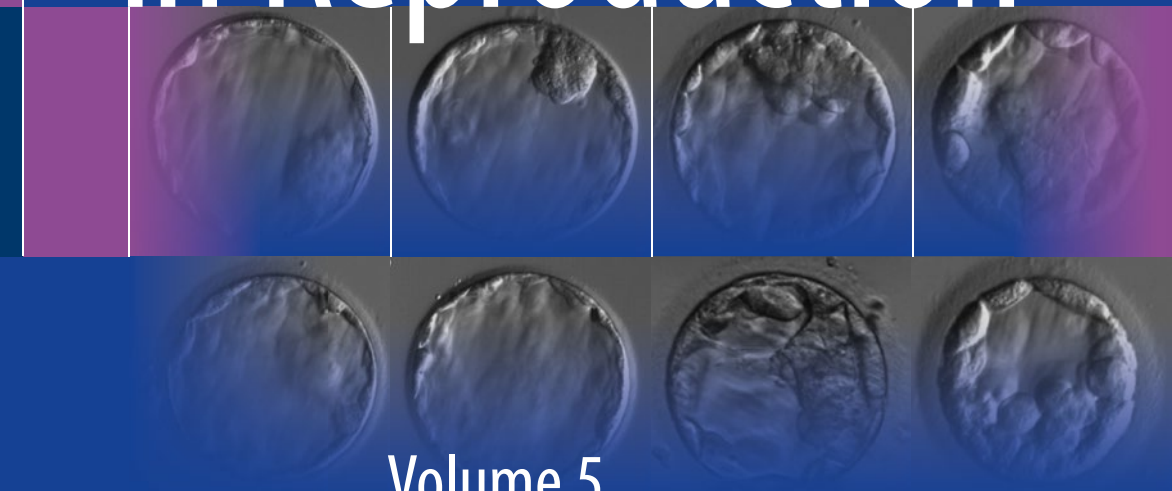
Emerging Topics in Reproduction



Volume 5

Douglas T. Carrell
Catherine Racowsky
Peter N. Schlegel
Alan H. DeCherney
Editors

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We dedicate Emerging Topics in Reproduction, Volume 5, to our patients, whose joy in successful outcomes we share and whose disappointments motivate us to further our quest for advancements that will benefit tomorrow's patients.

Preface

Volume 5 ushers in a new era for this book series. Previously, the book has been titled *Biennial Review of Infertility*, but in an effort to better reflect the cutting-edge nature and breadth of topics covered within the book, the editors have agreed to rename the series *Emerging Topics in Reproduction*. We are confident that this new title accurately describes the content within the book and our aim to provide both clinicians and researchers with cutting-edge reviews of emerging topics and possible clinical ramifications of evolving data and technologies.

Volume 5 addresses the pressing questions and controversies in modern reproductive medicine, as well as providing insights into emerging technologies and topics. For example, topics covered within the male reproductive health section of the book include the analysis of fertility preservation options in the adolescent male, the use of surgical retrieval of testicular sperm in patients with normal sperm DNA fragmentation rates, the role and future diagnostic potential of sperm RNAs, male infertility as a marker for future health concerns, and the emerging role of varicocelectomy in men with nonobstructive azoospermia. Each of these topics are of timely importance to clinicians, and the reviews provide a guide to possible future clinical paradigms.

Equally important topics are covered in the female reproductive section of the book, including the use of advanced imaging techniques, the treatment of subclinical hypothyroidism, and the effects of endocrine-disrupting chemicals on female fertility. Lastly, issues involving the care of patients undergoing assisted reproductive technologies (ART) are also covered in two foundational topics covered by long-time experts in the field. Swain and Roovers discuss embryo culture techniques and the issue of "the optimal" techniques labs should use. Huang and Alikani review the latest data and techniques for embryo selection during ART. The chapters are a balance of highlighting data critical for making the best clinical decisions today as well as introducing emerging ideas upon which data are being gathered now to improve practices of the future.

Perhaps the most popular section of *Emerging Topics in Reproduction* has been the "Controversies" section. Again, we highlight some of the most significant controversies and debates in our industry with the opinions and conclusions of experts

in the field. Topics covered in the book include whether it is good medicine and/or ethical to set a maximum body mass index for women desiring IVF treatment, the trend of freezing all embryos to be used in subsequent cryo cycles, the ongoing debate of broad use of PGS, and the question of if blastocyst-stage embryo transfer is really optimal as a standard practice for all patients. These are timely and important topics with opinions rendered by respected clinicians.

As always, the topics covered in the book are written by the leading experts in the field. They have strived to provide the latest data and their clinical judgment to help the readers make the clinical decisions best for patients, as well as highlighting the technologies of tomorrow.

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Part I
Male

Chapter 1
TESE for Cryptozoospermia with Normal Sperm DNA Fragmentation

Ahmad H. Al-Malki and Armand Zini

1.1 Introduction

The advent of intracytoplasmic sperm injection (ICSI) in 1992 revolutionized the management of couples with male factor infertility [1]. The report of Palermo et al. suggested that ICSI reproductive outcomes, such as clinical pregnancy and live-birth rates, were not influenced by impaired sperm characteristics [sperm concentration, morphology, or progressive motility] [2]. This claim was further supported by several subsequent publications. In their retrospective study of 966 ICSI cycles, Nagy et al. reported no effect of different sperm defects on ICSI outcomes [3]. Even in the most severe form of sperm impairment such as cryptozoospermia (spermatozoa only identified with extended centrifugation and microscopic search), severe OAT, or total asthenozoospermia, high fertilization and pregnancy rates were reported with ICSI [4]. However, Nagy et al. stated that the only sperm condition that could negatively affect ICSI outcome was the injection of immotile (presumably dead) sperm [3]. Subsequently, several authors reported similar findings [5, 6].

The initial optimism of ICSI's ability to overcome severe sperm impairment was later challenged by findings of more recent publications. Mitchel et al. studied 21 infertile patients with asthenozoospermia due to flagellar abnormalities and reported significantly lower pregnancy rates in couples with low sperm motility (<5%) compared to those with higher sperm motility (>5%) (22% vs. 84%, respectively, $p = 0.04$) [7]. Although earlier studies reported no effect of teratozoospermia on ICSI outcome, the effect of injecting sperm with abnormal morphology was later reassessed. De Vos et al. studied ICSI outcomes with the use of morphologically

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abnormal vs. morphologically normal spermatozoa and reported significantly poorer outcomes in the former vs. latter group with lower pregnancy (20% vs. 37%, respectively, $p = 0.018$), implantation (23% vs. 32%, respectively, $p = 0.013$), and live-birth rates (20% vs. 28%, respectively, $p = 0.006$) [8]. However, a more recent observational study by Van den Hoven et al. found no prognostic value of the degree of sperm morphology on ICSI outcomes [9].

Contrary to the initial promise of ICSI to overcome the effect of oligozoospermia, several more recent studies reported a negative effect of severe forms of oligozoospermia on ICSI outcomes. In 2010, Hashimoto et al. reviewed 908 ICSI cycles and reported a significant negative effect of severe oligozoospermia (<1 M sperm per mL) on ICSI fertilization rate with no effect on clinical pregnancy outcomes [10]. Similar findings were reported by Arikan et al. on a follow-up review of 506 ICSI cycles [11]. Moreover, Strassburger et al. reviewed 1076 ICSI cycles and found that cryptozoospermic couples had significantly lower fertilization and clinical pregnancy rates compared to couples with sperm concentration between 1×10^5 sperm/mL and 1×10^7 sperm/mL (46% vs. 61%, $p < 0.0001$ and 20% vs. 31%, $p < 0.05$, respectively) and a higher abortion rate (30% vs. 15%, $p < 0.03$) [12]. This notable effect of severe oligozoospermia on fertilization and the effect of cryptozoospermia on clinical pregnancy compared to earlier reports by Palermo's and Nagy's studies [2, 4] might be attributed to the difficulty in identifying morphologically normal sperm in men with very low sperm counts [13]. Additionally, men with severe oligozoospermia and cryptozoospermia show a higher incidence of chromosomal abnormalities that might negatively affect ICSI outcomes [14, 15].

1.2 Sperm DNA Damage Effect on ARTs

The genetic integrity of the spermatozoon is vital for proper embryonic development [16]. Men with compromised sperm parameters are typically those at greatest risk of harboring sperm DNA defects and those mostly likely to undergo intracytoplasmic sperm injection (ICSI) [17]. Given that natural selection barriers are bypassed with ICSI, the risk of transmitting a genetic defect is of concern in these men. Several studies have reported an association between impaired sperm parameters and underlying genetic defects in subfertile men [14, 15, 18–24]. Moreover, several investigators have reported on the negative influence of these genetic defects on outcomes following assisted reproductive techniques [25, 26].

In 2012, a systematic review and meta-analysis including 2969 IVF-ICSI cycles demonstrated that sperm DNA damage is associated with a high miscarriage rate (IVF/ICSI [risk ratio (RR) = 2.16 (1.54, 3.03); $p < 0.00001$)] [27]. A follow-up systematic review and meta-analysis reported a similar negative effect of high sperm DNA damage on miscarriage rates in 3106 couples undergoing ICSI (OR

2.68; 95% CI: 1.40–5.14; $p = 0.03$) [28]. In 2016, Oleszczuk et al. reported similar results on their series of patients [29]. Recently, a systematic review and meta-analysis including 8068 treatment cycles (3734 IVF, 2282 ICSI, and 2052 mixed IVF + ICSI) reported a negative effect of sperm DNA damage on clinical pregnancy, with a diagnostic odd ratio of 1.68 (95% CI: 1.49–1.89; $p < 0.0001$) [30]. Simon et al. also found that sperm DNA damage was significantly related to outcomes after different assisted reproduction cycles, with the following estimated diagnostic odds ratios for IVF: 1.65 (95% CI: 1.49–1.89; $p < 0.0001$), ICSI: 1.31 (95% CI: 1.08–1.59; $p = 0.0068$), and mixed IVF + ICSI: 2.37 (95% CI: 1.89–2.97; $p < 0.0001$) [30].

1.3 Sperm DNA Damage and Testicular Sperm

Greco et al. were the first to introduce the concept of using testicular sperm to overcome ejaculated sperm DNA damage [31]. Greco et al. hypothesized that sperm DNA damage begins after sperm are released from Sertoli cells with damage progressively increasing with time from sperm release. Accordingly, sperm harvested directly from testes would acquire less DNA damage than their ejaculated counterparts [31]. To test this hypothesis, they compared the DNA damage in both testicular and ejaculated sperm of infertile couples with two prior ICSI failures and high levels of ejaculated sperm DNA damage. They also investigated ICSI outcomes using both ejaculated and testicular sperm at the third and fourth ICSI cycles, respectively. They reported lower DNA damage in testicular sperm and higher pregnancy rates when using testicular compared to ejaculated sperm [31].

To test these findings (progressive DNA damage post-testicular release), Suganuma et al. conducted a study on an animal model comparing the outcomes of wild-type mice (normal spermatogenesis) and mutant mice (with abnormal spermatogenesis due to transition nuclear protein knockout). The investigators harvested spermatozoa from the testicles and from different levels of epididymis. Suganuma et al. reported that in mice with abnormal spermatogenesis, the passage of sperm through the epididymis was associated with loss of sperm DNA integrity and fertilizing capacity, and this was not observed in mice with normal spermatogenesis. They proposed that mutant mice produced spermatozoa with decreased sperm nuclear compaction (higher levels of residual histones and lower levels of disulfide bond) and that the sperm DNA of these mutant mice was not fully protected during epididymal transit. Accordingly, Suganuma et al. proposed that in some infertile men, the passage of sperm through the epididymis can compromise their sperm DNA integrity and fertilization capacity. These new findings challenged the previous concept on the protective role of the post-testicular (epididymal transit) environment.

1.4 Cryptozoospermia

Cryptozoospermia or virtual azoospermia is generally deemed to be a variant of non-obstructive azoospermia. In these men (with cryptozoospermia or virtual azoospermia), the presence of rare sperm in the ejaculate is presumed to arise from small foci of spermatogenesis or be the result of fluctuations in spermatogenesis [3, 32]. Men with cryptozoospermia on a first semen analysis will intermittently produce an azoospermic ejaculate on subsequent semen analysis making it necessary for some of these men to undergo testicular sperm retrieval at the time of ICSI [33, 34]. Indeed, Cui et al. studied a large cohort of cryptozoospermic men and found that 25% (71/285) of these men did not have viable sperm in two sequential ejaculates on the day of ICSI [34].

1.5 Cryptozoospermia and Sperm Genetic Integrity

Sperm DNA testing is not feasible for most men with cryptozoospermia because there are too few cells to evaluate sperm DNA damage. Nonetheless, men with cryptozoospermia are presumed to be at increased risk for sperm DNA damage and sperm chromosomal anomalies [17, 35]. Although oligozoospermia-cryptozoospermia is not commonly associated with sperm DNA damage, men with oligozoospermia-cryptozoospermia frequently have associated defects in sperm motility and viability, and these latter sperm defects increase the likelihood of sperm DNA damage [17, 36].

Several authors have reported an association between impaired spermatogenesis and sperm aneuploidy rates [14, 15, 18–24]. Also, sperm aneuploidy has been associated with poor outcomes after assisted reproductive techniques [15, 25, 26, 37] and an increased risk of recurrent pregnancy loss [23].

Additionally, testicular sperm have higher aneuploidy rates than ejaculated sperm [37], and this is more evident in men with impaired spermatogenesis [20, 38, 39]. Hence, the use of testicular sperm for ICSI, particularly, in cases with impaired spermatogenesis, might increase the incidence of genetic abnormality in the developed embryos [40], although some authors have reported no increase in the incidence of congenital abnormalities in children born with testicular sperm-ICSI [41, 42]. Therefore, more studies addressing the possible association between genetic consequences to the offspring and the use of testicular sperm in ICSI are needed.

1.6 Cryptozoospermia: Use of Ejaculated vs. Testicular Sperm for ICSI

Considering the initial report of Greco et al. and the reported association between impaired sperm parameters and elevated sperm DNA damage [43], several investigators have utilized testicular sperm for ICSI in men with

cryptozoospermia [32]. There is some evidence that testicular sperm-ICSI may be a better approach than ejaculated sperm-ICSI in men with oligozoospermia, but whether this applies to men with cryptozoospermia has not been demonstrated. Recently, Esteves et al. examined 147 couples and reported on outcomes of testicular sperm-ICSI compared to ejaculated sperm-ICSI in couples with oligozoospermia (mild to moderate) and persistently elevated sperm DNA damage (>30% sperm DNA fragmentation index post 3 months of medical treatment). They found that testicular sperm had lower levels of sperm DNA damage compared to ejaculated sperm (8.3% vs. 40.7%, respectively) [44]. They also found that testicular sperm-ICSI was associated with higher clinical pregnancy and live-birth rates and lower miscarriage rates than ejaculated sperm-ICSI (51.9% vs. 40.2, 46.7% vs. 26.4%, and 10% vs. 34.3%, respectively) [44]. Similarly, Mehta et al. reported that testicular sperm had lower sperm DNA damage and that testicular sperm-ICSI was associated with higher clinical and live-birth rates compared to ejaculated sperm-ICSI in oligozoospermic men with high ejaculated sperm DNA damage [45].

Given that sperm DNA testing is not feasible for most men with cryptozoospermia, studies of cryptozoospermic couples have not included data on sperm DNA integrity. In 2011, Hauser et al. examined ICSI outcomes with the use of testicular and ejaculated sperm in couples with cryptozoospermia and reported that the use of testicular sperm was associated with higher fertilization rate compared to ejaculated sperm [33]. Ben-Ami et al. evaluated 116 ICSI cycles and reported that testicular sperm-ICSI was associated with significantly higher implantation, clinical pregnancy, and live-birth rates compared to ejaculated sperm-ICSI (20.7% vs. 5.7%, 42.5% vs. 15.1%, 27.5% vs. 9.4%, respectively) [46]. Recently, a large retrospective analysis by Cui et al. found that couples undergoing testicular sperm-ICSI achieved higher implantation, clinical pregnancy, and live-birth rates compared to undergoing ejaculated sperm-ICSI [34]. However, other published studies failed to establish an advantage of testicular sperm over ejaculated sperm in men with cryptozoospermia [47]. A retrospective study by Gnoth et al. reported no difference between testicular and ejaculated sperm in ICSI outcomes for subset of patient with cryptozoospermia [48]. A recent meta-analysis by Abhyankar et al. examined 272 ICSI cycles using testicular or ejaculated sperm in men with cryptozoospermia and reported no difference in clinical pregnancy rates (relative risk [RR] 0.53, 95% CI: 0.19–1.42, I(2) = 67%) or fertilization rate (RR 0.91, 95% CI: 0.78–1.06, I(2) = 73%) between the groups [49]. However, the meta-analysis reported by Abhyankar et al. did not include the large study (n = 285) reported by Cui et al., where testicular sperm-ICSI was found to be associated better with reproductive outcomes than ejaculated sperm-ICSI [34].

It is presumed that the potential benefit of using of testicular rather than ejaculated sperm-ICSI in couples with cryptozoospermia is more significant in couples with an underlying sperm DNA damage. However, this remains to be demonstrated as the referenced publications did not measure the degree of sperm DNA damage in their study populations.

1.6.1 Sperm DNA Damage Limitations

Despite the numerous reports on the effect of sperm DNA damage on ARTs outcomes, the prognostic value of sperm DNA test is still questionable [50]. Several reasons are behind the poor acceptance of sperm DNA damage tests as a routine evaluation tools for couples with infertility. One of the major limiting factors is the marked heterogeneity of the studies characteristics. Studies differ in their designs, patient population characteristics, ARTs protocols, as well as the diversity in available sperm DNA damage testing tools [13]. Another limiting factor is the lack of standardized reference values that distinguish a normal from an abnormal test results [13]. Moreover, these tests exhibit poor precision (high inter-lab variability) and questionable accuracy. Considering the importance of sperm DNA damage measurement and the current uncertainties surrounding testing methods, additional studies are required to demonstrate the true clinical value of this test.

1.6.2 Cryptozoospermia and Testicular Sperm Retrieval (TSR)

Cryptozoospermia is considered a variant of nonobstructive azoospermia (NOA). In cryptozoospermia, rare spermatozoa are discovered after extended sperm preparation (ESP) method, while no spermatozoa are identified in the ejaculate before and often after centrifugation [12]. The presence of these rare spermatozoa is speculated to arise from small focal areas of active spermatogenesis [3, 51]. These spermatogenic foci are presumed to be present at variable testicular locations in men with spermatogenic failure [52, 53]. Indeed, cryptozoospermic men might intermittently produce an azoospermic ejaculate sample (no sperm identified even after ESP) [33].

Given the uncertainty of identifying sperm on the day of ICSI, some cryptozoospermic men may require a testicular sperm retrieval [TSR] even if the use of ejaculated sperm-ICSI was initially planned [33]. Several testicular sperm retrieval techniques are available and include testicular sperm aspiration (TESA), testicular sperm extraction (TESE), and microsurgical testicular sperm extraction (micro-TESE). The choice of technique depends on the underlying pathology, the anticipated success rate, the potential complication rate, and surgeon experience.

In their small series of men with cryptozoospermia, Alrabeeah et al. reported that micro-TESE was associated with a 96% (23/24) sperm retrieval rate, while TESA was associated with a 43% (3/7) sperm retrieval rate [54]. Shefi et al. reported a 78% sperm retrieval rate by fine needle aspiration (FNA) in a series of 36 cryptozoospermic men [55]. The differences in sperm retrieval rates between these studies can be explained by the variation in the techniques of sperm retrieval. Unfortunately, the limited data on the success rates of the different TSR techniques in men with cryptozoospermia does not allow us to define the optimal technique in these cases.

1.6.3 Testicular Sperm Retrieval Limitations

Many authors have reported on the short- and long-term physiological changes associated with testicular sperm retrieval (TSR). Schlegel and Su et al. reported a transient effect on spermatogenesis post-testicular biopsy [56]. Even in cases of unilateral testicular biopsy, the transient effect on spermatogenesis was reported on the contralateral untouched testis [57]. TESE has been associated with inflammatory reactions lasting up to 6 months [56], intratesticular bleeding [58], and complete testicular devascularization [56]. Other authors reported post-biopsy calcifications and fibrosis [59, 60]. Okada et al. also reported transient hypogonadism, testicular atrophy, and chronic testicular changes detected by ultrasound in up to 23% of cases [61]. Although these procedures are generally minimally invasive, the potential short- and long-term complications must be considered and weighed against the potential benefits of a testicular sperm retrieval.

1.7 Conclusions

There is ongoing controversy as to whether one should use ejaculated or testicular sperm for ICSI in men with cryptozoospermia and presumably normal sperm DNA integrity. Although several reports have shown improved ICSI outcomes with the use of testicular rather than ejaculated sperm, other reports have not demonstrated this benefit, and most studies in this field are small and retrospective in nature [49]. Moreover, there are no data on the genetic integrity of sperm from men with cryptozoospermia. Sperm DNA testing (if feasible) would help guide clinical management of these men and identify those men that would benefit most from testicular sperm-ICSI and those men at risk of transmitting a genetic or epigenetic abnormality to the offspring.

Testicular sperm retrieval (TSR) techniques are for the most part minimally invasive procedures that are associated with potential minor and, rarely, major complications. Some of the major complications are rare (e.g., complete testicular devascularization), but they may have significant deleterious effects on a man's fertility potential. Although unproven, the use of testicular sperm might potentiate the genetic and epigenetic risks to the offspring.

To conclude, there are currently no established criteria to guide decision-making for the use of testicular sperm in men with cryptozoospermia and normal sperm DNA integrity. Therefore, clinicians are encouraged to rely on their own judgment in deciding on whether to proceed to testicular sperm retrieval-ICSI (TSR-ICSI) or use ejaculated sperm-ICSI (Ej-ICSI). In doing so, clinicians need to consider the limited data on TSR-ICSI vs. Ej-ICSI, the TSR-associated risks, the uncertainty of TSR genetic and epigenetic safety profile on future embryos, and the inability to fully assess the sperm DNA integrity in these men.

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Chapter 2
The Argument for Varicocele Repair in Nonobstructive Azoospermia

Connor M. Forbes, Russell P. Hayden, and Marc Goldstein

Abbreviations

ART	Assisted reproductive technologies
FSH	Follicle-stimulating hormone
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilization
microTESE	Microsurgical testicular sperm extraction
NOA	Nonobstructive azoospermia
SRR	Sperm retrieval rate

2.1 Introduction

Varicocele represents an abnormal dilation of the pampiniform venous plexus. It is a common condition, occurring in approximately 15% of the general population, a prevalence that increases with age [1–3]. An association has long been recognized between the presence and severity of varicocele upon male fertility [4–6]. Historically, the presence of varicocele has been reported in 35% of men with primary infertility and 75% of men with secondary infertility, perhaps suggesting progressive testicular damage with a longer duration of exposure [7, 8]. Although the pathophysiology of the varicocele remains unclear, varicocele

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repair is associated with improved fertility outcomes [9, 10]. The documented advantages of varicocelectomy are largely based upon data involving men with oligoasthenoteratospermia.

In men with nonobstructive azoospermia (NOA), however, repair of varicocele is more controversial. NOA is defined as the absence of sperm in the ejaculate that cannot be explained by reproductive tract obstruction. It represents an extreme of male factor infertility, accounting for 10–20% of cases [11, 12]. Excluding hypogonadotropic hypogonadism, which represents an extra-testicular pathogenesis that can be treated with exogenous hormones, the remaining etiologies of NOA generally fall into a range of spermatogenesis failure. NOA is a particularly frustrating disorder to treat as the severe impairment of spermatogenesis often precludes successful retrieval of viable spermatozoa even with extensive microsurgical sampling of the testis [13].

Within the NOA cohort, 5–10% will be found to have a varicocele during physical exam [14]. This presents a therapeutic opportunity that may affect reproductive outcomes. Proponents of varicocele repair often cite small series that document either return of sperm to the ejaculate or improved sperm retrieval on surgical exploration [15–18]. From these data, meta-analyses have now been conducted, also providing clues as to the role of varicocele repair in men with NOA [19, 20]. Despite these promising results, controversy remains given that sperm retrieval and assisted reproductive technologies (ART) will often still be required whether or not varicocelectomy is performed [21]. In this chapter, we will review the data that addresses varicocele repair in this population and the controversies that surround it.

2.2 Return of Sperm to the Ejaculate

Facilitating return of viable sperm to the ejaculate is a logical goal for any treatment targeting the NOA population. Ejaculated samples serve as a reproducible source of fresh sperm for ART, can inherently capitalize upon the maturation and natural selection processes of the epididymis, and may even facilitate conception by natural means. Additionally, ejaculated sperm is cost-effective as it can preclude the need for surgical sperm retrieval and provides a means for multiple future pregnancies [22].

In terms of improved semen parameters, several groups have analyzed their experience of NOA patients who have undergone varicocelectomy. Early series were retrospective in nature and did not have comparison groups (Table 2.1). Matthews et al. published the first prospective series of nonobstructive azoospermic men undergoing microsurgical varicocelectomy [17]. Of their group of 22 patients with NOA, 55% developed motile sperm in the ejaculate post-repair. Even more encouraging, three of these men achieved unassisted pregnancy. This study was followed by Kim and colleagues, who observed 28 men up to 24 months following varicocelectomy [18]. Within their cohort, 43% had return of sperm to the ejaculate.

Table 2.1 Studies evaluating recovery of sperm in the ejaculate after varicocele repair in NOA

Study	Design	*n*	Return of sperm to the ejaculate	Number of pregnancies
Matthews et al. (1998) [17]	Prospective noncontrolled	22	12/22 (55%)	3
Kim et al. (1999) [18]	Retrospective noncontrolled	28	12/28 (43%)	2
Kadioglu et al. (2001) [23]	Prospective noncontrolled	24	5/24 (21%)	0
Cakan and Altug (2004) [24]	Retrospective noncontrolled	13	3/13 (23%)	0
Schlegel et al. (2004) [21]	Retrospective noncontrolled	31	7/31 (22%)	NA
Esteves and Glina (2005) [25]	Retrospective noncontrolled	17	6/17 (35%)	1
Gat et al. (2005) [26]	Prospective noncontrolled	32	18/32 (56%)	9
Pasqualotto et al. (2006) [27]	Retrospective noncontrolled	27	9/27 (33.3%)	1
Poulakis et al. (2006) [28]	Prospective noncontrolled	14	7/14 (50%)	3
Lee et al. (2007) [29]	Prospective noncontrolled	19	7/19 (36%)	1
Ishikawa et al. (2008) [30]	Retrospective noncontrolled	6	2/6 (30%)	0
Cocuzza et al. (2009) [59]	Prospective noncontrolled	10	3/10 (30%)	NA
Youssef et al. (2009) [60]	Prospective noncontrolled	51	14/51 (27%)	2
Abdel-Meguid et al. (2012) [31]	Prospective noncontrolled	31	10/31 (32%)	NA
Kirac et al. (2013) [32]	Retrospective noncontrolled	23	7/23 (30%)	3
Zampieri et al. (2013) [33]	Prospective noncontrolled	35	17/35 (48%)	0
Aboutaleb et al. (2014) [34]	Prospective noncontrolled	20	6/20 (30%)	NA
D'Andrea et al. (2015) [61]	Prospective noncontrolled	23	11/23 (48%)	NA
Ustuner et al. (2015) [62]	Prospective noncontrolled	19	1/19 (5%)	NA
Shiraishi et al. (2017) [16]	Prospective noncontrolled	83	20/83 (24%)	5

Reported rates were restricted for return of motile sperm when specified by the study. Pregnancies include assisted (using ejaculated sperm) and unassisted outcomes. When specified, results were restricted to men with absolute azoospermia.

These promising results were substantiated in subsequent series, although with slightly diminished percentages of men with improved semen parameters. Esteves et al. documented a rate for sperm recovery in 47% of men ($n = 17$), although only 35% had motility [25]. Another group contemporary to Esteves documented a 23% return of ejaculated sperm [24]. More recently, Kirac et al. and Shiraishi et al. documented recovery rates of 30% and 24% for their cohorts of 23 and 83 men, respectively [16, 32]. The wide variability of these results may reflect the known variability of semen parameters, a factor indirectly addressed by some groups, as well as variability in techniques of repair.

Kadioglu et al. followed 48 men postoperatively from varicocele repair [23]. They divided their cohort into two groups based on careful examination of a pelleted semen specimen: absolute azoospermia ($n = 24$) and virtual azoospermia ($n = 24$). Of men with absolute and virtual azoospermia (rare, non-motile), 21% and 85% had return of viable, motile sperm to the ejaculate, respectively. This study highlights the inherit variability of semen analysis, with intrasubject variation serving as the rule rather than the exception. Additionally, an extended search for sperm is highly dependent upon the length of time invested and the experience of the andrologist [35]. It is conceivable that many men in published cohorts may fall into the virtual azoospermia phenotype, thereby skewing results toward favorable outcomes [35]. Indeed, multiple sites have published mixed cohorts with consistent improvement in those with "few sperm" on pelleted analysis. Gat et al. utilized spermatic vein embolization of 101 patients with diagnoses ranging from azoospermia to "severe" oligospermia [26]. Their 82% improvement in semen analysis highlights the inherit danger of mixing NOA with men who produce sperm in the ejaculate. Smaller series contaminated by even a few subjects that have severe oligospermia, characterized as azoospermic by one or two semen analyses, will result in overly optimistic conclusions [28, 30].

Another concerning feature characterized in some studies is the limited durability of success after varicocele repair, defined as successful return followed by loss of ejaculated sperm. However, this too may be a symptom of intrasubject variability, as some men may indeed have sperm on future samples outside of the study period. Initial evidence of azoospermic relapse was discussed in 2003 by Pasqualotto and colleagues [36]. Among their study population of 15 men, 7 (47%) had return of sperm to the ejaculate. Five men redeveloped azoospermia, the majority of whom had germ cell aplasia on testis biopsy. An update in 2006 by the same group confirmed similar findings: 9 of 27 (33%) regained sperm in the ejaculate following varicocele repair, and 5 of those 9 relapsed by 6 months [27]. Another more contemporary series also documented a similar rate of relapse [29]. Countering accounts were also published. In a series of 33 men with confirmed azoospermia, Schlegel et al. documented that 22% of subjects continued to have sperm in the ejaculate at 14 months, far exceeding the 6-month window analyzed by Pasqualotto [21]. Similarly, in a series published in 2014, a cohort of 20 men with NOA demonstrated that 30% developed sperm in the ejaculate [34]. None of these individuals relapsed. Some of the variability in outcome may be due to variability in technique of repair, with microsurgical repairs yielding the best and most durable outcomes. Finally, Abdel-Meguid et al. attempted to formalize the relapse

rate with serial postoperative semen analyses [31]. Of their 31 study participants, 19.4% had persistent recovery, 6.5% had An intermittent recovery, and 6.5% appeared to have truly relapsed back to azoospermia. These data were bolstered by a reasonable cohort size, study duration, and a prospective design that was powered based on historical sperm recovery rates.

Multiple groups have utilized meta-analysis to strengthen the evidence for varicocele repair in the NOA population. Weedin et al. evaluated 11 studies, all of which were retrospective [20]. Their pooled analysis demonstrated that 39% of men had return of sperm to the ejaculate. A more recent review by Esteves et al. updated the results of Weedin [19]. Among the 18 studies that were included, 15 were used to examine improvement in semen parameters. They document a 44% rate of sperm return within the ejaculate. None of the included studies had control groups, again a reflection of the relative weakness of the evidence base and the resulting caution with which one must view these results. Nevertheless, the preponderance of evidence, produced by large high-volume centers with experienced andrology labs and now supported by two meta-analyses, maintains that varicocelectomy will return sperm to the ejaculate for some men with absolute NOA [12].

2.3 Pregnancy Achieved Without Sperm Retrieval After Varicocele Repair

Multiple observational studies have reported assisted and unassisted pregnancies using ejaculated sperm in men with NOA following successful varicocelectomy. One of the largest series, recently published by Shiraishi et al., followed 83 men with NOA prospectively across a mean follow-up of 18 months [16]. Five men within their cohort (6%) were able to contribute to a pregnancy using ejaculated sperm. These results included timed methods in addition to assisted insemination. Earlier studies had smaller cohorts with a wide variability of reported pregnancy rates, which again prompted subsequent attempts of a pooled analysis.

Two meta-analyses comment on pregnancy rates with ejaculated sperm following varicocele repair in men with NOA. A comprehensive review in 2010 summed the experience for 233 men, 14 of whom (6%) resulted in unassisted pregnancy [20]. Another analysis that included data up to 2015 further differentiated pregnancy outcomes into unassisted and assisted categories [19]. They identified 12 of 88 cases that resulted in unassisted pregnancy (13.6%). For couples who underwent ART with ejaculated sperm ($n = 58$), 18.9% achieved pregnancy. Finally, in an undifferentiated group (combining both natural conception and ART, $n = 88$), a pregnancy rate of 26% was reported.

Similar to the studies discussing return of sperm to the ejaculate, none of these studies included a comparison group in which no varicocelectomy was performed. Although it is highly unlikely that a man with NOA will conceive spontaneously, one cannot rigorously consider this rate negligible in the absence of a control group. The data do allow, however, reasonable evidence for counseling men with concomitant NOA and varicocele in terms of postoperative expectations.

2.4 Sperm Retrieval and ART Outcomes with Testicular Samples

For men with NOA, the possibility of paternity via testicular sperm was realized with the introduction of intracytoplasmic sperm injection (ICSI). However, even with extensive exploration of the testis, only 60% of all-comers can expect successful surgical sperm retrieval, a prognosis that is dependent upon histopathologic diagnosis, genetic background, and technique [13, 37–40]. Consequently, an alternative goal of any treatment for NOA, if not to return sperm to the ejaculate, is to optimize the likelihood of sperm retrieval.

Early retrospective literature characterizing sperm retrieval rate (SRR) benefited from matched controls, i.e., men who had palpable varicoceles but did not opt for varicocele repair prior to sperm retrieval (Table 2.2). In one of the first reports, Schlegel and Kaufmann examined a single center's experience spanning 6 years [21]. They identified 138 patients who underwent microTESE, 68 of which had undergone prior repair. Their results demonstrated an identical SRR of 60% between men who underwent repair and those who had not. These preliminary results were countered by Inci et al., who compared 66 men status post-varicocelectomy against 30 who decided against repair [41]. The intervention group had undergone repair at least 1 year prior to microTESE. An odds ratio for successful sperm retrieval of 2.6 (CI 1.05–6.6, $p = 0.03$) was found favoring varicocele repair, which translated forward to a statistically significant improvement in pregnancy rates. These data were strengthened by well-matched preoperative patient characteristics between cases and controls (most notably the prevalence and severity of concomitant female factors, preoperative FSH levels, and embryo fertilization/transfer rates).

Two more high-volume center experiences have since been published. Haydardedeoglu et al. retrospectively reviewed ICSI outcomes in couples with a male

Table 2.2 Studies evaluating sperm retrieval after varicocele repair in NOA

Study	Design	*n*	Retrieval after varicocelectomy	Retrieval without varicocelectomy
Schlegel et al. (2004) [21]	Retrospective case-control	138	41/68 (60%)	42/70 (60%)
Esteves and Glina (2005) [25]	Retrospective noncontrolled	9	4/9 (44.4%)	NA
Inci et al. (2009) [41]	Retrospective case-control	96	35/66 (53%)	9/30 (30%)
Haydardedeoglu et al. (2010) [42]	Retrospective case-control	269	45/74 (60%)	75/195 (38%)
Zampieri et al. (2013) [33]	Prospective randomized	35	11/19 (57%)	4/16 (25%)
Ustuner et al. (2015) [62]	Prospective noncontrolled	19	8/19 (42%)	NA
Shiraishi et al. (2017) [16]	Prospective noncontrolled	53	19/53 (36%)	NA

factor of NOA [42]. Of the 96 couples included in the analysis, 31 had undergone prior varicocelectomy. SRR increased in the varicocele repair group to 60% as opposed to 38%. Interestingly, pregnancy rate seemed to decrease with longer duration between varicocele repair and ultimate retrieval attempt (average duration of 42 months compared against 80). Zampieri et al. also examined the timing of varicocelectomy and the effect upon SRR [33]. In this prospective trial, men with a Grade III varicocele and NOA were either randomized to concomitant varicocele repair at the time of microTESE (n = 16) or staged varicocelectomy followed by microTESE 3 months later (n = 19). The staged arm had statistically significant improvement in SRR, 57% as opposed to 27%. However, the staged group also had significantly more individuals who had return of sperm to the ejaculate. It was not documented if the two arms had comparable histopathology, which may have confounded these results as evidenced by the differences in postoperative semen analysis. In other words, given their small sample size, randomization may have failed.

Conflicting results, small cohort size, and the lack of any multi-institutional accounts have led two groups to attempt a meta-analysis. Kirby et al. analyzed seven articles, although only two (Inci and Haydardedeoglu) were strictly based upon men with NOA [15, 41, 42]. Results for the NOA group demonstrated a statistically significant improvement in SRR, with a documented odds ratio of 2.5 ($p < 0.01$, CI not reported). The pregnancy rate also demonstrated a benefit with an odds ratio of 2.3 (CI 1.02–2.7). Live birth rate tended to favor varicocelectomy prior to sperm retrieval, although it did not obtain significance ($p = 0.051$). Esteves et al. performed a similar analysis with the addition of the Zampieri trial [19, 33]. They reported an improvement of SRR with an odds ratio of 2.65 (CI 1.6–4.1) and a similar trend toward significance for live birth rate (OR 2.1, CI 0.99–4.83). It should be noted that all three studies included in this meta-analysis applied strict criteria for nonobstructive azoospermia [33, 41, 42]. In combination, these data provide considerable evidence for the benefit of varicocele repair prior to sperm retrieval in men with NOA.

2.5 Prognostic Factors

Unnecessary varicocelectomy causes needless morbidity and adds cost in terms of both expense and time. The latter is most relevant for couples with advanced female age as timely intervention is required to optimize birth outcomes [43, 44]. In order to identify which NOA patients benefit most from varicocelectomy, multiple groups have attempted to identify preoperative criteria that may correlate with improved semen parameters, SRR, and pregnancy rate.

Within the NOA population, the strongest studies examining the utility of preoperative data involve surgical sperm retrieval. In one of the largest series, Ramasamy et al. studied 1026 patients undergoing microTESE at a single center [37]. They used multivariable logistic regression to assess the following variables: patient age, FSH, testis volume, history of cryptorchidism, Klinefelter syndrome, and presence

of varicocele. Only age, presence of Klinefelter syndrome, or history of cryptorchidism proved to be effective predictors for successful sperm retrieval.

Varicocele grade is another purported prognosticator for recovery of spermatogenesis after repair. Relatively few studies in the literature have stratified their cohort based upon varicocele grade. Esteves et al. reviewed five series that commented on varicocele severity [19]. Their meta-analysis included 76 patients with an endpoint of postoperative recovery of ejaculated sperm. A recovery rate was noted for Grade I, II, and III varicoceles of 7% (n = 13), 25% (n = 31), and 34% (n = 32), respectively. The trend did not reach statistical significance, possibly due to the small sample population. A subsequent study that followed Esteves examined 83 patients with either Grade II or III varicoceles [16]. They documented that 24% had return of sperm to the ejaculate for their combined Grade II and III cohort, consistent with the rates found in the above meta-analysis.

Testis biopsy remains the most consistent predictor of outcomes, both for recovery of sperm in the ejaculate and for SRR [13]. It is well known that NOA is a heterogeneous group of disorders, currently best characterized by testicular histopathology. In one series of testis biopsies of 37 patients with NOA, 30% of patients had complete spermatogenesis with evidence of disorganization, 38% showed arrested spermatogenesis (i.e., early or late maturation arrest), and 32% showed Sertoli-cell-only [45]. Kim et al. documented one of the first experiences comparing outcomes against testis biopsy [18]. Of their cohort of 28 men, only individuals with hypospermatogenesis or late maturation arrest had sperm return to the ejaculate (n = 14). Nonresponders were characterized as Sertoli-cell-only or early maturation arrest. Following this description, multiple small series were published documenting the value of testis biopsy to predict post-varicocelectomy outcome. These data were reviewed in a summative analysis by Elzanaty and colleagues [14]. From the five studies that met their inclusion criteria, they analyzed a cohort of 90 men. Sperm returned to the ejaculate in 60% of men with hypospermatogenesis (n = 30), 46% with maturation arrest (n = 26), and 3% with Sertoli-cell-only (n = 34). Recently, these data were revisited in a contemporary meta-analysis, now including 8 studies for a total cohort of 161 individuals [19]. Again, sperm returned to the ejaculate in a similar trend. When comparing hypospermatogenesis to early maturation arrest or Sertoli-cell-only syndrome, calculated odds ratios for return of ejaculated sperm were 2.35 (CI 1.04–5.29) and 12 (CI 4.34–33.17), respectively. These data compliment the well-documented association between SRR and baseline testis histopathology [46, 47].

Recent studies have attempted to address the benefit of varicocelectomy based upon emerging biochemistry techniques. A novel study, specifically addressing men with azoospermia and concomitant varicocele, utilized next-generation sequencing to analyze the transcriptome of testis biopsies procured at the time of varicocele repair [16]. Their analysis was limited to those men with maturation arrest, with resulting differential expression of several gene families involved in the cell cycle (upregulated) and antioxidants (downregulated). These preliminary

data dovetail nicely with prior accounts addressing varicocele pathophysiology, especially those positing the role of oxidative damage [48]. Future work addressing the impact of varicoceles on men with NOA will continue to address the interplay of genetics, clinicopathologic diagnosis, and response to treatment [49].

2.6 Cost Analysis

Couples facing a dual diagnosis of varicocele and NOA can choose either immediate microTESE or varicocelectomy with staged retrieval thereafter. The addition of varicocele repair can add considerable cost if microTESE ultimately becomes necessary regardless. However, early decision analyses for men with severe male factor infertility (not specific for NOA) have demonstrated cost savings of varicocele repair in lieu of immediate ART [50]. These estimates have encouraged other investigators to assess the cost-effectiveness of varicocelectomy in NOA.

Recovery of sperm in the ejaculate, with or without unassisted pregnancy, is one factor arguing toward initial varicocelectomy. Ejaculated sperm can be used for ART, may be obtained repetitively for multiple cycles, or may possibly obviate the need for sperm retrieval altogether should unassisted pregnancy occur. Early accounts estimated that viable sperm in the ejaculate, defined as suitable for ART, only occurred in approximately 9% of cases following successful varix repair [21]. In a more contemporary analysis, Esteves et al. documented an optimistic rate of 44% in the postoperative setting [19]. Taking a step beyond these data, one group conducted a direct comparison between immediate retrieval and a staged approach for treatment costs in 2005 [51]. This exhaustive analysis demonstrated that immediate microTESE resulted in less spending, $65,731, as opposed to $79,579 for staged treatment. This calculation included the scenarios of spontaneous post-varicocelectomy pregnancy and avoidance of retrieval via ejaculated sperm. However, they did not consider couples desiring multiple children and the cost savings that can be realized for men with sustained cryptozoospermia following varicocele repair [31].

The cost analysis for immediate retrieval versus staged treatment requires reevaluation for today's healthcare dollar. Indeed, Lee et al.'s review clearly demonstrated changes in relative cost burden independent of inflation rates between 1999 and 2005, foreshadowing further differences in the current practice environment [51]. Additionally, the financial advantages of varicocele repair for couples seeking large families remains unknown. Practitioners should remember, however, that for the couple desiring pregnancy, relative cost estimates will often be supplanted by the potential benefits of varicocelectomy, especially in regard to improved SRR. For this reason, many high-volume practitioners still offer staged treatment in couples with favorable preoperative characteristics [12].

2.7 Ancillary Indications for Varicocele Repair in NOA: Androgen Deficiency

Common alternative indications for varicocelectomy include pain, testis atrophy, and androgen deficiency, of which the latter will be discussed further. Low testosterone often accompanies NOA, as both processes result from primary testicular failure. Although literature specifically addressing the indication of hypogonadism in NOA is lacking, fruitful conclusions can be drawn from studies addressing the general population of men with both low testosterone and varicocele.

It is well recognized that the varicocele presents as a pan-testicular insult, with marked reduction in Leydig cell function [1, 52, 53]. The role for varicocele repair in low testosterone, however, has remained controversial [54]. Several groups have reviewed their experience regarding postoperative improvement of testosterone for subfertile men treated with varicocelectomy. The largest series documented a strong dependence upon preoperative serum testosterone, i.e., men with lower initial androgen levels had better outcomes [55–57]. A meta-analysis by Li et al. combined 9 of these early studies to span a total of 814 subjects [58]. The pooled data demonstrated a statistically significant increase in postoperative testosterone of 97.4 ng/dL (CI 43.7–151.2). Furthermore, they too observed that men with lower initial testosterone obtained greater benefit.

Improved testosterone levels may be of minimal immediate importance to couples concerned about fertility. Varicocelectomy in men with NOA can be a difficult decision in light of questionable cost-effectiveness, the promise of immediate sperm retrieval, and the prospect of two surgical procedures. However, the concomitant diagnosis of NOA and androgen deficiency, a common presentation, may clinch the decision to proceed to varicocele repair. Therefore, a discussion of androgen deficiency, its long-term sequelae, and the benefits of varicocelectomy are necessary components of counseling for men with NOA.

2.8 Conclusions

Men with NOA now have the chance to father biological children due to the development of microTESE and ICSI. However, even despite exhaustive intraoperative retrieval attempts, one in five individuals will not have sperm found. Some men in this scenario also have palpable varicoceles, presenting a modifiable risk factor related to fertility outcomes. In this chapter, we reviewed the evidence that relates varicocele repair to the NOA population. Varicocelectomy often results in return of viable sperm to the ejaculate, allowing IVF/ICSI with ejaculated sperm, rarely spontaneous pregnancy, increased surgical sperm retrieval rates, and improved testosterone production in hypogonadal men. However encouraging, these results are based upon data that is observational and often lacking appropriate control groups. For this reason, varicocele repair in

NOA remains controversial. This debate is also fueled by concerns of cost-effectiveness, the morbidity of a second procedure, and delays to sperm retrieval, which is an important issue for couples with advanced female age. As with any therapy, treatment of men with the dual diagnosis of NOA and varicocele requires a personalized approach. The data, although tempered by suboptimal study design, remains robust. A discussion between patient and urologist that will hinge upon the data outlined above will result in carefully selected men with concomitant varicocele and NOA benefitting from varicocelectomy.

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Chapter 3
Current and Future Perspectives on Sperm RNAs

Luke Simon and Douglas T. Carrell

3.1 Introduction

The structural organization of sperm nucleus proposes a highly condensed sperm chromatin [1]; lack of cytoplasm and absence of intact ribosomal RNA [2] suggest the notion that the sperm nucleus is transcriptionally inactive [3]. However, the presence of RNA in mature sperm raised questions such as "Why would sperm contain any RNA?" The detection of RNA in mature sperm was initially assumed to be either the remnant RNAs left behind after the spermatogenetic process or from contaminant cells and cytoplasmic droplets [4, 5]. In addition, sperm lack any translational activity, as most of their cytoplasm is extruded during sperm maturation process into a residual body, while sperm with a cytoplasmic droplet is considered as dysfunctional sperm and a product of faulty spermatogenesis [6]. Therefore, the ability of the sperm to deliver potentially important RNA to the oocyte on fertilization was met with skepticism.

The presence of RNA in mature sperm was first observed during the 1970s [7]. Since then, a number of authors have reaffirmed the presence of RNAs in mature sperm [4, 8], which was later termed as paternal RNA. With the help of advancement in molecular technologies, the first comprehensive profile of paternal RNA from normal fertile men was reported in 2002 [9]. Since then, a number of studies have identified diverse type of RNAs with a variety of function. The RNAs observed

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in sperm are functional, having translational capability and regulatory potential, and these paternal RNA is showed to involve in various biological processes [10]. In addition, the paternal RNA is known to successfully deliver to the oocyte upon fertilization.

Interestingly, most of the paternal RNA identified in sperm are completely absent in an unfertilized human or mouse oocytes [11]. Some of the paternal RNAs such as the RNAs related to protamine proteins (PRM family) are known to quick degradation after fertilization [3]. Whereas most paternal RNAs were observed to be present 5 h after fertilization, others were found to be present after the first embryonic cleavage in the mouse [12]. It is also showed that transcriptional activity first starts from the male pronucleus soon after decondensation of sperm chromatin [13]. Approximately, half the RNAs found in the testes are represented in sperm, and an average sperm contain 20–50 fg of RNA [14]. The ability of the paternal RNAs to actively participate during early embryogenesis, prior to the activation of zygotic genome, is a question to be fully answered. However, some studies have demonstrated the contribution of paternal RNA to fertilization and to early embryonic development [15–17]. In this chapter, we review the literature to illustrate the potential role of paternal RNA for its involvement during the early events of postfertilization that set embryonic development on its course and current evidence of mechanisms through which paternal RNAs can transfer paternal traits to their offspring.

3.2 Diversity of Paternal Sperm RNAs

The RNA population carried by sperm is large and varied. During the early days of RNA profiling, RNA extracted from sperm was subjected to cDNA cloning [18] or RT-PCR [19] followed by sequencing. These methods were able to survey only a small fraction of all transcripts. Later, microarray technique was developed that resulted in identification of 3000–7000 different RNA transcripts from human sperm [9]. In the last decade, the development of RNA-seq technique has depicted a much more complete picture of the diversity of sperm RNA [20]. The RNA-seq technique has also improved the identification, quantification, and characterization of known and unknown RNA sequences [5], resulting in the identification of coding and noncoding RNAs and their functions [20].

