sperm function and have the potential to be carried over to the next generation and affect the future progeny. Adoption of yoga/ meditation in our lifestyle may help in normalizing the dysregulation of sperm transcripts and thus may aid in exerting beneficial effects on the pregnancy outcomes.

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## Transfer of Mosaic Embryos – To do or Not to do?

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### ABSTRACT

The concept of mosaicism is not novel in the field of preimplantation genetic testing (PGT). The advancement in the technologies used for PGT has not only increased the sensitivity and accuracy of detection of mosaicism in embryos, but it has also helped in yielding important information on the viability of transfer of these embryos. Recently, researchers have attempted transfer of mosaic embryos and have reported birth of healthy babies. This has provided some hope to the women who fail to get euploid embryos after PGT for aneuploidies (PGT-A), especially those with the poor ovarian reserve and/or advanced maternal age. Although the data is limited, yet it proves that certain mosaic embryos do have a potential of resulting into healthy live births and they may be considered for transfer rather than being discarded instantly, particularly in cases where repeat cycle of IVF is not possible. Several recommendations and various scoring systems have been proposed to help in deciding the mosaic embryo which can be transferred and guiding the counselling of the patient undergoing such transfers.

### INTRODUCTION

The main aim of PGT-A is to identify aneuploidy in an embryo cohort. It helps in selecting euploid embryos for transfer in an IVF cycle to improve the outcomes mainly by increasing the chances of implantation, pregnancy rates and live birth per embryo transfer cycle. The concept of 'Mosaicism' is not new in the field of PGT. Chromosome mosaicism is characterised by the presence of two or more distinct cell lines in an embryo.<sup>1</sup> These different cell lines in the mosaic embryo may be abnormal (aneuploid/aneuploid), or a combination of a normal with an abnormal cell line (euploid/aneuploid). Mosaicism may result from a variety of mechanisms occurring during cell division and growth, which include anaphase lag, mitotic non-disjunction, inadvertent chromosome demolition, and premature cell division before DNA duplication.<sup>2</sup> It may be confined to a specific part of the embryo, like the trophectoderm cells of the blastocyst, or involve the entire embryo.<sup>2</sup> Sometimes it may be present only in a part of the trophectoderm. Such a variable distribution

of mosaic cells in the embryo affects the accuracy of PGT-A results and has a significant impact on clinical practice.

The ultimate goal of any IVF cycle is embryo transfer and decision are easier when a euploid embryo is present after PGT-A. However, having only mosaic embryos, especially in the patient who cannot undergo further IVF cycle due to any reason, can present a great deal of clinical and ethical dilemma. Earlier practise was to discard the mosaic embryo, but recently there have been many documented cases of live birth following the transfer of mosaic embryos. There is limited clinical data which suggests that the transfer of mosaic embryos may result in a healthy live birth in up to 30% cases. This may be attributed to the 'self-correction' of the chromosomal anomalies or selective growth of cells with a normal chromosomal pattern. Another explanation is that false interpretation of "technical noise" during PGT-A as mosaicism may lead to misdiagnosis of a euploid embryo as a mosaic.

It remains unclear that to what extent the degree of mosaicism in the embryo or the type of aneuploidy presenting in the mosaic, affects its developmental potential. However, the recent data shows that the embryos with low-level mosaicism (<40%) and those with a single/double monosomy may have a higher probability of implantation and subsequent viable pregnancy than those with 40-80% mosaicism or other chromosomal abnormalities.<sup>2</sup>

Repeated biopsy on the whole blastocysts (trophectoderm and Inner cell mass) has revealed that if the level of mosaicism in the embryo was less than 40% in the first biopsy, then there were lower chances of finding that mosaicism on repeated biopsy, which means that many of these embryos were later found to be uniform euploid; while if the level of mosaicism was greater than 40% on the initial biopsy, there was an increased probability of finding some level of mosaicism on repeated biopsy.<sup>1</sup> This discrepancy may be due to the technical limitations of the method employed for PGT-A.

