REVIEW

New Insights beyond Established Norms: A Scoping Review of Genetic Testing for Infertile Men

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Purpose: From a diagnostic standpoint, certain approaches to genetic screening in clinical practice remain ambiguous in the era of assisted reproduction. Even the most current guidelines do not provide definite guidance on testing protocols, leaving clinicians to carefully determine which tests best serve patients struggling with infertility. The lack of uniformity in the current practice of male fertility evaluation can prove to be quite costly, thus necessitating healthcare practitioners to carefully appraise the necessity and weigh the advantages against potential economic and psychological detriments. The objective of this review is to map the existing literature on the general topic of the clinical indications of routine karyotyping and/or AZF screening in infertile men, identify key concepts, determine where the gaps are, and lastly, provide an overview of the conclusions drawn from a body of knowledge that varies widely in terms of methodologies or disciplines.

Materials and Methods: A thorough search was conducted for the published findings up until July 2023, utilizing PubMed (MEDLINE). This comprehensive search involved the use of specific search keywords, either individually or in combination. The search terms employed were as follows: “Karyotype”, "Klinefelter" or "KS" or "47,XXY", "AZF" or "Azoospermi*" and/or "microdeletion*" in the title or abstract. Once the titles and abstracts of selected articles were obtained, the complete texts of linked papers were meticulously scrutinized.

Results: A total of 191 records were identified from PubMed. During screening, 161 records (84.3%) were eliminated. Finally, 30 papers were included in this scoping review, which was conducted in 18 countries. The number of sequence tag sites (STSs) used in the studies varied from 5 to 59. The rate of AZF deletions among patients with NOA ranged from 1.3% to 53%. The mean frequency was estimated to be 5.6%. The rate of YCM among patients with XXY karyotype was nil in 19 out of 30 studies (63%), whilst, in the remaining studies, the rate varied from 0.8% to 67%.

Conclusion: This review provides insights into managing male infertility. The presence of spermatozoa in ejaculation and successful surgical retrieval cannot be excluded for individuals with AZFb/AZFbc microdeletions. Screening for Y chromosome microdeletions is not needed for mosaic or classic KS. Only 1% of individuals with sperm concentration exceeding 1×10^6 sperm/mL and less than 5×10^6 sperm/mL exhibit AZF microdeletions; therefore, testing referral for such populations may need reassessment. Individuals with mosaic monosomy X karyotype and certain chromosomal anomalies should be referred for AZF deletion screening. These findings have implications for male infertility management and future research.

Keywords: Azoospermia; AZF; Karyotype; Klinefelter syndrome; Genetics; Y chromosome microdeletions; male infertility

INTRODUCTION

Infertility, currently characterized as a disease by the World Health Organization, affects around 15% of individuals worldwide. In nearly half of these cases, male infertility is solely responsible or a contributing factor[(1)]. Male infertility is a complex condition that can be influenced by multiple intrinsic or extrinsic factors, with a genetically proven etiology accounting for around 15% of cases. Genetic factors may impact various physiological processes related to fertility, including hormonal balance and sperm production, both in terms of quantity and quality[(2)]. Hence, genetic testing is of paramount significance in individuals experiencing infertility; not only allowing the prevention of passing on genetic disorders to progeny through assisted reproduction technologies (ART) but also providing insights into genetic defects that may hinder the successful retrieval of sperm through surgical extraction[(1)]. Moreover, as some genetic loci linked to infertility are also associated with oncogenesis, genetic screening can offer insight into one's prospect of developing certain cancers depending on the existence or lack of definite gene variants. This data may help guide medical decisions and optimize health outcomes overall[(3)]. Among the genetic tests, karyotyping and AZF microdeletions are commonly requested by clinicians as part of the in-

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Table 1. Summary of literature data on AZF microdeletion frequency in patients with NOA and KS

