CURRENT PERSPECTIVES OF PREIMPLANTATION GENETIC TESTING
by Irene Miguel-Escalada, PhD - Marie Curie Postdoctoral Fellow

HEMOGLOBINOPATHIES AND THALASSEMIAS – THE IMPORTANCE OF SCREENING AND GENETIC TESTING
by Jan Traeger-Synodinos, D.Phil (Oxon), ErCLG

IS THERE A SMALL RNA FINGERPRINT OF EMBRYO QUALITY AND HEALTH IN SPENT IVF MEDIA?
by Allison Tscherner, Leanne Stalker and Jonathan LaMarre

ASRM 2017, San Antonio, TEXAS
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KNOW YOUR REPRODUCTIVE RISKS & HAVE BETTER HEALTH FOR GENERATIONS TO COME!
Is there a small RNA fingerprint of embryo quality and health in spent IVF media?

by Allison Tscherner, Leanne Stalker and Jonathan LaMarre
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ABSTRACT: With improved technologies and enhanced understanding of cellular pathways that influence the success or failure of IVF procedures, there is an increasing demand for techniques to rapidly and non-invasively assess the health and developmental competence of individual embryos in clinical settings. These demands are further fueled by increasing age in those seeking ART to fulfill their reproductive aspirations (where the risk of embryo loss and genetic abnormalities is higher) and increasing demands by healthcare funders and regulators to perform single-embryo transfer in order to minimize potential complications associated with multiple gestation pregnancies. Promising strategies are emerging in which nucleic acids such as small regulatory RNAs secreted into the culture medium are measured to identify patterns and relative concentrations that may be related to the overall health and potential of those embryos to establish successful pregnancies leading to healthy children. This review describes some basic characteristics of one class of small RNAs known as microRNAs (miRNAs) and explores their potential as in-vitro biomarkers that reflect embryo quality and health.

Widespread societal changes have dramatically increased the demand for assisted reproductive technologies (ART), including in-vitro fertilization (IVF), as people frequently defer their reproductive ambitions until their period of optimal fertility has passed. Improvements in IVF, embryo culture and cryopreservation over the past decade have increased embryo implantation and cumulative pregnancy rates. This has, however, often been associated with concomitant increases in multiple gestation pregnancies (MGP) and their attendant complications. As a result, many government and private insurance programs have established funding strategies for IVF that are contingent upon the use of elective single embryo transfer (eSET) to avoid the health care costs and patient-related health risks associated with MGP. With this recent movement of IVF towards eSET, it has become increasingly important to develop strategies that evaluate both implantation potential and individual embryo quality in order to select the embryo that is most likely to result in a successful pregnancy and healthy child. Rapid, accurate and reliable techniques for optimizing embryo selection prior to transfer clearly have the potential to streamline IVF, increase efficiency, safely increase overall pregnancy success, and contribute to the long-term health of the children born from these procedures.

Current evaluation strategies that are routinely employed to determine the potential for pregnancy success with individual embryos are based primarily on the morphological characteristics of embryos or oocytes (Adamson et al., 2015). While these techniques are increasing in precision, ease and frequency of use, morphological assessments cannot universally identify the characteristics most strongly associated with either implantation or developmental potential, although they still hold considerable promise. Morphological evaluation is often paired with Preimplantation Genetic Screening (PGS) and/or Preimplantation Genetic Diagnosis (PGD), techniques by which trophectoderm lineage cells are biopsied from the blastocyst stage embryo and subjected to chromosomal or genetic analysis (Dahdouh et al., 2015, Palini et al., 2015). However, this technique entails some risk to the developing embryo, and, although it yields an accurate picture of the genetic and chromosomal status of the tested cells, it may not provide an accurate picture of the genetic and chromosomal status of the embryo as a whole, nor may it reflect overall embryo health and potential (Dahdouh et al., 2015, Cimadomo et al., 2016). Therefore, there remains considerable need for a diagnostic platform that could be paired with PGS/PGD, and by which embryo quality and developmental potential could be assessed prior to eSET. There are currently no routine biomarkers or comparable diagnostic indicators that can be used efficiently and non-invasively to determine the developmental potential and subsequent health status of an IVF embryo, or to identify those with the optimal potential from larger groups, leaving a considerable knowledge and practice gap in the IVF field.

In the preimplantation period, the embryo relies on diffusion and direct uptake for the exchange of gases and intake of metabolic substrates required for energy. As it continues to undergo the regular cell divisions necessary for early embryonic development, the breakdown
products (metabolites) of these substrates, as well as secreted proteins and nucleic acids, are released into the surrounding environment. For embryos cultured in vitro, these secreted products may reflect different aspects of the internal characteristics and potential quality of the embryo (Perkel et al., 2015). If changes can be detected and compared, there exists substantial potential for the development of a minimally-invasive diagnostic tool, in which the spent culture media is analyzed. This concept is not novel, however, the range of target molecules and our ability to detect and characterize them accurately from very small amounts of starting material has improved substantially over the past decade and continues to grow, further increasing the potential for the development of novel technologies.

The three major classes of molecules (recently reviewed by Rødggaard et al., 2015 in human IVF and Perkel et al., 2015 in domestic animals) with potential for non-invasive in vitro diagnostics are 1) the secretome: proteins secreted by the embryo that reflect the internal proteomic signature, 2) the metabolome: waste products of energy consumption, and 3) microRNAs: short strands of RNA released into media with a carrier protein, or packaged in small vesicles known as exosomes. The protein secretome and the metabolome were the first to be investigated as potential biomarkers, but the processing time, cost, and specialized equipment required to perform the relevant assays has significantly impeded their use in routine clinical settings. Most recently, studies have begun to examine the potential of small regulatory nucleic acids called microRNAs as specific biomarkers for the prediction of implantation success (Reviewed in: Traver et al., 2014).

MicroRNAs (miRNAs) are a class of short (~22 nucleotides), abundant, non-coding RNAs with numerous regulatory roles in development, including proliferation, developmental timing, cell fate determination, and apoptosis (Ambros, 2004). Their sequences are highly conserved among mammals and they have been detected in the gametes and embryos of many species including humans (Krawetz et al., 2011, Assou et al., 2013, Rosenbluth et al., 2013, McCullie et al., 2010). These single-stranded ribonucleic acids regulate gene expression by binding imperfectly to messenger RNA targets with complementary sequences and initiate mRNA destabilization or translational repression (Bartel, 2004). Estimates for the percentage of genes regulated by miRNAs have grown rapidly from 30% in 2005 (Lewis et al., 2005) to more than 60% of coding genes by 2014 (Cech et al., 2014).

miRNAs are ubiquitously expressed in mammalian cells and tissues, and abnormal increases or decreases in their expression play well-established roles in many diseases. Several types of cancer present distinct miRNA “signatures” (reviewed in Garzon et al., 2009) which are potentially predicitcive of disease progression. MiRNAs may also enter circulation, and serum/plasma miRNA profiles can be used to detect the associated disease states (Chen et al., 2008). The use of these circulating miRNA signatures as potential biomarkers has been investigated in many human pathologies including myocardial infarction (Ai et al., 2010, Wang et al., 2010) and liver disease (Arrese et al., 2015). As potent regulators of gene expression across a wide spectrum of cellular activities, their levels inside cells mediate profound effects on a wide range of cellular processes such as proliferation, apoptosis, and signalling. Importantly, in the context of ART, the presence and levels of miRNA in and around embryos is likely to provide a functionally relevant indicator of the “state of the embryo” from genetic, metabolic and developmental perspectives.

The evaluation of secreted miRNAs as non-invasive biomarkers of reproductive disease has recently become an intense focus of investigation, and miRNAs have been extensively profiled in many bodily fluids including amniotic and follicular fluids (Weber et al., 2010, Sang et al., 2013). Studies have demonstrated that secreted miRNAs have strong potential as useful prognostic metrics in clinical IVF settings: Rosenbluth et al. (2013) analyzed the miRNA content of human blastocysts and observed differential expression of several miRNAs between euploid and aneuploid embryos. A subsequent study by the same group revealed that some of these miRNAs are present in spent embryo culture media, and that specific miRNAs are associated with chromosomal status and pregnancy outcomes. In particular, high miR-191 expression in the culture medium is associated with aneuploidy, and high miR-372 in conjunction with miR-191 correlates with implantation failure when compared to medium from embryos that resulted in live births (Rosenbluth et al., 2014). These early findings suggest that miRNA abundance in culture media may be a highly relevant indicator of chromosomal content and implantation potential. Interestingly, miR-372 is one of the most abundant miRNAs present within euploid blastocysts (Rosenbluth et al., 2013). Taken together, the observations that miR-372 is abundant inside the blastocyst and is significantly more abundant in the spent medium of failed embryos raises interesting questions concerning whether the secretion of embryonic miRNAs into surrounding culture medium may be indicative of compromised embryo competency.

The presence of numerous specific, stable and detectable molecules secreted by embryos into their culture medium underlies the high potential utility of these molecules as non-invasive biomarkers of embryo quality. MiRNAs that specifically correlate with chromosomal status and developmental competence may represent useful adjunct diagnostic parameters for PGS, as well as novel targets in prognostic tests for overall embryo health and implantation potential. Research has demonstrated their resistance to degradation in the presence of changes in pH and after multiple freeze-thaw cycles (Chen et al., 2008, Mitchell et al., 2008), which helps offset concerns with respect to sample integrity in clinical settings, where storage and shipping may be necessary. Furthermore, miRNAs detected in circulation and other bodily fluids appear to be protected from degradation by several mechanisms: they are stabilized by binding to carrier proteins such as lipoproteins (Vickers et al., 2011), argonaute proteins that form the RNA-induced silencing complex (Turchinovich et al., 2011), or become
packaged into small vesicles known as exosomes that allow miRNA molecules to move through the extracellular milieu into recipient cells (Valadi et al., 2007).

The growing body of evidence described above strongly supports the potential use of miRNAs as promising non-invasive predictors of overall embryo health. Unlike secreted proteins and metabolites, miRNAs can be easily amplified and detected, suggesting that they can be readily assayed in small volumes of embryo culture medium. They are easily quantified by reverse-transcription polymerase chain reaction (q-RT-PCR), a method that is generally simpler and more cost-effective than the techniques required for detecting metabolites. Furthermore, these techniques can be readily employed either in-clinic by trained personnel or at medical diagnostic laboratories that routinely apply similar technologies to other medical problems. The intense research focus on miRNAs from functional and diagnostic perspectives, particularly in reproduction and development, strongly suggests that more miRNAs with additional diagnostic utility are likely to be identified in the future. In order to develop biomarker-based approaches to fertility problems, it will be essential to more definitively characterize the complex small RNA profiles that accompany embryos with robust health and developmental potential, compared to those with less favourable outcomes. Finally, because of their dynamic involvement in developing embryos, when multiple miRNAs are examined simultaneously in spent media, the profile or “fingerprint” that emerges may provide a more comprehensive picture of the many different elements that comprise overall embryo health.

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Female infertility contributes to a significant amount of infertility cases. Approximately 10% of female infertility has a genetic factor. Evolve Female FertilityReady™ Screen analyzes the most common genetic causes of infertility and factors that can impact reproductive success, either when attempting to conceive naturally or when undergoing assisted reproductive technologies (ART). Traditional physical and laboratory examinations for infertility cannot detect these genetic factors and often times misdiagnosis or delay diagnosis of an underlying genetic abnormality. Genetic screening allows for a more personalized and refined diagnosis and the potential for tailored fertility treatments.

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Leading the Way in Genetic Fertility Screening
An Overview of the Effects of Age on Fertility in Women

by Don Rieger, PhD
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I. INTRODUCTION

For a variety of social and economic reasons, women (and couples) are putting off having babies until they are in their mid-thirties, or later. As shown in Figure 1, this trend is common to most industrialized countries. In the United States, the average maternal age at first birth increased from 21.4 to 24.9 years of age from 1970 to 2000. Overall, birth rates in the United States decreased from 1970 to 1980, but since then, the birth rates for women over 30 years of age have increased significantly (Figure 2).

Although some women can, and do, have babies into their forties and even fifties, in general, fertility decreases markedly with a woman's age, particularly from 35 years onward. As shown in Figure 3, the chance of being infertile is approximately twice as great for women 40-44, compared with women 30-34. It is important to note that the onset of age-related infertility occurs approximately 10 years before menopause (te Velde and Pearson, 2002). The decrease of fertility with age, coupled with the tendency toward later child-bearing has led to the suggestion that “female ageing ... is now the main limiting factor in the treatment of infertility” (Ford et al. 2000).

This paper is intended as an overview of the information available on the relationships between age and fertility in women. It is by no means an extensive review of the literature, but rather, the data have been selected to illustrate general principles. Moreover, it is becoming increasingly evident that the fertility of a couple decreases with age of the male, as has been recently reviewed by Fisch (2005). The effect of male age is beyond the scope of this paper, but where relevant and whenever possible, studies have been chosen that have been controlled or adjusted for male age as well as frequency of intercourse and lifestyle factors.

Figure 1. The average maternal age at first birth in 1970 and 2000 for ten industrialized countries. (Mathews and Hamilton 2002)

Figure 2. The birth rate for women 30-34, 35-39 and 40-44 years of age in the United States from 1970 to 2000. (U.S. National Center for Health Statistics 2003)

Figure 3. The effect of a woman’s age on infertility and the chance of remaining childless. Adapted from Menken et al. (1986).
II. THE EFFECT OF AGE ON NATURAL CONCEPTION

The birth of a normal healthy baby requires that a woman be able to ovulate a mature, normal oocyte (egg) at the appropriate time, that a fertile sperm be present within the oviduct (Fallopian tube) for fertilization, that the oviduct and uterus be capable of supporting the development of the embryo, and that the embryo (later the fetus) reside in uterus until fully developed and delivered without major complications. Many of these processes have been shown to be affected by the woman’s age.

The development of the oocytes and the follicles in which they reside begins when the woman is herself a fetus. Primordial germ cells develop into oogonia which divide and differentiate into primary oocytes. The primary oocytes are enclosed in a single layer of granulosa cells to form primordial follicles, numbering approximately 7,000,000 in both ovaries at 4-5 months of gestation. From there onward, the oogonia stop dividing, and the primary oocytes are arrested in development until puberty. Most of the primordial follicles are lost to atresia such that only approximately 1,000,000 are left at birth, 40,000 at the time of puberty and 1,000 at menopause. Throughout reproductive life, groups of the primordial follicles spontaneously grow and develop into early antral follicles. Most of these are also lost, except for (usually) one follicle per menstrual cycle that continues to develop into a large antral (dominant) follicle in response to gonadotrophic hormones (FSH and LH) secreted by the anterior pituitary gland. A surge of LH at mid-cycle induces the final maturation of the oocyte in the dominant follicle and its release (ovulation) into the oviduct where it can be fertilized. (See Piñón, 2002)

The ability to produce and ovulate that one oocyte is directly related to the number of antral follicles on the ovaries at that time. As shown in Figure 4, the number of antral follicles present on the ovaries declines with age.

In addition to the significant decline in the number of antral follicles, increasing age is also associated with chromosomal and functional aberrations in the oocytes. Most notably, as shown in Figure 5, the frequency of aneuploidy (abnormal numbers of chromosomes) in human oocytes increases exponentially after 35 years of age. Although the sperm can also contribute to chromosomal defects in the embryo, defects in the aged oocyte are thought to be the major cause of Downs Syndrome and other chromosomal abnormalities in newborns (Figure 6). Increased maternal age is also associated defects in oocyte mitochondria, a structure responsible for energy production and many other important functions. These include an increased rate of point mutations in oocyte mitochondrial DNA (Barritt et al. 2000), and with a decreased ability of the mitochondria to produce energy (Wilding et al. 2002).

As discussed in the following section on assisted reproduction, there is evidence that maternal age can have effects on fertilization and on development of the embryo. In natural conception, it is difficult to be certain whether problems of fertility arise from deficiencies in the embryo or deficiencies in the reproductive tract. However, it is very clear that the ability to get pregnant, to maintain the
pregnancy, and to deliver a healthy baby decreases with maternal age.

Figure 7 shows that the probability of establishing a clinical pregnancy following intercourse on the most fertile day of the cycle decreases steadily with increasing maternal age. Even for women who do eventually become pregnant, the likelihood of achieving conception within six months is dramatically reduced for women 35 years of age and older (Figure 8).

![Figure 7](image)

**Figure 7.** The effect of a woman’s age on the probability of establishing a clinical pregnancy by intercourse on the most fertile day of the cycle. The data have been adjusted for paternal age and controlled for frequency of intercourse and lifestyle factors (Dunson et al. 2002).