### INCIDENCE

The percentage of mosaic embryos identified in an embryo pool is influenced by various factors which include maternal age, stage of the embryos, and the sensitivity of the technique used for PGT. Increased maternal age is associated with lower proportions of euploid/aneuploid mosaics because the aneuploidy associated with increasing maternal age results from meiotic non-disjunction and it affects all the cells of the embryo.

The level of mosaicism present in the cleavage-stage embryos varies largely between 15- 75%, while in the blastocyst stage, it may vary from 3-24%, depending on the method applied. Different techniques employed during PGT-A have different sensitivity for identifying the mosaic embryos. Array Comparative Genomic Hybridization (aCGH) and Next Generation Sequencing (NGS) are the most frequently used techniques presently and they have high sensitivity for identification of mosaic embryos. NGS is the preferred method as it may correctly identify the mosaicism in samples which has aneuploidy as low as 17%.<sup>3</sup>

### PRE-TEST GENETIC COUNSELLING<sup>4</sup>

- Counsel the patient about the risks and benefits of PGT-A, and limitations of the technologies used for it.
- Patients should be made aware that they can refuse PGT-A.

- All the possible outcomes of the test should be informed: euploid, aneuploid, mosaic and no result (test failure/insufficient DNA).
- Patients should be told about the limited availability of the data regarding the mosaic embryos.
- In cases of surrogacy, ask the surrogate if she is willing for the transfer the mosaic embryos.

### RECOMMENDATIONS FOR THE LABORATORY FOR REPORTING MOSAIC ANEUPLOIDIES<sup>1</sup>

#### ■ Biopsy Technique:

- Ideally, biopsy of 5-10 cells must be done, with minimum damage to the cells.
- Laser must be used cautiously with minimal contact points which should be at the cell junctions. Care should be taken to avoid the damage to the cell and its DNA.

#### ■ Methodology:

- Biopsy sample should be subjected to a validated NGS platform which can quantitatively measure copy number. It should accurately measure up to 20% mosaicism in a control sample and the results must be reproducible.

#### Reporting :

Level of Mosacism	Comment	Report
< 20%	Consider Normal	Euploid
20 - 80 %	Aneuploid/euploid	Mosaic
> 80%	Abnormal	Aneuploid

### THERE ARE CERTAIN RECOMMENDATIONS SUGGESTED AND SYSTEMS POSTULATED FOR EMBRYO TRANSFER (FIGURE 1; TABLE 1-3).

1) Consider the transfer of a euploid embryo when available.

2) Transfer of a mosaic blastocyst

The patient may be given the following options when only a mosaic embryo is present after PGT-A:

- (I) The patient can undergo a repeat IVF-PGT cycle to obtain euploid embryo for transfer,  
or  
(ii) Blastocyst with lower level of mosaicism may be transferred after appropriate counselling.

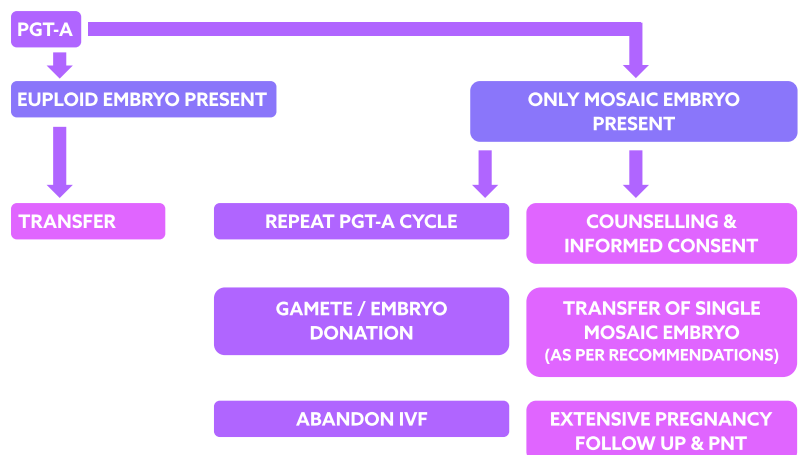


Figure 1: Recommendations for embryo transfer

### POST-TEST GENETIC COUNSELLING<sup>4</sup>

The following points must be discussed with the patient considering the transfer of a mosaic embryo:

- Explain mosaicism, interpretation of the PGT-A results, possible explanation for such results, and chances of misdiagnosis.
- There is very limited data to accurately explain the clinical significance of mosaicism.