References	KS Patients (n)		Rate of AZF deletions		STS[1]	Country
	Classic	Mosaic	KS	NOA		
(Fu, Chen et al. 2023)	80	0	2/80 (2.5%)	211/1980 (10.66%)	15	China
(Kalantari, Sabbaghian et al. 2023)	944	95	0	578/10,388 (5.6%)	6	Iran
(Chen, Chen et al. 2022)	17	0	2/17 (11.8%)	13/186 (7%)	6	Taiwan
(Gumus, Kati et al. 2021)	34	0	0	16/327 (4.9%)	6	Turkey
(Özdemir, Özyılmaz et al. 2020)	75	0	0	45/1696 (2.6%)	7	Turkey
(Sciarra, Pelloni et al. 2019)	116	2	1/118 (0.8%)	46/429 (10.7%)	9	Italy
(Abur, Gunes et al. 2019)	100	0	0	51/1300 (4%)	12	Turkey
(Akınsal, Baydilli et al. 2018)	191	0	0	35/547 (6.4%)	12	Turkey[2]
(Zhao, Gu et al. 2019)	8	0	0	21/57 (36.84%)	13	China[3]
(Kim, Lee et al. 2017)	92	4	0	67/556 (12%)	14	Korea
(Li, Dai et al. 2015)	111	0	28/111 (25%)	NA	24	China
(Zhang, Dai et al. 2014)	23	0	0	11/137 (8.03%)	8	China
(Zhang, Zhang et al. 2013)	78	2	0	NA	9	China
(Al-Achkar, Wafa et al. 2013)	11	5	1/16 (6.25%)***	45/146 (30.8%)	28	Syria
(Behulova, Varga et al. 2011)	12	0	0	8/226 (3.35%)	6	Slovakia
(Ceylan, Ceylan et al. 2010)	14	0	5/14 (35.7%)	NA	7	Turkey
(Alkhalaf and Al-Shoumer 2010)	15	1	0	9/126 (7.14%)	6	Kuwait
(Hadjkacem-Loukil, Ghorbel et al. 2009)	6	3	6/9 (67%)	1/18 (5.5%)	19	Tunisia
(Malekasgar and Mombaini 2008)	1	0	0	26/49 (53%)	32	Iran
(Balkan, Tekes et al. 2008)	7	0	0	1/73 (1.3%)	15	Turkey
(Simoni, Tüttelmann et al. 2008)	208	0	1/208 (0.5%)	39/3179 (2.4%)	15	Germany[4]
(Choe, Kim et al. 2007)	91	4	0	NA	5	Korea
(Mitra, Dada et al. 2006)	10	4	4/14 (28.6%) **	NA	18	India
(Hellani, Al-Hassan et al. 2006)	3	0	0	8/247 (3.2%)	19	Saudi Arabia
(Peterlin, Kunej et al. 2002)	NA	5	1/5 (20%)	8/92 (8.6%)	56	Slovenia
(Dohle, Halley et al. 2002)	6	0	0	8/150 (5.3%)	6	The Netherlands
(Lee, Kim et al. 2000)	6	0	0	1/9 (11%)	59	Korea
(Tateno, Sasagawa et al. 1999)	21	0	0	NA	31	Japan
(Oliva, Margarit et al. 1998)	2	1	1/3 (33%)*	8/45 (17.7%)	12	Span

*47,XXY[2]/46,XY[48]; **Three cases with 47,XXY/46,XY and 1 case with 46,XY/47,XXY/48,XXXY/48,XXYY chromosomal pattern; ***45,X/46,X,idic(Y)/47,XX-,+idic(Y)

[1] Internal controls (SRY/sY14 and ZFX/ZFY) are not included. [2]Mostly from Central Anatolia; [3]Included Hakka men from southern China; [4]77% of cases were German; ethnicity background of the rest were Turkish, Russian/Slavic, Italian, Hispanic, and Polish.