When pregnancy is established, increasing maternal age has severely detrimental effects on the outcome. Many of these effects probably result from the effects of maternal age on the oocyte/embryo/fetus or the embryonic contribution to the placenta. Both the miscarriage rate (Figure 9) and the frequency of preterm delivery (Figure 10) increase with increased maternal age. The risks of a wide variety of perinatal complications in the mother and the baby are significantly greater in women older than 35 years of age compared with women from 18-34 years of age (Figure 11). For example, compared with women 18-34 years old, the risk of an emergency Caesarian section was 1.5 times as great for women 35-40 years old and more than twice as great for women older than 40. Based on historic data and on animal studies, Tarin et al. (2005) have suggested that delayed motherhood may also have long-term effects on the health of the children, including impaired fertility and reduced lifespan.

![Figure 9](image)

**Figure 9.** The effect of maternal age on the incidence of miscarriage (Gindoff and Jewelewicz 1986).

![Figure 10](image)

**Figure 10.** The effect of maternal age on the frequency of preterm delivery (Astolfi and Zonta 1999).

![Figure 11](image)

**Figure 11.** The risk of perinatal complications for women 35-40 or >40 years of age compared with women 18-34 years of age (Jolly et al. 2000).
III. THE EFFECT OF AGE ON THE SUCCESS OF ASSISTED REPRODUCTION

Infertility can result from lack of ovulation, poor quality oocytes, blocked oviducts (Fallopian tubes), impotence in the man, inadequate sperm numbers, poor quality sperm, or a poor interaction between the sperm and the cervical mucus. Many of these problems (and infertility due to unknown causes) can be treated, or at least circumvented, by assisted reproductive technologies (ART). However, although ART procedures can improve the chances of having a baby, the success rate decreases markedly with increasing age of the woman.

1. Intrauterine Insemination and Donor Insemination

Intrauterine insemination (IUI) is the simplest form of ART. Semen is collected from the male partner by masturbation and then the sperm are usually washed to remove dead cells and other possible deleterious components of the seminal plasma. The washed sperm are then placed directly into the uterus via a catheter which has been passed through the cervix. This serves to avoid any problems of passage of sperm through the cervix or cervical mucus, and provides a greater number of sperm within the uterus to increase the chances of fertilization. Intrauterine insemination is also commonly used in conjunction with ovulation induction, in order to ensure optimal timing of insemination.

Figure 12 shows that the age of the man can have a significant effect on the clinical pregnancy rate following IUI, but for any given age of the man, the clinical pregnancy rate decreases markedly with increasing age of the woman. Similarly, the pregnancy rate resulting from IUI with sperm from fertile donors is also significantly reduced with increasing age of the woman (Figure 13).

2. In-Vitro Fertilization

In-vitro fertilization (IVF) was originally developed to overcome the problem of blocked oviducts but is now also used to treat male-factor infertility (low numbers or quality of sperm) and infertility for which there is no apparent cause. In general, the woman is treated with gonadotrophins to increase the number of antral follicles that fully develop. It is important to note that gonadotrophin treatment has no effect on the numbers of primordial follicles that develop to the antral stage – it only acts to rescue the follicles that have already developed to the antral stage and would normally be lost to atresia. When the follicles have reached the appropriate size, the woman is given human chorionic gonadotrophin to mimic the normal ovulatory LH surge, and induce final oocyte maturation. A needle is used to recover the oocytes from the mature antral follicles. For standard IVF, the oocytes are placed together with sperm from the partner or a donor and the sperm penetrate the oocyte naturally. In cases where only small numbers or immotile sperm are available, fertilization can be achieved by injection of a single sperm into each oocyte (ICSI). After fertilization, the resultant embryos are cultured for 2 to 6 days and then transferred back into the uterus of the woman.

As shown in Figure 4, the number of antral follicles present on a woman’s ovaries decreases with age and this results in a decreased number of oocytes that can be retrieved for following gonadotrophin treatment for IVF (Figure 14a). Moreover, the quality of the oocytes also decreases with increasing age (Figure 14b), resulting in a decreasing proportion of the oocytes that can be successfully fertilized in vitro (Figure 14c).

Embryo development in culture is also affected by the age of the woman. In the example shown in Figure 15,
The proportion of fertilized oocytes that developed to the blastocyst stage by Day 5 was significantly reduced with increasing age of the woman.

Overall the reduced implantation rate and increased fetal loss rate in older women resulted in only 5% of embryos transferred developing to a live baby in women 41-42 years old compared with 23% in women younger than 35 (Figure 16d). For women 41-42 years old, only 11% of cycles started yielded a live birth compared with 37% for women younger than 35 (Figure 16e). This would mean that on average, a woman 41-42 years old would need 12 IVF treatment cycles to have a 75% chance of one live birth, compared with only 3 treatment cycles for a woman younger than 35.

In addition to the decrease in live-birth rate with increasing age, pregnancies and babies resulting from ART in older women using their own oocytes are subject to the same problems of pre-term delivery, perinatal complications and chromosomal abnormalities seen with natural conception.

3. The Use of Donor Oocytes

In cases where a woman has no ovaries or is otherwise unable to produce her own viable oocytes, oocytes may be obtained from other women. The donors are most often anonymous fertile, young women but may be a relative or friend of the patient. The donor is treated with gonadotrophins and oocytes collected as described in the preceding section. Sperm from the patient’s male partner is usually used for fertilization and the resulting embryos cultured and then transferred into the patient. Interestingly, it appears that the patient’s age has no appreciable effect on the ability to support a pregnancy. The live-birth rate for women receiving embryos created from donor oocytes is approximately 50% at all ages from 25 to 45 (U.S. Department of Health and Human Services – Centers for Disease Control and Prevention 2004). Of course, the babies born from donated oocytes have no direct genetic relationship to the patient.

Clearly, common ART procedures can improve the chances of pregnancy but cannot overcome the deleterious effects of aging on numbers and quality of the oocytes. Based on a computer model, Leridon (2004) has calculated that ART can make up for only half of the births lost by postponing an attempt to become pregnant from 30 to 35 years, and less than 30% of the births lost by postponing from 35 to 40 years. Based on a literature review and their own data, Broekmans and Klinkert (2004) conclude that the prognosis for a successful pregnancy with IUI or IVF for women 44 or older “is flat zero.”

There are, however, two specialized ART procedures, pre-implantation genetic diagnosis and oocyte cryopreservation, that can, or have the potential to, circumvent the effects of aging on fertility.
IV. APPROACHES TO CIRCUMVENTING THE EFFECT OF AGE ON FERTILITY

1. Pre-Implantation Genetic Diagnosis

As noted above, the frequency of chromosomal abnormalities in oocytes increases with age in women, and this results in increased frequencies of chromosomal abnormalities in the embryos, fetuses, and babies born. An early approach to this was to obtain cells from the fetus by amniocentesis or chorionic villus sampling for evaluation of the chromosomes. Fetuses with abnormal numbers of chromosomes were then aborted, in order to prevent the birth of chromosomally abnormal babies. More recently, it has become possible to determine the chromosome status of early embryos produced by ART, before they are transferred back into the patient (preimplantation genetic diagnosis, PGD). In this case, only embryos with normal chromosome numbers are transferred.

A positive side effect of embryo selection following PGD is that the implantation and birth rates are increased because chromosomally abnormal embryos are often also developmentally compromised. An example is shown in Figure 17, where the implantation rate for embryos that had been tested and judged to chromosomally normal was 17.6% compared with 10.6% for embryos that had not been tested (and presumed to be a mixture of normal and abnormal embryos). Pre-implantation genetic diagnosis is usually used for couples with some history of chromosomal or other genetic defects, recurrent miscarriage, or in older women. Based on the improved rates of development following PGD, it has been suggested that all embryos should be tested.

Figure 16. The effect of a woman’s age on the a) cancellation rate, b) implantation rate, c) fetal loss, d) approximate babies born per embryo transferred, and e) live birth rate with in-vitro fertilization of non-donor oocytes in the United States in 2002. (Taken or derived from: U.S. Department of Health and Human Services – Centers for Disease Control and Prevention 2004).

Figure 17. Implantation rates for unselected embryos and for embryos judged as chromosomally normal by pre-implantation genetic diagnosis (Munné et al. 2003).

2. Oocyte Cryopreservation

When living tissues are deep-frozen (cryopreserved) under the appropriate conditions, all biological processes are arrested and aging of the tissue stops until it is thawed. This approach has been long used for the storage of sperm and embryos, and has recently been extended to oocytes. A major interest in oocyte cryopreservation is to preserve the possibility of fertility for young women that are due to...
undergo radiotherapy and chemotherapy for the treatment of cancer. Such treatments can have severely deleterious effects on the oocytes. By removing and freezing the oocytes, they are not exposed to the cancer treatments. After the patient has recovered from the cancer treatments and wants to start a family, the oocytes can be thawed and fertilized, and the embryos transferred back into her uterus.

In the same way that cryopreservation can protect oocytes from cancer treatments, it could also be used to protect oocytes from natural loss and degeneration due to aging. Stachecki and Cohen (2004) have suggested that this may offer an approach to preserving fertility for women wishing to delay reproduction. Oocytes would be collected from young women and then cryopreserved until they are ready to begin their families. Although as yet largely experimental, the pregnancy rates from cryopreserved oocytes are improving.

V. CONCLUSIONS

There is a tendency for women in industrialized countries to delay having babies until their mid-thirties or later. There are important social and economic reasons for doing so, but it is imperative that women be aware that fertility decreases significantly with age, particularly after 35 years of age. From a purely biological perspective, the best approach to ensuring fertility is for women to have their babies before they have reached their mid-thirties, but for many women, this is not a desirable or even practical option. At any given age, assisted reproduction techniques may improve the chances of becoming pregnant, but cannot make up for the loss of fertility due to the effects of aging on the numbers and quality of oocytes.

References


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Exploring Our Evolving Genetic World: How Genetic Screening Provides A New Era in Patient Care

by Rosalie Ferrari, BSc, MSc

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Introduction

In the last 15 years, the field of genetics has advanced rapidly and garnered new insights that have revolutionized medical practice. The advent of new genetic testing technologies has afforded patients more options in the delivery of personalized medicine. Reproductive medicine may benefit most from these advancements by offering patients a unique opportunity to understand their risk of genetic disease for themselves and their families and plan for the healthiest families for the future.

Genetic testing is becoming necessary in routine medical practice. More than 2,000 genetic tests are available not only to detect well defined genetic conditions but also to identify genes and genetic factors that contribute to complex, common diseases (12, 20, 35). Genetic testing can be carried out through a variety of methods, enhancing its clinical utility. Genetic testing can be done on tissue or tumor samples; or more commonly, genetic samples are obtained through blood and saliva samples, which provide enough DNA to determine an individual’s genotype (11). This method is used to determine the carrier status of an individual during genetic screening.

Accessibility to genetic testing has never been greater, particularly after the first draft of the Human Genome Project was reported in 2001 (12, 22, 32). The Human Genome Project was a large-scale global effort to interpret the human genome sequence and identify the locations of human genes (2, 18, 31). The Human Genome Project was quickly followed by the ENCODE (Encyclopedia of DNA Elements) Project which provided an integrated analysis into the functional organization and regulatory elements in the genome (18, 29). These projects have led to the expansion of genetic techniques, including genome-wide association studies and next-generation sequencing, that delve deeper into genetic variants affecting disease and risk for disease (22, 26, 32, 38, 39).

Recapping Key Concepts in Genetics

Genetics is characterized by the concept of variation. Variation refers to the genetic differences that distinguish individuals. Phenotypic variability refers to whether an individual has evident features from an underlying genotype (i.e. the penetrance) and the degree of affectedness (i.e. the expressivity) (21, 25). Beyond variability, there is a distinction that needs to be made between the three main categories of genetic disorders detected through genetic testing: single gene, chromosomal, and complex diseases (31, 36).

Single gene disorders are caused by mutations in a single gene. These disorders can be inherited by autosomal recessive or autosomal dominant patterns, or through X-linked inheritance (See Figures 1-3). An individual’s risk to pass on a genetic disorder differs depending on the inheritance pattern for the particular disorder. Chromosomal disorders are caused by deletions or duplications of chromosomes or regions of chromosomes. The most common chromosomal disorder is caused by an extra copy of chromosome 21, known more commonly as Down syndrome. Complex disorders are caused not only by genetic factors but also environmental factors. Complex disorders are more frequently seen in the general population and include diseases such as Alzheimer’s disease and cancer. Although chromosomal disorders typically occur spontaneously and across any population, single gene disorders as well as complex disorders can occur in higher frequencies depending on the population (34).

Approaches to Genetic Screening

Human evolutionary genetics unveils the history behind genetic diversity within the human population. Genetic sequences illustrate the migration patterns of humans during history and continue to dictate how certain populations are at increased risk for particular genetic disorders (34). In modern, practical health terms, a patient may have an increased risk for genetic conditions based on their ethnicity. For example, Tay-Sachs disease is a severe, progressive, and fatal metabolic genetic disorder that is rare in the general population but genetic mutations that cause it are more common amongst the Ashkenazi Jewish population (17, 18). Genetic screening for Tay-Sachs disease has resulted in many carriers being detected within this population and led to a decrease in the incidence of the disease (30).

Many genetic disorders can be screened for with genetic testing. Genetic screening identifies patients who may be at
Figure 1. Autosomal Recessive Inheritance (40). Generally, both parents are carriers for an autosomal recessive disease. There is a 25% chance offspring will be affected with an autosomal recessive disease and a 50% chance offspring will be carriers for an autosomal recessive disease.

Figure 2. Autosomal Dominant Inheritance (40). Generally, one parent is affected with an autosomal dominant disease by having a mutated or altered copy of a gene. There is a 50% chance offspring will also be affected with the autosomal dominant disorder.
risk for passing on a genetic condition or who may have a genetic condition. Screening benefits early treatment options (e.g. newborn screening), provides additional prevention measures (e.g. mammograms for breast cancer screening), or assists during reproductive decision making (5, 20, 28). Healthcare practitioners hold a special role working with patients to navigate the ever-evolving landscape of genetic screening services. Genetic screening is typically used on reproductive issues or when there is a strong family history of cancer. In the latter case, genetic screening can reveal an increased hereditary predisposition to cancer in a patient. This can affect management and, as mentioned previously, provide additional prevention measures (10).

All patients undergoing fertility treatments can benefit from determining their carrier status through genetic screening. A patient’s carrier status not only impacts a pregnancy but can also, at times, detect a genetic cause for infertility in the individual (3, 14). There are different times when genetic screening may be optimal for a patient. Patients could undergo genetic screening prior to a pregnancy to determine the risk of passing on a genetic condition. Patients could have genetic screening completed during a pregnancy to determine the risk a genetic condition was inherited. In fact, the American College of Obstetricians and Gynecologists (ACOG) recommend patients to have genetic screening, especially if an individual’s ethnicity places them at greater risk of being a carrier for a genetic condition. If patients are found to be carriers for genetic conditions, fertility clinics can offer patients preimplantation genetic screening.

Preimplantation genetic screening (PGS) is an alternative option for patients to improve the effectiveness of in vitro fertilization (IVF) by screening embryos for genetic conditions (11, 19, 23). It is estimated in the United States that 8% of IVF treatments involve PGS and the technique is quickly reemerging as an essential part of clinical practice (27). PGS significantly reduces the risks of transferring chromosomal abnormal embryos or embryos affected with a genetic disease (14, 27). PGS methods, such as blastocyst stage biopsy, are continuously being enhanced and employed using the safest techniques in order to diminish implantation failures (7).

PGS can be guided by knowing a patient’s genetic carrier status and eliminates the need for traditional prenatal diagnostic testing during a pregnancy. Prenatal diagnostic testing (i.e. amniocentesis and chorionic villus sampling) expose patients to a high risk of miscarriage and are often anxiety-producing procedures (7). Patients can benefit from determining their carrier statuses through genetic screening prior to utilizing PGS. PGS is expanding reproductive options for patients who are carriers for serious genetic conditions while improving the success of IVF procedures (6, 7).

Central to supporting informed decision-making, patients need to understand the risks and benefits involved in genetic testing (13, 22). Risks of genetic testing include incidental findings and the potential for further confirmatory diagnostic tests; for example if a pregnant woman has a positive genetic screen for Down syndrome, a diagnostic test (e.g. amniocentesis) would confirm a positive result (33). Genetic testing benefits patients by allowing them access to information that can transform their personal, family, and reproductive health. Ensuring patients understand the benefits and risks involved with
genetic testing is necessary for informed healthcare choices (1).

Results from genetic testing can be complex and it is imperative to have competent, reliable genetic specialists to interpret information for the patient. Often times, genetic counselors provide support and education while empowering patients during their decision-making processes (24). Genetic testing services which involve genetic counseling confirm a steadfast commitment to providing patients with quality healthcare.

Case Samples

Case 1

A female, age 33, and her male partner, age 34, are pursuing fertility treatments at a local clinic. Both are Caucasian and do not have significant family histories for any genetic disorders. However, their ethnicities place them at an elevated risk for Cystic Fibrosis, which is more common amongst individuals of Caucasian ethnicities, with a carrier rate of approximately 1 in 25.

