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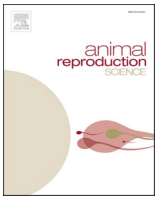

Review article

Evaluation of sperm quality and male fertility: The use of molecular markers in boar sperm and seminal plasma

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ABSTRACT

In pig production, the optimization of artificial insemination (AI) efficiency significantly relies on the accurate assessment of semen quality and fertility of boars. Traditional methods such as conventional seminogram techniques, although long-standing, exhibit limited sensitivity in predicting boar fertility, warranting the exploration of novel molecular markers. This review synthesizes the current knowledge on the utilization of molecular markers for semen quality evaluation and male fertility prediction in boars, providing an in-depth examination of molecular markers in this context. Specifically, the present work delves into the potential of OMICs technologies, encompassing genetic and genomic approaches, transcriptomics, proteomics, and metabolomics. A diverse array of molecular markers, including genomic regions associated with sperm quality and male fertility, chromatin integrity, mitochondrial DNA content, mRNA and non-coding RNA signatures, as well as proteins and metabolites in sperm and seminal plasma, are identified as promising molecular markers for fertility prediction in boars. Furthermore, the need of validating biomarkers and their practical implementation in AI centres is here emphasized. Addressing these considerations and integrating molecular markers within the swine breeding field holds the potential to enhance reproductive management practices and optimize productivity in boar breeding programs. This integration can significantly improve overall efficiency within the pig breeding industry.

1. Introduction

The evaluation of semen quality and male fertility is of outmost importance to maximize artificial insemination (AI) in pig production. Assessment of semen quality has traditionally been performed through the conventional seminogram, which evaluates sperm motility, morphology and concentration, as well as the volume of the ejaculate. This assessment has been substantially improved over the time by enabling the comprehensive evaluation of sperm quality and fertility parameters, thus enhancing boar selection and AI management (Jung et al., 2015). The seminogram, however, does not encompass molecular characteristics of sperm cells including DNA integrity, their oxidative status, or the presence of essential sperm proteins (Altmäe and Salumets, 2011), potentially hampering the identification of molecular causes of subfertility. Male infertility is extremely challenging to predict because of its multicausal nature, the complex physiology of sperm cells and the unknown details of how they interact with the female reproductive tract

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(Oehninger and Ombelet, 2019), consequently limiting the potential value of seminogram. Thus, despite the extensive utilisation of the seminogram for pig breeding, its application for the prediction of boar fertility is currently under debate due to its limited sensitivity and accuracy (Altmäe and Salumets, 2011; Kwon et al., 2014; Rodriguez-Martinez, 2013). Hence, the exploration of novel molecular markers with greater sensitivity and specificity for the prediction of boar fertility is of great interest for livestock industry.

OMICs encompass high-throughput measurements of specific molecular groups, allowing the acquisition of extensive datasets that depict the functionality of a particular biological system (Dai and Shen, 2022). OMICs technologies are in constant development, providing a comprehensive characterization of genes, transcripts, proteins and metabolites associated to sperm quality and male infertility (Kovac et al., 2013). Indeed, these advancements facilitate the identification of novel molecular determinants underlying male infertility. Nevertheless, while OMICs methodologies to uncover novel molecular markers of sperm quality and male infertility have been widely utilized in the human reproduction field (Carrell et al., 2016; Llavanera et al., 2022), the exploration of novel molecular markers remains relatively limited in the swine breeding field.

This review presents a comprehensive integration of the current knowledge regarding the utilization of molecular markers for assessing semen quality and fertility in boars. Promising OMICs technologies and specific molecular markers in sperm and seminal plasma are explored and discussed in order to advance the field towards novel predictive strategies to optimize AI in pig production. The utilization of novel molecular markers for semen quality and fertility assessment may offer a new instrument for AI management by facilitating boar selection.