RNA-seq has provided the most extensive analysis of sperm RNA profiling; 40% of these RNAs are small RNAs <200 bp, while approximately 60% are large RNA (> 200 bp) fractions. Approximately 85% of the sperm RNA is ribosomal RNAs [21]. However, there are no intact 28S and 18S ribosomal RNA in sperm. Therefore, presence of intact ribosomal RNA is often used as a biomarker to confirm the absence of somatic cell contamination [22]. Other categories of RNAs common in sperm are the mitochondrial RNAs (7%), annotated coding transcripts (4%), small noncoding RNAs (3%), and other RNAs (<1%) which include intronic retained elements, long nuclear RNAs, transcribed regions of unknown coding potential, short

expressed regions, transposable elements, and annotated noncoding RNAs [21]. Many of the relatively abundant RNAs in sperm are unannotated or of with unknown function and are uniquely to sperm.

Coding RNAs are common in sperm, but majority of the coding transcripts are fragmented [5]. Sandler et al. [5] showed that some of the coding RNAs are enriched during the later stages of spermatogenesis, and such genes are associated with male infertility, fertilization, and early embryonic development. A well-known example of such RNA is INTS I (integrator complex subunit I), which involve in the transcription and processing of small nuclear RNAs. INST I is observed in the embryo after fertilization prior to zygotic genome activation [23], and INTS I knockouts are embryonic lethal at the blastocyst stage [24]. Some of the coding RNAs are displayed as isoforms that are distinct from that found in whole testes, and such isoforms arise only in the final transcriptionally active stages of spermatogenesis. An example of such isoform is sperm-specific PKM2 (pyruvate kinase isozymes M1/M2), a key enzyme regulating glucose metabolism [5].

Sequence mapping of human sperm small noncoding RNA library shows that majority (65%) of sequences are associated with an unidentified repeat category [20]. Other small noncoding RNAs included in this category are transcription start site or promotors (10.8%), Piwi-interacting RNA (piRNA—16.9%), microRNA (miRNA—6.9%), small nucleolar RNA (snoRNA—0.3%), and small nuclear RNA (snRNA—0.1%) [20]. These small noncoding RNA reads correspond to unannotated regions of the genome and portions of coding and noncoding transcripts [21]. The noncoding sperm RNAs include transposable elements, annotated long noncoding RNAs, intronic retained elements, exonic elements, chromatin-associated RNAs, quiescent RNAs, and mature sperm-enriched tRNA and YRNAs [21]. These noncoding RNAs are known to act as epigenetic modifiers, used as biomarkers, and play an important role in RNA-mediated transgenerational epigenetic inherence.

3.3 Functions of Sperm RNA

The potential function of paternal RNA remained questioned until recent evidence suggest that the RNAs packed in sperm are not just remnants of discarded RNA during the spermatogenetic process. There is some evidence of translational activity in the sperm [25]. In addition, there is also evidence that sperm RNA contributes to fertilization and early embryonic development [26–28]. Studies suggest that the sperm not only transfer genetic material to the oocyte but also complements early embryonic development. For example, the nucleosomes retained in sperm are not randomly distributed, but are enriched at loci important for early embryogenesis [29]; specifically the nucleosomes are placed at the promoters of miRNAs and imprinted genes [30]. Similarly, paternal RNA may have also been strategically placed in the sperm; however their functions are yet to be discovered [25].

Hosken and Hodgson [31] elegantly proposed forth four novel hypothesis about potential functions of paternal RNAs. First, paternal RNAs facilitate relatedness signaling, either generally or at specific loci. Such signals would facilitate the evolution and maintenance of cooperation among the sperm within the ejaculate. Second, RNAs are packed into sperm by the diploid male to suppress the selfish interests of the haploid cell. Third, the RNA packed in sperm acts as a nuptial gift, where the paternal RNA contributes to early embryonic development. Fourth, the RNA packaged within sperm might act like the contents of a Trojan horse, where the paternal RNA may contribute specific traits benefiting the paternal genome [31].

Recently, small noncoding RNA has gained importance as they involve in post-transcriptional regulation of gene expression. A variety of small noncoding RNAs are identified in sperm; specifically miRNAs are known to involve in apoptosis, cell proliferation, and differentiation, and the potential target genes for miRNAs were identified during spermatogenesis [32, 33]. Ontological analysis of miRNA present in sperm suggests that their potential targets are related to cell differentiation, development, morphogenesis, and embryogenesis [34]. Approximately 90% of the miRNA targets identified by the sperm miRNAs were represented within unfertilized human oocyte and after fertilization [20]; however, about 260 miRNA identified in sperm are absent in oocytes [35].

A well-known example of sperm miRNA involved during embryogenesis is miR-34c, which is required for the first cellular division [21], through the modulation of Bcl-2 expression [28]. Injection of miR-34c inhibitor into zygotes inhibited DNA synthesis and significantly suppressed first cleavage division [28]. Positive correlation was observed between an increased level of miR-34c in sperm and a higher percentage of good-quality embryos on day 3 [36]. Interestingly, when miR-34c was expressed in embryonic chicken stem cells, an upregulation of germ cell-specific genes was detected [37]. These evidence suggests that maternal and embryonic genes are directly or indirectly under the control of miRNAs and both maternal and paternal miRNAs**Error! Bookmark not defined.** are essential for the earliest stages of embryonic development [38].

The other classes of functionally important small noncoding RNAs in sperm are piRNAs. piRNAs play an important role during spermatogenesis, and pachytene piRNA-deficient spermatocytes progress through meiosis, but become arrested at the postmeiotic round spermatid stage with an increased level of sperm DNA damage [39]. RNA-seq technology has identified about 1137 piRNAs from mature sperm [20]. The function of piRNAs is proposed as it protects the genome from the deleterious effects of invasive elements [40]. For example, the targeting repetitive elements of piRNAs can provide a platform for reverse transcription that is requisite for retrotransposition. The relative abundance of piRNAs suggests that they may assume a role in confrontation and consolidation. This may ensure the compatibility of the genomes at fertilization [20]. The complex and large populations of small noncoding RNAs in mature sperm have shown involvement in gene expression upon fertilization, but the function of the unique RNA molecules is yet to be discovered. However, these paternal RNAs have the potential to become biomarkers of male infertility and have the ability to play an important role during early embryogenesis.

3.4 Clinical Potential of Sperm RNAs

Infertility is a growing problem in the developed countries and accounts for 15% of the population in their reproductive aged couples. The evaluation of semen parameter is applicable for diagnosing some obvious forms of male infertility, but there is still a need for novel biomarkers to determine the fertility status. RNA during the spermatogenetic process is present in sperm; therefore it can be used to identify defects in spermatogenesis and is also useful as a biomarker to predict male infertility. For example, protamines play an important role in sperm chromatin condensation, and protamine (PRM1 and PRM2) mRNA is observed in mature sperm. The protamine genes are transcribed during the postmeiotic haploid spermatid, during the early stages of spermiogenesis [41], while protamine is translated during the elongated spermatid stage. It is well known that abnormal protamine ratio in ejaculated sperm results in poor chromatin condensation leading to increased sperm DNA strand break [42]. Similar to protamine content, the PRM1/PRM2 mRNA ratio in testicular spermatids and ejaculated sperm is showed to be altered in infertile men [43]. In addition, protamine mRNA levels are lower in the sperm of asthenozoospermic men compared to fertile controls [44]. Thus, the level and ratio of protamine mRNAs in mature sperm appear to show defects in protamine synthesis during spermatogenesis and could potentially be used as a noninvasive biomarker to investigate testis-specific infertility [45].

Paternal RNAs can also serve as a biomarker based on factors: relative abundance (ratio), integrity in sperm, and presence or absence [5]. It can be used as a noninvasive biomarker to diagnose patients with subfertility [32]. Differences in transcript expression profile (Table 3.1) suggest that sperm RNA can act as a strong potential marker for fertility evaluation. Analysis of paternal RNA using new technologies has contributed to the understanding of a variety of RNA types in mature sperm. The RNA expression pattern is used to identify male infertility and idiopathic infertility; differentiate pregnant and non-pregnant groups, fertile from infertile men, fresh and frozen sperm population, motile and non-motile sperm, and ejaculated and testicular sperm; and identify patients with conditions such as nonobstructive azoospermia, teratozoospermia, asthenozoospermia, and oligoasthenozoospermia and smoking, vasectomized, and cryptorchidism patients (Table 3.1).

Some studies have compared the paternal RNA profile in couples undergoing assisted reproductive treatment. Cui et al. [36] studied miRNA profile in 162 couples undergoing ICSI treatment. The level of two miRNAs miR-34b and miR-34c was significantly higher in the pregnant group compared to the nonpregnant group [36]. Bonache et al. [46] studied the RNA expression profile in donor sperm of couples undergoing IUI treatment. This study suggests that significant differences in the expression of individual genes were observed between groups of donors with the lowest and highest pregnancy rates [46]. Another study suggested the protamine mRNA expression levels are higher in the sperm of patients who were successful following IVF treatment [47]. Garcia-Herrero et al. [48] compared the RNA expression profile in sperm of men successful and unsuccessful after IUI treatment. This study identified differentially expressed transcripts: 756 transcripts

Table 3.1 A review of literature comparing the expression of paternal RNA in sperm of men with various infertility issues, sperm population, and ART outcomes

Study	Comparison groups	Key results
Cui et al. (2015) [36]	Pregnant and nonpregnant	miR-34b and miR-34c levels were higher in the pregnant group
Depa-Martynow et al. (2007) [47]	Pregnant and nonpregnant	Increase in PRM1 and PRM2 mRNA levels was observed in sperm obtained from patients with successful IVF
Bonache et al. (2012) [46]	Pregnant and nonpregnant	Significant differences in mRNA expression of six genes were observed between groups of lowest and highest pregnancy rates after IUI
Garcıa-Herrero et al. (2010) [48]	Pregnant and nonpregnant	About 741 expressed sequence tags were present only in pregnant group and 976 that were expressed only in nonpregnant group. In pregnant group, 756 differentially expressed transcripts presented increased expression, whereas 194 in nonpregnant group were overexpressed
Garcıa-Herrero et al. (2011) [49]	Pregnant and nonpregnant	Of the 19,229 transcripts analyzed, 44 were overexpressed in pregnant group and 5 were overexpressed in nonpregnant group. Exclusively expressed transcripts were 1358 in pregnant and 1836 in nonpregnant group. The differences in expression between pregnant vs. nonpregnant may explain ICSI failure associated with male factor infertility
Savadi-shiraz et al. (2015) [69]	Pregnant and nonpregnant	Normal protamine mRNA ratio was observed in more than 70% of men with successful ICSI cycles
Jodar et al. (2015) [70]	Normal and idiopathic infertility	About 30% of the idiopathic infertile couples presented an incomplete set of required sperm RNA elements
Wu et al. (2012) [71]	Normal and idiopathic infertility	The expression levels of two miRNAs (miR-19b and let-7a) were increased in the seminal plasma idiopathic infertile males
Montjean et al. (2012) [72]	Normal and idiopathic infertility	Transcription profile in germ cells of men with idiopathic infertility is different from that of fertile individuals
Bansal et al. (2015) [73]	Normal and idiopathic infertility	2081 transcripts were differentially expressed between the groups
Wang et al. (2011) [74]	Normal and nonobstructive azoospermia	RT-qPCR analysis identified miRNA miR-34c was associated with nonobstructive azoospermia. miRNAs were upregulated, and 14 were downregulated in the seminal plasma of azoospermia patient groups compared with normal control group
Lian et al. (2009) [75]	Normal and nonobstructive azoospermia	microRNA expression in nonobstructive azoospermia patients was downregulated in 154 miRNA and upregulated in 19 miRNAs in normal controls

Abu-Halima et al. (2014) [32]	Normal and nonobstructive azoospermia	One miRNA was overexpressed and four miRNAs were downregulated in nonobstructive azoospermia groups
Wu et al. (2012) [71]	Normal and nonobstructive azoospermia	The levels of two miRNAs were overexpressed in the seminal plasma of nonobstructive azoospermia patients
Platts et al. (2007) [76]	Normal and teratozoospermic men	Transcripts encoding components of various cellular remodeling pathways were severely disrupted in teratozoospermic patients
Savadi-shiraz et al. (2015) [69]	Normal and teratozoospermic men	A significantly downregulation of PRM1 and PRM2 mRNA was observed in teratozoospermic groups. TNP2 transcript was overexpressed in teratozoospermic group
Kempisty et al. (2007) [44]	Normal and asthenozoospermic men	The PRM1 and PRM2 mRNA transcripts were downregulated in sperm of asthenozoospermic men
Abu-Halima et al. (2013) [77]	Normal and asthenozoospermic men	50 miRNAs were upregulated and 27 miRNAs downregulated in sperm of asthenozoospermic men
Jodar et al. (2012) [78]	Normal and asthenozoospermic men	17 mRNA transcripts were downregulated, and 2 transcripts were upregulated in the sperm of asthenozoospermic men. 2081 are differentially expressed tags and were observed between the two groups
Wang et al. (2011) [74]	Normal and asthenozoospermic men	12 miRNAs were upregulated, and 5 were downregulated in the sperm of asthenozoospermia men
Abu-Halima et al. (2013) [77]	Normal and oligoasthenozoospermic men	42 miRNAs were upregulated and 44 miRNAs downregulated in the sperm of oligoasthenozoospermic men
Wu et al. (2012) [71]	Normal and oligozoospermic men	No difference in miRNA expression in the seminal plasma of the two groups
Hu et al. (2014) [79]	Normal and vasectomized men	84 seminal miRNAs were upregulated in normozoospermic men. 995 seminal piRNAs were identified in normozoospermic men but were absent in vasectomized men
Garrido et al. (2009) [80]	Fertile and infertile men	mRNA analysis between the groups suggested a single-gene expression difference in 27 transcripts
Montjean et al. (2012) [72]	Fertile and infertile men	Downregulation in genes involved in spermatogenesis, sperm motility, DNA repair, oxidative stress regulation, and histone modification in sperm of infertile men
Bansal et al. (2015) [73]	Fertile and infertile men	Transcripts that participate in a host of cellular processes, including reproduction ($n = 58$) and development ($n = 210$), were differentially expressed between the two groups. Some of these transcripts were related to heat shock proteins, testis-specific genes, and Y chromosome genes

(continued)

Table 3.1 (continued)

Study	Comparison groups	Key results
Avendano et al. (2009) [81]	Fertile and infertile men	Fertile group showed significantly higher levels of PSG1 and HLA-E mRNAs
García-Herrero et al. (2010) [48]	Fertile and infertile men	mRNA related to biological process, cellular components, and molecular function was differentially expressed between the groups
Liu et al. (2012) [28]	Fertile and infertile men	52 miRNAs were differentially expressed, 7 miRNAs were upregulated, and 6 miRNAs were downregulated in the semen of infertile men
Garcıa-Herrero et al. (2011) [49]	Fresh and frozen sperm population	A total of 19,229 transcripts were identified in fresh sperm compared to 18,095 transcripts from frozen sperm population
Valcarce et al. (2013) [82]	Fresh and frozen sperm population	mRNAs reported as markers of pregnancy success (ADD1) are reduced after cryopreservation
Ostermeier et al. (2005) [10]	Fresh and frozen sperm population	It is quite apparent that the population of paternal RNAs is adversely affected when semen is subjected to varying degrees of freeze-thaw. RNAs were likely subjected to random degradation during each freeze-thaw cycle
Lambard et al. (2004) [19]	Motile and nonmotile sperm	The amount of PRM-1 mRNA was significantly higher in low-density motile sperm than in the highly motile fraction. In most of high motile sperm samples, eNOS and nNOS transcripts were undetectable, but present in the low motile sperm
Wang et al. (2004) [83]	Motile and nonmotile sperm	Real-time PCR revealed that two sperm motility-related genes (testis-specific protein 1 and lactate dehydrogenase C transcript variant 1) have significant difference in the expression levels between the two groups
Steger et al. (2008) [43]	Ejaculated sperm and testicular sperm	Infertile men exhibit a more than tenfold higher Bcl2 mRNA in testicular biopsy and in ejaculated sperm
Linschooten et al. (2009) [84]	Smokers and nonsmokers	781 genes were found to be differentially expressed in spermatozoa of smokers compared to nonsmokers
Metzler-Guillemain et al. (2015) [85]	Smokers and nonsmokers	15 mRNA genes were differentially expressed: 5 were upregulated, and 10 were downregulated in smokers. 23 miRNAs were differentially expressed: 16 were upregulated, and 7 were downregulated in smokers
Nguyen et al. (2009) [86]	Control and cryptorchidism patients	43 genes were differentially expressed between the two groups. Thirty-eight genes were downregulated in the cryptorchidism patients

overexpressed in the pregnant group and 194 transcripts overexpressed in the nonpregnant group. In addition, they also found exclusive expressed transcripts: 741 that were expressed only in the pregnant group and 976 that were expressed only in the nonpregnant group [48]. Garcia-Herrero et al. [49] also studied the difference in RNA expression between pregnant and nonpregnant couples following ICSI treatment. The results show that among 16,035 sequences that were commonly expressed between the two groups, only 44 sequences were overexpressed in pregnant and 5 were overexpressed in the nonpregnant groups. The analysis on exclusively expressed transcripts showed that 1358 transcripts were only present in the pregnant and 1836 transcripts were only present in the nonpregnant groups [49]. These studies [36, 49] suggest that paternal RNA can be considered as a potential pregnancy success marker as ICSI treatment skips the normal sperm entry process of fertilization.

The development of bioinformatics and gene expression analysis reveals that a large number of transcripts have important roles in spermatogenesis, fertilization, and early embryonic development. Several classes of noncoding RNAs including lncRNAs, pri-miRNAs, novel elements, and mRNAs have been identified to be essential male factors critical to early postfertilization development [5]. Kawano et al. [50] identified two small noncoding RNAs (spR-12 and spR-13) in mature sperm that play an important role in early embryogenesis. In addition, paternal RNAs that encode several transcription factors and signaling molecules have also been identified. Siffroi and Dadoune [51] reported the existence of sperm-specific mRNAs coding for the transcription factor Stat 4, the cyclin B1 from the male pronucleus. Studies using the mouse embryos confirm the presence of transcription factors and proteins of signaling pathways that are involved in the developmental processes in the male pronucleus [52]. This evidence suggests the possible roles of paternal RNA during early embryonic development.

3.5 Sperm RNA as Epigenetic Modifiers

The effect of paternal RNAs is observed during fertilization and early embryo development [48–50], and some authors hypothesize that these RNAs can also perform as epigenetic modifiers. For example, it is shown that an offspring can be obtained using somatic cell nuclear transfer technique; however, placental and congenital defects in the fetus are frequent in such cases. This defects may arise from the inappropriate epigenetic programming of the donor and recipient cells resulting in aberrant inner cell mass and trophectoderm formation [53]. It is speculated that during somatic nuclear transfer, the contribution of sperm-specific elements is absent [20], which could include sperm-specific DNA methylation, sperm chromatin organization, and contribution of paternal RNA as epigenetic modifiers [20].

3.6 Transgenerational Heritability Through Paternal RNA

Transgenerational heritable change is defined as the effect that the offspring acquires from the parents without inherited through the parental genetic factor. Such heritable effect that can be acquired through paternal RNA is a mechanism by which the offspring inherits novel environmental traits from the parents in a non-Mendelian fashion. From evolutionary point of view, such mechanism is nature's way of coping to parents' exposure to toxic environment which could be lethal to their offspring. The transgenerational epigenetic effect is a form of paramutation and is common in plant kingdom. However, such mechanisms do occur in mammalian species and are of complex in nature.

For example, Rassoulzadegan et al. [54] demonstrated the role of paternal RNA in paramutation in a study showing that Kit mRNA microinjection into mouse oocytes conferred non-Mendelian inheritance by reverting the phenotype of a Kit tyrosine kinase receptor knockout (Fig. 3.1). Complete disruption of the mouse Kit gene was lethal to the offspring, while the heterozygote and paramutated animal maintained and transmitted the white-spotted phenotype characteristic to their mutant heterozygote progeny. Heterozygote and paramutated mice accumulated

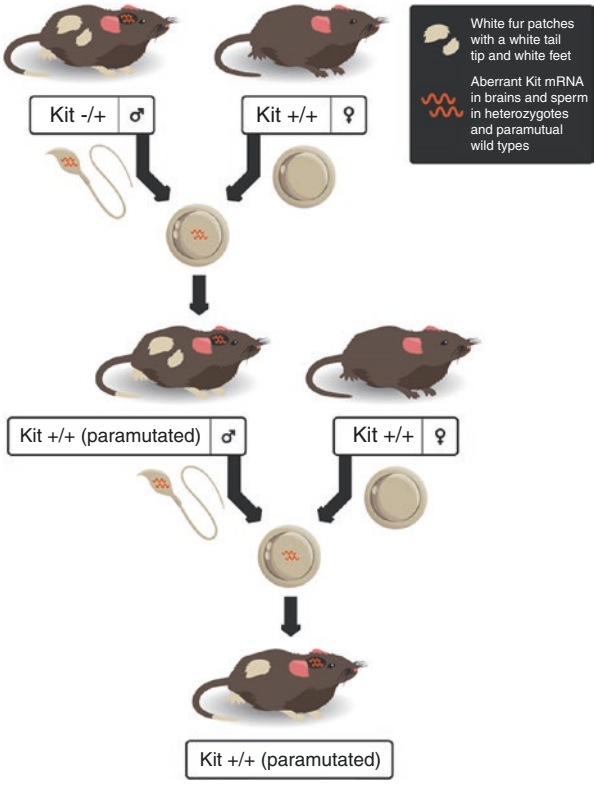

Fig. 3.1 An example of transgenerational inheritance of a modified phenotype via sperm RNA. White fur patches, a white tail tip, and white feet are characteristic of the mutant heterozygote mice. Mating of heterozygous Kit mice with wild-type mice results in paramutated offspring with Kit characters. When these mice are again mated with wild-type mice, a fraction of the offspring retains the phenotype, even with a wild-type genotype. This phenotype could also be induced by microinjection of RNA into fertilized oocytes, which suggests that RNA plays an important role in the mechanism of inheritance

abnormal Kit RNA in the mature epididymal sperm. Interestingly, microinjection of sperm RNA from heterozygous mutants or miRNAs that target Kit (miR-221, miR-222) into fertilized oocyte induced the heritable heterozygote phenotype. The inheritance of heritable change using Kit mRNA illustrates the RNA delivered at fertilization can facilitate RNA-mediated transgenerational effect [54].

Similarly, other examples of miRNAs that induce paramutation are identified in mouse. Heart hypertrophy could be induced by microinjection of miR-1 that paramutates and increases the expression of Cdk9 (cyclin-dependent kinase 9) resulting in cardiomyocyte precursors [55]. Similarly, microinjection of miR-124 or RNA fragments that targets Sox9 was done during fertilization, which results in paramutation of Sox9 during the first embryonic stages leading to increased proliferation of embryonic stem cells, increased body sizes during postnatal development, and twin pregnancies [56]. In these examples, the phenotypic modification was associated with an increase in the transcription rate of the targeted loci (Kit, Cdk9, and Sox9) using fragments of target RNA or microRNAs carried by the sperm and delivered to the oocyte upon fertilization. Unlike the genetic mutations, the paramutations described here have distinct characters: (a) paramutations could be induced at far greater frequencies; (b) the phenotypic changes occurred here are eventually reversible, but the effect is observed for three or more generations in crosses with wild-type partners; and (c) paternal RNA was responsible for the transgenerational effects [57].

For a successful transgenerational heritability, RNA must be present in the gametes. Even though the sperm is transcriptionally silent, recent studies show that a complex diversity of RNAs is present in the sperm and is delivered to the oocyte upon fertilization [10, 20, 21]. Although the amount of paternal RNA is low compared to the level of RNA present in the oocyte, a fertilized oocyte is initially equipped with RNAs inherited from both their parents [11]. Since paternal RNA is not protected due to the lack of cytoplasm, the question still remains: "how stable are the paternal RNAs delivered to the oocyte?" RNA from early embryos shows the presence of RNA-binding proteins, antisense RNAs, specific motifs for stability, and posttranscriptional RNA modifications, such as cytosine-5 methylation [58, 59]. A recent study shows that paternal RNAs do contain posttranscriptional RNA modifications, such as cytosine-5 methylation, which may increase stability of the inherited RNAs [60].

Several studies have suggested that a wide diversity of miRNAs are present in the sperm [20, 33]. miRNAs represent the class of small regulatory RNAs with a well-established role in the gene regulation programs. For example, paternal miR-34c is essential for early embryo development and is required for the first cellular division via modulation of Bcl-2 expression [28]. Another class of small regulatory RNAs with a well-established heritability is the short interfering RNA (siRNAs).

It is well-established in *Drosophila* that the endogenous siRNAs can be a candidate mechanism for developmental gene regulation by heritable siRNAs [61], while endogenous siRNAs are identified in mouse [62] and human sperm [20]. Other types of RNAs, such as long non-coding (lnc) RNAs and tRNAs, are capable of scaffolding protein complexes and recruiting chromatin modifiers to specific sites in the genome, thereby guiding epigenetic changes to specific loci and has the poten-

tial to modulate embryonic gene expression [63]. Although the mechanisms of gene regulation by these inherited RNA molecules are established, this area requires further research.

Experiment using mice models suggests that certain environmental exposure to the parents can influence the phenotype of the progeny. Such transgenerational transfer of information is called "inheritance of acquired character" or "Lamarckian inheritance" [64]. It was generally thought that the mother plays an important role in equipping the oocyte for the early embryonic development, but now it is apparent that the paternal contribution is also valuable for the inheritance of acquired character [65]. If the father can transfer the environmental stress information to the offspring, then what are the likely scenarios through which the sperm could deliver the information to the embryo? It is hypothesized that the sperm epigenome is responsible for the transfer of response to environmental conditions in the form of multiple epigenetic information carriers, such as cytosine methylation, transcription factors, chromatin structure, and RNA molecules [64].

The first study to demonstrate the successful transfer of environment response to the offspring through paternal RNA molecules was Gapp et al. [66]. They used a mouse model exposure to traumatic stress in early life induced by unpredictable maternal separation combined with unpredictable maternal stress (MSUS). In addition, MSUS males spent more time in the illuminated compartment of the light-dark box than controls, and on a Porsolt forced swim test, MSUS males spent more time floating than controls. The authors observed some of the behavioral traits were transmitted to the offspring. To identify the epigenetic information carrier molecule, they isolated total RNA from control and MSUS sperm and injected these populations into control zygotes. The offspring developed from injecting MSUS sperm RNA developed the behavioral traits identical to the offspring developed via natural MSUS mating. This study suggested that RNAs can act as a heritable molecule in transgenerational epigenetic inheritance and the first proof of evidence for RNA molecules being responsible for paternal passage of environmental information in mammals.

In a recent study, Chen et al. [67] showed that altered paternal diet affects the level of small RNAs in mouse sperm and such males could influence the metabolic phenotypes of offspring. In this study, a paternal mouse model given a high-fat diet and paternal RNA content was compared with control. The authors show that a subset of sperm transfer RNA-derived small RNAs (tsRNAs), mainly from 5′ transfer RNA halves and ranging in size from 30 to 34 nucleotides, exhibited changes in expression profiles and RNA modifications [67]. Injection of paternal tsRNA fractions from high-fat diet males to normal zygotes generated metabolic disorders in the offspring and altered gene expression of metabolic pathways in early embryos. The authors also observed that these changes were unrelated to DNA methylation. Results from this study [67] support the fact that sperm tsRNAs represent a paternal epigenetic factor that may mediate transgenerational inheritance of diet-induced metabolic disorders.

In another study, Sharma et al. [68] investigated the mechanism by which paternal diet affects offspring metabolism. In this model, offsprings obtained from male mice consuming a low-protein diet exhibited altered hepatic cholesterol biosynthesis. A comparison of small RNA level in mature sperm between the controls and

protein-restricted mice suggested a decrease in let-7 levels and an increase in the amounts of 5′ fragments of glycine tRNAs. Interestingly, the authors noticed that the tRNA levels are scarce in the testicular sperm, but they are abundant in the mature epididymal sperm. This led to the hypothesis that the tRNAs were packed in the mature sperm after the spermatogenic process. Further analysis revealed that in the epididymis, sperm fuse with small extracellular vesicles known as epididymosomes. These epididymosomes carry a large volume of the tRNAs that were able to fuse with the epididymal sperm and deliver the tRNAs to the mature sperm. The purified epididymosomes contained 87% of reads homologous to that of mature sperm but absent in the testicular sperm. This study for the first time identified novel pathways through which paternal RNAs are deliberately packed in mature sperm to regulate the endogenous retroelements active in the preimplantation embryos [68]. Although these studies are performed in animal models [66–68], further research in humans is necessary to understand the mechanisms by which progeny is affected by parental exposure to various environmental conditions.

3.7 Conclusions

Recent development in molecular technologies including RNA-seq has shed light on the diversity of RNA types present in sperm. These transcripts are showed to reflect the past spermatogenesis process, while some RNAs are known to be critical for fertilization and successful embryo development, while others are known to facilitate transgenerational inheritance of paternal traits to the offspring. An increasing number of comparative studies have analyzed the differential expression of paternal RNA in patients with a variety of infertility conditions, resulting in identification of unique RNAs and exclusively expressed RNAs. However, more studies are required to define which RNAs are universally present in the sperm of fertile males and which differentially expressed RNAs are responsible for specific infertility types. In addition, some animal models have reported specific paternal RNAs are essential for early embryonic development. These paternal RNAs have special interest in clinical tests to aid couples who seek treatment using assisted reproduction techniques and useful resource for improvements in the diagnosis and management of male infertility.

In the last few years, a number of animal studies have shown that early exposure to some dietary conditions and other environmental pollutants affect male gametogenesis and can change gene programming, thereby sometimes increasing risk of disease in the offspring. In addition, some animal models show that paternally acquired exposures can be captured and transmitted to the next generation using paternal RNAs. Most of these studies lack possible explanations of possible molecular mechanisms behind their observations, however, Sharma et al. [68] showed the incorporation of paternal RNAs and in sperm via epididymosomes, and these RNAs are then successfully transferred to the oocyte. Future research in toxicology is required to identify paternal RNAs that can serve as standard biomarkers for environmental exposure and may be key to understanding the transmission of epigenetic characteristics to subsequent offspring and to monitor male-associated reproductive risk.

The collective evidence presented in this chapter supports the differential expression of paternal RNA in various infertility types, and it involves in transmitting information about the paternal environment to the offspring. In addition, the presence or absence of the various stage-specific RNAs in the mature sperm is likely to provide the fidelity of past events of spermatogenesis and a start toward understanding the basis of unexplained or idiopathic infertility. Until now, there are only few animal models that had focused on transgenerational effect of paternal RNAs, but future studies in human subjects should understand the consequences of paternal stress to facilitate improved management of preconceptual stress in males and ultimately public health. With this understanding, paternal RNAs can be used as a diagnostic tool in the future to treat the underlying causes of infertility and to ensure the birth of a healthy child.

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Chapter 4
Male Infertility as a Marker of Future Health

Brent M. Hanson and James M. Hotaling

4.1 Introduction

Infertility is a common problem in the United States, affecting approximately 8–15% of couples. The male partner is identified as the sole cause of infertility in approximately 20% of infertile couples, but male factor infertility is believed to contribute to greater than 50% of infertility cases [1]. Traditionally, a primary focus has been placed on female infertility and the female partner's overall health status in order to achieve a healthy pregnancy. Publications within the medical literature have long neglected the male component of reproduction [2]. Recently, increased attention has been placed on the male partner, and it is becoming clear that a diagnosis of male factor infertility may be associated with long-term health consequences that go beyond the immediate reproductive needs of patients, particularly in the current environment of increasing paternal age [3]. Since approximately 15% of the male genome is involved in reproduction, it is entirely possible that problems related to fertility may also be linked to overall somatic health issues in men [3]. While the exact mechanism of these changes remains largely unknown, ongoing work has postulated that genetic, epigenetic, environmental, or hormonal changes may account for some of the associations between male infertility and somatic health problems. Further, another work has demonstrated that male infertility may not only be a biomarker of a man's health but also of the health of his family [4, 5].

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4.2 Genetics, Epigenetics, and Environmental Factors

Associations between male infertility and somatic health issues may be related to underlying genetic abnormalities. As stated above, nearly 15% of the male genome is involved with reproduction [3]. Spermatogenesis is a complex process that involves approximately 2300 genes which regulate the development and maturation of germ cells [6]. Underlying genetic abnormalities are common in infertile men. Nearly 8% of men with severe oligospermia and approximately 20% of men with nonobstructive azoospermia will have chromosomal abnormalities detected during an infertility workup [7, 8]. Variations in published prevalence of genetic abnormalities in the infertile male population appear to be related to differences in patient selection or the composition of the specific study population, but it is clear that men with infertility have a significantly elevated risk of genetic abnormalities.

In a large Chinese study from 2017, it was found that 27.3% of men with nonobstructive azoospermia and 15.9% of men with severe oligospermia had underlying genetic abnormalities. As a comparison, only 1.3% of normozoospermic controls possessed an underlying genetic abnormality. Of the men with nonobstructive azoospermia, the most common genetic abnormalities were sex chromosomal abnormalities such as Klinefelter syndrome and Y chromosome deletions. Autosomal translocations and inversions were less common. Men with severe oligospermia demonstrated similarly low rates of autosomal translocations and inversions. Klinefelter syndrome or mosaic was also less common in men with severe oligospermia, with the majority of chromosomal abnormalities being Y chromosome deletions [9].

Although infertile men with underlying genetic abnormalities may be able to father offspring with the use of assisted reproductive technologies such as microdissection testicular sperm extraction (microTESE) and intracytoplasmic sperm injection (ICSI), the risk of passing genetic aberrations to future generations is increased [9, 10].

Men with infertility related to known genetic syndromes such as Klinefelter syndrome, Kallmann syndrome, sickle cell anemia, Kartagener's syndrome, myotonic dystrophy, Fanconi anemia, and beta thalassemia face many somatic health risks related to their specific disease processes [11]. Historically, infertility limited the natural transmission of genetic abnormalities to future generations. While the use of assisted reproductive technologies has made it possible for these men to have families, it has also necessitated the development of genetic screening protocols for partners as well as embryos. Recently, genomic microarray tools have been increasingly used and have been applied to the entire male genome to assist in identifying novel genetic causes of infertility [12]. This recent data has proposed possible genetic links between male infertility and common health problems such as diabetes and obesity [13, 14]. Patients with genetic problems such as Klinefelter, Prader-Willi, and Laurence-Moon-Bardet-Biedl syndrome generally display both obesity and infertility. The specific genes involved with these syndromes are well documented, but it is possible that other, less conspicuous genetic abnormalities may explain the common correlations between infertility, obesity, and diabetes [15]. Emerging data

continue to show a likely genetic link between infertility and numerous disease processes which may develop years later. Early evidence suggests that specific genetic pathways may link infertility to testicular cancer, prostate cancer, nonfatal stroke, nonfatal coronary heart disease, psychosexual dysfunction, and mood disorders [16]. For infertile men with a normal chromosomal analysis, the use of microarray may identify subtle genetic abnormalities causing both infertility and possible long-term health consequences.

The study of epigenetics has also attracted increasing attention in recent years. Spermatogenesis involves several genes which are regulated through epigenetic mechanisms, and epigenetic aberrations such as hypermethylation, histone remodeling, and chromatin modification in many of these genes have been associated with poor sperm quality and male infertility [17]. Epigenetic changes can be transmitted to future generations, often with negative health consequences. See Fig. 4.1 regarding the impact of lifestyle and epigenetic changes on somatic health and fer-

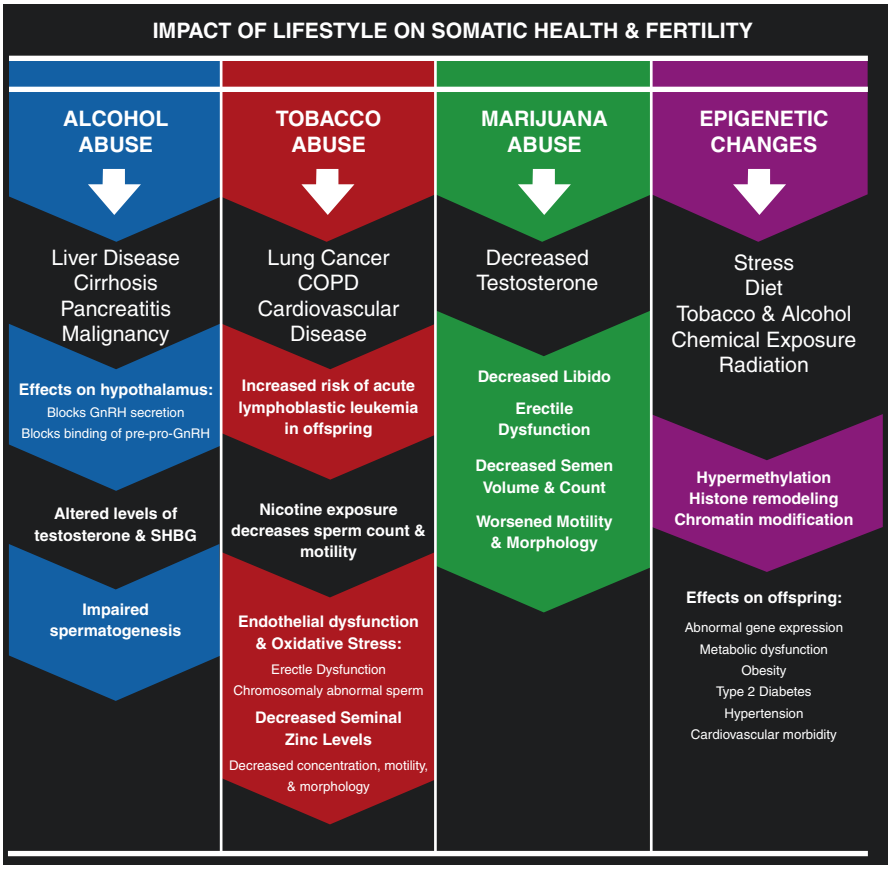

Fig. 4.1 The detrimental effects and proposed mechanistic roles of alcohol, tobacco, marijuana, and lifestyle on male fertility

tility. Paternal epigenetic changes may result in abnormal gene expression in children which could predispose offspring to metabolic abnormalities, obesity, type 2 diabetes, hypertension, and disruptions in body fat composition [18, 19]. Unlike genetic causes of infertility, many epigenetic causes of infertility are induced by lifestyle factors. Stress, physical activity, diet, alcohol intake, smoking, and sleep disturbances such as shift work have been associated with infertility in men and have been implicated with epigenetic changes [20, 21].

Specific environmental or occupational exposures may also result in epigenetic changes that impact future generations. Paternal exposure to pesticides may result in nervous system tumors in children, cigarette smoking may result in increased rates of childhood leukemia, and chemicals related to woodwork and wood processing may increase rates of leukemia in children [22–24]. Exposure to ionizing radiation may result in increased DNA methylation and impaired DNA repair processes which can lead to persistent instability of the male germ line [25].

Paternal diet may also play a role in gametogenesis. Human studies evaluating paternal obesity have demonstrated altered methylation in offspring, suggesting that developing sperm is susceptible to environmental insults related to diet. There is also some evidence that cardiovascular mortality in offspring may be related to epigenetic changes in the spermatozoa, although this effect may take multiple generations to manifest itself [26]. A recent comprehensive meta-regression analysis published in 2017 demonstrated a dramatic 50–60% decline in sperm counts in men in North America, Europe, Australia, and New Zealand between 1973 and 2011. While this downward trend in sperm counts has not been fully explained, lifestyle factors and epigenetic changes may play a large role in the overall reduction in male fertility in recent decades. Based on the epigenetic data discussed above, these factors may not only have negative consequences for male fertility and paternal health but may also negatively impact the health of future generations [27].

4.3 Mental Health and Substance Abuse

Certain associations have been documented between infertile men and mental health disorders. See Table 4.1 for details regarding key studies evaluating associations between male infertility and nonmalignant health risks. Male factor infertility appears to be associated with both depression and anxiety, although at relatively lower rates than in females who are diagnosed with infertility [28]. When evaluating the impact of male infertility on mental health, it is difficult to determine whether a cause and effect relationship exists since some men present at the time of the infertility diagnosis with known mental health issues and others will be diagnosed with mental health issues years later. Men with infertility undoubtedly experience a certain level of distress related to their diagnosis, and a portion of the mental health issues seen in infertile men may be explained by an emotional response to the infertility diagnosis. In a recent study, 51.8% of men reported a lack of willingness to discuss their reproductive problems with people other than their partner. A man's

Table 4.1 Key studies evaluating male infertility and nonmalignant health risks

Study author	Title	Year	Journal	Sample size	Main findings
Alvarez et al.	Do some addictions interfere with fertility?	2015	Fertil Steril	N/A (review article)	Tobacco abuse results in erectile dysfunction and chromosomal aberrations of sperm Sperm quality improves after 3 months of cessation 33% of chronic marijuana users have oligospermia Marijuana results in low libido, gynecomastia, erectile dysfunction Alcohol consumption >5 drinks per week has adverse effects on sperm with profound effects at >25 drinks per week Alcohol negatively impacts testosterone and SHBG
Babore et al.	Male factor infertility and lack of openness about infertility as risk factors for depressive symptoms in males undergoing assisted reproductive technology treatment in Italy	2017	Fertil Steril	340 participants (170 men and their female partners)	51.8% of males do not discuss ART treatments with people outside of their partners Male factor infertility increases depressive symptoms (Likert scale score 30.7 vs. 28.6) The decision to not share infertility experience was associated with higher levels of depressive symptoms (F1,162 = 5.115)
Calogero et al.	Klinefelter syndrome: cardiovascular abnormalities and metabolic disorders	2017	J Endocrinol Invest	Review of 56 publications	KS patients have increased risk of cerebrovascular disease (SMR 2.2), cardiovascular congenital anomalies (SMR 7.3), development of thrombosis and leg ulcers (SMR 7.9)[a] KS increases risk of obesity, metabolic syndrome, type 2 diabetes
Eisenberg et al.	Semen quality, infertility, and mortality in the USA	2014	Hum Reprod	11,935 men evaluated for infertility	Men with 2 or more abnormal semen parameters have a 2.3-fold higher risk of death compared to men with normal semen (95% CI 1.12–4.65)[b]
Eisenberg et al.	The relationship between male BMI and waist circumference on semen quality: data from the LIFE study	2014	Hum Reprod	501 couples attempting to conceive	Men in the highest category of waist circumference had a 22% lower total sperm count Linear association between higher BMI and low semen volume (<1.5 ml), low sperm concentration (<15 M/mL), and low total sperm count (<39 M) Waist circumference was correlated with low sperm concentration ($P = 0.025$) and low count ($P = 0.008$)

(continued)

Table 4.1 (continued)

Study author	Title	Year	Journal	Sample size	Main findings
Eisenberg et al.	Increased risk of incident medical conditions in infertile men: analysis of United States claims data	2016	Fertil Steril	13,027 men with male factor infertility	Men with infertility had a higher risk of developing diabetes (HR 1.30, 95% CI 1.10–1.53), ischemic heart disease (HR 1.48, 95% CI 1.19–1.84), alcohol abuse (HR 1.48, 95% CI 1.07–2.05), and drug abuse (1.67, 95% CI 1.06–2.63) compared with men who only received infertility testing[c]
Eisenberg et al.	Diabetes, medical comorbidities, and couple fecundity	2016	Hum Reprod	501 couples desiring pregnancy	Longer time to pregnancy for male partners with diabetes (0.35, 95% CI 0.14–0.86) Couples' medical comorbidity was associated with pregnancy status
Emanuele et al.	Alcohol and the male reproductive system	2001	Alcohol Res Health	Review of 30 publications	Alcohol is associated with low levels of hypothalamic LHRH and pituitary LH Alcohol use is associated with low testosterone and altered levels of additional reproductive hormones
Haring et al.	Prediction of metabolic syndrome by low serum testosterone levels in men: results from the study of health in Pomerania	2009	Diabetes	1004 men without baseline metabolic syndrome	Low testosterone predicted metabolic syndrome (RR 1.38 [95% CI 1.13–1.69]), particularly among men aged 20–39 years (2.06 [1.29–3.29]), even after adjustment for age, smoking, alcohol consumption, physical activity, waist circumference, self-related health, and time of blood sampling. DHEA-S levels were not related to incident MetS (0.99 [0.83–1.19])[d]
Jensen et al.	Habitual alcohol consumption associated with reduced semen quality and changes in reproductive hormones; a cross-sectional study among 1221 young Danish men	2014	BMJ Open	1221 young Danish men	Men with alcohol use >40 drinks per week had a 33% (95% CI 11–59%) reduction in sperm concentration compared to men with an intake of 1–5 drinks/week. A significant increase in serum-free testosterone was noted with increasing alcohol consumption

Katib et al.	Mechanisms linking obesity to male infertility	2015	Cent Eur J Urol	N/A (review article)	The prime hormonal defect in obese men is hypotestosteronemia, which results in impaired spermatogenesis leading to poor fecundability
Kovac et al.	The effects of cigarette smoking on male fertility	2015	Postgrad Med	Review of 31 publications	Men who smoked >20 cigarettes per day had a 19% reduction in sperm concentration compared with nonsmokers, even after controlling for other factors Smoking was associated with decreases in sperm density (15.3%), total sperm counts (17.5%), and total motile sperm (16.6%) compared with nonsmokers Morphology and volume were slightly affected by smoking
Krajewska-Kulak et al.	Thyroid function in male infertility	2013	Front Endocrinol	N/A (review article)	Hypothyroidism decreases SHBG, total serum testosterone, LH, and FSH Hyperthyroidism can lead to infertility through elevated levels of testosterone, LH, and FSH
Kulej-Lyko et al.	Could gonadal and adrenal androgen deficiencies contribute to the depressive symptoms in men with systolic heart failure	2016	Aging Male	226 men	Testosterone deficiency was related to higher prevalence of mild depression (OR = 3.6, 95% CI 1.2–10.63)
Kupelian et al.	Low sex hormone-binding globulin, total testosterone, and symptomatic androgen deficiency are associated with development of the metabolic syndrome in nonobese men	2006	J Clin Endocrinol Metab	1709 men	Lower levels of total testosterone and SHBG were predictive of metabolic syndrome, particularly in men with a BMI below 25 kg/m^2 with adjusted RRs for a decrease in 1 sd of 1.41 (95% CI, 1.06–1.87) and 1.65 (95% CI, 1.12–2.42)

(continued)

Table 4.1 (continued)

Study author	Title	Year	Journal	Sample size	Main findings
Liu et al.	Lower SHBG level is associated with higher leptin and lower adiponectin levels as well as metabolic syndrome, independent of testosterone	2017	Sci Rep	614 Taiwanese men	Subjects in the lowest quartile of testosterone and SHBG are exposed to a 1.58 and 3.22 times risk of developing metabolic syndrome compared to men in the highest quartile of testosterone and SHBG levels SHBG served as a major predictor for the risk of metabolic syndrome and was correlated with serum adiponectin and leptin levels
Liu et al.	Seminal plasma zinc level may be associated with the effect of cigarette smoking on sperm parameters	2010	J Int Med Res	79 nonsmokers, 68 smokers	Seminal plasma zinc concentrations were found to be significantly lower in smokers than in nonsmokers (1.84 vs. 2.38 mmol/L, $P < 0.05$) Concentration, motility, and morphology were found to be significantly lower among smokers than nonsmokers (58.6 vs. 75.7×10^6/mL, 10.4% vs. 16.6%, 8.2% vs. 12.5%)
Nguyen et al.	Men's body mass index and infertility	2007	Hum Reprod	26,303 couples	Increased infertility with increased BMI. OR for infertility was 1.20 for overweight men [BMI 25–29.9; 95% CI 1.04–1.38] and 1.36 for obese men (BMI 30–34.9; 95% CI 1.13–1.63) relative to men with low-normal BMI (20.0–22.4)[e]
Omani-Samani et al.	Evaluation on hope and psychological symptoms in infertile couples undergoing assisted reproduction treatment	2017	Int J Fertil Steril	180 infertile couples	Infertility is associated with increased anxiety, depression, and stress
Park et al.	Cannabis, cannabinoids, and reproduction	2004	Prostaglandins Leukot Essent Fatty Acids	N/A (review article)	Marijuana smoking decreases serum LH when compared to hormone levels in nonsmoking controls. Chronic marijuana use is associated with decreased plasma testosterone
Patel et al.	Thyroid dysfunction and male reproductive physiology	2016	Semin Reprod Med	N/A (review article)	Hyperthyroidism leads to decreased free testosterone (1.7 ng/mL vs. 3.1 ng/mL) After treatment for 12 months, 85% of males with seminal alterations had normalization of semen quality

Ramlau-Hansen et al.	Subfecundity in overweight and obese couples	2007	Hum Reprod	47,835 couples	In men with a BMI of 18.5 kg/m² or more, there is a dose-response relationship between increasing BMI and subfecundity (time to pregnancy of 12+ months): OR 1.19 (95% CI 1.14–1.24)
Rojdmark et al.	Hypothalamic-pituitary-testicular axis in patients with hyperthyroidism	1988	Horm Res	8 hyperthyroid men	Chronic hyperthyroidism makes the pituitary hypersensitive to exogenous GnRH LH and FSH responsiveness to GnRH was significantly larger in the thyrotoxic state compared with the euthyroid state (LH incremental areas, 3999 +/− 665 vs. 2640 +/− 430, $p < 0.02$; FSH incremental areas, 825 +/− 193 vs. 542 +/− 98, $p < 0.05$)
Sahin et al.	Psychologic and sexual dysfunction in primary and secondary infertile male patients	2017	Arch Ital Urol Androl	70 infertile men	Depression and erectile dysfunction seen in the patients with secondary infertility were significantly higher than the patients with primary infertility A statistically significant difference was detected between groups for Beck depression inventory scores ($p = 0.015$; $p < 0.05$)
Sallmen et al.	Reduced fertility among overweight and obese men	2006	Epidemiology	1329 men	A 3-unit increase in BMI was associated with infertility OR 1.12; 95% CI 1.01–1.25
Verze et al.	The link between cigarette smoking and erectile dysfunction: a systematic review	2015	Eur Urol Focus	Review of 13 publications	Smoking-related erectile dysfunction is mainly associated with endothelial impairment, reduction in nitric oxide availability, and imbalance between oxidative and antioxidative reactions increasing oxidative stress. Passive secondhand cigarette smoking negatively impacts erectile function
Wdowiak et al.	Impact of emotional disorders on semen quality in men treated for infertility	2017	Neuro Endocrinol Lett	112 subfertile males	Higher Beck depression inventory scores were correlated with significantly decreased testosterone ($p = 0.001$) and increased prolactin and cortisol ($p < 0.001$). Sperm count and volume were correlated with BDI scores

[a]*SMR* standardized mortality ratio
[b]*CI* confidence interval
[c]*HR* hazard ratio
[d]*RR* risk ratio
[e]*OR* odds ratio

decision to not discuss his infertility with other individuals was associated with increased depressive symptoms based on self-reported questionnaires [29]. However, there may be underlying differences in infertile men that predispose them to mental health diagnoses independent of the emotional stress of an infertility diagnosis, the process of achieving a pregnancy, and the emotionally taxing nature of infertility treatments. See Fig. 4.2 for details regarding male infertility and disease processes.