Cystic fibrosis is a single gene disorder, inherited in an autosomal recessive pattern, and affects many body systems primarily impacting digestive and respiratory functions. Although the disorder has seen advances in treatment, cystic fibrosis is typically fatal in adulthood.

A doctor recommends they consider genetic screening as part of their treatment due to their Caucasian ethnicities.

Results reveal both are carriers for cystic fibrosis. These individuals can consider more family planning options now that their carrier statuses have been disclosed. They may need additional fertility treatments to manage the CFTR mutation that has caused the male partner’s infertility. They may decide to pursue diagnostic testing during a pregnancy to determine if cystic fibrosis was inherited. They could also decide to pursue preimplantation genetic screening with IVF, to ensure cystic fibrosis is not a risk for a pregnancy.

Case 2

An African-American female, age 35, is considering IVF and makes an appointment at a fertility clinic. She mentions during the visit that her male partner, age 36, has sickle cell trait. Sickle cell trait is not a genetic disorder; sickle cell trait is a hemoglobin variant (HbS). There are many different forms of hemoglobin variants due to mutations of the HBB gene.

Sickle cell anemia is a genetic disorder that occurs when an individual inherits two copies of HbS variant. Sickle cell anemia causes characteristic sickle shaped red blood cells that can lead to painful vaso-occlusive crises. Life expectancy for individuals with Sickle Cell Anemia is lower than the general population. Sickle cell anemia is more common among individuals of African-American ethnicities, with a carrier rate of approximately 1 in 12.

A doctor recommends she undergo genetic screening to determine her sickle cell trait status.

Results reveal she is a carrier of a HbC variant. Although the risk of sickle cell anemia has decreased, the couple is at an increased risk to have a child with HbSC sickle cell disease. HbSC disease is generally less severe than sickle cell anemia; however, HbSC disease can still be clinically significant. These individuals can consider more family planning options now that their carrier statuses have been disclosed. If they opt to pursue preimplantation genetic screening with IVF, their carrier statuses can be used to guide the procedure.

Case 3

A female, age 34, and her male partner, age 37, have been trying to get pregnant for the previous 5 months. Neither have a family history of infertility but they want to assess whether there may be a genetic factor affecting their chances to conceive.

A doctor recommends they may benefit from genetic screening with male and female genetic fertility screens. Results reveal, the female partner is a Fragile X FMR1 premutation carrier, with 89 CGG repeats discovered upon analysis. This result may explain why they are having trouble conceiving, as a significant portion of Fragile X FMR1 premutation carriers are at risk for premature ovarian failure and therefore may have to consider other reproductive options. Fragile X FMR1 premutation carriers are common in the general population with approximately 1 in 150 women being carriers.

Fragile X FMR1 premutation carriers are not only at risk for infertility but they are also at risk to pass on the genetic disorder Fragile X syndrome to their sons. Fragile X syndrome is the #1 cause of inherited intellectual disabilities. This couple can use their genetic fertility screening results to plan and discuss what preconception reproductive options including IVF with PGD may be available for their family planning needs.

Conclusion

Genetic screening affords patients a new and exciting avenue for personalized medicine. Emerging laboratories are offering genetic screening panels that increase accessibility to meet patient demands (4). Patients receiving reproductive assistance are bound to take full advantage of these clinically relevant and practically meaningful genetic screening choices. Although current genetic screening focuses on preventing or predicting the risk of disease through genetic screening, future applications will likely lead to remarkable advances in healthcare such as curing cancer (37). Maintaining that patients receive understandable results after genetic screening will preserve the value of the information and retain genetic screening as an invaluable part of comprehensive patient care for years.
References

Next Generation Sequencing Brings New Opportunities For Noninvasive Prenatal Aneuploidy Screening

by Swan Cot, MSc

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The development of new sequencing and genotyping technologies has brought an ever-expanding menu of genetic tests into clinical practice over the past decade. The field of genetics has enabled healthcare providers to deliver a more personalized approach to patient care. There is no better application of personalized medicine than for the provision of reproductive care. From the pre-conception stage through to prenatal care, the field of genetics plays an important role in the evolving paradigms of healthcare options for prospective and expectant parents. The delivery of faster and more accurate information can help guide better decisions for the intended parents on their individual reproductive journey.

Non-invasive prenatal test (NIPT) is a method to screen for fetal chromosomal aneuploidies from maternal blood. It provides significantly better risk indication than traditional prenatal screens and poses no risk of miscarriage or fetal damage normally associated with invasive diagnostic procedures. With its high specificity and sensitivity for both high-risk and low-risk pregnancies [1,2], NIPT offers the greatest opportunity for reproductive autonomy by all women or parents.

Safer and More Reliable Aneuploidy Screening Strategies

It is important for prenatal care programs to have the capability to accurately identify women with high-risk pregnancies while allowing for timely monitoring and management strategies to be prescribed to best fit each clinical situation [3]. Current prenatal diagnosis for fetal aneuploidies typically relies on a mathematical risk assessment from first trimester screening, followed by the use of invasive diagnostic procedures to confirm fetal chromosome abnormalities in high-risk women. In general, first trimester screening options carry a detection rate of 75% - 96% and a false-positive rate of 5% -10% for Down syndrome [4 - 7]. Such high percentages of false-positives and false-negatives of these options can lead to a great deal of anxiety for the parents, and may result in many unnecessary invasive procedures, which carry a 1% - 2% risk of miscarriages [8]. Due, in large part, to these factors, the pursuit of safe and reliable strategies has, for the past decades, been the focus of many investigations and scientific inquiries. By reducing the need for multi-step screening and invasive procedures, non-invasive approaches can have a great impact on the delivery of optimized prenatal care. The rapid development of NIPT for aneuploidy detection was made possible by two main technical advancements: the discovery of fetal DNA in maternal circulation and the development of Next-generation sequencing.

Cell-Free Fetal DNA (cffDNA)

The presence of fetal cells in maternal blood has been a well-recognized phenomenon since 1969 [9-12], leading to the possibility of using these cells for noninvasive approaches to identify fetal genetic abnormalities [13]. However, the scarcity of intact fetal cells in maternal blood makes it technically challenging to implement on a large scale, thus, preventing its use in routine clinical practice [14].

In 1997, Lo and colleagues demonstrated the presence of male fetal DNA freely circulating in maternal plasma and serum. This was the first concrete evidence that cell-free fetal DNA (cffDNA) from maternal circulation during pregnancy could be reliably detected [15]. The advantage of using cffDNA is its abundance and stability in maternal circulation [16-19], providing a much better alternative genetic source than amniocentesis or chorionic villus sampling for prenatal detection of chromosomal aneuploidies which, in turn, translates into a simpler and more cost-effective process for scale-up applications. The cffDNA obtained is also most suitable for pregnancy-specific testing since it is cleared from maternal circulation within hours after delivery, excluding the possibility of contamination from previous pregnancies [20, 21].

The source of cffDNA found in maternal circulation has been demonstrated to originate from trophoblast cells in the placenta, these cells undergo apoptosis events releasing DNA fragments into maternal circulation [17, 22 - 24]. The fetal DNA can be detected in maternal serum as early as 5 to 7 weeks gestational age and continues to increase as pregnancy progresses, with a 21% weekly increase in the first trimester [24 - 26]. The minimum level of cffDNA needed to accurately identify fetal aneuploidy can be achieved at 10 weeks gestational age and represents approximately 10% of the total cell-free DNA population circulating in maternal blood (ranges 3 - 19%) [25, 26].

Next-Generation Sequencing

The Human Genome Project has led to the development of massively parallel sequencing approaches, also known as Next-Generation Sequencing (NGS) [27-29]. Such technologies can perform millions of DNA sequencing reactions simultaneously in a single run, thus acquiring large-scale genomic data at unprecedented speed and relatively low cost. There are three main NGS-based
approaches to NIPT for aneuploidy: whole-genome analysis; targeted chromosome panels; and targeted single nucleotide polymorphisms (SNPs). All three approaches can analyze the full cell-free fetal DNA complement in maternal circulation without the need for fetal DNA isolation or fetal fraction enrichment techniques. Detection sensitivity and specificity are high for all common aneuploidies, irrespective of the sequencing approach or the bioinformatic algorithm used [1, 2, 30]. In general, the fetal and maternal DNA are sequenced simultaneously and analyzed using sophisticated bioinformatics software. Accurate counting of the sequenced fragments for specific chromosomes are then compiled and aneuploidy is indicated by either an increase or a decrease in the count when compared to the expected threshold [26, 31, 32]. For example, if the fetus has trisomy 21 (Down syndrome), the sequencing count for chromosome 21 will yield a higher count than expected from maternal plasma (Figure 1). With genome-based techniques offering higher sensitivity and clearer resolution, we can now achieve the long-sought goal of non-invasive methods of testing for aneuploidy that can be put into routine practice.

Limitations of NIPT

Although the sensitivity and specificity are far better than conventional screening options for the common aneuploidies, NIPT should still be regarded as a highly sensitive screening test, rather than a diagnostic one [6, 33-36]. There are documented cases of false-positives and even rarer cases of false-negatives in the literature [37, 38]. These false-positives have been attributed to several biological factors: confined placental mosaicism; cotwin demise (vanishing twin); maternal aneuploidy; or maternal malignancy [39-45]. Due to these potential factors, it is recommended that a positive NIPT result should be confirmed by invasive diagnostic procedures such as amniocentesis or karyotyping of cultured chorionic villi [6, 33-36].

In up to 5% of the cases, low fetal fraction can contribute to an inconclusive (no call) result [46, 47]. There are three main contributors to low fetal fraction: testing before 10 weeks of gestation; high maternal body mass index; and fetal aneuploidy [48, 49]. It has been estimated that 22% of cases which failed to obtain a result through NIPT were, in fact, aneuploid pregnancies [50]. In the event of a no-call result, it is recommended that patients should be counseled on the options of repeat NIPT or diagnostic testing, and be made aware that repeat NIPT may delay diagnosis of fetal aneuploidy which may also affect the management of time-sensitive reproductive decisions [36].

NIPT Facilitates Autonomous Reproductive Choices

Non-invasive prenatal testing of cell-free DNA for aneuploidy identification has been regarded as a revolution in prenatal testing practice. It is not surprising that NIPT has garnered much commercial interest in the field. Since its inception into clinical practice in Hong Kong in 2011, NIPT has quickly spread to more than 60 countries around the world and counting [51, 52]. The global NIPT market has been estimated to reach $3.62 billion (USD) by 2019 [53].

Figure 1. Next-Generation Sequencing for the non-invasive prenatal detection of fetal chromosome aneuploidy.
NIPT is quickly changing the traditional framework of prenatal screening and diagnosis. Its rapid integration into routine prenatal care is prompting major ethical concerns on the responsible implementation and process of informed decision-making [54]. While all professional societies recognize the significant benefits offered by NIPT, maximum potential can only be achieved by educating patient and healthcare professionals on its utility, benefits, and limitations [33-36]. It is their opinion that genetic counseling is paramount to ensuring informed decision-making and reproductive autonomy. Special emphasis has been placed on the importance of providing accurate information prior to testing, for the purpose of informed consent, and also to mentally prepare the patients on the implications of what the test results could mean for them.

ACOG and SMFM updates guidance on NIPT

On March 1, 2016, The American College of Obstetricians and Gynecologists (ACOG) and Society for Maternal-Fetal Medicine released a joint Practice Bulletin to provide updated information regarding the available screening test options for fetal aneuploidy. Cell-free DNA screening, or NIPT, have now been added to the list of screening options recommended. The new recommendations strongly encourage physicians to discuss and offer aneuploidy screening or diagnostic testing to all women early in pregnancy, regardless of maternal age or risk factors. Women who have achieved IVF pregnancies, with or without preimplantation genetic screening, can also benefit from these options. Such discussions should take place at the earliest opportunity (ideally at the first prenatal visit), and discussion should include the benefits, risks, and limitations of the available screening and/or diagnostic test options. All management decisions should fit within the scope of the patients’ clinical circumstances, values, and goals. [36]

Future of NIPT

NIPT using cell-free DNA screening for aneuploidy is bringing the most radical change seen in prenatal care over the past decades. We have already seen a dramatic decline in invasive testing in countries with the clinical uptake of NIPT [55], which is met with overwhelming approval from patients and healthcare professionals alike [56, 57]. As molecular techniques continue to improve, NIPT promises to deliver increased use at a lower cost. The range of conditions it can detect is expected to expand and is likely to include microdeletions, microduplications, and single-gene disorders. As we move toward safer and earlier prenatal diagnosis, the opportunity for therapeutic interventions and treatments will have a significant impact on the delivery of fetal medicine. It will be crucial to educate clinicians on how to guide their patients through the complicated decisions as testing options increase. Autonomous decision-making is certainly attainable when patients and front-line healthcare providers are fully engaged in understanding the complexity of testing and interpretation of results. Responsible implementation of NIPT can have a far-reaching impact on the delivery of optimized prenatal care, with the best possible outcome for both mother and baby.

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ARTICLES

Donor Gamete Genetic Screening: Reducing Risk or Too Much Information?

by April O’Connor, MS, LCGC

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Introduction

Advancements in the field of genetics born out of discoveries made by the Human Genome Project and other similar projects have led to the expansion and implementation of genetic techniques, such as next-generation sequencing (NGS), in the diagnosis and treatment of human disease (1-5). Next generation sequencing technology has substantially facilitated the application of a form of genetic testing known as carrier screening. Traditionally, carrier screening was offered to patients based on their ethnic background or family history for a limited number of genetic conditions, but today with NGS technologies carrier screening can be offered for a much wider spectrum of inherited monogenic diseases with faster turnaround time but without significant increase in costs (4,5). Carrier screening in the context of reproductive medicine, involves genetic testing performed on asymptomatic adult individuals to determine their heterozygous status for mutations that can cause severe disease in their offspring (6-8). Genetic screening for reproductive purposes is recommended before conception whenever possible, so that patients can benefit from the greatest number of options and time for decision making regarding genetic testing and pregnancy management.

There has been a steady rise in the use of donor gametes in assisted reproductive technology (ART); however, recommendations regarding genetic screening of donors are still limited and based on the donors’ reported ethnicities (9). In contrast, pan-ethnic carrier screening is routinely offered to fertility patients under the guidance of medical society recommendations (10). This discrepancy in screening practices may result in patients receiving donor gametes which confer a high reproductive risk. Screening donors and patients for the same genetic conditions will allow patients planning to use donor gametes to make the most informed decisions throughout the IVF process. Given this concern of possible increased reproductive risk associated with genetic disease through the use of donor gametes, The American College of Obstetricians and Gynecologists (ACOG), The American Society for Reproductive Medicine (ASRM), and The Royal College of Obstetricians and Gynecologists all recommend that genetic screening for heritable diseases should be performed on potential sperm and oocyte donors (9-11).

The Importance of Donor Gamete Genetic Screening: Reproductive Risk Reduction

Genetic diseases are rare or extremely rare (affecting one in thousands or millions); however, they are common in the aggregate, with a gross estimate of the combined prevalence within the 0.4%–2.0% range for either single-gene or chromosomal disorders (4,5,8,21). Furthermore, almost 20% of pediatric mortality is due to genetic alterations, up to 70% of the children admitted to a pediatric intensive care unit carry genetically determined disorders, and 5%–12% of all child hospital admissions caused by them entail longer hospital stays, and increased morbidity and mortality (4,7).

Gamete donor eligibility at sperm and egg banks has traditionally been determined in part by infectious disease screening and family history risk assessments (9). However, routine testing for genetic disease is becoming an increasingly important part of the donor selection process due to the rising number and expanding availability of genetic tests (12,14). In addition, patient demands for donor genetic testing influence the screening and selection of donors at sperm and egg banks (12). The appropriate genetic testing panel for the gamete donor applicants may be determined using preconception and prenatal carrier screening guidelines that have been published by the American College of Medical Genetics (ACMG), and the American Society of Reproductive Medicine (ASRM) (9,10). Regardless of which diseases the donor is selected to be tested for, it is imperative to screen gamete donors for hereditary conditions that can be passed to the offspring and have a significant impact on quality of life and survival. This is evident in a recent study by Retamar, et. al. of 302 oocyte donors genetically screened for 250 autosomal recessive diseases (13). In this cohort, the donors screened positive for 35 of the total diseases screened (14%), 74.3% of these 35 diseases had a high impact over life expectancy and quality of life, and 25.7% had a moderate impact (13). Among the high impact diseases, 61.54% may have available medical treatment, but the remaining 38.46% do
not (13). Moderate impact diseases were the least present in the positive donors with 44.4% of these diseases having no associated treatment and 55.6% having a possible treatment available (13). A genetic assessment is a critical step in the evaluation of prospective oocyte donors, because almost one quarter of donors may have family history of a disease that could pose an increased fetal risk (14). Disclosure of ovum donor carrier status and, when applicable, testing of partners is imperative before accepting a potential ovum donor for assisted reproductive treatment.