Priority of Transfer	PGDIS 2016	COGEN 2016	Grati et al. 2018	PGDIS 2019
<p>Highest priority</p> <p>Lowest priority</p>	Full euploid	Full euploid	Full euploid	Full euploid
	Mosaic: Euploid/monosomy	Low level mosaic embryo (20-40% mosaicism)	Mosaic trisomies 1,3,10,12,& 19	Low-level mosaicism (<40%)
	Mosaic: Euploid/trisomy for chromosomes 1,3,4,5,6,8,9,10,11,12,17, 19,20,22,X,Y	Mosaicism involving chromosomes 1,3,4,5,6,8,9,10,11,12,17, 19,20	Mosaic: Euploid/trisomy for chromosomes 4,5, & 47 XXY	High-level mosaicism (40- 80%)
	Mosaic: Euploid/trisomy for chromosomes 2,7,14,15,16	Mosaic: Euploid/trisomy for chromosomes 2,7,16	Mosaic: Euploid/trisomy for chromosomes 2,7,11,17,& 22	Specific chromosomes linked to specific syndromes
	Mosaic: Euploid/trisomy for chromosomes 13,18,21	Mosaic: Euploid/trisomy for chromosomes 14,15	Mosaic: Euploid/trisomy for chromosomes 6,9,& 15	
		Mosaic: Euploid/trisomy for chromosomes 13,18,21	Mosaic: Euploid/trisomy for chromosomes 8,20,47 XXX, & 47 XXY	

Table 1: Various systems postulated for prioritising the transfer of mosaic embryos

- Mosaic embryos have a lower implantation potential and transfer of such embryos is often associated with higher chances of miscarriage.
- There are higher chances of prenatal and perinatal complications in the pregnancies resulting from the transfer of mosaic embryos which may be due to the chance of persisting placental mosaicism.
- There have been documented healthy live births following the transfer of mosaic embryos, but the number is significantly low.
- The patient should be counselled about the risk of live birth with persisting aneuploidy (uniform or mosaic) and the likelihood of Uniparental Disomy (UPD), which may result in various congenital anomalies.
- Mosaic containing full or partial aneuploidy involving chromosomes associated with a known syndrome or phenotype, (especially chromosomes 13, 18, 21, X, Y) may have a poor outcome. Patients must be given adequate clinical information and counselling.
- Refer the patients to a certified genetic counsellor if you are not familiar with the possible outcome listed.
- All patients must be counselled about the Prenatal Testing (amniocentesis) required for the pregnancy conceived after the transfer of the mosaic embryo, its possible outcome and further management.
- In cases of surrogacy, the surrogate should also be counselled about the possible clinical outcomes and need for prenatal testing.

**3) Embryos with low-level mosaicism/low risk (<40%) are given preference over the embryos with higher level (40-80%) mosaicism<sup>1</sup>**

- The relative percentage of mosaicism is found to be a better predictor of the outcome as compared to a specific
- Higher levels of mosaicism in the trophectoderm are associated with an increased probability of having aneuploidy in the inner cell mass, an increased risk of implantation failure and adverse pregnancy outcome. Such embryos should be transferred with caution and only after extensive genetic counselling.
- Counselling should be provided for the chromosomes which are linked to specific syndromes.

#### **4) Embryos mosaic for a single chromosome**

Mosaic embryos are prioritized for transfer primarily based on the level of mosaicism, followed by the type of chromosome involved. Chromosomes which are associated with no/fewer adverse outcomes are given priority over those associated with known syndromes or significant adverse outcome (Table 1).

#### **5) Two mosaic embryos with similar levels of mosaicism**

Mosaic embryos with affected chromosomes that have potential for liveborn syndromes, uniparental disomy or severe intrauterine growth restriction may be given lower priority.

#### **6) Fully aneuploid embryos**

They may result in adverse obstetric or paediatric outcomes and should not be transferred.