Infertile men have been shown to have lower secretion of sex hormone-binding globulin (SHBG) and dehydroepiandrosterone sulfate (DHEA-S), significantly decreased testosterone levels, as well as elevated secretion of cortisol and prolactin [30]. These hormonal abnormalities appear to result in decreased semen volume and

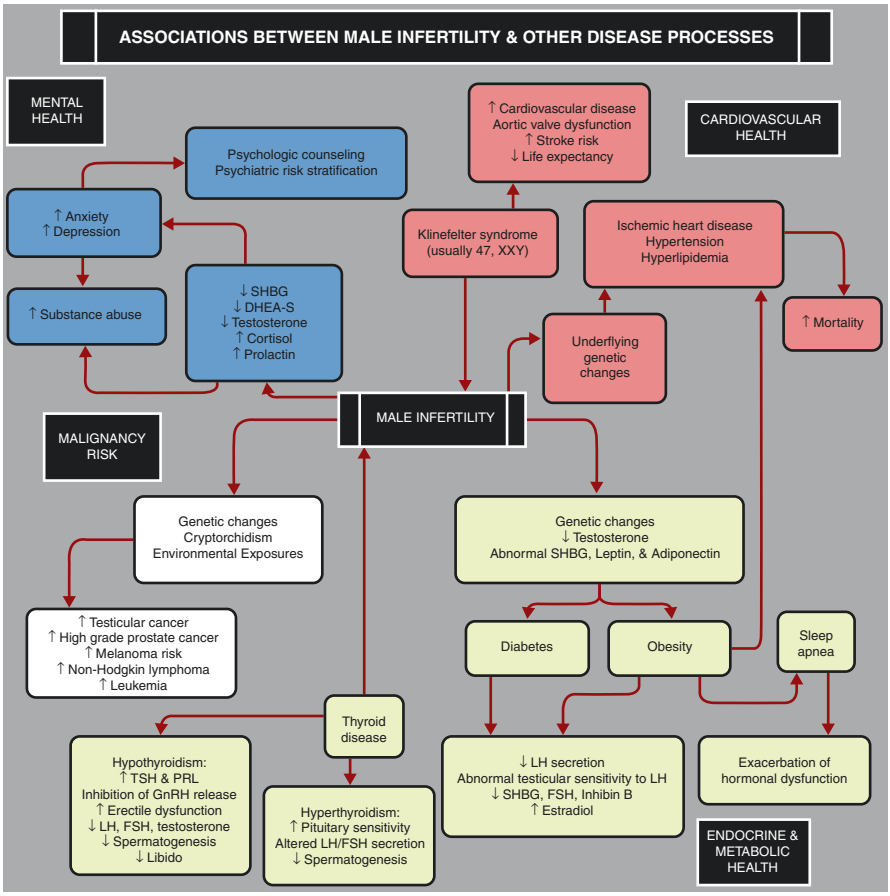

Fig. 4.2 The associations between male infertility and various aspects of somatic health, including cardiovascular disease, endocrine and metabolic derangement, malignancy risk, and mental illness. *SHBG* sex hormone-binding globulin, *FSH* follicle-stimulating hormone, *LH* luteinizing hormone, *TSH* thyroid stimulating hormone, *PRL* prolactin, *GnRH* gonadotropin-releasing hormone, *DHEA-S* dehydroepiandrosterone sulfate

sperm density, but they have also been associated with increased rates of anxiety and depression. Overall, a higher incidence of depression appears to exist in infertile men when compared to the general population, and men who are diagnosed with secondary infertility have been found to have significantly higher rates of depression than men with primary infertility [31]. Depression and anxiety in otherwise healthy individuals who are diagnosed with male factor infertility may be the result of hormonal deficiencies in testosterone and DHEA-S rather than simply a response to the distress of the diagnosis [32]. Psychological counseling sessions may be beneficial in addressing the mental health needs of male patients who are diagnosed with infertility, although there has traditionally been a reluctance among men to seek out mental health resources [2, 29, 33].

It has been demonstrated that older men with infertility may be more likely to have children diagnosed with autism, schizophrenia, and bipolar disorder, particularly in the setting of conception achieved through intracytoplasmic sperm injection (ICSI) [34, 35]. The use of genetic sequencing technologies has highlighted associations between male factor infertility, particularly in men of advanced age, and mental health consequences in offspring [36]. However, there has been virtually nothing published regarding rates of these psychiatric disorders in the infertile men themselves. To date, it remains unknown whether rates of autism, schizophrenia, and bipolar disorder are different in the infertile population than the general male population. Studies evaluating the likelihood of developing these psychiatric illnesses among infertile men would be beneficial in determining future risk to patients since an association has been demonstrated in future generations.

In addition to depression and anxiety, substance abuse disorders appear to be more common in men who are diagnosed with infertility. Based on US claims data, both alcohol abuse and drug abuse appear to be elevated (HR 1.48 and 1.67, respectively) in men with infertility compared to men who underwent infertility testing but had normal semen analyses [37]. See Fig. 4.1 for the impact of lifestyle on somatic health and fertility. A clear explanation for the increased risk of drug and alcohol abuse among infertile men is lacking in the literature. The association between alcohol abuse and male infertility has been evaluated, with studies demonstrating that alcohol acts on the hypothalamus and blocks the secretion of gonadotropin-releasing hormone (GnRH) as well as the binding of the GnRH precursor pre-pro-GnRH to the functionally active GnRH hormone, resulting in impaired spermatogenesis [38]. Even modest alcohol consumption of more than five drinks per week has been shown to negatively impact sperm quality, although the most significant impact on semen analysis parameters is seen with heavy drinking of greater than 25 drinks per week [39]. Alcohol consumption in men has also been linked to changes in testosterone and SHBG levels, so identification of infertile men who use alcohol is an important aspect of a comprehensive male infertility workup [40]. Lifestyle modifications among infertile men who consume alcohol may be warranted to improve sperm quality, but interventions or recommendations to prevent the future development of alcohol abuse in patients with male factor infertility are an area which remains uninvestigated. Since infertile men have a higher likelihood to develop alcohol abuse in the future, further studies that provide insight into the mechanistic link between male infertility and predisposition to these risks are vital.

Men who use tobacco are known to have higher rates of erectile dysfunction and increased levels of chromosomally abnormal sperm, and smoking rates have been shown to be higher in infertile men than the general population [37]. Additionally, the offspring of men who smoke near the time of conception has an elevated risk of developing acute lymphoblastic leukemia in the future (OR 1.1) [24]. A mechanistic explanation for the association between smoking and male infertility may be related to lower seminal zinc levels observed in smokers when compared to non-smokers. Lower seminal zinc levels appear to be associated with decreased sperm concentration, motility, and morphology [41]. There is also some evidence that nicotine itself, rather than the additional toxins associated with cigarettes, may lead to adverse effects on fertility. In animal models, exposure to oral nicotine has been associated with decreased sperm motility and sperm count [42]. Cigarette smoking is a well-established risk factor for erectile dysfunction, with endothelial dysfunction and increased oxidative stress representing the primary pathophysiologic mechanisms for this association [43]. 21.6% of American men smoke cigarettes, and it is clear that smoking has a detrimental effect on male fertility [44]. Smoking cessation in men can lead to improvements in semen parameters in as little as 3 months, so tobacco habits should be addressed in all men who are diagnosed with infertility [40]. The increased use of tobacco among infertile men predisposes this population to the known health consequences of smoking such as emphysema, lung cancer, COPD, and cardiovascular disease. Tobacco cessation in the infertile population may lead to both improvements in reproductive health as well as overall somatic health.

Additionally, men who abuse marijuana have been found to have decreased libido, increased rates of erectile dysfunction, decreased semen volume, decreased sperm count, and higher rates of abnormal sperm morphology and motility. Marijuana also decreases the production of testosterone, and more than 33% of men who abuse marijuana on a regular basis will present with oligospermia [45]. While associations between male factor infertility and other drugs of abuse have been published, large studies often lack details about specific substances being abused and the frequency of use. The fertility impact of many recreational drugs is not known, but thorough patient counseling about possible risks and avoidance of addictive substances is prudent.

4.4 Cardiovascular Health

Both congenital and acquired causes of male infertility have been associated with an increased risk of cardiovascular disease. One of the most common congenital causes of male factor infertility is Klinefelter syndrome, affecting approximately 1 in 1000 males. This disease process results in an overall lower life expectancy and is associated with an increased risk of cerebrovascular disease or stroke (standardized mortality ratio (SMR), 2.2) and cardiovascular congenital anomalies such as aortic valve disease (SMR 7.3) [46]. Cardiovascular health for this specific patient

population appears to be improved with long-term testosterone replacement therapy, which may also be beneficial for psychosocial morbidity and behavioral problems seen in patients with Klinefelter syndrome [46, 47].

Acquired or unexplained male factor infertility is more commonly encountered than congenital infertility in the clinical setting, with approximately 40–50% of male infertility cases being classified as idiopathic or unexplained [48]. Despite the frequent lack of an identifiable cause for infertility, infertile men as a whole still appear to be at an elevated risk for the development of cardiovascular disease later in life. When compared to previously fertile men who had undergone vasectomy, men diagnosed with infertility were at a higher risk to develop ischemic heart disease (HR 1.41), hypertension (HR 1.09), and hyperlipidemia (HR 1.14) [37]. Associations between infertility and obesity as well as higher smoking rates in infertile men appear to provide the most plausible explanations for the association between male factor infertility and relatively poorer cardiovascular health.

Increasing rates of obesity in the United States and other developed nations have coincided with rising rates of poor sperm quality and male infertility [49]. In the United States, rates of obesity among men of reproductive age have tripled since the 1970s, with recent data estimating an obesity rate of 33.9% for individuals over age 20 [19]. There appears to be a direct relationship between increasing body mass index (BMI) and higher rates of male factor infertility. This relationship has been reproduced in multiple studies [50–52]. See Table 4.1 for key studies evaluating associations between male infertility and nonmalignant health risks. Increases in BMI and waist circumference have been shown to have a linear, negative impact on semen volume, concentration, and sperm count [53]. The mechanisms by which obesity results in infertility are complex and incompletely understood, but it is known that obesity can negatively impact the secretion of luteinizing hormone (LH) from the pituitary gland as well as testicular sensitivity to LH. Reductions in SHBG, follicle-stimulating hormone (FSH), and inhibin B and elevations in estradiol (E2) can all result in decreased sperm quality. Hormonal changes seen in obese men may be exacerbated by comorbidities such as sleep apnea and diabetes, which are frequently observed together [19].

While the hormonal changes seen in obesity may result in infertility for many men, there is emerging evidence that infertility itself may actually cause or worsen obesity. Low testosterone levels may induce changes that result in the metabolic syndrome, leading to increased risk of obesity in the future [14]. A German study from 2009 demonstrated that in men who did not meet criteria for the metabolic syndrome, low levels of testosterone were predictive of a subsequent diagnosis of the metabolic syndrome during a 5-year follow-up period [54]. In a 2017 study from Taiwan, men with low serum testosterone and low SHBG levels were found to have a 1.58–3.22 times increased risk of developing the metabolic syndrome in the future when compared to men with higher levels of testosterone and SHBG [55]. This study also demonstrated that lower SHBG levels were associated with higher leptin levels and lower adiponectin levels, which can both exacerbate weight gain [55–57]. Similarly, a 2006 study from Massachusetts demonstrated that low serum SHBG, low total testosterone, and androgen deficiency are associated with an increased risk

for the development of metabolic syndrome in the future. This increased risk was seen in men of normal BMI (less than 25) at the time of initial presentation. Low testosterone, low SHBG, and male infertility in general may be early warning signs for future risk of obesity in men who are not obese at the time of their infertility diagnosis [58].

Overall, men with infertility appear to have higher mortality than the general population, with a 2.3-fold increased risk of death compared to men with normal semen analyses [59]. While increased mortality cannot be entirely attributed to cardiovascular health consequences, the effects of obesity, ischemic heart disease, and cardiovascular disease should not be overlooked since they play a significant role in the general health status of infertile men. It is vital to recognize that infertility may be the primary marker in a man's life for these future health complications and the hormonal changes related to infertility may be the underlying cause for subsequent health issues.

4.5 Endocrine Dysfunction

Male infertility can also result in significant endocrine abnormalities [3]. Men with impaired semen parameters as well as ejaculatory and erectile dysfunction are frequently diagnosed with concomitant diabetes [60, 61]. As discussed previously, infertility can result in abnormalities in serum testosterone, SHBG, leptin, and adiponectin which predispose patients to insulin resistance and metabolic syndrome [55–57]. See Table 4.1 for a list of key studies evaluating associations between infertility and nonmalignant health risks. The insulin resistance that is a component of the metabolic syndrome greatly increases a man's risk of developing diabetes. Patients frequently present with both diabetes and obesity, which often makes it difficult to fully characterize the reproductive impact of these two disease processes independently. In the United States, 3.4% of the general population is diagnosed with both diabetes and obesity [62]. Infertility can lead to a positive feedback loop in which endocrine abnormalities related to infertility worsen diabetes and obesity, which subsequently worsen semen parameters. This cycle appears to result in reduced synthesis of SHBG in the liver, resulting in elevations in free testosterone and subsequent downregulation of the androgenic axis. While data are somewhat conflicting, this appears to result in further decreased fecundity and semen quality [19, 63].

Because of the endocrine abnormalities described above, men with infertility and no prior diagnosis of diabetes are at an elevated risk to develop diabetes later in life. A large prospective cohort study published in 2017 evaluated 39,516 men without diabetes who were undergoing IVF for male factor infertility and found that with a median follow-up time of 5.6 years, 1.6% of subjects were ultimately diagnosed with diabetes during the study period (HR 1.45) [13]. These findings indicated that compared to men with normal semen analyses or sterilized men, men with male factor infertility were at a statistically significant increased risk to subsequently

develop diabetes. Implementation of diabetes screening in men who are diagnosed with infertility and long-term follow-up appear to be important methods to provide appropriate care for this group of patients.

In addition to diabetes, proper thyroid function appears to be important for the maintenance of male reproductive health. Infertility in men may be a marker of underlying thyroid pathology. In initial studies, there was felt to be little association between male fertility and the thyroid [64]. More recently, multiple studies have shown that abnormal semen analysis parameters and poor male sexual function may be a sign of improper thyroid function [65]. Both hyperthyroidism and hypothyroidism have been associated with problems related to male fertility. Chronic hyperthyroidism may result in a state of pituitary hypersensitivity which can impact LH and FSH secretion as well as testicular function [66]. In an opposite manner, hypothyroidism may result in increased secretion of TSH and prolactin. Increased prolactin can inhibit the release of GnRH which results in a decrease in LH and FSH. Decreases in LH and FSH can negatively impact testosterone levels and spermatogenesis, resulting in loss of libido, erectile dysfunction, and infertility [65]. In order to appropriately screen men with infertility for thyroid disease, the American Society for Reproductive Medicine recommends measurement of the thyroid-stimulating hormone (TSH) in men undergoing a thorough endocrine evaluation [1].

4.6 Malignancy

Many early studies evaluating the risk of male factor infertility on long-term health focused on a man's risk of developing cancer. The most well-documented association between infertility and cancer is that of testicular germ cell tumors, but men with infertility may also be at an increased risk to develop prostate cancer, melanoma, non-Hodgkin's lymphoma, and other types of malignancy [67]. See Table 4.2 for details regarding key studies evaluating a link between male infertility and malignancy. Multiple studies have uniformly shown that men with abnormal semen analysis parameters are at increased risk to develop testicular cancer. A retrospective study from Cornell evaluating 3800 men with abnormal semen analyses demonstrated that the study population had a significantly elevated risk for a subsequent diagnosis of testicular cancer following their infertility diagnosis (standardized incidence ratio (SIR) 18.3) [68]. Similarly, a large Danish cohort study of 32,442 men undergoing semen analysis documented statistically significant elevations in risk of testicular germ cell tumors in men with abnormal semen analyses compared to the general population (SIR 1.6) [69]. A large cohort study linking 15 fertility centers in California to the California Cancer Registry also reported that men with male factor infertility were 2.8 times more likely to develop testicular cancer than the general male population [70]. A population-based study in Utah showed increased rates of testicular cancer in men with oligospermia (HR 11.9), reduced sperm motility (HR 1.3), and lowest quartile morphology (HR 4.2) compared to fertile controls [71]. This strong association is important in the counseling and screening of patients with male factor infertility.

Table 4.2 Key studies evaluating male infertility and malignancy

Study author	Title	Year	Journal	Sample size	Main findings
Eisenberg et al.	Increased risk of cancer in infertile men: analysis of US claims data	2015	J Urol	76,083 infertile men	Infertile men had a 49% higher risk of all cancers compared to controls and a twofold higher risk of testicular cancer The risks of melanoma, prostate cancer, bladder cancer, thyroid cancer, HL, NHL, and leukemia were all higher in infertile men
Hanson et al.	Subfertility increases risk of testicular cancer	2016	Fertil Steril	20,433 men undergoing semen analysis	Men undergoing semen analysis have increased risk of testicular cancer (HR 3.3). Increased risk of testicular cancer in men with oligozoospermia based on concentration (HR = 11.9) and sperm count (HR = 10.3). Men in the lowest quartile of motility (HR = 4.1), viability (HR = 6.6), morphology (HR = 4.2), or total motile count (HR = 6.9) have higher risk of testicular cancer
Jacobsen et al.	Risk of testicular cancer in men with abnormal semen characteristics: cohort study	2000	BMJ	32,442 men undergoing semen analysis	Elevated risk of testicular cancer with low semen concentration (SIR 2.3), poor motility (SIR 2.5), and high proportion of abnormal morphology (SIR 3.0)[a]
Jorgensen et al.	Fatherhood status and prostate cancer risk	2008	Cancer	All men born in Denmark 1935–1988	Childless men were found to be at a 16% reduced risk of prostate cancer compared with fathers (rate ratio [RR] 0.84; 95% CI 0.73–0.95)
Raman et al.	Increased incidence of testicular cancer in men presenting with infertility and abnormal semen analysis	2005	J Urol	3800 men with infertility	SIR of testicular cancer was 22.9 (95% CI 22.4–23.5) when comparing the infertile group to the control population
Rogers et al.	Male infertility and risk of cancer	2017	Semin Reprod Med	N/A (review article)	Elevated risk of testicular germ cell tumors, prostate cancer, melanoma, non-Hodgkin's lymphoma in infertile men

Venn et al.	Cancer risks associated with the diagnosis of infertility	2003	Best Pract Res Clin Obstet Gynaecol	Review of six publications	The strongest and most widely recognized risk of testicular cancer is cryptorchidism, associated with RRs of 4–9 Increased risk of testicular cancer in men with reduced fertility
Walsh et al.	Increased risk of testicular germ cell cancer among infertile men	2009	Arch Intern Med	22,562 male partners	Men seeking infertility treatment had an increased risk of testicular cancer (SIR 1.3; 95% CI 0.9–1.9). Markedly higher risk among men with known male factor infertility (2.8; 1.5–4.8). In multivariable analysis, men with male factor infertility were 3 times more likely to develop testicular cancer compared with those without (HR, 2.8; 95% CI 1.3–6.0)[b]
Walsh et al.	Increased risk of high-grade prostate cancer among infertile men	2010	Cancer	22,562 men evaluated for infertility	Male factor infertility patients were found to be 2.6 times more likely to be diagnosed with high-grade prostate cancer (hazard ratio, 2.6; 95% CI, 1.4–4.8)
Walsh et al.	Male reproductive health and prostate cancer risk	2011	Curr Opin Urol	N/A (review article)	Studies evaluating male infertility and prostate cancer are inconsistent. Despite this, there is an association between reproductive health in a man's fourth decade (30s) and his development of aggressive prostate cancer in his sixth decade (50s)
Wiren et al.	Fatherhood status and risk of prostate cancer: nationwide, population-based case-control study	2013	Int J Cancer	117,328 prostate cancer patients	Childless men had a decreased risk of prostate cancer compared to fathers, OR 0.83 (95% CI 0.82–0.84), and risk was lower for low-risk prostate cancer, OR = 0.74 (95% CI = 0.72–0.77), than for metastatic prostate cancer, OR = 0.93 (95% CI = 0.90–0.97)

[a]*SIR* standardized incidence ratio
[b]*HR* hazard ratio

The risk of prostate cancer may also be elevated in men with infertility, although publications related to this topic are somewhat conflicting. A large population-based study of 20,433 men in Utah did not demonstrate an association between male factor infertility and prostate cancer [71]. Some European studies have shown decreased rates of prostate cancer in childless men, but these studies are limited by the fact that childless men are not necessarily infertile [72, 73]. On the other hand, a California study of 22,562 men with infertility found that male factor infertility was associated with an increased risk of developing high-grade prostate cancer (SIR 2.0) [74]. Additionally, a study evaluating 76,083 infertile men based on claims data also found an increased risk of prostate cancer associated with male factor infertility [75]. A clear explanation for the possible association between male infertility and prostate cancer is lacking, although reproductive disorders such as cryptorchidism, environmental factors, abnormal hormonal function, and genetics may be involved with the development of both infertility and prostate cancer [76].

The literature is relatively lacking regarding associations between male infertility and other types of malignancy, although there appears to be a positive correlation between abnormal semen analysis parameters and melanoma (1.37), leukemia (HR 1.82), and non-Hodgkin's lymphoma (HR 1.76) [67, 75]. When evaluated as a whole, infertile men appear to be at an elevated lifetime risk of malignancy.

4.7 Conclusions

The relationship between male reproductive health and overall somatic health remains complex and largely poorly understood. Numerous environmental, genetic, and lifestyle factors likely influence a man's overall health as well as his ability to reproduce. However, it is clear that men with infertility may be predisposed to develop other health conditions which may not be present at the time of their infertility diagnosis. Using infertility as a marker of future health can allow for appropriate health screening, detailed counseling of risks, and long-term follow-up for men with infertility. As men delay fatherhood and as problems such as obesity, diabetes, and infertility become more common, healthcare providers should be aware of the specific risks that patients face to provide the most up-to-date information and provide the most appropriate interventions for patients.

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Chapter 5
Fertility Preservation in the Male Adolescent Patient

Ron Golan and James A. Kashanian

5.1 Introduction

Adolescence is a period of time prior to early adulthood that encompasses a child's physical, intellectual, and social development throughout and beyond puberty. During this time, the child undergoes significant physical developments which include growth and development of secondary sexual characteristics. The child also matures cognitively, socially, and emotionally, developing an evolving sense of identity. A diagnosis of cancer during this critical point in development can and will have significant and long-lasting effects on that person's development.

Over 15,000 children will develop cancer in the United States (US) each year [1] with a 5-year survival rate for childhood cancer being roughly 80% [2]. This extrapolates to over 385,000 childhood cancer survivors living in the United States [3]. With a growing number of childhood cancer survivors, oncologic counseling has seen a shift in focus from concentrating solely on short- and long-term survival benefits of treatment regimens to survivorship and long-term effects of treatments. With this shifting paradigm, short- and long-term gonadotoxic effects of cancer treatment have become a major concern for clinicians treating and patients diagnosed with malignancies. Because of this, fertility preservation (FP) among adolescent and young adult (AYA) cancer patients has become a significant area of interest and research.

Although fertility preservation in AYA patients has become a relatively new concept in coordinated medical care, the American Society of Clinical Oncology (ASCO) has recommended education and informed consent regarding cancer treatment and infertility for over the past decade. In its newest, updated recommendations, published in 2013, ASCO recommends that all patients should be counseled

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on the possibility of infertility due to oncology treatments and that clinicians should be either able to discuss fertility preservation options or offer early referral to reproductive specialists [4]. Similarly, the American Society for Reproductive Medicine (ASRM) ethics committee recommends all physicians should inform cancer patients about options for fertility preservation and future reproduction prior to treatment and only offer established methods of fertility preservation [5]. Being that the only reliable method of fertility preservation in male cancer patients is sperm cryopreservation with subsequent use with assisted reproductive techniques [6–8], all committees agree that experimental procedures should be offered only in a research setting with IRB oversight.

With the growing number of FP or oncofertility programs nationwide, the seamless coordination and integration of fertility and oncologic care have become a cornerstone with timely diagnosis and treatment. In settings where formalized programs have been established, FP discussions are much more likely to occur [9]. Possibly the most important aspect of treating AYA patients is the aspect of a full-disclosure informed consent regarding all aspects of treatment outcomes and effects. When asking cancer survivors to look back on their treatment, one of the biggest regrets they have is not being offered or not fully discussing effects of treatment on fertility and options for fertility preservation [10]. Because of this, fertility preservation should be incorporated into all patient treatment plans [11]. Counseling should also include the expectation of a high likelihood of morbidity in childhood cancer survivors including impaired cognitive function, limitations in activity, pain, and anxiety [3]. There are the additional concerns among AYA cancer survivors that cancer treatment may impact future romantic relationships and sexual function [12]. Since post-therapy fertility impairment is variable and grossly unpredictable, the best that can be done is proper counseling and planning in anticipation.

In this chapter, we will discuss FP management options, outcomes, and expectations in the AYA patient population. Questions that will be addressed include barriers to FP, attitudes and perspectives of cancer survivors, short- and long-term effects of cancer treatment on spermatogenesis, quality of sperm in the setting of localized or systemic malignancies, optimal timing for FP, conventional and experimental options for FP, and utilization of sperm banked. These topics among others will be covered throughout.

5.2 Barriers to Fertility Preservation

Even with the aforementioned increase in FP programs nationwide, many barriers still exist to fertility preservation in the AYA population leading to delayed or lack of proper fertility risk counseling. In one study, less than half of pediatric patients receiving hematopoietic stem cell transplantation, known to be among the highest risk to fertility impairment, received fertility preservation counseling, and less than one-third received a FP procedure [13]. One reason for this may be the lack of formal training in FP for health-care providers. Fuchs et al. reported that 93%

of pediatric oncology providers received no formal training on FP, though 84% of all providers reported that formal training on the topic would be useful [14]. In another study, 91% of all pediatric endocrinologists surveyed routinely saw patients at risk for infertility, but only 36% felt adequately trained in fertility and 25% in sexual function [15].

Beyond proper training, pediatric and adolescent oncology providers often report at least one additional barrier to fertility preservation [16]. The most common barriers are related to patient characteristics: age, health status, and urgency of cancer treatment [16]. Furthermore, discussion of infertility risk is much less likely to occur in patients with metastatic disease, those undergoing low-risk chemotherapy, and in certain cancer types [9]. Another common reason for the absence of FP counseling is the lack of time [17]. Other barriers to fertility preservation include cost of cryopreservation, lack of proper counseling by a qualified provider [18], and lack of access to a fertility specialist [16]. Expectantly, but not appropriately, those who already have children are also less likely to be counseled on fertility preservation [9].

In order to overcome these barriers, systems can be put in place to ensure clinicians can access appropriate channels to offer FP treatments to their AYA cancer patients [9]. Being that recent research on the topic has helped elucidate areas of needed improvement, improved quality and integration of care have ensued. For instance, when a multidisciplinary team was put in place, 85% of AYA patients attempted to bank sperm [19], and 78% were successful in providing a sample [19]. In addition, since there may be knowledge gaps regarding FP treatments among nurses and allied health-care professionals [16], web-based curriculums have been developed to help build knowledge base and communication skills [20]. Educational pamphlets have been developed by various organizations for patients that may tend to prefer verbal and written information on fertility preservation over videos and in-class education [21]. Future work should continue to explore barriers and define actionable interventions clinicians can take during fertility discussions.

5.3 Patient and Parent Concerns Regarding Fertility Preservation

The decision process necessary to pursue FP in the pediatric and adolescent population is unique from that of the adult population. Key concepts unique to this population include adolescent involvement in decision-making and ethical considerations in this population [22]. In a systematic review of adolescent, parent, and provider perspective and experiences regarding fertility preservation, four common themes were encountered [23]: first, the notion of "fertility in trust" whereby patients and parents put their trust in providers to supply them with relevant information and recommendations regarding FP and to keep future options for fertility open. Second, there are decision-making challenges that occur at the onset of a cancer diagnosis including the sensitivity of fertility discussions and balancing FP with the time

constraints for treatment. Two other large themes encountered include gaps in provider knowledge and discrepancies between patient and parent expectations of FP counseling and actual experiences. Focus groups of adult survivors of pediatric cancers and their parents also revealed concerns regarding long-term treatment effects and a retrospective desire for fertility concerns to have been addressed with input from experts in fertility preservation [10]. Other common concerns for young cancer survivors include confusion about their fertility potential posttreatment [24]. Moreover, young cancer survivors are concerned about how possible infertility may affect future relationships and self-esteem [24].

In contrast to adult cancer patients, treatment of AYA males requires a much more coordinated attempt on the part of the treating provider, primary caretaker, and patient. Additionally, parents need to take more of a leadership role, which may be facilitated through the guidance of providers. Because parents may feel overwhelmed by the diagnosis and treatment decisions, they believe it is the responsibility of the treating physician to broach the topic of FP [14]. Parents are also unsure about when it is developmentally appropriate to discuss fertility with their children [24].

Implementation of adolescent and young adult services, which address these concerns, significantly increases rates of FP discussions. This has been especially instrumental in AYA patients with leukemia and lymphoma [9]. Provider comfort and knowledge regarding fertility preservation significantly increases sperm collection attempts in adolescents. Furthermore, adolescents who speak to a fertility specialist are five times more likely to successfully bank sperm [25]. Leading to the conclusion that increased training in provider communication regarding fertility preservation will significantly increase the proportion of at-risk males who preserve fertility prior to receiving gonadotoxic treatment [14, 25].

5.4 Semen Characteristics and Utilization Among Oncology Patients

Sperm cryopreservation is the only proven means for male fertility preservation at this time. Sperm banking via ejaculation occurs most commonly in patients diagnosed with testicular cancer, leukemia, and lymphoma, and most cancer survivors continue storage of banked sperm [26, 27]. Because chemotherapy can easily penetrate the blood-testis barrier, replicating cells with high mitotic activity, i.e., spermatogonial stem cells, are at greatest risk for damage by chemotherapeutic agents [28]. For this reason, semen cryopreservation prior to initiation of any gonadotoxic treatment with one to three sample collections is recommended [8].

The rapid increase in spermatogonial density and testicular volume associated with increasing gonadotropin levels starts at an average age of 11 years old (yo) [29]. However, when assessing one's ability to produce a semen specimen, the focus should be on Tanner stage and not on chronological age [19]. In a study from Children's Hospital of Philadelphia assessing semen quality in AYA patients, 64.5%, 80.5%, and 90.3% of patients had sperm present on semen analysis and successfully

cryopreserved sperm in age groups 11–14 yo, 14–17 yo, and 17–30 yo, respectively [19]. The average sperm concentration was 20.0, 33.8, and 40.0 million sperm/mL in age groups 11–14 yo, 14–17 yo, and 17–30 yo, respectively [19]. In the study, 16.9% of patients were azoospermic [19]. It has been suggested that overall tumor burden and tumor stage can negatively affect bulk semen parameters as well [30, 31]. And although there is a known decrease in sperm cryopreservation survival rate in cancer patients as compared to fertile controls [32], overall tumor stage or histology does not seem to directly influence post-thaw semen characteristics [33].

Men who bank sperm are more likely to be younger and single, and they are less likely to have children before cancer diagnosis [34, 35]. They are also more likely to have a new child after treatment, whether it be by natural means or IVF. Even though a minority of males will use cryopreserved sperm in their lifetime [27, 36], a large majority of patients choose to maintain their frozen sperm long after completion of treatment [36]. Utilization of frozen sperm has been noted to be 4.5% at 4 years, 8.7% at 8 years, and 11.8% at 12 years [37]. In a systematic review on sperm cryopreservation and reproductive outcomes in male cancer patients, utilization rate of cryopreserved sperm ranged from 2 to 60% with an aggregate value of 8% [38]. Among men who used their cryopreserved sperm for assisted reproductive techniques, 49% reached parenthood [38]. There was a 30% pregnancy rate per cycle with IVF and a 25% ongoing pregnancy and live birth rate [38]. With IUI, there was a 13% pregnancy rate and 8% ongoing pregnancy and live birth rate [38]. Congenital anomalies in newborns were reported to be 4% based on reports from eight studies [38]. Of note, only 16% of men discarded their cryopreserved sample [38].

Not all males are able to cryopreserve via ejaculated sperm. In a systemic review from 2016, 7% of AYA cancer patients were unable or failed to cryopreserve, the majority of these secondary to azoospermia [38]. In another study, 11.6% of men who were referred for cryopreservation of sperm prior to commencing cancer treatment were azoospermic [37]. But even in the most challenging cases, options for FP are available. Live births have been seen in couples where an azoospermic man with a testicular tumor in a solitary testicle underwent sperm retrieval with microdissection testicular sperm extraction and subsequent use with IVF [39]. These alternative treatment options used to aid in FP will be discussed later in this chapter.

5.5 Testicular Cancer and Semen Parameters

Testicular cancer is one of the most common malignancies treated by urologists, and treatment has the potential to significantly impact fertility and male sex hormones. Overall, patients with testicular germ cell tumors (TGCT) have significantly lower semen concentrations compared to fertile controls [40]. Less than one-third of patients with TGCT will have normal sperm concentrations [41], and 6–12% of patients will be azoospermic [41–44]. Unilateral TGCT seems to affect spermatogenesis bilaterally. This was demonstrated by examining histology of contralateral testicular biopsies in patients with TGCT. In a study of 218 men with TGCT,

contralateral testicular biopsies revealed a histologic pattern of testicular dysgenesis in 25% of patients and Sertoli cell only pattern in 13.8%. Only 51.4% had normal spermatogenesis [45]. When further subdividing patients with testicular cancer, those with seminoma have lower semen concentrations and total sperm numbers compared to men with non-seminoma TGCT [46]. Similarly, patients with TGCT are at high risk for impaired semen quality even when compared to other malignancies [27, 41].

Roughly one-quarter of men presenting with testicular cancer will have low testosterone [47], a finding that often worsens after treatment. Anywhere between 33 and 50% of men surviving testicular cancer at 12 months have low testosterone, and an additional one-quarter of men have low-normal testosterone levels, a finding that does not seem to be related to treatment type [47–49]. Additionally, half of testicular cancer survivors will have at least one long-term abnormality in testosterone, LH, or FSH following treatment [50]. The best predictors of subfertility and low testosterone in these men are associated with patient age and residual testicular volume < 12 cc [47].

5.6 Treatment Effect on Fertility

It is well known that any form of cancer treatment can significantly affect testicular function and spermatogenesis with a resulting increase in post-treatment LH and FSH levels [50]. In the Childhood Cancer Survivor Study, infertility was reported in 46% of cancer survivors versus 17.5% of siblings. Overall, adult male childhood cancer survivors were roughly 50% less likely to contribute to a pregnancy as compared to their siblings. Multivariate analysis demonstrated that alkylating agent dose, surgical excision of any GU organ, and testicular radiation therapy (RT) > 4 Gy are independent risk factors for infertility [51]. Those who are at the highest risk for fertility impairment are patients who undergo total body irradiation, pelvic or testicular RT, and chemotherapy for bone marrow transplant, patients with Hodgkin's disease, and patients with soft tissue and Ewing's sarcoma.

5.6.1 Chemotherapy

The highest risk for gonadotoxicity is with the chemotherapeutic agents cyclophosphamide, ifosfamide, chloromethine, busulfan, melphalan, procarbazine, and chlorambucil [52]. Alkylating agents have the most significant impact on long-term sperm production, and the rate of azoospermia is directly proportionate to the dose of alkylating agent. The relative risk of azoospermia is 1.22 with each increase of 1000 mg/m2 of cyclophosphamide equivalent dose (CED) with impairment of spermatogenesis unlikely if CED is less than 4000 mg/m2 [52]. These findings were among the first to highlight the dose-dependent negative effect that chemotherapy

has on spermatogenesis. Overall, in cancer survivors who receive alkylating agents, the long-term rate of oligospermia and azoospermia are 28% and 25%, respectively [52]. In the case of testicular cancer, Howell et al. demonstrated that treatment of males with cisplatin-based chemotherapy resulted in temporary oligo and azoospermia in the majority of men. Although recovery to normospermia occurred in 80% of patients by 5 years, many semen parameters remained below baseline [53]. Similarly, Bujan et al. showed that quantitative semen characteristics also returned to baseline 12 months following two or fewer cycles of chemotherapy and 24 months after greater than two cycles of BEP or RT [40].

The effects of chemotherapy on sperm DNA quality have also been well documented in the literature. Recommendations for posttreatment contraception come from multiple studies demonstrating aneuploidy and increased DNA fragmentation index (DFI) immediately following treatment with chemotherapy [30, 54–56]. DFI significantly increases up to 12 months and continues to decline until 24–34 months following chemotherapy [40, 57]. Paoli et al. demonstrated an increase in DFI from 18.0 ± 12.5% at baseline to 27.7 ± 17.4% at 3 months and 23.2 ± 15.3% at 6 months following chemotherapy. The authors also showed a significant decline in DFI at 12 months and 24 months, 14.0 ± 8.9% and 14.4 ± 10.3%, respectively [57]. This suggests not only a return to baseline but even an improvement from baseline in sperm DNA quality following completion of cancer treatment. These findings have also been corroborated in other studies with a decrease in DFI after completion of cancer treatment when compared to baseline [30, 58]. Smit et al. and Ribeiro et al. both showed no significant difference in pretreatment DNA fragmentation in TGCT patients compared to controls [30, 59], but Smit did find significantly reduced DNA damage posttreatment at a median follow-up of 1.1 years [30]. Interestingly, following treatment, there does not seem to be a significant difference in sperm DNA integrity between cancer survivors and control groups (9%, 5–13, vs. 11%, 7–16; $p = 0.06$) [60].

Regarding chromosomal makeup, patients will commonly have significantly increased aneuploidy frequencies 6 months after chemotherapy, specifically chromosome 16, 18, and XY disomy and chromosome 13 and 21 nullisomy. In general, aneuploidy frequencies decline to pretreatment levels 18 months after treatment initiation, but increased aneuploidy frequencies may persist in some chromosomes for up to 24 months [54, 56].

5.6.2 Radiation Therapy

Radiation therapy is known to cause testicular dysfunction in a dose-dependent manner as well [61]. Transient effects on spermatogenesis have been seen after low doses of RT [62, 63], with cumulative fractionated doses as low as 2 Gy causing permanent azoospermia [62]. RT as a single dose greater than 4 Gy may also cause permanent azoospermia, with higher doses greater than 12 Gy almost uniformly causing permanent azoospermia [64]. Higher doses of RT are needed to impact

Leydig cell function. Doses of RT in excess of 20 Gy have been shown to affect testosterone production and cause primary hypogonadism [65].

Regarding sperm DNA quality, Paoli et al. also showed DFI increased at 3 and 6 months after radiotherapy, with DFI reaching baseline values at 12 and 24 months posttreatment [57]. Smit et al. did, however, find a significantly higher DFI in patients treated with radiation therapy at a median follow-up of 1.1 months [30]. Others have found that radiation increases sperm DNA damage 1–2 years after treatment, and even 38% of irradiated patients with normozoospermia have high rates of DNA damage [55].

5.6.3 Surgery: Retroperitoneal Lymph Node Dissection

Retrograde ejaculation (RE) and failure of emission (FOE) have classically been the most untoward long-term complication of retroperitoneal lymph node dissections (RPLNDs) with incidences as high as 65% [66–68]. With the adoption of nerve sparing and modified template RPLND, the frequency of RE and FOE has drastically declined to less than 10% [69–71]. Even in patients undergoing post-chemotherapy RPLND (PC-RPLND), antegrade ejaculation is preserved in roughly 80% of patients [72]. In worst-case scenarios, among patients presenting with infertility after PC-RPLND in whom azoospermia is thought to be secondary to RE or FOE, 50% of men with RE can be converted to anterograde ejaculation with medical therapy, and 80% of patients overall will have enough sperm for assisted reproduction via electroejaculation or testicular sperm extraction [73].

5.7 Alternatives to Semen Cryopreservation

As per the American Urological Association guidelines, testosterone and FSH are minimum baseline hormones needed to assess fertility status. In the case of FP, hormonal levels may be of less significance. Being that the presence of most AYA malignancies requires immediate intervention, the time necessary to correct hormonal imbalances, if present, would be too great to have an impact on spermatogenesis [74]. Nevertheless, these hormone levels can be helpful in counselling and setting expectations.

Because cancers may affect the systemic environment, sperm banking via ejaculation alone may not be successful or feasible, and so other means may be necessary. Roughly 10–15% of males with a cancer diagnosis will be unable to bank sperm secondary to psychosocial issues, anejaculation, necrospermia, or azoospermia [27, 41]. In this situation, alternative options of sperm retrieval can be employed with varying success rates. The use of vibratory stimulation, electroejaculation (EEJ), testicular sperm aspiration, or testicular sperm extraction (TESE) can be used with success rates as high as 60% [75]. In patients with FOE, sperm can be

successfully retrieved 75% of the time with EEJ [73]. When EEJ is unsuccessful, TESE can be performed with high success [73].

In postpubertal AYA patients with testicular cancer who cannot cryopreserve semen, active spermatogenesis is usually present in the cancerous testicle, with no significant difference between testicular cancer histology [76]. Multiple case reports have demonstrated feasibility of simultaneous sperm extraction at time of orchiectomy in azoospermic patients [77–79]. An onco-TESE in this setting would allow the surgeon to examine the testicle on a back table for presence of focuses of sperm production within the orchidectomized specimen. The only known predictors of onco-TESE outcome are an inverse relationship to tumor size and presence of spermatogenesis. Overall, a large majority of males who are unable to provide an ejaculated specimen for cryopreservation can have sperm retrieved for use with assisted reproduction.

Hormonal gonad protection with gonadotropin releasing hormone agonists (GnRHa) has been studied extensively in females and may be considered an option in certain malignancies for preserving fertility in hopes of preserving ovarian function. This strategy has not been shown to be effective in men and is not recommended [8]. However, in some instances, where patients are scheduled to receive future gonadotoxic treatments (i.e., for planned bone marrow transplant in thalassemia major or aplastic anemia), induction of puberty with hormonal manipulation may be feasible [80]. It is important to note that in most instances, bone marrow transplant is undertaken within a much shorter time schedule (urgently). When puberty induction is considered, HCG is initiated to drive testicular production of testosterone with subsequent addition of recombinant FSH 3–6 months later to support spermatogenesis [81].

When there has been a failure to cryopreserve sperm or patients are considered too young to have active spermatogenesis, testicular tissue and spermatogonial stem cell cryopreservation may be the only option [8]. At this time, testicular tissue harvesting for cryopreservation of spermatogonial stem cells should only be performed within a research setting under an institutional review board-approved protocol.

5.7.1 Prepubertal Testicular Tissue Cryopreservation

The testicle possesses a complex microenvironment well suited to supporting the developmental needs and self-renewal of spermatogonial stem cells (SSCs) and other cells critical to spermatogenesis. The field of fertility preservation has made large gains in the past several decades for both male and female patients, but for prepubertal boys who do not yet have mature sperm, therapies remain experimental with great interest in developing effective treatment options. Current strategies of obtaining and preserving SSCs or testicular tissue depend upon the future development of successful techniques that will allow in vitro maturation and/or in vivo transplantation of these cells. Proper informed consent must be obtained in this adolescent and prepubertal population. Obtaining testicular tissue is a relatively

straightforward procedure but must be performed under an IRB-approved protocol. Procedures to obtain testicular tissue should be coordinated with alternate procedures in order to decrease the frequency of treatments requiring anesthesia.

Cryopreservation of testicular tissue or SSCs allows for the ex situ storage and preservation of human cells and tissue. As these prepubertal boys mature and ultimately desire offspring, it is possible that specimens will require preservation over several decades prior to utilization. As such, the techniques whereby these tissues and cells are handled, stored, and thawed are critical to successful fertility preservation [82, 83]. Cooling of the specimens is performed in a controlled manner to slow metabolic processes. It is important to prevent the formation of intracellular ice crystals which may be deleterious to the preservation of these specimens. The importance of thawing cannot be underestimated, as improper rewarming may result in recrystallization of damaging ice crystals [84]. The specimens are commonly stored within a cryoprotective extracellular medium such as dimethyl sulfoxide (DMSO), glycerol, or propanediol, as well as within a storage vessel. Growth factors and bioengineered scaffolds may also be supplemented to optimally preserve biological activity of these cells [85]. The size and anatomic structure of the preserved testicular tissue may play a role in future tissue viability, as may the cell count or density of SSCs within their vessels [86, 87].

Animal models have demonstrated that mammalian testicular tissue may be either autotransplanted or xenografted into immunodeficient recipients in order to both preserve spermatogonial stem cells and facilitate sperm development. Honaramooz and colleagues transplanted testicular tissue from neonatal mice, pigs, and goats into the backs of immunodeficient mice and observed that grafts which survived had both increased in volume and were capable of producing mature sperm [88]. Jahnukainen and colleagues obtained testicular tissue from prepubescent primates prior to irradiation and castration. They then ectopically transplanted the testicular fragments subcutaneously on the scrotum or shoulder and observed relatively low graft survival rates of 5%, though one-quarter of these possessed spermatozoa [89]. Viable mammalian offspring has subsequently been produced from several of such studies [90, 91]. Complete spermatogenesis has not yet been achieved with xenografted human testicular tissue. Goossens et al. and Wyns et al. attempted xenografting of prepubertal human male tissue into immunodeficient mice [92, 93]. Though there was graft survival with rare spermatogonia observed, no mature sperm was identified. At present, concerns about transmission of infectious diseases from animal hosts, as well as varying microenvironments, limit the utility of xenografting human tissue into immunodeficient animal recipients [94]. Ultimately, testicular tissue autografting or xenografting may not entirely restore an individual's organic fertility but may help generate mature sperm which may be used for in vitro fertilization with intracytoplasmic sperm injection (ICSI) [95].

Isolation of SSCs from testicular tissue requires careful processing from surrounding testicular stromal cells [96]. Given that many of these patients will have malignant cells with in the testicle, several studies have demonstrated that malignant cells may be successfully sorted from the SSCs [97, 98]. The isolated SSCs may then be either cryopreserved until fertility is desired, cultured in vitro to pre-

serve the stem cells, or transplanted in order to regenerate spermatogenesis. Langenstroth and colleagues isolated nonhuman primate SSCs from somatic cells and were able to establish cell cultures [99]. Utilizing adult human SSCs, Sadri-Ardekani and partners were able to culture and propagate stem cells for several months [100]. Maturing viable human sperm in vitro has proven challenging [101]. Sato et al. generated spermatids in vitro from mouse germ cells that were successfully inseminated to produce fertile offspring [102]. Human in vitro spermatogonial differentiation will require a greater understanding of the culture mediums, hormones, growth factors, and scaffolds in order to successfully induce meiosis of germ cells into haploid sperm while ensuring genetic and epigenetic stability [103].

There is additional exploration into whether transplanted testicular cells may be able to reorganize into sperm-producing entities either in vivo or in vitro. Xenotransplantation of testicular cells has demonstrated the ability to organize into structures similar to testicular tissue [104, 105]. The development of human testicular organoids in vitro may be a promising approach to re-creating a testicular microenvironment optimized for ex vivo spermatogenesis [106]. In conclusion, there are many exciting and promising areas of research, but to date none has been successful in producing viable human sperm. Thus, prepubescent testicular tissue and spermatogonial stem cell cryopreservation remain experimental.