In addition, screening semen donors for a number of genetic conditions that are passed silently from generation to generation is also warranted since family history alone cannot identify them (15-20). A recent study by Isley, et al. demonstrated this point through an 8.5-year retrospective review of outcome reports and donor management summarizing the medical risks to donor-conceived offspring that presented after the sperm donors were qualified for participation in the donor program (17). Records from these operations for the 8.5-year period from January 2007 through June 2015 were reviewed (17). Distribution of vials from 108 donors were restricted from January 2007 through June 2015, and approximately one third of the restrictions involved risks for 21 different autosomal recessive (AR) conditions (17). Pregnancies and births were reported for each of the 35 donors who were confirmed or suspected to be carriers for specific AR disorders before identification of the AR disease risk (17). For the 20 cases in which a child was diagnosed with the disorder, the child’s biological mother and the donor were both previously unidentified carriers of mutations for the same recessive disorder (17). Restricting distribution of vials and notifying clients of the donors’ carrier statuses allowed recipients to be informed about the new information, and educated about risks before use of additional specimens to reduce the risk of having a child with that specific disorder (17).

It is imperative that genetic screening of gamete donors be comprehensive and assess autosomal recessive carrier risks as well as the risk for chromosome abnormalities in the offspring. Approximately 1 in 500 individuals is a carrier of a balanced chromosome translocation (7,8,21). Thus, screening gamete donors for chromosome abnormalities such as balance translocations by way of karyotype is an important consideration to help improve the likelihood of a health pregnancy outcome. This important aspect of screening in addition to carrier assessment for autosomal recessive disease was discussed in greater detail in the article by Reh et al. where the results of genetic screening of 1303 oocyte donors were evaluated (22).

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of donors</th>
<th>General Population Risk (%)</th>
<th>Average risk to donors’ offspring (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achromatopsia</td>
<td>1</td>
<td>1/38,000 (0.003)</td>
<td>1/390 (0.26)</td>
</tr>
<tr>
<td>Congenital adrenal hyperplasia (due to 21-hydroxylase deficiency)</td>
<td>3</td>
<td>1/15,000 (0.006)</td>
<td>1/248 (0.40)</td>
</tr>
<tr>
<td>CF</td>
<td>3</td>
<td>1/3,136 (0.03)</td>
<td>1/112 (0.9)</td>
</tr>
<tr>
<td>DFN1B1 (nonsyndromic hearing loss)</td>
<td>1</td>
<td>1/7,259 (0.014)</td>
<td>1/170 (0.59)</td>
</tr>
<tr>
<td>Galactosemia</td>
<td>3</td>
<td>1/40,000 (0.0025)</td>
<td>1/100 (1)</td>
</tr>
<tr>
<td>Glycogen storage disorder type 1a (GSD1a)</td>
<td>1</td>
<td>1/100,000 (0.001)</td>
<td>1/832 (0.16)</td>
</tr>
<tr>
<td>Joubert syndrome</td>
<td>1</td>
<td>1/80,000 (0.001)</td>
<td>1/564 (0.18)</td>
</tr>
<tr>
<td>Medium-chain acyl-CoA dehydrogenase deficiency</td>
<td>1</td>
<td>1/14,600 (0.007)</td>
<td>1/242 (0.41)</td>
</tr>
<tr>
<td>Phenylalanine hydroxylase deficiency</td>
<td>2</td>
<td>1/10,000 (0.01)</td>
<td>1/200 (0.5)</td>
</tr>
<tr>
<td>Pompe disease</td>
<td>1</td>
<td>1/40,000 (0.0025)</td>
<td>1/100 (1)</td>
</tr>
<tr>
<td>Smith Lemli Opitz syndrome</td>
<td>1</td>
<td>1/18,604 (0.005)</td>
<td>1/273 (0.37)</td>
</tr>
<tr>
<td>Spinal muscular atrophy</td>
<td>8</td>
<td>1/12,986 (0.008)</td>
<td>1/228 (0.44)</td>
</tr>
<tr>
<td>Very-long-chain acyl-CoA dehydrogenase deficiency</td>
<td>1</td>
<td>1/31,500 (0.003)</td>
<td>1/374 (0.27)</td>
</tr>
<tr>
<td>Other disorders</td>
<td>8</td>
<td>1/40,000 (0.0025)</td>
<td>1/100 (1)</td>
</tr>
</tbody>
</table>


Figure: Newly identified risks for autosomal recessive (AR) disorders in donor-conceived offspring (17).
the risk of children born from these pathways of assistive reproductive treatment. It can be inferred that the genetic screening of recessive mutations in people who donate their gametes will further reduce the risk of certain genetic diseases transmission.

Too Much or Too Little Donor Information? Solution: Customized Genetic Screening

A gamete donor facility faces many challenges when incorporating new tests into its donor screening program. Although the approach of screening all active donors is costly, time consuming, and potentially distressing to recipients, there is significant benefit to this process. It is not possible nor is it reasonable for gamete donor facilities to perform every available genetic test on donor applicants, nor would it eliminate the risk for birth defects in donor conceived offspring even if it were possible. However, since gametes from an individual donor may be used by several recipients, there is an increased risk for autosomal recessive disease in the offspring of that donor due to the multiple pairings, as opposed to the risks to offspring of a single couple. Hence, it is reasonable to perform screening of potential gamete donors for common and severe disorders such as SMA due to the early onset of symptoms, significant suffering, and current lack of established, effective treatment (18).

Test requests on gamete donors has increased dramatically and is complicated by the donor’s availability and willingness to participate. Testing frequently delays clients’ plans and causes anxiety especially if the client is already pregnant (16,25). It can be costly to the couple if he or she is a carrier for a rare disorder and testing on the donor for the specific disease is only available through a few laboratories. Expanded carrier screening on donors during qualification would increase the detection of carriers and reduce some of these challenges (17,18,25). However, with expanded carrier screening a greater percentage of donors would be identified as carriers for recessive diseases (25). Gamete providers would be unable to continue excluding these applicants as it may lead to diminishing pools of donors. Gamete providers would also be unlikely to expand screening if the inventory from these donors is unlikely to be used. Therefore, the use of customized smaller carrier screening panels specifically designed for reproductive donors can provide a solution for gamete donor programs, as a means to effectively screen their donor population while minimizing the potential drawbacks of expanded carrier screening.

However, it is likely that these evaluations would identify specific genetic risks for some donors, because many reproductive screening tests are designed to detect individuals who carry mutations for common genetic disorders that are inherited in an autosomal recessive manner and donors are just as likely as any other individual to test positive on these tests. (12,13). Rodriguez, et al. calculated and compared the observed carrier frequencies of select high-impact conditions in the donor and non-donor patient populations (25). No significant differences in carrier rates between the two populations were observed (25). These results demonstrate that, as predicted, gamete donors are equally as likely to be carriers as general fertility patients. Knowing this, the greatest reduction in risk for those receiving donated gametes will come from ensuring both donor and patient undergo similar levels of carrier screening. Hence, it is important to ensure that the manner in which screening is conducted does not unnecessarily eliminate healthy carriers from the donation process.

Conclusion

Gamete donor facilities do not currently have mandated regulatory requirements to perform extensive genetic testing on their donors, and there are significant costs associated with such testing (26). However, the objective of reproductive medicine is to help recipients achieve their goals of having children, and it is important to support those goals through practices that emphasize the well-being of those children. Resources for genetic disease risk reduction should be allocated towards family medical history evaluation, genetic screening, ongoing risk assessment services, and proper education of gamete donor recipients in order to achieve the greatest benefit to the broadest population. These practices, combined with the application of carrier screening for recessive genetic disease and karyotype evaluation for chromosome abnormalities are desirable for the evaluation of inherited risks in donor-conceived individuals. Consequently, by harnessing the technological advancements born out of the mapping of the human genome, the field of reproductive medicine now has access to higher quality donor gametes with lower associated reproductive risks by way of comprehensive genetic screening availability.

References

The Relationship Between Judaism and Assisted Reproductive Technology

by Ronit Lebor, MS, LCGC

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I am not a rabbinic authority. If one has a question regarding appropriate assisted reproductive technology or regarding the process of assisted reproductive technology in terms of permissibility within Judaism, a Rabbi or Yoetzet Halacha (female advisor in Judaism) should be consulted.

Introduction

Judaism is a religion based on tradition. The traditional Jewish legal system begins with the Torah, written law, and the Talmud oral law. These laws are interpreted by various Jewish scholars to give Jews the laws that are practiced today. Therefore, the Jewish legal system is built upon hundreds and hundreds of years of interpretation of Talmudic law, taking the principles and applying them to the modern world we now live in. Additionally, these laws may be different for Jews who identify as different sects within Judaism (reform, conservative, orthodox, ultra-orthodox etc.) as rabbis within those different sects may interpret the Torah and/or Talmud differently. Even within the same sect of Judaism, scholars interpret the Torah and Talmud differently, ruling differently as to what is permissible.

Anytime there is a question in Jewish law that does not have a precedent, such as assisted reproductive technology which was not present during the time of the Torah and Talmud, medieval scholars and current scholars look for a case that is somewhat analogous and apply it to the modern situation. In this way, the chain of Jewish law is essentially case law. Regarding assisted reproductive technology, modern day scholars probe the sources to understand what potential conflicts exist. Originally, when assisted reproductive technology was first being introduced, scholars had to determine if these technologies were permissible. Since the purpose of this technology is to have children, which Judaism promotes, currently the question is not so much if this is allowed but rather which methods are most preferable within the parameters of the laws in the Torah and Talmud.

Male Fertility Work Up

One issue that arises during the male fertility work up is the process of sperm extraction. One concern in Judaism for sperm extraction is the prohibition of “wasting seed” (1, 2, 3). This prohibition applies to masturbation, use of condoms, as well as other methods used to collect sperm. However, some modern-day scholars have ruled that in an IVF setting, the goal of sperm extraction is for procreation, and, therefore, the methods used are not prohibited. However, scholars may have a hierarchy of which methods are more ideal to use given the prohibition of “wasting seed” (4). Not only do rabbinic authorities differ in the best method for sperm extraction, but they also differ about the best time for the male fertility workup (12).

Another issue that arises during the process of sperm extraction is the prohibition to mutilate genitalia. This prohibition causes debate of permissibility for procedures such as testicular sperm extraction (TESE). However, many scholars permit these procedures as the injury will likely heal and result in enhanced fertility (5).

In these cases, genetic fertility testing may be useful. Since looking at genetic causes of male infertility can guide the patient to a specific type of sperm extraction or specific type of assisted reproductive technology, Jewish individuals may find it beneficial to have genetic fertility screening prior to undergoing sperm extraction or treatment. Having this testing can provide the utility to waste the least amount of seed, by utilizing the assisted reproductive technology that is most likely to result in pregnancy due to genetic contributions to the couples’ infertility.
Timing of Treatment

The timing of treatment is important in Judaism due to laws of “Niddah”. Niddah refers to family purity laws. While a woman is bleeding from her uterus, and seven “clean” days afterwards (7 days of no bleeding), intercourse is prohibited (6, 7). Once the seven clean days have passed, the woman immerses herself in a mikvah (7, 8). A mikveh resembles a large bath where ritual purifications occur. There is debate among modern Jewish scholars as to if this prohibition for intercourse extends to conception as well. If it does indeed extend to conception, then a woman cannot undergo intrauterine inception (IUI) until after the seven clean days and immersion in the mikvah (9). Additionally, based on these laws, it would be ideal when performing implantation of an embryo that it does not occur while a woman is considered in Niddah (10). The question also arises as to if any assisted reproductive technology procedures cause uterine bleeding. If a procedure does cause uterine bleeding, the follow up question of ‘Is this woman now in Niddah?’ arises.

Another consideration that arises for timing of treatment is the administration of treatment and monitoring on Sabbath or Jewish holidays. On Sabbath and holidays, there are many restrictions to everyday activity that are not applicable on other days. Although many scholars rule that it is ideal if treatment/supervision be avoided on Sabbath/holidays, logistic and medical factors must be considered as well before a final decision is made.

Donors and Surrogacy

There is controversy within modern day scholars if utilizing a donated egg/sperm or using a surrogate is permissible. For those scholars who rule that using an egg donor is permissible, there are many questions to follow. For example, who is considered the “mother” from the Jewish law perspective? If the donor is not Jewish, is a conversion required for the child (11, 12)? Although, the question of conversion is not as pertinent for donor sperm as it is believed that the status of Jew is passed down maternally, there is still the question of does the sperm donor need to be Jewish or should the sperm specifically be from a gentile donor? Additionally, these questions apply in the case of surrogacy. Does the surrogate need to be Jewish? If so, are there certain standards the surrogate must meet? Is a conversion required if the surrogate is not Jewish (12)? Depending on which authority is asked, these answers may differ.

Supervision in the Laboratory

Although there are high standards for laboratories to ensure that each sample goes to the intended place, many scholars rule that another level of supervision is necessary (13). This process is called Halachik (Jewish law) supervision. This extra supervision is performed to testify that the chain of custody of each sample, and making of embryo, are done without human error. For some, it may be comforting to have an individual from their religious organization present during the steps where the patient is not (11). However, there is a wide variety of practice for supervision and not all individuals hold that this is necessary.

Conclusion

Judaism is thought about by Jews daily. It is not only a religion, but also an identity. Judaism is not trying to prevent individuals from having children. In fact, it is just the opposite. Having children is a major focus in Judaism. In regard to assisted reproductive technology, solutions must be found that are compliant with Talmudic law. Communication is key between the triad of patient, doctor, and religious leader, as the preferences and allowances are not identical from patient to patient. Rulings within Judaism are individualized based on the specific circumstances of the couple. Support is present for individuals trying to navigate this unfamiliar territory with organizations such as Puah and Yesh Tikvah. Judaism can work hand in hand with these new technologies and clinic staff, taking into consideration the Jewish laws, logistics, and medical factors, to find a method that works for the patient to provide the agreed upon outcome – a child.

Thank you to the Halacha (Jewish Law) and Infertility program YeshTikvah.org for providing consultation during the preparation of this article.

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Current perspectives of Preimplantation Genetic Testing

by Irene Miguel-Escalada, PhD - Marie Curie Postdoctoral Fellow

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In recent years, modern demographics and delayed motherhood have resulted in an increased use of assisted reproduction technologies as a method of conception. This has inevitably led to the improvement of existing in vitro fertilization (IVF) techniques, including extended in-vitro culture, blastocyst biopsy and the development of novel vitrification methods and time-lapse imaging systems. These advances, coupled with a deeper understanding of embryology and rapidly evolving genetic tools have resulted in the implementation of accurate genetic diagnostic testing in fertilization cycles.

Preimplantation genetic diagnosis (PGD) is a procedure that involves the genetic testing of one or more cells from an oocyte or an embryo prior to transfer. PGD may be used to avoid the transmission of genetic disorders to the offspring and it is thus indicated for carriers of single-gene disorders and known structural chromosomal abnormalities.

Preimplantation genetic screening (PGS) uses PGD methods in order to analyze the chromosomal number of biopsied cells with the aim of selecting euploid embryos for transfer. Chromosomal aneuploidy is very common in human preimplantation embryos and it is thought to be a major cause of IVF failure (Morales et al. 2008). Thus, PGS has been commonly used in chromosomally normal patients with a history of recurrent miscarriages, implantation failure, severe male factor or advanced maternal age, with the main objective of improving their clinical outcome. Compared to PGD, its practice is a bit more controversial.

PGD was first successfully performed in 1990 to avoid the transmission of chromosome X-linked disorders (Handyside et al. 1990). In these cycles, a region of chromosome Y was amplified by polymerase chain reaction (PCR) in order to select unaffected female embryos. Shortly afterward, a genetic testing method alternative to PCR that used a single biopsied blastomere and DNA probes labeled with fluorochromes was developed: fluorescent in-situ hybridisation (FISH). FISH became an extended diagnostic tool not only for embryo sexing but also for the analysis of aneuploidy and chromosomal rearrangements (Munne et al. 1993b; Munne et al. 1993a). However, FISH-based methods have serious technical limitations: there is only a small number of chromosomes that can be screened per cell and there can be errors of interpretation caused by overlapping or split signals in single nuclei. These factors, combined with the use of FISH on cleavage stage embryos, a time where mitotic errors can lead to mosaicism, are some of the reasons why initial randomized PGS studies failed to enhance IVF outcomes (Mastenbroek et al. 2011). Therefore, in the last years, the focus has shifted towards the use of molecular genetic techniques that allow a comprehensive analysis of all 23 pairs of chromosomes and overcome the aforementioned limitations.