fertility workup for patients with a sperm concentration of less than 5×10^6/mL, prior implementation of ART [(2)]. The human Y chromosome accounts for 2% to 3% of the estimated haploid genome size (~60 Mb) and conceals the genes encoding for the proteins that are not only involved in testicular development but also in triggering and maintaining normal spermatogenesis [(4)]. A unique feature of the human Y chromosome is the presence of inverted duplicates with a high degree of sequence identity, also called palindromes, on the long arm of the chromosome (Yq11). This peculiarity facilitates the process of gene conversion to modulate genetic diversity in equilibrium while maintaining the structural integrity of the genome[(5)]. Nevertheless, it has the potential to predispose the region to intra-chromosomal loss of genetic material during meiosis, causing a spectrum of outcomes ranging from common polymorphisms to pathological alterations in a set of genes involved in spermatogenesis and male infertility[(6)]. This scoping review was conducted to elucidate and combine scholarly articles on the utilization of routine genetic testing in the evaluation of infertile men; and finding any interconnection(s) between Y-chromosome microdeletions and chromosomal aberrations.

METHODS

Design

A scoping review was employed, which involved the integration of summaries, explications, and elucidations derived from accessible quantitative and qualitative investigations to address the inquiries posed in the review. This approach permits the review to extract diverse information and elaborate on it in a manner that is meaningful, transparent, and systematic[(7)]. The reporting adhered to the PRISMA-ScR checklist[(8)].

Search methods

The present scoping review was carried out in conformity with the suggestions advanced by Arksey and O'Malley[(9)]. The reasons that the current scoping review was undertaken were not only to summarize and disseminate research findings but also to identify research gaps in the existing literature. In this review, the electronic database PubMed, as the optimal tool in biomedical electronic research[(10)], was searched to retrieve the available literature evidence on the frequency of AZF microdeletions and the used STSs for screening (**Table 1**) among azoospermic subjects with Klinefelter syndrome (KS; XXY) published until July 2023. The search terms included “Karyotype”, "Klinefelter" or "KS" or "47,XXY", "AZF" or "Azoospermi*" and/or "microdeletion*" in the title or abstract.

The papers eligible for selection in this scoping review were determined based on the following inclusion criteria: (a) original articles papers were the main focus to guarantee a comprehensive and unbiased summary of the evidence; (b) studies on human subjects; (c) all of languages were included to omit publication bias and increase the comprehensiveness of the review; (d) all of the cohort, irrespective to their karyotype status, had to be screened for AZF deletions. Exclusion criteria were denoted in the flowchart (**Figure 1**). 30 original articles were included as a consequence of the literary investigation.

