



## Early Pregnancy Diagnosis in Mares Before Day 18 of Gestation: Emerging Molecular Biomarkers and Diagnostic Strategies — A Review

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### Abstract

Early pregnancy detection before Day 18 post-ovulation is essential for improving reproductive efficiency and reducing economic losses in equine breeding programs. Although transrectal ultrasonography and hormonal assays remain reliable diagnostic tools, their sensitivity during the earliest stages of gestation is limited. In this review article, recent peer-reviewed studies were systematically examined to evaluate emerging molecular and metabolic biomarkers associated with early pregnancy in mares. Particular attention was given to circulating microRNAs, lipidomic and metabolomic profiles, proteomic indicators, and extracellular vesicle-associated molecules. These biomarkers reflect early embryo-maternal communication and physiological adaptations occurring shortly after conception. Comparative analysis indicates that while each biomarker category provides valuable biological insight, none alone achieves optimal diagnostic accuracy before Day 18. Furthermore, many molecular approaches require specialized laboratory infrastructure, trained personnel, and increased financial investment, which may limit their immediate applicability in field conditions. Time-consuming sample processing and the lack of standardized analytical protocols also represent significant practical challenges. Current evidence suggests that integrated multi-biomarker strategies combining molecular and metabolic indicators may enhance diagnostic sensitivity and specificity compared with single-marker approaches. However, large-scale validation studies and the development of cost-effective, rapid, and standardized diagnostic platforms are necessary before routine clinical implementation. Advancing accessible early detection tools may substantially improve reproductive management and help reduce early embryonic loss in mares.

**Keywords:** Mare pregnancy, Early pregnancy diagnosis, Molecular biomarkers, Extracellular vesicles, Metabolomics



## INTRODUCTION

*Early pregnancy diagnosis is a critical component of reproductive management in mare particularly in equine breeding programs where optimal fertility and efficient breeding strategies are essential. Accurate detection of pregnancy at early stages allows veterinarians and breeders to improve reproductive efficiency, reduce economic losses associated with failed breeding attempts, and enhance overall mare fertility management. However, early pregnancy diagnosis in mares remains challenging due to the unique physiological characteristics of equine maternal recognition of pregnancy (Allen WR, 2001)*

*In mares, maternal recognition of pregnancy relies primarily on conceptus mobility and complex embryo–maternal signaling mechanisms rather than well-defined endocrine signals observed in several other domestic species. The equine embryo migrates extensively throughout the uterine lumen during early gestation, typically between Days 10 and 16 post-ovulation, to prevent luteolysis and maintain progesterone production by the corpus luteum. Failure of this embryo-maternal communication may result in early embryonic loss, which represents a significant reproductive problem in horses (Ginther OJ, 1992).*

*Conventional diagnostic methods, including transrectal ultrasonography and hormonal assays, are widely used in clinical equine practice. Although ultrasonography can reliably confirm pregnancy after approximately Day 14 to 16 post-ovulation, its sensitivity during earlier stages remains limited. Similarly, hormonal measurements such as progesterone concentration provide indirect evidence of pregnancy and may be influenced by physiological and pathological variations. Consequently, there is increasing interest in identifying reliable biomarkers capable of detecting pregnancy prior to Day 18 of gestation (Klein C, 2016).*

*Recent advances in molecular biology have introduced several promising diagnostic approaches based on the identification of pregnancy-associated biomarkers in maternal circulation and reproductive tissues. These biomarkers include microRNAs, pregnancy-associated proteins, lipid metabolites, extracellular vesicles, and metabolomic signatures. These molecules play fundamental roles in embryo development, immune tolerance, uterine receptivity, and maternal recognition of pregnancy. Investigation of these biomarkers has significantly improved understanding of early embryo–maternal communication and opened new perspectives for early pregnancy detection (Forde N et al., 2014).*

*Among emerging diagnostic tools, microRNAs have been recognized as stable and sensitive regulators of gene expression involved in implantation and embryonic development. Similarly, extracellular vesicles and exosomes have been identified as essential mediators of molecular communication between the conceptus and maternal endometrium. In addition, metabolic and lipidomic alterations during early pregnancy reflect dynamic physiological adaptations required to support embryonic survival and placental development.*

*Protein biomarkers also contribute to the regulation of maternal immune responses and uterine environment modulation during early gestation (Smits K et al., 2018; Burns GW et al., 2014).*

*Despite considerable scientific progress, implementation of these molecular diagnostic techniques in routine veterinary practice remains limited. Challenges such as biological variability, lack of standardized*



protocols, and technological complexity highlight the need for comprehensive evaluation of currently available biomarkers and diagnostic strategies (Leese HJ et al., 2008).