#### **7) Complex mosaicism**

Transfer is not recommended when mosaicism is observed across multiple chromosomes.

**Table 2: Prioritisation of mosaic embryo for transfer (PGDIS)**

<b>*Preimplantation Genetic Diagnosis International Society (PGDIS) Position Statement 2016</b>		
<b>Mosaic with trisomies for chromosomes 13, 18, 21, 22</b>	Live born viability	<b>Lowest priority</b>
<b>Mosaic with trisomies for chromosomes 14, 15</b>	Uniparental Disomy	<b>Low priority</b>
<b>Mosaic with trisomies for chromosomes 2, 7, 16</b>	Intrauterine growth retardation	<b>Low priority</b>
<b>Mosaic with trisomies for chromosomes 1, 3, 4, 5, 6, 8, 9, 10, 11, 12, 17, 19, 20, 22, X, Y</b>	Adverse outcomes are seen only when mosaicism is present in the fetus	
<b>Mosaic euploid/monosomy</b>	As monosomic embryos (excepting 45, X) are not viable euploid/trisomy embryos are preferred	

**Table 3: Prioritisation of mosaic embryo transfer (Grati et al) <sup>5</sup>**

<b>Scoring system for mosaic embryo transfer</b>			
Mosaic chromosomal anomalies	Composite score	Priority	Risk of adverse outcomes
<b>Mosaic trisomies 1, 3, 10, 12 and 19</b>	0	Highest	Low
<b>Mosaic trisomies 4 and 5 and 47,XYY</b>	1	Second highest	Slightly increase in miscarriage Or risk of aneuploidy (47,XYY).
<b>Mosaic trisomies 2, 7, 11, 17 and 22</b>	2	Third highest	Risk of miscarriage is slightly higher; relatively lower risk for UPD (trisomies 7 and 11).
<b>Mosaic trisomies 6, 9 and 15</b>	3	Consider for transfer with caution & after detailed discussion with the patient	Risk of miscarriage, UPD or viable aneuploidy is higher
<b>Mosaic trisomies 8, 20, 47,XXX and 47,XXY</b>	4-5	Once the couple is explained in detail then only tnsfer could be considered	Higher risk of affected foetus and risk for miscarriage and viable aneuploidy is slightly higher
The remaining mosaic aneuploidies such as trisomies 13, 14, 16, 18, 21 and 45,X are best avoided.			



**PRENATAL TESTING AFTER THE TRANSFER OF A MOSAIC EMBRYO**

- Prenatal testing is highly recommended in the pregnancy conceived after the transfer of a mosaic embryo.
- Amniocentesis, from 14 weeks onwards, is a preferred method as it represents the foetal genetics most accurately.
- NIPT (Non-invasive Prenatal Testing) analysing placental copy number of all 24 chromosomes may be advised at 10 weeks.
- Ultrasound may help in detecting foetal anomalies, while PAPP-A level and Doppler Ultrasound may pick up placental malfunction if confined placental mosaicism exist.

**CONCLUSION**

NGS is the preferred method for doing PGT-A as it has a high sensitivity for detecting mosaicism. The patient undergoing PGT-A must be counselled about the possibility of getting mosaic embryos. Only euploid embryos must be considered for transfer when available. In case of non-availability of a euploid embryo, if the patient insists for transfer of the mosaic embryo, proper counselling regarding the possible outcome must be done before planning the transfer. Mosaic embryos must be prioritised for transfer as per available evidence and recommendations. Pregnancy conceived after the transfer of mosaic embryo must undergo early amniocentesis (around 14 weeks) to rule out chromosomal abnormalities in the foetus.

