Fertility and Sterility

A systematic review identifying fertility biomarkers in semen: a clinical approach through -OMICs to diagnose male infertility

--Manuscript Draft--

Manuscript Number:	FandS34614R3
Article Type:	Systematic Review or Meta-Analysis
Keywords:	sperm; seminal plasma; infertility; molecular biomarkers; OMICs
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Abstract:	Objective: To identify the most robust molecular biomarkers in sperm and seminal plasma for the diagnosis of male infertility, and to evaluate their clinical use. Design: Systematic review. Setting: N/A. Patients: Accessible studies reporting well-defined (in)fertile populations and semen molecular biomarkers were included in this review. Intervention: A systematic search of the literature published in MEDLINE-PubMed and EMBASE databases was performed, following PRISMA guidelines. Main outcome measure: The primary outcome was the content, expression, or activity of molecular biomarkers in human semen samples. Only studies reporting ROC analysis were included. Results: Eighty-nine studies were included. Direct evaluation of sperm DNA damage has high potential as a diagnostic biomarker of fertility and ART outcomes (area under the curve; AUCs median=0.67). Regarding strand break-associated chromatin modifications, γH2AX levels show good predictive value for the diagnosis of male infertility (AUCs median=0.93). Some non-coding RNA exhibit excellent predictive values; miR-34c-5p in semen is the most well-characterized and robust transcriptomic biomarker (AUCs median=0.78). While many proteins in semen show fair diagnostic value for sperm quality and fertilizing capacity, the levels of some such as TEX101 in seminal plasma have an excellent diagnostic potential (AUCs median=0.69). Although both individual metabolites and metabolomic profiles in seminal plasma present good predictive value, the latter seem to be better than the former when inferring sperm quality and fertilizing capacity. Conclusions: The current review supports that some OMICs (e.g., DNA structure and integrity, genomics and epigenomics, transcriptomics, metabolomics, and proteomics) could be considered relevant molecular biomarkers that may help identify infertility etiologies and fertilization prognosis with cost-effective, simple, and accurate diagnosis.

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Running title

Fertility molecular biomarkers in semen

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ABSTRACT

Objective: To identify the most robust molecular biomarkers in sperm and seminal plasma for the diagnosis of male infertility, and to evaluate their clinical use.

Design: Systematic review.

Setting: N/A.

Patients: Accessible studies reporting well-defined (in)fertile populations and semen molecular biomarkers were included in this review.

Intervention: A systematic search of the literature published in MEDLINE-PubMed and EMBASE databases was performed, following PRISMA guidelines.

Main outcome measure: The primary outcome was the content, expression, or activity of molecular biomarkers in human semen samples. Only studies reporting a Receiver-Operating Characteristic (ROC) analysis values were included.

Results: Eighty-nine studies were included. Direct evaluation of sperm DNA damage has high potential as a diagnostic biomarker of fertility and ART outcomes (area under the curve; AUCs median=0.67). Regarding strand break-associated chromatin modifications, γH2AX levels show good predictive value for the diagnosis of male infertility (AUCs median=0.93). Some non-coding RNA exhibit excellent predictive values; miR-34c-5p in semen is the most well-characterized and robust transcriptomic biomarker (AUCs median=0.78). While many proteins in semen show fair diagnostic value for sperm quality and fertilizing capacity, the levels of some such as TEX101 in seminal plasma have an excellent diagnostic potential (AUCs median=0.69). Although both individual metabolites and metabolomic profiles in seminal plasma present good predictive value, the latter seem to be better than the former when inferring sperm quality and fertilizing capacity.

Conclusions: The current review supports that some OMICs (e.g., DNA structure and integrity, genomics and epigenomics, transcriptomics, metabolomics, and proteomics) could be considered relevant molecular biomarkers that may help identify infertility etiologies and fertilization prognosis with cost-effective, simple, and accurate diagnosis.

***Keywords*:** Sperm, seminal plasma, infertility, molecular biomarkers, OMICs.

Capsule: The present study is the first in compiling semen robust biomarkers that show high diagnostic value for male sperm quality and fertility disorders.

INTRODUCTION

In humans, infertility affects 8-12 % of couples worldwide. As a male factor is involved in 50 % of cases (1), prediction of men fertility is very important (1,2). Despite the growing interest in reproductive health, there is still a lack of biomarkers able to predict male fertility with high accuracy and sensitivity. Traditionally, the prognosis of men fertility has been performed through conventional semen analysis, which provides general information on quantitative parameters such as the ejaculate volume, sperm morphology, count and concentration, and motility. While the spermiogram is a relatively simple, fast, informative and economical assessment, it does not provide information about sperm physiology, because it leaves aside essential molecular aspects such as DNA integrity, sperm oxidative status and the presence of sperm-oocyte binding proteins, amongst others (3,4). The application of conventional semen analysis for the prognosis and diagnosis of male fertility has been under debate for many years (3,5). In this regard, exploring new and robust molecular biomarkers in sperm providing additional information on their functional status is of great interest for assisted human reproduction.

OMICs are high-throughput measurements of specific molecular groups, such as proteins, deoxyribonucleic acid (DNA), ribonucleic acid (RNA) or metabolites among others, which are increasingly studied in the andrology field. OMICs technologies and their derivatives are in constant development, allowing for the characterization of proteins, genes, metabolites and epigenetic traits associated to male infertility (6). Along these lines, the advent of OMICs has uncovered relevant molecular biomarkers that may help identify infertility etiologies and fertilization prognosis with cost-effective, simple and accurate diagnosis. The aim of this systematic review, therefore, is to identify the most robust molecular biomarkers in sperm and seminal plasma for the diagnosis of male infertility, and to evaluate their potential clinical use. A comprehensive review of high-quality studies published to date investigating reliable molecular biomarkers in semen may assist physicians in the diagnosis of the conditions causing sperm quality impairments and male fertility disorders. To the best of our knowledge, this is the first well-designed systematic review of observational studies, based on Receiver-Operating

Characteristic (ROC) analysis outcomes, that critically appraises the quality of the current body of literature on relevant molecular biomarkers in human semen.

MATERIAL AND METHODS

Systematic review registration

The protocol of the present systematic review has been registered in the international database for the prospective registration of systematic reviews (PROSPERO 2020: CRD42020176417).

Data sources and search strategy

A systematic search of the literature published in MEDLINE-PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and EMBASE databases (https://www.embase.com/#search) was performed, in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (7,8). The strategy performed for literature search combined keywords and Medical Subject Heading (MeSH) terms. The search was focused on "semen", "male (in)fertility" and "biomarkers" related words. The full search strategy and applied filters are available in **Supplemental File 1**.

Study selection, eligibility, and data extraction

Titles and abstracts were screened by two expert authors in the field of male fertility (AD-B and ML), and discrepancies were re-evaluated together with two additional authors (AS-H and MY). Moreover, a PICOS (Patient, Intervention, Comparator, Outcome, Study) design structure was set to develop the study questions and the inclusion/exclusion criteria (**Supplemental Table 1**). The accessible studies reporting well-defined (in)fertile populations and semen molecular biomarkers were included in this review. The primary outcome of the present article was the content, expression, or activity of molecular biomarkers in human semen samples; only studies reporting a ROC analysis were considered. We excluded animal studies, review articles, editorial/opinions, case-reports articles, and studies measuring (in)fertility biomarkers in blood or samples other than semen. After primary screening (assessing the scope of study) and evaluating the quality in

accordance with inclusion/exclusion criteria, the full text of selected articles was obtained. The following information was extracted from each selected study: author/s, year of publication, study design, infertility status, sample size, sample type, measured biomarkers, measurement method, area under the curve (AUC), sensitivity, specificity, *p*-value and main conclusion.

Quality assessment

The quality of selected observational studies was evaluated and scored using the quality assessment tool of NHLBI-NIH for Case-Control studies to assess the quality of each study (https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools). The quality scores were assessed in parallel by two authors (AD-B and ML), and discrepancies were re-evaluated altogether to reach a consensus. Studies with a score between 0 and 5 points were considered of low-quality (excluded) and those with a score greater than 5 were considered as of moderate to high-quality (included for subsequent analysis).

RESULTS

Identification and selection of the articles

The primary search by MEDLINE-PubMed and EMBASE identified a total of 37,854 articles. We screened the titles and abstracts, and excluded a total of 36,979 studies for duplication or not meeting the scope of the study; 875 articles were selected for full text evaluation of the inclusion/exclusion criteria and quality assessment. Out of these articles, 786 were excluded because they did not meet the inclusion/exclusion criteria or because of deficient quality scoring. After applying eligibility parameters, 89 studies were included for qualitative synthesis. The 89 selected articles were case-control studies reporting ROC analysis of fertility molecular biomarkers in sperm and seminal plasma. These studies were classified in five groups: DNA structure and integrity (n = 41), genomics and epigenomics (n = 6), transcriptomics (n = 8), proteomics (n = 17) and metabolomics (n = 20). Three reports belonged to more than one group. The PRISMA Flow Diagram is shown in **Figure 1**.

Primary outcomes of interest

DNA structure and integrity

A total of 41 studies reporting nine different DNA structure and integrity biomarkers to predict fertilizing capacity were selected (**Supplemental Table 2**). Diagnostic potential of all biomarkers was evaluated through ROC curve analysis. Structure and integrity were exclusively evaluated in sperm DNA but not in cell-free DNA.

The terminal deoxytransferasemediated deoxyuridine triphosphate (dUTP) nick end labeling assay (TUNEL) uses a terminal TdT transferase to label the 3' free DNA ends; therefore, sperm with fragmented DNA become highly labelled (9). The TUNEL assay measures DNA damage directly without a prior denaturation step and is coupled with flow cytometry, which makes this test reliable and reproducible (10). The TUNEL assay was analyzed as a potential fertility biomarker in 14 different works. The potential of the TUNEL assay as a discriminator of infertile patients from men with proven fertility has been extensively demonstrated with AUCs of 0.930 (11), 0.903 (12), 0.757 (13) and 0.608 (14). In addition, Sharma *et al.* (2010, 2016) (15,16) and Kabartan *et al.* (2019) (17) explored its potential as a diagnostic biomarker to identify idiopathic infertility due to male factor (with AUCs of 0.820, 0.556 and 0.892, respectively. Hichri *et al.* (2018) confirmed its potential to differentiate between samples from patients that conceived after an IUI and samples of patients with oligoasthenoteratozoospermia that ended up being enrolled in IVF or ICSI cycles; the results of the TUNEL test, therefore, would be a good prognostic biomarker to choose the best ART strategy (AUC = 0.790). When considering the potential of the TUNEL test as a prognostic biomarker of pregnancy, results were found to be variable. On the one hand, Vončina *et al.* (2016) (19) proved its good potential as a prognostic biomarker of natural conception in normospermic patients that had not been able to conceive after a year of unprotected intercourse (AUC = 0.700), and Avendaño *et al.* (2010) (20) determined that it should be useful as a moderate prognostic biomarker of clinical pregnancy in ICSI cycles (AUC = 0.700). On the other hand, Jin *et al.* (2015) (21) reported poor predictive power of TUNEL as a prognostic biomarker of successful clinical pregnancy in couples with a reduced

ovarian reserve (AUC = 0.594). Moreover, the TUNEL test showed no predictive power as a predictor of clinical pregnancy in IUI cycles (AUC = 0.675) (22). In addition, Benchaib *et al.* (2003), Esbert *et al.* (2011), Thomson *et al.* (2011) and Jin *et al.* (2015) (21–24) concluded this test does not have a significant power as a prognostic biomarker of clinical pregnancy in ICSI cycles (AUCs of 0.500, 0.552, 0.530 and 0.510, respectively). Similarly, Esbert *et al.* (2011) (24) explored the potential of the TUNEL test as a prognostic biomarker of successful clinical pregnancy in IVF/ICSI cycles with couples' or donor oocytes, and concluded that this assay does not have a significant power for this purpose (AUCs of 0.559 and 0.528, respectively). Finally, Esbert *et al.* (2011) (24) found that TUNEL has no significant power to predict successful delivery following IVF/ICSI cycles, regardless of whether all cases are considered together (AUC = 0.666) or cycles with own and donor oocytes are envisaged separately (AUCs of 0.652 and 0.670, respectively).