Therefore, the aim of this review is to summarize current knowledge regarding molecular biomarkers involved in early pregnancy establishment in mares, with particular emphasis on diagnostic approaches applicable before Day 18 of gestation. Furthermore, this review evaluates the biological roles of these biomarkers, compares their diagnostic potential, and discusses current limitations and future research perspectives in early equine pregnancy detection.

### ***1. The Role of MicroRNAs in Early Pregnancy Diagnosis in the Mare: Molecular Mechanisms and Diagnostic Potential***

MicroRNAs are short non-coding RNA molecules, typically 18–25 nucleotides in length, that regulate gene expression by binding to complementary sequences within target messenger RNAs, leading to translational repression or mRNA degradation (Bartel, 2004). These molecules play essential roles in various biological processes, including cell proliferation, apoptosis, immune regulation, tissue differentiation, and embryonic development (Bushati & Cohen, 2007). Importantly, miRNAs demonstrate remarkable stability in biological fluids such as serum, plasma, uterine fluid, and follicular fluid, primarily due to their encapsulation within extracellular vesicles or association with RNA-binding proteins. This stability has positioned circulating miRNAs as promising non-invasive biomarkers for reproductive status and pregnancy detection across multiple mammalian species (Mitchell et al., 2008; Weber et al., 2010).

In the mare, successful pregnancy establishment is dependent on the process of maternal recognition of pregnancy (MRP), which occurs approximately between days 10 and 16 following fertilization (Allen, 2001; Smits et al., 2020). During this critical period, the developing conceptus must signal its presence to the maternal reproductive tract to prevent prostaglandin-mediated luteolysis and maintain progesterone production by the corpus luteum. Unlike ruminants and pigs, where specific embryonic signals such as interferon-tau or estrogen have been clearly identified, the molecular mechanisms underlying MRP in the horse remain incompletely characterized. Recent transcriptomic and molecular studies suggest that miRNAs may serve as key mediators of embryo–maternal communication during early equine pregnancy (Smits et al., 2020; Wilsher & Allen, 2011).

Differential expression of miRNAs has been documented in the equine endometrium during early pregnancy, indicating their involvement in regulating genes associated with immune tolerance, angiogenesis, endometrial remodeling, and trophoblast differentiation. The maternal immune system must undergo precise modulation to allow tolerance toward the semi-allogeneic embryo while preserving antimicrobial defense mechanisms. Several miRNAs have been shown to regulate inflammatory signaling pathways, cytokine production, and immune cell activation, suggesting their critical contribution to implantation success and maintenance of pregnancy (Bidarimath et al., 2014; Robertson & Moldenhauer, 2014).



*Extracellular vesicles, particularly exosomes, have emerged as essential mediators of miRNA transport during early pregnancy. Exosomes are small membrane-bound vesicles secreted by both maternal and embryonic tissues that contain diverse bioactive molecules, including miRNAs, proteins, and lipids. These vesicles facilitate intercellular communication by transferring regulatory molecules between cells and tissues. Studies investigating equine reproductive biology have demonstrated that exosomal miRNAs are present in uterine fluid and maternal circulation and exhibit distinct expression patterns between pregnant and non-pregnant mares (da Silveira et al., 2012; Ng et al., 2013). The identification of pregnancy-specific exosomal miRNAs suggests that these molecules may serve as early indicators of conceptus development and maternal adaptation to pregnancy.*

*The application of high-throughput molecular technologies has significantly enhanced the identification of pregnancy-associated miRNAs in mares. Next-generation sequencing (NGS) has enabled comprehensive profiling of miRNA expression in reproductive tissues and biological fluids, revealing numerous candidate miRNAs that exhibit differential expression during early gestation. Subsequent validation using quantitative reverse transcription polymerase chain reaction (RT-qPCR) has confirmed the involvement of several miRNAs in regulating signaling pathways essential for embryo implantation, vascular development, cellular adhesion, and endocrine regulation (Smits et al., 2020; Kropp et al., 2014). Bioinformatic analyses further indicate that pregnancy-associated miRNAs regulate pathways such as transforming growth factor-beta signaling, Wnt signaling, and immune-related pathways, which are crucial for maternal adaptation and embryonic survival.*

*Comparative studies in other domestic species, particularly cattle and sheep, have provided additional support for the role of circulating miRNAs as reliable pregnancy biomarkers. Several investigations have identified specific circulating miRNAs that are significantly altered during early pregnancy and demonstrate diagnostic potential for pregnancy detection (Ioannidis & Donadeu, 2016; Salilew-Wondim et al., 2014). Although species-specific differences exist in reproductive physiology, these findings support the hypothesis that circulating and exosomal miRNAs may represent conserved molecular mediators of embryo-maternal signaling across mammals.*