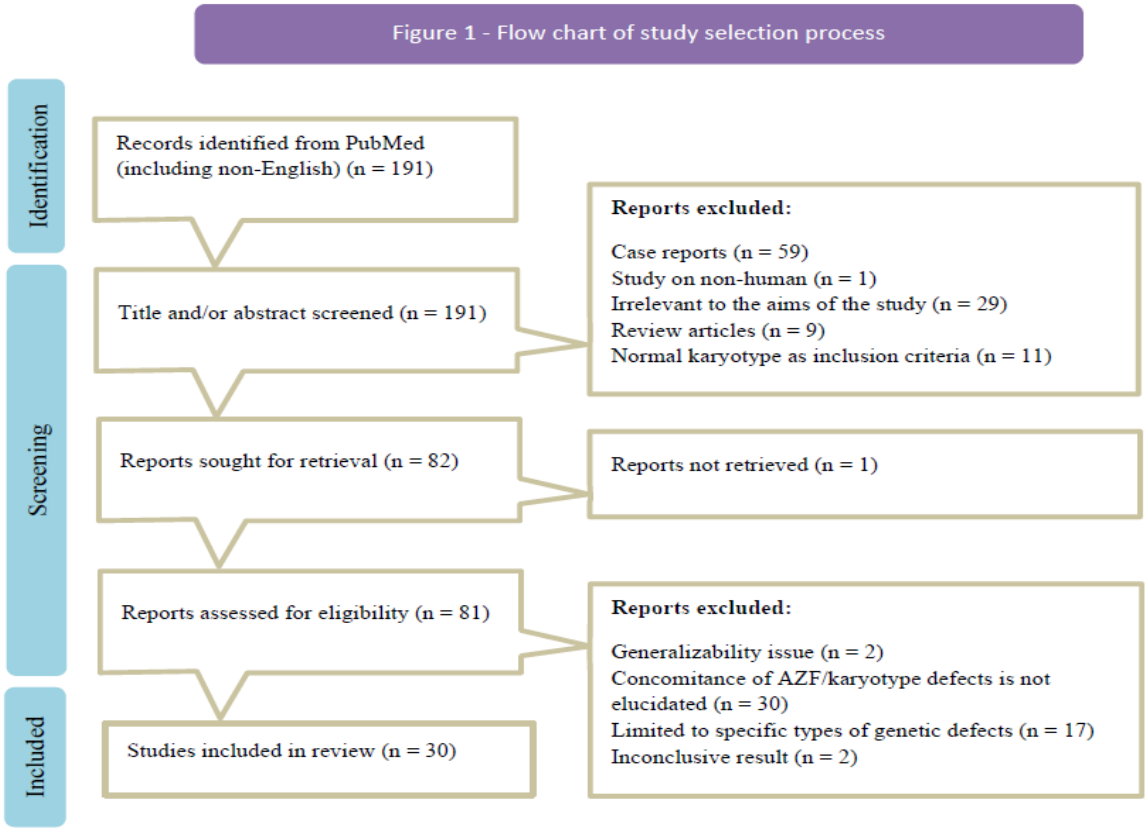

Figure 1. Flow chart of study selection process

Search outcome

The process of selecting is illustrated in Figure 1. A total of 191 titles were retrieved by searching through the PubMed database. After conducting an initial evaluation of the titles and abstracts, 82 reports remained for full-text review. Finally, 30 included original articles were scoped to identify their key concepts, gaps, and conclusions.

RESULTS

Bibliographic Overview

A total of 191 records were identified from PubMed. During the title/abstract analysis, 109 records (57%) were eliminated (**Figure 1**). The remaining records were screened for eligibility, which led to the exclusion of 51 records (63%). Finally, 30 papers were included in this scoping review (**Table 1**), which was conducted in 18 countries: China, Iran, Taiwan, Turkey, Italy, South Korea, Syria, Slovakia, Kuwait, Tunisia, Germany, India, Saudi Arabia, Slovenia, the Netherlands, Japan, Spain, and the United States.

The number of STSs used in the included studies varied from 5 to 59. The rate of AZF deletions among patients with NOA ranged from 1.3% to 53%; however, the mean frequency (after excluding the out-rangers) was estimated to be 5.6%. The rate of YCM among patients with XXY karyotype was calculated as nil in 19 out of 30 studies (63%); in the remaining studies, the rate ranged from 0.8% to 67% (**Table 1**).

DISCUSSION

1.The clinical indication of routine AZF screening in patients with KS

KS is the most common form of sex-chromosome numerical abnormalities, occurring in approximately 1 in 400-600 male births and 1 in 7 infertile cases with non-obstructive azoospermia (NOA)[2]. Y-chromosomal microdeletions have emerged as a leading genetic cause of male infertility, affecting around 1 in 14 (7%) of NOA patients globally[11,12]. The value of screening for Y-chromosome microdeletions in KS cases remains open to discussion, though it is important for NOA. Some studies suggest that individuals with a 47,XXY karyotype may harbor Y-chromosomal microdeletions and warrant screening[13-23], others argue against it, citing the absence of such deletions (**Table 1**)[24-45]. In our prior study on 1,039 cases encompassing both mosaic and classic forms of KS, we found that there is no concurrent occurrence of KS and YCMs[26]. This discovery positively aligns with the guidelines established by EMQN/EAA[27,28]; thus, males affected by KS do not warrant YCM screening. Notably, our data extend this conclusion beyond KS, encompassing autosomal inversions/translocations, Robertsonian translocations, and Jacobs's syndrome[26]. Although the underlying reasons for these observations remain somewhat unclear, these findings pose intriguing questions for future exploration.