5.8 Conclusions

Addressing the topic of fertility preservation is a cornerstone of patient-centered pediatric and adolescent cancer treatment. Providers are encouraged to discuss the impact of cancer treatments on fertility and sexuality with patients and their caregivers and to refer to specialists when necessary. Chemotherapy, radiation therapy, and surgery may impact an individual's fertility potential, and so patients are encouraged to consider cryopreservation of semen or testicular tissue prior to initiation of such treatments. Utilization of testicular tissue and spermatogonial stem cells remain experimental and should be performed within the structure of an institutional review board-approved protocol.

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Part II
Female

Chapter 6
Advanced Imaging Techniques Used in the Infertile Female

Erica Boiman Johnstone and Jeffrey Dee Olpin

6.1 Uterine Imaging

Uterine factor infertility comprises a small portion of infertility diagnoses. Uterine abnormalities that may impact fertility and pregnancy outcomes include congenital uterine anomalies, uterine leiomyomata, adenomyosis, endometrial polyps, and uterine synechiae. No single imaging modality is optimal for evaluating all of these, and when there is a finding of concern, multiple imaging tests may be required to ascertain the correct diagnosis and determine optimal management.

6.1.1 Uterine Anomalies

The uterus and fallopian tubes are formed in utero by fusion of the bilateral Mullerian ducts, followed by canalization and resorption of the septum between these two tubes, under the influence of the HOX family of genes [1]. Approximately 5.5% of women have an anomaly of the formation of the uterus, and thus these are among the most common congenital anomalies [2]. There is wide variability among anomalous uteri, as well as varying impact on reproduction and options for treatment. Multiple classification systems for uterine anomalies have been developed over the past 40 years [3–7], with increasing emphasis on objective imaging findings for classification. It is vital to correctly classify each uterine anomaly in order to counsel a woman about her risks and select appropriate candidates for surgery. In

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Table 6.1 Uterine anomalies, implications, and management [2, 7]

Uterine anomaly	ESHRE definition	Reproductive implications	Management
Arcuate uterus	Internal indentation ≥1 cm; ≤ 1.5 cm External cleft <1 cm	Increased second trimester loss, RR 2.39 (1.33–4.27) Malpresentation RR2.53 (1.54–4.18)	Expectant
Septate uterus	Internal indentation ≥1.5 cm External cleft <1 cm	First trimester miscarriage RR 2.89 (2.02–4.14) Preterm birth RR 2.14 (1.48–3.11) Malpresentation RR 6.24 (RR 4.05–9.62) Conception RR 0.86 (0.77–0.96)	Surgical (hysteroscopic metroplasty)
Bicornuate uterus	Internal indentation ≥1.5 cm External cleft ≥1 cm	First trimester miscarriage RR 3.40 (1.18–9.76); Second trimester miscarriage RR 2.32 (1.05–5.15); preterm birth RR 2.55 (1.57–4.17) Malpresentation RR 5.38 (3.15–9.19)	Expectant
Didelphys uterus	Two separate unicornuate uterine cavities Two corpus bodies with double cervix	Preterm birth RR 3.58 (2.00–6.40) Malpresentation RR 3.70 (2.04–6.70)	Expectant
Unicornuate	Single well-formed uterine cavity with a single interstitial portion of the fallopian tube and concave fundal contour Asymmetric ellipsoidal shape with our without smaller horn	First trimester miscarriage RR 2.15 (1.03–4.47) Preterm birth 3.47 (1.94–6.22)	Expectant

Table 6.1, the most common types of uterine anomalies are described according to the ESHRE/with their impact on reproductive outcomes and amenability to surgical repair. Reproductive outcomes appear to be improved after hysteroscopic surgery for uterine septum [8], and this procedure may be considered in women with a history of infertility, pregnancy loss, or poor pregnancy outcome [9]. Historically, reunification procedures were commonly performed for bicornuate and didelphys uteri, but these invasive procedures have not been demonstrated to increase the chances of a live birth and have largely been abandoned [10].

Imaging techniques for uterine anomalies are summarized in Table 6.2, including their sensitivities and specificities as well as positive predictive value (PPV) and negative predictive value (NPV), relative to a gold standard of laparoscopy and hysteroscopy. In the evaluation of a woman with infertility, two-dimensional (2D), transvaginal ultrasound is often the first test performed, due to its availability, low cost, and lack of radiation, as well as its ability to provide information on a number

Table 6.2 Sensitivity and specificity of imaging modalities for uterine anomalies [11]

Modality	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	Comments
2D ultrasound	67.3 (51.0–83.7)	98.1 (96.0–100)	94.6 (89.4–99.8)	86.0 (73.7–98.3)	86.6 (81.3–91.8)	Inexpensive and easily accessible
3D ultrasound	98.3 (95.6–100)	99.4 (98.4–100)	99.2 (97.6–100)	93.9 (84.2–100)	97.6 (94.3–100)	Requires specialized transducer
Hysterosalpingo-contrast sonography (saline infusion sonohysterography)	95.8 (91.1–100)	97.4 (94.1–100)	97.8 (93.3–100)	94.6 (87.6–100)	96.5 (93.4–99.5)	
Hysterosalpingography	84.6 (74.4–94.9)	89.4 (80.0–100)	83.6 (74.6–92.6)	89.1(79.7–98.5)	86.9 (79.8–94.0)	Limited to internal uterine contour; painful; requires radiation
MRI					85.8	

of anatomic findings that impact fertility, as discussed later in the chapter. 2D ultrasound offers high specificity, but relatively poor sensitivity for uterine anomalies, and is dependent upon the skill of the operator. Three-dimensional ultrasound improves sensitivity in the diagnosis of uterine anomalies but is less widely available than 2D ultrasound. Image acquisition is typically simple, but processing and interpretation of images requires training and experience [11]. If available, a 3D ultrasound image may be acquired at the time of standard 2D ultrasound and reviewed later if concerns arise. Alternatively, 3D ultrasound may be used as an adjunct to 2D sonographic imaging in the case of indeterminate findings or clinical suspicion.

Saline infusion sonohysterography, also known as hysterosalpingo-contrast sonography (HyCoSy), similarly provides improved sensitivity and accuracy in diagnosing uterine anomalies, as compared with 2D ultrasound. It is relatively low cost and requires specialized training of the operator. Compared with hysterosalpingography, it is less likely to cause pain or infection [11]. This technique is also advantageous in definitively diagnosing other intracavitary lesions. Some authors advocate the use of HyCoSy as a standard, first-line tool in the investigation of infertility [12], while others suggest this modality should be reserved for those with concerning findings on 2D ultrasound or hysterosalpingography [9].

Hysterosalpingography (HSG) is frequently performed in the evaluation of the infertile woman, as a primary assessment of tubal patency. This test is performed in the radiology suite. The cervix is canalized with a catheter, and radio-opaque contrast material is injected through the cervix to fill the uterus and ultimately spill from the fallopian tubes into the uterine cavity. An abnormal internal uterine contour

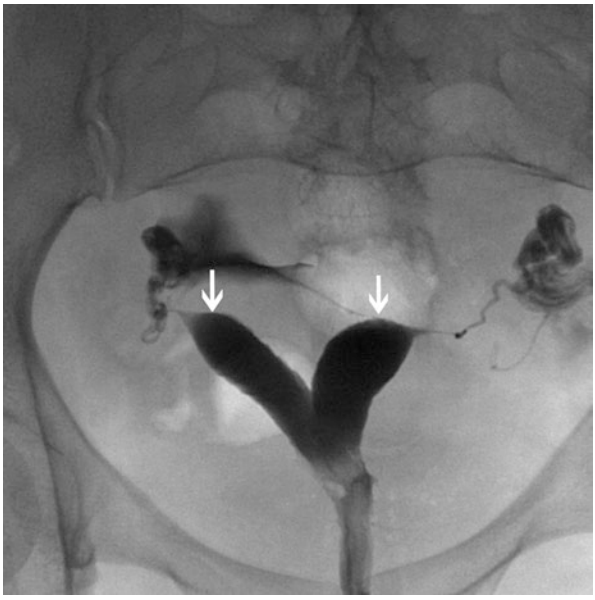

Fig. 6.1 Nonspecific Mullerian duct anomaly. A hysterosalpingogram shows divergent uterine horns (arrows), suggestive of either a septate or bicornuate uterus. Because the external contour of the uterus is not visible, these two anomalies cannot be differentiated on HSG

may be noted. However, because HSG does not define the external contour of the uterus, this technique cannot distinguish a bicornuate from a subseptate uterus (Fig. 6.1) nor a unicornuate uterus from a uterine didelphys or complete uterine septum [11].

MRI (magnetic resonance imaging) offers a slight increase in accuracy of diagnosis of Mullerian anomalies relative to 3D ultrasound [13]. The internal and external contours of both uterus and cervix can be completely imaged with MRI. While intravenous contrast is not necessary, T2-weighted images and use of vaginal gel allow for optimal contrast imaging. Both axial and oblique coronal images should be obtained and reviewed, with 4–5 mm slice thickness and a 24–26 cm field of view. Because of the association between uterine and renal anomalies, consideration should be given to imaging the kidneys as well at the time of MRI [14]. Because of increased cost, MRI should be reserved for cases in which less expensive techniques have not adequately defined uterine and cervical anatomy, and a key management decision relies on the distinction, e.g., a complete uterine septum (Fig. 6.2) versus didelphys uterus (Fig. 6.3) in a patient with recurrent pregnancy loss. In this case, MRI imaging confirmation would enable hysteroscopic septum incision.

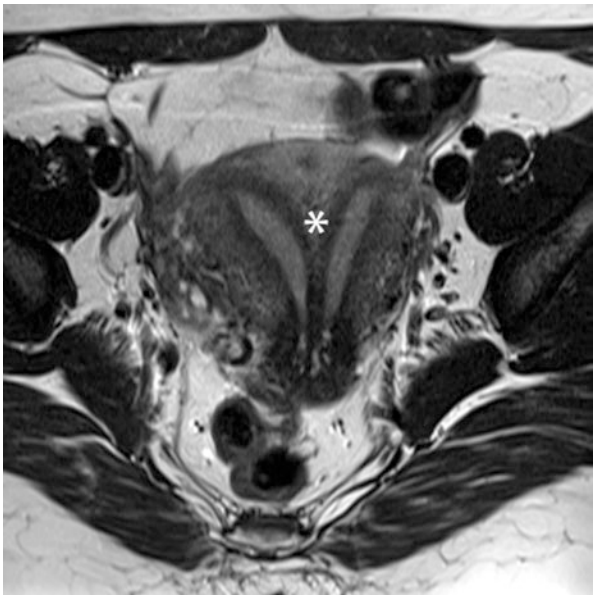

Fig. 6.2 Septate uterus. Axial T2-weighted MR shows a prominent septation (asterisk) with separation of the uterine cavities extending through the cervix

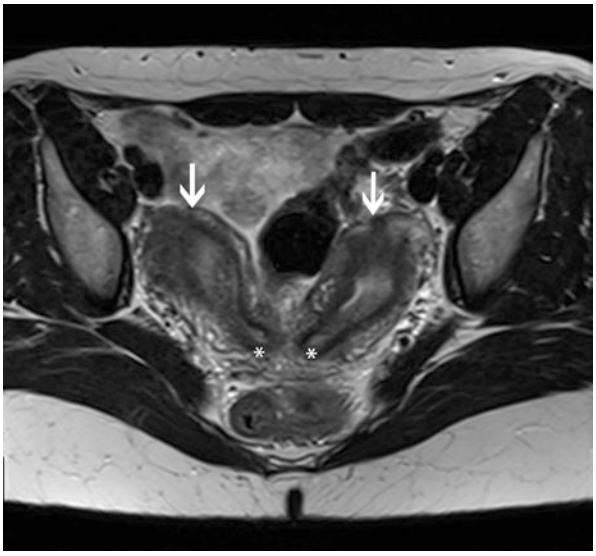

Fig. 6.3 Uterine didelphys. Oblique axial T2-weighted MR demonstrating two widely divergent uterine horns (arrows) and two separate cervices (asterisk) in the setting of uterine didelphys

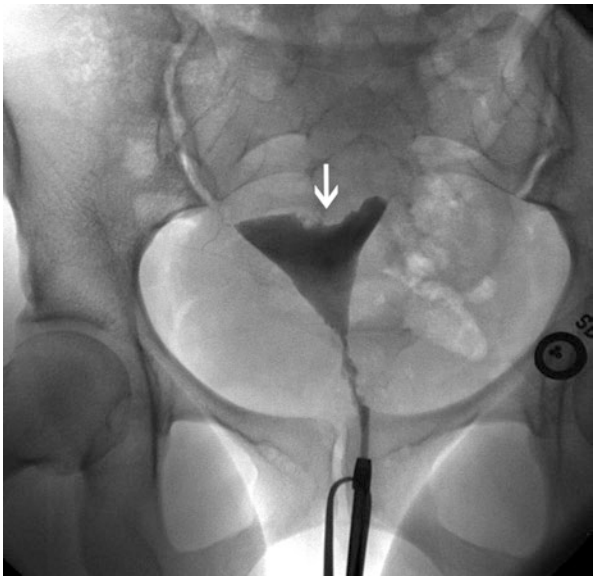

Fig. 6.4 Uterine leiomyoma. A hysterosalpingogram shows focal contour irregularity along the fundal aspect of the endometrial cavity (arrow) consistent with a partially submucosal fibroid

6.1.2 Leiomyoma

Uterine leiomyomata are an extremely common finding among women of reproductive age, with a cumulative incidence of nearly 60% in black women and approximately 30% in white women by age 40 [15]. Leiomyoma are classified as submucosal, intramural, or subserosal based on their location relative to the myometrium, with subclassifications for the proportion of each fibroid in each location [16]. The impact of intramural and subserosal uterine fibroids on fertility is somewhat controversial, and it is not certain whether myomectomy for these types of fibroids improves the chances of successful pregnancy. In contrast, hysteroscopic myomectomy for submucosal leiomyomata has been demonstrated to increase the chances of clinical pregnancy in infertile women [17–19]. Therefore, a precise delineation of the location of uterine is imperative for selecting candidates who are likely to benefit from surgical management.

Uterine leiomyomata are often initially diagnosed on 2D ultrasound, which allows measurement of the size of fibroids but is often inadequate for classification of the location. Hysterosalpingography may identify intracavitary filling defects that might represent leiomyoma but cannot differentiate submucosal fibroids from endometrial polyps, as noted in Fig. 6.4 [20]. Saline infusion sonohysterography has nearly 100% sensitivity, and approximately 90% specificity for diagnosing submucosal fibroids [21, 22], and is the optimal second imaging study for characterization of uterine fibroids.

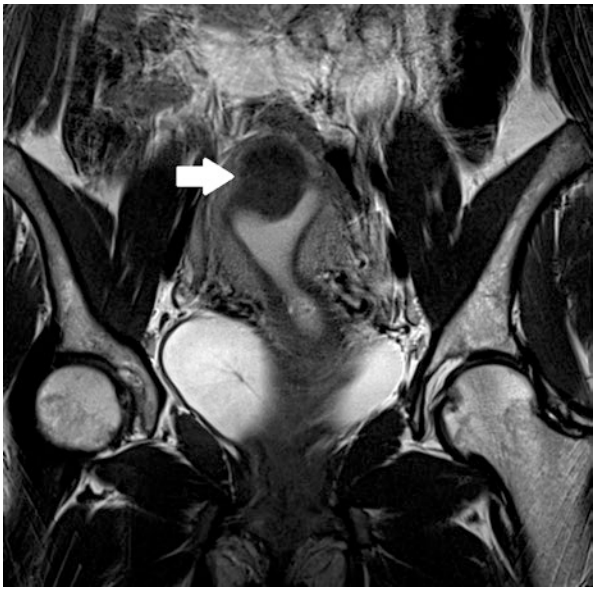

Fig. 6.5 Uterine leiomyoma. Coronal T2-weighted MR shows a prominent intramural hypointense mass (arrow) within the uterine fundus

While MRI provides high resolution imaging of uterine fibroids with clear classification of location (Fig. 6.5), its high cost mandates selective utilization. T2-weighted images provide the greatest contrast between leiomyoma and surrounding myometrium; T1-weighted images should also be obtained to differentiate uterine fibroids from other types of pelvic masses [23]. Unlike ultrasound, MRI has high accuracy in differentiating benign leiomyoma from uterine malignancies [24] and adenomyosis [25, 26], particularly with the use of diffusion-weighted imaging. Utilization of MRI for confirmation of an ultrasound diagnosis of fibroids prior to abdominal or laparoscopic myomectomy may avoid unnecessary surgeries for adenomyosis and inadvertent spread of malignant cells.

6.1.3 Adenomyosis

Adenomyosis is the growth of endometrial glands and stroma within the uterine myometrium, which can lead to menorrhagia and dysmenorrhea. It may be focal, creating an adenomyoma, or diffuse throughout the uterus. While it is more commonly found in parous women, adenomyosis has been hypothesized to contribute to infertility [27] and is associated with decreased chances of clinical pregnancy among women undergoing in vitro fertilization [28, 29]. Hysterectomy is the definitive treatment of adenomyosis, but in infertile women, conservative uterine surgery

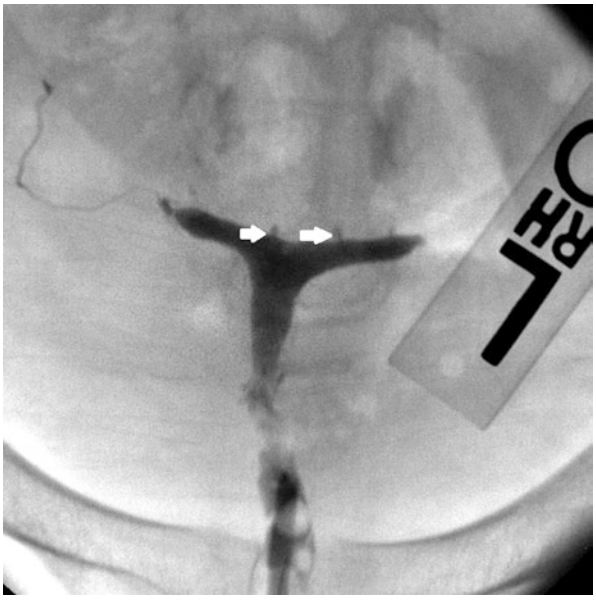

Fig. 6.6 Adenomyosis. A hysterosalpingogram shows multiple outpouchings from the endometrial cavity (arrows), consistent with heterotopic endometrium in the setting of adenomyosis

[30, 31] and the use of GnRH agonists [32, 33] have been found to be beneficial in small studies.

Hysterosalpingography may provide evidence of adenomyosis with small outpouchings from the uterine cavity representing the invasion of endometrial glands into the myometrium (Fig. 6.6). Transvaginal ultrasound findings suggestive of adenomyosis include myometrial heterogeneity and myometrial anechoic cysts [34], as well as enlargement of the uterine corpus and asymmetric anterior or posterior myometrial thickening [35]. A meta-analysis found transvaginal ultrasound to be 82.5% sensitive and 84.6% specific for the diagnosis of adenomyosis.

MRI offers decreased sensitivity (46%) but increased specificity (99%) in the diagnosis of adenomyosis, leading to a positive predictive value of 92% among women with an enlarged uterus planning hysterectomy [36]. MRI findings include a junctional zone between the endometrium that is thicker than 12 mm, poorly defined margins of the lesion, and the absence of deformity of the endometrium, as demonstrated in Fig. 6.7. In contrast, Fig. 6.8 depicts MRI findings consistent with focal adenomyosis. When managing adenomyosis in a patient with infertility, the high specificity of MRI allows certainty in diagnosis to facilitate appropriate medical or surgical treatment.

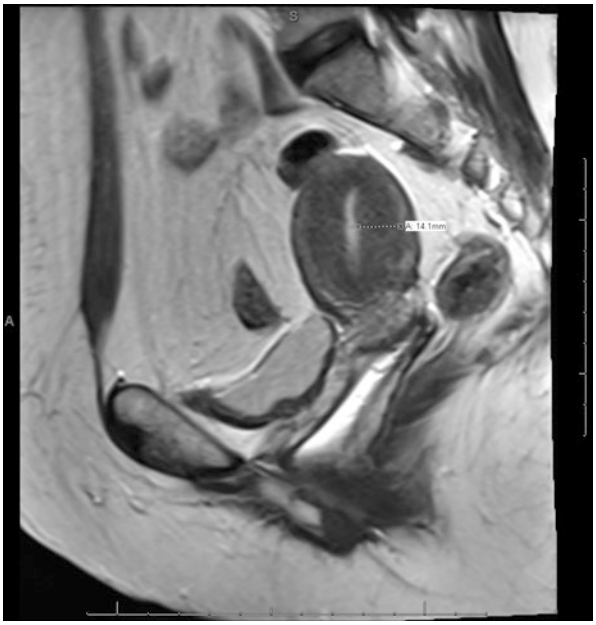

Fig. 6.7 Adenomyosis. Sagittal T2-weighted MR shows diffuse thickening of the junctional zone up to 14 mm, consistent with adenomyosis

6.1.4 Endometrial Polyps

Endometrial polyps are outgrowths of the endometrium that project into the uterine cavity. They are found in approximately 10% of women with infertility [37] and may present with abnormal uterine bleeding. Among premenopausal women, over 98% are benign, while less than 2% represent cancerous or premalignant lesions [38]. While polyps may spontaneously regress, and clearly pregnancy can be achieved in the presence of polyps, a randomized controlled trial has demonstrated higher pregnancy rates with intrauterine insemination among women who underwent hysteroscopic resection of endometrial polyps than in those whose polyps were left in situ [39]. Because hysteroscopic resection of endometrial polyps is a low risk procedure that can be performed in an office setting, many authors advocate that endometrial polyps be removed prior to fertility treatment [40, 41].

Saline infusion sonohysterography offers the optimal visualization of endometrial polyps, with sensitivity and specificity at 90% or greater, as shown in Fig. 6.9. This technique has greater sensitivity than transvaginal ultrasound, and there is no statistically significant increase with the use of 3D versus 2D sonohysterography [22, 42]. HSG cannot differentiate endometrial polyps from submucosal fibroids. MRI is not indicated in the evaluation of suspected endometrial polyps.

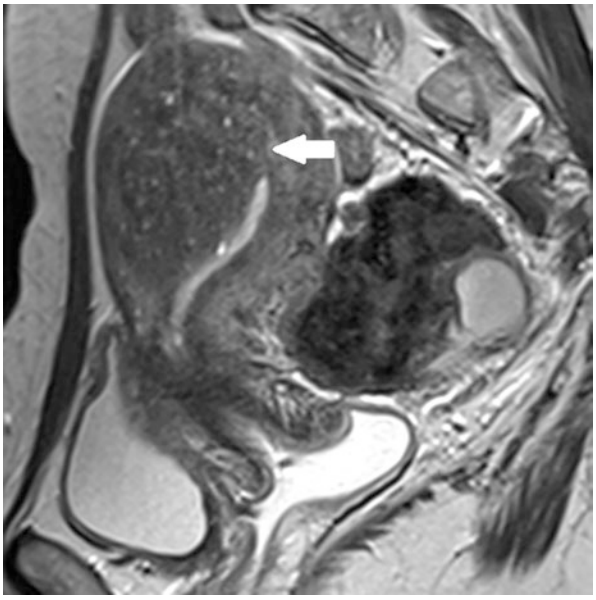

Fig. 6.8 Adenomyosis. Sagittal T2-weighted MR shows poor delineation of the uterine zonal anatomy with marked thickening of the junctional zone within the anterior myometrium (white arrow). Multiple punctate hyperintense foci are noted within the myometrium, consistent with heterotopic endometrial glands

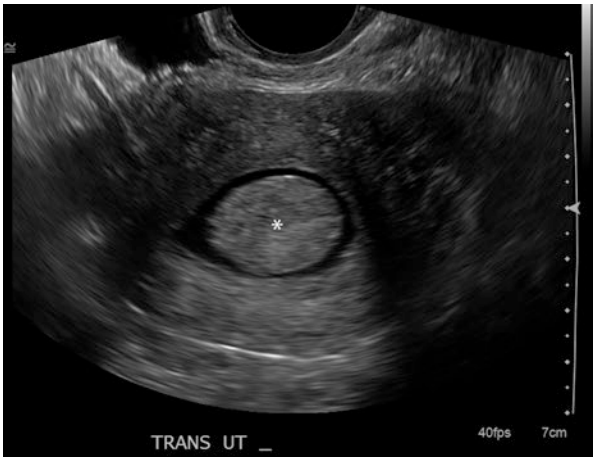

Fig. 6.9 Endometrial polyp. A transvaginal US from a saline-infused sonohysterogram shows fluid outlining a prominent endometrial polyp (asterisk)

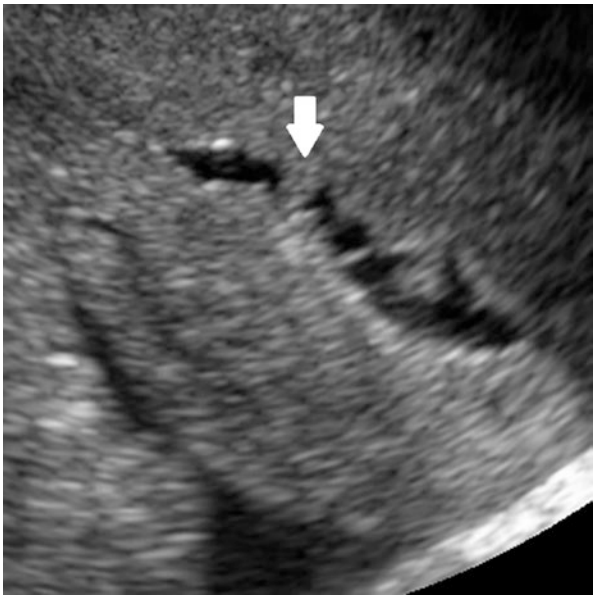

Fig. 6.10 Uterine synechiae. A transvaginal US from a saline-infused sonohysterogram shows multiple echogenic nodules lining the endometrial cavity (white arrow) consistent with uterine synechiae

6.1.5 Uterine Synechiae

Uterine synechiae are adherent fibrous bands crossing the uterine cavity, initially described by Asherman in 1948 in association with amenorrhea [43]. They are found in approximately 7% of women with infertility. Surgical procedures involving the uterine cavity are the primary risk factor, with the highest rates of adhesion formation after dilation and evacuation procedures for intrauterine fetal demise, dilation, and curettage for retained products of conception and postpartum dilation and curettage [44]. The risk of uterine synechiae among women who have undergone dilation and curettage to manage a miscarriage may be as high as 19% [45]. Uterine synechiae may present clinically with changes in menstrual bleeding patterns including amenorrhea or hypomenorrhea, infertility, cyclic pelvic, or recurrent pregnancy loss [46]. Treatment is surgical, with hysteroscopic lysis of adhesions.

Saline sonohysterography is the optimal imaging method for uterine synechiae, with sensitivity of 82–100% and specificity of 99–100% [47]. Uterine synechiae appear as hyperechoic bands crossing the uterine cavity (Fig. 6.10). Hysterosalpingography has demonstrated approximately 80% sensitivity and specificity for uterine synechiae, leading to a positive predictive value of 63% and negative predictive value of 84% in infertile women [48]. While 2D ultrasound lacks adequate sensitivity, relatively new data suggests that 3D ultrasound findings including irregularity at the endometrial margin, partial thinning and endometrial defects,

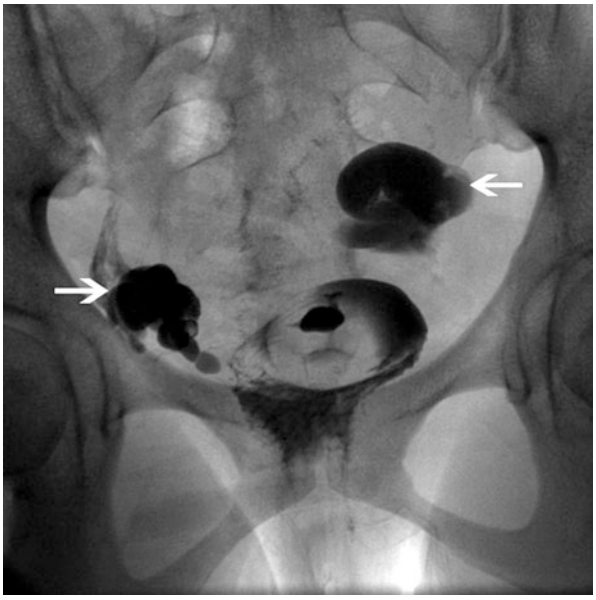

Fig. 6.11 Hysterosalpingogram with bilateral hydrosalpinges. The fallopian tubes are markedly dilated bilaterally (white arrows) with minimal spillage of contrast material from the right tube. The uterine cavity is poorly visualized on this delayed image

and hyperechoic lesions are highly predictive of a hysteroscopic diagnosis of intrauterine adhesions [49]. While MRI can be considered when the severity of intrauterine adhesions prevents catheter passage for saline-infused sonohysterography, this test has not been demonstrated to provide additional value in ascertaining individuals who may benefit from hysteroscopic evaluation and treatment.

6.2 Fallopian Tube Imaging

Evaluation of fallopian tube patency remains a cornerstone of the female fertility assessment. Hysterosalpingogram has long been the mainstay of this evaluation. Distal tubal obstruction with and the degree of hydrosalpinx can clearly be delineated by HSG (Fig. 6.11). Women with bilateral hydrosalpinges are presumed to have extremely low chances of conception without in vitro fertilization, and women with unilateral hydrosalpinx are 75% less likely to conceive with intrauterine insemination than those with bilateral tubal patency [50]. In women with hydrosalpinges, salpingectomy improves the odds of ongoing pregnancy with IVF [51]. Salpingectomy for proximal tubal obstruction has been hypothesized to improve the chance of spontaneous pregnancy in women with unilateral hydrosalpinx [52].

However, the specificity of HSG for proximal tubal obstruction is limited, as 60% of women with unilateral proximal tubal obstruction on initial HSG will have bilateral tubal patency on repeat HSG [53]. Tubal spasm is the hypothesized mechanism for this [54].

Various radiologic techniques have been proposed for the evaluation and management of proximal tubal obstruction. These include selective salpingography, in which a catheter is fluoroscopically guided to the cornu where proximal obstruction is detected and contrast material directly injected, tubal catheterization with a soft, Teflon catheter, and canalization with a guide-wire. Each of these techniques has relatively high success in achieving tubal patency, and approximately 40% of treated patients achieve spontaneous pregnancies within 1 year [55, 56]. However, an absence of randomized controlled trials or high quality observational studies comparing treated to untreated patients makes it impossible to assess whether these treatments actually improve the odds of successful pregnancy. Indeed, Ferraiolo et al. [57] found that 21% of women with bilateral proximal tubal obstruction that could not be relieved by selective salpingography spontaneously conceived an intrauterine pregnancy within 1 year after the procedure. Women with untreated unilateral proximal tubal obstruction on HSG have similar rates of clinical pregnancy with intrauterine insemination to women with bilateral tubal patency [50].

Assessment of tubal patency by HyCoSy requires specialized training, and the technique involves instillation of air bubbles, which appear hyperechoic on ultrasound, through the fallopian tubes after completion of the uterine cavity assessment. Two meta-analyses have demonstrated 95–98% sensitivity and 90–93% specificity of HyCoSy for tubal obstruction relative to the gold standard of laparoscopic evaluation, which was equivalent to HSG [58, 59]. In the first meta-analysis, the addition of contrast media to HyCoSy did not improve diagnostic accuracy. Use of this technology to diagnose hydrosalpinx to select candidates for surgical treatment prior to in vitro fertilization may also prevent unnecessary surgeries, as only hydrosalpinges visible on ultrasound appear to impact the outcome of IVF [60, 61]. Some authors have noted higher pain scores in women undergoing HyCoSy relative to HSG [62], while others found the opposite with hysteron-foam-sonography [63]. Some authors advocate for the use of HyCoSy rather than HSG for assessment of tubal patency due to its ability to evaluate all pelvic anatomy and avoidance of radiation [64].

6.3 Ovarian Imaging

6.3.1 Infertility Evaluation and Monitoring

Transvaginal ultrasound is the standard of evaluation of the ovaries in the infertile female. Initial ultrasound is best performed in the early follicular phase in order to assess antral follicle count, a key measure of ovarian reserve, as well as ovarian volumes, in the absence of a dominant follicle or corpus luteum cyst that could impact these measurements (Fig. 6.12). Antral follicle count predicts response to

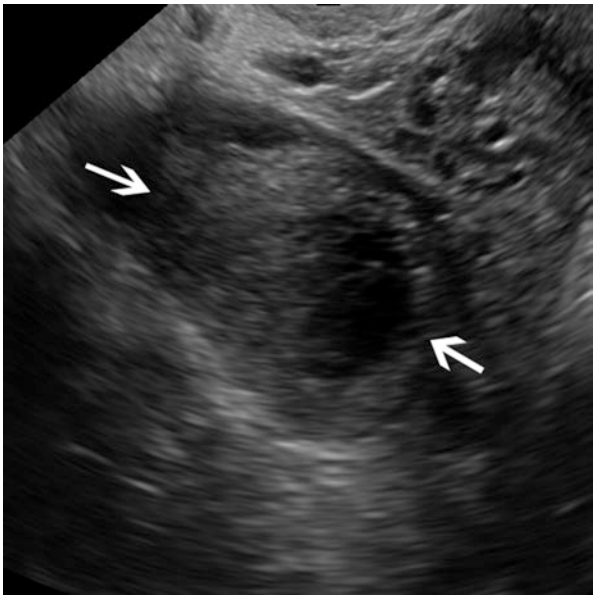

Fig. 6.12 Normal ovary. Transvaginal US demonstrating a typical appearance of a normal ovary (white arrows)

gonadotropins in in vitro fertilization cycles [65] and has been correlated with chances of live birth [66].

Antral follicle count and ovarian volume are elements of the ovarian morphology criterion for polycystic ovary syndrome, although societies debate on the antral follicle count that defines polycystic ovaries. The Endocrine Society requires an antral follicle count of 12 per ovary [67], while the Androgen Excess and Polycystic Ovary Syndrome Society utilizes 25 per ovary [68]. Both concur on an ovarian volume of at least 10 mL.

Ultrasound monitoring of the number and size of follicles in in vitro fertilization is the standard of care, for determining cycle cancelation, gonadotropin dose changes, risk of ovarian hyperstimulation syndrome, and timing of ovulation trigger [69]. In Fig. 6.13 is an ovary with multiple maturing follicles after treatment with gonadotropins, while Fig. 6.14 demonstrates the appearance of the ovary in ovarian hyperstimulation syndrome, with multiple corpus luteum cysts. Ultrasound monitoring may be used with clomiphene citrate in order to determine the timing of intrauterine insemination, but this has not been demonstrated to improve the chances of pregnancy compared to urinary LH monitoring [70, 71].

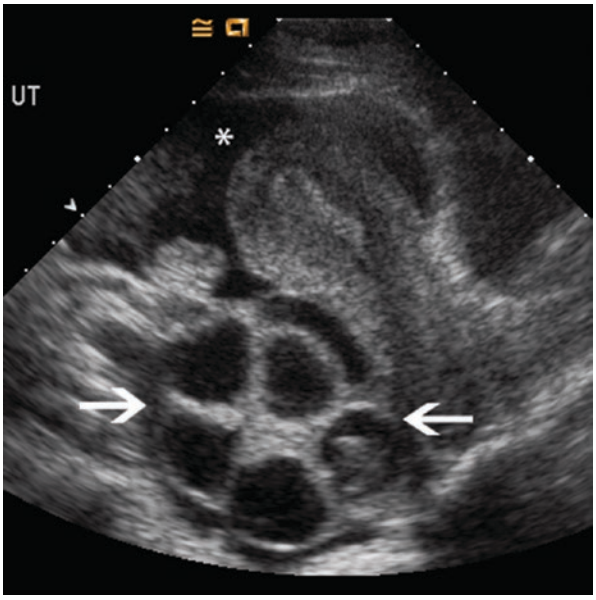

Fig. 6.13 Ovarian hyperstimulation. Transabdominal US demonstrating an enlarged ovary with multiple follicles (white arrows) in a patient undergoing controlled ovarian hyperstimulation for in vitro fertilization. Free fluid is noted (asterisk)

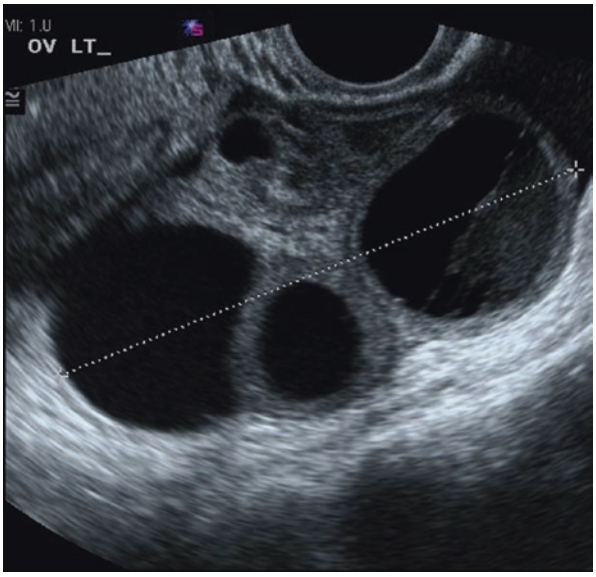

Fig. 6.14 Ovarian hyperstimulation. Transvaginal US image of an enlarged ovary with multiple prominent follicles in a patient with ovarian hyperstimulation syndrome

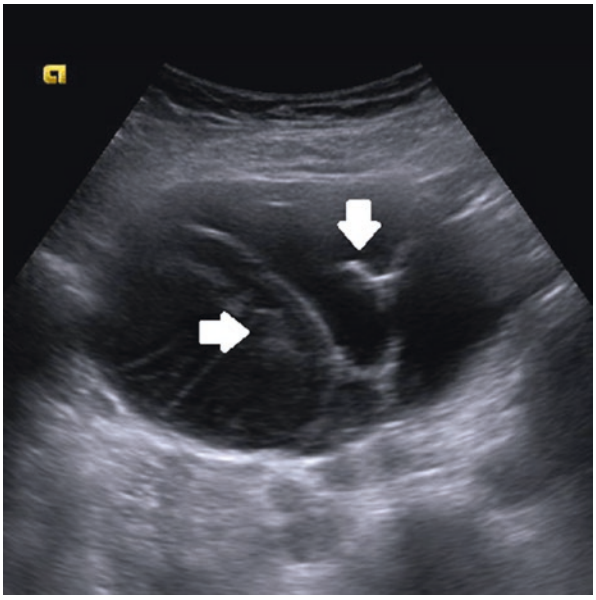

Fig. 6.15 Complex ovarian cyst. Transabdominal US image of a complex ovarian cyst concerning for malignancy. The horizontal arrow indicates a small, solid area, while the vertical arrow indicates a papillary excrescence

6.3.2 Ovarian Cysts

Ovarian cysts and other adnexal masses are common findings in women with infertility. While they may present with pain, often they are found incidentally, most commonly with transvaginal ultrasound performed in the evaluation of infertility [72]. The primary goal of imaging of ovarian cysts is to determine which cysts present with a significant risk of malignancy so that they may be appropriately managed surgically. A secondary goal is to determine whether an ovarian cyst contributes to the patient's infertility, and if so, determine an optimal management strategy.

The LR2 prediction model, developed from the International Ovarian Tumor Analysis study, uses patient age and ultrasound findings to predict the likelihood of malignancy in adnexal masses. Ascites, blood flow in a papillary projection, maximal diameter of the solid component, irregular internal cyst walls, and acoustic shadows each increase the risk of malignancy [73]. The LR2 model has demonstrated 94% sensitivity and 82% specificity for ovarian cancer, significantly better than a model that incorporates the serum markers CA-125 and HE4 [74] and can be performed by the clinician to determine which patients should be referred to gynecologic oncology. Figure 6.15 depicts a complex cyst with cystic and solid components and a papillary projection, highly concerning for malignancy.

Follicular cysts, corpora lutea, endometriomas, and dermoid cysts (mature cystic teratomas) are among the most common benign ovarian cysts found on ultrasound.

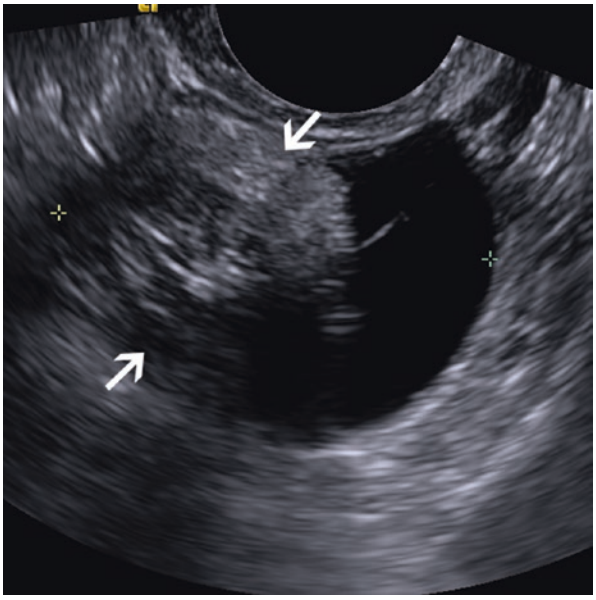

Fig. 6.16 Ovarian dermoid. Transvaginal US demonstrating a heterogeneous solid and cystic mass. An echogenic shadowing Rokitansky nodule or dermoid plug (white arrows) is a characteristic feature of a dermoid

Thirty to fifty percent of those who have both dysmenorrhea and infertility have endometriosis, and 20–40% of women with endometriosis will have an endometrioma [75]. Endometriomas may be unilocular or multilocular, and most commonly have a homogeneous, ground-glass appearance on ultrasound (Fig. 6.16), and may have hyperechoic wall nodules [76]. Transvaginal ultrasound is highly sensitive (87–99%) and specific (92–99%) for endometrioma, with a predictive value equivalent to MRI [77].

Endometriomas may have a negative impact on the surrounding ovarian stroma and follicles, as decreased follicular number and density has been reported in ovaries with endometriomas [78]. While measures of ovarian reserve are lower in women with endometriomas [79], the presence of an endometriomas does not decrease the chances of live birth with in vitro fertilization [80]. Moreover, it is well established that surgical excision of endometriomas decreases ovarian reserve [72], and a meta-analysis found that neither medical nor surgical treatment of endometrioma prior to in vitro fertilization improved the odds of clinical pregnancy [81].

Dermoid cysts, also known as mature cystic teratomas, are comprised of a variety of cell types and have a complex appearance on ultrasound, with bright calcifications and echogenic sebaceous material, as shown in Fig. 6.17 [82]. While studies of the impact of dermoid cysts on fertility are very limited, surgical resection of dermoid cysts has been reported to decrease ovarian reserve to a greater degree than cystectomy for endometrioma [83], and the presence of a dermoid does not appear

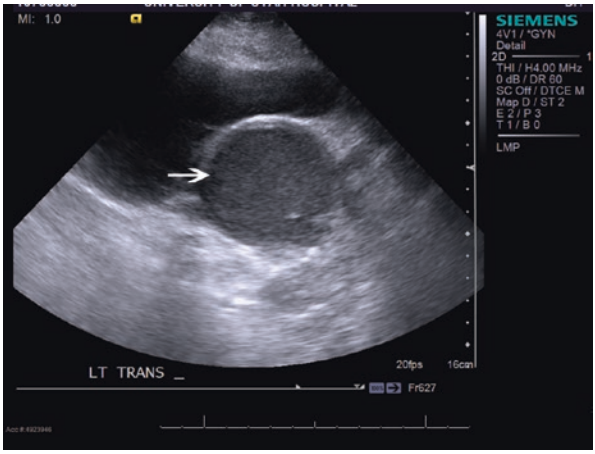

Fig. 6.17 Endometrioma. Transabdominal US demonstrating an ovarian endometrioma (arrow)

to decrease ovarian response to gonadotropin stimulation for IVF [84]. Therefore, dermoid cysts do not require surgical treatment in the infertile patient.

6.4 Endometriosis

As previously noted, the majority of cases of endometriosis are not associated with an ovarian endometrioma. Transvaginal ultrasound may detect large nodules of endometriosis, and the sliding sign technique (applying pressure of the vaginal ultrasound transducer to the posterior fornix to determine if the uterus moves independently from the rectum) demonstrates high sensitivity and specificity for deeply infiltrating endometriosis in the rectovaginal septum [85]. However, MRI ultimately demonstrates greater sensitivity for deeply infiltrating endometriosis than ultrasound, 94% versus 79% [77], as well as for posterior implants involving the uterosacral ligaments and cul-de-sac [76]. MRI can detect small endometrial implants on T1-weighted images, and adhesions resulting from endometriosis are visible on both T1- and T2-weighted images. In Fig. 6.18, a posterior endometrioma can be visualized on T2-weighted imaging, as well as deeply infiltrating endometriosis in the rectovaginal septum, as indicated by the arrow.

MRI may also improve identification of hematosalpinx due to endometriosis, as seen in Fig. 6.19. The detailed, 3-dimensional imaging provided by MRI is highly predictive of surgical findings [76]. Prospective studies are needed to demonstrate the utility of MRI assessment for endometriosis among infertile women, in order to select the optimal treatment for each woman.

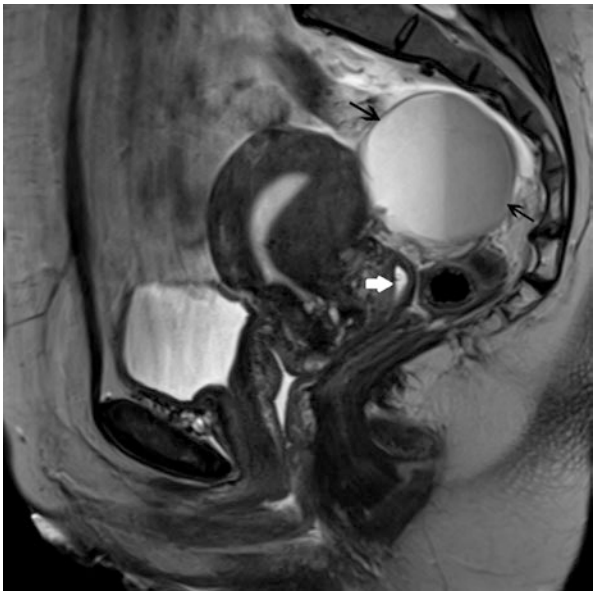

Fig. 6.18 Endometriosis. Sagittal T2-weighted MR shows a large posterior presacral endometrioma (black arrows) and deeply infiltrating endometriosis in the rectovaginal septum (white arrow)

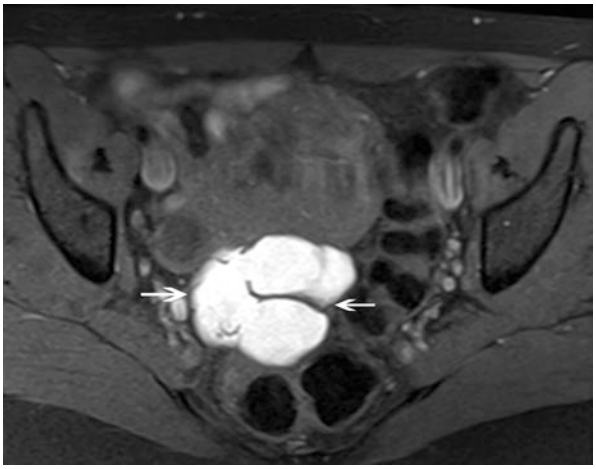

Fig. 6.19 Hematosalpinx. Axial T1-weighted MR with fat saturation shows a large, posterior hematosalpinx (arrows) due to endometriosis

6.5 Conclusions: A Practical Approach to Imaging

In a woman presenting with infertility, HyCoSy offers an opportunity to evaluate the uterus, fallopian tubes, and ovaries with a single, relatively low cost, minimally invasive test that can be performed in the office and does not require radiation exposure. As such, many authors advocate for this as the first-line test for all new infertility patients [12]. Because of the high sensitivity and specificity of this test, clinical management of abnormalities including adnexal masses, hydrosalpinges, and uterine anomalies can often be determined from HyCoSy alone. However, when specific abnormalities are suspected due to clinical history or uncertain findings, additional imaging may be beneficial to make a definitive diagnosis, and prior to surgery or other invasive procedures. Radiation exposure, need for contrast, patient discomfort, and cost are all important considerations in weighing imaging techniques, and a personalized approach is paramount, with selection of only those tests whose results will change clinical management.

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Chapter 7
Treatment of Subclinical Hypothyroidism in the Infertile Female

Mohamad Irani and Samantha M. Pfeifer

7.1 Introduction

Hypothyroidism can be either overt or subclinical. Overt hypothyroidism is diagnosed when high TSH is accompanied with low free T4 levels. Overt hypothyroidism may have adverse impact on reproduction including oligo-anovulation leading to subfertility and may complicate pregnancy increasing the risk of miscarriage, placental abruption, preterm delivery, preeclampsia, and fetal demise [1]. Adequate thyroid hormone supplementation before and during pregnancy for women with overt hypothyroidism is important as it reduces the risk of complications.