2. Genetic approaches for the evaluation of sperm quality and male fertility

2.1. Quantitative trait loci (QTL)

Due to the advancements in high-throughput DNA sequencing technology and the widespread availability of genetic marker panels, associations between genetic markers and phenotypes can be identified. In this regard, quantitative trait loci (QTL) are specific regions within the genome associated with quantitative complex traits, such as semen quality and fertility. QTLs can be identified through genome-wide association studies (GWAS), thus spotting genomic regions linked to reproductive traits. Xing et al. (2009) described, for the first time, 18 QTLs for traits associated to semen quality and ejaculation time and duration in a White Duroc × Erhualian population. Another study carried out by Diniz et al. (2014) aimed to identify single nucleotide polymorphisms (SNPs) associated with sperm motility, and to determine candidate genes within these QTL regions. Six SNPs on chromosome 1 were found to be associated with sperm motility in Large White-based breeds, suggesting MTFMT as a candidate gene in this region influencing sperm motility. Similarly, using QTL mapping, Gao et al. (2019) identified several candidate genes associated to semen quality parameters, such as sperm count, motility and morphology, in a Duroc population. Accordingly, QTLs are promising molecular markers to select early-aged boars for genetic improvement in terms of semen quality and fertility. This genetic marker-assisted selection approach through QTLs could potentially reduce production costs and enhance efficiency by circumventing the need of rearing pigs until puberty for semen quality assessment. Nevertheless, only few studies with limited sample sizes have evaluated and discovered potential QTLs to predict semen quality in pigs (Diniz et al., 2014; Gao et al., 2019; Xing et al., 2009); thus, the validation of their use as molecular markers in this species is still pending.

2.2. Chromatin integrity of sperm

There is consensus that infertile males exhibit increased chromatin damage compared to fertile ones across mammalian species, with sperm DNA damage significantly impairing fertility outcomes (Kumaresan et al., 2020). A range of methods is utilized to assess sperm chromatin damage, each targeting different types of DNA defects. This diversity in methodologies leads to inconsistencies in the reproducibility of results, especially in farm animals, where large-scale studies are limited (Kumaresan et al., 2020). Recently, few reports shed some light on this matter. Sperm chromatin defects can be evaluated by direct methods, which directly assess DNA damage, or by indirect methods, which determine the amount of DNA damage through the differential chromatin decondensation of fragmented DNA. The most common direct methods for the evaluation of chromatin integrity in sperm are TUNEL and Comet assays, whereas Sperm Chromatin Structure Assay (SCSA) and Sperm Chromatin Dispersion test (SCD) are classified as indirect methods (Dutta et al., 2021; Ribas-Maynou and Benet, 2019). A recent study comparing the aforementioned methods in pigs evidenced a correlation between the proportion of sperm exhibiting fragmented DNA and the degree of chromatin impairment evaluated with direct methods (TUNEL and Comet assay), but not with indirect methods (SCSA and SCD) (Ribas-Maynou et al., 2021c). It is worth mentioning that, contrary to other mammalian species, complete decondensation of chromatin is required for direct methods to show reliable DNA fragmentation levels in species containing protamine P1 only, such as pigs (Ribas-Maynou et al., 2021b, 2021a). These findings agree with the fact that whereas high levels of DNA fragmentation evaluated through the SCSA are not indicative of subfertility in boars (Waberski et al., 2011), the DNA damage evaluated through the Comet assay is not only associated with sperm quality but also with embryo development in pigs (Mateo-Otero et al., 2022). Collectively, these results suggest that direct rather than indirect methods are more suitable to evaluate DNA fragmentation in pigs. Yet, further studies are needed to validate the TUNEL and Comet assays as biomarkers for sperm quality and fertility, as well as to design appropriate tools for implementing this technology in boar studs.

2.3. The mitochondrial genome

Mitochondria contain their own genome known as mitochondrial DNA (mtDNA), which is structured in a circular, double-stranded DNA molecule (Rackham and Filipovska, 2022). These organelles, located within the midpiece of sperm, are known to carry out a range of cellular functions in this cell type, from energy production to calcium homeostasis (Boguenet et al., 2021). Although numerous investigations in humans evidenced a negative association between the content of mtDNA in sperm and their quality parameters (Boguenet et al., 2022; Popova et al., 2021; Shi et al., 2022), few studies have been conducted in farm animals. To date, only two studies delved into the role of mtDNA in sperm as a molecular marker for sperm quality and fertility in pigs. Specifically, Guo et al. (2017) observed that sub-motile sperm exhibited greater content of mtDNA as well as mitochondrial activity compared to motile sperm within the same ejaculate. A different approach was adopted by Llavanera et al. (2024), conducting an inter-ejaculate analysis of mtDNA content. In that study, higher content of mtDNA was found to be associated to greater mitochondrial activity and intracellular ROS levels, and to reduced motility and poorer farrowing and conception rates. Interestingly, both studies converged on the conclusion that sperm cells showing higher content of mtDNA have increased mitochondrial activity and ROS production, and impaired motility and fertility. These findings suggest that high mtDNA content in sperm can indicate a compromised motility and fertilization potential owing to heightened mitochondrial activity and ROS generation. It can thus be suggested that mtDNA content in sperm, assessed by real-time quantitative PCR (RT-qPCR), may be used as a novel molecular marker for evaluating sperm quality and fertility performance in swine.