Currently, the most common method to diagnose monogenic disorders is based on the use of single-cell multiplexed amplification of highly polymorphic markers (Short Tandem Repeats, STR) that are close to the mutation site (Harton et al. 2011). This approach has been also extended to human leukocyte antigen (HLA) typing, which can facilitate the birth of a healthy HLA-matched infant, a potential donor for the affected sibling (Fiorentino et al. 2004). Although highly accurate, these tests are customized per patient or locus, which makes them labor-intensive and time-consuming.

The arrival of whole-genome amplification protocols and single cell-based library preparation methods that generate sufficient quantities of DNA have made genome-wide analysis possible. Recently, a highly efficient method named karyomapping was introduced in the clinical practice (Handyside et al. 2010). Karyomapping allows the simultaneous detection of chromosomal abnormalities and monogenic disorders in a single test (Konstantinidis et al. 2015; Natesan et al. 2014; Thornhill et al. 2015). It involves the genotyping of thousands of single nucleotide polymorphisms (SNPs) spread throughout the genome in the parents and a relative of known disease status, thereby identifying heterozygous informative SNPs loci and their inheritance pattern in the IVF embryo. The main advantage is that karyomapping offers a highly accurate universal combined test and provides relatively rapid protocol development, which promises to simplify PGD cycles in the near future.

PGS techniques have also changed dramatically in the last 25 years. In order to provide a more accurate screening of embryos, several techniques that allow a comprehensive analysis of the entire chromosomal complement have been developed (Handyside 2013). Some of them include real-time quantitative PCR (qPCR) (Treff et al. 2012), microarray-based methods such as array comparative genomic hybridization (aCGH) (Gutierrez-Mateo et al. 2011) and SNP arrays (Treff et al. 2010), and more recently,
next generation sequencing (NGS) (Wells et al. 2014). aCGH is particularly robust and was the first technique to be widely available for reliable copy number analysis of all chromosomes with a short turnaround time. Multiple randomized clinical trials have validated these methods and provided strong evidence that aneuploidy screening through PGS can be translated into a better clinical outcome (Schoolcraft and Katz-Jaffe 2013; Scott et al. 2013; Yang et al. 2012).

Particularly exciting is the implementation of NGS to the PGD/PGS field. NGS involves fragmenting genomic DNA from the biopsied cells, followed by parallel sequencing until a sufficient number of reads is achieved. This level of coverage will determine the application of NGS. Low depth of genomic coverage has been shown to be sufficient for aneuploidy detection (Fan et al. 2015; Fiorentino et al. 2014; Wells et al. 2014; Yin et al. 2013). Deeper sequencing offers the possibility of a more powerful and comprehensive analysis, which can lead to the detection of single-gene defects (Treff et al. 2013). Although some technical limitations remain, due to rapidly evolving sequencing technologies and declining costs, NGS of the entire embryo genome might become a reality of the clinical practice in coming years. This will unavoidably raise ethical questions and pose challenges for data interpretation and patient counseling. Nevertheless, it will provide an unprecedented amount of information that will help geneticists and clinicians gain a deeper understanding of the biology of the embryo.

Although the development of accurate non-invasive methods for assessing embryo aneuploidy is desirable, PGD/PGS remains the only reliable approach to guarantee the transfer of a chromosomally normal and unaffected embryo. Continuous advances in genetic testing open up exciting venues for the future and the implementation of cost-effective, highly-accurate diagnostic methods hold the potential to have profound implications in the way embryo normalcy is determined, to strengthen the position of PGD in IVF cycles, and to ultimately benefit patients and treatment success rates.

References

Multicentre study of the clinical relevance of screening IVF patients for carrier status of the annexin A5 M2 haplotype

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Abstract Thrombophilia and impaired placental vasculature are a major cause of adverse pregnancy outcome. In 2007, a new hereditary factor for obstetric complications and recurrent pregnancy loss (RPL) was identified as a sequence variation in the core promoter of the annexin A5 gene, ANXA5, called the M2 haplotype. M2 carriership has been demonstrated in couples with recurrent miscarriage and its origin is embryonic rather than specifically maternal, confirmed by subsequent papers. The M2 haplotype is the first report of a hereditary factor related to pregnancy pathology caused by embryonic-induced anticoagulation. It has been demonstrated that couples with RPL had equal and significantly increased M2 carriership and that maternal and paternal carriership confers equal risk. Given its importance for patients with RPL, and potentially implantation failure, this study assessed the incidence of carrier status for the M2 ANXA5 haplotype in both the male and female of couples attending five CARE IVF centres. In 314 patients (157 couples), 44% of couples (one or both partners), 24% of females, 26% of males and 37% of couples with unexplained infertility were M2 carriers. This high incidence has provoked further urgent studies on specific patient populations and on the value of post embryo-transfer therapy.

KEYWORDS: ANXA5, infertility, miscarriage, recurrent pregnancy loss, thrombophilia

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Introduction

Thrombophilias are a major cause of adverse pregnancy outcome (Markoff et al., 2011) and there is increasing evidence to suggest that impairment of placental vasculature increases the risk of recurrent pregnancy loss (RPL), intrauterine fetal death, gestational hypertension, preeclampsia, venous thromboembolism, fetal growth restriction and small-for-gestational-age (SGA) newborns (Chinni et al., 2009; Grandone and Margaglione, 2003; Grandone et al., 2010; Tiscia et al., 2009, 2012; Younis and Samueloff, 2003).

Normal pregnancy is an acquired hypercoagulable state and therefore women with a genetic predisposition to thrombophilia may develop clinical signs of coagulation defects de novo during pregnancy or during the postpartum period (Chunilal and Bates, 2009; Rey et al., 2003). The predisposing role of hereditary thrombophilic factors has been reported in several clinical studies (Rodger et al., 2010), and historically, in the majority of patients, the hereditary factor has been Factor V Leiden or prothrombin (Bick, 2000). However, in 2007 a new hereditary factor
for RPL and additional thrombophilia-related obstetric complications was identified (Bogdanova et al., 2007; Chinni et al., 2009). This defect, termed the M2 haplotype, is a sequence variation in the core promoter of the annexin A5 gene, ANXA5. It consists of four consecutive nucleotide substitutions in the core promoter and results in reduced expression of ANXA5 in placentas from M2 haplotype carriers when compared with noncarriers.

Annexin A5 is a member of the annexin protein family which share the properties of binding calcium and phospholipids. It is distributed abundantly and ubiquitously, mostly in the kidney, liver and placenta (Morgan et al., 1998). It is most abundant on the apical membranes of placental syncytiotrophoblasts, the interface between maternal and fetal circulation. ANXA5 was originally named ‘placental anticoagulant protein’. It has been extensively studied both in vivo and in vitro (Romisch et al., 1991; Thigagarajan and Tait, 1990). It has potent anticoagulant properties associated with its phospholipid-binding activity and is one of the few annexins to be found extracellularly (Gerke et al., 2005). The ability of ANXA5 to form two-dimensional aggregates on cell membranes has led to the development of the ANXA5 ‘protective shield’ model that postulates that ANXA5 shields phospholipids at this site from availability for coagulation reactions and thus contributes to the maintenance of blood fluidity in the placenta. Annexin 5 is deficient in placentas of patients with antiphospholipid syndrome (APS), and antiphospholipid antibody-mediated reduction of annexin 5 on vascular endothelium may also contribute to systemic thrombosis (Rand, 1999). Bogdanova et al. (2012) revisited the annexin A5 protective shield model and reported that preliminary genotyping analysis of a cohort of 30 lupus anticoagulant-positive patients with obstetric APS revealed that 11 out of the 30 were M2 carriers and this would correspond to a 3-fold relative risk to develop obstetric antiphospholipid antibodies.

Markoff et al. (2010) reported not only that decreased ANXA5 expression in M2 ANXA5 placentas (including those from women with fetal growth restriction and or pre-eclampsia) is the result of carriage of the M2 haplotype, but that this occurred regardless of parental origin, with obvious consequences for embryonic- rather than wholly maternal-induced risk. They observed that the normal ANXA5 allele does not compensate for observed M2 allele-specific decreased mRNA concentrations and made the significant finding that, unlike Factor V Leiden and prothrombin where paternal thrombophilic genes are not associated with RPL (Toth et al., 2008), the M2 ANXA5 allele acts via the embryo.

The work of Markoff et al. (2010) led to a pilot study of 30 RPL couples where all other causes of RPL had been excluded (including inherited thrombophilias and APS; Rogenhofer et al., 2012). The study confirmed that male and females in these RPL couples had equal and significantly increased M2 carriehship when compared with control populations. The authors concluded that paternal and maternal carriage of the M2 ANXA5 haplotype associate with RPL and confer equal risks. They further reported that M2 ANXA5 is the first instance of a hereditary factor causing pregnancy pathology by affecting embryonic anticoagulation (Rogenhofer et al., 2012).

Tüttelmann et al. (2012) undertook a risk stratification study of an IVF cohort of 695 German women compared with 500 fertile female controls and 533 population controls. The carriers of the M2 haplotype had a higher relative risk (1.4) of belonging to the IVF group in comparison with fertile female controls and a higher relative risk (1.2) compared with population controls. This overall risk was due to a subgroup of women with previous pregnancy losses and for this group the relative risks were 3.8 and 2.3, respectively. The authors reported that there was no association with biochemical pregnancy loss, implantation rate, ovarian reserve, hormone status, number and quality of egg cells and general embryonic development. However, there was no male partner genotyping data available.

Ueki et al. (2012) in their knockout murine model found significant reductions both in litter size and fetal weight in ANXA5-null mice (ANXA5-KO) and thus demonstrated that the maternal supply of ANXA5 to the circulation was crucial for maintaining normal pregnancy. They further observed that cross-breeding of ANXA5-KO and wild-type mice showed that only litters bred using ANXA5-KO females had reduced numbers of pups. They also demonstrated that administration of heparin on pregnancy days 12, 14 and 16 to ANXA5-KO mice significantly increased litter size.

Evidence to date suggests that maternal and paternal carriage of the M2 ANXA5 haplotype confers equal risks and acts via the embryo, causing pregnancy pathology by affecting embryonic coagulation unlike the other wellcharacterized thrombophilias. Additionally there is a high incidence of carrier status in both control and subfertile populations, including patients with RPL. In the context of the IVF population, it is essential to understand potential endometrial and/or blood-borne factors responsible for IVF failures. Thus, this work performed a multicentre study of the incidence of carrier status of the M2 ANXA5 haplotype in both partners attending IVF clinics and to ascertain the potential relevance to pretreatment screening.

Materials and methods

Study population

Patients were recruited between March 2012 and February 2013 from patients attending five CARE fertility clinics. Informed consent was obtained from all patients. During this period, 314 patients (157 couples) presented with at least one previously failed IVF cycle (mean 1.9 IVF and 0.2 intrauterine insemination). A detailed clinical history was obtained, and the genotyping for presence or absence of carriage of the M2 ANXA5 haplotype formed
part of the diagnostic investigations for infertility.

The mean (range) age of women was 36.3 years (23–49 years) and that of their partners 38.6 years (23–64 years). The mean body mass index of the women was 25.5 kg/m² (19–40.5 kg/m²) and that of their partners was 33.7 kg/m² (21–36 kg/m²). The selection of patients for screening was based on their prior history and the patients’ willingness to be tested, following the detailed nature of the study being provided to them at consultation. Women were screened for antiphospholipid antibodies.

With regard to their infertility status, the majority of the male population had oligospermia (48%), asthenospermia (27%) or azoospermia (13%). These varied according to carrier status with an incidence in the noncarriers of 41%, 26% and 11%, respectively, and for the carriers 35%, 12%, and 12%, respectively. With regard to women, the most prevalent causes of infertility were unexplained (27%), poor ovarian reserve (17%), polycystic ovary syndrome (PCOS; 11%) and endometriosis (6%); according to carrier status, incidence in the noncarriers was 30%, 16%, 16% and 3%, respectively, and for the carriers 26%, 9%, 18% and 8%, respectively.

The majority of patients were white British (77% men and 75% women) and Indian/Pakistani (8%) the remainder being of diverse ethnicity. As a whole, this cohort is representative of the demography of the UK and Eire. DNA was collected from couples either by a blood sample (the first cohort) or buccal cell analysis on specific collection paper (the remaining cohort) from September 2012. Extensive laboratory tests were undertaken to ensure the transfer to buccal cell collection caused no deterioration in the quality of the DNA. DNA was extracted from white blood cells using QIAmp DNA Blood Mini kit (Qiagen, Hilden, Germany) or from elution from the collecting paper. PCR reactions were carried out on 100 ng genomic DNA isolated from blood samples using the QIAmp Blood Mini kit or from purified collecting paper punches. Amplification was carried out using Biotaq Polymerase (Bioline Reagents, London, UK) in a volume of 25 μl containing 10x NH₄ reaction buffer: 160 mmol/l (NH₄)₂SO₄, 670 mmol/l Tris–HCl (pH 8.8), 50 mmol/l MgCl₂ (final concentration 1.5 mmol/l), 50 pmol/l forward and reverse primers, 200 mmol/l dNTP, PolyMate Additive (Bioline) and 2.5 U Biotaq polymerase. The cycling conditions were 94°C for 45 s, 30 cycles of 94°C for 30 s, 60°C for 30 s and 68°C for 1 min and a final extension step of 7 min. Amplification products were purified using standard column purification methods (Zymo ZR-96DNA Clean and Concentrator kit; Zymo Research, Irvine, CA, USA). Purified amplicons were sequenced using ABI BigDye Terminator chemistry version 3.1 using standard conditions and electrophoresis on an ABI 3730xl DNA analyser and traces were analysed and genotyped using ABI Seqscape version 2.5. (Applied Biosystems, Foster City, CA, USA). The presence of the M2 haplotype (a set of four consecutive nucleotide substitutions in the ANXA5 promoter: 19G>A (rs112782763), +1A>C (rs28717001), 27T>C (rs28651243) and 76G>A (rs113588187)) was investigated. When only two of the four variants (+1A>C, 27T>C) were present, the haplotype was defined as M1.

Quality control

All genotype calls were made using Seqscape software (Applied Biosystems) with a 25% mixed-base calling threshold. Seqscape was programmed to analyse nucleotide variations at four specific bases, as described in the literature (Bogdanova et al., 2007). Results were generated in the form of a mutations report that detailed mutations across the region of interest. Report production was carried out by means of an in-house laboratory information management system, which was programmed to only allow certain combinations of mutation. Any sample that gave an unexpected result was flagged by the system and checked by an operator before repeating the test on a fresh sample.

Genotyping and statistical analysis

Patients who were heterozygous carriers or homozygous for the M2 ANXA5 haplotype were recorded as affected heterozygous or affected homozygous. Tests for deviations from Hardy–Weinberg equilibrium (HWE) were performed using the method of Guo and Thompson, 1992 (also used by Bogdanova et al., 2007 and Rogenhofer et al., 2012). This test was performed within the male and female groups and overall.

This work also tested all individuals not classified as white British or white Irish to see whether this affected the results. To check whether the significant deviation from HWE observed in the female subgroup could be attributed to chance, 155 individuals were subsampled at random from the entire set (men and women combined) and the P-value for deviation from HWE was estimated using the same method. This procedure was performed 1000 times, and of these, only three P-values were more extreme than those observed for the all-female group, thus suggesting that the deviation from HWE in women was real and not attributable to chance.

The controls used for comparison were those used by Rogenhofer et al. (2012) from a population control sample drafted from the PopGen biobank at University Clinic Schleswig–Holstein Kiel (n = 533). PopGen population controls were from northwest Germany and were healthy subjects identified through official population registers (Krawczak et al., 2006). The sample used in this study comprised approximately equal numbers of men and women distributed among three age groups (18–30, 30–50 and 50–80 years). The cohort of Muenster fertile controls were anonymized individuals from the institute’s registry (Rogenhofer et al., 2012), all with successful pregnancies and no documented history of RPL.
**Results**

Six patients were not genotyped: four men (two azoospermia, one oligospermia and one aged 65) and two women (one early menopause and one menopause). Of the remaining 314 patients (157 couples), the overall M2 carriage rate was 25% (n = 78) and was of similar incidence in women (24%, n = 37) and men (27%, n = 41). However, in couples, there was a high incidence of M2 carriage (defined as one or both partners being M2 carriers or homozygotes; 44%, n = 69). None of these patients tested positive for APS.

Among these carrier couples were small subsets of couples in which one partner was a noncarrier and one was homozygous (4%, n = 7), both partners were carriers (4%, n = 6), or one partner was a carrier and one was homozygous (2%, n = 3). There were nine homozygotic women and one homozygotic man. The genotype frequencies of ANXA5 promoter haplotypes observed in this study and expected under HWE in men and women are presented in Table 1. There was no significant deviation from HWE in men, but there was significant deviation from HWE in women (P = 0.005). Restricting the analysis to only those individuals classified as white British or white Irish gave similar results (data not shown).