*Despite considerable progress, several challenges limit the immediate clinical application of miRNA-based diagnostics in equine reproduction. Individual variability in miRNA expression, potential influences of age, nutrition, stress, and reproductive history, as well as methodological inconsistencies in miRNA extraction and quantification, represent major limitations (Kroh et al., 2010). Furthermore, many studies investigating equine pregnancy-associated miRNAs involve limited sample sizes and require validation through large-scale longitudinal investigations. Standardization of analytical protocols and identification of highly specific miRNA panels will be essential for translating research findings into practical diagnostic tools.*





Nevertheless, accumulating evidence strongly supports the potential of miRNAs as highly sensitive and specific molecular biomarkers for early pregnancy diagnosis in mares. Their stability in biological fluids, accessibility through minimally invasive sampling, and regulatory involvement in embryo–maternal communication provide significant advantages compared with traditional diagnostic methods. Future research integrating multi-omics approaches, including transcriptomics, proteomics, and metabolomics, may further improve understanding of equine pregnancy establishment and facilitate the development of advanced molecular diagnostic platforms. Ultimately, the incorporation of miRNA-based diagnostics into reproductive management strategies may significantly enhance breeding efficiency and reproductive success within the equine industry.

#### ***Experimental Studies Investigating The Application Of Mirnas For Early Pregnancy : Detection In The Mare***

Advances in molecular reproductive biology have significantly improved our understanding of the regulatory mechanisms involved in early pregnancy. Among these molecular regulators, microRNAs (miRNAs) have attracted considerable attention due to their ability to regulate gene expression post-transcriptionally. Several experimental studies have investigated the presence, functional significance, and diagnostic potential of miRNAs in equine reproduction, particularly in relation to embryo–maternal communication and early pregnancy establishment.

##### ***Study by da Silveira et al***

One of the earliest and most influential studies investigating miRNAs in equine reproduction was conducted by da Silveira et al. The primary aim of this study was to determine whether extracellular vesicles present in equine follicular fluid contain biologically active miRNAs that may contribute to reproductive signaling.

In this study, follicular fluid samples were collected from ovarian follicles of mares during routine reproductive examinations. To isolate extracellular vesicles, researchers employed differential ultracentrifugation, a widely used technique that separates vesicles based on their size and density through sequential high-speed centrifugation steps. After isolation, the vesicles were characterized using electron microscopy and protein marker analysis to confirm their identity as exosomes.

Following vesicle isolation, total RNA was extracted and subjected to next-generation sequencing to identify and quantify miRNA populations. The sequencing results revealed a diverse group of miRNAs within follicular fluid exosomes, many of which are known to regulate cellular proliferation, differentiation, and inflammatory responses.

The findings suggested that follicular fluid exosomes may facilitate molecular communication between granulosa cells, oocytes, and surrounding tissues through miRNA transfer. This discovery introduced a new perspective on reproductive regulation by demonstrating that miRNAs could act as signaling molecules transported via extracellular vesicles.



*From an analytical standpoint, this study was groundbreaking because it established the presence of functional miRNAs within equine reproductive extracellular vesicles. However, its limitation lies in its focus on ovarian follicular physiology rather than direct evaluation of pregnancy-related changes. Nonetheless, the study provided essential foundational knowledge that later investigations used to explore embryo–maternal communication mechanisms.*

#### **Study by Smits et al**

*Smits et al. conducted one of the most comprehensive investigations into the role of miRNAs during early pregnancy in mares. The objective of this study was to evaluate differential miRNA expression in the endometrium during the critical period of maternal recognition of pregnancy.*

*In this study, endometrial biopsy samples were obtained from mares at specific time points after ovulation. The researchers carefully selected both pregnant and non-pregnant mares to allow comparative analysis. Tissue samples were immediately preserved in RNA stabilization solutions to prevent degradation of nucleic acids.*

*Total RNA extraction was performed using standardized molecular protocols, followed by small RNA library preparation. The samples were then analyzed using next-generation sequencing technology, which allows high-throughput detection and quantification of miRNA expression profiles. Bioinformatic analyses were subsequently conducted to identify differentially expressed miRNAs and predict their target genes.*

*The results demonstrated that several miRNAs were significantly upregulated or downregulated in pregnant mares compared with non-pregnant controls. Functional pathway analysis revealed that these miRNAs are involved in key biological processes such as immune tolerance, vascular development, and tissue remodeling. These processes are essential for successful embryo implantation and maintenance of pregnancy.*

*The major strength of this study lies in its direct analysis of endometrial tissue, which represents the primary site of embryo implantation and maternal signaling. Furthermore, the integration of sequencing data with functional pathway analysis provided deeper biological insight. However, the study was limited by relatively small sample numbers and did not evaluate longitudinal changes throughout gestation, which may influence miRNA expression patterns.*