3. Transcriptomics as a tool for the evaluation of sperm quality and male fertility

Transcriptomics, which is the analysis of RNA expression profiles in cells and tissues, has emerged as a powerful tool for understanding the underlying molecular mechanisms associated with sperm function and fertility outcomes (Wang et al., 2009). Accordingly, transcriptomics opens the door to explore into RNAs as molecular determinants of semen quality and fertility in mammals. Specifically, coding RNA (or messenger RNA; mRNA) and small non-coding RNAs, such as microRNAs (miRNAs), piwi-interacting RNA (piRNAs) or circular RNA (circRNA), may serve as potential molecular markers for assessing sperm quality and fertility performance in pigs, providing information to optimize reproductive management strategies.

The study of Pang et al. (2023) demonstrated that specific mRNAs in sperm were able to predict sperm quality and male fertility. Receiver Operating Characteristic (ROC) curves were used to assess several sperm mRNAs expression for predicting male fertility and sperm motility. Some sperm mRNAs, such as *PRDX4* (AUC of 0.90 and 0.76) and *HSPD1* (AUC of 0.65 and 0.83), showed good predictive values for litter size and total motility, respectively. Similar findings were reported by Pang et al. (2022a), who identified several mRNAs in pig sperm as excellent predictors of male fertility in terms of litter size. *EQTN* (AUC of 0.80), *ZP4* (AUC of 0.90), and *SPACA3* (AUC of 0.80) mRNAs, for example, emerged as promising molecular markers of boar fertility. Additionally, the authors presented multiple mRNA models combining two mRNAs for predicting boar fertility. This combination of individual mRNAs further enhanced sensitivity and specificity in fertility predictions, demonstrating remarkable predictive values for litter size. Other authors identified several mRNAs as relevant fertility markers in boars. Alvarez-Rodriguez et al. (2021) conducted porcine-specific microarrays followed by qPCR validation of sperm mRNAs to identify potential mRNA with predictive value for boar fertility in terms of farrowing rate and litter size. Among others, the authors reported *GPx4* and *ATOX1* mRNA levels in sperm to be highly associated with boar fertility. On the other hand, expression levels of *PSP-I* and *PSP-II* mRNAs in boar sperm showed the potential to predict male fertility in terms of litter size, demonstrating greater accuracy when compared to conventional semen parameters (Kang et al., 2019). While most studies did not carry out ROC curve analysis to assess the potential of mRNA as predictors of male fertility in pigs, Kim et al. (2019) proved that the expression levels of *SLC9A3R1* in sperm exhibited an overall accuracy of 90 % in predicting the fertility of Yorkshire boars. Thus, while further validation is necessary, mRNAs show promise as molecular markers for the diagnosis of boar subfertility.

miRNAs, on the other hand, are short molecules comprising 20–25 nucleotides, which control gene expression by attaching to the 3'-UTR of specific target mRNA (Bartel, 2009). Because sperm are believed to be transcriptionally silent, the presence of miRNAs in these cells and their association with fertility is tentatively attributed to functions related to spermatogenesis rather than sperm physiology. Multiple miRNAs within boar sperm have been recognized as potential fertility predictors. Alvarez-Rodriguez et al. (2020) conducted a porcine-specific microarray analysis enabling the detection of two miRNAs (miR-221 and miR-621) that showed differential abundance in sperm of boars with high and low fertility following AI, as evaluated by farrowing rate and litter size. Similarly, Martinez et al. (2022) conducted a study that pinpointed five previously unidentified miRNAs (miR-10386, miR-10390, miR-6516, miR-9788–1, and miR-9788–2) that showed differential abundance in sperm of boars having high and low fertility after AI. Conversely, additional porcine-focused investigations showed associations between other non-coding RNAs, such as circRNAs, and sperm quality parameters. Gòdia et al. (2020) used RNA-seq coupled with RT-qPCR validation, enabling the identification of two circRNAs (ssc_circ_1458 and ssc_circ_1321) that exhibited significant correlation with sperm motility in pigs. Nonetheless, literature concerning this type of non-coding RNAs remains limited, and their relationship with fertility is largely unexplored.