Subclinical hypothyroidism has been classically defined as elevated TSH (>4.5 mIU/L) with normal free T4 levels. According to the National Health and Nutrition Examination Survey (NHANES III) using TSH threshold of 4.5 mIU/L, the overall prevalence of subclinical hypothyroidism is approximately 4.3% [2]. The problem with using a generalized threshold is the presence of differences in the TSH levels between males and females, white and black, and young and old individuals [2]. These physiologic variations may need to be used for defining normal references that are gender-, age-, and ethnicity-specific. Moreover, the upper limit of normal might be lower than 4.5 mIU/L when using the third-generation assay and excluding patients with occult autoimmune thyroid disease and positive thyroid peroxidase antibodies (TPOAb) from the studied population to determine the normal range [3]. Therefore, the National Academy of Clinical Biochemistry (NACB) guidelines consider that 95% of individuals with no thyroid dysfunction have TSH $\leq$ 2.5 mIU/L and suggest the use of this threshold as the upper range of normal for everyone [3, 4]. They recommend repeating the measurement 3–4 weeks after the initial TSH > 2.5 mIU/L and diagnose subclinical hypothyroidism should the

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second level be also elevated [3, 4]. If TSH is found to be >2.5 mIU/L, then the recommendation is to repeat the measurement 3–4 weeks after the initial TSH > 2.5 mIU/L and diagnose subclinical hypothyroidism should the second level be also elevated [3, 4]. However, lowering the threshold to 2.5 mIU/L would dramatically increase the prevalence of hypothyroidism in the United States from 4.3 to 15% [5]. This change will lead to treating many more individuals in the United States for subclinical hypothyroidism, potentially unnecessarily. In addition, given that treating individuals with TSH 5–10 mIU/L did not improve the lipid profile or cardiovascular outcomes, the value of treating those with TSH < 5 mIU/L is challenged [6]. The adverse effects of the potential overtreatment such as iatrogenic hyperthyroidism and its effect on bone metabolism are also of concern. Thus, the Endocrine Society does not endorse lowering the threshold to 2.5 mIU/L [7]. It also recommends that each laboratory determine its upper limit of normal for the third-generation TSH assay taking into account patient's age [7–9].

During pregnancy, the reference range is modified due to the effects of the ample amounts of human chorionic gonadotropin (hCG) especially in the first trimester [10]. The American Thyroid Association recommends using trimester-specific reference ranges for TSH: 0.1–2.5 mIU/L for the first trimester, 0.2–3 mIU/L for the second trimester, and 0.3–3 mIU/L for the third trimester [11–13]. Some advocate using the first trimester reference range, TSH < 2.5 mIU/L, for women who are attempting pregnancy in order to assure that TSH will be less than 2.5 mIU/L in the first trimester [14]. However, this suggestion has been controversial owing to the lack of studies on the impact preconception TSH levels on the pregnancy outcomes (11). Several issues arise. For instance, serum TSH levels have been shown to exhibit up to 40% variation when performed serially during the same time of day and may fluctuate [7, 15]. Thus, a slightly abnormal TSH level can become within the normal range when repeated in few days to weeks even without any therapy. There are also racial differences in TSH values and changes in early pregnancy [14]. Additionally, TSH typically decreases in the first trimester to levels <2.5 mIU/L due to the effect of hCG produced by the corpus luteum and subsequently the placenta, and thus a slightly higher TSH level prior to conception might become spontaneously less than 2.5 mIU/L by the time hCG is detected in the serum [12]. As TSH reaches its nadir around 10 weeks gestation, TSH levels early in the first trimester may not reflect true abnormality and there may not need to be treated [16]. Moreover, TSH may be transiently increased during episodes of controlled ovarian hyperstimulation and ovarian hyperstimulation syndrome. The presumed mechanism is attributed to the increase in thyroid-binding globulin levels as a result of the elevated serum estrogen levels [14, 17–21]. Hence it is advised to measure TSH levels either before or 2 weeks after the stimulation [14]. Therefore, the typical variability in TSH levels using different assays, the fluctuations with ovarian hyperstimulation, and the decline in the first trimester make the assessment for the presence of subclinical hypothyroidism more challenging.

To determine the indications of treatment of subclinical hypothyroidism, we will now review the association between subclinical hypothyroidism and infertility and adverse obstetric and fetal outcomes. Overall, there are conflicting data regarding

the association between subclinical hypothyroidism and infertility and reproductive outcomes. The literature consists primarily of retrospective studies with different definitions of subclinical hypothyroidism and inadequate controls used. Two recent systematic literature reviews have evaluated the association between subclinical hypothyroidism, defined as TSH > 4–5 mIU/L or between 2.5 and 4–5 mIU/L, and both infertility and reproductive outcomes along with the effect of treatment. The American Society for Reproductive Medicine published guidelines regarding subclinical hypothyroidism and the infertile female population, and the American Thyroid Association updated their recommendations regarding diagnosis and management of thyroid disease during pregnancy [9, 14].

7.2 Subclinical Hypothyroidism and Infertility

There are conflicting data regarding the association between subclinical hypothyroidism and infertility. This is in part due to different definitions of subclinical hypothyroidism and inadequate controls used. Overall, subclinical hypothyroidism does not appear to be increased in women with infertility compared to those without regardless of TSH cutoff used. A prospective study including 538 women showed that, despite the higher median TSH level in the infertile group (1.3 mIU/L) compared to controls (1.1 mIU/L), they had comparable prevalence of elevated TSH level (defined as TSH > 4.2 mIU/L) [22]. Likewise, in a prospective study including 1228 women attempting pregnancy, Plowden et al. showed that there are no significant differences in time to pregnancy, pregnancy loss rates, or live birth rates between those with preconception TSH ≥ 2.5 mIU/L and those with TSH < 2.5 mIU/L [23].

In some studies, subclinical hypothyroidism is more commonly seen in those women with infertility and ovulatory dysfunction. In one study evaluating TSH levels in 704 infertile women with no known thyroid disease, 2.3% were noted to have a TSH > 4.09 mIU/L and of those with an elevated TSH, 69% had ovulatory dysfunction [24]. Similarly, Arojoki et al. [25] showed a relatively high prevalence of elevated TSH levels among women with ovulatory dysfunction (6.3%) and unexplained infertility (4.8%) but not with tubal (2.6%) or male infertility (1.5%). On the other hand, Abalovich et al. [26] showed a much higher prevalence of thyroid dysfunction (TSH > 5 mIU/L) among infertile women (13.9%) especially those with primary ovarian insufficiency (40%), tubal factor (18.2%), and ovulatory dysfunction (15.4%) compared to controls (3.9%).

Given the lack of evidence demonstrating any impact of subclinical hypothyroidism, defined as TSH between 2.5 and 4 mIU/L or TSH > 4 mIU/L in women who do not have thyroid antibodies, it is not recommended to treat with levothyroxine to improve fertility [9, 14]. However, the American Thyroid Association does recommend considering treatment in women attempting pregnancy with TSH > 2.5 mIU/L with a low dose of levothyroxine given its ability to prevent the progression to a more significant hypothyroidism once pregnancy is achieved [14]. The American

Society for Reproductive Medicine recommendation differs: treatment with levothyroxine may be initiated, or alternatively TSH levels may be monitored prior to pregnancy and treating when TSH > 4 mIU/L [9].

7.3 Subclinical Hypothyroidism and Adverse Pregnancy Outcomes

Untreated over hypothyroidism is associated with an increase in miscarriage rate during pregnancy [27]. However, the evidence supporting the association between untreated subclinical hypothyroidism diagnosed during pregnancy and adverse pregnancy outcomes is fair [9, 10, 14, 28–31]. Literature evaluating this association is predominantly retrospective studies, and evaluation of the literature is difficult for several reasons: (1) different TSH cutoff values have been used; (2) most studies evaluated women in the late first trimester or early second trimester with little or no information regarding early first trimester losses; (3) there is a paucity of data assessing whether subclinical hypothyroidism prior to pregnancy is associated with adverse pregnancy outcomes. This is particularly important given the recent trend of treating TSH values >2.5 mIU/L in women with infertility prior to pregnancy with levothyroxine to maintain TSH < 2.5 mIU/L. As discussed earlier, TSH will reach a nadir by 10 weeks' gestation, and thus it is unclear what is the proportion of women with preconception TSH > 2.5 mIU/L will actually have a persistently elevated TSH during pregnancy [13].

Benhadi et al. [28] conducted a large retrospective study including 2497 women with no overt thyroid disease and showed that mean TSH level in the late first trimester (mean gestational age: 13 weeks) of women who had pregnancy loss was significantly lower than those without pregnancy loss (1.48 vs. 1.11 mIU/L). They also concluded that the incidence of pregnancy loss increase by 60% for every doubling in TSH level. However, the reported miscarriage rate was 1.08%, which is a relatively very low rate. In addition, the clinical significance of this finding may be questioned as both of these TSH values are well within the normal range and < 2.5 mIU/L [28]. Similarly, Ashoor et al. [29] showed that the 202 pregnancies resulting in miscarriage had higher TSH and lower free T4 compared to the 4138 normal pregnancies. It is important to note that the authors assessed thyroid function at 11–13 weeks, which is usually after the occurrence of most miscarriages [29]. Negro et al. [30] evaluated the pregnancy outcomes of 4123 thyroid peroxidase antibody-negative women with TSH less than 5 mIU/L in the mid-first trimester (mean gestational age: 8.8 weeks) who did not receive thyroid hormone supplementation. Women with TSH 2.5–5 mIU/L had higher rate of pregnancy loss compared to those with TSH < 2.5 mIU/L (6.1% vs. 3.6%, respectively; $P = 0.006$) [30]. Wang et al. [31] also showed that women who were diagnosed in the first trimester of pregnancy (mean gestational age: 6 weeks) with subclinical hypothyroidism, defined as TSH ≥ 2.5 mIU/L with normal free T4, had a higher incidence of

miscarriage compared to those with TSH < 2.5 mIU/L (15.4% vs. 8.8%, respectively; $p < 0.05$). More recently, Ma et al. [32] conducted a randomized controlled trial concluding that screening for and treatment of subclinical hypothyroidism (TSH 2.5–10 mIU/L with normal free T4) in early pregnancy (median gestational age = 11 weeks) reduce the incidence of miscarriage (odds ratio = 0.3; 95% CI = 0.21–0.56). In addition, a large prospective study concluded that although treating subclinical hypothyroidism (TSH 2.5–10 mIU/L with normal free T4) in early pregnancy may increase some pregnancy complications, it results in a decrease in miscarriage rate [33]. Furthermore, a retrospective study of 25,756 women who delivered singleton infants showed that subclinical hypothyroidism, defined as TSH > 97.5% for gestational age with normal free T4, was associated with a 3-fold increase in placental abruption and 1.8-fold increase in preterm delivery [16].

In summary, there is fair evidence that subclinical hypothyroidism defined as TSH > 4 mIU/L with negative TPO antibodies, during pregnancy, is associated with miscarriage, but insufficient evidence that TSH levels between 2.5 and 4 mIU/L are associated with miscarriage [9]. In addition, while there is good evidence that treatment in women with subclinical hypothyroidism with TSH > 4 mIU/L is associated with improvement in pregnancy and miscarriage rates, there is insufficient evidence that levothyroxine therapy in women with TSH between 2.5 and 4 mIU/L is associated with improvement in pregnancy and miscarriage rates [9]. The American College of Obstetricians and Gynecologists (ACOG) does not recommend routine screening for subclinical hypothyroidism during pregnancy [10]. Targeted screening for those likely to have overt hypothyroidism is indicated, and when overt hypothyroidism is diagnosed, treatment is indicated with the goal to keep TSH less than 2.5 mIU/L in the first trimester, and less than 3 mIU/L in the second and third trimesters [10].

The data evaluating subclinical hypothyroidism diagnosed before pregnancy is primarily comprised of infertile women undergoing IVF, with a few studies evaluating women undergoing intrauterine insemination. There are two randomized controlled trials investigating the influence of treating preconceptional subclinical hypothyroidism, defined as TSH > 4 mIU/L with normal free T4, in women undergoing IVF [34, 35]. Kim et al. [34] randomized 64 infertile women on the first day of controlled ovarian hyperstimulation for IVF into two groups: treatment (levothyroxine 50 μg daily) and placebo. Both groups had comparable clinical pregnancy rate, but the treatment group had a significantly higher number of high-grade embryos, higher implantation and live birth rates, and lower miscarriage rates compared to the placebo group (0% vs. 33.3%, respectively; $P = 0.021$) [34]. Abdel Rahman et al. [35] also randomized 70 infertile patients 1 month prior to their IVF cycles into two groups: treatment (levothyroxine 50–100 μg daily) and placebo group. They found that both groups had comparable number of retrieved oocytes, but women who received treatment had significantly lower miscarriage rates (9% vs. 13%, respectively) and higher clinical pregnancy and live birth rates compared to the placebo group [35]. The findings of these two trials promote a beneficial impact of treating preconception subclinical hypothyroidism with TSH > 4 mIU/L.

In contrast, studies examining the implementation of strict TSH thresholds (<2.5 mIU/L) in the preconception period showed a substantial increase in the prevalence of subclinical hypothyroidism with no clinical benefit of treatment [9, 23, 36–38]. Michalakis et al. [36] studied 1231 women undergoing assisted reproductive technologies and found that the prevalence of women with preconception TSH level of 2.5–4 mIU/L was 23%. These women had similar pregnancy and assisted reproductive technology outcomes compared to those with TSH < 2.5 mIU/L [36]. Likewise, Reh et al. [37] investigated a group of 1055 patients pursuing their first-cycle IVF and showed that lowering the cutoff from 4.5 to 2.5 mIU/L increased by nearly fivefold the prevalence of subclinical hypothyroidism (5.3% vs. 24%), while it did not change the clinical pregnancy, live birth, or miscarriage rates. More recently, Karmon et al. [38] studied 1477 patients who underwent 4064 intrauterine insemination cycles and found that preconception TSH of 2.5–4.9 mIU/L may be associated with higher live birth rates and lower miscarriage rates compared to TSH of 0.4–2.4 mIU/L. A large retrospective study showed that preconception TSH < 2.5 mIU/L had similar miscarriage and live birth rates following intrauterine insemination (IUI) compared to those with higher TSH levels [39]. Similarly, a randomized controlled trial including women with history of one or two miscarriages revealed comparable pregnancy loss rates, time to pregnancy, and live birth rates between women with TSH < 2.5 mIU/L and those with TSH ≥ 2.5 mIU/L [23]. Interestingly, a recent study by Korevaar et al. showed that higher maternal free T4 levels are associated with lower child IQ and lower gray matter cortex volume. These findings suggest that thyroid hormone therapy for subclinical hypothyroidism to reach high-normal free T4 levels may negatively affect the child neurodevelopment [40].

Although the above studies show no clear difference in pregnancy and miscarriage rates following IVF or IUI when comparing patients with TSH < 2.5 mIU/L to those with TSH between 2.5 and 4.0 mIU/L prior to achieving a pregnancy, there is a discrepancy in recommendations regarding treatment. The American Thyroid Association has made the recommendation to treat women with subclinical hypothyroidism who are undergoing infertility treatment for any TSH elevation >2.5 mIU/L [14]. The rationale of these recommendations was based on the combination of the two following findings: (1) the improvement of outcomes when treating preconception TSH > 4.5 mIU/L and (2) the variability in TSH levels. Thus, they concluded that it would "seem prudent" to recommend treating women undergoing ART for TSH > 2.5 mIU/L [14]. In contrast, the American Society for Reproductive Medicine, in the practice committee guideline document on subclinical hypothyroidism, proposed two options for managing patients with TSH levels between 2.5 and 4 mIU/L [11]. The first is to monitor TSH levels and treat when TSH is >4 mIU/L. No specific interval for testing TSH is recommended, but as there is variability, one could justify monitoring after 4–8 weeks and as needed thereafter. TSH should then be monitored in pregnancy, but as the nadir for TSH is in the late first trimester, it is unclear when treatment should be initiated. The second option is to treat aiming to maintain TSH < 2.5 mIU/L. The ASRM Guideline recommends treatment for TSH > 4 mIU/L [9].

7.4 Subclinical Hypothyroidism and Children Developmental Outcomes

Maternal thyroid hormones are transported to the fetus throughout the pregnancy and play an important role in the fetal brain development [41, 42]. They are critical especially in the first trimester because the fetal gland starts concentrating iodine and synthesizing thyroid hormone at approximately 12–13 weeks of gestation [43]. The impact of maternal overt hypothyroidism on the children neurodevelopmental outcomes has been well established [44–46]. Haddow et al. [44] measured TSH in stored serum samples collected from 25,216 pregnant women and compared the performance of children of 62 women with overt hypothyroidism (TSH > 99.7th percentile or the combination of TSH of 98–99.6th percentile with T4 of 7.75 ug/dL) with those of 124 matched control women. They showed that the 7- to 9-year-old children of women with hypothyroidism performed slightly less well on 15 tests related to intelligence, attention, language, school performance, reading ability, and visual-motor performance compared to those with lower TSH levels. Moreover, Pop et al. [45] assessed the neurodevelopment of 202 healthy children at 10 months of age using Bayley Scales of Infant Development and correlated the scores to maternal TSH and free T4 levels at 12 and 32 weeks' gestation. Children of women with free T4 less than 10th percentile at 12 weeks' gestation had lower scores compared to children of mothers with higher free T4 levels. In an attempt to provide evidence of causality between maternal hypothyroidism and low intelligence quotient (IQ) in children, Klein et al. [46] compared the IQ of children at 8 years of age between those of mothers with TSH < 98th percentile, 98th to 99.85th percentile, and ≥ 99.85th percentile at 17 weeks' gestation. They showed a negative correlation between the severity of maternal hypothyroidism and IQ of children supporting a causal relationship.

In order to investigate whether subclinical hypothyroidism is associated with children neuropsychological development, Li et al. [47] compared the intellectual and motor development scores of children at 25–30 months of age of mothers with subclinical hypothyroidism (TSH > 4.2 mIU/L with normal free T4 at 16–20 weeks' gestation) to controls. They found that children of the subclinical hypothyroid mothers had lower scores than those of the control group. This study is limited by its retrospective nature and the small number of mothers with subclinical hypothyroidism (n = 18) [47]. Williams et al. [48] also showed that higher maternal TSH levels at delivery of infants born preterm were associated with lower scores on the general cognitive index of children at 5.5 years of age. Of note, 27% of these mothers had subclinical hypothyroidism (euthyroid with TSH ≥ 3 mIU/L) and 28% had elevated thyroglobulin antibody.

In contrast, Momotani et al. [49] reported five women who were diagnosed with overt hypothyroidism and started treatment at 6–16 weeks' gestation. One women remained subclinical hypothyroid, while the other four restored their euthyroidism status by 20 weeks' gestation. The children of these mothers had comparable development scores to those of euthyroid mothers suggesting that low maternal T4 in

early pregnancy does not affect neurodevelopment in iodine-sufficient areas [49]. Behrooz et al. [50] also examined the effect of subclinical hypothyroidism on children's intellectual development by comparing children of 19 mothers with subclinical hypothyroidism (TSH > 3 mIU/L before 20 weeks' gestation) to those of 19 mothers with normal TSH levels while receiving thyroid hormone supplementation. Children in both groups had similar cognitive performance and IQ levels suggesting that subclinical hypothyroidism does not influence children's intellectual development [50]. Likewise, Chen et al. [51] compared 106 children of mothers with subclinical hypothyroidism to 106 children of euthyroid mothers in terms of neurodevelopment using Gesell development test. There was no difference in the scores between the two groups at 12 and 24 months of age.

In the setting of these conflicting findings on the impact of subclinical hypothyroidism on the developmental outcomes, there were two randomized controlled trials investigating whether treatment of this condition would improve outcomes [52, 53]. In the first trial, Lazarus et al. [52] measured TSH and free T4 levels of 21,846 women in the first 16 weeks of gestation. They divided participants with subclinical hypothyroidism (TSH > 97.5th percentile and/or free T4 < 2.5th percentile) randomly into treatment group (levothyroxine supplementation to reach TSH 0.1–1 mIU/L; $n = 390$) and screening group (no treatment; $n = 404$). The IQ scores of children at 3 years of age were comparable between the two groups, suggesting that levothyroxine treatment that was initiated at a median gestational age of 13 weeks and 3 days did not improve cognitive function. In the second trial, Casey et al. [53] randomly allocated 677 women with subclinical hypothyroidism (TSH > 4 mIU/L with normal free T4) between 8 and 20 weeks' gestation to receive levothyroxine or placebo. There was no significant difference in the IQ scores of children at 5 years of age between the two groups. Given the non-beneficial effects of levothyroxine treatment, it is valuable to mention a large prospective study that investigated the association between child IQ and maternal thyroid function [40]. They found that high-normal maternal free T4, which is usually achieved after treating subclinical hypothyroidism, was associated with lower child IQ and lower gray matter and cortex volume. In addition, a recent retrospective study compared the pregnancy outcomes of 843 treated to 4562 not treated subclinical hypothyroid women [33]. Although treated women had lower rates of miscarriage (odds ratio [OR] 0.62), they had higher rates preterm delivery (OR 1.60), gestational diabetes (OR 1.37), and preeclampsia (OR 1.61) [33]. Therefore, overt hypothyroidism is likely to be associated with adverse developmental outcomes, and it requires treatment. However, subclinical hypothyroidism is unlikely to be linked to a delay in neurodevelopment, and thus it should not be a reason to treat subclinical hypothyroidism in pregnancy.

While there is a clear association between overt hypothyroidism and neurodevelopment issues in offspring, there is only fair evidence linking subclinical hypothyroidism and neurodevelopmental issues. According to the two guidelines, there is fair or insufficient evidence that treatment of subclinical hypothyroidism, defined as TSH outside the normal pregnancy range, does not improve neurodevelopmental outcomes [9, 14]. However, there are no data evaluating the impact of TSH between

2.5 and 4.0 mIU/L either preconceptionally or in early pregnancy on neurodevelopment of the offspring [9, 14]. However, the lack of data does not exclude a potential harmful effect nor a potential beneficial effect of treatment especially as overt hypothyroidism during pregnancy is correlated with adverse neurodevelopmental effect. Of note, there is no evidence that preconception TSH between 2.5 and 4 mIU/L are correlated with adverse neurodevelopmental outcomes [11].

7.5 Thyroid Antibodies and Female Reproduction

Thyroid antibodies include antithyroid peroxidase (TPOAb) and anti-thyroglobulin (anti-Tg) antibodies. These antibodies are present in 2–17% of unselected pregnant women and prevalence varies with ethnicity [14]. The vast majority of studies evaluating the effect of thyroid antibodies used only TPOAb; therefore, it is recommended to assess only TPOAb when testing for thyroid autoimmunity [14]. Thyroid autoimmunity is associated with an increased risk of hypothyroidism during pregnancy as the thyroid gland is less able to increase thyroid hormone production. However, the data concerning the significance of thyroid antibodies in female reproduction are mixed.

Studies evaluating the presence of thyroid autoimmunity in women with infertility are mixed. In a prospective study, Poppe et al. [22] compared the prevalence of thyroid autoimmunity between 438 women of infertile couples and 100 age-matched parous women. Women with infertility of female origin, especially endometriosis, had a significantly higher rate of TPOAb-positive compared to controls [22]. However, this association was not identified by Kutteh et al. [54] who found that the prevalence of TPOAb-positive was comparable between 688 infertile women pursuing IVF and 200 healthy controls. In addition, TPOAb-positive euthyroid women undergoing IVF had comparable pregnancy rates to those with negative TPOAb [55]. Also, levothyroxine supplementation for TPOAb-positive euthyroid women did not improve their pregnancy rates [55].

Some studies have suggested that TPOAb are associated with an increased risk of miscarriage [56–58]. A study of 552 women screened for thyroid antibodies in the first trimester showed that 19.6% had positive thyroglobulin or thyroid peroxidase antibodies, which were associated with higher miscarriage rates compared to autoantibody-negative women [56]. The investigators mentioned that the difference in miscarriage rates could not be explained by the differences in thyroid hormone levels, maternal age, previous obstetric history, or the presence of cardiolipin autoantibodies [56]. A meta-analysis of 31 studies showed that the presence of thyroid autoantibodies was associated with a significant increase in miscarriage rates and preterm birth rates [57]. A more recent meta-analysis of 12 studies showed that the presence of thyroid autoantibodies may be associated with higher miscarriage rates and lower live birth rates [58]. However, the investigators quoted a cautious interpretation of the findings owing to the heterogeneity among studies. Negro et al. [55] conducted a prospective study showing that levothyroxine supplementation to

TPOAb-positive women reduces the miscarriage rates [55]. However, the same group showed more recently that levothyroxine supplementation did not affect the miscarriage or preterm delivery rates in euthyroid women (TSH < 2.5 IU/L) with positive thyroid autoantibodies [59].

There are several studies that do not demonstrate an adverse effect of thyroid antibodies on miscarriage and liver birth rates. A large retrospective study of 3143 infertile women undergoing IUI found that those with TPOAb-positive women had similar miscarriage and live birth rates to TPOAb-negative women [39]. A large prospective study of women with history of one or two pregnancy losses showed that time to conception, pregnancy loss rates, and live birth rates following natural conception were comparable between those with and those without thyroid autoimmunity [23]. Furthermore, Lukaszuk et al. [60] compared the pregnancy outcomes between 114 TPOAb-positive and 495 TPOAb-negative infertile women undergoing IVF-intracytoplasmic sperm injection (IVF-ICSI). They both had comparable fertilization, implantation, pregnancy, and live birth rates. Sakar et al. [61] also found no significant differences in pregnancy and miscarriages rates between 49 TPOAb-positive and 202 TPOAb-negative women following IVF. Similarly, Tan et al. [62] showed that thyroid autoimmunity did not affect IVF-ICSI outcomes of 835 women.

While there is good evidence that thyroid antibodies are associated with miscarriage, the mechanism underlying the association is not clear. Hypotheses include antibody-mediated mild thyroid hypofunction, cross-reactivity of antithyroid antibodies with hCG receptors, the presence of concurrent autoimmunity, and increased levels of endometrial cytokines [14]. However, there is insufficient evidence to conclude that thyroid replacement reduces miscarriage rates or preterm delivery rates in euthyroid women with TPOAb. It is recommended that euthyroid women who are thyroid antibody positive have serum TSH tested at the time of pregnancy and every 4 weeks through midpregnancy [14]. Levothyroxine treatment may improve pregnancy outcomes in TPOAb-positive women with TSH > 2.5 mIU/L [9].

7.6 Who Should Be Screened for Thyroid Disease?

Whether to routinely screen for thyroid disease before or during pregnancy remains controversial. While screening women for thyroid disease prior to conceiving may be beneficial, there are currently no data to support this tactic. The Endocrine Society Clinical Guideline on the management of thyroid dysfunction during pregnancy recommended that women with infertility should have screening with TSH as part of their infertility evaluation [63]. The American Thyroid Association in 2011 recommended that all women with infertility should have screening TSH as part of their infertility work-up as the prevalence of hypothyroidism, including subclinical hypothyroidism ranges from 1 to 43% [11]. However, this statement fails to acknowledge that the majority of infertility patients with hypothyroidism will also have ovulatory dysfunction [24, 25]. In the 2017 updated guideline on thyroid

disease by the American Thyroid Association, screening serum TSH is recommended for all women who are at high risk for thyroid disease including those with a history of infertility, pregnancy loss, preterm delivery, thyroid disease, or age > 30 years among others [14]. However, it is noted in the guideline that there is insufficient evidence to recommend for or against preconception screening with the exception of women planning assisted reproduction or those known to have thyroid peroxidase antibodies (TPOAb) [14]. The recommendation to screen all women with infertility is based on the association of infertility with hypothyroidism and the low risk associated with treatment of subclinical hypothyroidism. In those high risk women, TSH should be rechecked as soon as pregnancy is confirmed. Universal screening for hypothyroidism in pregnancy and in all women attempting pregnancy (in the absence of infertility) is not recommended [10, 14]. Targeted screening based on clinical signs or symptoms that suggest overt hypothyroidism is recommended [10].

Screening for thyroid antibodies should be performed when TSH > 2.5 mIU/L in both infertile and pregnant women [14].

7.7 Conclusions

Subclinical hypothyroidism is typically defined as TSH > 4 mIU/L with a normal T4. Each laboratory should determine its upper limit of normal for the third-generation assay taking into consideration patient's age. For the laboratories that don't have this capability, a threshold of 4 mIU/L can be considered. The upper limit of reference range during pregnancy has been well established: 2.5 mIU/L for the first trimester and 3 mIU/L for the second and third trimesters.

Lowering the upper limit of TSH to 2.5 mIU/L has been advocated for certain high risk groups including women with infertility. While subclinical hypothyroidism with TSH > 4 mIU/L has been associated with adverse pregnancy outcomes, data are lacking regarding the impact of TSH between 2.5 and 4 mIU/L with respect to fertility, pregnancy outcomes, or fetal neurodevelopment. Current recommendations by the Endocrine Society and the American Thyroid Society recommend treating TSH > 2.5 mIU/L in this population, despite lack of convincing evidence of benefit, as these women may be at increased risk of developing hypothyroidism and the risks associated with treatment are low [14]. However, in the latest guideline, they reserve the strong recommendation for women undergoing ART and allow for monitoring TSH in women with infertility not undergoing IVF. The American Society of Reproductive Medicine recommends two treatment algorithms for infertility patients with TSH between 2.5 and 4 mIU/L based on lack of evidence showing adverse outcomes: treating TSH > 2.5 mIU/L with levothyroxine or monitoring TSH and treating if TSH > 4 mIU/L [9]. More data are needed to evaluate the impact of TSH between 2.5 and 4 mIU/L on reproductive outcomes and determine the best treatment algorithm that maximizes therapeutic effect and minimizes unnecessary treatment.

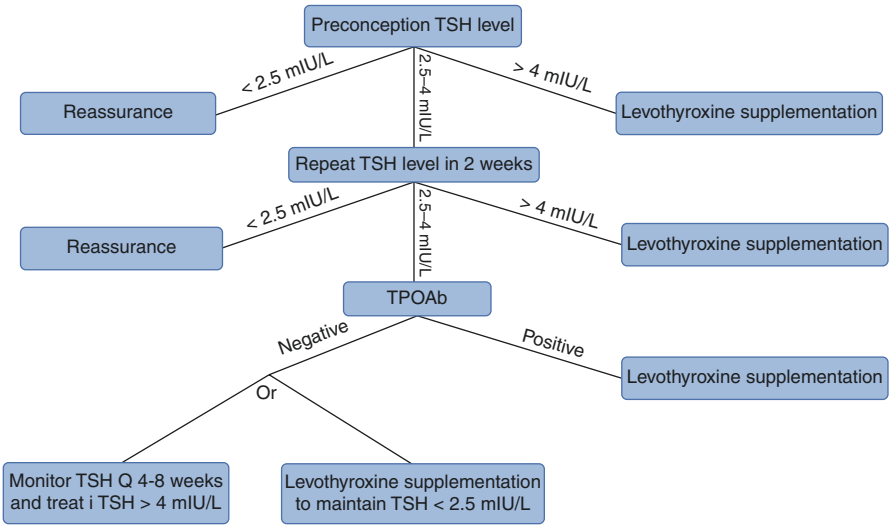

Fig. 7.1 Assessment of thyroid function in infertile women. *TSH* thyroid-stimulating hormone, *TPOAb* thyroid peroxidase antibodies

The association between the presence of thyroid peroxidase antibody and infertility or miscarriage is controversial. Thyroid hormone supplementation may be beneficial for women who have both positive thyroid peroxidase antibodies and TSH > 2.5 mIU/L owing to their suspected higher rate of progression into hypothyroidism. Therefore, it may be useful to assess for the presence of TPOAb in women with TSH > 2.5 mIU/L (Fig. 7.1). Also, patients with TSH < 2.5 mIU/L but who are positive for TPOAb need to monitor TSH every 4 weeks during the first 20 weeks of pregnancy [14].

Screening for thyroid disease is reasonable for infertile women with ovulatory dysfunction and those with other risk factors such as personal history of thyroid disease, symptoms of thyroid disease, and the presence of goiter or a thyroid nodule. However, the American Thyroid Society and the Endocrine Society have recommended screening for all infertility patients as they are considered "high risk" of developing hypothyroidism. This recommendation is confusing, as many studies have shown that infertility patients have a similar risk of hypothyroidism when compared to controls, especially in the absence of ovulatory dysfunction. Despite this observation, TSH screening test has become a standard test in the basic evaluation of infertility, and clinicians are obligated to decide how to manage TSH valued between 2.5 and 4 mIU/L without strong evidence in the literature. It is interesting that universal screening for thyroid disease is not recommended for all women attempting pregnancy nor for all women who are newly pregnant according to ASRM and ACOG guidelines. More high-quality studies are needed that evaluate subclinical hypothyroidism and reproductive outcomes in women with infertility and the benefits of thyroid hormone replacement.

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Chapter 8
Endocrine-Disrupting Chemicals and Female Fertility

Ronit Machtinger

8.1 Introduction

Growing evidence suggests that exposure to low levels of environmental toxins at several different life stages may impair animal and human fertility [1–3]. Some of these chemicals can alter the endocrine system either by simulating or blocking endocrine actions and are therefore known as endocrine-disrupting chemicals (EDCs). EDCs can be estrogenic or antiandrogenic or impact thyroid function. They are also known to bind to nuclear, nonnuclear, steroid, and nonsteroid receptors, leading to alteration of pathways such as those involved in steroidogenesis and hormone metabolism [4–6].

Among the EDCs with ubiquitous exposure in western countries are the plasticizers bisphenol A (BPA) and phthalates [7, 8]. BPA is commonly used in the lining of food cans and beverages, polycarbonate bottles, thermal receipts, and dental sealants [7, 9–12] while phthalates can be found in cosmetics, personal care products, children's toys, food packaging, building materials, and household furnishings [13]. Low levels of BPA and phthalates have been detected in human follicular fluid [14–16]. While most of the data concerning how phthalates impact fertility have been reported in males, because some of phthalate metabolites are antiandrogenic, BPA has been primarily linked to female infertility as it mimics the structure and function of the hormone estradiol and has the ability to bind and activate estrogen receptors. This chapter summarizes the effects of EDCs, specifically BPA, on female fertility.

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8.2 Oocyte Meiosis

Oocytes arise from primordial germ cells during the development of the female fetus. Following DNA replication, oocytes enter prophase I of the first meiotic division during which the chromatin condenses and recombination occurs. Shortly thereafter, the chromosomes decondense, and the oocytes become surrounded by a single layer of granulosa cells (primordial follicles) [17]. Oocytes remain arrested at prophase I (i.e., at the germinal vesicle (GV) stage), until adolescence. After puberty, cohorts of oocytes and their surrounding follicles enter the growth phase and resume meiosis during ovulation in response to the luteinizing hormone (LH) surge. The stages of meiotic initiation in the fetal ovary and recommencement of meiosis in the adult ovary can be specifically vulnerable to EDCs [3].

8.3 BPA and Oocyte Meiosis

The effects of BPA on the early stages of the first meiosis have been tested both in rodents and rhesus monkeys. Treatment of mice with a low dose of BPA [400 ng (1.7 μM) daily] in mid-gestation results in synaptic defects and increased rates of recombination in the oocytes of the offspring [18]. Similar effects were reported in rhesus monkeys as exposure to BPA during the second and third trimesters of pregnancy [mean levels of bioactive (unconjugated) BPA measured in maternal serum <1 ng/mL] resulted in alterations in the meiotic prophase and impaired follicular formation in the exposed fetuses [19].

Brieño-Enríquez et al. assessed the effects of BPA on human fetal oocytes from ovaries cultured for 7, 14, and 21 days with or without BPA [20]. Oocytes exposed to ≥1 μM of BPA showed increased rates of degeneration, and those exposed to 10 μM of BPA showed a delay in meiotic progression [20]. These findings are in line with the previously mentioned animal studies, although the concentrations of BPA were much higher compared to the levels reported in human follicular fluid (1–2 ng/mL) [15]. The same group also showed that exposure of human fetal oocytes to a concentration of 30 μM BPA resulted in impairment of the expression of *Spo11*, *H2ax*, *Blm*, and *Rpa*, all of which are genes known to be associated with double-strand break generation, signaling, and repair [21].

Studies in rodents have indicated that exposure to BPA during the final stages of oocyte maturation leads to a decreased number of MII oocytes, abnormal spindle formation, malalignment of chromosomes [22–25], and even aneuploidy in mice [26]. Hunt et al. was the first to report a sudden, spontaneous increase in meiotic disturbances, including aneuploidy in mice accidentally exposed to BPA [26]. Can et al. tested the effect of BPA on meiotic maturation in cumulus-oocyte complexes (COCs) from mice. In vitro exposure of oocytes to BPA (10 and 30 μM) during the first meiotic division leads to meiotic and spindle abnormalities [25]. Lenie et al. incubated follicles from mice with various concentrations of BPA (3 nM–30 μM) for 12 days and found that exposure to 30 μM BPA resulted in a significant increase in MI-arrested oocytes with spindle aberrations and unaligned chromosomes. The

authors reported a nonlinear dose-dependent effect of the chemical on the meiotic spindle with malalignment of chromosomes in MII oocytes that were exposed for prolonged periods to low concentrations of BPA (3 nM–3 μM) [24]. In addition, mouse oocytes cultured in medium containing 10 μg/mL BPA showed a significantly higher incidence of cytoskeletal aberrations compared to controls [22].

Based on the results from the above animal studies, human studies have been conducted to determine whether BPA also interferes with meiotic maturation in humans. GV-stage oocytes from patients undergoing IVF were cultured with BPA at concentrations ranging from 20 ng/mL (0.09 μM) to 20 μg/mL (88 μM). As the BPA dose increased, there was a significant decrease in the likelihood of an oocyte progressing to MII and a significant increase in the percentage of degenerated oocytes or that had undergone abnormal activation. Increased BPA dose also resulted in a significant trend for a decreased incidence of bipolar spindles ($P < 0.0001$) and aligned chromosomes ($P = 0.02$) in MII oocytes [27].

8.4 BPA and Steroidogenesis

Previous animal studies have shown that granulosa cell steroid hormone synthesis is compromised after BPA exposure, but the findings have been difficult to replicate in humans due, in part, to the limited amount of available discarded biological material [28]. In our recent work, treatment of granulosa cells with high doses of up to 20 μg/mL BPA impairs progesterone and estradiol production in a human in vitro model [29]. These concentrations also significantly reduce the mRNA levels of 3β-hydroxysteroid dehydrogenase (*3β-HSD*), *CYP11A1*, and *CYP19A1* without affecting *StAR* and 17β-hydroxysteroid dehydrogenase mRNA expression. Similarly, 3β-HSD, CYP11A1, and CYP19A1 protein levels have been found to be reduced after administration of 20 μg/mL BPA [29]. These results support the idea that only supraphysiologic concentrations of BPA (100-fold that baseline exposure) affect granulosa cell steroidogenesis.

8.5 BPA Exposure, Embryo Quality, and Blastocyst Formation

The effect of BPA on the development of embryos has been assessed in several animal models. In mice, pregnant females gavaged daily with 800 mg/kg/day of BPA have been found to have significantly impaired blastocyst development and hatching rates compared to controls. Moreover, the rate of the apoptotic cell number of hatched blastocysts in mice that were exposed to 400–800 mg/kg/day of BPA was greatly increased compared to controls [30]. In bovine, acute exposure of embryos (days 3.5 to 7.5 postfertilization) to 10 ng/mL of BPA decreased both blastocyst formation rate and the percentage of top-quality embryos compared to embryos that were not exposed to BPA [31]. In another study, bovine oocytes were matured

in vitro with several concentrations of BPA up to 30 ng/mL. Exposure of oocytes to 30 ng/mL BPA during in vitro maturation resulted in decreased embryo cleavage and blastocyst rates, increased apoptosis, and a skewed sex ratio with a higher proportion of female embryos at blastocyst stage [32].

8.6 BPA and Implantation

Several studies have investigated the effect of BPA on uterine receptivity in rodents. In mice, injection of a single dose of 10.125 mg BPA on the day of insemination, or a single dose of 6.75 or mg/10.125 mg BPA on the day after insemination, resulted in a decrease in the number of implantation sites [33]. Pregnant female mice treated subcutaneously with 100 mg/kg/day BPA from gestation days 0.5 to 3.5 showed retention of embryos in the oviduct and delayed embryo development on day 3.5 with no implantation sites detected on day 4.5. The authors of this study also describe an absence of implantation sites when untreated healthy embryos are transferred to pseudopregnant females treated with 100 mg/kg/day BPA, thus implicating BPA-induced disruption in uterine receptivity. Treatment with 40 mg/kg/day BPA is associated with delayed implantation and increased perinatal lethality of offspring while implantation is normal in mice exposed to lower BPA concentrations [34]. Chronic exposure (34 days) of prepubertal female mice to 60 or 600 μg/kg/day of BPA (a dose of 50 μg/kg/day is considered safe for human consumption) results in delayed implantation, a dose-dependent decline in the number of implantation sites, and a marked impairment in decidualization in the BPA-exposed uteri compared to controls [35]. Martínez-Peña et al. recently assessed the effect of exposure to BPA during the perinatal period on the fertility of the first generation (F1) and on the expression of tight junction proteins in the uterus during early pregnancy in a rat model. Pregnant rats (F0) received either a low dose of BPA (0.05 mg/kg/day), a high dose of BPA (20 mg/kg/day), or a vehicle only. BPA treatment induced alterations in implantation rate. BPA treatment during the perinatal period altered the expression of tight junction proteins in the endometrium and decreased the number of implantation sites in F1 animals that reached puberty and became pregnant [36]. Taken together, these studies suggest that exposure to BPA at high doses can alter implantation either by mismatch between the timing of blastocyst formation and the window of uterine receptivity, or by direct perturbation of uterine receptivity due to the estrogenic properties of BPA.

8.7 Clinical Studies

8.7.1 BPA and Ovarian Reserve Parameters

A few studies have investigated the association between BPA, ovarian reserve parameters, and IVF outcome. Souter et al. tested possible associations between

urinary levels of BPA, antral follicle count (AFC), day 3 serum follicle-stimulating hormone levels (FSH), and ovarian volume in a cohort of 209 women undergoing IVF. BPA was measured in most of the urine samples at an average concentration of 1.6 μg/L. While there was an average decrease in AFC of 12%, 22%, and 17%, in the second, third, and fourth BPA quartiles (respectively) compared to the first quartile, no associations between BPA and day 3 FSH or ovarian volume were reported [37]. Bloom et al. also investigated the effect of BPA on ovarian reserve parameters in a cohort of 44 women undergoing IVF. No associations were detected between fasting serum BPA levels and FSH levels and baseline AFC. The dissimilarities between the results of the two studies might be attributable to the different body fluids in which BPA was assessed (urine vs. serum) or to the relatively small number of patients [38]. Zhou et al. investigated the effects of BPA on FSH, AFC, anti-Müllerian hormone (AMH), and inhibin B (INHB) in 268 infertile women diagnosed with polycystic ovary syndrome (PCOS). Exposure to BPA was ubiquitous in this study group, with a median concentration of 2.35 ng/mL. BPA was significantly associated with a decrease in AFC but not with AMH and day 3 FSH levels nor INHB [39].

8.7.2 BPA and IVF Outcomes

Several epidemiological studies have investigated possible associations between BPA concentrations and IVF outcomes. Mok-Lin et al. followed 84 women that underwent 112 IVF cycles as part of the Environment and Reproductive Health (EARTH) Study [40]. Each participant provided two urine samples during the cycle for the measurement of BPA concentrations. BPA was identified in 85% of the urine samples with a range between <0.4 and 25.5 μg/L. BPA concentrations were inversely associated with the number of oocytes retrieved and peak estradiol levels on the day of hCG triggering. Another study from the same group in a larger cohort of 174 women (237 IVF cycles) showed a consistent decrease in E_2 levels as well as a significant inverse correlation between BPA concentrations and the number of oocytes retrieved and mature oocytes (metaphase II oocytes) [41]. Bloom et al. [38] evaluated fasting unconjugated BPA concentrations in serum and follicular response to exogenous ovarian stimulation in another cohort of women undergoing IVF. Forty-four women undergoing IVF were included in this preliminary study. BPA was detected in the serum of 86.4% of the women with a median concentration of 2.5 ng/mL (range = 0.0, 67.4 ng/mL) [38]. BPA levels were inversely correlated with estrogen levels although no association was found between BPA levels and the number of oocytes retrieved [38]. Another study from the same group investigated the effect of serum unconjugated BPA concentrations on oocyte maturation [42]. Considering the whole study population of 57 women, no association was found between BPA concentrations and oocyte maturation. However, in a sub-analysis, the authors showed a 9% decrease in the probability of mature oocytes among nine Asian women undergoing ICSI [42]. The difference between the two cohorts regarding the association between BPA levels and number of oocytes retrieved might be attributed

to different assessment strategies [38]. In fact, for that reason, comparing the results of these two studies might be associated with bias [43]. BPA measured in the urine consists of both conjugated and unconjugated fractions and is dominated by the biologically inactive conjugated form [44], while BPA measured in the serum (unconjugated BPA) consists mainly of the biologically active fraction [45].

Ehrlich et al. reported an inverse correlation between BPA concentrations and normal fertilization among women from the EARTH study. However, urinary BPA concentrations were not associated with day 3 embryo quality. Women with higher urinary BPA had less blastocyst formation, but the results were not statistically significant (trend test P value = 0.08). The same group also tested the relationship between urinary BPA levels and implantation failure [46]. One hundred and thirty-seven women undergoing 180 IVF cycles were included in the study. The authors reported increased odds of implantation failure with higher quartiles of urinary BPA concentrations compared to the lowest quartile and showed a positive linear dose-response association between urinary levels of BPA and implantation failure.

Recently, a new study from the EARTH group including 256 women (n = 375 IVF cycles) from 2004–2012 contrasts the previously published results. Urinary BPA concentrations were not associated with endometrial thickness, peak E_2, or fertilization rates. Moreover, urinary BPA concentrations were not associated with implantation, clinical pregnancy, or live birth rates [47].

8.8 BPA Substitutes and Female Fertility

Increasing evidence of the possible harmful effects of BPA to human health has prompted the industry to use alternative chemicals such as the bisphenol analogs of bisphenol S (BPS) and bisphenol F (BPF) [48]. Liao et al. detected BPF and BPS in 55 and 78% of a sample of 100 Americans [48, 49]. While ideally the substitute chemicals would be inert, it is not the case with these replacements [48]. The effects of these substitutes are not well characterized, and their specific effects on female fertility remain unknown with a recent study showing similar or even worse negative effects of BPS on oocyte maturation and spindle formation in pigs [50]. Currently, the effects of these chemical substitutes on female fertility and IVF outcomes are still unexplored.

8.9 Conclusions

Current data indicate that exposure to BPA is associated with impairment to oogenesis, meiosis, and steroidogenesis, both in vitro and in vivo and in both animal models and human. Clinical data have suggested a possible inverse correlation between urinary BPA levels and peak estrogen levels, number of oocytes retrieved, and implantation rates among IVF patients, although a recent study in a larger

cohort of women contrast previous results. Increasing concern over the harmful effects of bisphenol A (BPA) on human health has prompted the use of other chemicals for BPA replacements such as BPS and BPF. However, it is unknown whether these are less harmful than BPA. Lack of data regarding a chemical's health hazard does not indicate that it is safe but simply suggests that no data are available to point toward damage. The risks are potentially high [51], and continuing research is necessary in this evolving field of reproductive toxicology.

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Part III
ART

Chapter 9
Is There an Optimum System for Culturing Human Embryos?

Jason E. Swain

9.1 Introduction

Multiple factors influence the success of an IVF program. Obtaining good quality gametes and ensuring appropriate uterine receptivity are paramount. However, despite the often large variability in these clinical aspects or the inherent properties of patients themselves, immense focus and critique are often placed in the processes that occur within the confines of the IVF laboratory. It is often viewed that much of what drives IVF success, or failure, is a result of what occurs during the time that the gametes and embryos reside within the laboratory. The IVF laboratory takes extreme measures to ensure consistency and repeatability: tracking inventory, monitoring equipment function, and assessing technician efficacy on a regular basis. This attention to detail and quantification of quality data may explain why such credit, and blame, is often assigned to the laboratory.

Certainly, when embryo development is poor or outcomes are low, one of the first suspected culprits is a suboptimal culture system. Indeed, laboratory conditions are one key component than can impact embryo quality. As a result, immense amounts of time and resources are devoted to identifying the "optimal" system to culture embryos. The key word here, however, is "system," as culturing embryos consists of several variables. Importantly, within the context of the entire culture system and interactions between variables, "optimal" conditions may vary slightly from laboratory to laboratory.

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9.2 Gamete Processing

Quality of preimplantation embryos is dependent upon quality of the gametes used to create the embryo. Unfortunately, a significant component of gamete quality is inherent and beyond influence of laboratory conditions. Thus, it should be mentioned, that if gamete quality is poor, even an "optimal" culture system is likely unable to overcome inherent abnormalities. That being said, sperm and oocytes are processed and cultured briefly within the laboratory, and these brief periods can impact quality. A detailed discussion regarding the processing of oocytes and sperm is beyond the scope of this review. However, some key factors should be considered. Care should be taken to ensure appropriate environmental conditions, including temperature and pH stability, during handling and manipulation of oocytes and sperm. This includes use of appropriate media formulated for the respective gametes. Precautions should also be taken to avoid excessive physical stress during manipulations occurring during procedures like sperm isolation/centrifugation and oocyte denuding.