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## Role of KNDy Neurons in Hypogonadotropic Hypogonadism

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### ABSTRACT

Hypothalamus-pituitary-gonads are the integrated system that is responsible for the release of the GnRH and gonadotropin which are required for the proper functioning of the reproductive axis. Multiple neuronal and non-neuronal cell populations work in synchronization to carry out reproduction, the onset of puberty and fertility. KNDy neurons are characterized by the co-expression of Kisspeptin, Neurokinin B (NKB) and Dynorphin. GnRH neurons act as a central channel in sending the signal from the brain to pituitary and gonads that are important for spermatogenesis, ovulation and reproductive cycle. GnRH neurons are not directly receiving most of the exogenous and endogenous cues. In hypothalamus afferents from different neuronal population interact with GnRH neurons in the preoptic area and median eminence of the hypothalamus to transmit various signals to these neurons. One such neuronal population is KNDy neurons which interact with GnRH neurons in the median eminence and are responsible for the release of the GnRH and LH/FSH that are important for the onset of puberty and to maintain the reproductive function of an organism. Kisspeptin secreted from these neurons acts as an output signal to GnRH while NKB and Dynorphin are the stimulator and terminator of GnRH pulse respectively. Earlier, the known role of these KNDy neurons is the GnRH pulse generation and participates in estrogen-mediated negative feedback. Two critically important neuropeptides KISS1 and NKB mutations result in Idiopathic Hypogonadotropic Hypogonadism (IHH) which are also co-expressed by KNDy neurons. Low level of the sex steroids due to the insufficient level of the secretion of the LH/FSH from the pituitary is the characteristics of Hypogonadotropic Hypogonadism (HH). Reduction in the sizes of ovaries, atrophy, constant diestrus, and nadir level of LH was observed in the KNDy ablated rat. These observations provide the direct pieces of evidence that dysfunction of KNDy neurons can cause HH. The new finding in context with the KNDy neurons will allow finding out medical interventions that can cure and prevent multiple neuroendocrine disorders.

### INTRODUCTION

The perpetuation of the species depends upon the reproductive capacity of the organism. Hypothalamic-pituitary-gonadal (HPG) axis is an integral system that is required for the onset of puberty and to make organisms competent for reproduction. There are lots of neuronal as well as non-neuronal circuits that work in a synchronized manner to carry out reproduction on which the continuation of any species depends. In the hypothalamus, one of the most important components of the HPG axis is GnRH neurons which act as a central tunnel in conveying most of the cues from the brain to the pituitary and gonads for the normal functioning of the reproductive axis. Most of the cues are transmitted to these neurons through the afferents from other neuronal populations which are in the proximity of GnRH neurons. The main neuronal populations are Kisspeptin, KNDy, and nNOS neurons. These neuronal populations work together for the normal release of the GnRH, which is required for the release of LH/FSH from the anterior pituitary that leads to ovulation and spermatogenesis. In 2007, a subpopulation of neurons gained a lot of attention because of the co-expression of the three important neuropeptides kisspeptin, neurokinin B (NKB), and dynorphin as the role of these each neuropeptide is already known and widely accepted in the regulation of the GnRH neurons. These neurons are conserved across many species including humans and rodents and are present in the infundibular nucleus and arcuate nucleus (ARC) of the hypothalamus. Co-expression of three neuropeptides kisspeptin, NKB, and dynorphin by a single neuronal subpopulation in ARC leads to the development of the "KNDy hypothesis" and for the ease of understanding these neurons were later named as "KNDy neurons". According to KNDy hypothesis, kisspeptin acts as the output signal to GnRH neurons, whereas NKB act as a stimulatory signal, and dynorphin acts as an inhibitory signal in GnRH pulse generation.<sup>1-4</sup>

### REGULATORY GENES AND HYPOGONADOTROPIC HYPOGONADISM

Ablation of the KNDy neuropeptides results in many neuroendocrine pathogenesis that associated with the failure in the normal secretion of GnRH and gonadotropins. One of such pathogenesis that directly related to the malfunctioning of these neurons is hypogonadotropic hypogonadism (HH).<sup>5</sup> Insufficient or low levels of gonadotropins and sex steroids are the clinical characteristics of HH.<sup>6</sup> There are two types of HH: congenital and acquired. Congenital HH may have some genetic origin best known is Kallmann syndrome.<sup>7</sup> Acquired HH can be caused by many factors, some of these are abusive alcohol, pituitary lesions (abscess, granuloma, and tumour), anabolic steroids, illicit drug intake, and various systematic diseases such as sarcoidosis, hemochromatosis.<sup>7</sup> Based on the intact sense of the smell congenital hypogonadotropic hypogonadism is further divided into two types: anosmic HH (Kallmann syndrome) and congenital normosomic hypogonadotropic hypogonadism (IHH).