H2AX is a histone variant that has different post-translational modifications. Phosphorylation of Ser-139 from H2AX, a variant known as histone γ-H2AX, is involved in the cell response to the induction of DNA double-strand breaks (DSBs) during spermatogenesis, which consists of recombination (25). This post-translational modification spreads several bases on the DNA strand from the break, acting as an epigenetic mark to start DNA repair (26). Some DSBs, nevertheless, might remain unrepaired in mature sperm until the male pronucleus meets the DNA repair molecules provided by the oocyte after fertilization. Levels of γ-H2AX in mature sperm, therefore, are proportional to the number of DSBs. Remarkably, Zhong *et al.* (2015) (27) confirmed the excellent potential of γ-H2AX levels as a biomarker capable to differentiate between fertile and infertile men (AUC = 0.930).

Oxidative DNA damage was previously investigated in seven studies. The Comet assay can be performed under alkaline or neutral conditions, which allows for the differentiation between single and double strand DNA breaks (ssSDF and dsSDF, respectively) induced by oxidative damage (28). The alkaline Comet assay was used in four studies to evaluate oxidative DNA damage in terms of ssSDF. Its power as a diagnostic biomarker of idiopathic male infertility

was proven to be excellent by Simon *et al.* (2011), Ribas-Maynou *et al.* (2013) and Fernandez-Encinas *et al.* (2016) (12,29,30) with AUCs of 0.970, 0.937 and 0.994, respectively. It was also confirmed as a predictor of clinical pregnancy in IVF cycles with AUCs of 0.648 and 0.905 (Simon *et al.*, 2010; 2011) (29,31), but not in ICSI cycles (AUC = 0.601; Simon *et al.*, 2010) (31). The predictive power of dsSDF evaluated through the neutral Comet assay was also assessed in two different studies; neither Fernandez-Encinas *et al.* (2016) (30) nor Ribas-Maynou et al. (2013) (12) were able to confirm its power as a good fertility biomarker, with AUCs of 0.373 and 0.516, respectively. Finally, oxidative DNA damage can also be determined through the measurement of 7,8-dihydro-8-oxo-2′-deoxoguanosine (8-OHdG), which is a modified DNA base that is prone to become a DSB and is thus a marker of latent DNA damage (32,33). Its potential as a fertility biomarker was investigated in two different works, and the potential of 8-OHdG as a prognostic biomarker of clinical pregnancy was good in IUI, but failed in ICSI cycles (22) (AUCs of 0.794 and 0.496, respectively). Considering that this biomarker is indicative of latent DNA damage, Simon *et al.* (2010) (31) evaluated whether the treatment of sperm samples with formamidopyrimidine DNA glycosylase (FPG), which converts 8-OHdG into strand breaks before the alkaline Comet assay, improved the power of this technique. Indeed, this pre-treatment of samples increased the power of the alkaline Comet assay as a prognostic biomarker of clinical pregnancy in IVF cycles (AUC = 0.776) (31).

Another interesting parameter is sperm toroid integrity (STI), whose evaluation allows determining the compaction of sperm DNA in toroids. It can detect latent DNA damage and abnormal or unstable chromatin structures that might not be detected through other tests only sensible to DNA strand breaks (34). It was evaluated as a fertility biomarker by Chan *et al.* (2015) (34), who confirmed it as a good predictor of miscarriage (AUC = 0.710), but not of pregnancy, after an ICSI treatment.

Other methods base the detection of SDF on the denaturing capacity of sperm chromatin. The Sperm Chromatin Structure Assay (SCSA) is based on the use of acridine orange (AO) to stain dsDNA and ssDNA in different colors (35), whereas sperm with intact DNA present a halo that is absent from those with fragmented DNA in the Sperm Chromatin Dispersion (SCD) test

(36). Jiang et al. (2011) (37) showed that the percentage of AO-stained sperm evaluated through fluorescence microscopy was a good predictor of clinical pregnancy in ICSI cycles (AUC = 0.750). Six studies analyzed AO staining through flow cytometry. Chromatin decondensation evaluated through SCSA was proven to be an excellent diagnostic biomarker of infertility in Venkatesh *et al.* (2011) (38), and its potential was confirmed to be good by Ribas-Maynou *et al.* (2013) (12), with AUCs of 0.919 and 0.792, respectively. In addition, this assay was found to provide a robust diagnostic and prognostic biomarker in idiopathic recurrent pregnancy loss (RPL) following spontaneous conception (39,40) or IVF treatment (41), with AUCs of 0.830, 0.752 and 0.713, respectively. Its power as a predictor of spontaneous clinical pregnancy in patients with varicocele was also good with an AUC of 0.762 (42).

The potential of the sperm chromatin dispersion (SCD) test as a fertility biomarker was evaluated through Halosperm G2 in twelve different works. The SCD test exhibited a good potential to distinguish between samples presenting normal sperm characteristics according to WHO criteria and samples not meeting these quality standards with an AUC = 0.753 (43). Ribas-Maynou *et al.* (2013) (12) confirmed SCD was also an excellent diagnostic biomarker of fertility with an AUC of 0.869. In addition, Esteves *et al.* (2015) (44) evaluated the diagnostic potential of SCD in infertile men with varicocele, which was confirmed to be excellent (AUC = 0.942). The SCD test also proved its ability to predict fertilization rates ≥50% in conventional IVF cycles (45) and in ICSI cycles (46) (AUCs of 0.664 and 0.680, respectively). Breznik *et al.* (2013) (45) and Tandara *et al.* (2014) (47) found that the percentage of sperm presenting chromatin dispersion had a good predictive value for embryo quality after conventional IVF cycles (AUCs of 0.771 and 0.710, respectively); Tandara *et al.* (2014) (47) also reported the percentage of sperm with undamaged DNA as an excellent predictor of embryo quality (AUC = 0.830). Finally, seven works evaluated the potential of SCD as a predictor of pregnancy after conventional IVF and ICSI cycles, but the conclusions of these studies were not consistent. The percentage of sperm with chromatin dispersion was found to be a good prognostic biomarker for clinical pregnancy after IVF cycles by Tandara *et al.* (2014), Bounartzi *et al.* (2016) and Comhaire *et al.* (2018) (with AUCs of 0.670, 0.700 and 0.830, respectively) (47–49), similarly to the percentage of sperm with

undamaged DNA (47) (AUC = 0.750). Yet, Sun *et al.* (2018) (50) did not confirm this parameter as a good predictor for the achievement of clinical pregnancy after IVF (AUC = 0.543). When considering ICSI cycles alone, Gosálvez *et al.* (2013) (51) reported SCD as a good prognostic biomarker of clinical pregnancy when using donor oocytes (AUC = 0.711), but again the results from Sun *et al.* (2018) (50) were in disagreement (AUC = 0.477). When IVF and ICSI cycles were considered together, López *et al.* (2013) (52) found SCD was a poor predictor of clinical pregnancy with an AUC = 0.546, and the results from Muriel *et al.* (2006) (53) evidenced it did not have a significant power to predict clinical pregnancy (AUC = 0.597).

Chromatin maturity can be assessed through the evaluation of the successful replacement of histones by protamines. Whereas CMA3 binds to the DNA's minor groove similarly to protamines and allows for the detection of deficient protamination (54), AB stains persistent histones in the sperm nucleus (55). Chromatin protamination assessed through the CMA3 assay was evaluated as a fertility biomarker in four different works. Esterhuizen *et al.* (2000a, 2000b) (56,57) determined the good potential of CMA3 to predict a fertilization rate >60% in IVF cycles (AUCs of 0.740 and 0.760, respectively), and Tarozzi *et al.* (2009) (58) evaluated the good potential of CMA3 as a predictor of successful fertilization after IVF (AUC = 0.769). Finally, Marchiani *et al.* (2017) (59) evaluated the potential of CMA3 as a predictor of embryo quality, and demonstrated its good potential as a biomarker (AUC = 0.778). Histone persistence measured through aniline blue (AB) staining as a fertility biomarker was evaluated in two different works. Marchiani *et al.* (2017) (59) determined the fair prognostic value of AB as a predictor of good fertilization rate in males of infertile couples with an AUC of 0.611. In addition, AB staining proved its fair potential as a prognostic biomarker of successful clinical pregnancy in couples with male factor infertility after IUI cycles with an AUC of 0.653 (60).

In summary, whereas the TUNEL test and the levels of histone variant γ-H2AX were confirmed to identify infertile patients, the oxidative damage measured through the alkaline Comet assay showed slightly higher sensitivity and specificity. The TUNEL assay showed a highly variable potential to identify the etiology of infertility and was also observed to predict the success of IUI. While the TUNEL assay was also useful to predict clinical pregnancy in natural

conceptions, almost all works concurred that this test did not have a good prognostic power in IVF and ICSI treatments. On the contrary, the alkaline Comet assay was found to exhibit a variable prognostic value to predict clinical pregnancy after IVF. In addition, the measurement of 8-OHdG, when used alone, had a good diagnostic power for infertility but, when combined with the alkaline Comet assay, it also exhibited a good prognostic power of clinical pregnancy after IVF and ICSI cycles. Both SCSA and SCD tests are excellent diagnostic biomarkers for infertility. In patients with varicocele, both might be excellent as a non-invasive diagnostic tool and a good prognostic biomarker of spontaneous pregnancy, respectively. These two techniques, therefore, could be utilized to assess sperm quality in fertility clinics and establish a proper treatment to solve this potential cause of infertility, thus helping decide which ART strategy is better for each patient. The SCD test was found to show a fair power as a predictor of fertilization rates following IVF and ICSI, and a good to excellent power as a biomarker of embryo quality. As there is evidence for and against the prognostic value of SCD as a clinical pregnancy biomarker in IVF and ICSI cycles, it could be important for each laboratory to establish their own cut-off values; the standardization of this technique, therefore, would be more difficult than others. Regarding SCSA, there is evidence supporting its potential as a good pregnancy biomarker in ICSI cycles. Finally, both CMA3 and AB have proven their power as prognostic biomarkers of the outcome of different ARTs, both being accurate to predict successful fertilization after IVF and ICSI. In addition, CMA3 has good potential as an embryo quality biomarker after IVF, and AB is a fair prognostic biomarker of clinical pregnancy after IUI. In the light of the aforementioned, it is evident that reproductive clinics would benefit from the use of these techniques during sperm quality assessment, thus contributing to the election of the best ART strategy.