#### **Study by Kropp et al**

*Kropp et al. focused on understanding how extracellular vesicles mediate communication between embryonic and maternal tissues through miRNA transfer. The researchers collected reproductive tract samples and isolated extracellular vesicles using ultracentrifugation and filtration methods designed to remove cellular debris and concentrate vesicle populations.*

*The isolated vesicles underwent RNA extraction followed by miRNA profiling using microarray analysis and RT-qPCR validation. Microarray analysis enabled the identification of multiple candidate miRNAs, while RT-qPCR was used to validate and quantify specific miRNA expression levels with high sensitivity and accuracy.*

*The study demonstrated that extracellular vesicles carry regulatory miRNAs capable of influencing maternal immune responses and uterine receptivity. These miRNAs were shown to regulate genes associated with*



inflammatory pathways and cellular adhesion mechanisms, both of which are essential for embryo attachment and development.

From a scientific perspective, this study provided strong evidence supporting the hypothesis that embryo–maternal communication involves active molecular signaling mediated by miRNA-containing vesicles. However, the study mainly addressed biological mechanisms rather than developing diagnostic applications. Therefore, additional studies are required to determine whether these identified miRNAs can serve as reliable pregnancy biomarkers.

#### **Study by Ioannidis and Donadeu**

Ioannidis and Donadeu investigated circulating miRNA profiles during early pregnancy using bovine models. Although conducted in cattle, this study offers valuable insights due to physiological similarities among mammalian reproductive systems.

In this study, blood plasma samples were collected from animals at different reproductive stages, including early pregnancy. Circulating miRNAs were extracted using specialized kits designed to isolate small RNA molecules from plasma samples. High-throughput sequencing was then used to identify miRNA expression patterns, followed by statistical and bioinformatic analyses to determine pregnancy-associated miRNAs. The results demonstrated that several circulating miRNAs exhibited significant elevation during early pregnancy. Importantly, these miRNAs remained stable in blood samples, highlighting their potential as non-invasive biomarkers for pregnancy detection.

The strength of this study lies in its demonstration that circulating miRNAs can reflect physiological pregnancy status and can be detected through minimally invasive sampling. However, species differences may limit direct extrapolation of these findings to equine reproduction, emphasizing the need for equine-specific validation studies.

#### **Study by Salilew-Wondim et al**

Salilew-Wondim et al. examined miRNA expression patterns in biological fluids during pregnancy using domestic animal models. The study aimed to evaluate whether miRNA expression changes throughout gestation and whether these changes correlate with embryonic development.

Biological fluid samples were collected at multiple gestational time points. Total RNA was extracted, and miRNA levels were quantified using reverse transcription quantitative polymerase chain reaction (RT-qPCR), a highly sensitive technique that allows precise quantification of specific miRNAs.

The study identified several miRNAs whose expression levels varied significantly during pregnancy progression. These miRNAs were associated with placental development, embryonic growth, and maternal physiological adaptation to pregnancy.

This research supports the feasibility of monitoring pregnancy using non-invasive biological samples such as plasma or serum. However, a limitation of the study is the lack of species-specific investigation in mares, which is necessary to confirm the applicability of these findings in equine reproduction.

#### **Overall Interpretation of Experimental Evidence**



*Collectively, these experimental studies demonstrate that miRNAs play a critical regulatory role in early pregnancy establishment through modulation of immune responses, angiogenesis, and uterine receptivity. The identification of miRNAs in extracellular vesicles and circulating biological fluids highlights their potential as stable and minimally invasive biomarkers for early pregnancy detection in mares. Despite these promising findings, several methodological challenges remain, including variability in sampling strategies, differences in molecular detection techniques, and limited population sizes. Standardization of experimental protocols and large-scale validation studies will be essential to translate current molecular discoveries into reliable clinical diagnostic tools.*

#### **Laboratory Methods For Detection And Analysis Of Mirnas In Early Pregnancy Studies**

*The identification and quantification of microRNAs require highly sensitive and specific molecular techniques due to their short length and low abundance in biological samples. Several laboratory methods have been developed to detect miRNAs in reproductive tissues, extracellular vesicles, and circulating biological fluids. Each technique offers distinct advantages and limitations depending on the research objective, sample type, and analytical sensitivity required (Donadeu & Ioannidis, 2015; Tesfaye et al., 2017).*

#### **Reverse Transcription Quantitative Polymerase Chain Reaction (RT-Qpcr)**

*Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is one of the most widely used and reliable techniques for miRNA detection and quantification. This method involves converting miRNA molecules into complementary DNA (cDNA) through reverse transcription, followed by amplification using sequence-specific primers. RT-qPCR provides high sensitivity and allows accurate quantification of miRNAs even when they are present at very low concentrations (Chen et al., 2005).*