2.AZF deletions in KS: the factors affecting the prevalence discrepancies

The prevalence of YCMs among azoospermic males

with KS varies significantly across studies, ranging from zero to 66.7% (**Table 1**) and this wide variation arises due to the implementation of different diagnostic protocols, differentiations in sample size, participant selection criteria, and ethnic/geographic differences [13-26,30-45]. However, the primary confounding factor contributing to the wide range of deletion rates is the composition of the study population, particularly the clinical context of cases recruited from a tertiary-referral fertility center compared to other settings with less selective referrals[28,46]. The diagnostic protocols that have been embraced, concerning the markers that have been employed, may also significantly influence the findings. Literature suggests that researchers have shown interest in employing additional STSs, beyond those recommended by the guidelines, in the hope of enhancing the resolution of screening and mapping, thereby increasing the likelihood of identifying YCMs [26]. It is important to note that the rate of YCM is not affected by the number of STSs used[12]; however, STSs can have both benefits and risks due to their varying clinical significance in assessing male factor infertility. Indeed, according to the guidelines, using polymorphic/repetitive markers for STS-PCR screening may result in inaccurate PCR results[11,12,28,47]. As a result, this may challenge the methodological accuracy of studies demonstrating a high incidence of YCMs in KS patients due to the choice of STSs employed[14,15,24-26,28,35,46].

3.Unprecedented prevalence of AZF deletion in Iran: A Global Record

The findings of Simoni et al.[20], which indicate a significantly elevated prevalence of YCMs (24.2%) in Iranian infertile patients, necessitate the examination of factors that contribute to this notable disparity when compared to other nations globally. The data originate from a study with a limited sample size of 99 participants, specifically recruited from the northwest region of Iran. Consequently, the generalizability of the results to other regions within the country and its diverse local populations is limited[48]. Indeed, later studies with larger groups of patients who were referred to a specialized infertility clinic have revealed a lower occurrence of AZF microdeletions at about 5% [26,48,49]; which aligns with the figures reported by Simoni et al. [12], suggesting an approximate prevalence of 7.3% of Caucasian patients with NOA or severe oligozoospermia harboring AZF deletions. It is therefore, imperative to consider geographical and populational variations previously reported, if these indicate an alternate prevalence than of general population, in order to offer adequate patient management.

4.Genetic test indications in NOA: Insights from the EMQN/EAA Guidelines

The patient management priorities in the context of genetic tests during infertility workup are discussed based on the EMQN/EAA guidelines, which recommend AZF screening as the first-tier test for cases with a sperm count of $< 5\times10^6$/mL, while karyotyping is necessary to rule out 46,XY/45,X mosaicism. In contrast, according to the same guidelines, cases with abnormal karyotypes, excluding 46,XY/45,X, do not require AZF screening [28]. Hence, within the realm of these guidelines, the specific genetic test that clinicians ought to prioritize remains obscure. It is noteworthy to mention that there are two distinct perspectives established among clinical geneticists and andrologists/urologists. From the viewpoint of a clinical geneticist, there may be justification for ordering a karyotype before YCM testing, however, in routine clinical practice, physicians usually opt to request both karyotype and AZF assessment simultaneously in patients with NOA or severe oligozoospermia, irrespectively of the guideline recommendations. The purpose of conducting both the karyotype and AZF assessment simultaneously has two aspects: to optimize patient satisfaction by reducing wait times for screening results and consequently infertility treatments and to address the lack of clarity within the present guidelines that occurs due to the limited existing literature, the potential gaps on genetic marker discovery, and the complexity of male infertility itself. Therefore, it is essential for guidelines to offer clear and consistent instructions or endorse a simultaneous testing approach.