Gene expression and epigenomics

Regulation of gene expression can be evaluated through the detection of epigenetic marks or through the measurement of the products of gene expression. There are few studies, nevertheless, evaluating this type of biomarkers in semen samples. A total of six studies reporting nine different gene expression and epigenomics biomarkers for predicting fertilizing ability were selected

(**Supplemental Table 2**). ROC curve analysis was used to assess the diagnostic potential of all these biomarkers. Gene expression and epigenomics features were evaluated in both sperm DNA and cell-free DNA as potential biomarkers.

In terms of sperm DNA, Bonache *et al.* (2012) (61) defined an expression signature that was an excellent biomarker of clinical pregnancy rate after IUI (AUC = 0.910), which included the evaluation of the expression of *EIF5A, RPL13, RPL23A* and *RPS27A*. This panel includes genes that encode a translation factor and three ribosomal proteins and, thus, they are essential for the basic cell function (62,63). The other three studies evaluating sperm DNA identified different methylation patterns as fertility biomarkers. On the one hand, global methylation patterns showed fair power to differentiate between fertile and infertile men, as well as to predict embryo quality after IVF (64) (AUCs of 0.670 and 0.640, respectively). Furthermore, the top 500 most differentially methylated CpGs identified in the aforementioned study showed an excellent power as a diagnostic biomarker of male infertility (64) (AUC = 0.930). These results evidence that the analysis of differential methylation in site-specific CpG has higher diagnostic power as a biomarker of infertility than the global methylation pattern. Finally, whereas sperm methylation status of the *MLH1* promoter was proposed as a fair candidate biomarker for oligozoospermia, this was not the case of *MSH2* (65) (AUCs of 0.610 and 0.600, respectively). In fact, *MLH1* and *MSH2* are involved in DNA mismatch repair system, but only *MLH1* is related to a decrease in sperm production in mice, which is in agreement with the relationship with these results (66,67). Finally, the methylation status of the *MTHFR* promoter was confirmed as a good diagnostic biomarker of infertility (68) (AUC = 0.730). This gene encodes the enzyme methylenetetrahydrofolate reductase, which is essential in folate metabolism, that is crucial for methionine synthesis which, in turn, is the donor of the methyl group in DNA methylation (69).

With regard to cell-free DNA, two different studies evaluated the potential of cell-free DNA in semen as a fertility biomarker. Whereas cell-free DNA concentration in semen did not have a significant predictive value as a clinical pregnancy success biomarker in IVF cycles (48) (AUC = 0.600), the methylation pattern of the *CCNA1* promoter from cell-free DNA showed a fair power to predict successful sperm retrieval in non-obstructive azoospermia (NOA) patients

(70) (AUC = 0.670). This gene encodes cyclin A1, which is essential for the passage of spermatocytes into meiosis I and, in fact, null mice for this gene are healthy but infertile due to cell cycle arrest in spermatogenesis (71). This last finding has a potential application in IVF clinics, because this determination could provide a non-invasive establishment of NOA etiology.

The elevated cost of these techniques and the difficult translation into the clinical environment might be a reason for the lack of exhaustive studies regarding the potential of these biomarkers in semen samples.

Transcriptomics

Non-coding RNAs are known to be essential regulatory elements in biological systems, and reproductive biology processes do not escape from their regulation (72). Specifically, microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs) are short and single-stranded non-coding RNAs that act as regulatory elements. miRNA regulate gene expression at the transcriptional level in many cell types, whereas piRNA represses transposons at the transcriptional or post-transcriptional level in the germline and gonadal somatic cells (73,74). In this regard, non-coding RNAs present in semen could evidence dysregulations in spermatogenesis leading to semen quality and fertility disorders. Some studies, indeed, suggested the potential of non-coding RNAs as biomarkers for reproductive diseases or for reproduction success (75). Herein, we compiled robust miRNAs and piRNAs biomarkers present in semen samples showing high diagnostic value for sperm quality and fertility disorders. A total of eight studies reporting 31 high-quality non-coding RNA biomarkers (22 miRNAs and 9 piRNAs) to predict semen quality and/or fertility were selected (**Supplemental Table 2**). All RNAs were validated by qRT-PCR. To date, miRNAs and piRNAs are the only types of non-coding RNAs showing robust diagnostic value in semen samples. While miRNAs exhibited high-quality prediction results in both sperm and seminal plasma, piRNAs were reported to exclusively perform as good quality biomarkers in seminal plasma but not in sperm.

Few studies in the literature reported robust transcriptomic biomarkers for male idiopathic infertility. A panel of 5-miRNA (hsa-miR-34b-5p, 34b-3p, 34c-5p, 122-5p and 429) (76) and a

panel of four independent sperm miRNAs (hsa-miR-122-5p, 34b-3p, 34b-5p and 34c-5p) (77) were found to be strong predictors of semen quality and ICSI pregnancy rates. Specifically, a study by Cui *et al.* (2015) (77) tested sperm hsa-miR-34c-5p and hsa-miR-34b-3p in 162 patients with idiopathic male infertility undergoing ICSI cycles, and established hsa-miR-34c-5p, but not hsa-miR-34b-3p, as an indicator of embryo quality, implantation, clinical pregnancy, and live birth following ICSI. Thus, sperm hsa-miR-34c-5p is the only non-coding RNA in semen showing a robust predictive value for male idiopathic infertility undergoing ICSI cycles, being associated to embryo quality, implantation, clinical pregnancy and live birth in ICSI treatment outcomes. On the other hand, Abu-Halima *et al.* (2014) (76) validated a set of five microRNAs (hsa-miR-34b-5p, 34b-3p, 34c-5p, 122-5p and 429) using qRT-PCR in a cohort of 226 men attending an infertility clinic and reported these miRNAs to show good diagnostic value for both oligozoospermia and non-obstructive azoospermia, whereas hsa-miR-429 was described exclusively as a biomarker for oligozoospermia. The potential application of these non-coding RNAs as non-invasive semen biomarkers could minimize or avoid surgical sampling for the diagnosis of this type of sperm disorders.

On the other hand, a greater number of miRNA predictors of semen quality have been described in seminal plasma. A total of ten seminal plasma miRNAs (hsa-miR-122-5p, 146-5p, 181a-5p, 205-5p, 210-3p, 31-5p, 34c-5p, 374b-5p, 509-5p and 513a-5p) and a panel of three miRNA (hsa-miR-141, 429 and 7-1-3p) were reported to have good to excellent diagnostic potential for sperm quality disorders (60-62). Good diagnostic power was reported for azoospermia (hsa-miR-122-5p, 146-5p, 181a-5p, 34c-5p, 374b-5p, 509-5p and 513a-5p) (78), non-obstructive azoospermia (panel of 3-miRNA [hsa-miR-141, 429 and 7-1-3p]) (80), asthenozoospermia (hsa-miR-122-5p, 146-5p, 181a-5p, 34c-5p, 374b-5p, 509-5p and 513a-5p) (78) and varicocele-induced dyszoospermia (hsa-miR-210-3p) (79). Furthermore, seminal plasma miRNAs showed predictive value for discriminating among different types of sperm quality conditions, such as asthenozoospermia and azoospermia (hsa-miR-122-5p, 146-5p, 181a-5p, 34c-5p, 374b-5p, 509-5p and 513a-5p) (78), as well as non-obstructive and obstructive azoospermia (hsa-miR-31-5p, hsa-miR-205-5p) (81). The same study, however, reported four miRNAs (hsa-

miR-182-3p, 449a, 539-5p, 941) not to be sufficient to discriminate between non-obstructive and obstructive azoospermia.

Other research tested the diagnostic potential of sperm and seminal plasma piRNAs. Seminal plasma piRNAs showed good diagnostic potential for azoospermia and asthenozoospermia. Hong *et al.* (2016) (82) performed high-throughput sequencing technology in a cohort of 302 men and identified a panel of 61 piRNAs differentially expressed between normozoospemic donors and infertile patients. After qRT-PCR validation, a panel of five individual piRNAs (piR-30198, 31068, 31925, 43771 and 43773) were found to be significantly down-regulated in seminal plasma of both azoospermia and asthenozoospermia patients, when compared to normozoospermic men. The same authors also tested the diagnostic potential of these piRNAs combined in a panel of five piRNAs (piR-31068, 31925, 43771, 43773 and 30198) and another of four piRNAs (piR-31068, 31925, 43771 and 43773). They found increased diagnostic value for azoospermia in the panel of five piRNAs. Furthermore, these authors reported piR-30198 to be able to discriminate between asthenozoospermia and azoospermia patients, whereas Barceló *et al.* (2018) (81) showed piR-58527 as a useful biomarker to differentiate non-obstructive from obstructive azoospermia. On the contrary, although piRNAs from sperm were found to correlate with sperm concentration and fertilization rates after ICSI, they did not show a good diagnostic value either for sperm quality or male fertility (83).

Along these lines, several non-coding RNA (miRNAs and piRNAs) in semen have shown excellent predictive values, showing good to excellent diagnostic value for sperm quality disorders, such as azoospermia, oligozoospermia, asthenozoospermia, oligoasthenozoospermia and varicocele-induced dyszoospermia. The potential application of these semen biomarkers in fertility clinics could lead to faster and reliable diagnostics for men reproductive disorders, as they show high sensitivity and specificity (AUCs ranging from 0.730 to 0.990). Yet, hsa-miR-34c-5p in sperm and seminal plasma is the most well-characterized and robust transcriptomic biomarker for the diagnosis of sperm quality disorders and male factor infertility. In this regard, the relatively cheap and simple quantification methods for non-coding RNAs make these molecular biomarkers suitable candidates for their implementation in fertility clinics and should

be tested before assisted reproduction procedures to predict their success. Further validation and implantation strategies, however, should be envisaged before their clinical use in fertility clinics.

Proteomics

The role of semen proteins as a diagnostic tool for male reproduction disorders was also reviewed in the present study. Contrary to other molecular biomarkers, proteins are the most time-saving, cheaper and simpler diagnostic tools since they can be measured using cheap, simple and quick techniques such as rapid colloidal gold immunochromatography, ELISA or spectrophotometry. In the present study, 17 studies reporting a total of 32 semen protein biomarkers for different parameters predicting sperm quality, functionality and/or fertilizing capacity were selected (**Supplemental Table 2**). Protein concentration was measured using highly sensitive and specific technics such as ELISA, LC-MS/MS, Western blot and flow cytometry, whereas their activity was quantified through spectrophotometry.