The genotype frequencies of ANXA5 promoter haplotypes in the current study are compared with two control groups in Table 1. The abundance of the M2 haplotype was enriched in both men and women compared with both the Muenster controls (women) and the PopGen controls (men and women).

The IVF female patients were not in HWE (P = 0.0052) owing to the excess of M2 heterozygotes but particularly M2 homozygotes (9 observed versus 3.4 expected). To check whether the significant deviation from HWE observed in women could be attributed to chance, this work subsampled 155 individuals at random from the entire set (men and women combined) and estimated the P-value for deviation from HWE using the same method and recorded the P-value. We performed this procedure 1000 times, and of these only three P-values recorded were more extreme than those observed for the all-female group, thus suggesting that the deviation from HWE in women was real and not attributable to chance.

The patients’ previous IVF, intrauterine insemination and pregnancy histories are shown in Table 2. The numbers of previous failed IVF cycles were highest in couples who had one homozygotic partner and one noncarrier (mean 3.1 previous IVF) and in couples where the male partner was a carrier (mean 2.1 previous IVF).

Previous live births were very low in all carrier/homozygous groups (range 0–4) and a slightly higher incidence was observed in noncarrier couples (n = 13). The patients’ most recently reported miscarriage in carrier couples occurred at a mean of 10.1 weeks (range 5–23 weeks) in the 17 miscarriages where date of loss was reported. In noncarrier couples, miscarriage (n = 53) occurred at a mean of 9 weeks (range 5–26) in 25/53 miscarriages.

### Male infertility and M2 frequency

Overall, 63 of 157 men (40%) had associated infertility factors. Carriage incidence in this group was 27% (n = 17). Overall, oligospermia was the most frequent finding (40%, 25 infertile men) followed by oligoasthenoteratozoospermia (13%, eight infertile men). However there is unlikely to be any relationship or causal linkage between the existence of the M2 haplotype and male infertility.

Of 157 women, 93 (59%) had a diagnosis of infertility...
other than unexplained or male factor. Additionally, 25 of the 93 women with a diagnosis (27%) were also found to be M2 carriers. Unexplained, poor ovarian reserve/ovulation failure often linked to age plus PCOS were the most frequently cited causes of infertility in both groups. However, male infertility was cited as the primary cause of infertility in 21% of noncarrier couples but noted in only one of 37 women who carried the M2 haplotype. Six out of 17 PCOS cases (35%) were also carriers.

**Unexplained infertility and M2 frequency**

Overall, 104 patients (33%) presented as having no explanation for infertility. Of these, 38 patients (37%) were identified as M2 carriers: 25 men (24%) and 13 women (13%). There were nine homozygotic women (6% of all women) There was also one homozygotic man aged 49 for whom the couple had no other known diagnosis although his partner had had two IVF cycles which had resulted in miscarriage.

**Discussion**

Carriership of the M2 ANXA5 haplotype in this cohort of patient couples was 44%, representing a very high incidence. Furthermore it was present in 27% of male infertility patients, 27% of female infertility patients and in 37% of patients with previously unexplained reasons for infertility. Additionally, it was present in 35% of PCOS patients, which has been reported by Rogenhofer et al. (2013) who note that the M2 ANXA5 haplotype is independently associated with RPL in PCOS patients. Of the patients who carried the M2 haplotype in the present study, none tested positive for APS. Bogdanova et al. (2012), in a cohort of 30 lupus anticoagulant-positive patients with obstetric APS, reported 11 as M2 carriers; It is possible that the observed variance is a result of the infertility cohort in this study being a different group of patients than those with ‘obstetric complications’.

The genotype distribution in men and women was similar to that reported by Rogenhofer et al. (2012) where a RPL cohort was compared with three different control groups. Genotype M1/M1 was absent in the RPL cohort and rare in controls. Genotype M1/M2 was not observed in the RPL cohort and seen only in a total of eight from control groups and in only four patients in the current IVF cohort. However, the incidence of M2 homozygotic women was elevated at 6% in this cohort and one M2 homozygotic man was recorded. Female homozygote frequency was 3-times higher than that reported from other control groups and double that of RPL women (Rogenhofer et al., 2012).

The use of the PopGen and Muenster controls is justified as Nelis et al. (2009) concluded that four areas could be identified – namely: (i) central and western Europe; (ii) the Baltic countries, Poland and Western Russia; (iii) Finland; and (iv) Italy – which, if not corrected for, the interpopulation differences would affect the significance of disease gene associations. The incidence in controls from published studies from Germany, southern Italy and Bulgaria – representatives of three of these regions – have all shown consistency in the M2 haplotype frequency. The majority of the IVF patients were white British (77% men, 75% women), which correspond to the central and western Europe region. This study had no Finnish patients and analysis with and without the subset of Indian/Pakistani
and others still showed the significant departure from HWE in women but not in men, mainly due to the abundance of M2 homozygotes.

In terms of ethnicity, this study found M2 carriers in a wide range of ethnicities, including Jewish, Turkish and Middle Eastern in addition to Indian and Pakistani patients. The possible differences in carriage rate and clinical effects in these ethnicities warrants further investigation since there may be significant differences in incidence and pathology. The incidence in Caucasian populations of Europe is well established (Markoff et al., 2011) and Miyamura et al. (2011) reported that carriage of the haplotype resulted in risks for RPL in the Japanese population similar to that observed in the populations of central Europe; however, the incidence of RPL was lower in Japan (5.5 versus 15%). Thus further study of different ethnicities other than white Europeans and Japanese is warranted.

M2 is a hereditary factor that causes various pathologies during pregnancy by adversely affecting embryonic anticoagulation (Markoff et al., 2010; Rogenhofer et al., 2012). A very recent paper on RPL in German and Bulgarian patients by Tüttelmann et al. (2013) provides further evidence that paternal carriage contributes similar risk to that of maternal carriage, as reported by Markoff et al. (2010) who showed nonpreferentially and equally reduced ANXA5 mRNA expression in chorionic placenta carrying maternal or paternal alleles.

Although Ueki et al. (2012) could only demonstrate a maternal influence on pregnancy viability from their ANXA5-KO murine model, the human placental study of Markoff et al. (2010), which has been further confirmed by Rogenhofer et al. (2012), supports earlier work on the embryonic influence on placental function (Rand et al., 1997). Rand et al. (1997) demonstrated that the fetal component has a characteristically evident pattern of ANXA5 expression on the apical surface of the syncytiotrophoblast layer lining the chorionic villi. Furthermore, as concluded in Malassine’ et al. (2003), there should be caution in extrapolating data from experimental models, particularly in studies of the pathophysiology of complications of pregnancy with a placental origin.

Any impairment of embryonic coagulation is of particular importance in IVF practice since the focus is often on managing and providing for healthy gametes and embryos, selecting for optimal embryo viability and ensuring a healthy uterus able to sustain a pregnancy. However, although the largest single cause of miscarriage is believed to be the aneuploid embryo, other factors are clearly of significance, especially in RPL cases, where it can remain an issue even after the transfer of euploid embryos following IVF. The relatively recently discovered genetic factor M2 ANXA5 is alone in influencing placental function via adverse effects on embryonic anticoagulation and, if undetected, could negate the considerable work and cost incurred to establish a healthy pregnancy via IVF. In this study, there were a significant number of patients, equally distributed between men and women, where M2 carriage was either an additional factor to those already determined or it was present in a significant number of patients with no other infertility diagnosis. There is a growing body of evidence of the risks of carriage of the M2 ANXA5 haplotype to maternal health (RPL, venous thromboembolism, pre-eclampsia, gestational hypertension, APS; Bogdanova et al., 2012; Grandone et al., 2010; Tiscia et al., 2009). Bogdanova et al. (2012) postulated that carriage of the M2/ANXA5 haplotype leads to reduced ANXA5 cover of exposed phosphatidylserine surfaces, and this reduced shielding would allow coagulation factors to compete for phospholipid binding. Secondly, there would be greater exposure of phospholipid antigenic factors, that would then lead to antiphospholipid antibody development, which in turn would further disrupt the ANXA5 shield. Sifakis et al. (2010) demonstrated significant differences in mRNA expression between normal and fetal growth restriction pregnancies but no difference in ANXA5 protein concentration. However, the authors did not genotype their samples for M2 ANXA5.

A significantly higher prevalence of the M2 haplotype in a group of women with a history of idiopathic SGA babies has been reported (Tiscia et al., 2012), demonstrating a 2-fold higher risk of giving birth to a SGA newborn. All the M2 homozygotes in this study (there were no homozygotes in the controls) had a history of a severe SGA (below the 3rd percentile).

Recently, a large cross-sectional study (Henriksson et al., 2013) was determined the incidence of pulmonary and venous thromboembolism in pregnancies after IVF and reported an increased risk of thromboembolism and, importantly, pulmonary embolism. The risk of venous thromboembolism increased during all trimesters, particularly during the first trimester, as did the risk of pulmonary embolism. The study concluded that ‘efforts should focus on the identification of women at risk of thromboembolism, with prophylactic anticoagulation considered in women planning to undergo in vitro fertilization.’

Nelson and Greer (2008) conducted an extensive review of the similarities of heparin and heparan, the haemostatic changes induced by ovarian stimulation and the risk of thrombosis, the contribution of thrombophilia to pregnancy and infertility outcomes, early embryonic–maternal dialogue and how these various aspects of assisted conception may be modified by heparin. The authors concluded that heparin has the potential to improve pregnancy rates and outcomes. Recently, Seshadri et al. (2012) conducted an extensive meta-analysis of observational and randomized studies on the effect of heparin on the outcome of IVF treatment. The meta-analysis of the observational studies showed a significant increase in clinical pregnancy and live birth rates and the authors concluded that that the role of heparin as an adjuvant therapy during IVF treatment required further evaluation in adequately powered high-
quality randomized studies. They further suggested that such studies could either target the general IVF population or a specific subgroup of patients including those with known thrombophilia or recurrent implantation failure. In the absence of such studies and in view of the recent important findings from Henriksson et al. (2013) and the high incidence of the M2 ANXA5 haplotype within the current IVF cohort, this study’s fertility centres have taken a pragmatic view to identify and treat patients who are carriers of the M2 ANXA5 haplotype, which is now known to be an inherited thrombophilia adversely affecting embryonic anticoagulation. In 2001, the Royal College of Obstetricians and Gynaecologists reviewed four randomized controlled trials in women with two or more pregnancy losses treated with low-dose aspirin with and without low-molecular-weight heparin (Scientific Impact Paper 26). It noted that these studies failed to demonstrate improvement in live birth outcome. They further noted that these studies were underpowered to be able to confirm or refute effects in women with three or more losses or those with thrombophilia. However, when this opinion was advanced there was no knowledge of the existence of the M2 ANXA5 haplotype in women with RPL. Indeed the authors stated that ‘there remain unidentified inherited thrombophilias’. Furthermore the findings that paternal carriage contributes a similar risk to that of maternal carriage and that the defect is conveyed embryonically were also unknown, reflecting the need to understand an appropriate stratification of patients. This study’s fertility centres are adopting the approach of offering screening of patients for carriage of the M2 haplotype with a view to identifying women at risk not only of pregnancy loss but for the additional risks conferred by this thrombophilic genetic defect. While appreciating that this is an incidence study only, the current practice advice for women identified at risk (either because she and/or her partner are carriers) in this study’s fertility centres is that they be treated from implantation to near term with low-molecular-weight heparin. If the woman is a carrier, treatment for 6 weeks post partum is advised to reduce the risk of maternal venous thromboembolism. In terms of risk to the fetus, a recent case–control study (Tiscia et al., 2012) reported that carriage of the M2 ANXA5 haplotype was an independent risk factor for idiopathic SGA newborns and that women carrying the M2 haplotype had a 2-fold higher risk of giving birth to an SGA baby. In addition they reported a 6% incidence of homozygotes which is similar to the 6% incidence in the current cohort. In their study, all M2 homozygotes had a history of a severe SGA (below the 3rd percentile).

It is possible to speculate that M2 homozygotic women may be at greater risk of thrombotic events by virtue of the decrease in their own endogenous ANXA5 during pregnancy; thus identification of this subset of patients before IVF treatment is important since from this study their IVF cycle failure rate is higher than for noncarriers. This study reports a single homozygotic man with no other infertility diagnosis whose partner had had two previous failed IVF cycles. Rogenhofer et al. (2012) interestingly noted no M2 homozygotic men in their cohort of 30 RPL couples. It is already well established (RCOG–SAC Opinion Paper 8, 2007) that the risk of low birthweight for IVF singletons is significantly higher than for naturally conceived singletons (incidence of SGA 12.6% versus in England 7.5%, reported by the London Health Observatory (2002–2004)). Thus identifying and treating women who are themselves M2 carriers or whose partner is a carrier may assist in reducing the incidence of SGA by mitigating the adverse effects on embryonic anticoagulation. There are long-lasting health costs associated with low birthweight in infants and this aspect warrants further study.

In conclusion, since the defect is conveyed embryonically and affects embryonic anticoagulation and also the risk is independent of any specific parental transmission (i.e. it can be induced whether the transmission is maternal or paternal or both), screening of both partners presenting for IVF for carriage of the M2 ANXA5 haplotype ought to be considered as routine and early in the diagnostic work up of couples being treated with their own gametes. The M2 haplotype appears to be an additional independent factor that contributes to the risk of pregnancy failure.

Further work accessing trio genotyping data of paternal, maternal and infant origin together with outcome is required to determine whether there are differences in outcome if both mother and child are carriers of the M2 haplotype. Additionally further consideration should be given to a test-and-treat critical pathway for those receiving donated gametes, embryo donors and surrogate mothers.

References


AMH and AMHR2 genetic variants in Chinese women with primary ovarian insufficiency and normal age at natural menopause

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Abstract The aim of this study was to investigate the role of the anti-Müllerian hormone (AMH) signalling pathway in the pathophysiology of idiopathic primary ovarian insufficiency (POI) and age at natural menopause (ANM) using a genetic approach. DNA sequencing was used to detect the genotype distribution and allele frequency of the genes AMH and AMHR2 in 120 cases of idiopathic POI and 120 normal-ANM women. Fourteen sequence variants of AMHR2, including 10 novel variants, were identified. Two novel exonic missense variants were p.I209N and p.L354F. The missense variant p.I209N, which is conserved in different species, was predicted to have functional and structural impacts on the AMHR2 protein. The clinical significance of one additional variant (p.L354F) remains arguable pending functional studies. The genotype frequencies of AMH and AMHR2 were similar in distribution for POI patients and normal-ANM women. These findings suggest that POI patients and normal-ANM women in China share AMH and AMHR2 genetic variants. The AMH signalling pathway associated with ANM also may contribute to POI.

KEYWORDS: age at natural menopause, AMH receptor, anti-Müllerian hormone, primary ovarian insufficiency

Introduction

Primary ovarian insufficiency (POI) is defined as the cessation of ovarian function under the age of 40 and is characterized by amenorrhoea, hypo-oestrogenism and elevated serum gonadotrophin concentrations (Santoro, 2003; Timmreck and Reindollar, 2003). The cause of POI remains undetermined in the majority of the cases. While there is a strong genetic association with POI, familial studies also have indicated that idiopathic POI may be genetically linked (Goswami and Conway, 2005). However, in women with POI, there is usually premature depletion of the primordial follicle pool. This might be caused by defects in oocyte apoptosis mechanisms, leading to either a decrease in follicular formation, resulting in a reduction of oocytes formed during ovarian development, or accelerated follicle loss.

Primordial follicle recruitment is regulated predominantly by intraovarian factors. One of the factors known to regulate initial recruitment in mice is anti-Müllerian hormone (AMH). AMH, a member of the transforming growth factor-β family, is involved in the regulation of follicular growth (di Clemente et al., 2003). It is produced by the granulosa cells of early developing follicles in the ovary. Studies in AMHnull mice have demonstrated that, in the absence of AMH, follicles are recruited at a faster rate and they are more sensitive to FSH (Durlinger et al., 2001). This expression pattern suggests that AMH can inhibit both the initiation of primordial follicle growth and FSH-induced follicle growth. The absence of AMH results in a prematurely exhausted follicle pool and, subsequently, an earlier cessation of the oestrous cycle (Durlinger et al., 1999).

Research has shown that genetic variations in AMH have been associated with age at natural menopause (ANM). Kevenaar et al. (2007) demonstrated correlations of
genetic variants in the gene for AMH receptor II (AMHR2) with ANM in two large cohorts of Dutch post-menopausal women. Voorhuis et al. (2011) investigated genetic variants in genes involved in initial follicle recruitment in association with ANM among 3445 Dutch women participating in Prospect-EPIC. They observed an association between ANM and two singlenucleotide polymorphisms (SNP) in AMHR2. Recent studies in natural-menopause women have demonstrated that a pairwise interaction between two SNP in AMH and AMHR2 in association with ANM (Braem et al., 2013). Women with idiopathic POI or early ANM differ in age of menopause onset and are considered to represent variable expression of the same genetic pattern (Tibiletti et al., 1999). It is highly plausible that certain genes contribute to both. Furthermore, previous studies in POI women have demonstrated that serum AMH concentration was significantly decreased to very low concentrations in POI (Li et al., 2011).