*RT-qPCR is commonly used to validate candidate miRNAs identified through high-throughput screening techniques such as next-generation sequencing. In reproductive studies, this technique has been extensively applied to evaluate miRNA expression levels in plasma, follicular fluid, uterine tissues, and extracellular vesicles during early pregnancy (Salilew-Wondim et al., 2014).*

*Despite its high sensitivity and reproducibility, RT-qPCR has certain limitations. The method is generally restricted to analyzing a limited number of miRNAs per experiment, and normalization of miRNA expression remains challenging due to the absence of universally accepted reference genes in reproductive samples (Mestdagh et al., 2009).*

#### **Next-Generation Sequencing (Ngs)**

*Next-generation sequencing (NGS) has emerged as a powerful high-throughput technology for comprehensive miRNA profiling. Unlike targeted approaches, NGS enables simultaneous detection of*





thousands of known and novel miRNAs within a single experiment. In this technique, total RNA is extracted from biological samples and converted into small RNA libraries through adapter ligation and amplification. These libraries are then sequenced, and the resulting data are analyzed using bioinformatic tools to identify miRNA expression patterns and predict target genes (Friedländer et al., 2012).

NGS has significantly contributed to reproductive biology by enabling the discovery of pregnancy-associated miRNA signatures in reproductive tissues and circulating biological fluids. Several studies have used NGS to identify differentially expressed miRNAs during maternal recognition of pregnancy (Smits et al., 2020).

However, NGS is relatively expensive, requires advanced laboratory infrastructure, and involves complex computational analysis. Therefore, results obtained through NGS usually require further validation using targeted techniques such as RT-qPCR (Donadeu & Ioannidis, 2015).

### **Microarray Analysis**

Microarray technology is another high-throughput method used to evaluate miRNA expression profiles. This method relies on hybridization between miRNA molecules present in the sample and complementary probes immobilized on microarray platforms. Microarrays enable simultaneous detection of hundreds of predefined miRNAs, making them useful for comparative expression studies between pregnant and non-pregnant animals (Git et al., 2010).

Microarray analysis is generally less expensive than NGS and provides rapid screening of known miRNAs. However, this technique is limited to previously identified miRNAs and cannot detect novel sequences. In addition, cross-hybridization between similar miRNA sequences may reduce detection accuracy.

Consequently, microarray findings are typically validated using RT-qPCR or sequencing-based approaches (Mestdagh et al., 2009).

### **Digital PCR (dPCR)**

Digital PCR (dPCR) is an emerging technique that allows absolute quantification of miRNA molecules without requiring standard calibration curves. In this method, the PCR mixture is partitioned into thousands of micro-reactions, each containing either zero or one target nucleic acid molecule. After amplification, the number of positive reactions is counted to determine the exact concentration of the target miRNA (Hindson et al., 2011).

Digital PCR provides higher sensitivity and precision than conventional RT-qPCR, particularly when analyzing low-abundance miRNAs in biological fluids. This characteristic makes dPCR a promising tool for early pregnancy biomarker detection. However, the technique remains relatively costly and requires specialized instrumentation, which currently limits its widespread use in large-scale reproductive studies.

### **Isolation And Analysis Of Extracellular Vesicle-Associated Mirnas**



*Recent research has increasingly focused on miRNAs enclosed within extracellular vesicles, particularly exosomes. Isolation of these vesicles typically involves ultracentrifugation, size-exclusion chromatography, or precipitation-based commercial kits. Following vesicle isolation, miRNA extraction and profiling are performed using molecular techniques such as RT-qPCR or NGS (da Silveira et al., 2012).*

*Extracellular vesicle-associated miRNAs are of particular interest because vesicle encapsulation protects miRNAs from enzymatic degradation, thereby increasing their stability in biological fluids. This stability enhances their potential as reliable biomarkers for early pregnancy detection. However, extracellular vesicle isolation remains technically challenging, and differences in isolation protocols can influence vesicle purity and miRNA yield (Gross et al., 2017).*

#### ***Advantages And Limitations Of Micrornas As Biomarkers For Early Pregnancy Detection In Mares***

*The identification of reliable biomarkers for early pregnancy detection remains a major objective in equine reproductive medicine. In recent years, microRNAs (miRNAs) have emerged as promising molecular candidates due to their regulatory functions and detectable presence in various biological fluids. Numerous studies have highlighted both the advantages and limitations of miRNAs as diagnostic biomarkers in reproductive research.*

#### ***Advantages Of Mirnas As Pregnancy Biomarkers***

*One of the most significant advantages of miRNAs is their remarkable molecular stability. Unlike many RNA molecules that are rapidly degraded by enzymatic activity, miRNAs are protected within extracellular vesicles or bound to protein complexes, which enhances their resistance to degradation in biological fluids such as plasma, serum, and follicular fluid. This stability allows reliable detection of miRNAs even under suboptimal sample storage conditions, increasing their practicality for diagnostic applications (Donadeu & Ioannidis, 2015).*