The concentration or activity of sperm or seminal plasma candidate biomarkers were characterized to explore their putative diagnostic value. Seminal plasma levels of Testis-expressed protein 101 (TEX101) (84) (AUC = 0.990) and enzymatic activity of N-acetyl-b-D-hexosaminidase (85) (AUC = 0.800) were identified as robust biomarkers for the diagnosis of azoospermia. In addition, the concentration of NT-proCNP (86) (AUC = 0.733) and PELP1 (87) (AUC = 0.781) in seminal plasma was established as a putative diagnostic tool for asthenozoospermia and oligozoospermia, respectively. Furthermore, Intasqui *et al.* (2016) (88) conducted a proteomic analysis using a cohort of 156 normozoospermic men and revealed some seminal plasma proteins that were able to identify ejaculated sperm showing low mitochondrial activity (proteomic profile of Annexin A7 and CD63; AUC = 0.993), altered acrosome integrity (proteomic profile of PLTP and COL12A1; AUC = 0.972) and high DNA fragmentation (CRISPLD1; AUC = 0.882). In this regard, the previously mentioned protein biomarker candidates showed high diagnostic value for sperm disorders, such as azoospermia (TEX101 and N-acetyl-b-D-hexosaminidase), asthenozoospermia (NT-proCNP,) and oligozoospermia (PELP1), as well as for sperm physiology alterations, such as mitochondrial activity (Annexin A7

and CD63, combined), acrosome stability (PLTP and COL12A1, combined) and DNA integrity (CRISPLD1), thus being promising candidate biomarkers for clinical diagnosis of male reproduction disorders.

Other sperm and seminal plasma proteins have been demonstrated to predict ejaculate fertility. Concentration of sperm BAG6 and HIST1H2BA (89), the nuclease activity corrected by sperm count (30) and PON-1 activity in seminal plasma (90) were able to discriminate between fertile and infertile men showing abnormal semen analysis, with AUCs of 0.921, 0.935, 0.705 and 0.950, respectively. Similarly, PON-1 activity in seminal plasma did also show an excellent predictive value for subfertility diagnosis (90) (AUC = 0.950). The study of Marsillach *et al.* (2011) (91), however, explored the subfertility diagnostic power of PON-1 concentration and activity in a cohort of 93 men attending an infertility clinics, but found no significant diagnostic value. Moreover, the study of Korbakis *et al.* (2017) (84) explored the potential of TEX101 as a diagnostic tool for idiopathic male infertility, although they did not show a significant predictive value. Along these lines, although lacking conclusive results, PON-1, BAG6 and HIST1H2BA are putative protein biomarker candidates to predict male (in)fertility, even though further studies with larger cohorts would be warranted.

Similarly, some proteins in semen did show robust results as predictors of the success of assisted reproduction techniques (ART). The study of Martinez-Soto *et al.* (2018) (92) reported that total but not active uPA in seminal plasma was able to predict ART outcomes with an AUC of 0.720. Similarly, the study of Bøllehuus Hansen *et al.* (2019) (93) disclosed the percentage of CYP24A1-positive sperm as a good biomarker of the IUI-clinical pregnancy success (AUC = 0.710). Moreover, the study of Ovayolu *et al.* (2016) (94) identified the levels of Presepsin (a soluble CD14 subtype) in seminal plasma as a significant biomarker for live birth and chemical pregnancy after ICSI, although it showed limited robustness (AUCs of 0.634 and 0.677, respectively). Finally, CATSPER1 expression in sperm measured by fluorescence intensity using flow cytometry showed predictive value for ART success, being able to discriminate between poor quality- and good quality-embryos (59).

Additionally, protein biomarkers in semen were observed to be able to predict the etiology

of male fertility disorders, discriminating between non-obstructive and obstructive azoospermia as well as predicting the etiology of azoospermic men (vasectomy or testicular defects). In effect, seminal plasma levels of TEX101 (66), CRISP1/PAP (71) and α-glucosidase (71) were reported to be able to discriminate between non-obstructive and obstructive azoospermia, showing AUCs of 0.670, 0.929 and 0.609, respectively. Moreover, seminal plasma proteins showed the ability to predict the success of surgical procedures such as testicular sperm extraction (TESE) and to verify the success of vasectomy. Concentrations of TEX101 (84) and inhibin B (95) in seminal plasma were found to predict the success of TESE, showing AUCs of 0.690 and 0.910, respectively. Similarly, TEX101 concentration (84) and α-glucosidase activity (96) were reported to be significant validators of successful vasectomy procedures, showing AUCs of 1 and 0.730, respectively. Finally, the activity of α-glucosidase (96) and concentration of transferrin receptors (97) in seminal plasma were related to the etiology of azoospermic men (vasectomy or testicular defects), with AUCs of 0.760 and 0.630, respectively.

In short, protein biomarkers for the prediction of male fertility and sperm quality should be further tested in clinical trials, as they could facilitate and improve diagnosis and prognosis of male fertility disorders.

Metabolomics

Recently, the sperm metabolome has been increasingly studied in both humans and farm animals. Anabolic and catabolic reactions are known to be essential processes in sperm, and their substrates, products, and byproducts could be used as indicators of sperm metabolic status. In this regard, the application of metabolomics as a promising tool to uncover biomarkers of sperm quality and fertilizing capacity is of great interest for the field of andrology (98). Twenty studies exploring the predictive potential of metabolites for sperm quality and/or fertility disorders were selected (**Supplemental Table 2**). All biomarkers were analyzed by specific techniques such as chemiluminescence or complex and sensitive analytical procedures such as MALDI-TOF MS and ^{1}H-NMR.

Oxidative stress (OS) reflects the imbalance between the production of reactive oxygen species and the antioxidant capacity of sperm (99). The diagnosis of male OS with highly sensitive metabolomic biomarkers is essential to achieve better clinical outcomes. In this study, three methods for the detection of sperm OS - reactive oxygen species (ROS), total antioxidant capacity (TAC) and oxidation-reduction potential (ORP) - were reviewed. Physiological ROS levels are known to be essential for an adequate sperm function. In the present review, six studies were found to report ROS to show high diagnostic potential for male infertility. ROS levels in the entire ejaculate measured by chemiluminescence exhibited fair to excellent diagnostic value for infertility, reporting AUCs of 0.683 (100) and 0.833 (101). When measured in sperm cells, ROS also exhibited a good predictive value with an AUC of 0.789 (102). Regarding the diagnostic potential for idiopathic infertility, ROS levels in the entire ejaculate, despite exhibiting limited robustness, were reported to be able to predict recurrent pregnancy loss, with an AUC of 0.630 (103), and to diagnose teratozoospermia, with an AUC of 0.614 (104). An excellent diagnostic potential, however, was observed to discriminate between ejaculates with high and low number of polymorphonuclear leukocytes (PMN; AUC of 0.888) (105). Finally, a fair diagnostic power was seen for seminal plasma ROS to differentially diagnose varicocele (AUC of 0.689), and an excellent diagnostic power in the diagnosis of varicocele with prostatitis (AUC of 0.948) (106). On the other hand, total antioxidant capacity (TAC) is known to be a parameter that indicates sperm oxidative status. TAC in seminal plasma was studied by Sharma *et al.* (1999) (106) to explore its diagnostic value in male reproduction. They reported seminal plasma TAC to differentially diagnose between varicocele with prostatitis (AUC = 0.828) and varicocele (AUC = 0.802), as well as to predict normozoospermic idiopathic infertility (AUC = 0.818). Yet, another study from Roychoudhury *et al.* (2016) (107), even though significant, reported fair diagnostic power for seminal plasma TAC in the prediction of male infertility (AUC = 0.608). Furthermore, because of the relationship between ROS and TAC, both indicating sperm oxidative status, as well as their good diagnostic power when assessed individually, Sharma *et al.* (1999) (106) explored their male fertility diagnostic potential when combined. They reported an excellent diagnostic value for ROS-TAC in seminal plasma to predict idiopathic infertility (AUC = 0.845)

and to differentially diagnose varicocele with prostatitis (AUC = 0.932) and varicocele (AUC = 0.808) from normozoospermic men. Finally, oxidation–reduction potential (ORP) is a measure reflecting the balance between ROS and TAC of sperm, which is determined by the MiOXSYS™ System based on a galvanostatic measure of electrons (108). ORP in the entire ejaculate has been explored as a new diagnostic tool by Agarwal and collaborators (108–112). Semen ORP, evaluated through a MiOXSYS™ system, has been established as a good diagnostic tool for oligozoospermia (109) (AUC = 0.754), asthenozoospermia (AUCs of 0.751 (109) and 0.648 (110)) and teratozoospermia (109) (AUC = 0.693). The differentiation between normozoospermic and abnormal sperm using ORP in seminal plasma was also explored in different studies, reporting AUCs of 0.765 (111), 0.596 (112) and 0.809 (109). Finally, they also explored the potential of seminal plasma ORP to diagnose primary or secondary male infertility and found a good predictive AUC value (113) (AUC = 0.770). Along these lines, increased ROS formation and/or decreased TAC, however, were found to lead to an unbalanced oxidation-reduction and subsequent sperm quality and fertility disorders (114). Based on the reviewed literature, ROS and TAC in the ejaculate exhibit good to excellent diagnostic value for male factor infertility and varicocele. Interestingly, the combination of both biomarkers shows better predictive values for both reproductive disorders. Notwithstanding, ROS levels in the ejaculate are able to diagnose the presence of PMN in semen. Similarly, the reviewed literature supports that ORP is a robust biomarker for oligozoospermia, asthenozoospermia, teratozoospermia and primary or secondary male factor infertility. Remarkably, the prediction of male infertility using ORP shows similar results than the combination of ROS and TAC. Along these lines, OS biomarkers such as ROS, TAC and ORP seem to be strong predictors of sperm quality and fertility disorders, as they are indicators of sperm metabolic status.

On the other hand, metabolomic fingerprints in seminal plasma evaluated by MALDI-TOF MS and ^{1}H-NMR were established as excellent biomarkers for male infertility disorders such as idiopathic infertility (115) (AUC = 0.994), oligozoospermia (115) (AUC = 0.993) and asthenozoospermia (116) (AUC = 0.927), as well as indicators of sperm physiological status, such as high sperm DNA fragmentation (117) (AUC = 0.925) and lipid peroxidation (117) (AUC =

0.974). The study of Rivera-Egea *et al.* (2018) (118) tested sperm lipids as biomarkers for pregnancy rates following ICSI through UHPLC-time-of-flight (TOF) MS. Some ceramides (e.g., Cer(d18:1/24:0)), phosphatidylcholines, phosphatidylethanolamines and sphingomyelins showed a good predictive value for ICSI clinical pregnancy, with AUCs ranging from 0.700 to 0.770. Another study by Tang *et al.* (2017) (119) used gas chromatography mass spectrometry (GC/MS) to determine the potential of fatty acids in seminal plasma (L-valine, cholecalciferol, D-pinitol, hexadecanoic acid, Oleic acid, nonadecanoic acid and benzoic acid) to diagnose asthenozoospermia, with AUCs ranging from 0.729 to 0.862. Lipid hormones such as testosterone or estradiol in seminal plasma showed a good predictive value for sperm recovery in non-obstructive azoospermia patients (120). Whereas testosterone showed an AUC of 0.771 for discriminating between NOA patients with and without successful sperm recovery, the relation testosterone/estradiol AUC was 0.886. Finally, the study of Tunc *et al.* (2010) (121) reported nitro blue tetrazolium in the entire ejaculate normalized by sperm concentration to be able to predict male factor infertility with high sensitivity and specificity, showing an AUC of 0.880. Thus, sperm metabolomic analysis for predicting male fertility disorders has been proven as a robust technique, as it provides relevant information for the diagnosis of male factor infertility.

In short, the available literature supports the high diagnostic value of ROS, TAC, ORP, metabolite profiles and individual lipids in sperm and seminal plasma for male sperm quality and fertility disorders. Moreover, the quantification of metabolite profiles in seminal plasma by ^{1}H-NMR or MALDI-TOF MS has been shown to be better than that of individual metabolites when predicting sperm quality and fertility. Nevertheless, the need of highly specialized technicians and expensive instruments required for the analysis of metabolomic biomarkers may hinder its implementation in fertility clinics.