Laboratories should permit adequate oocyte maturation prior to insemination/ICSI, often 2–4 h post-retrieval. It has been shown that completion of nuclear maturation does not occur until ~1 h following polar body extrusion and injection of oocytes prior to 2–4 h after retrieval yielded lower fertilization rates [1]. The culture of cumulus intact oocytes for 3–6 h prior to injection yielded superior fertilization and cleavage embryo development compared to those cultured <3 h [2]. Similarly, using patient randomization in a prospective trial, waiting for 4 h to inject cumulus intact oocytes following retrieval, yielded superior fertilization and blastocyst development compared to injection immediately after retrieval [3]. It should be noted that these timings are likely dependent upon the type of stimulation and trigger used and timing between the trigger and oocyte retrieval.

It is known that separation and processing techniques can impact sperm quality and DNA integrity and likely resulting embryo quality [4, 5]. Various studies exist comparing common sperm isolation practices such as density gradient separation, swim-up and also newer technological approaches [6–9]. A clear consensus as to the superior approach is not apparent from the literature and conflicting results may stem from subtle variations in techniques, such as gradient used, centrifugation speed and time of centrifugation, number of washes, media used, swim-up technique and other variables [10]. Appropriate sperm concentrations should be used for standard insemination cases, with 50,000–100,000 motile sperm/mL as standard accepted concentrations. Brief co-incubation of sperm with eggs has been shown to be sufficient and may avoid possible negative impacts of extended sperm exposure [11–13]. Elevated sperm concentrations and/or extended exposure could be detrimental to oocyte quality and embryo development due to increased ROS, lowered pH, or other media alterations. With regard to ICSI, at a minimum, normal morphology sperm should be utilized for injection with sperm assessment at 400×. Use of higher magnification approaches, such as IMSI, does not appear overly beneficial, though this may depend on patient population and what magnification is

normally used for ICSI [14–17]. Emerging sperm selection approaches, such as PICSI, may also be useful [18], though this likely depends on the patient population and semen characteristics [19].

9.3 Embryo Culture

Once a sperm has been successfully joined with an egg, formation of a zygote occurs. Continued development of the resulting preimplantation embryo in vitro, often to the blastocyst stage, is a requirement for a successful IVF program. Therefore, the culture conditions used in the laboratory must support this development. However, numerous variables are present in the embryo culture system. Thus, it is extremely difficult to do a properly controlled comparison of culture systems, considering the number of variables involved. Without an exhaustive comparison within the same laboratory, identification of an "optimal" system is practically unattainable.

That being said, there are key components of the culture system that have been found to be superior to alternative approaches and that are viewed as "best practices." Some of these key components, as well as other relevant variables, will be discussed.

9.3.1 Incubators

Minimizing environmentally induced stress within the IVF laboratory is crucial in creating a culture system optimized for embryo development and to achieve maximal assisted reproductive outcomes. Key environmental variables to consider include temperature, humidity and atmospheric/gas stability. These can subsequently impact properties of embryo culture media, such as pH and osmolality. Importantly, all of these potential stressors are regulated or impacted by the laboratory incubator. As a result, the incubator is arguably the most important piece of equipment in the embryo culture system.

Multiple culture incubator types exist with varying capabilities and differing methods of regulating their internal environment. Box-type incubators, initially developed to hold multiple flasks of somatic cells, have been long used for clinical IVF. These were later adapted to smaller box-type incubators, with lower volume and faster gas/atmosphere recovery following openings/closings. Most recently, a plethora of embryo-specific benchtop incubators have been development. These newer incubators tend to have several small chambers for individual patients to reduce the number of openings/closing and to speed gas recovery. They tend to also include direct heat for faster temperature recovery and various accessories, such as air filtration and other technologies aimed at improving environmental stability. As a result, selection of an appropriate culture incubator for the IVF laboratory has

become a complex process. To date, there is no clear consensus as to a superior incubator type, as exhaustive controlled comparisons have not been done, and as efficacy and environmental stability rely heavily on incubator management [20] (Table 9.1).

For any incubator, optimal function requires proper use and daily quality control (gas and temperature measurements/validation). An appropriate number of units is required to avoid overcrowding with patient samples and prevent excessive openings/closings. Reduced incubator openings appears to benefit mouse embryo development [21, 22], as well as improving human blastocyst development [23]. Importantly, it should be noted that while single-step culture media (discussed later) and new time-lapse incubator technology permit uninterrupted culture of embryos with no disturbance over 5/6 days of development, no clear benefit of this approach has been shown, indicating that other variables in the culture system must also be considered [24–26]. A mix of incubator types within an individual laboratory permits a wide variety of uses to accommodate different dish or test-tube types, as well as implementation of emerging culture technology.

9.3.2 Atmosphere

Today's optimal culture system requires the use of low oxygen within the incubator. Culturing embryos in reduced oxygen concentrations (~5%), compared to atmospheric concentrations of ~21%, have been long recognized as superior in various animal IVF systems [27–33]. This makes sense when one considers the female reproductive tract appears to have oxygen levels between 2 and 8% (see reviews [31]). Numerous studies have also demonstrated improved human embryo development, implantation, and pregnancy rates when using reduced oxygen concentrations [34–46]. Importantly, use of low oxygen throughout the entire culture period through day 5/6 appears to be required to see the most benefit [34, 36, 46]. While 5% is often the standard concentration used for low oxygen culture, it is currently unknown if even lower oxygen concentrations or differential oxygen concentrations during embryo development may yield superior outcomes [47, 48].

There is no consensus as to the superior method of achieving low oxygen conditions. Nitrogen gas is used to depress oxygen concentration down from atmospheric levels of 21%. Nitrogen gas can be supplied via a nitrogen generator, nitrogen gas siphoned from a high-pressure liquid nitrogen tank, or compressed cylinders of nitrogen or premixed cylinders of gas.

The exact mechanism of the benefit of low oxygen use for embryo culture is unknown. Use of low oxygen may be superior due to improved embryo metabolism, reduced ROS, or perhaps even improved air quality (reduced levels of VOCs).

Maintenance of an appropriate and stable CO_2 concentration by the laboratory incubator is also required to ensure a highly effective embryo culture system. The carbon from CO_2 can be utilized by developing embryos for biosynthetic processes [49–51]. Additionally, the CO_2 concentration inside the incubator is important in

Table 9.1 Comparison of embryo culture incubators

Study	Incubator comparison	Method	Outcome: results (statistical significance)	Conclusion and notes
[21]	MINC vs. forma large-box (water jacketed, TC CO_2 sensor)	Compared time to temperature recovery from 35 °C to 37 °C of 1.0 mL media w/ 0.1 mL oil overlay and 50 μL media w/1 mL oil overlay	MINC recovered temp within 5.5–6.5 min depending on media volume Forma did not recover temp by 20 min for either volume (reached 36.2 and 36.7 °C)	Direct heat transfer of the benchtop unit resulted in faster temperature recovery than the indirect warming system of the larger incubator An aluminum block can help with temperature recovery in the large indirect warming incubator
[22]	K-MINC benchtop vs. ASTEC small-box (TC CO_2 sensor, galvanic O_2 sensor, water jacketed)	RCT: Sibling embryos randomized after 2PN check 5 s door opening, repeated 10× and averaged	Temp recovery time: 5 min vs. 30 min *($p < 0.01$)* O_2 recovery time: 3 min vs. 8 min *($p < 0.01$)* Early "good" embryo rate: 40% vs. 38% *($p < 0.05$)* "Good" blast formation rate:15% vs. 8% *($p < 0.05$)*	Benchtop incubators offer improved culture environment recovery times Recovery time may influence the formation of good quality embryos
[23]	K-MINC benchtop vs. Forma large-box (high oxygen, TC CO_2 sensor, water jacketed, inner doors)	RCT: Sibling embryos randomized 10 s door opening and outcomes compared	Temp recovery time: 1 min vs. 180 min ($p < 0.01$) CO_2 recovery time: 8 min vs. 120 min ($p < 0.01$) Humidity recovery: 12 min vs. 180 min ($p < 0.01$) Fert rate: 72% vs. 67% ($p < 0.05$) Day 3 grade: (ns) Impl rate: 39% vs. 32% (ns) Clin preg rate: 64% vs. 54% (ns)	Benchtop incubators offer improved culture environment recovery times No differences were found between incubators in respect to embryo quality or outcomes

(continued)

Table 9.1 (continued)

Study	Incubator comparison	Method	Outcome: results (statistical significance)	Conclusion and notes
[24]	EmbryoScope vs. HeraCell 150 large-box (air jacketed, high O_2) EmbryoSlide individual culture vs. microdrop individual culture	RCT: Sibling embryos were randomized after 2PN check Morphological assessment was made at d2 and d3 Embryos were *not* removed from EmbryoScope for assessment Outcomes compared	Blast formation rate: 55% vs. 51% (ns) Blast viability: 29% vs. 35% (ns) Ongoing preg rate: 43% (6/14) vs. 42% (8/19) (ns)	No difference between the EmbryoScope and a standard incubator in blastocyst formation blastocyst viability rate or ongoing pregnancy rate
[25]	EmbryoScope vs. Galaxy R 170 large-box (direct heat, high O_2, IR CO_2 sensor) EmbryoSlide individual culture in small microvolume vs. group culture in Nunc wells of larger volume	RCT: Sibling embryos were randomized after insemination Morphological assessment at d2, d3, d5 Embryos were removed at equal time points for *both* incubators and outcomes compared.	Number of 4-cell embryos on d2: (ns) Number of 7–8-cell embryos on d3: (ns) Number of blasts on d5: (ns) Impl rate: 37% (7/19) vs. 33% (6/18) (ns) Clin preg rate: 38% (8/21) vs. 30% (7/23) (ns)	No difference was found between the EmbryoScope and standard incubator for embryo development, implantation rate, or clinical pregnancy rate
[26]	"Standard incubator" vs. EmbryoScope 20μl microdrops vs. Embryoslide	Patient randomization Transfers on D2 Embryos removed in the standard incubator 3×—not removed in EmbryoScope	Good-quality embryos: (ns) Fertilization (ns) 4-cell embryos on D2 (ns) Implantation (ns) Pregnancy (ns) Miscarriage (10.2% vs. 33.3% ($p < 0.01$) Ongoing pregnancy (ns)	No differences in embryo development or pregnancy were apparent. The EmbryoScope had higher rates of miscarriage

[27]	Standard incubator (20μl microdrops) vs. EmbryoScope (embryoslide)	Patient randomization	*Poor prognosis patients* Grade A and B embryos: (ns) Pregnancy rate (ns) Implantation rate (ns) *Donor patients* Grade A embryos (81.2 vs. 55.8%) ($p < 0.005$) Grade B embryos (36.8 vs. 7.7%) ($p < 0.01$)	No differences in day 3 embryo development implantation or pregnancy rates were apparent between incubators in poor-quality patients. The EmbryoScope yielded more poor-quality day 3 embryos in egg donor cases
[28]	Sanyo 170 L box incubator (high oxygen, Nunc 4-well dish) vs. EmbryoScope (low oxygen, embryoslide)	Retrospective matched pair analysis Patients cultured in EmbryoScope vs. "standard incubator"	Clinical pregnancy rate (36.5 vs. 44.8%) ($p < 0.02$) Implantation rate (322.2 vs. 39.3%) ($p < 0.03$) Live birth rate (33.8 vs. 43.1%) ($p < 0.01$) Miscarriage rate (244.4% vs. 18.9%) (ns)	Use of a time-lapse incubator and selection system yields superior outcomes to a standard incubator and morphological assessment
[29]	G185 (dry) vs. G185 (humidified)	Sibling oocyte split	Top quality embryo D3 (83 vs. 65%) ($p < 0.0001$) D3 compaction (22 vs. 55%) ($p < 0.0001$) Blastocyst rate (51 vs. 73%) ($p < 0.0001$) High-quality blastocysts (35 vs. 61%) ($p < 0.0001$) Cryopreserved blastocysts (33 vs. 55%) ($p < 0.0001$) Biochemical pregnancy (49 vs. 61%) ($p < 0.043$ Clinical pregnancy (43 vs. 57%) ($p < 0.017$) Ongoing pregnancy (37 vs. 52%) ($p < 0.008$) Miscarriage (12 vs. 9%) (ns)	The humidified incubator produced more high-quality embryos and higher pregnancy rates than the dry incubator

establishing the pH of the embryo culture media [52–54]. The CO_2 gas dissolves in the media and reaches equilibrium with the sodium bicarbonate concentrations to establish a set point. As CO_2 is increased, pH decreases and vice versa. This is important because the pH of culture media can impact embryo development. No clear consensus exits as to the optimal pH requirement of embryo culture media to optimize development. This may vary between media, due to the impact of ingredients, such as lactate [54], and may vary between embryo developmental stages [54, 55]. The value usually falls between 7.2–7.4 and may change if using a sequential cutlure medium. The amount of CO_2 required to achieve the desired pH will vary between labs based on lab elevation, type of culture media, and other variables (protein concentrations, etc.). Rapid and accurate CO_2 recovery and maintenance of CO_2/pH stability is paramount. Thus establishment of the desired pH range by the laboratory is required, with pH measurement to determine the required CO_2 concentration with routine verification.

9.3.3 Temperature

Temperature is another variable of the culture system that may impact efficacy. It is well known that temperature can impact various aspects of gamete and embryo function, most notably, meiotic spindle stability [56–58] and possibly embryo metabolism [59]. Studies have reported that maintaining a warm temperature around 35–37 °C compared to 25–30 °C during retrieval is beneficial for bovine embryo development and mouse embryo gene expression [60, 61]. Maintaining temperature stability around 37 °C during human oocyte injection also improved fertilization rates and embryo development [57]. However the optimal temperature at which to culture embryos remains unknown.

Commonly, 37 °C is used to culture embryos and is based on the estimate of human core body temperature. Importantly, body temperature varies and is likely not exactly 37 °C. Temperature can vary based on time of day, between the sexes and between individuals. Furthermore, temperature of the female reproductive tract may actually be slightly less than the core body temperature. The temperature of the human follicle is ~2.3 °C cooler than surrounding stroma, and the fallopian tube from animal models carries a temperature gradient ~1.5 °C cooler [62–67]. As a result, the question has been poised "whether human IVF and related procedures should be carried out, at, say, 35.5–36°C rather than at 37°C" [59]. Use of a 1.5 °C lower temperature would presumably lower the metabolic rate of the embryo ~15%, which may result in a more "quiet" metabolism, thereby possibly benefiting embryo development [59]. However, this hypothesis has not been proven.

Recently, a prospective randomized controlled trial utilized patient embryo splits and examined the effect of culture human embryos at either 37 or 36 °C [68]. Controlling for temperature variations, incubator type, and pH, authors demonstrated that culture of human embryos at 37 °C yields higher average cell numbers at day 3 of development, higher blastocyst formation, and higher useable blastocyst

Table 9.2 Comparison of culture temperature on development of human preimplantation embryos [69]

	MII's (n)	Fert rate	Day 3 cell number	Blast rate	Usable blast rate	Aneuploidy rate	Implantation rate
36.0 ± 0.07 °C	399	86.2%	7.0 ± 0.1[a]	51.6%[a]	41.2%[a]	42.5%	67.4%
37.0° ± 0.04 °C	406	82.0%	7.7 ± 0.1[b]	60.1%[b]	48.4%[b]	46.1%	73.3

Different superscripts within a column represent statistical significance, $p < 0.05$

rates compared to 36 °C (Table 9.2). No differences were observed in rates of aneuploidy or implantation. More detailed analysis of the optimal temperature for embryo culture is required, but the best practice at the moment appears to be maintenance of a stable temperature around 37 °C.

There is no clear advantage of one incubator type over another in terms of temperature regulation, though some types may recover temperature faster or maintain temperature better than others under various circumstances [20]. In addition to incubators, temperature stability should be monitored on other lab equipment, such as warm ovens and heating stages. The type of culture dish, as well as other factors that can impact heat exchange, should be considered. Careful monitoring of temperature and proper handling of embryos during use is imperative to ensure the desired conditions are achieved and maintained.

9.3.4 Group Culture

It is known that embryos can modify their surrounding microenvironments during culture though secretion or depletion of various proteins. Indeed, examination of the embryo secretome through analysis of spent culture media has become an active area of research [69–78]. Modification of their own microenvironment may impact embryo development during the culture period. It is therefore perhaps not surprising that group embryo culture has been shown to be beneficial over individual embryo culture for animal embryos, from both mono- and polyovulatory species. Various studies have shown a benefit of group culture of embryos from the rodent [79–83] as well as domestic species like the cow, sheep, and pig [84–91].

Similarly, studies exist that indicate human embryos may also benefit from group embryo culture [92]. Grouping of human embryos significantly enhanced cleavage rates but not morphology grade as compared with embryos grown individually [93]. Group embryo culture resulted in significantly improved pregnancy rates, with embryos grown in groups producing a 43% pregnancy rate per transfer, compared to a 24% pregnancy rate for embryos that were cultured individually [94]. A retrospective analysis of patients who had embryos cultured from day 1 to day 3, either individually or in groups of three to five embryos, had no differences in pregnancy or implantation rates, but those cultured in groups did result in more usable blastocysts [95]. This is similar to a prior prospective report that indicated no benefit of group culture of human embryos prior to day 2 or day 3 transfer [96], suggesting extended culture may be required to observe the full benefits of group embryo cul-

ture. Finally, prospective trial patients had their embryos split between three treatment groups using sibling embryos [9]. Embryos were cultured individually or in groups where embryos were physically separated but able to exchange media, or in groups without physical separation. Importantly, all treatments were included within the same culture dish to help control for variables. Group culture of embryos without physical separation resulted in greater rates of compaction on day 4 and blastocyst formation on day 5 compared to group culture with physical separation and embryos cultured individually. Group culture yielded a trend toward higher live birth rate compared to individual culture. This indicates that extended group culture of embryos may be beneficial for human preimplantation embryo development and that physical contact or proximity of embryos seems to be an important factor.

9.3.5 Culture Dishes

Various factors involved in group embryo culture can impact results, including volume of media and shape/size of the culture area. These factors can impact the ratio of media volume used to the number of embryos (embryo density) and embryo spacing. Importantly, these factors may be influenced by the type of culture dish utilized. The type of culture dish may impact heat exchange during embryo manipulations due to surface contact with the bottom of the dish. The type of culture dish may also be dictated by the type of incubator. Notably, new time-lapse incubator systems have proprietary culture dishes, many of which promote single embryo culture or that are aimed at trying to utilize a beneficial microenvironment created by the culture area. The type of culture dish could therefore have an impact on embryo quality and outcomes [97–99]. Currently, there is no consensus as to a superior type of culture dish or volume of media used for embryo culture.

Directly related to optimizing the culture system is an assurance that the culture dish, as well as other contact materials, is nontoxic. Contact material testing is one of the most important aspects of laboratory QC/QA and is paramount for improving embryo development. It is well documented that not all products, despite being packaged or sold as sterile, are inert in terms of the impact on embryo development [100, 101]. Thus, verification of material safety is required prior to clinical use. This is usually performed using a relevant bioassay, such as the mouse embryo assay (MEA) or the human sperm survival assay (HSSA), also known as the human sperm motility assay (HSMA). Comparisons and the merits of these two assays have been discussed elsewhere [102–108], and each can be useful in their own respect, with factors such as cost and availability warranting attention. Perhaps more importantly, the sensitivity of the bioassay is important, and approaches can be modified to increase this sensitivity to ensure that subtle material toxicity can be detected. Examples of approaches used to increase sensitivity are use of the one-cell vs. the two-cell MEA, outbred vs. inbred vs. hybrid mouse strains, blastocyst cell counts vs. simple blastocyst formation, exclusion of

protein from media vs. protein inclusion, and use of a simple media vs. a more robust complex media [109–111]. Importantly, thresholds must be set for an assay to "pass," and these thresholds should be set to ensure rigorous criteria. Each laboratory can determine which assay and threshold suits their needs regarding sensitivity and cost. A commonly used and often recommended sensitive assay includes using the one-cell MEA, noting time-appropriate embryo development with the rate of expanded or hatching blastocyst >70% at 96 h (4 days) of culture. Real-time assessment of morphokinetics may also be useful in helping improve sensitivity of the MEA [112].

9.3.6 Culture Media

In trying to discern key components of the "optimal" embryo culture system, focus often immediately turns to culture media. Unquestionably, culture media plays a significant role in embryo development and quality and therefore, clinical outcomes. However, in terms of identifying or selecting the single "optimal" medium for use within IVF laboratories, this is likely a futile endeavor. As mentioned, numerous variables are present within the laboratory and can impact culture media performance [113]. Thus, the same medium can perform differently between laboratories. The inability to identity a single optimum media is reflected by examining the numerous studies in the literature comparing one medium against another [114]. Indeed, when examining prospective trials using sibling oocytes splits or patient randomization, comparison of different sequential media [115–121], as well as comparisons between single-step culture media and sequential media [122–127], many studies fail to identify a clearly superior approach or product. No trial exists comparing every available media against each other in a controlled fashion, which would be required to identify the superior medium. It is also unclear whether changing media at 48 or 72 h intervals or utilizing uninterrupted culture yields superior results with modern media formulations. Individual laboratories should perform their own trials/splits to determine which product and methodology performs most successfully under their specific conditions. (see Table 9.3).

Modern human embryo culture media must contain some assortment of amino acids. Amino acids support numerous cellular processes, including acting as metabolites, osmolytes, antioxidants and buffers, and likely help alleviate stress in the embryo culture system [136]. Brief period of culture with no amino acids present can impair mouse embryo development [21]. While animal studies have given some insight [128, 129, 137], it is likely impossible to determine the optimal mixture and concentrations of specific amino acids for human embryo culture. Studies have identified glycine, taurine, and glutamine as important amino acids for human embryos [131–133]. If glutamine is present, modern embryo culture media should include the dipeptide form of the amino acid. Use of the stable dipeptide-glutamine helps avoid ammonium accumulation and the associated detrimental effects on

Table 9.3. Comparison of single-step and sequential media for culturing human embryos in randomized controlled studies focused on pregnancy, implantation, and live birth

A

Study	Media compared	Total number of transfers	Transfer day (2/3 vs. 5/6)	Clin preg rate/ transfer (seq vs. single)	Implantation rate—total	Implantation rate day 3 (seq vs. single)	Implantation rate day 5/6 (seq vs. single)
[128]	Sydney IVF cleavage vs. GM501	70 vs. 77	Day 3 or day 5		20 vs. 28%	19.6 vs. 26%	20 vs. 43%
[129]	ECM/multiblast vs. global	38 vs. 40	Day 5/6	52.6 vs. 72.5	36.8 vs. 57.5 ($p < 0.08$)	No data	36.8 vs. 57.5[a] ($p < 0.08$)
[130]	ISM1 vs. GM501	83 vs. 84	Day 2/3	26.5 vs. 22.6%	17.8 vs. 20.0	17.8 vs. 20.0	No data

B

Study	Media compared	Total number of transfers	Number of transfers for each media	Transfer day (3 vs. 4/5)	CPR D3 (seq vs. single)	CPR day 4/5 (seq vs. single)	Implantation rate D3 (seq vs. single)	Implantation rate D4/5 (seq vs. single)
[131]	G5 series vs. global	80	7 vs. 21 (52 had embryos from both)	Day 3 or day 5				
[132]	ECM/multiblast vs. SSM	49	13 vs. 17 (19 had embryos from both)	3, 4, 5	0 vs. 40% (0/1 vs. 2/5)	66.6 vs. 58.3%	0 vs. 28.6%	57.1 vs. 52.6%
[133]	Sage vs. global	73	17 vs. 25 (31 had embryos from both)	Day 3	58.8 vs. 48%		38.2 vs. 42%	
[134]	G1/G2 vs. G-TL	94	46 vs. 41 (7 had embryos from both)			50.0 vs. 56.1%		41.3 vs. 48.8%

C

Media system	Usable blast rate	Euploid rate (%)	Sustained implantation (%)
Quinns cleavage /blast assist	55.2%a	70.3	52.8
CSC	46.9%b	69.1	47.2

(A) Studies using patient randomization. (B) Studies using sibling embryo splits. (C) Study using pair comparison and genetic fingerprinting of sibling embryos from different media within the same patient [135]

[a]For the purpose of this review, media that were formulated to only support embryo development through day 3 of development, such as P1, IVF-50, or HTF, are not viewed as single-step media. Single-step media are media formulated to support embryo development throughout development to the blastocyst stage. These other media, often modifications of HTF, are commonly used with a separate second-stage media to grow blastocysts and, though not developed as a sequential system per se, will be viewed as a sequential media approach

human embryo development and appears superior to glutamine [130, 134, 135, 138]. No clear consensus exists regarding whether glycyl-glutamine or alanyl-glutamine is the superior dipeptide for human embryo culture. Whether use of other dipeptide amino acids may be beneficial is unknown.

The pH of culture media should be monitored and used adhering to manufacturers recommendations. As mentioned, CO_2 concentrations should be adjusted to achieve the desired/recommended pH. No clear consensus exists as to the ideal pH for embryo culture, whether this be a single pH set point throughout culture or whether pH should change during embryo development.

9.3.7 Protein

Protein supplementation to culture media can have a dramatic impact on embryo development and resulting outcomes. The macromolecule supplementation can act as a surfactant, nitrogen source to stabilize membranes, modulate physical microenvironment, act as a carrier molecule for other compounds, or even bind trace elements/toxins. A recent report even indicates that protein in culture media can impact human birth weight [139], though results of the study should be interpreted with caution due to lack of control over other system variables. Regardless, it is irrefutable that protein supplements are the ones least defined components of the culture system and thus are a large source of potential variation. For example, stabilizers/preservatives, such as octanoic acid, used when making protein solutions, may be embryotoxic [140]. Additionally, as mentioned, other proteins, growth factors, and hormone "contaminants" may be present at varying levels in protein preparation [141–143]. Some clinical IVF media currently include growth factors, though limited data exists to support their purposeful use [144–146], and this remains a controversial topic [147]. Thus, to optimize performance of the culture system, specific lot numbers of protein should be screened for efficacy prior to clinical implementation.

Primary protein supplements used in clinical embryo culture media include human serum albumin (HSA), as well as complex protein supplements containing HSA and a combination of alpha and beta globulins [148, 149]. Recombinant albumin is also available, as well as a dextran-based macromolecular supplement. Data suggest that a complex protein supplement with globulins is superior to HSA for animal embryos [148, 150], human embryo development [151, 152], and even live birth [153]. This may be due to growth hormones and other components in the complex protein products [142]. Recombinant albumin gave comparable embryo development to HSA in a prospective randomized trial and may reduce variability in the culture system [154]. Optimal concentrations of protein are unknown, though 5% of albumin or 10% of complex protein products are commonly used and higher concentrations of up to 20% of a globulin supplement may be beneficial in certain circumstances [155].

Care should be taken to ensure pH of the culture media is still within expected ranges after adding protein, as supplementation can cause pH to shift and may require adjustment of incubator CO_2 levels.

9.3.8 Oil

Oil overlay is commonly used during embryo culture to prevent media evaporation, which can result in media osmolality increase and compromised embryo development. Oil is required when using modern benchtop incubators with no humidification. However, open culture, or culture without oil overlay, may also be used if humidification is present and sufficient volume of media is used. Similar to protein, despite improvements in refinement and testing, oil overlay products can differ significantly due to contaminants and are a large source of variation within the culture system. Washing of oil with either water or culture media can help alleviate some toxicity concerns [156] and a good practice to ensure that all oil is washed. Additionally, proper storage is required to avoid peroxidation of oil, which can lead to embryo toxicity [156–159]. Recommendations often include keeping oil out of direct light and storing at 4 °C.

There are several oil products for embryo culture including paraffin oil and light mineral oil. Products are sold in plastic or glass bottles. There is no clear consensus on a superior oil type or packaging method. Regardless of the oil, testing using an approved bioassay is recommended prior to use.

9.4 Conclusions

It is evident that there are numerous variables to consider when attempting to optimize an embryo culture system. Interactions between variables can impact outcomes. Thus, an "optimum" system may vary slightly from laboratory to laboratory. Key variables that appear consistent between high-performing culture systems include use of low-oxygen, proper incubator management to yield stable CO_2/pH and temperature and proper quality control. Of course, even if a culture system is optimized, embryo selection remains a critically important factor to maximize outcomes. In fact, if a system is optimized to maximize embryo development, then selection of embryos becomes even more difficult, as there are more "good quality" embryos to choose from. Embryo assessment, noting key morphological features or morphokinetic timings/selection algorithms, or use of embryo biopsy/CCS become very important.

With advances in technology, culture "systems" have been introduced which consist of advanced incubator systems with imaging technology, proprietary customized cultureware, and embryo selection algorithms. Even if selecting one of these systems, differing culture media and other variables, such as protein and oil, often vary. This is likely at least one reason why there appears to be difficulty in identifying a single embryo selection algorithm that is applicable between laboratories. Regardless, existing data does not demonstrate that any of these emerging commercial systems is superior to another or to more traditional methods of culturing embryos to the blastocyst stage [160–164].

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Chapter 10
Evidence-Based Approaches to Embryo Selection by Morphology and Kinetics

Thomas Huang and Mina Alikani

10.1 Introduction: A Word About "Evidence-Based" Embryology

Evidence-based medicine (EBM) purports to objectively determine the efficacy of medical treatments in order to reduce cost and avoid unnecessary interventions. Randomized controlled trials (RCTs) and meta-analyses (or systematic reviews) that combine and analyze the results of multiple RCTs are the cornerstones of EBM [1]. The design of RCT studies is central to EBM and must incorporate the principles of randomization, blinding, allocation concealment, and full data reporting. Designing RCTs to evaluate the efficacy of drugs is rather unambiguous, but this is not the case with non-pharmacological interventions [2], and interventions in clinical embryology fall under this category [3].

Relevant to the present discussion, subjective assessment of embryo quality involves the skill of the observer, but the observer is rarely considered in study design or the analysis and interpretation of results. Moreover, in some cases, randomization of patients may not sufficiently address selection bias. Gametes and embryos add a layer of complexity to RCT design in embryology since, in most cases, each patient produces multiple embryos; observations related to sibling

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embryos are often treated as independent, but in fact, they are not. Thus, without proper statistical corrections or randomization based on embryos rather than (or in addition to) patients, the effect of interest may be skewed. Blinding is also difficult as embryologists may have visual and other clues to treatment groups.

Another major issue in clinical embryology is the choice of the primary outcome measure. Clinical pregnancy, while both important and relevant, is only partly a function of embryo quality; it is in major part a function of uterine receptivity. Embryo transfer technique and luteal phase support are other confounding clinical variables. Yet, many studies choose pregnancy, ongoing pregnancy, or clinical pregnancy (in fresh cycles) as primary outcome measure when assessing the efficacy of embryo selection methods. The same may be said about live birth rate, which is significantly influenced by number of embryos transferred and many other variables.

Left without clear guidelines, investigators continue to use a wide range of methodologies and approaches to clinical embryology RCT design. Again, in the context of this chapter, studies assessing the efficacy of embryo selection using time-lapse microscopy (TLM) have been particularly prone to design flaws, as highlighted in recent systematic reviews and commentaries [4–8]. Naturally, study design flaws reduce reliability of the conclusions of meta- analyses that include them, and that is where TLM finds itself today. Additionally, the safety of these technologies in terms of obstetric and perinatal health remains essentially an unexplored area, with only one study broaching the issue so far [9]. Clearly, more remains to be done [6].

To promote better-designed clinical trials and retrospective observational studies with sufficient power and the potential to provide high-quality evidence, clinical embryology professional groups could take on the task of constructing and issuing, through focused discussion and debate, specific consensus guidelines, and an effective framework within which investigators in our field may be persuaded to design clinical embryology studies [3].

We have two other disclaimers: first, many time-lapse microscopy platforms are currently available. We do not advocate for or against any of these systems. Our focus on the two systems described here is based on the number of studies citing their use, which is most likely a function of the timing of their clinical introduction. In the USA, these two platforms reportedly are being utilized by a majority of laboratories that use TLM [10]. Second, we do not specifically focus on selection of vitrified/warmed (frozen/thawed) embryos for transfer for two main reasons. In our view, with the increased efficiency of cryopreservation technologies and survival rates of nearly 100% in most laboratories, the main concern is selection of embryos *for* cryopreservation. Embryos that are judged as being suitable for transfer would, presumably, also be suitable for cryopreservation and transfer following warming/thaw. Furthermore, with high survival rates and emphasis on single embryo transfer, the need for warming/thawing of multiple embryos (and selection among those embryos) has been largely obviated.

10.2 The Standard Morphology Approach to Embryo Selection

Most (if not all) published studies on the relationship between morphology and viability have been retrospective observational studies. These studies have involved morphological "grading" of embryos during development in vitro via brief microscopic observations at discreet time points. Different grading systems have been developed and promoted through professional societies in the interest of standardization (e.g., Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology) [11, 12].

A number of morphological features of developing embryos have been associated with implantation or pregnancy. However, some of these features may be considered less relevant or practical because (1) they occur rarely (e.g., planar embryos) [13], (2) they require additional or prolonged observations (e.g., nucleolar patterns) [14, 15], (3) they are not routinely checked (e.g., Day 4 morphology) [16], or (4) they have produced contradictory results in different studies, due to methodology and/or study design (e.g., zona pellucida thickness and variation) [17, 18]. We will not discuss these features here.

The most relevant morphological features on Days 1–3 of development are pronuclear configuration (number, size, and location), cell number and size, degree of fragmentation, and blastomere nucleation status. Although the subject of much debate in terms of priority, during Days 5–7 in culture, the parameters include inner cell mass (ICM) shape and size, trophectoderm (TE) cell number, and cohesion and the degree of blastocyst expansion. Deviations from "normal" constitute transfer or cryopreservation exclusion criteria, based on association with diminishing blastocyst formation and/or implantation potential [19] (Table 10.1).

The limitations of embryo morphology as a measure of embryo quality are well known. Embryos with atypical morphology and "low" quality evidently have lower implantation potential than their normal morphology counterparts, but this potential is not null; on the other hand, "high" morphological quality embryos may still fail to implant. Moreover, studies suggest that obstetric and perinatal outcomes following transfer of embryos of varying "quality" are not negatively impacted by atypical morphology. One example is the study of Bouillon et al. [22]. These investigators assessed 799 singleton clinical pregnancies following single fresh Day 5 blastocyst transfer, categorizing the embryos as good, fair, poor, or early blastocysts. They confirmed previous findings that poor morphology was associated with diminished pregnancy and live birth rates. However, once a pregnancy was established, and after adjusting for confounders, there were no differences between the groups with respect to mean birth weight or length, low birth weight, small/very small for gestational age, or large/very large for gestational age, neonatal complications, or congenital malformations.

Table 10.1 Key morphological parameters on Days 1–6 in culture, associated with reduced development potential

Day	Morphology	Possible biological origin	Consequences
1	0 PN; 1PB	Failure of fertilization	No development potential
1	1 Large PN	Altered pronuclear dynamics or parthenogenetic activation	Aneuploidy[a]
1	>2 Full PN	Polyspermic (IVF) or digynic (ICSI) fertilization	Aneuploidy[a]
1	Separate PN	Abnormal nuclear-cytoplasmic dynamics	Aneuploidy; reduced development potential
1	Uneven PN size	Abnormal nuclear-cytoplasmic dynamics	Aneuploidy; reduced development potential
2	Uneven 2-cell (overt)	Abnormal chromosome segregation/spindle location	Aneuploidy; failed blastulation; reduced development potential
2	3-Cell (even)	Abnormal spindle formation and first division	Aneuploidy; reduced development potential
2, 3	Multi-, bi-, micro-nucleation	Abnormal chromosome segregation and nuclear formation or cell division	Aneuploidy; reduced (but not null) development potential
3	>10-cell	Abnormally short cell cycle length	Aneuploidy; reduced development potential
3	<4-cell	Abnormally long cell cycle	Aneuploidy; reduced development potential
3	>35% fragmentation	Abnormal cell division (cytokinesis)	Aneuploidy; blastulation failure; reduced development potential
5–6	Failed blastulation	Failed genomic activation; abnormal early divisions; abnormal compaction; catastrophic early loss of cytoplasmic volume, etc.	Complex aneuploidy[b]

[a]Some recent reports indicate that a proportion of embryos with these pronuclear configurations may be euploid [20, 21], although euploidy does not necessarily equal viability
[b]Aneuploidy affecting multiple chromosomes

Another retrospective study by Wirleitner et al. [23] examined birth outcomes following 1010 fresh and 1270 vitrified/warmed blastocyst transfers originating from the same stimulation cycle, as well as 636 fresh and 304 vitrified/warmed transfers involving blastocysts with a 1- to 2-day developmental delay. Similar live birth rates were obtained whether "top- quality" or non-top-quality embryos were transferred. Furthermore, even blastocysts with delayed development fared well if vitrified following extension of culture to Day 7 and warmed and transferred in a subsequent cycle [23].

10.2.1 Morphometrics

The main limitation of all morphology-based grading systems, whether pertaining cleavage stages or blastocysts, is their subjectivity, which can lead to intra- and interobserver variability [24]. To address this issue, semiautomatic and automatic image analysis techniques have been devised, but they are not widely used owing to their complexity and labor intensity [25]. The technologies use segmentation software to assess embryo "metrics," including size of blastomeres and nuclei, total embryo volume, blastocyst diameter, ICM diameter, zona pellucida thickness, blastocyst total surface area, ICM surface area, and ICM size.

Two recent morphometric studies are of interest. Paternot et al. [26] used a semiautomatic system to calculate the total cytoplasmic volume of Day 1, 2, and 3 embryos. Logistic regression analysis showed that both lower and higher volumes were associated with a lower probability of successful pregnancy [26]. However, the authors also pointed out that while the results showed good negative predictive value, other measures of reliability including sensitivity, specificity, positive predictive value, accuracy, and c-statistics of the volume range were considered relatively poor.

While Paternot et al. [26] focused on cleavage stage embryo morphometrics, Lagalla et al. [27] studied morphometrics of blastocysts, expanding on earlier work by Richter et al. [28] among others. Again, using digital image analysis software, Lagalla et al. [27] measured area and mean diameter of early and expanded blastocysts as well as ICM size and (calculated) TE cell number; they confirmed the positive relationship between the degree of blastocoel expansion and implantation and further suggested that while ICM size alone was not predictive of implantation potential, expanded blastocysts also showed larger ICM area; thus ICM size was significantly related to implantation only in expanded blastocysts.

For a concise review of other morphometric studies, the reader is referred to Ziebe [29].

10.2.2 Morphology and Ploidy

The prevailing embryo-centric view of implantation and pregnancy failure attributes this outcome primarily to aneuploidy. Despite being seriously questioned [30], the validity of this view has been reinforced by the reported success of single euploid blastocyst transfer [31, 32]. Thus, embryo selection strategies are now often assessed based on their ability to estimate the "risk" of aneuploidy. To this end, morphology and morphokinetics are correlated to results from embryo biopsy and aneuploidy testing. However, we note that these studies are limited by the limitations of current PGT-A technologies, including the potentially high incidence of false-positive results, compounded by the problem of mosaicism.

This caveat notwithstanding, currently, the weight of evidence suggests that only a limited correlation exists between blastocyst morphology and ploidy as determined by TE biopsy.

Alfarawati et al. [33] reported that >50% of blastocysts with top expansion grades (of 5 and 6) were euploid, compared with only 37.5% with lower expansion grades of 1 and 2. Minasi et al. [34] also reported that among 1730 biopsied blastocysts, significantly more top- quality blastocysts were euploid than aneuploid. But a high proportion of both euploid and aneuploid blastocysts were fully expanded (grades 5–6), and a significant proportion of aneuploid blastocysts also had top-quality ICM and TE grades.

This appears to be a recurring theme in many other studies attempting to correlate morphology and ploidy. Capalbo et al. [35] classified 956 blastocysts from 213 patients into 4 groups of excellent, good, average, and poor quality. They reported that euploidy correlated with blastocyst quality; however, euploid blastocysts selected from all of these morphology groups could establish sustained pregnancies. Using the same classification system, Irani et al. [36] also reported pregnancies from different morphological classes; however, they found a significantly higher implantation rate using embryos with the highest ("excellent") morphological grade. Their data also showed that "poor" grade euploid embryos had the highest chance of leading to spontaneous abortion. Fragouli and co-workers [37] compared standard morphological features of 858 euploid and aneuploid blastocysts from 161 patients. They concluded that aneuploidy had "little if any effect on morphological score" of blastocysts (although they did not provide specific statistical comparisons or include pregnancy rates in their study).

These studies show that while high-grade morphology may be more represented among euploid blastocysts, those embryos that can form sustained pregnancy actually occupy all morphological classes. Conversely, aneuploidy is common among high morphological grade blastocysts.

These conclusions are in general agreement with those drawn from studies seeking to correlate standard morphological features of TE with sustained pregnancy in non-PGT-A cycles. Ahlstrom et al. [38] analyzed 1117 live births following SET and concluded that both expansion grade and good TE morphology best correlated with pregnancy. The importance of TE morphology has been corroborated by Honnma et al. [39] in over 1000 single blastocyst frozen embryo transfers. Similarly, Ebner et al. [40] reported that TE quality and cell number were the only parameters predicting ongoing pregnancy after evaluating 254 fresh single blastocyst transfers.

Combining the evidence from both PGT-A and non-PGT-A studies, it is reasonable to hypothesize that morphology assessment and aneuploidy screening are complementary methods of embryo selection.

10.3 The Kinetics Approach to Embryo Selection

Morphology and morphometric-based grading schemes generally do not take into account the precise timing of developmental events; rather, they place embryos within classes, as discussed above. For example, a grade 4AA blastocyst, based on

the Gardner scoring system [41], is not graded differently depending on whether it forms on Day 5 or on Day 6. The question at this point is whether time-lapse microscopy and morphokinetics, alone or in combination with morphology/morphometrics, can offer better predictive value for pregnancy.

TLM has been adopted by many IVF laboratories over the past 10 years [10, 42, 43]. The technology allows precise timing of developmental events, and TLM studies have sought to correlate these events with specific endpoints, including blastocyst formation and implantation.

10.3.1 The Early Embryo Viability Assessment (Eeva™) System

The pioneering work of Wong et al. [44] identified kinetics of the first two cleavage divisions, namely, first cytokinesis duration, time interval between 2-cell and 3-cell, and time interval between 3-cell and 4-cell, as predictors of blastocyst formation. Based on these parameters, an algorithm was developed and became the basis for the commercial application, Eeva™. The Eeva™ system heralded the arrival of time-lapse image analysis purposed for automated and standardized assessment of kinetics using expert system software for "class ranking" of embryos.

A later version of Eeva™ categorized embryos as having "high," "medium," or "low" potential to form blastocysts [45–48]. In one multicenter prospective study [45], Eeva™ performed better than Day 3 morphology, the latter assessed by a single static image, in predicting blastocyst formation. (It's important to note here that morphology assessment using a single static two-dimensional image cannot be considered "standard" morphology assessment.) Moreover, embryos scored as Eeva™-high and Eeva™-medium led to a significantly higher clinical pregnancy rate compared to Eeva™-low embryos. More recently, Aparicio-Ruiz et al. [49] presented a retrospective study in which embryo selection was based on Eeva™ scoring in combination with standard morphology. The study included 521 donor egg embryos for which implantation results were known. Ongoing pregnancy rate in cycles with transfer of at least one Eeva™-high embryo (about 30% of the embryos transferred) was 2.5 times higher than cycles in which only Eeva™-medium and Eeva™-low embryos were transferred; this observation was independent of day of transfer (Day 3 or Day 5) and patient (oocyte recipient) characteristics. Of note, ongoing pregnancy rate in the "no Eeva™-high" group was still nearly 50%! This is perhaps not surprising considering the very narrow definition of Eeva™-high embryos as those in which time between cytokinesis 1 and 2 (P2 [t3-t2]) and cytokinesis 2 and 3 (P3 [t4-t3]) were 9.20–11.28 h and 0–1.44 h, respectively. Indeed, it has been shown that manual annotation could "upgrade" embryos from a lower to a higher Eeva™ rating, improving the sensitivity for predicting blastocyst formation [50].

Aparicio-Ruiz et al. [49] concluded that the Eeva™ classifications were complementary to standard morphology and that the Eeva™ algorithm had a better predictive

value for pregnancy than morphology alone. We would point out that because this study was conducted in an oocyte donation program, the results, while interesting, cannot be extrapolated to patients using autologous oocytes. Moreover, the actual number of embryos transferred and overall results in all patients were not given, but it can be deduced that multiple embryos were transferred in these oocyte recipients, which, as expected, led to a high pregnancy rate but presumably also a high multiple pregnancy rate (data were not provided). Ironically, this outcome would effectively defeat the purpose of embryo selection and platforms such as Eeva™.

The latest published study on the utility of Eeva™ is a prospective study by Kaser et al. [51], in which the authors set out to "determine whether adjunctive use of the Eeva™ test on [Day 3 or Day 5] improves the [clinical pregnancy rate] per transfer, as compared to [Day 5] selection with conventional morphology (CM) alone." The study showed that clinical, ongoing, and cumulative pregnancy rates per randomized patient were similar with or without Eeva™, but the authors also cautioned that the sample size was limited and the study was not sufficiently powered [51].

10.3.2 The EmbryoScope

The EmbryoScope is a TLM platform that combines an incubation system with time-lapse microscopy equipment enabling analysis of images captured at frequent intervals [52]. It is equipped with both standard and customizable annotation software; however, unlike the Eeva™ system, it was not initially purposed with embryo grading and ranking software.

Several groups have sought to exploit the annotation features of the EmbryoScope to develop algorithms that predict blastocyst formation and in turn chances of clinical pregnancy [52–58]. Results from these studies have been the focus of several excellent reviews and commentaries to which we refer the interested reader [49, 59–63].

Thus far, the ability of these algorithms to predict or improve clinical pregnancy remains controversial.

A small prospective cohort study in 92 patients [55] concluded that kinetic parameters associated with the first two cell cycles (duration of the first cytokinesis, duration of the three-cell stage, and absence of direct cleavage to three cells) predicted blastocyst formation but did not predict pregnancy outcomes after blastocyst transfer.

Thus although cleavage stage kinetics may have predictive value for blastocyst formation, the potential of algorithms built on kinetics of fertilization and early cleavage stages to improve pregnancy rates may be limited to Day 3 transfers and obscured after extended culture and blastocyst transfer.

To overcome this limitation, a shift has occurred from algorithms that predict blastocyst formation to those that predict implantation. In one retrospective study [53] involving 8414 cycles, a set of ten early development morphokinetic parame-

ters were used to construct a "hierarchical classification model" that was shown to improve clinical pregnancy rates over a standard grading system. This model was refined and evaluated by the same group in the study of Rubio et al. [64] which compared pregnancy rates following Day 3 or Day 5 transfer of embryos selected via morphokinetics. Overall, implantation rate was higher with morphokinetic selection (44.9%; CI 41.4–48.4) compared to standard morphology (37.1%; CI 33.6–40.7). There were also fewer early pregnancy losses with morphokinetic selection (16.6% vs. 25.8%). In a retrospective multicenter study involving 1664 cycles, Basile et al. [65] categorized embryos into five groups based on a hierarchical model built on three early cleavage stage morphokinetic variables (plus data on direct cleavage, multinucleation, and blastomere size). Implantation rates in the different groups were significantly different, ranging from 32% to 17%.

Liu et al. [66] presented a time-lapse deselection model involving both qualitative and quantitative parameters. To develop the model, a total of 270 embryos with known implantation status after Day 3 transfer were retrospectively analyzed, and the model was validated using 66 additional embryos with known implantation status. Embryos were classified into seven grades (A+ through F). Qualitative deselection parameters included poor conventional Day 3 morphology, abnormal cleavage patterns identified via time-lapse monitoring, and < 8 cells at 68 h post insemination. Quantitative parameters, independent of method of insemination, included time from pronuclear fading to 5-cell stage and duration of 3-cell stage. Implantation rate decreased with decreasing grade, reaching zero in grade F embryos, with the model showing AUC of 0.762 (95% CI, 0.701–0.824). The prospective validation study also showed significant predictive ability for implantation with AUC of 0.750 (95% CI, 0.588–0.912) to AUC of 0.820 (95% CI, 0.671–0.969) for different culture media.

The importance of independent (preferably multicenter) validation of morphokinetic algorithms cannot be overemphasized. This point is well demonstrated in the study of Barrie et al. [67], in which the robustness of six published algorithms was examined using a large data set consisting of 977 embryos with known implantation status. The authors reported that the positive predictive value for these algorithms never exceeded 45%, while the negative predictive value was between 60 and 70%. AUC values were below 0.65 indicating a low predictive capacity. The authors argued correctly that the development of algorithms "thus far has not involved the control of confounding factors such as media type, patient age, and treatment type"; hence, each algorithm corresponds to specific patient populations and the conditions specific to the laboratory in which the algorithm was developed and may not be relevant or predictive in other laboratory settings [67, 68].

In an effort to address this particular weakness of the existing algorithms, Peterson et al. [63] developed an algorithm based on morphokinetic data for Day 3 embryos with known implantation data (KID), compiled from an impressive 24 different clinics. The authors drew distinction between this algorithm and those that preceded it by noting its generalizability, that is, its effectiveness in ranking embryos based on blastocyst formation, independent of culture conditions (high or low oxygen and choice of media) and fertilization method (ICSI or standard). The algorithm

(KIDScore) incorporates six annotations between Day 1 and Day 3 of development: presence of two pronuclei on Day 1, time to pronuclear fading, and time from insemination to the two-, three-, five-, and eight-cell stages. Testing in a second independent set of embryos demonstrated that the "KIDScore" algorithm predicted blastocyst formation with an AUC of 0.745 and blastocyst quality with an AUC of 0.679. Moreover, a relative low AUC of 0.650 was obtained for implantation potential. Based on the latter, the new algorithm needs further development and modification and, ultimately, validation through a multicenter RCT. Nonetheless, the authors' innovative approach to development of this algorithm is of great interest and their initial results are encouraging.