This study investigated, as far as is known, for the first time, whether these genetic variants of the AMH signalling pathway are associated with idiopathic POI in China.

Materials and methods

Subjects

A total of 120 patients (age, mean ± SD, 31.58 ± 6.01 years) with idiopathic primary ovarian insufficiency (POI) and 120 women (age 49.2 ± 5.1 years) with normal ANM were included. The study was approved by the University’s Institutional Ethics Committee (reference no, 20120020, approved 15 March 2012) and informed consent was obtained from all participants.

The diagnostic criteria for POI (Qin et al., 2011) was as follows: at least 6 months of amenorrhoea before the age of 40, with at least two serum FSH concentrations >40 IU/l. Patients with associated endocrinopathies, autoimmune disorders, iatrogenic agents such as pelvic surgery, chemotherapy and radiotherapy and infections were excluded.

The normal ANM in Chinese women is approximately 49 years (Nie et al., 2011). ANM was defined as the age at the last menstrual period, which can only be defined retrospectively after at least 12 consecutive months of amenorrhoea. This last menstrual period should not be induced by surgery or other obvious causes, such as irradiation or hormone therapy. Women who reported hormone use during the onset of menopause were excluded to avoid uncertainty on menopausal age. Each subject were assessed clinically, with a complete medical and gynaecological history, including the history of menses, age at menopause, LH and FSH concentrations (twice at 1-month intervals) and pelvic ultrasound. Karyotyping with high-resolution GTG banding to check for chromosomal anomalies was performed in all subjects. Those with abnormalities were excluded from the study. All patients were sporadic cases and were interviewed by investigators regarding their biological parents and grandparents. They were classified as individuals of Han ethnicity whose families had resided in southern China at least since their grandparents’ generation.

DNA extraction and karyotyping

Peripheral blood was collected in EDTA vacutainers for genomic DNA isolation (5 ml) and in heparin vacutainers for chromosomal analysis (5 ml). Genomic DNA was extracted from lymphocytes using standard proteinase K/chloroform extraction methods (Shelling et al., 2000). Chromosomal analysis was performed on phytohaemagglutinin-stimulated peripheral lymphocyte cultures using standard conventional cytogenetic methods.

PCR protocol

Primers for all the exons were designed using Genefisher software (http://bibiserv.techfak.uni-bielefeld.de/genefisher). The thermal program was 98°C for 3 min and was 35 cycles of denaturation at 94°C for 45 s and extension at 72°C for 45 s. All primer sequences with their corresponding annealing temperatures are summarized in Table 1. The presence of all sequence variants was confirmed by performing three independent PCR reactions and subsequent DNA sequencing.

DNA sequencing

Samples were sequenced using a BigDye Terminator Cycle Sequencing Kit 3.1 and run on a 3730xl ABI DNA Analyser (Applied Biosystems, USA). The sequencing results were analysed using Chromas version 2.3 (Technelysium, Australia) and compared with reference sequences in the National Centre for Biotechnology Information database (http://www.ncbi.nlm.nih.gov).

Statistical analysis

SNPAlayer 5.0 software (DYNACOM, Japan) was used to evaluate Hardy–Weinberg disequilibrium. Chi-squared test was performed using SPSS 13.0 (SPSS, Chicago, IL, USA). Three web-based programs were used to evaluate the possible biological effects of amino acid substitution on the structure and function of the AMHR2 protein: (i) Polymorphism Phenotyping (PolyPhen; http://genetics.bwh.harvard.edu/pph/); (ii) Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/); and (iii) Prediction of Polymorphism Phenotyping (PMut; http://mmb2.pcb.ub.es:8080/PMut/). Possible alterations of protein motifs and functional sites were assessed by scanning through the PROSITE database (ScanProsit; http://www.expasy.org/prosite/). This work also examined evolutionary conservation of the amino acid residues in these variants by multiple sequence
alignment using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Results

The sequencing data collection and analysis were successfully performed for the coding regions and respective flanking intronic regions of AMH and AMHR2 in all subjects. The populations were in Hardy–Weinberg equilibrium for all of the genetic variations. The clinical characteristics of the two populations are presented in Table 2.

Sequence variants detected in AMHR2

Sequence analyses of 120 idiopathic POI patients and 120 normal-ANM women revealed 14 sequence variations, including 10 novel SNP in AMHR2. These novel variants included two missense variants (Figure 1) and two synonymous variants. Eight intronic variants, including six novel substitutions, were identified (Table 3). Four novel exonic variants were discovered: c.627T>A (p.I209N), c.993C>T (p.H332H), c.1038G>T (p.S347S) and c.1060C>T (p.L354F). The p.I209N and p.L354F variants were not detected in any of the 120 normal-ANM women. The sequencing electrophoretograms of the two novel missense variants of AMHR2 are shown in Figure 2. One patient was heterozygous for the p.I209N variant and the other was heterozygous for the p.L354F variant. Genetic variants in p.I209N and p.L354F were not found in the mothers of these two cases.

Three web-based programs were used to evaluate possible biological effects of the amino acid substitution on the structure and function of the AMHR2 protein (Table 4). For the p.I209N variant, the predictions were ‘Possibly damaging’ and ‘Intolerant’ by PolyPhen and SIFT, and ‘Pathological’ (reliability index of 2) by PMut. PROSITE scanning showed that the variant p.I209N was predicted to affect one ATP binding domain on the AMHR2 protein, which can interact selectively and noncovalently with ATP, a universally important coenzyme and enzyme regulator. Therefore, p.I209N has a high potential to cause disease.

The impact of variant p.L354F was predicted to be ‘Probably damaging’ by PolyPhen, but was predicted to be ‘Tolerated’ by SIFT (Table 4). Note that the predictions of the PolyPhen and SIFT scores are opposite. Therefore, the PMut program was applied to provide additional assessment. The impact of variant p.L354F was predicted to be ‘Neutral’ by PMut, with a maximum reliability index of 8. PROSITE scanning showed that no structural or functional
Figure 1. Schematic overview of the anti-Müllerian hormone receptor II (AMHR2) gene. Exons, indicated by rectangles, are numbered from 1 to 11. The two novel missense variants, p.I209N and p.L354F, detected by sequence analysis in 120 Chinese women with idiopathic primary ovarian insufficiency are shown.

Table 2. Clinical features of women with POI compared with normal-ANM women.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>POI (n = 120)</th>
<th>Normal ANM (n = 120)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>31.58 ± 6.01</td>
<td>49.20 ± 5.10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body mass index (kg/cm²)</td>
<td>20.69 ± 2.60</td>
<td>20.78 ± 2.79</td>
<td>NS</td>
</tr>
<tr>
<td>Age at menarche (year)</td>
<td>13.87 ± 1.59</td>
<td>13.92 ± 1.55</td>
<td>NS</td>
</tr>
<tr>
<td>Age at menopause (year)</td>
<td>28.25 ± 6.97</td>
<td>49.92 ± 1.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FSH (miU/ml)</td>
<td>89.88 ± 35.02</td>
<td>85.95 ± 10.62</td>
<td>NS</td>
</tr>
<tr>
<td>LH (miU/ml)</td>
<td>43.32 ± 16.89</td>
<td>40.41 ± 5.53</td>
<td>NS</td>
</tr>
<tr>
<td>Oestradiol (pg/ml)</td>
<td>27.68 ± 27.57</td>
<td>31.29 ± 25.66</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SD. P-values calculated using chi-squared. ANM = age at natural menopause; NS = not statistically significant; POI = primary ovarian insufficiency.

Table 3. Sequence variants detected in anti-Müllerian hormone receptor II (AMHR2) in women with POI compared with normal-ANM women.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Nucleotide change</th>
<th>Residual change</th>
<th>POI (n = 120)</th>
<th>Normal ANM (n = 120)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Promoter</td>
<td>-482A&gt;G</td>
<td>-</td>
<td>31</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>Promoter</td>
<td>-443delC</td>
<td>-</td>
<td>84</td>
<td>63</td>
</tr>
<tr>
<td>3</td>
<td>Intron 1</td>
<td>49+10T&gt;G</td>
<td>-</td>
<td>55</td>
<td>39</td>
</tr>
<tr>
<td>4</td>
<td>Intron 3</td>
<td>424→29C&gt;T</td>
<td>-</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>Intron 4</td>
<td>502+84A&gt;G</td>
<td>-</td>
<td>36</td>
<td>45</td>
</tr>
<tr>
<td>6</td>
<td>Intron 4</td>
<td>502+85T&gt;G</td>
<td>-</td>
<td>30</td>
<td>39</td>
</tr>
<tr>
<td>7</td>
<td>Intron 4</td>
<td>502+90T&gt;G</td>
<td>-</td>
<td>36</td>
<td>42</td>
</tr>
<tr>
<td>8</td>
<td>Exon 5</td>
<td>627T&gt;A</td>
<td>I209N</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Intron 5</td>
<td>622→6C&gt;T</td>
<td>-</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>Intron 5</td>
<td>622→24C&gt;A</td>
<td>-</td>
<td>31</td>
<td>24</td>
</tr>
<tr>
<td>11</td>
<td>Exon 8</td>
<td>993C&gt;T</td>
<td>H322H</td>
<td>43</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>Exon 8</td>
<td>1038G→T</td>
<td>S3475</td>
<td>30</td>
<td>26</td>
</tr>
<tr>
<td>13</td>
<td>Exon 8</td>
<td>1060C&gt;T</td>
<td>L354F</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>Intron 10</td>
<td>1425+77A&gt;G</td>
<td>-</td>
<td>25</td>
<td>42</td>
</tr>
</tbody>
</table>

ANM = age at natural menopause; POI = primary ovarian insufficiency.
Figure 2. Sequencing electrophoretograms illustrating the two novel missense variants of anti-Müllerian hormone receptor II (AMHR2), p.I209N and p.L354F, found among 120 Chinese women with idiopathic primary ovarian insufficiency.

Domain was present at or around the p.L354F residue. Based on these assessments, the clinical significance of one additional variant (p.L354F) remains arguable, pending functional studies.

Determination of the evolutionary conservation of p.I209N and p.L354F by multiple amino acid sequence alignment of AMHR2 with the protein sequences derived from human (hg19), marmoset (calJac1), gorilla (gorGor1), mouse lemur (micMur1), horse (equCab2), tarsier (tarSyR1), bat (pteVam1), microbat (myoLuc1), elephant (loxAfr3), cat (felCat3), dog (canFam2), cow (bosTau4), rabbit (oryCun2), pig (cavPor3), dolphin (turTru1), rock hyrax (proCap1), mouse (mm9) and kangaroo rat (dipOrd1) showed that both residues 209 and 354 in AMHR2 were highly conserved (Figure 3).

The significance of the analysis was represented following the Bonferroni’s correction guidelines. Analyses were adjusted for multiple testing (14 SNP; \( P < 0.05/14 = 0.0036 \)). On the basis of Fisher’s exact test, genotypic and allelic frequencies for 14 variants were not significantly diverse in POI cases as opposed to normal-ANM women. The details of the genotypic analysis are summarized in Table 3.

### Sequence variants detected in AMH

Table 5 compares the genotypic and allelic frequencies of AMH c.146T>G (p.I49S), c.303G>A (p.G101G) and c.546G>A (p.P182P) among the 120 idiopathic POI patients and 120 normal-ANM women. These sequence variants included one missense variant and two synonymous variants. The genotypic and allelic frequencies of all these SNP were not significantly different between the two groups.

#### Genotype distributions in different populations

This work compared AMH p.I49S and AMHR2 c.–482A>G polymorphisms in different populations. The genotypic and allelic frequencies of AMH p.I49S and AMHR2 c.–482A>G polymorphisms in the Rotterdam cohort, the LASA cohort, Italian, Dutch and Korean cohorts and the current cohort were similar and did not differ from the frequencies in normal-ANM women (Table 6).

#### Discussion

It has been proposed that variation of menopausal age is largely influenced by genetic factors (Voorhuis et al., 2010). Moreover, genes involved in primary follicle recruitment were associated with timing of menopause in a genetic association study with a large menopausal cohort (Voorhuis et al., 2011). Although it is unclear whether or not POI and menopause have the same genetic mechanisms, there may be a chance that the genes involved overlap each other. AMH clinical research in women has become extensive. Fauser and his colleagues were the first to stress the value of AMH measurements for the assessment of follicular reserve (de Vet et al., 2002; van Rooij et al., 2002; Weenen et al., 2004). In contrast, the role of AMH in human ovarian function appears crucial. Animal studies have demonstrated that AMH represses the LH receptor and aromatase of granulosa cells and the recruitment of primary follicles (Durlinger et al., 1999), leading to premature cessation of ovarian cycling in AMH-knockout
mice (Gruijters et al., 2003). Because AMH has an inhibitory effect on follicle recruitment, polymorphisms on \( \text{AMH} \) or its receptor \( \text{AMHR2} \) might lead to POI.

In this study, both of the genetic variants analysed in \( \text{AMH} \) and \( \text{AMHR2} \) captured all the exons and flanking regions of the gene, including 1 kb of the promoter region. This study provides, as far as is known, the first germ-line case–control status of \( \text{AMHR2} \) in Chinese idiopathic POI patients and normal-ANM women and presents a spectrum of 14 sequence variants that includes four novel exonic variants and six novel intronic variants. Two novel missense changes were identified in \( \text{AMHR2} \) (p.I209N and p.L354F) that were not found in the normal-ANM women. Four web-based programs (PolyPhen, SIFT, PMut, and PROSITE) were used to evaluate possible biological effects of the amino acid substitution on the structure and function of the AMHR2 protein. The impact of the variant p.I209N was likely to be a deleterious substitution, with consistent predictions by PolyPhen, SIFT, and PMut. PROSITE scanning showed that the variant p.I209N was predicted to affect one ATP-binding domain on the AMHR2 protein. Mutations located in the intracellular domain, for instance p.I209N (Figure 1), which is thought to disrupt the substrate-binding site of the kinase domain (Messika-Zeitoun et al., 2001), migrate normally to the cell surface but are unable to transduce the AMH signal. Determination of the evolutionary conservation of this site found that it was highly conserved among different species. Therefore, p.I209N is potentially disease causing. By using these tools, one additional variant (p.L354F) is expected to be clinically significant but remains arguable pending functional studies.

The AMHR2 –482A>G homozygous mutation could result in diminished AMH signalling and, because AMHR2 –482A>G is located at the promoter region, it could possibly cause disequilibrium with several other SNP (Kevenaar et al., 2007). However, the nature of the disequilibrium with other SNP remains unknown. There have been reports that this polymorphism is associated with age at menopause. Nulliparous women with the GG genotype had 1.9–2.6 years’ earlier onset of menopause compared with women with the AA genotype (Kevenaar et al., 2007; Voorhuis et al., 2011), and carriers of \( \text{AMH} \) 49Ser and \( \text{AMHR2} \) –482G alleles had higher follicular phase oestradiol concentrations compared with noncarriers (Kevenaar et al., 2007). An in-vitro study also showed that the bioactivity of AMH is diminished in the AMH 49Ser protein compared with the AMH Ile49 protein (Kevenaar et al., 2008). Interestingly, in the present study, the genotype distributions and allele frequencies for AMH Ile49Ser and \( \text{AMHR2} \) –482A>G were similar between the groups. This is in agreement with a previous study (Yoon et al., 2013), in which AMH Ile49Ser and \( \text{AMHR2} \) –482A>G did not show any association with idiopathic POI in Korean women. This finding suggests that variants in genes of the AMH signalling pathway are not associated with the timing of menopause.

The genotype distributions for both polymorphisms in this population were consistent with other reports. For AMH Ile49Ser, the genotype distributions were similar to
those of Asians in the HapMap database (http://hapmap.ncbi.nlm.nih.gov). For the AA genotype in AMHR2 -482A>G polymorphism, the distribution was 62.5% in this study versus 65.2% in the Rotterdam Cohort, 64.1% in the LASA cohort, 63.0% in an Italian cohort, 65.0% in a Dutch cohort and 60.9% in a Korean cohort (Kevenaar et al., 2007; Rigon et al., 2010; Voorhuis et al., 2011; Yoon et al., 2013).

Because POI is a complex trait like menopause, genetic interaction with other factors or other genetic variants of the AMH signalling pathway might also influence the development of POI. Braem et al. (2013) searched for pairwise interactions between the SNP in five genes (AMH, AMHR2, BMP15, FOXL2, GDF9). They found a statistically significant interaction between rs10407022 in AMH and rs11170547 in AMHR2 (\(P = 0.019\)) associated with age at natural menopause. These might imply that complex interactions between AMH and AMHR2 play a role in POI and early ANM.