DISCUSSION

The current review supports that some OMICs (e.g., DNA structure and integrity, genomics and epigenomics, transcriptomics, metabolomics, and proteomics) could be considered relevant molecular biomarkers that may help identify infertility etiologies and fertilization prognosis with

cost-effective, simple, and accurate diagnosis.

The identification of reliable molecular biomarkers in semen could help in the diagnosis and treatment of conditions causing semen quality and male fertility disorders. In this sense, exploring semen biomarkers showing high diagnostic value is of great interest to determine their potential to become clinical tools for the diagnosis of sperm quality dysfunctions and predict male fertility and ART success. Implementation of these novel tools in fertility clinics may be translated into non-invasive, time-saving, cheaper and robust diagnostics for men reproductive disorders. In the present study, many molecular biomarkers of sperm and seminal plasma showing high diagnostic power have been evaluated. To the best of our knowledge, the present study is the first in compiling semen robust biomarkers that show high diagnostic value for male sperm quality and fertility disorders (**Figure 2**).

The differential diagnosis and/or etiology of male factor infertility by conventional semen analysis often remains elusive, which makes the prognosis and treatment of infertile patients difficult. The advent of OMICs techniques, nevertheless, has uncovered relevant molecular biomarkers that may help identify infertility etiologies with cost-effective, simple and accurate diagnosis. Although highly specific and robust (in)fertility biomarkers have been unearthed using OMICs techniques, an accurate prediction of sperm physiological status and/or (in)fertility should envisage the integration of data from each OMICs field. The combination of independent biomarkers to reach a system biology approach is currently in its infancy in the andrology field. The advent of diagnostic tools incorporating independent molecular biomarkers, however, could be of great interest for spermatology because it would improve the identification of male fertility disorders through highly accurate and robust diagnostics.

The use of these novel biomarkers in IVF clinics could be useful since their implementation might be translated into cost-effective, non-invasive, time-saving and accurate diagnosis of men reproductive disorders. Yet, although OMICs techniques have uncovered a wide range of highly accurate molecular biomarkers, the translation of such results into a clinical setting is often challenging. The high costs of some OMICs techniques and the need of highly specialized technicians are two major limitations for the implementation of such diagnostic tools.

In spite of this, the continuous innovation, simplification, and cost-reduction of some OMICs can make them to become a routine tool for the diagnosis of male fertility disorders.

Limitations and strengths

The observational studies revised in the present review reported ROC analysis of the molecular biomarker candidates. ROC analysis provides an objective statistical method to assess the diagnostic accuracy of biomarkers and indicate the diagnostic power for the assessment of male (in)fertility (122). Nonetheless, some of the studies reported herein had limited sample size, which could generate biases in ROC analysis. Furthermore, some of the articles evaluated for inclusion did not report essential information regarding the studied population and ROC analysis and, consequently, could not be included even though corresponding authors were contacted by e-mail to ask for those data. Moreover, there is a very limited number of studies linking biomarkers with clinical outcomes using ROC and OR/RR and therefore a diagnostic test accuracy is, unfortunately, not possible. On the other hand, we also detected a disparity in the analytical techniques and methodology thus hindering the comparison between molecular biomarkers. Finally, due to the heterogeneity of studies, approaches, and methodologies, it was not possible, despite intended, to undertake a meta-analysis. Further studies and clinical trials with a larger number of samples, standardized methodology and well-characterized cohorts are required before these molecular diagnostic tools may be implemented in IVF clinics.

CONCLUSIONS

The current review presents the largest evidence to date supporting that some OMICs have revealed relevant molecular biomarkers that may help identify infertility etiologies and fertilization prognosis with cost-effective, simple, and accurate diagnosis.

ACKNOWLEDGEMENTS

The authors acknowledge the support from the Ministry of Science and Innovation, Spain (Grants: IJC2019-039615-I to A.S-H.; PRE2018-083488 to A.D-B.; FPU18/00666 to M.L.), the European Union's Horizon 2020 research and innovation scheme under the Marie Skłodowska-Curie grant agreement nº 801342 to J.R-M. (Techniospring INDUSTRY; TECSPR-19-1-0003) and the Regional Government of Catalonia (2017-SGR-1229 to M.Y.). Figure 2 created with BioRender, http://biorender.io.

AUTHOR CONTRIBUTIONS

A.S-H. and M.Y. initiated the idea and designed the review. A.D-B. and M.L. collected and selected the data, assessed the articles, and wrote the manuscript. A.S-H. and M.Y. contributed to the drafting and critically reviewed the article for important intellectual content. J.R-M. critically revised the Manuscript. All the authors approved the final manuscript.

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Table 1. Summary table of the statistically significant biomarkers identified in the systematic review.

BIOMARKER	TYPE	CASE	CONTROL	METHODS	AUC	REF.
A. DNA structure and integrity						
Sperm						
SDF - 3' free ends	SDF	Infertile (n=348)	Fertile (normo) (n=86)	TUNEL	0.757	(13)
		Infertile (no clinical pregnancy after 1 year) (n=427)	Fertile (proven fertility) (n=40)		0.608	(14)
		Infertile (n=72)	Fertile (proven fertility) (n=21)		0.903	(12)
		Infertile (no pregnancy after 1 year) (n=66)	Fertile (proven fertility) (n=47)		0.930	(11)
		Infertile, seeking for treatment (n=194)	Fertile (normo) (n=25)		0.820	(15)
		Infertile, seeking for treatment (n=261)	Fertile (normo) (n=95)		0.556	(16)
		Infertile (no pregnancy after 1 year), no clinical pregnancy within observation period (n=59)	Infertile (no pregnancy after 1 year), clinical pregnancy within observation period (n=26)		0.700	(19)
		Infertile, no clinical pregnancy after ICSI (n=21)	Infertile, clinical pregnancy after ICSI (n=15)		0.700	(20)
		Infertile, undergoing IVF/ICSI cycles (n=65)	Clinical pregnancy through natural conception or IUI (n=57)		0.790	(18)
		Partners with reduced ovarian reserve, no clinical pregnancy after ICSI (n=255)	Partners with reduced ovarian reserve, clinical pregnancy after ICSI (n=72)		0.594	(21)
		Infertile, idiopathic oligoasthenoteratozoospermia (n=73)	Fertile (normo) (n=20)		0.892	(17)
γ-H2AX levels	SDF	Infertile (n=100)	Fertile (proven fertility) (n=100)	Flow cytometry with FITC–conjugated anti-γH2AX	0.927	(27)
Single strand DNA breaks (ssSDF)	SDF - oxidative DNA damage	Normo idiopathic infertile, astheno, terato, asthenoteratozoospermic, azoospermic (n=83)	Fertile (normo) (n=11)	Alkaline Comet	0.994	(30)
		Infertile, no clinical pregnancy after IVF (n=180)	Infertile, clinical pregnancy after IVF (n=39)		0.648	(31)
		Infertile, enrolled in IVF (n=70)	Fertile (normo) (n=28)		0.970	(29)
		Infertile, no clinical pregnancy after IVF (n=50)	Infertile, clinical pregnancy after IVF (n=20)		0.905	(29)
		Infertile (n=133)	Fertile (proven fertility) (n=50)		0.937	(12)
8-OHdG bases	SDF - oxidative DNA damage	Infertile, no clinical pregnancy after IUI (total n=53)	Infertile, clinical pregnancy after IUI (total n=53)	Immunohistochemistry using FITC-labeled 8-OHdG-specific protein	0.794	(22)

		Infertile, no clinical pregnancy after IVF (n=63)	Infertile, clinical pregnancy after IVF (n=10)	Alkaline comet+formamidopyrimidine DNA glycosylase treatment	0.776	(31)
		Infertile, no clinical pregnancy after ICSI treatment (n=38)	Infertile, clinical pregnancy after ICSI (n=15)		0.704	(31)
STI	Chromatin structure	Infertile, miscarriage after ICSI (n=6)	Infertile, pregnancy with livebirth after ICSI (n=15)	STI	0.710	(34)
Chromatin decondensation	SDF	Infertile, idiopathic recurrent pregnancy loss (n=45)	Fertile (normo) (n=20)	SCSA	0.830	(39)
	SDF	Infertile with varicocele, no clinical pregnancy (n=66)	Varicocele, spontaneous pregnancy (n=22)		0.762	(42)
	SDF	Infertile (n=74)	Fertile (proven fertility) (n=49)		0.792	(12)
	SDF	Idiopathic infertility (n=100)	Fertile (recently proven fertility, 2 years) (n=100)		0.919	(38)
	SDF	Infertile, recurrent spontaneous abortion (n=139)	Fertile (recently proven fertility, 2 years) (n=200)		0.752	(40)
	SDF	Infertile, recurrent pregnancy loss (RPL) after IVF (n=461)	Infertile, clinical pregnancy after IVF (n=411)		0.713	(41)
	SDF	Infertile, no clinical pregnancy after ICSI (n=39)	Infertile, clinical pregnancy after ICSI (n=24)	Acridine orange	0.750	(37)
	SDF	Infertile, no clinical pregnancy (n=88)	Fertile (proven fertility) (n=58)	Halosperm (SCD)	0.546	(52)
	SDF	Infertile (n=74)	Fertile (proven fertility) (n=49)		0.869	(12)
	SDF	Infertile, embryos of low quality (CES 1, 2 or 3) after IVF (n=43)	Infertile, embryos of high quality (CES 4 or 5) after IVF (n=37)		0.710	(47)
	Undamaged DNA (big halo)	Infertile, embryos of low quality (CES 1, 2 or 3) after IVF (n=43)	Infertile, embryos of high quality (CES 4 or 5) after IVF (n=37)		0.830	(47)
	SDF	Infertile, no clinical pregnancy after IVF (n=61)	Infertile, clinical pregnancy after IVF (n=27)		0.670	(47)
	Undamaged DNA (big halo)	Infertile, no clinical pregnancy after IVF (n=61)	Infertile, clinical pregnancy after IVF (n=27)		0.750	(47)
	SDF	Infertile, poor fertilization rates after ICSI (total n=135)	Infertile, good fertilization rates after ICSI (total n=135)		0.680	(46)
	SDF	Infertile, no clinical pregnancy after IVF (n=44)	Infertile, clinical pregnancy after IVF (n=11)		0.700	(48)
	SDF	Infertile, fertilization rate <50% after conventional IVF (n=29)	Infertile, fertilization rate ≥50% after conventional IVF (n=104)		0.664	(45)
	SDF	Cycles with total embryo development arrest (n=15)	Cycles of at least one developed blastocyst (n=97)		0.771	(45)
	SDF	Infertile with varicocele (n=98)	Fertile (n=80)		0.942	(44)
	SDF	Non-normo (n=434)	Fertile (normo) (n=234)		0.753	(43)
	SDF	Infertile, no clinical pregnancy after ICSI (n=32)	Infertile, clinical pregnancy after ICSI (n=49)		0.711	(51)