It is noteworthy that, in addition to the RNAs identified in sperm, some studies have also assessed the potential role of RNAs coupled with exosomes and extracellular vesicles as molecular markers of sperm quality in pigs. The study conducted by Dlamini et al. (2023) unveiled differentially expressed extracellular vesicle-coupled miRNAs in boar seminal plasma between samples of poor and good sperm quality. These miRNAs were found to be associated with spermatogenesis and sperm function. Similarly, Zhao et al. (2024) reported comparable findings, identifying six exosomal miRNAs that were differentially expressed between samples with high sperm motility and those with low motility. These exosomal miRNAs were purported to be involved in biological processes such as regulation of gene expression and signalling pathways. These findings provide evidence of an association between the abundance of miRNAs in

extracellular vesicles and sperm quality in terms of motility and morphology, suggesting their potential utility as molecular markers of boar sperm quality.

In light of the above, RNAs are emerging as promising molecular markers for assessing semen quality and fertility in swine. These

Table 1
Putative protein markers for sperm quality and fertility in swine. The source from where the protein comes from (sperm or seminal plasma), the analytical method (methods), the predicted parameter (parameter), and the inclusion of ROC curve analysis in the study (ROC curve analysis) is specified for each protein.

Protein	Sample	Methods	Parameter	ROC curve analysis	References
Ras-related protein 3 A (RAB3A)	Sperm	ELISA	Litter size and sperm quality	Yes	(Bae et al., 2022b, 2022a)
Ras-related protein 5 (RAB5)	Sperm	ELISA	Litter size and sperm quality	Yes	(Bae et al., 2022b, 2022a)
Ras-related protein 14 (RAB14)	Sperm	ELISA	Litter size and sperm quality	Yes	(Bae et al., 2022b, 2022a)
Ras-related protein 8 A (RAB8A)	Sperm	ELISA	Litter size	Yes	(Bae et al., 2022a)
Ras-related protein 9 (RAB9)	Sperm	ELISA	Litter size	Yes	(Bae et al., 2022a)
Ras-related protein 25 (RAB25)	Sperm	ELISA	Litter size	Yes	(Bae et al., 2022a)
Ras-related protein 2 A (RAB2A)	Sperm	2D SDS-PAGE coupled with ESI-MS/MS, validated with Western blot and ELISA	Litter size	Yes	(Kwon et al., 2017; Kwon et al., 2015)
Ras-related protein 11 (RAB11)	Sperm	ELISA	Sperm quality	No	(Bae et al., 2022b)
Ras-related protein 27 A (RAB27A)	Sperm	ELISA	Sperm quality	No	(Bae et al., 2022b)
Glutathione S-transferase kappa 1 (GSTK1)	Sperm	Western blot	Sperm quality	No	(Zeng et al., 2022)
Glutathione S-transferase Mu 3 (GSTM3)	Sperm	2D SDS-PAGE coupled with LC-MS/MS, validated with Western blot	Litter size	No	(Kwon et al., 2015)
Glutathione peroxidase 4 (GPx4)	Sperm	2D SDS-PAGE coupled with LC-MS/MS, validated with Western blot	Litter size	No	(Kwon et al., 2015)
Peroxiredoxin 4 (PRDX4)	Sperm	Western blot	Litter size	Yes	(Ryu et al., 2021)
Cysteine-Rich Secretory Protein 2 (CRISP2)	Sperm	ELISA	Litter size	No	(Gao et al., 2021)
Heat shock protein family D member 1 (HSPD1)	Sperm	Western blot	Litter size	No	(Pang et al., 2022b)
Ceruloplasmin (CP)	Sperm	LC-MS/MS (iTRAQ), validated with Western blot	Litter size	No	(Zeng et al., 2021)
Carboxypeptidase E (CPE)	Sperm	LC-MS/MS (iTRAQ), validated with Western blot	Litter size	No	(Zeng et al., 2021)
Serine protease inhibitor family A member 12 (SERPINA12)	Sperm	LC-MS/MS (iTRAQ), validated with Western blot	Litter size	No	(Zeng et al., 2021)
Sperm acrosome associated 4 (SPACA4)	Sperm	LC-MS/MS (iTRAQ), validated with Western blot	Farrowing rate	No	(Chen et al., 2020)
IZUMO family member 2 (IZUMO2)	Sperm	LC-MS/MS (iTRAQ), validated with Western blot	Farrowing rate and litter size	No	(Chen et al., 2020)
Triosephosphate isomerase (TPI)	Sperm	Western blot	Sperm quality	No	(Vilagran et al., 2016)
Cytochrome b-c1 complex subunit 1 (UQCRC1)	Sperm	2D SDS-PAGE coupled with ESI-MS/MS, validated with Western blot and ELISA	Litter size	Yes	(Kwon et al., 2015)
Cytochrome b-c1 complex subunit 2 (UQCRC2)	Sperm	2D SDS-PAGE coupled with ESI-MS/MS, validated with Western blot and ELISA	Litter size	Yes	(Kwon et al., 2015)
Arginine vasopressin receptor 2 (AVPR2)	Sperm	2D SDS-PAGE coupled with LC-MS/MS, validated with Western blot	Litter size	No	(Kwon et al., 2015)
15-lipoxygenase (15-LOX)	Sperm	Western blot	Sperm quality	No	(Lovercamp et al., 2007)
Ubiquitin (UBI)	Sperm	Western blot	Sperm quality	No	(Lovercamp et al., 2007)
Paraoxonase 1 (PON1) activity	Seminal plasma	Photometry	Sperm quality and farrowing rate	No	(Barranco et al., 2015; Lucca et al., 2022; Nedić et al., 2023)
Glutathione peroxidase 5 (GPx5)	Seminal plasma	2D SDS-PAGE coupled with LC-MS/MS, validated with Western blot	Farrowing rate and sperm quality	No	(Novak et al., 2010; Vilagran et al., 2016)
Major seminal plasma (PSP-I)	Seminal plasma	2D SDS-PAGE coupled with ESI-Q-TOF	Sperm quality	No	(De Lazari et al., 2019)
Cathepsin B (CTSB)	Seminal plasma	2D SDS-PAGE coupled with ESI-Q-TOF	Sperm quality	No	(De Lazari et al., 2019)
N-acetyl-β-hexosaminidase (β-HEX) activity	Seminal plasma	Photometry	Sperm quality	No	(Wysocki et al., 2015)
Major seminal plasma glycoprotein (PSP-I)	Seminal plasma	2D SDS-PAGE coupled with LC-MS/MS	Litter size and sperm quality	No	(Novak et al., 2010)