In conclusion, these results suggest that POI patients and normal-ANM women in China share AMH and AMHR2 genetic variants. The AMH signalling pathway associated with ANM may also contribute to POI. However, the number of patients studied limits the interpretation of the results obtained, as does the fact that no molecular study has been performed to support the functional significance of AMH and AMHR2 genetic variants. More research is required to confirm such findings.

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References


Hemoglobinopathies and thalassemias – the importance of screening and genetic testing

by Jan Traeger-Synodinos, D.Phil (Oxon), ErCLG, Associate Professor of Genetics, Laboratory of Medical Genetics, National and Kapodistrian University of Athens, Greece.

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Introduction

Hemoglobinopathies and thalassemias are a diverse group of inherited disorders that disrupt the normal synthesis of the globin polypeptide chains of the hemoglobin molecule. Hemoglobinopathies usually refer to structural variants and thalassemias usually refer to quantitative variants (usually a reduction), although a few globin chain variants are also synthesized at reduced levels (e.g. HbE). Overall, this group of disorders constitute the most common life-threatening monogenic conditions worldwide, and, with very rare exceptions, they are inherited in an autosomal recessive manner (1, 2).

It is estimated that 7% of the world’s population carry a mutation leading to defective synthesis of hemoglobin (3). Alpha thalassemia is more common globally than beta-thalassemia; in some populations, the carrier frequency of alpha-thalassemia is over 50%! Typically, heterozygotes (carriers) of any hemoglobin disorder are clinically asymptomatic. However, the inheritance of either two alpha-thalassemia alleles or two variant HBB genes lead to clinically relevant disease states (Figure 1). Overall the global burden of beta-globinopathies is more prominent, with approximately 300,000-400,000 new patients born every year to carrier-couples, of whom around 40,000 have transfusion-dependant beta-thalassemia major and approximately 300,000 have sickle cell disease (SCD). With respect to alpha-globinopathies, patients with HbH disease have a milder clinical condition compared to most patients with beta-thalassemia, and the severest form of alpha-thalassemia (Hb Bart’s Hydrops fetalis) is usually incompatible with post-natal life (Figure 1) (1, 3).

Hemoglobinopathies and thalassemias are traditionally endemic in all the sub-tropical regions of the world (3, 4), extending from the Mediterranean area, through the Middle East and India, to Southeast Asia. Specifically, the clinically relevant forms of beta-thalassemia are most common in the Mediterranean basin, the Middle East and Asia. The clinically relevant forms of alpha-thalassemia are found mainly in Southeast Asia. With respect to hemoglobin variants, HbE is very frequent in Thailand, Laos and Cambodia, and quite prevalent in India, Sri Lanka and Malaysia. The sickle-cell syndromes (most common of which is sickle cell disease, SCD) are found most predominantly in sub-Saharan Africa and populations originating from there, such as in countries of South America, the Caribbean, and Central America. However, HbS is also frequent in parts of India and to a lesser degree in the Mediterranean basin and the Middle East.

There is now substantial evidence that links the occurrence of hemoglobinopathies and thalassemias to a process of natural selection, whereby carriers tend to survive better than non-carriers when infected by P. falciparum malaria (3). In most populations where hemoglobinopathies and thalassemias are endemic, alpha- and beta-thalassemias co-exist along with various abnormal hemoglobins. However, today hemoglobinopathies and thalassemias are found in almost all regions of the world, even where they were not originally endemic, due to global migration and genetic admixture (5, 6).

Molecular genetics and clinical relevance

Hemoglobinopathies and thalassemias are caused by DNA mutations in or near the genes that code for globin polypeptide chains of hemoglobin (7, 8). All normal hemoglobin molecules are tetramers, consisting of two alpha-like and two beta-like globin chains. In healthy adults the major hemoglobin is HbA (α²β²), with a minor hemoglobin fraction (<2.5%-3.0%) of HbA₂ (α₂β₂). Before birth, the major hemoglobin is HbF (α²γ²), but in normal individuals HbF levels rapidly decline after birth (inversely related to the rise in HbA), usually falling to levels below 1-2% Hb F in normal adults. (Figure 2).

According to which globin gene(s) are mutated, and in turn which globin chain(s) have reduced synthesis, thalassemias are grouped as alpha-thalassemias and beta-thalassemias (the latter including also delta-thalassemias). Common hemoglobin variants with clinical significance all affect the beta-globin chain and include HbS, HbC, HbE, HbD-Punjab and HbO-Arab.

Approximately 1600 DNA variations have been reported which affect normal globin gene expression (9). Mutation categories include deletions that remove functional genes (and/or their regulatory regions), or nucleotide variants within or nearby globin genes. In the chromosome region 16p13.3 a normal allele includes two,
almost identical, α-globin genes (HBA1 and HBA2) within the so-called alpha-gene cluster: 5’ HBZ, HBA2, HBA1 3’.
The HBA1 and HBA2 genes both contribute to the synthesis of the α-globin polypeptide chains (Figure 2). The β-globin chains are synthesized by the β-globin gene (HBB), located in the chromosome region 11p15.5. The HBG1, HBG1 and HBD genes are positioned close to HBB and together form the so-called HBB gene cluster: 5’ HBE, HBG2, HBG1, HBD, HBB 3’ (Figure 2). With respect to the thalassemias, it has been traditional to classify DNA variants that abolish the synthesis of globin chains as “α-zero” or “β-zero” (e.g. αβ-thalassemia or β0-thalassemia) and those that partially abolish synthesis of globin chains as “α-plus” or “β-plus” (e.g. αγ-thalassemia or ββ-thalassemia).

**Alpha-globinopathies**: Around 200 different mutations have been described to cause α-thalassemia. Most commonly α-thalassemia is caused by deletions that partially or entirely remove the α-globin genes; nucleotide variations within the α-globin genes (so called non-deletion α-thalassemia) are less frequent. Due to the presence of two α-globin genes on each normal allele, the molecular genetics of α-thalassemia is more complicated than that of β-globinopathies. The most common variant in humans, found in all populations where α-thalassemia is (or was) endemic, involves the deletion of a single α-globin gene from the α-globin gene cluster (so-called “α-zero” or “α-thalassemia carriers”). Homozygotes are clinically asymptomatic, but hematologically they usually have mildly microcytic, hypochromic red blood cells, comparable to individuals who carry α-globin gene deletions that remove both α-globin genes from chromosome 16p13.3 (αα-thalassemia alleles – Figure 1). However, αα-thalassemia alleles have clinical significance when co-inherited in the compound heterozygous or homozygous state with another α-thalassemia variant. Homozygosity for αα-thalassemia leads to Hb Bart’s Hydrops Fetalis (Figure 1), which is quite common in countries of SE Asia, where αα-thalassemia variants have a significant carrier frequency. Although babies with Hb Bart’s Hydrops Fetalis generally do not survive the neonatal period, it is still clinically relevant to identify at-risk pregnancies for the sake of the pregnant mother, to predict and manage complications (including preeclampsia, premature delivery and abnormal bleeding). HbH disease occurs when α-thalassemia variants interact to reduce the α-globin production to levels approximately equivalent to the output a single α-gene and there are many underlying genotypes (10, 11) (Figure 1). HbH disease gets its name from the detection of β-globin tetramers formed by the excess of β-globin chains in the red blood cells. The β-globin tetramers are called HbH and account for 5-30% of hemoglobin in the peripheral blood of HbH disease patients. Although HbH is a chronic, hemolytic anemia, with pronounced microcytic hypochromic red blood cells, most patients survive without regular blood transfusions and usually require only sporadic clinical support.

Finally, the α-gene cluster is susceptible to copy number variations leading to alleles with additional HBA1/2 genes. Most common are triplicated α-gene arrangements (αααα) that have arisen on the reciprocal chromosome following the deletion of a single HBA1/2 during unequal crossover in the α-gene cluster during meiosis. Alleles with additional functional HBA1/2 genes, are the opposite of “α-thalassemic” in so much as they produce more α-globin chains than normal. However, in some cases they may be of clinical significance when they are co-inherited with heterozygous β-thalassemia, as they may lead to forms of β-thalassemia of intermediate severity (12, 13, 14).

**Beta-globinopathies**: In contrast to α-thalassemia, the vast majority of mutations described in HBB are single nucleotide variations, or indels affecting up to a few nucleotides, with over 300 described. Beta-thalassemia mutations less commonly involve deletions from within HBB gene cluster, removing from a few hundred to over 60,000 base-pairs (9). Large gene deletions also cause most forms of ββ-thalassemia, which may interact with other β-globinopathies and cause clinically significant conditions.

The most severe clinical manifestations of the β-globinopathies are β-thalassemia major, sickle cell syndromes (homozygous Hb S, but also Hb S interacting with β-thalassemia or many of the other clinically significant variants) and Hb E/β-thalassemia. All β-globinopathies require life-long symptomatic treatment (15, 16, 17, 18, 19). For patients inheriting two β-thalassemia mutations, this primarily involves regular blood transfusions (to address the severe anemia) and iron chelation therapy (to reduce iron overload). HbE homozygotes usually have only mild clinical features but patients who have HbE interacting with β-thalassemia often have severe clinical presentation, comparable to thalassemia major. For sickle-cell syndromes, patient management aims to minimize, or otherwise treat, a tendency towards painful crisis, infections, anemia, and pulmonary hypertension, amongst other symptoms. HLA-compatible bone marrow transplantation potentially offers long-term cure (20, 21, 22). However, availability and access is currently limited for most patients, partly due to lack of suitable donors, but additionally due to the high costs and specialized infrastructure required for treatment procedures.

**Approaches for carrier screening and testing**

Due to the high prevalence of the hemoglobinopathies and thalassemias, many countries acknowledge the benefit of minimizing the incidence of new cases by applying programmes for carrier screening (2, 23). Carriers are clinically asymptomatic, but carrier-couples who have defects in the same globin gene group (either both an α-globinopathy or a β-globinopathy) have a 25% risk of transmitting a clinically significant condition to their offspring each time they conceive. Carrier-couples identified following screening have the chance to select a
reproductive option that supports them to have children who are not affected by a hemoglobin disorder, or at least prepare them for the birth of an affected child. (Note that any option offered should be within the framework of accepted local practices and legislation). Carrier screening programmes were pioneered in the 1970’s and 1980’s in several Mediterranean countries, such as Italy, Greece and Cyprus, and have proven extremely effective in reducing the incidence of new cases (24).

Traditional carrier screening for the hemoglobinopathies and thalassemias involves a range of hematological tests, which together provide an essentially presumptive diagnosis. DNA analysis of globin genes supports definitive characterization of carriers. Best practice guidelines recommend that screening with hematological methods should accompany DNA testing, such that DNA results can be evaluated in the light of hematological findings (23, 24). In most regions of the world, α- and β-thalassemia co-exist alongside various abnormal hemoglobins, whereby there are numerous potential genotype interactions and thus phenotypes. Finally, it is appropriate that screening be offered with appropriate genetic counselling.

Of the 1600 or so variants underlying the hemoglobinopathies, around 500 have clinical significance (9, 25). However, in each population where these diseases were (or are) endemic, the spectrum is usually limited to around 20 variants each for α-thalassemia and β-thalassemia, along with at least one clinically significant hemoglobin variant. Thus to date, DNA analysis focused on population-specific testing strategies, based on targeted mutation-specific methods. However, since worldwide migration has lead to multi-ethnic populations in many countries, there is a trend towards the use of more generic DNA analytical methods, which facilitate the potential detection of almost any mutation, irrespective of ethnicity (26, 27).

Traditional procedures used for detecting globin gene mutations for almost 3 decades involve the use of techniques based on the polymerase chain reaction (PCR) to investigate the presence or absence of specific nucleotide variants. These so-called targeted methods included e.g. allele-specific oligonucleotide probe (ASO) hybridization, the amplification refractory mutation system (ARMS) and restriction endonuclease (RE) analysis of PCR products (24, 26, 27). For common globin gene cluster deletions, PCR methods were developed which amplify across the deletion junction fragment when present (so-called GAP-PCR). (24, 26, 27). However, none of these methods can detect new or unknown mutations. DNA “scanning” methods were subsequently introduced to evaluate the presence of nucleotide variants in gene regions of up to several hundred nucleotides at a time; these methods require the subsequent use of DNA methods that specifically characterize any variant(s) indicated in a sample. DNA “scanning” methods include denaturing gradient gel electrophoresis (DGGE) or, more recently, high resolution melting analysis (HRMA).

Each method has certain advantages and limitations (24, 26, 27). Most PCR-based targeted methods are relatively simple to apply and require only basic laboratory infrastructure (thermocyclers, accurate pipettes, agarose gel electrophoresis and recording equipment), making them suitable for use in laboratories where resources are limited. However, they are somewhat ineffective when there is a need to analyse many samples and/or many variants. Although some DNA “scanning” methods are potentially more automated, and have a higher throughput capacity, they usually require more costly infrastructure, and are technically more demanding to run.

Many labs have introduced a more generic approach to address the challenge when screening ethnically diverse populations. Automated Sanger sequencing represents a generic method with the capability to characterize any nucleotide variation, and Multiplex Ligation-dependent Probe Amplification (MLPA) or targeted Array Comparative Genome Hybridization (aCGH) can characterize the majority of deletions within the globin gene clusters. Conveniently, both sequencing and MLPA can be run on a single platform, namely an automated capillary electrophoresis nucleotide analyzer (automatic sequencer). Overall, screening and genetic testing for hemoglobinopathies and thalassemias is not without challenges! (23, 24, 26, 27).

However, we are witnessing a big step forward with the introduction of Next Generation Sequencing (NGS) for diagnostic genome and exome sequencing (28, 29, 30, 31, 32). There are many NGS platforms and NGS strategies vary, including the simultaneous sequencing of many targeted genes (gene panels), Whole Exome Sequencing (WES) which focuses on sequencing the approximately 30 million base pairs encoding all functional proteins, and finally analysis of the entire human genome (roughly 3 billion base pairs), known as Whole Genome Sequencing (WGS). The strategy chosen will be influenced by the laboratory infrastructure and also the requirements and objectives of the analysis. As relevant to any method when introduced into a clinical setting, the NGS strategy must be validated for all laboratory steps, as well as the bioinformatics analysis of data and data interpretation (28, 29, 30, 31, 32). When using NGS for detecting DNA variations that are more complex than single nucleotide variants, such as large deletions and insertions, there are still outstanding technical challenges (33).

With respect to detecting the clinically relevant DNA variants in the hemoglobinopathies and thalassemias, NGS remains challenging. This is partly due to the high homology of the sequences between globin genes, which complicates the preparation of sequencing “libraries”, and additionally the alignment of the short sequence reads during the bioinformatics analysis steps. In addition, copy number variants are frequently associated with certain thalassemias (most notably α-thalassemia – see Figure 1) and this category of variants is still more reliably detected using other methods, such as MLPA, a, quantitative or
digital PCR (23, 26). Despite the technical difficulties, a few recent studies demonstrate the emerging application of NGS for genotyping globin gene variants (34, 35).

Conclusion

Hemoglobinopathies and thalassemias comprise the commonest recessive monogenic disorders worldwide, and the treatment of affected individuals is challenging. Thus carrier screening and genetic testing represent valuable procedures that can identify couples that have a risk of having affected children. In this way carrier-couples can be offered reproductive options most appropriate for them. For hemoglobinopathies and thalassemias, optimal carrier screening should include hematological and DNA testing so that the latter can be evaluated with in the light of the former, overall supporting comprehensive counseling when providing the test results (23). Traditional DNA testing methods are gradually being replaced by more advanced methods, including NGS. Once the technical challenges associated with applying NGS in routine clinical practice are resolved, it is predicted that NGS will become the method of choice for testing the majority of clinically relevant DNA variants, delivering precision, and relative to traditional DNA testing, speed and cost-effectiveness. For any method(s) and strategy aimed at clinical application, it is paramount that every stage of the procedure (screening, testing and counselling) fulfils all criteria associated with the highest standards of quality assurance.

References


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<td>Clinical expression</td>
<td>Normal</td>
<td>Asymptomatic</td>
<td>Asymptomatic</td>
<td>HbH disease with chronic, hemolytic anemia</td>
<td>Hb Bart’s Hydrops Fetalis</td>
</tr>
<tr>
<td>Hematology</td>
<td>Normal</td>
<td>Normal or borderline-reduced microcytosis &amp; hypochromasia</td>
<td>Moderate microcytosis &amp; hypochromasia</td>
<td>Pronounced microcytosis &amp; hypochromasia &amp; HbH levels 5-30%</td>
<td>(NA)</td>
</tr>
</tbody>
</table>

**Figure 1:** Alpha thalassemia genotypes and associated phenotypes

![Diagram of hemoglobin genes and their expression](image1)

**Figure 2:** The globin gene clusters and their hemoglobin

![Diagram of globin gene clusters](image2)
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