	SDF	Infertile, no pregnancy after IVF (total n=38)	Infertile, pregnancy after IVF (total n=38)		0.830	(49)
Deficient protamination	Chromatin maturity	Infertile, fertility rate ≤60% after IVF (total n=140)	Infertile, fertility rate >60% after IVF (total n=140)	CMA3 staining	0.760	(57)
		Infertile, fertility rate ≤50% after IVF (n=28)	Infertile, fertility rate >50% after IVF (n=43)		0.740	(57)
		Infertile, <50% embryos of A quality after IVF/ICSI (n=133)	Infertile, ≥50% embryos of A quality after IVF/ICSI (n=9)		0.778	(59)
		Infertile, no fertilization after IVF (n=37 non-fertilized oocytes of a total of 235 inseminated oocytes from 82 different patients, each in a single IVF cycle)	Infertile, fertilization after IVF (n=198 fertilized oocytes of a total of 235 inseminated oocytes from 82 different patients, each in a single IVF cycle)		0.769	(58)
Histone persistence	Chromatin maturity	Infertile with mild male factor or unexplained infertility, no clinical pregnancy after IUI (n=221)	Infertile with mild male factor or unexplained infertility, clinical pregnancy after IUI (n=22)	Aniline blue staining	0.653	(60)
		Infertile, fertilization rate <80% after IVF/ICSI (n=89)	Infertile, fertilization rate ≥80% after IVF/ICSI (n=117)		0.611	(59)
B. Gene expression and epigenomics						
Sperm						
Expression model (EIF5A + RPL13 + RPL23A + RPS27A)	Expression signature	Low IUI clinical pregnancy rate (<13.6%) (n=17)	High IUI clinical pregnancy rate (>13.6%) (n=51)	TaqMan Arrays	0.910	(61)
Sperm global DNA methylation pattern	Methlylation pattern	All IVF patients (female factor previously excluded) (n=127)	Normo, fertile donors (n=54)	GW sperm DNA methylation	0.670	(64)
		IVF patients (female factor previously excluded) with poor embryogenesis (n=72); either with positive (n=42) or negative (n=30) chemical pregnancies after IVF	IVF patients (female factor previously excluded) with good embryogenesis and positive chemical pregnancy (n=55)		0.640	(64)
Sperm site-specific DNA methylation pattern	Methlylation pattern	All IVF patients (female factor previously excluded) (n=127)	Normo fertile donors (n=54)	Bisulphite conversion and methylation microarray	0.930	(64)
Methylation of *MLH1* promoter	Methylation pattern	Oligo men (n=10)	Samples from normo men (n=29)	Bisulphite modification and methylation-specific polymerase chain reaction	0.611	(65)
Hypermethylation of MTHFR promoter region	Methylation pattern	Idiopathic infertile men (n=40)	Samples from donors with proven fertility (n=40)	Bisulfite Pyrosequencing	0.734	(68)
Seminal plasma						
CCNA1 promoter methylation from cell-free DNA	Cell-free DNA methylation	NOA patients with SCO and with round spermatid MA (n=42)	NOA patients with hypospermatogenesis (HO) (n=26)	Bisulfite treatment of DNA and MethyLight analysis	0.668	(70)
C. Transcriptomics						
miRNA in sperm						

hsa-miR-122-5p	miRNA	Oligo (n=80)	Normo (n=90)	qRT-PCR	0.777	(123)
		NOA (n=40)	Normo (n=16)		0.988	(123)
hsa-miR-34b-3p	miRNA	Oligo (n=80)	Normo (n=90)	qRT-PCR	0.944	(123)
		NOA (n=40)	Normo (n=16)		0.852	(123)
hsa-miR-34b-5p	miRNA	Oligo (n=80)	Normo (n=90)	qRT-PCR	0.798	(123)
		NOA (n=40)	Normo (n=16)		0.948	(123)
hsa-miR34c-5p	miRNA	Oligo (n=80)	Normo (n=90)	qRT-PCR	0.776	(123)
		NOA (n=40)	Normo (n=16)		0.978	(123)
		No clinical pregnancy after ICSI (n=97)	Clinical pregnancy after ICSI (n=65)		0.750	(77)
hsa-miR-429	miRNA	Oligo (n=80)	Normo (n=90)	qRT-PCR	0.980	(123)
hsa-miR-34b-5p, 34b-3p, 34c-5p, 122-5p, 429	miRNA profile	NOA (n=40)	Normo (n=16)	qRT-PCR	0.999	(123)
		Oligo (n=80)	Normo (n=90)		0.987	(123)
miRNA in seminal plasma						
hsa-miR-122-5p	miRNA	Azoo (n=73)	Normo fertile (n=68)	Seq and qRT-PCR	0.921	(78)
		Astheno (n=73)	Normo fertile (n=68)		0.733	(78)
		Astheno (n=73)	Azoo (n=73)		0.967	(78)
hsa-miR-146-5p	miRNA	Azoo (n=73)	Normo fertile (n=68)	Seq and qRT-PCR	0.825	(78)
		Astheno (n=73)	Normo fertile (n=68)		0.789	(78)
		Astheno (n=73)	Azoo (n=73)		0.963	(78)
hsa-miR-181a-5p	miRNA	Azoo (n=73)	Normo fertile (n=68)	Seq and qRT-PCR	0.875	(78)
		Astheno (n=73)	Normo fertile (n=68)		0.781	(78)
		Astheno (n=73)	Azoo (n=73)		0.976	(78)
hsa-miR-205-5p	miRNA	Secretory azoo (n=14)	OA (n=13)	miRCURY LNA™ array and qRT-PCR	0.838	(81)
hsa-miR-210-3p	miRNA	Varicocele (dyszoospermia) (n=204)	Normo (n=30)	qRT-PCR	0.940	(79)
hsa-miR-31-5p	miRNA	Secretory azoo (n=14)	OA (n=13)	miRCURY LNA™ array and qRT-PCR	0.963	(81)
hsa-miR-34c-5p	miRNA	Azoo (n=73)	Normo fertile (n=68)	Seq and qRT-PCR	0.894	(78)
		Astheno (n=73)	Normo fertile (n=68)		0.783	(78)

		Astheno (n=73)	Azoo (n=73)		0.990	(78)
hsa-miR-374b-5p	miRNA	Azoo (n=73)	Normo fertile (n=68)	Seq and qRT-PCR	0.839	(78)
		Astheno (n=73)	Normo fertile (n=68)		0.813	(78)
		Astheno (n=73)	Azoo (n=73)		0.984	(78)
hsa-miR-509-5p	miRNA	Azoo (n=73)	Normo fertile (n=68)	Seq and qRT-PCR	0.822	(78)
		Astheno (n=73)	Normo fertile (n=68)		0.836	(78)
		Astheno (n=73)	Azoo (n=73)		0.983	(78)
hsa-miR-513a-5p	miRNA	Azoo (n=73)	Normo fertile (n=68)	Seq and qRT-PCR	0.825	(78)
		Astheno (n=73)	Normo fertile (n=68)		0.806	(78)
		Astheno (n=73)	Azoo (n=73)		0.966	(78)
hsa-miR-141, 429, 7-1-3p	miRNA profile	NOA (n=96)	Normo fertile (n=96)	TLDA and qRT-PCR	0.833	(124)
piRNA in seminal plasma						
piR-30198	piRNA	Azoo (n=52)	Normo fertile (n=58)	Seq and qRT-PCR	0.986	(82)
		Astheno (n=74)	Azoo (n=52)		0.955	(82)
piR-31068	piRNA	Astheno (n=74)	Normo fertile (n=58)	Seq and qRT-PCR	0.985	(82)
		Azoo (n=52)	Normo fertile (n=58)		0.996	(82)
piR-31068, 31925, 43771, 43773	piRNA profile	Astheno (n=74)	Normo fertile (n=58)	Seq and qRT-PCR	0.894	(82)
piR-31068, 31925, 43771, 43773, 30198	piRNA profile	Azoo (n=52)	Normo fertile (n=58)	Seq and qRT-PCR	0.991	(82)
piR-31925	piRNA	Astheno (n=74)	Normo fertile (n=58)	Seq and qRT-PCR	0.932	(82)
		Azoo (n=52)	Normo fertile (n=58)		0.967	(82)
piR-43771	piRNA	Astheno (n=74)	Normo fertile (n=58)	Seq and qRT-PCR	0.903	(82)
		Azoo (n=52)	Normo fertile (n=58)		0.954	(82)
piR-43773	piRNA	Astheno (n=74)	Normo fertile (n=58)	Seq and qRT-PCR	0.796	(82)
		Azoo (n=52)	Normo fertile (n=58)		0.880	(82)
piR-58527	piRNA	Secretory azoo (n=14)	OA (n=13)	miRCURY LNA™ array and qRT-PCR	0.744	(81)
D. Metabolomics						
Sperm						

ROC (AU)	ROS	Infertile patients (n=133)	Normo (n=91)	Chemiluminescence (luminol)	0.789	(102)
Cer(d18:1/24:0)	Ceramide	No clinical pregnancy after ICSI (n=16)	Clinical pregnancy after ICSI (n=22)	UHPLC-time-of-flight (TOF)-MS	0.700	(118)
Cer(d18:1/22:0)	Ceramide	No clinical pregnancy after ICSI (n=16)	Clinical pregnancy after ICSI (n=22)	UHPLC-time-of-flight (TOF)-MS	0.710	(118)
Cer(d18:1/23:0)	Ceramide	No clinical pregnancy after ICSI (n=16)	Clinical pregnancy after ICSI (n=22)	UHPLC-time-of-flight (TOF)-MS	0.700	(118)
Phosphatidylcholines (O-22:0/20:4)	Phosphatidylcholines	No clinical pregnancy after ICSI (n=16)	Clinical pregnancy after ICSI (n=22)	UHPLC-time-of-flight (TOF)-MS	0.770	(118)
Phosphatidylethanolamines (P-16:0/18:2)	Phosphatidylethanolamines	No clinical pregnancy after ICSI (n=16)	Clinical pregnancy after ICSI (n=22)	UHPLC-time-of-flight (TOF)-MS	0.700	(118)
Sphingomyelins (38:1)	Sphingomyelins	No clinical pregnancy after ICSI (n=16)	Clinical pregnancy after ICSI (n=22)	UHPLC-time-of-flight (TOF)-MS	0.700	(118)
Sphingomyelins (d18:1/22:0)	Sphingomyelins	No clinical pregnancy after ICSI (n=16)	Clinical pregnancy after ICSI (n=22)	UHPLC-time-of-flight (TOF)-MS	0.720	(118)
Sphingomyelins (42:1)	Sphingomyelins	No clinical pregnancy after ICSI (n=16)	Clinical pregnancy after ICSI (n=22)	UHPLC-time-of-flight (TOF)-MS	0.720	(118)
Sphingomyelins (d18:1/25:0)	Sphingomyelins	No clinical pregnancy after ICSI (n=16)	Clinical pregnancy after ICSI (n=22)	UHPLC-time-of-flight (TOF)-MS	0.740	(118)
Phosphatidylcholines (0:0/20:0)	Phosphatidylcholines	No clinical pregnancy after ICSI (n=16)	Clinical pregnancy after ICSI (n=22)	UHPLC-time-of-flight (TOF)-MS	0.710	(118)
Seminal plasma						
ROC (AU)	ROS	Idiopathic infertility (n = 28)	Normo fertile (n=24)	Chemiluminescence	0.743	(106)
		Infertile varicocele (n = 55)	Normo fertile (n=24)		0.689	(106)
		Infertile varicocele with prostatitis (n = 8)	Normo fertile (n=24)		0.948	(106)
TAC (AU)	TAC	Idiopathic infertility (n = 28)	Normo fertile (n=24)	Chemiluminescence	0.818	(106)
		Infertile varicocele (n = 55)	Normo fertile (n=24)		0.802	(106)
		Infertile varicocele with prostatitis (n = 8)	Normo fertile (n=24)		0.828	(106)
TAC (uM Trolox equivalent)	TAC	Infertile (n=279)	Normo fertile (n=46)	TAC assay kit	0.608	(107)
ROS-TAC (AU)	ROS/TAC	Idiopathic infertility (n = 28)	Normo fertile (n=24)	Chemiluminescence	0.845	(106)
		Infertile varicocele (n = 55)	Normo fertile (n=24)		0.808	(106)
		Infertile varicocele with prostatitis (n = 8)	Normo fertile (n=24)		0.932	(106)
Lipid fingerprint 1	Metabolomic profile	High lipid peroxidation levels (spectrophotometry)	Low lipid peroxidation levels (spectrophotometry)	MALDI-TOF MS	0.974	(117)
Lipid fingerprint 2	Metabolomic profile	High SDF (Comet Assay)	Low SDF (Comet Assay)	MALDI-TOF MS	0.925	(117)