molecules, detectable via RT-qPCR, hold potential as novel molecular markers for male fertility within the pig breeding industry. Nevertheless, the implementation of RNAs as molecular markers in swine breeding facilities requires thorough assessment of intra- and inter-individual variability using larger cohorts (Alvarez-Rodriguez et al., 2021), along with validation through the performance of ROC curves. Furthermore, future investigations should explore the combination of diverse RNAs to enhance the overall accuracy and robustness of fertility predictions (Kang et al., 2019).

4. Molecular markers within the proteome of sperm and seminal plasma

The proteome of sperm and seminal plasma has been investigated not only to get a deeper understanding of sperm physiology, but also as a potential source for molecular markers of sperm quality and fertility (Bustamante-Filho et al., 2022). Indeed, specific seminal proteins used as molecular markers in porcine species are undoubtedly the most studied aspect among OMICS approaches. Table 1 shows the most relevant sperm and seminal plasma protein markers in pigs.

The proteome screening of sperm and seminal plasma has unveiled an extensive array of candidate proteins to serve as molecular markers for sperm quality and fertility in the porcine species. While validation of these biomolecules as molecular markers through ROC curves is virtually non-existent in the literature for other types of biomolecules, the case for proteins is different. A range of proteins in sperm (RAB3A, RAB5, RAB14, RAB8A, RAB9, RAB25, RAB2A, PRDX4, UQCRC1, and UQCRC2), but not in seminal plasma, have undergone validation as molecular markers of sperm quality and fertility through ROC curve analysis. In this context, it is worth mentioning that various studies have validated the sperm-specific Ras-related proteins (RAB) as predictive markers for sperm quality and fertility in pigs (Bae et al., 2022a, 2022b; Kwon et al., 2017; Kwon et al., 2015). Regarding the methods used to determine the potential of seminal proteins as markers of sperm quality and male fertility, high-throughput proteomics using tandem mass spectrometry (MS/MS) predominates for screening, whereas enzyme-linked immunosorbent assay (ELISA) and Western blot are used for validation. Validating these proteins as markers through antibody-based antigens detection assays, such as ELISA or Western blot, is crucial for their practical application in AI centers, facilitating their adaptation to other, more practical and cost-effective methods, such as immunochromatographic assays. Despite the significant progress made in the identification of seminal proteins as potential markers of male fertility, much remains to be done for their validation and practical application in AI centers. In this regard, seminal proteins hold promise for predicting boar fertility in a cost-effective manner, thereby enhancing reproductive management practices.