Kallmann syndrome is linked with mutation of the ANOS1 (KAL1) gene, which disrupts the migration of forebrain GnRH neurons.<sup>8</sup> Lack of the regular episodic release of GnRH is commonly happening in HH which results in infertility and delayed puberty.<sup>7-8</sup> Earlier morphological evidence demonstrated the highest expression of NKB in the ARC neurons, which co-express dynorphin with estrogen receptor- $\alpha$  (Er $\alpha$ ), which are KNDy neurons.<sup>9</sup> Later in 2009, Topaloglu et al. found that mutations of the gene TAC3 (encode NKB) and TAC3R (encode NK3R) in humans results in IHH.<sup>10</sup> On the other hand administration of the GnRH in a pulsatile manner restores the normal secretion of LH as well as

testosterone in males that are necessary for the normal ovulation and normal birth in females.<sup>11</sup> Results from this finding suggest that low level of serum gonadotropins is mainly occurred due to defects in NK3R signaling at the hypothalamus level but not at the level of pituitary and gonads.

Similarly, mutations of gene encoding G protein-coupled receptor (GPR54) that encode kisspeptin receptor in humans, and *gpr54* (encode kisspeptin receptor in mice) also results in IHH, sexual infantilism and delays puberty which highlight the role of kisspeptin.<sup>12</sup> Exogenous administration of GnRH can also restore these effects. This study found that *gpr54* <sup>-/-</sup> male mice lack the development of the secondary sex glands (prostate, preputial glands, seminal vesicles) and halt in the spermatogenesis. Female *gpr54* <sup>-/-</sup> mice have very thin uterine horns like thread and small ovary as compared to control. Defects in sexual development and lack of estrous cycle were also found in mutant female rats. In addition to this low circulating level of the LH and FSH were observed in the mutant mice as compared to wild type mice.

Another study shows the role of ARC kisspeptin neurons in reproductive function. In this study, Intra-ARC administration of the rAAV kisspeptin antisense (rAAV-Kisspeptin-AS) was used to KO kisspeptin within the ARC region of the brain. However, the kisspeptin knockout (KISS1- KO) doesn't show significant changes in the level of the serum LH level but influences the gonadotropin frequency which is required for the normal follicular development.<sup>13</sup> This finding is also supported by evidence which demonstrated that intra-ARC and intra-mPOA administration of kisspeptin antagonist reduced the LH pulse frequency in ARC but not in mPOA in rats.<sup>14</sup> In short, these studies describe and emphasize the role of these ARC kisspeptin neurons in the LH pulse generation.

Earlier the exact mechanism of the inhibition of the GnRH pulse by Dynorphin is not clearly understood. A recent finding shows that dynorphin regulates GnRH neurons by directly acting on these neurons and through KNDy neurons.<sup>15</sup> New research also suggests that the trafficking and internalization of kappa opioid receptors (KOR) by KNDy neurons are important for GnRH pulse termination in ewes.<sup>16</sup> There was a direct evidence provided for the role of KNDy neurons in HH in a study based on the ablation of KNDy neurons in ARC by stereotaxic injection of ribosome inactivating toxin saporin (SAP) conjugated with selective neurokinin B receptor (NK3R) agonist.<sup>17</sup> Overall the data from this research work demonstrated that the KNDy neurons are important for the release of LH after the removal of the E2. Ablation of these neurons is associated with the decrease in the level gonadotropins, which is one of the causes of HH. Another study was done in rats demonstrated the effects of loss of the KNDy on the estrous cycle, gonadotropin secretion, estradiol and, progesterone-induced gonadotropins surge, ovarian morphology, as well on body weight.<sup>5</sup> Similarly, the women with the HH with the mutations in the gene of NKB, kisspeptin and their respective receptors also show such characteristics of small ovaries, reduced LH serum level and absence of menstrual cycle.<sup>10,11,12,18</sup>

Besides the role of KNDy in pulse generation, these neurons may also act as mediators in conveying stress and nutritional status to various components of the reproductive axis. The recent findings also suggest that these KNDy neurons participate in the thermoregulation as well as in cutaneous vasodilation.<sup>19</sup> All the above mentioned studies support the role of the KNDy neurons in control of the pulsatile release of the GnRH, which conveys a signal to downstream components of the reproductive axis and controls the release of gonadotropins.