Lactate, alanine, choline, citrate, glycerophosphocholine, glutamine, tyrosine, histidine, phenylalanine, and uridine	Metabolomic profile	Infertile normo (n=65)	Normo fertile (n=60)	H-NMR	0.994	(115)
Lactate, alanine, choline, citrate, glycerophosphocholine, glutamine, tyrosine, histidine, phenylalanine, and uridine	Metabolomic profile	Infertile oligo (n=60)	Normo fertile (n=60)	H-NMR	0.993	(115)
Cholesterol, 5a-cholesterol, 7-ketocholesterol, lipids, citrate, a-ketoglutaric acid, creatine, choline, phosphocholine, glycerophosphocholine, uridine, cytidine, cysteine, glutamine, glutamate, phenylalanine, tyrosine, histidine, taurine	Metabolomic profile	Astheno (n=10)	Normo (n=11)	H-NMR	0.927	(116)
L-Valine	Aminoacid	Astheno (n=30)	Normo (n=30)	GC/MS	0.801	(119)
Cholecalciferol	Vitamin D	Astheno (n=30)	Normo (n=30)	GC/MS	0.762	(119)
D-Pinitol	Cyclic polyol	Astheno (n=30)	Normo (n=30)	GC/MS	0.804	(119)
Hexadecanoic acid	LCFA	Astheno (n=30)	Normo (n=30)	GC/MS	0.813	(119)
Oleic acid	LCFA	Astheno (n=30)	Normo (n=30)	GC/MS	0.729	(119)
Nonadecanoic acid	Straight-chain fatty acid	Astheno (n=30)	Normo (n=30)	GC/MS	0.820	(119)
Benzoic acid	Aromatic carboxylic acids	Astheno (n=30)	Normo (n=30)	GC/MS	0.862	(119)
Testosterone (nM)	Hormone	NOA patients without sperm (n=26)	NOA patients with sperm (n=30)	Competitive immunoassay	0.771	(120)
Testosterone/E2	Hormone	NOA patients without sperm (n=26)	NOA patients with sperm (n=30)	Competitive immunoassay	0.886	(120)
Ejaculate						
ROS (mv/30min/million sperm)	ROS	Infertile (n=48)	Normo fertile (n=41)	Chemiluminescence	0.833	(101)
ROS (RLU/s/10^6 sperm)	ROS	Infertile (normo and abnormal sperm) (n=318)	Normo fertile (n=56)	Chemiluminescence	0.683	(100)
		Partners of women with RPL (n=50)	Normo (n=33)		0.630	(103)

		Terato (n=79)	Normo (n=56)		0.614	(104)
		High number of PMN (n=41)	Normo (n=94)		0.888	(105)
ORP (mV/10^6 sperm/mL)	ORP	Primary or secondary infertility (n=106)	Normo (n=51)	MiOXSYSTM System	0.770	(113)
		Abnormal motility (<40%) (n=15)	Normal motility (>40%) (n=44)		0.648	(110)
		Abnormal sperm (at least 1 abnormal WHO semen parameter; n=152)	Normo (n=42)		0.809	(109)
		Oligo (n=92)	Normo (n=42)		0.754	(109)
		Astheno (n=102)	Normo (n=42)		0.751	(109)
		Terato (n=95)	Normo (n=42)		0.693	(109)
		Abnormal sperm (n=292)	Normo fertile (n=15)		0.596	(112)
		Abnormal sperm (at least 1 abnormal semen parameter; n=1893)	Normo (n=199)		0.765	(111)
NBT (µg formazan/10^7 sperm)	NBT	Infertile (n = 36)	Normo fertile (n=21)	Photometry (colorimetric nitro blue tetrazolium)	0.880	(121)
E. Proteomics						
Sperm						
BAG6	Regulatory protein	Infertile (n=16)	Normo fertile (n=7)	LC-MS/MS and Western blot	0.921	(89)
CatSper1	Calcium channel	Bad quality embryos (EQA>50%) (n=16)	Good quality embryos (EQA<50%) (n=120)	Flow cytometry (fluorescence intensity)	0.682	(59)
CYP24A1	Enzyme	No clinical pregnancy after IUI (n=68)	Clinical pregnancy after IUI (n=15)	Immunocytochemistry (% positive cells)	0.710	(93)
HIST1H2BA	Histone	Infertile (n=16)	Normo fertile (n=7)	LC-MS/MS and Western blot	0.935	(89)
Nuclease activity corrected by sperm count	Enzimatic activity	Idiopathic infertile, astheno, terato, asthenoteratozoospermic, azoo (n=83)	Normo fertile (n=11)	SRED method	0.705	(30)
PELP1	Scaffolding protein	Oligospermic (n=26)	Normo (n=17)	ICC + FACS analysis	0.781	(87)
Seminal plasma						
CRISP1/PAP	Glycoprotein	NOA (n=14)	OA or pre-vasectomy (n=14)	Western blot	0.929	(125)
Inhibin B	Hormone	Failed TESE (n=45)	Successful TESE (n=17)	Two-site ELISA	0.910	(95)
PON1 activity	Antioxidant enzyme	Subfertile (n=32)	Normo fertile (n=30)	Spectrophotometry	0.950	(90)
PON1 activity (U/L)	Antioxidant enzyme	Infertile with abnormal semen parameters (n=32)	Normo fertile (n=30)	Spectrophotometry (colorimetric test)	0.950	(90)

Presepsin	CD24 subunit (inflammatory response)	Non-live birth (n=86)	Live birth (n=28)	PATHFAST chemiluminescence immunoassay analyzer	0.634	(94)
		No chemical pregnancy after ICSI (n=81)	Chemical pregnancy after ICSI (n=33)		0.677	(94)
Annexin A7 i CD63	Proteomic profile	Low mitochondrial activity (n=12)	Normal mitochondrial activity (n=12)	LC-MS/MS	0.993	(88)
CRISPLD1	Protein	High SDF (Comet Assay) (n=12)	Low SDF (Comet Assay) (n=12)	LC-MS/MS	0.882	(88)
PLTP and COL12A1	Proteomic profile	Altered acrosome integrity (PNA) (n=12)	Normal acrosome integrity (PNA) (n=12)	LC-MS/MS	0.972	(88)
TEX101	Glycoprotein	NOA (n=81)	OA or pre-vasectomy (n=93)	ELISA	0.670	(84)
		Azoo (n=137)	Normo (n=64)		0.990	(84)
		Fertile pre-vasectomy (n=64)	Fertile post-vasectomy (n=57)		1.000	(84)
		No sperm retrieval in NOA (n=11)	Sperm retrieval in NOA (n=15)		0.690	(84)
Total uPA	Enzyme	No clinical pregnancy after AI (AIH or ICSI) (n=23)	Clinical pregnancy after AI (AIH or ICSI) (n=23)	ELISA	0.720	(92)
Transferrin receptors	Protein receptors	Azoo (n=25)	Post-vasectomy (n=40)	ELISA	0.630	(97)
α-glucosidase	Enzymatic activity	Azoo (vasectomy) (n=27)	Azoo (testicular defects) (n=33)	Spectrophotometry	0.760	(96)
		Successful vasectomy (n=27)	Unsuccessful vasectomy (n=11)		0.830	(96)
α-glucosidase	Enzyme concentration	NOA (n=14)	OA or pre-vasectomy (n=14)	Immunoblotting	0.610	(125)

Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; γ-H2AX, Gamma-H2A.X; AI, Artificial insemination; AIH, Artificial insemination by husband; Astheno, Asthenozoospermic; Azoo, Azoospermia; BAG6, BAG Cochaperone 6; CMA3, Chromomycin A3; CRISP1, Cysteine-rich secretory protein 1; CYP24A1, Vitamin D inactivating enzyme; E2, Estradiol; ELISA, Enzyme-linked immunosorbent assay; FACS, Fluorescence-activated cell sorting; GW, genome wide; HIST1H2BA; Histone H2B type 1-A; H-NMR, Proton nuclear magnetic resonance; HO, hypospermatogenesis; ICC, Immunocytochemistry; ICSI, Intracytoplasmic sperm injection; IUI, Intrauterine insemination; IVF, *In vitro* fertilization; LCFA, Long-chain fatty acids; LC-MS/MS, Liquid Chromatography with tandem mass spectrometry; MA, Maturation arrest; miRNA, MicroRNA; NBT, Nitro blue tetrazolium; NOA, Non-obstructive azoospermia; Normo, Normozoospermic; OA, Obstructive azoospermia; Oligo, Oligozoospermic; ORP, oxidation-reduction potential; PELP1, Proline, Glutamate And Leucine Rich Protein 1; piRNA, piwi-interacting RNA; PMN, Polymorphonuclear Leukocytes; PNA, Peanut agglutinin; PON1, Paraoxonase 1; qRT-PCR, Real-Time Quantitative Reverse Transcription polymerase chain reaction; ROS, Reactive oxygen species; RPL, recurrent pregnancy loss; SCD, Stearoyl Coenzyme A Desaturase; SCO, Sertoli cell only; SDF, Sperm DNA fragmentation; Seq, Sequencing; STI, Sperm toroid intergrity; T, Testosterone; TAC, total antioxidant capacity; Terato, Teratozoospermic; TESE, Testicular sperm extraction; TEX101, Testis expressed protein 101; TLDA, TaqMan Low Density Arrays; TUNEL, Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling assay; UHPLC, Ultra-High-Performance Liquid Chromatography; uPA, Urokinase-type plasminogen activator.

FIGURE CAPTIONS

Figure 1. Flowchart of the systematic review.

Figure 2. Schematic summary of proposed well-characterized molecular fertility biomarkers in semen with a potential clinical application.