5. Metabolites to predict sperm quality and male fertility

Metabolomics involves the comprehensive analysis of substrates and products of cell metabolism, known as metabolites (Egea et al., 2014). The presence or concentration of specific metabolites could provide insights into the physiological state of the cell. By studying the metabolome using analytical techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, metabolomics contributes to the understanding of biological processes, as well as the discovery of novel molecular markers. The application of metabolomics in livestock reproduction could potentially increase the efficiency in breeding programs (Chakraborty et al., 2022). In the context of boar reproduction, two investigations specifically examined the metabolome of seminal plasma with the dual objective of interrogating how it modulates sperm physiology and identifying novel molecular indicators of semen quality and male fertility. A comparative metabolomic analysis through Ultra-high Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-qTOF-MS) revealed new metabolomic markers in seminal plasma (Zhang et al., 2021). These markers are 4-aminobenzoate, Pro-Asn, Ile-Tyr, homoveratric acid, D-biotin, L-serine, butoxyacetic acid, S-methyl-5'-thioadenosine, capsaicin, and 1-O-(cis-9-octadecenyl)-2-O-acetyl-sn-glycero-3-phosphocholine (PAF), and have been proposed to be associated to boar fertility. Similarly, studies utilizing NMR spectroscopy have been conducted for the same purpose. Notably, levels of glutamate, methanol, trimethylamine N-oxide, carnitine, and isoleucine have been found to correlate with sperm quality (Mateo-Otero et al., 2021b), whereas carnitine, hypotaurine, sn-glycero-3-phosphocholine, glutamate, and glucose have demonstrated discriminatory potential for litter size (Mateo-Otero et al., 2021a). Despite the need for further validation, the assessment of these metabolites in seminal plasma holds promise as a non-invasive approach to predict boar fertility.

6. Separation methods for the assessment of biomarkers in semen

As demonstrated throughout this review, most biomarkers of seminal quality are analyzed solely in one of the components that constitute semen: either sperm or seminal plasma. In this context, it is crucial to use an appropriate separation method to obtain reliable results in predicting the seminal quality of ejaculates and/or the fertility of boars. Various methods have been developed to achieve this separation effectively. Firstly, the standard centrifugation method involves concentrating sperm into a pellet by centrifuging the ejaculate, allowing their separation from the seminal plasma (Carvajal et al., 2004). However, this method can cause sperm damage. To prevent this cellular damage resulting from the centrifugation process, a modified method, called cushioned centrifugation, has been designed. This method employs a dense, inert, and isotonic solution at the bottom of the centrifuge tube to cushion the sperm during centrifugation, thereby reducing cell damage during separation (Matás et al., 2007). Other sperm selection methods include Density Gradient Centrifugation (DGC) and Swim-Up. DGC uses density gradients to separate sperm from seminal plasma, selecting motile and morphologically normal sperm (Noguchi et al., 2015). Swim-Up, on the other hand, allows the most motile sperm to swim up into a new medium, separating them from the seminal plasma (Magdanz et al., 2019). Both methods introduce bias into the sample by selecting motile and morphologically normal sperm, making these them unsuitable for the acquisition of sperm destined for biomarker analysis. Additionally, the use of gradients and media prevents the use of these methods for the separation of seminal

plasma destined for molecular marker analysis, as contact with the media may alter the composition of the seminal plasma and consequently the results. Similarly, filtration-based separation methods have also been developed, which are effective in separating seminal plasma without damaging sperm viability (Bussalleu et al., 2009). These methods show comparable results to centrifugation but with less damage to sperm cells. Filtration methods using Sephadex and other chromatographic resins, however, can improve sperm quality by selecting viable and morphologically normal spermatozoa (Bussalleu et al., 2009; Ramió-Lluch et al., 2009), thus introducing bias in sperm quality while potentially altering the composition of the seminal plasma. Taking all the aforementioned into consideration, it is essential to carefully select the method for biomarker analysis in sperm or seminal plasma, as it is crucial to avoid biases and other alterations in marker analysis. In this regard, standard centrifugation seems to be the most suitable method for analyzing biomarkers in boar ejaculate, as it does not introduce sperm quality bias nor alters the composition of the seminal plasma.