5.YCM occurrence with sex chromosome mosaicism: an exploration

In the existing literature, certain studies have documented an increased occurrence of AZF deletions in cases of sex chromosome mosaicism, such as 45,X/46,XY or 47,XXY/46,XX [50,51]. Notably, the guidelines developed by EMQN/EAA have shed light on the interplay between 45,X/46,XY mosaicism and YCMs, suggesting that expanded YCMs may serve as a predisposing factor for Y chromosome instability and the development of the 45,X cell line within mosaic individuals [52]. In our previously reported findings from a cohort of 10,388 consecutive patients with NOA and severe oligozoospermia, intriguing results were observed, indicating that while patients with specific types of sex chromosome mosaicisms, such as 45,X/46,X,del(Y); 45,X/46,X,inv(Y)(p11.2q12); or even 45,X/46,XY, indeed exhibit a higher prevalence of YCMs, this correlation does not appear to apply for individuals harboring 47,XXY/46,XX mosaicism[26].

6.Association between inv(Y) and YCMs

The presence of inv(Y) in individuals with Y chromosome microdeletions (YCMs) depends on the specific breakpoints. While the pericentric inversion inv(Y)(p11q11) is considered a normal polymorphism with a prevalence of 0.1%-0.2% [53], the association between inv(Y) and YCMs is negligible in cases with inv(Y)(p11q11), but positive in cases with inv(Y)(p11.2q12) or 45,X/46,X,inv(Y)(p11.2q12), where AZFbc/AZFc deletions are commonly found[26].

The presence of inv(Y)(p11q11) and other chromosomal heteromorphisms in males with severe spermatogenesis defects raises a question about their clinical significance[26]. It may be tempting to regard these findings as mere coincidental occurrences or as a reflection of previous observations on the potential role of genetic variations in infertility[53]. However, to draw a definitive conclusion, additional evidence from future studies is required.

7. Sperm concentration vs. AZF deletion

According to the EMQN/EAA guidelines, AZF screening is the initial diagnostic approach in situations where sperm count is less than 5×10^6/mL during semen analysis. In line with data from North America and Europe [47], a cohort analysis of 10,388 patients with male infertility revealed that 1% of men with a sperm con-

centration of more than 1×10^6 sperm/mL (and < 5×10^6 sperm/mL) had YCMs. This leads to a reassessment of the thresholds for sperm concentration in the guidelines for male infertility, considering the screening of YCMs during investigations into infertility[26].

8. Testicular sperm extraction in men with AZFb/ AZFbc deletions

Patients with deletions in the AZFb and AZFbc regions of the Y chromosome commonly experience azoospermia, as a result of Sertoli cell-only syndrome and/ or maturation arrest. However, in rare cases, these deletions are observed in men who have sperm in their ejaculate[1-3]. Therefore, the possibility of surgical sperm retrieval should not be dismissed for men with AZFb/ AZFbc deletions. However, it is important to consider two crucial aspects within this particular framework. Firstly, the evaluation of AZFb deletions involves the application of two anonymous markers, namely sY127 and sY134, which are situated within the median and distal regions of the AZFb region, respectively.

According to the most recent edition of EAA/EMQN guidelines[27], the simultaneous deletion of both markers generally indicates a complete deletion of the AZFb region. However, the guidelines emphasize the necessity of conducting a mandatory deletion extension analysis using an additional set of five markers to establish the prognostic significance of the deletion in terms of TESE outcomes. Herein, one may question what would happen if a center solely utilizes the two first-line EAA/EMQN recommended STSs, neglecting the extended portion of the detection procedure. In this particular context, Stouffs et al. have introduced two infertile men, wherein the initial examination indicates the absence of STS sY127 and sY134. Nevertheless, the extension analysis revealed an irregular pattern and their unconventional AZFb deletions are in accordance with the potential for sperm production, albeit occurring at significantly diminished levels[54]. Secondly, the lack of any documentation regarding chemical and/or clinical pregnancies following intracytoplasmic sperm injection (ICSI) in individuals with complete AZFb/b-c microdeletions is noteworthy[55]. These factors must be considered when guiding infertile couples grappling with fertility issues, particularly in cases where the male partners exhibit such Y-chromosome microdeletions (YCMs).