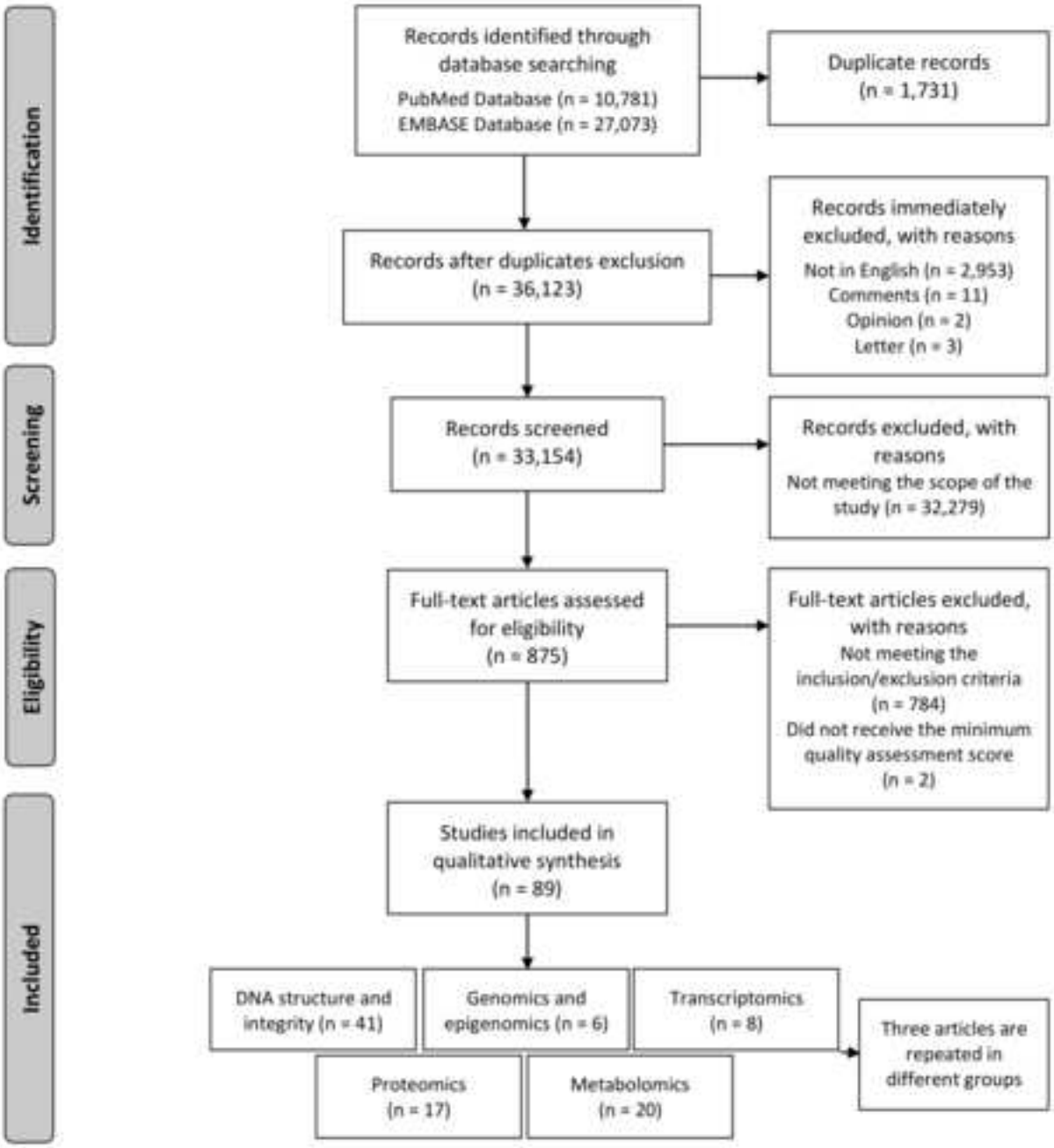
Abbreviations: 8-OHdG, 8-hydroxy-2' -deoxyguanosine; CMA3, Chromomycin A3; Me3, tri-methylation; miRNA, micro ribonucleic acid; NT-proCNP, N-terminal C-type natriuretic propeptide; ORP, oxidation-reduction potential; PELP1, proline-, glutamic acid- and leucine-rich protein 1; piRNA, piwi ribonucleic acid; PON-1, Paraoxonase 1; ROS, reactive oxygen species; SCSA, sperm chromatin structure assay; STI, sperm toroid integrity; TAC, total antioxidant capacity; TEX101, testis-expressed protein 101; TUNEL, terminal deoxy-transferase-mediated deoxy-uridine triphosphate (dUTP) nick end labeling.

SUPPLEMENTAL DATA

Supplemental File 1. Complete search strategy.

Supplemental Table 1. PICOS Table.

Supplemental Table 2. Studies included in the systematic review.


Identification
Screening
Eligibility
Included
Records identified through database searching
PubMed Database (n = 10,781)
EMBASE Database (n = 27,073)
Duplicate records (n = 1,731)
Records after duplicates exclusion (n = 36,123)
Records immediately excluded, with reasons
Not in English (n = 2,953)
Comments (n = 11)
Opinion (n = 2)
Letter (n = 3)
Records screened (n = 33,154)
Records excluded, with reasons
Not meeting the scope of the study (n = 32,279)
Full-text articles assessed for eligibility (n = 875)
Full-text articles excluded, with reasons
Not meeting the inclusion/exclusion criteria (n = 784)
Did not receive the minimum quality assessment score (n = 2)
Studies included in qualitative synthesis (n = 89)
DNA structure and integrity (n = 41)
Genomics and epigenomics (n = 6)
Transcriptomics (n = 8)
Proteomics (n = 17)
Metabolomics (n = 20)
Three articles are repeated in different groups

Figure(s)

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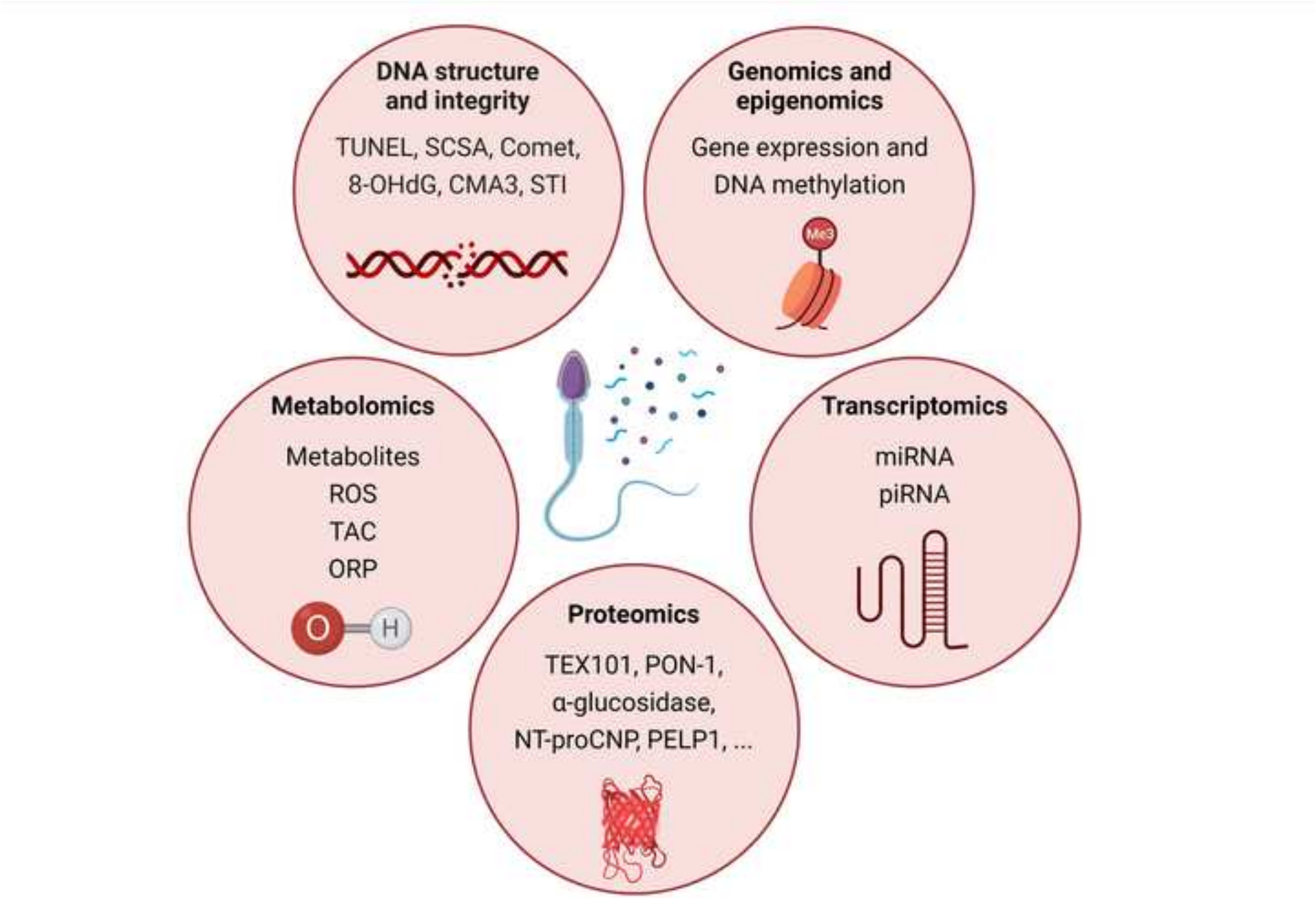

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Date: January 17, 2022 **Manuscript # (if available):**

Manuscript title: A systematic review identifying fertility biomarkers in semen: a clinical approach through -OMICs to diagnose male infertility

Corresponding author: Albert Salas-Huetos and Marc Yeste

Authors may either sign the same form or submit individually

I am an author on this submission, have adhered to all editorial policies for submission as described in the Information for Authors, attest to having met all authorship criteria, and disclosed all potential conflicts of interest for inclusion on the title page of the submission.

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Typed or CLEARLY Printed Name:

Signature:

PRISMA 2020 Checklist

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	3-4
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	3-4
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	4-5 and Additional File 2
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	4
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Additional File 1
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	4-5
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	4-5
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	4-5 and Additional File 2
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Additional File 2
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	5 (quality assessment)
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	N/A
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	4-5 and Additional File 2
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	4-5 and Additional File 2
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	4-5 and Additional File 2
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	4-5 and Additional File 2
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	N/A
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	N/A
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	5 (quality assessment)

PRISMA 2020 Checklist

Section and Topic	Item #	Checklist item	Location where item is reported
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	N/A
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	5 and Figure 1
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	N/A
Study characteristics	17	Cite each included study and present its characteristics.	5-17 and Additional File 3
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	N/A
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	5-17 and Additional File 3
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	5-17 and Additional File 3
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	N/A
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	N/A
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	N/A
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	N/A
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	N/A
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	17-25
	23b	Discuss any limitations of the evidence included in the review.	25
	23c	Discuss any limitations of the review processes used.	25
	23d	Discuss implications of the results for practice, policy, and future research.	24-25
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	4 and PROSPERO 2020: CRD42020176417
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	4 and PROSPERO 2020: CRD42020176417
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	4 and PROSPERO 2020: CRD42020176417
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	26
Competing interests	26	Declare any competing interests of review authors.	26

PRISMA 2020 Checklist

Section and Topic	Item #	Checklist item	Location where item is reported
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	PROSPERO 2020: CRD42020176417

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71

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