## CONCLUSION

KNDy neurons are the integral component of the GnRH system which is required for the normal reproductive function. Alteration in the normal functioning or ablation of these neurons can cause some serious neuroendocrine disorders. Additional research on KNDy neurons is required to accurately know the mechanisms of KNDy neurons in GnRH pulse termination and gonadotropins release. Further studies will also allow exploring and finding the new upstream and downstream components with which KNDy neurons interplay that could help in developing medical intervention which may prevent and cure HH and other pathogenesis associated with the GnRH system such as infertility, precocious puberty and polycystic ovarian syndrome.

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## WEBINAR SERIES - FERTILITY AND GENOMICS : HOPES & HYPES



Dr Sudha Prasad  
President, IFS



Dr Neena Malhotra  
General Secretary, IFS



Dr Sarabpreet Singh  
Convenor, SIG  
Applied Genetics



Dr Rakesh Kumar  
Co-Convenor, SIG Applied  
Genetics



DR B. N. Chakravarty  
Key Note Speaker  
Medical Director - IRM

**BLOCK  
YOUR  
DATE**  
**14.08.2020**

### HIGHLIGHTS OF THE WEBINAR

- ▶ Molecular Markers in Fertility Practice - Dr B.N. Chakraborty, Medical Director - Institute of Reproductive Medicine (IRM), Kolkata.
- ▶ Understanding of Genetics, their diagnostics value for clinician and patients - Dr Neena Malhotra, General Secretary, IFS.
- ▶ Micro-deletions and Epigenetic Screening in reproductive disorders- Dr Rajendra Singh, Central Drug Research Institute Lucknow.
- ▶ A leap from Pre-Implantation Genetics to Genomics: Solutions & Myths - Dr Biren Banerjee, inDNA Life Sciences Pvt Ltd (Invited Speaker).
- ▶ Question & Answers Session.



DR B. N. Chakravarty  
Key Note Speaker  
Medical Director - IRM



Dr Neena Malhotra  
General Secretary, IFS



Dr Rajendra Singh  
MALE INFERTILITY & EPIGENETICS, IFS



Dr Biren Banerjee  
Founder, inDNA Life  
Sciences

14TH OF AUGUST' 2020

FRIDAY || 5:30 PM ONWARD

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### PRE-CONCEPTION

- General Karyotyping
- Bad Obstetric History (BOH) Karyotyping - For Couple
- Y chromosome microdeletion (YMD)
- Sperm DNA Fragmentation Assay
- Endometrial Receptivity Test

### PRE-IMPLANTATION

- Pre-implantation Genetic Screening (PGS)
- Pre-implantation Genetic Diagnosis (PGD)

### PRE-NATAL

- Non-Invasive Pre-Natal Test (NIPT)
- Amniocentesis FISH
- Amniocentesis Karyotyping
- Prenatal diagnosis for Genetic Disorders and Syndromes

### POST-NATAL

- Abortus Karyotyping
- Abortus FISH (5 Probes)
- Five Probe Chromosomal Involvement (13,18,21,X,Y)
- Cocktail Probe (13,21 or 18,X,Y) - FISH
- Karyotyping for Screening of Genetic Disorders

### PEDIATRIC

- Karyotyping for Screening of Genetic Disorders
- DMD Mutation Analysis
- Mutation analysis for SMA
- Mutation analysis for Thalassemia
- Factor II mutation study
- Factor V Leiden mutation study
- Fragile X mutation analysis

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- Personalized and Customized Panels with Mandatory Genetic Counselling Session.
- Real-Time Monitoring for customized Fertility Panels with Clinician & Patient.
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