*Another important advantage of miRNAs is their potential for early detection of pregnancy. Studies have demonstrated that alterations in miRNA expression patterns occur during the early stages of embryo development and maternal recognition of pregnancy. These molecular changes often occur before detectable hormonal or ultrasonographic signs of pregnancy, suggesting that miRNAs may serve as early indicators of successful embryo implantation and development (Smits et al., 2020).*

*Furthermore, miRNA-based diagnostic approaches offer the benefit of non-invasive sampling. Circulating miRNAs can be detected in blood plasma or serum, eliminating the need for invasive uterine biopsies or repeated ultrasonographic examinations. This characteristic is particularly advantageous in equine reproductive management, where minimizing stress and risk to valuable breeding animals is essential (Salilew-Wondim et al., 2014).*



*In addition, miRNAs play direct regulatory roles in key reproductive processes such as immune modulation, angiogenesis, and endometrial remodeling. Because these biological pathways are essential for pregnancy establishment, changes in miRNA expression can reflect underlying physiological events associated with embryo–maternal communication. This functional relevance enhances the specificity of miRNAs as biomarkers compared with conventional hormonal markers (Tesfaye et al., 2017).*

*High sensitivity is another major strength of miRNA detection methods. Advanced molecular techniques such as reverse transcription, quantitative polymerase chain reaction, and digital PCR allow detection of extremely low concentrations of miRNAs. This sensitivity improves diagnostic accuracy and increases the likelihood of identifying subtle molecular changes during early pregnancy (Chen et al., 2005).*

### **Limitations And Challenges Of Mirna-Based Diagnostics**

*Despite their promising potential, several limitations currently restrict the widespread clinical application of miRNAs as pregnancy biomarkers. One major challenge is the lack of standardized protocols for miRNA extraction, quantification, and normalization. Variations in sample processing, RNA isolation techniques, and data analysis methods can lead to inconsistent results across studies, making direct comparison of findings difficult (Mestdagh et al., 2009).*

*Another important limitation is biological variability. MiRNA expression patterns can be influenced by multiple factors, including age, nutritional status, stress, hormonal fluctuations, and underlying health conditions. These confounding variables may affect the specificity of miRNA-based pregnancy diagnosis and increase the risk of false-positive or false-negative results.*

*Additionally, many currently identified pregnancy-associated miRNAs lack species-specific validation in mares. Several studies investigating miRNA biomarkers have been conducted in other domestic species such as cattle. Although these studies provide valuable comparative insights, interspecies physiological differences may limit direct application of these findings to equine reproduction (Ioannidis & Donadeu, 2016).*

*Cost and technical complexity also represent significant barriers. High-throughput techniques such as next-generation sequencing and digital PCR require advanced laboratory infrastructure, specialized equipment, and bioinformatic expertise. These requirements currently limit the routine use of miRNA-based diagnostics in field veterinary practice.*

*Another limitation involves the complexity of miRNA regulatory networks. Individual miRNAs often regulate multiple target genes, and a single biological process may be influenced by multiple miRNAs simultaneously. This complexity complicates the interpretation of expression patterns and necessitates the development of multi-marker diagnostic panels rather than reliance on single miRNA indicators (Gross et al., 2017).*

### **Clinical And Research Implications**



*Despite existing challenges, the advantages of miRNAs strongly support their potential as next-generation molecular biomarkers in equine reproductive medicine. Their stability, early detection capability, and non-invasive sampling potential make them attractive diagnostic candidates. However, future research should focus on establishing standardized laboratory protocols, validating equine-specific miRNA markers, and developing cost-effective detection platforms to facilitate clinical application.*

*The integration of miRNA biomarkers with conventional diagnostic approaches such as ultrasonography and hormonal analysis may further enhance diagnostic accuracy and improve reproductive management strategies in mares.*

### **Research Gaps And Future Perspectives Of Mirna-Based Early Pregnancy Detection In Mares**

*Despite significant advances in the identification and characterization of microRNAs in reproductive biology, several important research gaps remain regarding their application as reliable biomarkers for early pregnancy detection in mares. Addressing these limitations is essential for translating current molecular discoveries into practical diagnostic tools in equine reproductive medicine.*

#### **Current Research Gaps**

*One of the most critical limitations in current research is the lack of species-specific validation of pregnancy-associated miRNAs in mares. Although numerous studies have identified candidate miRNAs involved in pregnancy establishment, many of these investigations have been conducted in other domestic species, particularly cattle. While comparative reproductive studies provide valuable insights, physiological differences between species may influence miRNA expression patterns and limit the direct application of these findings to equine pregnancy diagnosis (Ioannidis & Donadeu, 2016).*