9. The significance of haplogroup distribution in Y-DNA research

A haplogroup is a group of closely related haplotypes that share a common ancestor, which can be either mitochondrial DNA or non-recombining regions of the Y chromosome; these distinct patterns of inheritance allow for tracing direct maternal or paternal ancestral lineages[56]. In Y-DNA research, one of the primary concerns revolves around population stratification and the uniformity of haplogroup distribution, which can be a confounding factor; even when seemingly homogeneous groups display differences in Y-DNA haplotypes due to population history when analyzed using mathematical algorithms like principal-component analysis [57]. The role of Y-DNA haplogroups in the prevalence of Y-chromosome microdeletions remains uncertain, with some studies finding no evidence supporting this concept[46], while others suggesting that individuals belonging to haplogroup E may be more susceptible to AZFc microdeletions[58].

When a research investigation is undertaken within a specific population, uncertainties may arise regarding the comprehension of the outcomes about their external validity and applicability to other geographic or ethnic cohorts. This argument originates from the variances in Y chromosome haplotypes observed across diverse populations, which can potentially impact the applicability of the study's findings. For instance, some populations have an inherently high diversity of haplogroups due to different migratory events throughout history, and vice versa[59]. It is important to keep this in mind during the interpretation of the literature on Y DNA.

10. The extra-gonadal implications of the AZF genes

Most of the AZF genes are indeed involved in spermatogenesis, however, some of the AZF genes (14%), mainly those in AZFa and AZFb loci, are widely expressed in multiple tissues and are considered to be involved in processes such as gene regulation, transcription and translation. Amongst these genes, *RBMY* which regulates the splicing of a transcription factor named SMAD5, as well as the cell cycle regulators of histone-lysine N-methyltransferase (EHMT1) and protein lin-9 homolog (LIN9); and *UTY* that encodes a histone H3 Lys27-specific demethylase. These genes play important roles in different phases of germ cell development, including mitotic, meiotic, and post-meiotic phases. These microdeletions can lead to lower fertilization rates, compromised quality of male embryos in assisted reproduction[60,61], a higher prevalence of neuropsychiatric disorders[62], and copy number variations, and altered expression of AZF genes in several cancers[4]. The provided data are preliminary and observational, necessitating the need for systematic investigations to elucidate the impact of genetic modifications in the Y chromosome on the overall well-being of males beyond their fertility status as well as their offspring.

As a scoping review, the current study encompasses some strengths and limitations that should be acknowledged. The study provides a broad overview of the evidence on the crosslink between karyotyping and AZF deletions, irrespective of study quality. The provided results may help identify research gaps in the literature to aid in the planning and commissioning of a forthcoming systematic review or research. Moreover, the study concisely encapsulates the research discoveries amidst a compilation of research data that is disparate and intricate. However, our study has some limitations. It does not assess the quality of the included studies, unlike systematic reviews. It may also be limited by the quality and quantity of available literature on the topic. Additionally, it may not provide a comprehensive synthesis of the evidence, unlike systematic reviews.

CONCLUSIONS

The findings of this review can be summarized as follows:

1- The presence of spermatozoa within the ejaculate, but also the successful surgical retrieval of spermatozoa cannot be excluded for individuals with AZFb/AZFbc microdeletions and should be considered in future management guidelines.

2- The current literature suggests that only 1% of individuals with a sperm concentration exceeding 1×10^6

sperm/mL and less than 5×10^6 sperm/mL exhibit AZF microdeletions. Consequently, it is justified to reassess the inclusion of testing referrals for such populations in forthcoming guidelines.

3- Screening for Y chromosome microdeletions is not warranted for either mosaic or classic forms of KS. The lack of indication for AZF testing is not restricted solely to KS but also encompasses autosomal inversions/translocations, and Jacobs's syndrome.

4- Research has demonstrated that individuals exhibiting mosaic monosomy X karyotype, along with certain chromosomal anomalies such as inv(Y)(p11.2q12), idic(Y)(q11.2), 46,XY,r(Y), idic(Y)(p11.2), and der(Y;Autosome), should be referred for AZF deletion screening.

5- In contrast with previous discrepancies, more recent extensive investigations reveal that among Iranian patients affected by NOA or severe oligozoospermia, those with YCMs represent approximately 5%.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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