Some abnormal patterns of cleavage have been defined based on time-lapse monitoring of cell division, and these have been correlated with ploidy, development, and/or implantation potential. Lagalla et al. [69] presented a retrospective study including 791 embryos from 141 patients aged 26–48 years undergoing PGT-A. Cleavage abnormalities that were assessed included direct cleavage (1–3-cell and 1–5-cell), rapid cleavage, and reverse cleavage. These abnormalities affected some 14% of the embryos examined and the majority arrested in development, but a few did develop to blastocysts and a proportion was found to be euploid. Although viability in utero was not assessed, the authors concluded that embryos with these qualitative morphokinetic abnormalities can be euploid and therefore viable.

The study of Barrie et al. [70] assessed the developmental potential of embryos affected by one of five abnormal cleavage patterns: direct cleavage, reverse cleavage, absent cleavage, chaotic cleavage, and cell lysis. A total of 15,819 embryos from 4559 treatment cycles were included. Overall, some 11% of the embryos examined showed one of these abnormalities with the most common being chaotic cleavage and direct cleavage. None of the embryos with reverse cleavage or cell lysis implanted while implantation rates for the other abnormalities ranged from 2 to 17%. Compared to a control group without any of these abnormalities, overall implantation rate for all abnormal embryos was shown to be significantly lower (6.9% vs. 38.7%). In contrast to the study of Lagalla et al. [69] in which the authors highlighted the potential of abnormal embryos for development, Barrie et al. [70] highlighted the reduced developmental capability of embryos with abnormal cleavage evidenced by reduced quality and implantation potential.

Athayde Wirka et al. [71] described four "groups of atypical phenotypes." These included abnormal syngamy (defined as "disordered" pronuclear movement and "delayed dispersion" of the nuclear envelopes), abnormal first cytokinesis (defined as irregular and ruffled oolemma just before and during the first cytokinesis), abnormal cleavage (defined as more than two cells originating from a single cell division), and chaotic cleavage (defined as erratic first two cell divisions resulting in unevenly sized blastomeres and/or fragments). The authors correlated the occurrence of these phenotypes with Day 3 morphology and development potential. They observed a very high incidence for these abnormalities (15–31%) and reported that while a significant number of such embryos was classified as "good quality" on Day 3 of development, significantly fewer blastocysts were formed compared to a control group without these abnormalities. Figures 10.1 and 10.2 show time-lapse images representing normal and abnormal development.

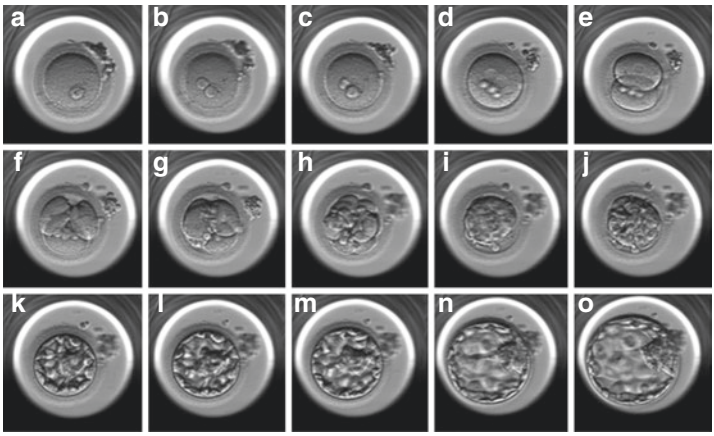

Fig. 10.1 Time- lapse images of an embryo that resulted in a term delivery: all hours are post-ICSI. (**a**) 1.8 h post-ICSI; (**b**) second polar body at 3.8 h; (**c**) PN appearance at 8.5 h; (**d**) 2PN full at 18.0 h; (**e**) two-cell at 24.2 h; (**f**) three-cell at 33.8 h; (**g**) four-cell at 37.0 h; (**h**) eight-cell at 65.6 h; (**i**) morula at 91.0 h; (**j**) cavitation at 95.4 h; (**k**) blastocyst formation (tB) at 103.9 h; (**l**) tB + 4 h; (**m**) tB + 6 h; (**n**) tB + 8 h; (**o**) tB + 10 h, fully expanded blastocyst

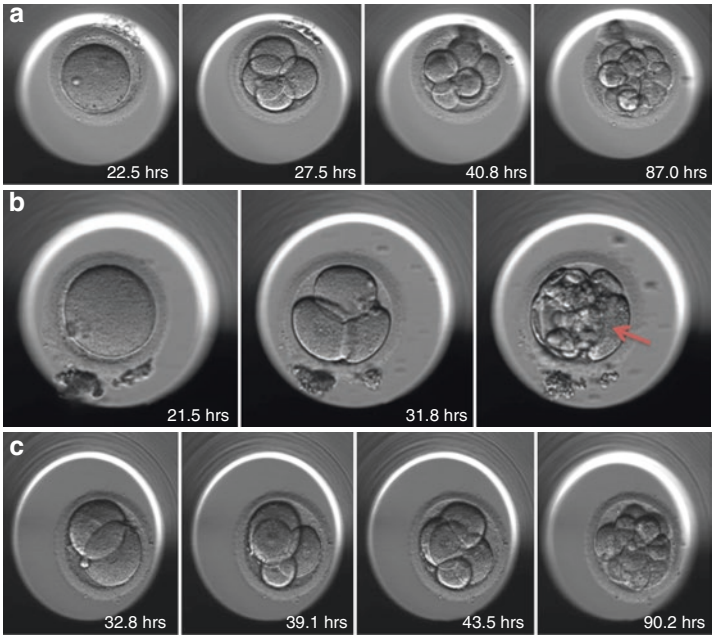

Fig. 10.2 (**a**) This embryo shows direct cleavage to four similarly sized blastomeres that continued to divide until it arrested before compaction. (**b**) This embryo shows "immediate" unequal cleavage from one to three cells at 31.8 h. One of the three cells (possibly a fragment; shown by arrow) did not participate in subsequent compaction and cavitation. (**c**) This embryo underwent unequal division of one blastomere at the two-cell stage (lower right) into three cells at 39.1 h, just prior to a normal division of the remaining blastomere into two daughter cells at 43.5 h. Development arrested at 90.2 h

10.4 Morphokinetics and Genetic Integrity

A number of excellent reviews have focused on the question of whether morphokinetics can predict the genetic quality of an embryo [72–74]. Once again, notwithstanding the potentially significant limitations of the studies (as discussed above), current evidence leads to the conclusion that late stages in the preimplantation period are better in predicting ploidy [72]. Campbell and co-workers [75, 76] were first to investigate the ability of morphokinetics to predict ploidy. They found that while there was no discernible relationship between ploidy and early cleavage stage events, two specific late stage parameters, namely, time to start of blastulation (tSB) and time to full blastulation (tB), were indicative of "aneuploidy risk." However, subsequent studies have not conclusively corroborated these observations. In a study that included 455 blastocysts from 138 "poor prognosis" patients, Rienzi et al. [77] found no statistically significant association between ploidy and any of 16 morphokinetic events, including those identified by Campbell and co-workers [75, 76]. Kramer et al. [78] were also unable to confirm the aneuploidy risk model of Campbell et al. [75, 76]. After analyzing 149 blastocyst biopsies, they found no significant differences in ploidy in the three risk groups identified by Campbell and co-workers, concluding that "patient variability even for euploid embryos is so great that it will be impossible to use morphokinetic parameters to select euploid embryos using universal criteria [78]."

Perhaps somewhat contradictory to the Kramer et al. [78] and Rienzi et al. [77] studies, the study of Minasi et al. [34], examining 530 PGT-A cycles, found that mean time to achieve several blastulation milestones (tSB, tB, tEB, tHB) was shorter in euploid embryos than aneuploid embryos. Notably, euploidy was found to be more prevalent among those embryos showing the highest expansion grades according to standard morphology [34].

Mumusoglu et al. [72] have recently published a review of ten studies providing a clarifying perspective on the attempts to correlate morphokinetics with ploidy. By analyzing and correcting for the statistical effects of clustering through calculation of intra-class coefficients, these authors found that only 5 of 23 kinetic parameters retained even a limited ability to discriminate between euploid and aneuploid embryos. Those parameters *excluded* were largely related to early cleavage stage dynamics, while those that remained were mostly related to later developmental events involving blastocyst formation (tM, tSB, tB, tEB). However, the AUC calculations from ROC analysis ranged from 0.55 to 0.63, implying only low to moderate predictive ability.

Taken together, the above studies suggest that morphokinetic features of euploid and aneuploid embryos may share a problematic degree of overlap, as it appears to be the case with standard morphology.

10.5 Aneuploidy in Somatic Cells and Relevance to Embryos

The impact of aneuploidy on human reproduction is well established and aneuploidy appears to be highly detrimental in all organisms [79, 80]. Not only does aneuploidy originate from chromosome instability, it also leads to further

segregation errors and, ultimately, to cell cycle arrest, not immediately, but over time [81]. Aneuploid cells of diverse origin exhibit what is called an "environmental stress response" (ESR), consisting of about 300 upregulated and 600 downregulated genes related to heat shock and oxidative stress. This common response has been seen across many species.

Of interest to clinical embryology, this response appears largely independent of specific chromosomes involved and the net result is always a decrease in the proliferative capacity of the affected cell. Decreased proliferation results at least partly from what has been termed "proteotoxicity" [81–85]. For example, trisomic cells have one extra chromosome copy that is actively transcribed. This creates a subsequent protein translational overload that burdens the cell's chaperone system and impairs cell function. Thus, cell lines with single chromosome trisomies (e.g., 1, 13, 16, or 19) all exhibit metabolic alterations and prolonged cell cycle times compared to euploid cell lines [83, 86].

Both simple and complex aneuploidies in blastomeres are often accompanied by cleavage arrest [87], although some embryos continue to develop to the blastocyst stage, successfully circumventing aneuploidy-induced arrest [88]. However, metabolic alterations that result in slower cell division rate could reasonably be hypothesized to impair or delay other developmental events such as compaction, blastocyst formation, and blastocoel expansion. The latter may also be affected through dysfunction of junctional complexes or blastocoel fluid transport, resulting in delayed blastocyst formation or deficits in subsequent expansion that might manifest as episodes of blastocoel collapse. Our laboratory (TH) has preliminary evidence in support of this hypothesis [89]. Blastocyst expansion (BE) was measured in 188 blastocysts from 34 consecutive PGT-A cases involving infertility patients (age 24–42 years). Expansion was defined as the total cross- sectional area of the blastocoel cavity circumscribed by the trophectoderm and was measured over a 10-h period at 2-h intervals beginning from the time of blastocyst formation (tB; see Fig. 10.1k–o). There were a total of 89 euploid (47.3%) embryos and 99 (52.7%) aneuploid embryos in the study group. Blastocyst expansion rate was significantly faster in those classified as euploid versus aneuploid, as reflected in the expansion slopes (Fig. 10.3).

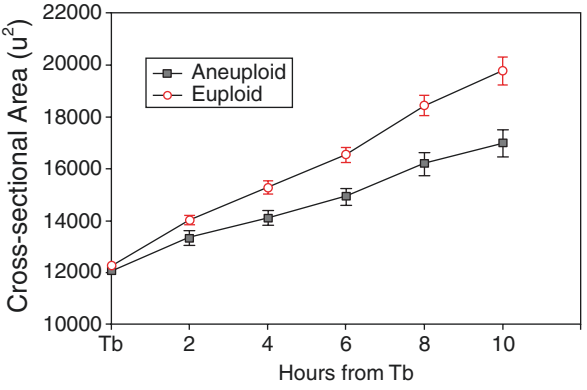

Fig. 10.3 The mean expansion curves during the first 10 h of expansion after blastocyst formation in 89 euploid and 99 aneuploid blastocysts after trophectoderm biopsy. Euploid embryos expand significantly faster (red circles; slope = 836.9) than aneuploid embryos (black squares, slope = 547.5; $p = 0.001$). Error bars represent SEM

The differences between euploid and aneuploid blastocysts were independent of the absolute time of blastocyst formation on either Day 5 or Day 6 of development [89]. Based on this observation, we proposed that the expansion slope values of individual blastocysts could be used to rank order embryos to improve the chances for euploid embryo selection. This may be of practical use in differentiating between blastocysts that are currently classified as having Gardner expansion grades 4 or 5 using standard morphology.

The "stress-strain" nature of zona expansion may act as a de facto "stress test" for euploidy that might be seen in the oscillatory nature of the expansion process [90]. Repeated blastocoel collapse may be a risk factor associated with impaired development in animal models [91, 92] and implantation in the human [93]. This interpretation is also consistent with evidence, discussed above, the basic premise of which dates back to the early days of IVF [94, 95] that expansion and TE grade are predictors of implantation potential. Current conventions of standard morphology do not place value on the rate or efficiency of blastocyst expansion, but morphokinetics can help dissect and define this process and, in turn, the risk of aneuploidy.

10.6 Assessing the Efficacy of Morphokinetics Approaches

The first RCT in morphokinetics was published by Rubio et al. [64] and included 930 patients. The RCT showed that blastocysts selected based on morphokinetics led to higher implantation rates and lower spontaneous miscarriage rates compared to blastocysts selected (after standard incubation) and based on standard morphology.

Returning briefly to the topic of "evidence- based" embryology and study design, a close examination of the trial by Rubio et al. [64] reveals that despite the significant first-time effort that it represents, this study is at high risk of bias in a number of areas both on the basis of CONSORT guidelines [5, 7, 74] and with respect to aspects unique to clinical embryology. Patients were removed from the control arm after randomization, which defeats the purpose of randomization. Reporting was also biased since the authors deviated from what they had intended to report; clinical pregnancy rates were not reported and ongoing pregnancy (primary outcome) was assessed differently from that which had been originally planned by the authors. Randomization was carried out by an embryologist active in the day-to-day laboratory operations. However, this task is best assigned to a designated staff member without direct involvement in clinical work. Embryo transfer was not limited to Day 3 or Day 5 of development but included both, and patients received single and double embryo transfers. Additionally, both autologous and donor oocyte cases were included in the trial, confounding the results.

A more recent RCT was published by Goodman et al. [96] involving 235 patients. The design of this study was quite interesting in that embryos in both experimental and control arms of the study were cultured in the EmbryoScope and therefore exposed to identical conditions. Embryo selection was done, either using an algorithm that incorporated early stage and blastocyst kinetics or morphology. Morphology assessments were performed daily at specific time points by scrolling through all seven focal planes captured by the EmbryoScope, without viewing the time-lapse video. The study showed increased clinical pregnancy and implantation rates with the addition of kinetic parameters to embryo selection; however, the differences did not reach statistical significance. Moreover, the investigators found that time to blastulation, the absence of multinucleation, and a combined score based on morphology and kinetics were significant predictors of implantation.

Overall, the risk of bias in the CONSORT elements in this study is quite low, except in the area of patient attrition [6]. Moreover, the trial clearly represents a major effort on the part of the investigators and uses a logical approach to the treatment of experimental and control arms in order to eliminate confounding of results by the incubation system. The limitations of the study design are that (1) up to four (and a minimum of two) embryos were transferred rather than single embryos, (2) timing of embryo transfer was not predetermined and was depended on quality and number of available embryos, and (3) both Day 3 and Day 5 transfers were included, with the latter representing patients with a better prognosis and much better outcomes as expected.

Four systematic reviews of RCTs assessing the efficacy of morphokinetics have been published since 2014. The first of these included only two RCTs and concluded that TLM was "unlikely to have a large effect on the chance of achieving clinical and/or ongoing pregnancy when transferring embryos at the blastocyst stage" [97]. In a Cochrane Review, Armstrong et al. [98] included three trials involving 994 women, comparing TLM with or without the use of algorithms to select embryos and conventional incubation and embryo assessment. The authors concluded that there was no conclusive evidence of a difference in clinical pregnancy rate per couple randomly assigned to the TLM and conventional incubation arms [98]. In another systematic review and meta- analysis by Racowsky et al. [5], four studies were included. The authors highlighted limitations of the existing studies and concluded that "uncertainty remains regarding the effect of this intervention on ongoing pregnancy rate." This was despite the fact that the overall result considering three of the four studies in which the effect of both the time-lapse incubation system and the use of time-lapse images for embryo selection were assessed suggested a benefit of this intervention.

A meta-analysis including three of the trials assessed by Racowsky et al. [5] and an additional two new trials was recently published [7]. These authors concluded that TLM with morphokinetic embryo selection improves pregnancy and live birth rate and reduces early pregnancy loss. Their findings, however, were challenged for being potentially biased due to study inclusion/exclusion criteria and conflict of interest issues (see [8]). It remains to be seen whether future meta- analyses confirm or contradict the findings of Pribenszky et al. [7].

10.7 Conclusions

The last section of this chapter brings us full circle back to the point made in the first section: we need a change in the landscape of clinical embryology research from studies providing low to moderate quality evidence to those providing high-quality evidence. Specific study design guidelines that incorporate the principles of EBM but also consider the complexities of embryology interventions could help bring about this change. This issue notwithstanding, the multitude of studies published to date, a fraction of which was reviewed here, has established that the addition of morphokinetics to classical morphology and morphometrics does improve our ability to predict blastocyst formation, but whether it also helps identify embryos with high implantation potential and improve clinical outcomes is a subject of continued debate. Blastocyst formation does not necessarily predict implantation since a significant proportion of blastocysts may be aneuploid or suffer other deficits. Thus, morphology- and kinetic-based embryo selection platforms must be able to predict the risk of aneuploidy or, alternatively, predict implantation potential to be clinically efficacious. Evidence obtained at the laboratory of one of the authors (TH) suggests that the period of blastocyst expansion represents a potentially fruitful area for research. While this is a period difficult to quantify using standard morphology, its dynamic nature is perfectly suited to morphokinetic approaches; however, its value also remains to be demonstrated in a large unselected patient population.

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Part IV
Controversies

Chapter 11
Is It Good Practice/Ethical to Set a Max BMI Before IVF?

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11.1 Introduction

Increasing rates of obesity (BMI $\geq$ 30 kg/m^2) are being recognized broadly, fueling discussions on the topic in medical, academic, political, and popular forums. Based on direct measurement in US field studies, the prevalence of obesity increased by over 50% from 1988–1994 to 2013–2014 with over a third of adults being defined as obese at the end of the study period [1]. Further, the prevalence increased faster in women than in men (40.4% vs. 35.0%, respectively), and has not plateaued yet in the female population [2]. While obesity rates vary widely between countries, the preponderance of data shows increased rates globally over the past several decades. Obesity is recognized as a chronic disease, with known associated morbidity and mortality. Based on these considerations, virtually all medical providers are going to face decisions related to this topic. The focus of this article will be whether it is good practice and ethical to set a maximum BMI before IVF treatment.

11.2 Trends in Obesity

Before exploring a BMI threshold for the IVF procedure, it is important to consider the underlying repercussions on health and reproduction. The prevalence of obesity has been steadily increasing worldwide and has taken epidemic dimensions.

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The American Medical Association (AMA) recently classified obesity as a disease, rather than a condition, and there is common consensus that treating, or more importantly preventing, obesity is a critical goal.

The World Health Organization (WHO) definition of obesity is a condition of excess body fat accumulation to the extent that health is impaired. Because of its simplicity, the body mass index (BMI) is the most widely accepted measure to describe the severity of obesity and is calculated using the individual's weight and height (kg/cm^2). A BMI of 25–29.9 kg/m^2 is considered overweight. Obesity is defined as a BMI ≥30 kg/m^2 and further subclassified into class I obesity (30–34.9 kg/m^2), class II obesity (35–39.9 kg/m^2), and class III obesity (≥40 kg/m^2); each class increase carries a correlative health risk increase [3]. However, BMI has been challenged as the appropriate measure of health risk related to obesity, driven by several findings. Interestingly, certain populations (such as Asians) are at greater risk for cardiovascular disease and type 2 diabetes at lower BMI cutoff values [4, 5]. Additionally, certain types of obesity (central and intra-abdominal versus peripheral) have different health risk implications. Metabolic syndrome was defined to provide greater predictive value than BMI for future health risks (specifically, cardiovascular disease and type 2 diabetes) by clustering increased blood pressure, high blood glucose levels, excess central body fat, and abnormal cholesterol or triglyceride levels. For all its shortcomings though, BMI is unlikely to be supplanted anytime soon in the framework for obesity research and discussions.

Only 30% of the adult US population is considered normal weight (BMI ≤ 25 kg/m^2); paradoxically, only 36% of the population believe they need to lose weight [6]. Trailing only tobacco use, obesity is a leading cause of morbidity and mortality in the USA [7]. Women are more likely to be obese compared to men, which has been attributed to weight gain during pregnancy and shorter average height. Non-Hispanic blacks have the highest age-adjusted rates of obesity (48.1%), followed by Hispanics and non-Hispanic whites. Asians are least likely to be obese (11.7%) [8]. By 2030, nearly 40% of the world's population is anticipated to be overweight, and one in five people will be obese [9]. Obesity-related costs to the overall US economy were estimated to be more than $1.4 trillion in 2014 and constituted over 10% of total healthcare expenditures [10].

A newer concern is the prevalence of obesity in children, who will add to an ever-growing prevalence in adults. In 2003, the estimated prevalence of obesity in the USA was 90 million individuals, 13% of which were children. Childhood obesity leads to lasting endocrine, cardiovascular, gastrointestinal, and psychological consequences. As it relates to reproduction, adolescent obesity is associated with a threefold increase in lifetime nulliparity and fourfold increase in lifetime nulligravidity [11]. With increasing focus on obesity prevention in children through introduction of healthier school lunch programs and encouragement of physical exercise, the incidence of obesity in children has recently plateaued. Such initiatives are promising and provide compelling proof that obesity is largely a preventable disease and mostly caused by an affordable and readily available diet rich in highly processed, high-fat, and sugar-laden foods. This is what has been referred to as the "obesogenic environment" of our modern society. Children have been viewed as a major market

force by the food industry because of their influence on their parents' spending patterns and their potential as future adult consumers. More than $10 billion per year are spent on advertising and marketing foods directly to children through television, youth-targeted promotions, and school relations [6]. In 1991, Sweden implemented the strictest rules in Europe, banning all television and radio advertising targeted at children under the age of 12 on ethical and moral grounds [12].

There is growing evidence that the foundation for obesity may be laid even earlier than childhood. Maternal nutrition and endocrine profile during pregnancy are potential critical determinants of fetal metabolic programming in future life. Infants born to mothers with diabetes are at higher risk for being overweight or obese themselves and have higher rates of metabolic syndrome, type 2 diabetes, and cardiovascular disease as adults [13]. This has been attributed to possible epigenetic modifications in utero [14–20]: murine models have demonstrated differences in placental gene expression and methylation patterns and evidence of aberrant mitochondrial dynamics passed transgenerationally from the obese maternal germline to F1 and F2 pup generations [21–23]. These findings regarding in utero programming are quite early in development and have been challenged by the inability to separate the antepartum and postpartum influences of obesity on children.

In spite of the importance of setting the course for a healthy adult life as early as pregnancy and childhood, most excess weight accumulation occurs as an adult, predominantly resulting from a high-calorie diet in combination with a sedentary lifestyle. Genetics plays a smaller role than the environment, but certain genes may increase an individual's risk of weight gain if immersed in an obesogenic lifestyle [24, 25]. These are considered susceptibility genes, on which environmental factors act to trigger obesity. There are genes described as the primary cause of obesity, such as mutations in the LEP gene causing leptin deficiency, but these conditions are rare [24–26].

11.3 Impact of BMI on Conception and ART

The adverse effects of female obesity on the reproductive system have been extensively documented [27]. Women with BMI $\geq$30 kg/m^2 are more than twice as likely not to have children compared to normal-weight women [11]. Numerous studies indicate that obesity is associated with ovulation dysfunction, infertility, and increased miscarriage rates [10, 28, 29]. Polycystic ovarian syndrome (PCOS) is the classic phenotype that comes to mind. However, there is substantial evidence that the impact of obesity on reproduction extends beyond ovulation dysfunction [28–31]. Two large Danish studies showing an inverse relationship between BMI and fecundability demonstrated that this effect persisted for women with regular menstrual cycles [32, 33]. A 2015 prospective cohort study of almost 2000 women showed a 5% increase in time to pregnancy for every 5-kg body weight increase. Interestingly, 90% of women in this study had regular menstrual cycles during the study period [31].

Assisted reproductive technologies (ART) can overcome ovulatory disorders, but efficacy of these treatments is negatively affected by obesity, with overall lower pregnancy and live birth rates with IVF treatment. They require higher doses of gonadotropins [34, 35] and have higher cancelation rates [36]. Oocyte yields and maturity are lower [37] and embryo quality poorer [38–40]. They have lower rates of fertilization [30], blastulation, implantation, and live birth [41–44] and increased rates of spindle apparatus disorganization, metaphase alignment abnormalities, and miscarriage [45, 46].

Much of the literature assessing the effect of obesity on conception has focused on the female partner, though more recent research has linking male-factor infertility to obesity. Overweight and obese men are at higher risk for oligozoospermia, lower total motile sperm counts, and higher DNA fragmentation index (DFI) values compared to men with normal body weight [47–51]. Large epidemiologic studies have confirmed a direct correlation between BMI and male infertility: controlling for female factors, the odds ratio (OR) for infertility was 1.2 and 1.36 for overweight and obese men, respectively [33, 52, 53]. ART treatments are not spared from the negative contribution of male obesity, and it is now recognized that, similar to their female counterparts, male obesity impacts clinical pregnancy, miscarriage, and live birth rates (controlling for female variables, OR 0.65, 95% CI 0.44–0.97) [43, 54]. Proposed mechanisms include affected spermatogenesis, thermal effects from insulating adipose tissue, hypothalamic-pituitary-gonadal axis dysfunction, sexual dysfunction, and possible sperm epigenetic abnormalities [55].

11.4 Impact of BMI on Pregnancy

Obese women have higher rates of maternal and fetal complications during the antepartum, intrapartum, and postpartum period. The risk of maternal obesity on the developing fetus is not just the result of excess adipose tissue but also relates to the metabolic diseases and altered background physiology that accompany obesity. The following risks represent a comparison of the obese to the normal-weight population unless otherwise noted.

Miscarriage has been an area of considerable research. A pooled analysis of retrospective studies including almost 30,000 women who conceived spontaneously revealed higher miscarriages rates in obese (13.6%) compared to the normal-weight women (10.7%; OR 1.31; 95% CI 1.18–1.46) [56]. A nested case-control study of 1644 obese and 3288 age-matched normal-weight controls showed the risk of miscarriage and recurrent pregnancy loss were significantly higher among obese patients (OR 1.2 and 3.5, 95% CI 1.01–1.46 and 1.03–12.01, respectively) [57]. Chromosomal analysis of the products of conception (with exclusion of maternal cell contamination) from obese women with recurrent miscarriages demonstrates a

high rate of euploid losses (58% compared with 37% in nonobese women; relative risk (RR) 1.63, 95% CI 1.08–2.47) [58]. This suggests that obesity potentially adversely impacts the endometrium.

Offspring from obese mothers are at higher risk for congenital abnormalities (controlling for diabetes), including neural tube defects, cardiovascular anomalies, and limb reduction, among others, with statistically significant odds ratios ranging from 1.20 to 2.24 [59]. Antenatal testing with ultrasound is at least 20% less likely to detect these anomalies. Ultrasonographic detection of markers for aneuploidy screening has lower sensitivity and higher false-negative rates (obese, 22% sensitivity/78% false-negative rate; normal-weight, 32% sensitivity, 68% false-negative rate) [60]. They also have increased risk of macrosomia and growth restriction, described further below. Obese pregnant women are 40% more likely to experience stillbirth compared to nonobese counterparts (adjusted hazard ratio (HR) 1.4, 95% CI 1.3–1.5), and unfortunately there is no evidence that increased antepartum surveillance improves outcomes [61]. Neonatal and infant death have increased hazard ratios with each increase in obesity class, such that women with a BMI ≥50 have a 13.6-fold greater risk of stillbirth at 41 weeks gestational age compared to normal-weight individuals [62].

Other obstetrical complications encountered at higher rates in obese women include gestational diabetes (OR 2.8, 95% CI 2.54–3.08), hypertensive disorders including preeclampsia (OR 2.38, 95% CI 2.24–2.52), cardiac dysfunction, nonalcoholic fatty liver disease, and medically induced preterm (OR 1.46, 95% CI 1.39–1.54) and very preterm (OR 1.49, 95% CI 1.34–1.65) birth [13, 63, 64]. In an effort to minimize the increased pregnancy complications, many clinicians induce labor at 39 weeks gestation, but these patients are also at higher risk for induction failure, resulting in higher rates of cesarean section [65–67]. A meta-analysis demonstrated an unadjusted OR for cesarean section of 2.05 (95% CI 1.86–2.27), and severely obese women had an even further increased rate compared to normal-weight women (OR 2.89, 95% CI 2.28–3.79). Another factor contributing to dysfunctional labor is the increased rate of fetal macrosomia (birthweight over 4000 g): 13.8% in the obese population compared to 8.3% in the normal-weight population [68]. This in turn results in a higher rate of shoulder dystocia (OR 1.51, 95% CI 1.32–1.74), which predisposes the infant to brachial plexus injuries/palsies, clavicular/humeral fractures, and ischemic encephalopathy or death and the mother to operative deliveries, perineal lacerations, and postpartum hemorrhage [68].

Postpartum, obese women are at increased risk for endometritis, venous thromboembolic events (OR 5.3, 95% CI 2.1–13.5), and wound infections (OR 1.67, 95% CI 1.38–2.0) or dehiscence (OR 1.85, 95% CI 1.1–3.1) resulting from poor vascularization of subcutaneous adipose tissues and seroma formation [63, 69, 70]. Wound infection rates approach 20% of all obese women with cesarean sections (OR 1.43, 95% CI 1.09–1.88, after adjusting for diabetes mellitus and primary cesarean section) [71].

11.5 Good Practice and Ethics of BMI and IVF

In terms of whether it is good practice and ethical to use a BMI threshold, most people would accept that, at some level, completing an elective medical procedure just is not feasible. The topic could be reasonably turned around ("Is it ethical *not* to have a BMI threshold for IVF?") if carried out far enough – at some point the infertile woman would be taking very high risk with the procedure and pregnancy, and the fetus would face overwhelming intra- and peripartum risk. To address the alternative position that a BMI threshold is not justifiable, some would claim that it is discriminatory against obese individuals, which is itself ethically unacceptable, and asserted not to be the intent of the discussion. Rather, the purpose of this chapter is to purely consider it from the perspective of medical outcomes, which is our role as providers. In this light, the difficulty of the topic is determining not *if* but *where* that BMI threshold should be.

The threshold is on solid ground when fewer individuals are excluded; if that is where composite risk significantly changes, that is an appropriate place to consider a threshold. Unfortunately, wherever the threshold is applied, some individuals will be excluded who would have an uncomplicated outcome. The proportion of "good" outcomes increases with a lower BMI threshold, though the total number would be smaller. Here, the "good practice" concept diverges from the "ethical" concept in where to best apply a BMI threshold, with the latter applying upward pressure. Each of these perspectives will be considered in turn.

11.5.1 Good Practice

Distilling the issue purely to a medical decision of how to best maximize conception, minimize pregnancy loss, and optimize maternal and fetal outcomes, the above sections on the impact of BMI on conception and maternal-fetal outcomes indicate that using a lower threshold would provide overall better outcomes.

Hypertension, diabetes, asthma, inflammatory bowel disease, nephropathy, smoking, psychiatric diseases, hepatitis, and lupus – most would agree that these should be medically optimized prior to pregnancy, and the same bar would be appropriate prior to IVF. Is it consistent for a fertility clinic to have a no-smoking policy for treatment, but not a BMI policy? If we have acknowledged obesity as a chronic disease, should we treat this disease, like others, before pregnancy is pursued? Is obesity as a chronic disease different from these other examples? Obesity can be managed with effective medical and surgical interventions, and there is evidence to suggest that weight reduction improves medical comorbidities that impact, as well as direct measures of, reproductive and maternal-fetal outcomes [72–79]. From a magnitude of effect perspective, the above sections demonstrate not insignificant increases in risk of significant maternal and fetal complications in the obese population. The aggregate of these factors support the use of lower BMI thresholds.

11.5.2 Ethics

As with other questions of medical ethics, using the principles defined by Beauchamp and Childress provides an appropriate framework to consider the ethics of a BMI threshold with IVF treatment [80]. Specifically, the goal is to frame the question around autonomy, nonmaleficence, beneficence, and justice.

11.5.2.1 Autonomy

Autonomy, as defined by the obligation to respect the decisions made by another, including the negative duty to not interfere with the decisions of a competent adult, is a principle in conflict.

Reproductive liberty frequently surfaces as supporting evidence for this principle, though it would be important to consider the nuances of how this applies. Reproductive liberty is defined as the liberty or right to decide whether or not to reproduce. Most moral rights in society, such as this one, have generally been legally interpreted as a negative right, wherein it exists as a right protected from interference by another group (think of the first constitutional amendments), rather than a positive right, wherein another group is obligated to act to protect that right. This has clearly not been the only interpretation though; states have chosen to uphold procreative liberty by supporting services such as abortion and infertility treatment.

It would be difficult to justify a BMI threshold if considering patient autonomy and reproductive liberty in isolation. They are not the only party involved in these considerations though. The fetus is fundamentally impacted by the decision-making, but has no voice in this discussion on autonomy. The medical provider is another party, who plays a pivotal role in the process. Here, the ASRM statement provides a basis for applying a BMI threshold when physician autonomy is considered:

> When patients face increased risks that are modifiable in ways that reduce risks, efforts should be made to decrease these risks. Some examples include weight loss, smoking cessation, and blood sugar regulation in diabetics. In cases where the patient is unable or unwilling to modify their risk, physicians may differ regarding whether or not to treat the patient. So long as treatment decisions are based on reasonable medical considerations and applied without bias, physician autonomy should be respected [81].

While this statement would not serve as justification for a uniform BMI threshold in all circumstances, it does support the concept that there are situations when a threshold can be ethically justified. One such circumstance is when the individual physician makes the unbiased decision that the risk exceeds their threshold for providing safe and appropriate medical care. This indicates that *different* BMI thresholds may be possible, which will be inspected more carefully in subsequent sections.

11.5.2.2 Nonmaleficence

In their responsibility for their patients' well-being, physicians are bound by a second ethical principle. *Primum non nocere* ("first, do no harm") was not derived verbatim from the Hippocratic Oath but is a near derivation. It is a reminder that it may be better not to do something, or even do nothing, rather than risk causing more harm than good. In an elective situation that can be improved upon, nonmaleficence is a classic principle that ethicists reference, typically taking a leading role over autonomy (as emphasized by *primum*), and in this case providing the strongest basis for lower BMI thresholds. Based on the above review of the impact of BMI on reproduction and maternal-fetal outcomes, harm will be encountered as increasingly higher treatment BMIs are considered. The question then again becomes what magnitude risk sufficiently justifies a given BMI threshold.

Nonmaleficence also brings up practical considerations. Is it appropriate for a stand-alone surgical center to perform elective procedures on high BMI patients without immediate access to the resources of a tertiary care medical center? The Ethics Committee of the American Society for Reproductive Medicine (ASRM) states, "When clinicians determine that a fertility treatment or the resulting pregnancy may pose increased risk, consideration should be given to providing care in a setting that can best meet the patient's needs. Often, the involvement of a center with expertise in treating her particular medical condition during part of all of her care will be helpful in achieving this goal." The American College of Obstetrics and Gynecology (ACOG) states that accommodation of the physical needs of obese pregnant patients is necessary in inpatient and outpatient settings; this requires consideration of blood pressure cuffs, wheelchairs, labor beds, and operating room tables of appropriate size to accommodate the size and weight of these patients, along with the additional staff to safely assist in patient care. An anesthesia consult is also important to properly plan monitoring, access, and management of comorbidities of obesity, which include increased risks of hypoxia, hypercapnia, and sudden death [82]. Perhaps high BMI patients should be referred to tertiary medical centers to ensure their best outcome, creating a second circumstance where different BMI thresholds appropriately apply.

Clearly, the practical considerations extend beyond the immediate risks of oocyte retrieval though. The maternal-fetal unit needs to navigate implantation, miscarriage, myriad obstetrical risks, delivery, and postpartum recovery and development. Again, this would not justify a fixed threshold in all circumstances, but it highlights the necessity to carefully consider whether the patient is receiving the appropriate level of care in their particular setting to justify initiating IVF treatment.

11.5.2.3 Beneficence

As the counterpoint to nonmaleficence, beneficence is the obligation to bring about good. The good for a woman facing infertility is the ability to deliver an infant. Achieving this good involves traveling a path with many obstacles and hardships.

There are medical and surgical therapies directed at obesity that can make the obstacles achievable to overcome, but these are neither guaranteed to durably reduce weight nor guaranteed to facilitate an uncomplicated reproductive outcome.

Regarding the efficacy of weight loss interventions, results of currently available medical treatments are relatively modest (10–15% in combination with lifestyle modification), and some of these interventions rely on newer medications with limited safety data. Bariatric surgery effectively reduces weight in individuals with BMI $\geq$40 kg/m^2 but is expensive, invasive, and rarely accessed and creates near-term gastrointestinal malabsorption problems that are a relative contraindication to pregnancy for up to a year following the procedure. Discouragingly, of those who achieve weight loss, more than half will gradually regain this weight [3, 83, 84].

Regarding the impact of weight loss in obese individuals on reproductive outcomes, the absence of demonstrated benefit is an important consideration articulated in a recent article: though it is frequently cited that even a 5% weight loss can improve fertility [85, 86], there are no published dose-response studies demonstrating a relationship between weight loss and fertility in obese patients. Additionally, a meta-analysis of 49 randomized trials and 11,444 women compared various interventions (dietary only, exercise only, and a combination) to no intervention in preventing excessive gestational weight gain. While the interventions were successful in reducing the risk of excessive weight gain by 20% compared to the control group, there were no differences between the groups in cesarean section, preterm delivery, or macrosomia rates [87]. The absence of adequate studies to identify reproductive benefits accrued with weight loss in obese individuals does not imply the effect does not exist but is an important consideration when making an evidence-based evaluation of the topic.

With these facts in mind, mandating weight loss becomes a barrier to achieving a delivery, and thus a BMI threshold could be an ethical barrier to beneficence if considering in isolation. However, this considers an obligation to bring about good from the perspective of the woman facing infertility. Arguably, the concept of beneficence could be extended to her partner and family, which would be significantly impacted if the woman incurred a significant medical complication resulting from treatment. Zoomed out further, societal implications become relevant. Perspective is clearly important to the interpretation and measure of beneficence.

11.5.2.4 Justice

Justice indicates there is an ethical obligation to treat all people equally, fairly, and without bias. The challenge of assessing justice is that the parties involved may interpret the situation and risks differently. The inherent, emotional desire for offspring makes a well-informed, externally uninfluenced decision challenging, impacting how a patient interprets risk. The practitioner conversely has to consider their ability to provide the appropriate care the patient desires without encountering risks to the patient or fetus above the provider's threshold for comfort. Layered upon this, medical providers may differ in the level of risk they are comfortable accepting, potentially influenced by their own biases.

This implication of bias is a critical point when considering justice. As discussed above in the section on autonomy, physician autonomy can justify denying patient treatment due to the interpreted risk. However, the most important aspect of this is closely inspecting the conclusion to ensure it is not biased. Again drawing from the ASRM statement *Provision of fertility services for women at increased risk of complications during fertility treatment or pregnancy: an Ethics Committee opinion*:

> When declining to provide treatment, physicians must ensure that these decisions are made after careful consideration of the medical facts and without bias toward the woman or her partner. Such bias could include the physician's feelings towards the patient's age, ethnicity, socioeconomic status, parenting unit, medical condition or disability. In cases where the underlying disease is caused by behavioral factors such as smoking or alcohol intake, the physician may have bias as well. It is important for physicians to fully assess their reasons for denying care, and ensure that it is not discriminatory [81].

Appropriate measures to avoid making biased decisions include accessing the opinions of related providers (e.g., an obstetrician and/or maternal-fetal medicine) and obtaining a second opinion.

Justice is better achieved by having a consensus within a given medical practice on a specific BMI threshold, rather than case-by-case decision-making. A "rule" serves as a unified decision by the providers and is understandable to patients and protective of mother and fetus. It can be debated, decided, and applied fairly, to minimize conscious or unconscious bias between providers. It also allows for justifiable differences in inter-practice BMI thresholds – a higher risk threshold (due to resources, expertise, or other factors) can justify a higher BMI threshold. And as long as an obese patient is referred to another provider comfortable with the considerations, it is justifiable for a provider with lower-risk tolerance to decline care.

11.6 Conclusions

Obesity is a pervasive challenge in medicine. In this "obesogenic environment" our society has structured, it is optimistic to expect willpower to overcome the evolutionary and physiologic forces that result in accumulation of excess weight [88]. Yes, obesity is considered to be largely preventable, but unless society truly commits itself to reducing the caloric density of our highly refined diet and limit many of the conveniences that make our life more sedentary, the obesity trend is likely to continue. This will require substantial changes on a cultural, economic, and political level. Do we have the willpower for that?

In the meantime, we must contend with how to best address the present and, from the perspective of this chapter, how to best consider application of a BMI threshold for IVF. From the good practice and ethical perspective, most people would agree that a BMI threshold for IVF is necessary to apply at some point. Further, a threshold is readily justified from a good practice perspective and supported by the ethical principles of autonomy, nonmaleficence, and justice.

Finally, arguments for a specific BMI threshold (whether that is 35 kg/m^2, 40 kg/m^2, or 50 kg/m^2, all of which are used in US fertility practices) can be met by counterarguments, but since it becomes the provider's responsibility to decide where the threshold best applies as it relates to medical outcomes, providing a framework for making these decisions is a goal of this chapter. First, it is important to acknowledge that the appropriate threshold may vary, and it would in fact be worse to use a single, uniform threshold. Different practices have different resources, and more complicated circumstances can and should be handled accordingly. Second, it is important for providers to individually decide what level of risk they are comfortable with. It is entirely acceptable to have variation in risk tolerance in the appropriate context. The Ethics Committee of the ASRM provides the following guidance:

> Whenever possible, physicians should encourage patients to reduce their modifiable risk factors. In cases where the patient is unable or unwilling to modify her risk, physicians may differ regarding whether or not to treat the patient. Treatment decisions must be based on medical considerations and applied without bias [or discrimination]. It is acceptable for physicians to decline to provide fertility treatment. Clinicians may differ ethically about what constitutes a reasonable level of risk to the pregnant woman….It is ethically acceptable for clinicians, based on their unbiased assessments of risk, to decline fertility treatments to women at high risk of complications to themselves or their resulting children [81].

With respect to bias, it is important to have awareness of what drives a decision and have a low threshold for getting additional medical input and alternate opinions. And lastly, tied to individual differences in risk tolerance, it is useful to seek consensus within a provider group on the appropriate BMI threshold. This is ideally reached with a detailed review of the relevant medical literature and risks and consideration of the resources and experience available to the providers. Using a common "rule" allows bias to be minimized and provides an ethical basis for treatment decisions.

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Chapter 12
Are We Closer to "Freeze-All" for ART?

Daniel J. Kaser and Jason Franasiak

Abbreviations

COS	Controlled ovarian stimulation
FET	Frozen embryo transfer
PPE	Premature progesterone elevation
WOR	Window of receptivity

12.1 Introduction

Embryo cryopreservation is critical to the success of any in vitro fertilization (IVF) program. With a conventional approach to IVF, the top-quality embryo(s) is transferred in a fresh cycle, and the remaining supernumerary embryos are cryopreserved. A freeze-all is performed only in cases of preimplantation genetic testing (PGT) for single-gene conditions or aneuploidy screening, fertility preservation, or concern for ovarian hyperstimulation syndrome (OHSS). Over the last 10 years, though, a different strategy has emerged, in which the IVF cycle is intentionally segmented into two phases: (1) controlled ovarian stimulation (COS) with planned cryopreservation of all suitable embryos and (2) frozen embryo transfer (FET) after at least one intervening menses. With increasingly compelling data, it has become apparent that not only are implantation rates higher with a planned freeze-all strategy, but also pregnancy and neonatal outcomes may likewise be improved. Indeed, the incidence of adverse events such as OHSS, miscarriage, ectopic pregnancy, and low birth weight may all be reduced following FET [1–3]. This chapter reviews the

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effect of COS on endometrial development, indications for, and evidence supporting a planned freeze-all strategy including premature progesterone elevation (PPE) and delayed embryo development, the optimal developmental stage and method for cryopreservation, and programmatic changes to consider when transitioning to a freeze-all program.

12.2 Effect of COS on Endometrial Development

Through the normal menstrual cycle, the endometrium undergoes an orderly and predictable series of histologic changes in the glandular, stromal, and vascular compartments in preparation for implantation. This series of events is orchestrated by sequential variation in the local concentration of ovarian sex steroids, and COS can profoundly impact the expression of key genes involved in this process. A paired microarray analysis of midsecretory endometrial biopsies from the same patients following unstimulated and stimulated cycles demonstrated that COS leads to marked disruption in the transcriptional program of the endometrium [4]. Furthermore, the choice of COS protocol (i.e., GnRH agonist vs. GnRH antagonist) can likewise alter expression of key genes involved in implantation, including cyclins, cyclin-dependent kinases, members of the E2F transcription factor family, TGF-β signaling, complement and coagulation cascades, and leukocyte migration [5, 6]. Notably, transcriptomic signatures vary even in the absence of histological changes in the uterine epithelium [7].

A novel test called the endometrial receptivity array sequences RNA from 236 genes that are differentially regulated in the peri-implantation period. This test can distinguish a receptive state from a pathologic pre-receptive or post-receptive state, in which the window of receptivity (WOR) is displaced [8]. Using this test, it is clear that the trigger method (GnRH agonist vs. hCG) and type of luteal support (vaginal progesterone, recombinant LH, or dual trigger with low-dose hCG) can likewise affect endometrial gene expression [9].

12.3 Indications for Freeze-All

Freeze-all cycles are commonly limited to patients undergoing PGT and fertility preservation or if there is a concern for OHSS. These indications are well-established and will not be discussed further. Here, we focus on other indications for freeze-all, including premature progesterone elevation (PPE) and embryo-endometrial dyssynchrony. The uncoupling of COS and embryo replacement eliminates the known deleterious effects that supraphysiologic steroid concentrations exert on the endometrium and allows embryo transfer to occur in a programmed cycle in which both the endometrial and embryo development may be more tightly controlled.

12.3.1 Premature Progesterone Elevation

Progesterone (P4) mediates endometrial decidualization with attendant glandular dilation, endothelial proliferation, and hypercoiling of spiral arteries. A group of steroid-responsive genes is likewise activated post-LH surge in preparation for implantation, including interleukins, such as leukemia inhibitory factor, integrins, cadherins, and mucins [10, 11]. Epigenetic alterations like DNA methylation and histone acetylation likewise occur in the glandular, luminal, and stromal compartments of the endometrium in response to P4 rise [12]. When P4 rise occurs prior to surge, these same series of events transpire, and the result is a shift in the WOR (Fig. 12.1).

Mid-follicular P4 rise has been shown to be detrimental to live birth rates following fresh ET in several cohort studies. In a meta-analysis of 63 studies (n = 55,199 fresh transfers), PPE was inversely associated with ongoing pregnancy and live birth rates [13]. The effect of PPE on fresh cycle outcomes appears to be dose-dependent: even modest elevations to 0.8–1.1 ng/mL may impair pregnancy rates (OR 0.79; 95% CI 0.67–0.95), and higher levels may be associated with a more pronounced effect (1.2–1.4 ng/mL: OR 0.67; 95% CI 0.53–0.84; 1.5–1.8 ng/mL:

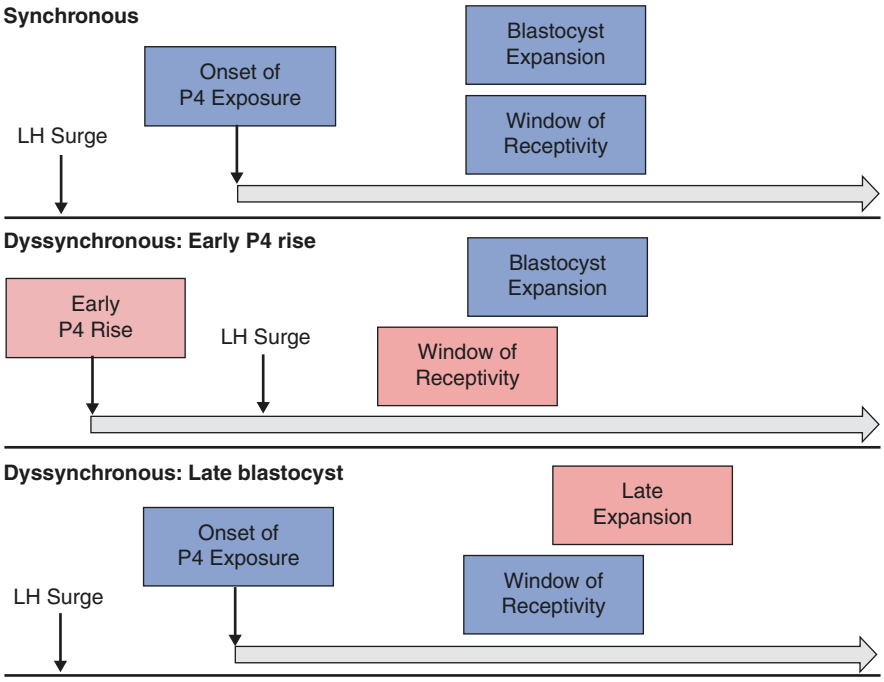

Fig. 12.1 Schematic depictions of (**a**) synchronous cycle, (**b**) dyssynchronous cycle due to premature progesterone elevation with the resulting shift in the window of receptivity, and (**c**) dyssynchronous cycle due to delayed blastocyst development may fail to implant despite an appropriately timed window of receptivity

OR 0.64; 95% CI 0.54–0.76; Fig. 12.2). Importantly, the threshold for determining what degree of PPE has a meaningful impact on clinical outcomes is likely specific to the local center and will depend on the assay used, but most data would indicate that cycles with P4 > 2 ng/mL at trigger have higher pregnancy rates with a freeze-all and subsequent FET [14]. Indeed, in this retrospective paired analysis of 608 women undergoing one fresh ET and one subsequent FET, live birth rates were significantly lower in fresh cycles with PPE ≥ 2 ng/mL compared to FET cycles (10% vs. 47%; $P = 0.02$; Fig. 12.2). Not surprisingly, the P4 concentration on day of surge in the fresh cycle did not have an effect on live birth rates in the subsequent FET cycle (< 2 ng/mL vs. ≥ 2 ng/mL: 45% vs. 47%; $P = 0.99$) [14]. These authors conclude that cryopreservation of all embryos with subsequent FET in cycles in which there is PPE ameliorates the detrimental effect of P4 rise on implantation.