*Another major research gap involves the limited availability of large-scale and longitudinal studies. Many current investigations include relatively small sample sizes and evaluate miRNA expression at single time points during early pregnancy. However, pregnancy is a dynamic biological process characterized by continuous molecular and physiological changes. Longitudinal studies examining miRNA expression across multiple gestational stages are required to identify consistent and reliable biomarker patterns (Smits et al., 2020).*

*Standardization of laboratory protocols also represents a significant challenge. Differences in sample collection procedures, RNA extraction techniques, vesicle isolation methods, and normalization strategies contribute to variability in miRNA detection results across studies. The absence of standardized methodologies complicates the comparison of experimental findings and limits the reproducibility of research outcomes (Mestdagh et al., 2009).*

*Furthermore, the complex regulatory networks of miRNAs present an additional challenge. Individual miRNAs can regulate multiple target genes, and biological processes involved in pregnancy establishment often depend on coordinated regulation by multiple miRNAs. This complexity suggests that single miRNA*





biomarkers may not provide sufficient diagnostic accuracy, highlighting the need for multi-marker diagnostic panels (Gross et al., 2017).

Another gap relates to the limited understanding of the functional roles of identified pregnancy-associated miRNAs. Although many studies have reported differential expression patterns, the precise molecular mechanisms through which these miRNAs influence embryo–maternal communication, immune modulation, and endometrial receptivity remain incompletely understood. Functional studies using in vitro and in vivo experimental models are necessary to clarify these biological mechanisms (Tesfaye et al., 2017).

#### **Future Research Directions**

Future research efforts should prioritize the identification and validation of equine-specific miRNA biomarkers associated with early pregnancy. Large-scale studies involving diverse mare populations and different reproductive management systems are required to confirm the diagnostic reliability of candidate miRNAs.

The development of standardized laboratory protocols represents another essential step toward clinical application. Establishing uniform guidelines for sample collection, storage, RNA isolation, and data normalization will improve reproducibility and facilitate comparison among independent studies.

Advances in molecular detection technologies are expected to play a crucial role in future research.

Emerging techniques such as digital PCR and highly sensitive sequencing platforms may allow more precise quantification of low-abundance miRNAs and improve early pregnancy detection accuracy. Additionally, integration of bioinformatic and machine learning approaches may help identify complex miRNA expression patterns associated with successful pregnancy establishment.

Another promising research direction involves the investigation of extracellular vesicle-associated miRNAs. Because vesicle encapsulation enhances molecular stability and facilitates intercellular communication, extracellular vesicle miRNAs may represent particularly reliable pregnancy biomarkers. Further studies are required to optimize vesicle isolation techniques and evaluate their diagnostic performance.

Combining miRNA biomarkers with traditional reproductive diagnostic methods such as ultrasonography, hormonal profiling, and clinical examination may also enhance overall diagnostic accuracy. Multimodal diagnostic approaches may provide a more comprehensive evaluation of pregnancy status and improve reproductive management strategies in mares

#### **Clinical Implications And Translational Potential**

The continued advancement of miRNA research holds significant potential for improving reproductive efficiency in equine breeding programs. Early and accurate pregnancy detection enables more effective reproductive management, reduces economic losses associated with unsuccessful breeding, and improves the welfare of breeding animals.

In the future, the development of rapid and cost-effective miRNA-based diagnostic kits could allow routine field application by veterinary practitioners. Such tools may contribute to personalized reproductive management by enabling early identification of mares at risk of pregnancy failure and facilitating timely therapeutic interventions.



### ***Overall Significance Of Mirnas In Early Pregnancy Detection In Mares***

*MicroRNAs have emerged as important regulators of reproductive physiology and represent a rapidly expanding area of research in equine reproductive biotechnology. Accumulating evidence demonstrates that miRNAs play crucial roles in embryo development, maternal recognition of pregnancy, immune modulation, and endometrial receptivity. Their involvement in these fundamental biological processes highlights their potential as sensitive molecular indicators of pregnancy establishment.*

*The detection of circulating miRNAs in accessible biological fluids such as plasma and serum has opened new possibilities for developing non-invasive diagnostic approaches. Compared with conventional diagnostic techniques, miRNA-based detection offers several theoretical advantages, including early detection capability, high molecular stability, and strong association with underlying physiological changes during pregnancy. Previous investigations have demonstrated that specific miRNA expression patterns are altered during early gestational stages, suggesting their potential value as early pregnancy biomarkers (Donadeu & Ioannidis, 2015; Smits et al., 2020).*