7. Validation and practical application of molecular markers

As discussed throughout the present review, a comprehensive list of molecular markers associated with sperm quality and male fertility has been reported in the pig. Yet, and as previously mentioned, validation of these molecular markers is required before their utilization in AI centers. Validation entails ensuring its adequate performance for its intended use. Biomarker validation is, therefore, of outmost importance prior to its application, as it must ensure that its sensitivity, specificity, and accuracy are appropriate. As reviewed in Ou et al. (2021), biomarker validation should envisage both analytical and clinical angles. Analytical validation involves the evaluation of sensitivity, specificity, accuracy, precision, and reproducibility of the marker's assessment. On the other hand, clinical validation entails verifying the predictive capacity of the marker for the clinical outcome, through the use of large and independent sample sets obtained at different times, and from separate centers and geographical regions. In this regard, as emphasized by Davis et al. (2020), transparency, external validation, generalizability, ease of deployment, and cost-effectiveness are particularly important in biomarker validation. Furthermore, it is crucial to consider the applicability of biomarkers within the context of AI centers. The assessment of molecular markers using methods such as qPCR and RT-qPCR for genes and transcripts, respectively, as well as ELISA and Western blot for proteins, ensures high sensitivity and specificity. Nevertheless, their technical requirements, high cost, and the need for specialized technicians hinder their application as routine tools for the prediction of male fertility in AI centers. In this regard, the development of rapid and cost-effective *in vitro* diagnostic (IVD) kits for boar fertility, such as immunochromatographic assays, is warranted. Although immunochromatographic tests are routinely used for pig disease surveillance (Cui et al., 2008; Wang et al., 2023; Xu et al., 2022), their use for enhancing reproductive management practices has not yet been applied in swine AI centers. A possible reason for not evaluating these molecular markers - despite their potential benefits to enhance reproductive management practices in the pig breeding industry - is that the development and commercialization of IVD kits is expensive and time-consuming (Bustamante-Filho et al., 2022). Indeed, although commercially available IVD kits based on immunochromatography, such as SpermCheck®, have been developed in humans (Coppola et al., 2010; Klotz et al., 2008), the 4MID® kit is, to the best of the author's knowledge, the only ELISA-based commercially available kit for boar fertility (Delehedde, 2019).

8. Conclusions and future perspectives

Advancements in biotechnology coupled to cost-effectiveness of OMICs have facilitated significant strides in biological understanding as well as biomarker discovery and development. While a considerable number of promising molecular markers of diverse origins have been identified for predicting seminal quality and male fertility in swine, substantial progress is needed before these markers can be effectively used in AI centers. First, the integration of molecular markers from distinct chemical nature emerges as pivotal for comprehensive fertility prediction. Each OMICs technology shows distinct advantages and disadvantages for sperm quality and fertility marker analysis. Genomics offers insights into genetic predispositions but may not reflect real-time fertility status. Transcriptomics provides information on gene expression patterns, though it can be influenced by a range of external factors. Proteomics allows for the study of protein abundance and modifications, which are direct effectors of cellular function, yet is often complex and resource-intensive. Metabolomics captures metabolic changes, providing a snapshot of physiological states, but may be less specific in pinpointing fertility-related biomarkers. Considering these aspects in detail and integrating molecular markers from distinct chemical nature will, therefore, have a greater impact on the analysis of fertility markers. Second, robust validation studies, using large sample sets and rigorous statistical analyses, are of outmost importance to ensure the reliability and practical applicability of biomarkers. Finally, the need for the development of cost-effective diagnostic tests tailored for implementation within AI centers, such as those based on immunochromatography, emerges as crucial for facilitating the routine use of biomarker-based fertility assessments in swine breeding. By addressing these considerations, the livestock industry stands to significantly improve reproductive management practices, optimize productivity, and enhance overall efficiency in boar breeding programs.

CRediT authorship contribution statement

Marc Llavanera: Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of Competing Interest

The author declares no competing interests.

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