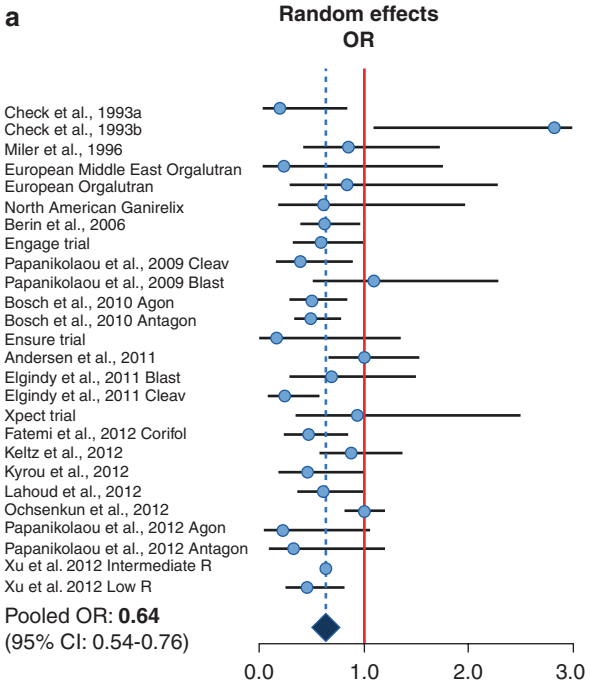

Fig. 12.2 (**a**) Forest plot of 26 studies showing the pooled odds ratio (OR) of the effect of premature progesterone elevation (1.5–1.8 ng/mL) on clinical pregnancy rate. Reprinted with permission from Venetis et al. Hum Reprod Update 2013;19:433–57 [13] (**b**) Implantation and (**c**) live birth rates in a paired analysis of 608 women undergoing a fresh and frozen embryo transfer cycle according to serum progesterone concentration on the day of surge. Reprinted with permission from Healy et al. Fertil Steril 2016;105:93–9 [14]. (**d**) Effect of female age and progesterone concentration at trigger on the odds of ongoing pregnancy for freeze-all vs. fresh cycles. Age (years) is indicated in the panel above each graph. Reprinted with permission from Wang et al. Fertil Steril 2017;108:254–61 [15]

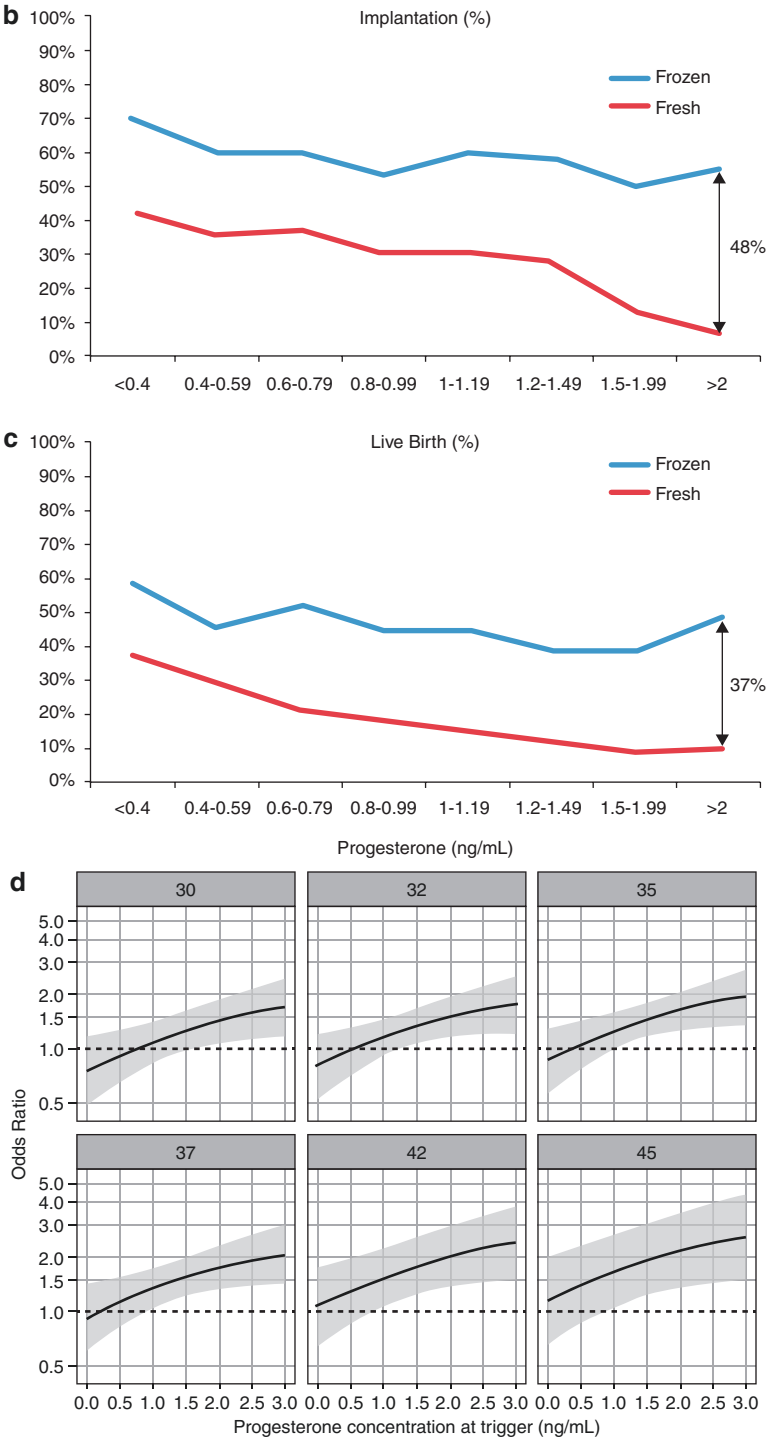

Fig. 12.2 (continued)

More stringent cutoffs, as low as >1 ng/mL, have also been proposed [15]. Receiver operating characteristic (ROC) curve analysis indicates that this threshold maximizes the sensitivity and specificity for ongoing pregnancy (area under the curve 0.526). This retrospective analysis also demonstrated that the P4 thresholds may vary according to female age (Fig. 12.2d). These data highlight the relatively limited subset of patients that is optimally positioned for a fresh transfer.

Certain stimulation protocols, such as the GnRH microflare regimen, may lead to PPE through rescue of a corpus luteum. Likewise, higher follicle counts [13] and an exogenous LH:FSH ratio < 0.3 or > 0.6 are associated with a higher risk of late P4 rises [16]. Thus, clinicians may be able to minimize the likelihood of PPE through protocol selection. Of note, a spurious cause of PPE is dehydroepiandrosterone (DHEA) supplementation, as conversion to DHEA-sulfate has been shown to interfere with some progesterone immunoassays [17]. Thus, P4 levels in patients taking DHEA should be interpreted with caution so as not to misguide clinical management.

12.3.2 Delayed Embryo Development

Not only can PPE impair implantation rates but so can embryos undergoing delayed cavitation and/or expansion. The so-called "slow-growing" embryo, even when the WOR is not displaced, can still fail to implant due to embryo-endometrial dyssynchrony (Fig. 12.1).

Blastocyst morphology is scored at approximately 116–120 h post-insemination on day 5 and approximately 140 h post-insemination on day 6 according to the degree of cavitation and expansion and the quality of the inner cell mass (ICM) and trophoectoderm (TE) [18]. Blastocysts with a greater degree of expansion have higher implantation rates as compared to early blastocysts (43% vs. 17%; $P < 0.001$) [19]. This correlation between higher blastocyst stage and implantation rates in fresh transfers has been corroborated in other studies as well [20–23].

Indeed, when an expanded blastocyst is available for transfer on day 5, implantation rates are likely similar between fresh and FET cycles. This was demonstrated by Wirleitner et al. [24] in a retrospective analysis of the same cohort of patients undergoing fresh ($n = 1010$) and vitrified/warmed blastocyst transfer ($n = 1270$). For patients undergoing transfer of a stage 4 or stage 5 blastocyst, there was no difference in implantation rates according to cycle type (fresh: 29.8% vs. frozen: 27.4%; $P > 0.05$; Fig. 12.3). However, when an earlier stage 2 or stage 3 blastocyst was available, implantation rates were significantly lower in fresh cycles compared to freeze-all with subsequent FET (24.8% vs. 45.0%; $P < 0.001$; Fig. 12.3). Similarly, when a compacting cleavage-stage embryo, morula, or early blastocyst was transferred in a fresh cycle, implantation rates were low (3.1–8.0%); in contrast, if such embryos were cultured for an additional day, vitrified, and transferred in a subsequent warmed cycle, implantation rates improved (21.9%–24.7%; $P < 0.001$ for the comparison of fresh vs. frozen; Fig. 12.3).

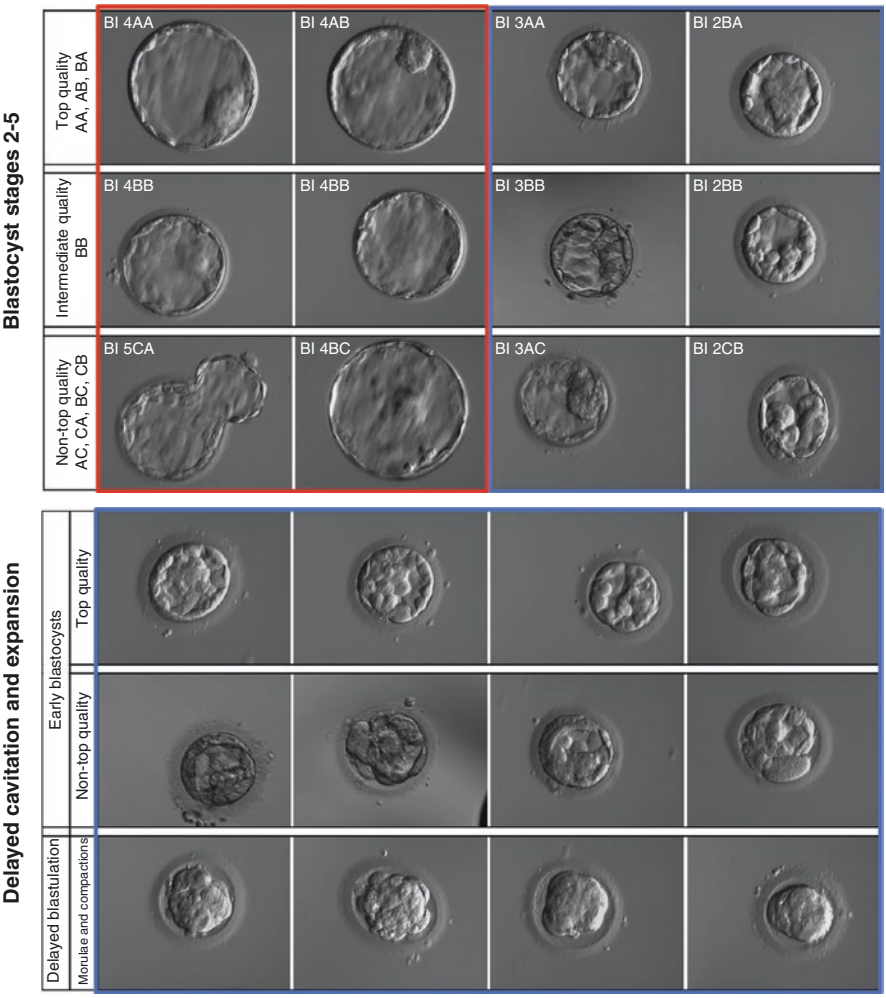

Fig. 12.3 Bright field images of day 5 embryos with varying developmental stages. Red box represents the stages of embryos in which implantation rates were similar between fresh and frozen transfer; blue boxes represent the stages of embryos in which prolonged culture, vitrification, and subsequent FET resulted in higher rates of implantation. Reprinted with permission from Wirleitner et al. Hum Reprod 2016;31:1685–95 [24]

Thus, the timing of blastocyst expansion (day 5 vs. day 6) may be a primary determinant of implantation rates in fresh transfers. Several other retrospective studies likewise indicate that delayed blastulation has a detrimental effect on implantation, due to a shift in embryo-endometrial synchrony (Fig. 12.1). For example, Shapiro et al. [25] reported that implantation rates were nearly twofold higher following the transfer of an expanded blastocyst on day 5 as compared to an equivalent embryo on day 6 (36.3% vs. 19.0%; $P < 0.001$). Similarly, time to cavitation and time to expansion have both been shown to be somewhat predictive of implanta-

tion potential in time-lapse imaging studies [26, 27]. The detrimental effect of "slow-growing embryos" is further exacerbated by PPE in fresh cycles, as shown in a retrospective analysis of day 5 ($n = 4120$) and day 6 ($n = 230$) transfers [14]. This analysis showed that day 6 live birth rates were 8% lower than day 5 when P4 was not elevated ($P = 0.04$) and 17% lower with PPE ≥ 1.5 ng/mL ($P < 0.01$).

In addition to the Wirleiner et al. study described above, other studies have confirmed that cryopreservation of embryos with delayed blastulation and subsequent FET may be preferred to fresh transfer of these embryos [28–30]. Such embryos were once deemed to be of lesser quality, but accumulating evidence suggests that the observed lower implantation rates are likely due to embryo-endometrial dyssynchrony, not inferior embryonic competence. Prolonged culture with cryopreservation of embryos with delayed blastulation and expansion may be one method to overcome these impaired rates.

12.4 Outcomes Following FET

Data compiled by the Center for Disease Control in the 2014 National ART Summary Report [31] indicate that rates of pregnancy, live birth, and singleton live birth are higher in all age categories following FET ($n = 56,259$), as compared to fresh ET ($n = 67,070$) (Fig. 12.4). An important limitation of how this data is reported is it is not stratified by whether or not the transferred embryos had undergone PGT. Still, this consistent increase in rates from a heterogeneous group of clinics supports the notion that FET may be associated with improved cycle outcomes.

Accumulating class I data likewise support FET as the preferred strategy for embryo replacement to optimize pregnancy rates per transfer. There have been four randomized controlled trials (RCTs) that randomized patients to undergo fresh ET vs. freeze-all with subsequent FET, and all have shown improved pregnancy rates with freeze-all [32–35]. Notably, one study [32] has been retracted due to serious methodological flaws. Of the remaining three, two reported statistically significant results [2, 33]. Shapiro et al. randomized 137 normal responders <41 years undergoing their first IVF cycle to fresh day 5 or day 6 ET vs. bipronucleate (2PN) freeze-all with subsequent blastocyst transfer in a downregulated FET cycle [33]. Per-protocol analysis revealed that rates of implantation (70.8% vs. 38.9%, $p < 0.0001$) and ongoing pregnancy (78.0% vs. 50.9%, $p < 0.01$) were significantly higher with FET. Intention-to-treat analysis was not reported in this study, but using the data available, a 15% improvement in ongoing pregnancy rates was still apparent with FET (55.7% vs. 40.3%, $P = 0.09$). In a follow-up study of 122 high responders, the same group reported a 12% increase in ongoing pregnancy in the FET group, but the study was underpowered to detect statistical significance at this effect size [34].

More recently, Chen et al. [2] randomized 1508 women with PCOS according to the modified Rotterdam criteria undergoing their first IVF cycle to fresh day 3 transfer vs. cryopreservation of all embryos at the cleavage-stage with subsequent frozen

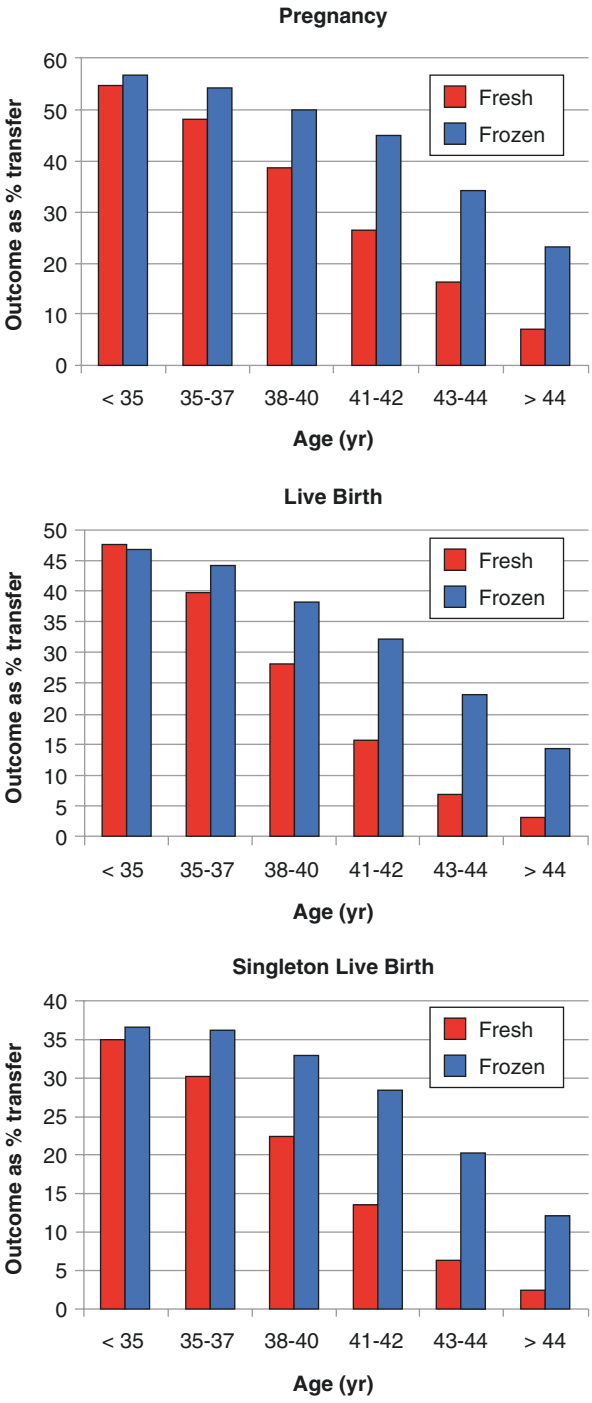

Fig. 12.4 SART outcomes of fresh vs. frozen embryo transfers according to patient age. Data from the Centers for Disease Control and Prevention, American Society for Reproductive Medicine, and Society for Assisted Reproductive Technology. 2014 Assisted Reproductive Technology National Summary Report. Atlanta (GA): US Dept of Health and Human Services; 2016 [31]

day 3 transfer. The study had 80% power to detect an absolute difference of 10% in live birth rates ($\alpha = 0.01$). These authors observed a significantly higher live birth rate with vitrified-warmed cleavage-stage transfer (49.3% vs. 42.0%; RR 1.17, 95% CI 1.05–1.31). It is important to note that all of these studies have been restricted to good-prognosis patients undergoing their first IVF cycle. Studies of low responders or those with multiple failed IVF cycles are lacking. A fifth randomized study (NCT02471573) of fresh vs. freeze-all that specifically excludes patients with PCOS is currently ongoing.

Other outcomes, including miscarriage, OHSS, and ectopic pregnancy, may also be improved with a freeze-all approach. Chen et al. reported that women allocated to planned FET had a lower miscarriage rate (22.0 vs. 32.7%; RR 0.67, 95% CI 0.54–0.83) and a lower rate of OHSS (1.3 vs. 7.1%; RR 0.19, 95% CI 0.10–0.37). Several studies indicate that the rate of ectopic pregnancy may be lower with FET [1, 36, 37]. Among 31,925 women undergoing IVF, the absolute risk of ectopic pregnancy per transfer was 1.97% in the fresh group and 1.01% in the FET group ($P < 0.001$) [38]. In another analysis of 103,700 pregnancy cycles, FET was associated with a 65% reduction in the odds of ectopic pregnancy (OR 0.35, 95% CI 0.29–0.42, $p < 0.001$); this association was upheld in a paired subanalysis of women who underwent both a fresh and frozen transfer [1].

Several cohort studies have assessed the incidence of adverse neonatal outcomes following fresh and frozen transfers [39, 40]. A consistent observation is that the risk of low birth weight is lower with FET, independent of the gestational age at delivery. This was shown most robustly in a SART registry study of 56,792 singleton live births by Kalra et al. [3], in which the incidence of preterm and term low birth weight was compared according to cycle type. Importantly, these authors reported FET was associated with a lower odds of preterm low birth weight (23.8% vs. 34.1%, adjusted OR 1.49, 95% CI 1.24–1.78) and term low birth weight (1.2% vs. 2.5%, adjusted OR 1.73, 95% CI 1.31–2.29). When data were restricted to a paired analysis of women who delivered one infant from a fresh transfer and another infant from a frozen transfer, the higher odds of low birth weight from fresh transfer was further increased (11.5 vs. 5.6%, adjusted OR 4.66, 95% CI 1.18–18.38). Notably, when analysis was restricted to donor oocyte cycles, the effect of fresh transfer on low birth weight was mitigated (11.5% vs. 11.3% adjusted OR 0.99, 95% CI 0.82–1.18), arguing that embryo replacement into a stimulated endometrium may be the causal factor.

A recent systematic review and meta-analysis of 13 cohort studies including 126,911 women likewise demonstrated that fresh ET was associated with a higher risk of low birth weight (OR 1.48, 95% CI 1.37–1.60) [41]. These authors also reported a higher risk of preterm birth with fresh transfer (OR 1.14, 95% CI 1.02–1.28). There were no differences in stillbirth (OR 1.01, 95% CI 0.76–1.35) or perinatal mortality (OR 1.11, 95% CI 0.85–1.46).

In summary, freeze-all cycles significantly improve rates of implantation and live birth among good-prognosis patients and may also be associated with a reduction in miscarriage, ectopic pregnancy, preterm birth, and low birth weight.

12.5 Optimal Stage and Method for Cryopreservation

Embryos can be cryopreserved at the zygote, cleavage, or blastocyst stage. There is no consensus about the preferred stage of cryopreservation, and likely in a high-performing laboratory, there is little difference in cryosurvival rates [42]. In the only RCT assessing the effect of stage of cryopreservation (2PN vs. blastocyst) on rates of cryosurvival and subsequent implantation, there was no difference in either end point according to the day of freeze [43]. Freezing at the blastocyst stage offers the important advantage of trophectoderm biopsy for PGT and also more accurately reflects the embryo inventory to patient and clinician alike. Furthermore, 2PN freezes are still at risk for embryonic dyssynchrony, which voids much of the benefit of doing a freeze-all in the first place. For these reasons, it is our opinion that programs that elect to pursue a freeze-all strategy should cryopreserve embryos at the blastocyst stage.

Two methods of cryopreservation are used in contemporary practice: equilibrium cooling (otherwise known as slow freezing) and vitrification [42]. While both methods can safely and effectively store embryos for an unspecified amount of time, vitrification seems to offer improved rates of cryosurvival. A 2016 meta-analysis of seven randomized studies ($n = 3615$ embryos) demonstrated higher embryo survival rates following vitrification/warming (RR 1.59, 95% CI 1.30–1.93; $p < 0.001$) [44]. Likewise, clinical pregnancy rates were reported to be higher (RR 1.89, 95% CI 1.00–3.59; $p = 0.051$; 3 RCTs; $n = 638$). Vitrification is also quicker and less expensive than slow freezing. Given these improvements in laboratory and clinical outcomes, most contemporary laboratories have transitioned to vitrification as the preferred method for embryo cryopreservation.

12.5.1 Types of FET Protocols

Uterine preparation for FET may be conducted using a natural cycle, a modified natural cycle, or a synthetic cycle [45]. While there are certain advantages and disadvantages to each in terms of medication requirements and ease of scheduling, clinical outcomes appear to be similar. Indeed, a 2016 systematic review and meta-analysis of 33 studies evaluating the various methods of endometrial preparation for FET demonstrated that there was no difference in live birth rates according to protocol choice [46]. Further study, in particular, whether one method of luteal replacement is superior to another for FET cycles, should be undertaken.

A common question from patients following a freeze-all cycle is how long they should wait until undergoing FET; limited data are available, but the one retrospective analysis that compared immediate FET after the trigger-induced withdrawal bleed to delaying FET by at least one intervening menses indicated that clinical pregnancy rates were similar between the two [47].

12.6 Should Programs Universally Adopt a Freeze-all Strategy?

Elimination of fresh ET in favor of a freeze-all strategy is poised to improve the safety and efficacy of ART. Whether or not programs will adopt this as a universal approach to IVF remains to be determined. There are increasingly fewer circumstances under which a fresh embryo transfer may be appropriate. At a minimum, a freeze-all approach should be used for primary prevention of OHSS among high responders and PGT cycles, in the face of PPE and/or a cohort of embryos with delayed blastulation and expansion. For programs that are considering a transition to the routine use of cycle segmentation, a SWOT analysis of strengths, weaknesses, opportunities, and threats is presented in Fig. 12.5 [48]. Ultimately, some questions remain: How will patients accept the recommendation for freeze-all? Are improved outcomes generalizable to poor responders and those who have failed multiple IVF cycles? How will staffing, laboratory flow, inventory, and cryostorage capacity be affected by the increased demand for freezing? Will insurance providers in mandated states penalize patients who take this approach? And perhaps most salient, who actually benefits from a *fresh* transfer, and how can they be reliably identified?

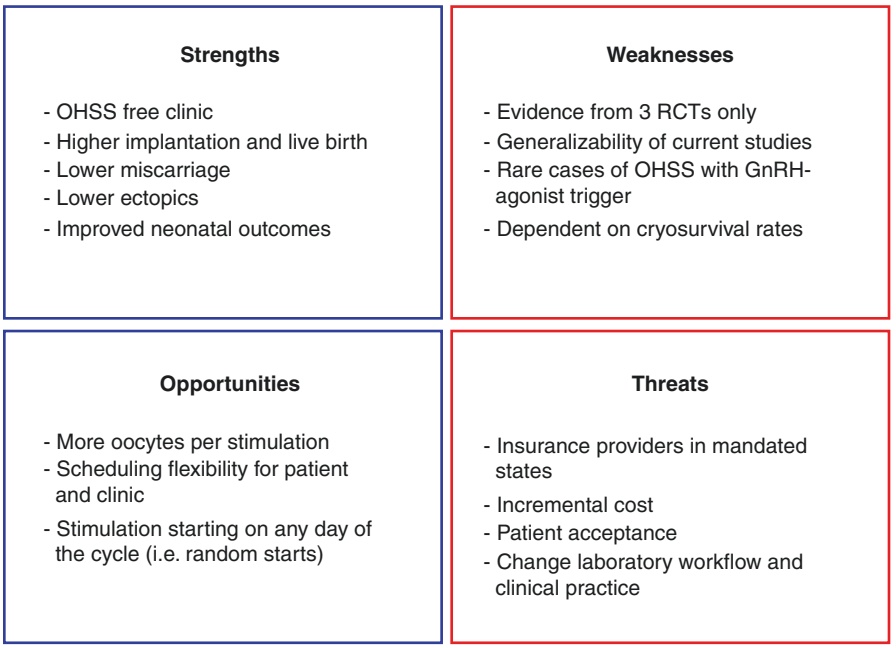

Fig. 12.5 SWOT analysis of a freeze-all IVF program. Reprinted with permission from Blockeel et al. Hum Reprod 2016;31;491–7 [48]

12.7 Conclusions

IVF cycle segmentation with COS, planned freeze-all, and subsequent FET has become an increasingly popular strategy in contemporary practice. This approach not only virtually eliminates the risk of OHSS and allows the time necessary for PGT, it also circumvents problems with embryo-endometrial dyssynchrony due to PPE and delayed embryo development. Furthermore, rates of implantation and live birth have been shown to be higher in RCTs of good-prognosis patients, and pregnancy and neonatal outcomes, including miscarriage, ectopic pregnancy, and low birth weight may all be reduced. It is our opinion that the freeze-all approach will become the dominant strategy for IVF in the not too distant future.

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Chapter 13
Preimplantation Genetic Screening: Not for Everyone

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13.1 Introduction: Preimplantation Genetic Screening

Preimplantation genetic screening (PGS) is a tool used during assisted reproductive technology (ART) in which an embryologist removes a small number of cells from an embryo and send those cells for DNA evaluation to determine the ploidy status of the embryo. The technology was developed with the hope of removing aneuploidy as a factor in ART and thereby greatly reducing in vitro fertilization (IVF) implantation failure or pregnancy loss [1]. PGS initially involved blastomere biopsy of day 3 embryos, which typically contain 6–10 cells, followed by fluorescent in situ hybridization (FISH) analysis. Though initially thought to be a way to improve in vitro fertilization (IVF) outcomes by eliminating aneuploidy as a cause of implantation failure or early pregnancy loss [2], PGS performed in this manner not only failed to improve pregnancy rates or minimize miscarriage rates, but also it lowered live birth rates following ART [3].

The reincarnation of PGS introduced a number of modifications designed to improve safety and accuracy. Embryos are currently biopsied on day 5 or 6, at the blastocyst stage of development where the embryo contains approximately 100 cells. At this point the trophectoderm, destined to become the placenta and membranes, has differentiated from the inner cell mass, so cells destined to become the fetal component of the embryo are not perturbed. Because embryos at this stage of development have many more cells, sampling of three to five cells instead of just one theoretically reduces error rates. The status of all chromosomes is now assessed as part of PGS, so-called comprehensive chromosomal screening (CCS).

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Reminiscent of its predecessor, PGS has been touted as a way to improve IVF outcome for all patients undergoing ART [4]. A meta-analysis published in 2015 concluded that the use of PGS of blastocysts increased clinical and implantation rates, especially in patients with normal ovarian reserve [5]. However, this conclusion was drawn from observational studies. Three randomized control trials (RCTs) have tested the efficacy of PGS on blastocysts. All three, however, included only young women with normal ovarian reserve, so the results may not be generalizable to the full range of patients who undergo ART, who increasingly tend to be older and to have diminished ovarian reserve. Also, subjects were randomized at the time of embryo transfer rather than at cycle initiation. Therefore, they did not follow the intention-to-treat principle expected of well-designed RCTs. Furthermore, the embryo quality criteria for trophectoderm biopsy was stringent with patients requiring 2 or more Gardner stage 3 or higher blastocysts in order to be eligible for inclusion in the study. Presumably, patients with diminished ovarian reserve and/or older women would be less likely to have sufficient numbers of blastocysts and therefore would be underrepresented in this sample.

Though the technology's safety profile has improved considerably with this latest iteration with presumably minimal harm to the embryo [6], critics voice concerns about its widespread applicability in the general IVF population [7]. Indeed, with the widespread acceptance and utilization of PGS technology, improvements in live birth rates have not been shown uniformly across the heterogeneous IVF population [8–10]. Thus, PGS is likely better suited to be a tool to address specific goals, such as minimizing multiple gestations, minimizing time to pregnancy in older patients with high rates of aneuploidy, facilitating gender balancing, or screening embryos in women with a history of recurrent pregnancy loss (RPL).

PGS has been successfully utilized in minimizing the risk of multiple gestations. In the BEST trial by Forman et al. in 2014, women up to age 42 undergoing IVF at a large private center were randomized to receive either one euploid embryo selected by CCS or the standard of care at the time, which were two non-biopsied blastocysts for transfer. This study demonstrated that single embryo transfer (SET) following CCS produced live birth rates comparable to double embryo transfer (DET) but reduced the risk of multiple gestations from 47 to 1.6%. The risks of multiple gestations, including preterm delivery, low birthweight, and NICU admission, also were reduced following PGS [11]. However, the applicability of these results is limited to women who have biopsiable blastocyst stage embryos. Thus, older women, who have high rates of aneuploidy, and women with diminished ovarian reserve were excluded from the study because they tended not to produce any blastocysts or blastocyst stage embryos of sufficient quality to be biopsied. The evidence in support of the utility of PGS is heavily weighted toward women with robust ovarian reserve.

PGS is predicated on the concept that culturing embryos to the blastocyst stage itself is benign. The assumption is that embryo culture screens out the less robust embryos, which cannot survive the stress of the in vitro culture system, and if embryos do not survive the stress of the in vitro environment, they are not destined to produce a live birth. However, evidence suggests that some embryos that do not survive to day 5 or 6 in vitro still may establish normal pregnancies if transferred on

day 3 [12–14]. Indeed, two randomized controlled trials reported a higher cumulative ongoing pregnancy rate per oocyte recovery in day 2/3 transfer as compared with day 5 transfer [15, 16]. The benefit of day 2/3 transfer is not consistent across the literature: other randomized controlled trials have found the opposite to be true [17, 18]. A recent meta-analysis comparing day 3 to day 5 embryo transfers found no significant difference between live birth rates, though the quality of evidence was considered poor [19]. Thus, if a patient has a history of poor blastocyst conversion rate or has few embryos, a day 3 transfer without PGS may provide the best chance of a successful pregnancy.

The role for PGS in embryo selection for donor egg cycles remains an open question. A 2014 study examining aneuploidy rates in 305 donor oocyte embryos found that even in donors aged <30 years old, only 53% of the blastocysts were aneuploid [20]. The authors suggested that PGS might offer a way to minimize the transfer of abnormal donor egg embryos. A retrospective single-center study published by Coates et al. in 2017 evaluated the role for PGS in donor egg embryos and found aneuploidy rates to be lower than the Sills et al. study. In the Coates et al. study, patients who had SET with PGS-screened embryos had a higher ongoing pregnancy rate than non-screened embryos (58% vs. 36%) but the difference did not reach statistical significance. In the same study, PGS embryos did better than non-PGS embryos in gestational carriers in SET, again falling just shy of statistical significance [21]. Another retrospective study by Haddad et al., published in 2015, had similar findings – PGS-screened donor egg embryos had an aneuploidy rate of 39.1%. The PGS-screened embryo group had increased implantation and pregnancy rates and reduced miscarriage rates compared to the unscreened embryo group, but this difference did not reach statistical significance [22].

The turnaround time for most CCS methods requires that the blastocyst be cryopreserved pending the results of the PGS. This cryopreservation step may be especially stressful for embryos that previously had been frozen and/or embryos derived from previously cryopreserved donor oocytes. A retrospective study by Deng and Wang published in 2015 found that only 15.8% of blastocysts created from 764 frozen donor eggs were aneuploid and aneuploidy rates varied among donors. Moreover, transfer of blastocysts screened by PGS resulted in lower implantation and clinical pregnancy rates compared to unscreened blastocysts [23]. Recently, Barad et al. published a retrospective cross-sectional study using SART data with 392 first preimplantation genetic screening cycles compared to 20,616 control cycles from 2005 to 2013. The authors found that PGS in donor oocyte cycles was not associated with improved odds of live birth or reduction in miscarriage rates [10]. The authors suggest that the role for PGS in donor egg embryos needs to be explored further in prospective, randomized trials to determine if PGS significantly improves outcomes.

The first international multicenter, blinded, randomized controlled trial evaluating the role of PGS embryos from women aged 25 to 40 was presented at the annual meeting for the American Society of Reproductive Medicine 2017. This study randomized patients who met eligibility criteria on day 5 or 6 of embryo culture, meaning that they did develop blastocysts, to either trophectoderm biopsy and PGS with

NGS or embryo selection based on morphology. The primary outcome was ongoing pregnancy rate at 20 weeks gestation. The study found no significant improvement in pregnancy or miscarriage rates for women less than 35 years of age. In women 35 years and older, the PGS group had a superior ongoing pregnancy rate [24]. Thus, PGS is not applicable across all IVF populations but may provide benefit to older women with good ovarian reserve and embryo development who have higher rates of aneuploidy.

Though PGS does not appear to benefit the general IVF population, in theory, it offers help to those with recurrent pregnancy loss secondary to aneuploidy to minimize the risk of miscarriage. However, not all studies support this assumption. A study by Perfetto et al., published in 2015, noted that time to pregnancy was increased with the use of PGS from an average of 3 months in spontaneous pregnancy to 5 months with IVF and PGS. In the 6-month follow-up of study participants, there was no significant difference in ongoing pregnancy rate [25].

PGS has not been found to be a cost-effective strategy for increasing live birth. In a 2015 study by Murugappan et al., the live birth rate for IVF/PGS in a non-insurance mandated state would have had to be 91% for the screening to be cost-effective compared to expectant management. However, the live birth rate with IVF/PGS was only 53% (vs. 67% in expectant management), and clinical miscarriage rate was 7% (vs. 24% in expectant management group) [26]. The same group published a retrospective cohort study of an intent-to-treat analysis of IVF/PGS vs. expectant management in non-infertile RPL patients. The 2016 study found that PGS success rates are limited by the high incidence of canceled PGS in cycles or cycles that do not reach transfer.

In the older patient population, some have argued that employing PGS can decrease the risk of clinical miscarriage because of the higher rates of aneuploidy with advancing age, thus overall minimizing the time to pregnancy in a population where time is of utmost importance. This theoretical benefit has been found only in older women with robust ovarian reserve. In a retrospective study by Lee et al. in 2015, implantation rates were compared between fresh IVF cycles with day 5 embryo transfers without PGS, frozen embryo transfer without PGS, and frozen embryo transfer with PGS in women ages 40–43. Implantation and clinical pregnancy rates were higher for the PGS frozen embryo transfer cycles [27]. Though promising, the analysis was performed according to embryo transfer rather than intention-to-treat, i.e., from cycle start. Thus, it is difficult to extrapolate to a population consisting of many 40- to 43-year-old women who will not have embryos that meet criteria for biopsy or euploid embryos to transfer.

A recent iteration of PGS uses next-generation sequencing technology (NGS) to assess embryo ploidy status and has introduced a layer of complexity that makes clinical interpretation of PGS data more challenging than ever before. Mosaicism is quite common during early development, and the increased resolution of NGS detects mosaicism in blastocysts that would have been called euploid by array comparative genomic hybridization (aCGH), the immediate predecessor to NGS. Mosaicism is when two or more genetic variants exist within a single embryo. It is detected when analysis of the biopsied cells reveals a mixture of aneuploidy and

euploidy. Mosaicism had been demonstrated by florescent in situ hybridization (FISH) in cleavage stage embryos [2]. The enhanced sensitivity of NGS now demonstrates mosaicism in blastocyst stage embryos [28, 29]. The immediate and long-term consequences of embryo mosaicism are poorly understood. Faced with the moral imperative to first do no harm and the legal threat from producing an abnormal fetus, many centers refuse to transfer mosaic embryos, further reducing the probability of pregnancy per cycle start.

Rates of mosaicism vary considerably by center, from 69% [30] to just 20% [31]. Maternal age does not affect rates of mosaicism. Fragouli et al. found that approximately one-third of blastocysts from his center were mosaic [32]. Northrop et al. 2010 reported that only 16% of embryos were mosaic [33]. Studies of mosaicism later during development demonstrated that the placenta is more tolerant of mosaicism, implying that biopsy of the trophectoderm, the precursor of the placenta, may not reflect the genetic status of the embryo. Huang et al. 2017, however, examined the genetic composition of both the inner cell mass (ICM) and the trophectoderm cells of discarded embryos and noted concordance between the ICM and trophectoderm in seven out of eight embryos. In only one embryo did trophectoderm differ from ICM [28]. These results contrast with those of Orvieto et al. 2017, who reported three out of eight embryos showed discordance between the ICM and trophectoderm [29]. Both studies are limited by small sample sizes, so additional studies of the relationship between ploidy status of trophectoderm and inner cell mass are needed.

What threshold of mosaicism is clinically relevant remains unclear. Some evidence suggests that abnormal cells may be forced out of the fetal component of the blastocyst and is confined to the placenta so the aneuploidy does not persist in the developing fetus [34]. Thus, trophectoderm biopsy of only a few of the >100 cells in the blastocyst may label the blastocyst as abnormal when, in fact, it may have the potential to progress into a healthy pregnancy. This is especially relevant in women who have few embryos available for biopsy and risk having no embryos for transfer after testing.

Variability in biopsy technique provides a further limitation of PGS. Trophectoderm biopsy is a delicate micromanipulation procedure. The safety of TE biopsy depends on the training and experience of the clinical embryologist. A reassuring cohort study by Capalbo et al. published in 2015 showed that seven embryologists across three IVF centers had high consistency and reproducibility of blastocyst biopsy coupled with qPCR-based PGS for both genetic and clinical outcomes [35]. The embryologists in this study had undergone rigorous training and met quality metrics prior to participating in the study. These results are reassuring, but may not reflect the reality of heterogeneity in embryologists' training and experience in most ART centers. Indeed, a 2017 study by Munne et al. examined euploidy rates for donor egg embryos across 42 infertility clinics using the same NGS methodology and found that euploidy rates varied by ART center, independent of almost all parameters except donor age and testing technology [36]. This suggests that euploidy rates on trophectoderm biopsy are influenced by other factors besides the embryo itself. Some have speculated that stimulation protocols may influence ploidy status.

A retrospective study by Sekhon et al. found no increased risk for embryo aneuploidy based on gonadotropin dose. However, a small subset of patients who were stimulated past 12 days showed a small but significant effect of gonadotropin dose on rate of aneuploidy. This group exhibited a 16.4% increase in the odds of aneuploidy for each 1000-unit increase in cumulative gonadotropin exposure. This relationship did not hold if the analysis was limited to low responders, regardless of gonadotropin dose [37]. Whether stimulation protocol, culture systems, trophectoderm biopsy procedure, or other aspects of the embryo growth and manipulation affect results, PGS remains a highly complex process, whose results vary markedly from center to center.

13.2 Conclusion

Preimplantation genetic screening has revolutionized IVF in many ways, providing insight into the chromosomal makeup of the embryo. While it has yielded improved implantation and ongoing pregnancy rates per embryo transfer in older women with robust ovarian reserve, it does not provide a panacea for all patients undergoing IVF, especially women under the age of 35 years old, the majority of whose embryos are euploid, or women older than 35 with diminished ovarian reserve. The role of PGS is still evolving and further research is needed to clarify which specific patient populations are likely to benefit as it does not appear to be an appropriate universally applicable procedure.

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Chapter 14
Should All Patients Undergo Blastocyst Transfer? No

Wellington P. Martins and Catherine Racowsky

14.1 Arguments Against Always Transferring Embryos at the Blastocyst Stage

- The in vitro environment undoubtedly does not perfectly simulate the environment in vivo, and it is likely that some embryos not forming blastocysts in the lab would do so in vivo and develop into fetuses if they were transferred in an earlier stage. Actually, we are still far from developing in vitro culture systems that are similar to the intrauterine environment, as our knowledge regarding the in vivo conditions remains very limited [1].
- Although at least theoretically extended embryo culture improves embryo selection, there is no robust evidence that such a strategy is able to increase the reproductive outcomes, even when considering only the first embryo transfer [2, 3].
- When there are only a few oocytes, the possibility of improved embryo selection becomes even less important as frequently, all the viable embryos are transferred. Such a scenario is very common in women with poor ovarian response or after mild ovarian stimulation, and it is the rule when performing IVF without ovarian stimulation [4–6].
- The number of available embryos progressing normally in development after extended culture is more difficult to predict then that at the cleavage stage. The average proportion of embryos that reach the blastocyst stage on days 5/6 after fertilization is approximately 40% [2], but the rate is very variable

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among women. Usually, more oocytes or zygotes at the fertilization check are required when deciding on such strategy which, in turn, may result in a higher number of surplus embryos compared with that following cleavage stage transfer. Although this may be beneficial for overall efficacy, a high number of surplus embryos frequently lead to ethical problems regarding what to do with abandoned embryos [7–9].

- By always transferring embryos at the blastocyst stage, more women will have no embryos to be transferred, and more women will not have surplus embryos to be transferred in a subsequent cryo cycle [2].
- The cumulative pregnancy rate per oocyte retrieval is very likely to be lower when performing blastocyst stage embryo transfer, since embryos that fail to develop into blastocysts by days 5/6 in culture are usually discarded. It is quite possible that some of these embryos would have been "rescued" and proceeded in development if they have been transferred at the cleavage stage. On the other hand, there is good evidence that embryos that have developed into blastocysts in vitro by 7 days after fertilization still have a considerable chance of evolving into a healthy baby [10, 11]. Therefore, in laboratories performing exclusively blastocyst transfer, it may be beneficial to maintain more slowly developing embryos until day 7. Although embryo selection is important for reducing the time until pregnancy, the cumulative pregnancy rate per oocyte retrieval is also an important outcome, since this is the most expensive and risky step of the treatment [4, 12].
- There is considerable evidence that blastocyst stage embryo transfer increases some adverse perinatal outcomes, particularly preterm birth and perinatal mortality [13, 14]. The increased perinatal mortality was reported by a large national registry consisting of data from 2002 to 2013: the perinatal mortality rate in singleton pregnancies following blastocyst embryo transfer was 1.0% (0.9% for fresh and 1.2% for frozen), which was significantly higher than the 0.6% rate observed in singleton pregnancies following cleavage embryo transfer (0.5% for fresh and 0.7% for frozen) and the 0.7% observed following natural conception [14]. Although such data is based on observational studies that are prone to bias, the maternal age was slightly lower in women that transferred embryos at blastocyst stage, and therefore the expected complication rate should be lower in this group.
- Extended embryo culture with blastocyst transfer is associated with increased expenses compared with cleavage stage transfer, as more incubators and human resources are needed for the same number of couples being treated. Additionally, some other refinements and expenses, including use of low oxygen tension, are frequently only employed in centers performing extended embryo culture, resulting in further increases in the total lab expenses [15]. And all these extra costs will ultimately increase the total costs of IVF, making the treatment even less accessible, particularly in low- and middle-income countries.
- There is a longer delay between oocyte retrieval and embryo transfer. This may not be desirable if the couple is being treated in a different city from that

in which they live, increasing the total expenses and the days off work. Additionally, there are the associated uncertainties regarding if and when they may have embryos to transfer: although the embryo transfer is likely to occur 5 days after oocyte retrieval, it is possible that embryos will only reach the blastocyst stage at 6 or 7 days after fertilization.

14.2 Conclusions

The most important argument in favor of exclusively performing blastocyst embryo transfer is that such an approach reduces the time to pregnancy and delivery of a baby (Table 14.1). While such an argument makes intuitive sense since extended culture offers the opportunity for further embryo selection, to date there is limited evidence to support such conclusion. Blastocyst transfer is associated with an obvious increase in costs, and there is some evidence of worse obstetric outcomes, including more perinatal deaths, and probably less pregnancies per oocyte retrieval. Therefore, transfer at the cleavage stage should continue to be considered the reference standard, or, at least, it should not be abandoned. We feel that this should be the case even if one day the evidence supports blastocyst embryo transfer for improving the outcomes of the first embryo transfer.

Table 14.1 Main concerns regarding always transferring embryos at blastocyst stage

Efficacy
– No strong evidence of reduced time until achieving an ongoing pregnancy [2]
– Reduced number of women with cryopreserved embryos after the fresh embryo transfer [2]
– Probably reduced cumulative live birth rate per oocyte retrieval [2, 3]
Safety
– Longer exposure to in vitro conditions, not being possible to perfectly simulate the in vivo conditions [1]
– Evidence of increased preterm delivery (<37 weeks): 10.9% vs. 12.2% [13]
– Evidence of increased preterm delivery (<37 weeks): 1.7% vs. 1.9% [13]
– Evidence of increased perinatal death: 0.53% vs. 0.79% [13]
Costs
– Increased laboratory costs: extra time from embryologists and incubators
– Extra time between oocyte retrieval and embryo transfer cause troubles for couples not being treated in their own cities: additional expenses with accommodation and more days off work
Other issues
– Increased risk of not having embryos to be transferred, what can be a source of psychological distress
– Increased risk of having several cryopreserved embryos after pregnancy as frequently more eggs are fertilized to avoid not having embryos to be transferred. There are several psychological/ethical/religious issues regarding what to do with the extra/abandoned embryos [7, 9]

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