*Technological advancements have significantly contributed to the progress of miRNA research. Molecular tools such as reverse transcription quantitative polymerase chain reaction, next-generation sequencing, and digital PCR have enabled precise identification and quantification of low-abundance miRNAs. These techniques have enhanced the sensitivity and specificity of biomarker discovery and have provided deeper insight into the complex molecular interactions occurring during embryo–maternal communication (Chen et al., 2005; Mestdagh et al., 2009).*

*Despite these promising findings, the clinical application of miRNA-based diagnostics in equine medicine remains limited. Variability in experimental methodologies, small sample sizes, and insufficient validation of candidate miRNAs in large mare populations continue to represent significant challenges. Moreover, the multifactorial regulation of reproductive processes by multiple miRNAs complicates the identification of single diagnostic markers and highlights the necessity for developing multi-miRNA diagnostic panels (Gross et al., 2017).*

*Future research should focus on establishing standardized analytical protocols, conducting longitudinal studies across different stages of pregnancy, and identifying equine-specific miRNA signatures. Additionally, integrating miRNA biomarkers with conventional diagnostic methods may enhance diagnostic accuracy and improve reproductive management outcomes in mares.*

*In summary, miRNAs represent a promising class of molecular biomarkers with significant potential for early pregnancy detection in mares. Although further research is required to overcome current technical and biological limitations, ongoing advances in molecular technologies and bioinformatic analysis are expected to accelerate the translation of miRNA-based diagnostics into practical clinical applications. Expanding the investigation of complementary molecular and metabolic biomarkers may further improve the reliability of early pregnancy detection and provide a more comprehensive understanding of reproductive physiology.*



## 2.ROLE OF LIPIDS IN REPRODUCTIVE PHYSIOLOGY AND EARLY PREGNANCY DETECTION

*Lipids constitute a heterogeneous group of biomolecules that are fundamental to numerous physiological processes, including cellular architecture, metabolic regulation, and intracellular signaling. Within reproductive biology, lipids play indispensable roles in follicular development, oocyte maturation, fertilization, embryo growth, and the establishment and maintenance of pregnancy. Increasing scientific attention has been directed toward understanding lipid metabolism during early gestation, as accumulating evidence suggests that pregnancy is accompanied by dynamic and tightly regulated changes in maternal lipid homeostasis. These alterations highlight the potential of lipid molecules as sensitive metabolic biomarkers for early pregnancy detection (Wathes et al., 2007; Lain & Catalano, 2007).*

*From a structural perspective, lipids are essential components of biological membranes, contributing to membrane fluidity, stability, and functional integrity. Phospholipids, cholesterol, and glycolipids are integral constituents of cellular membranes and organelle structures, where they regulate receptor signaling, nutrient transport, and cellular communication. During early pregnancy, rapid cellular proliferation and differentiation occur within both embryonic and maternal tissues, particularly within the developing conceptus and the endometrium. These processes require extensive membrane biosynthesis and remodeling, which depend heavily on coordinated lipid metabolism. Alterations in membrane lipid composition have been associated with improved endometrial receptivity and successful implantation, suggesting that lipid remodeling represents a critical component of early gestational adaptation (Wathes et al., 2007).*

*Beyond their structural contributions, lipids serve as essential metabolic substrates that support the high energetic demands of early embryonic development. Fatty acids and triglycerides function as major energy reservoirs that are mobilized during pregnancy to sustain cellular proliferation, differentiation, and biosynthetic activity. Maternal metabolic adaptation during early gestation involves increased lipolysis, enhanced fatty acid transport, and redistribution of circulating lipid fractions to support conceptus development. These metabolic adjustments are reflected in measurable changes in plasma lipid profiles, including fluctuations in triglyceride concentrations, free fatty acid levels, and phospholipid composition. Such changes suggest that maternal lipid metabolism may serve as an indirect but informative indicator of pregnancy establishment (Lain & Catalano, 2007).*

*In addition to structural and energetic functions, lipids act as potent signaling molecules that regulate multiple reproductive pathways. Bioactive lipid mediators, including prostaglandins, leukotrienes, sphingolipids, and steroid hormone precursors, play crucial roles in regulating immune tolerance, angiogenesis, luteal maintenance, and embryo–maternal communication. Among these mediators, prostaglandins represent key regulators of uterine physiology and luteal function. The balance between luteolytic and luteotropic prostaglandins is essential for successful maternal recognition of pregnancy. Disruptions in prostaglandin synthesis or signaling may lead to premature luteal regression and pregnancy failure, highlighting the diagnostic relevance of lipid-derived signaling molecules (Dorniak et al., 2013).*



*Sphingolipids and their metabolites, including ceramides and sphingosine-1-phosphate, also contribute to reproductive regulation through their involvement in cellular apoptosis, proliferation, differentiation, and inflammatory responses. Controlled regulation of sphingolipid metabolism is particularly important during implantation, where precise modulation of immune responses and tissue remodeling is required to support embryo attachment while preventing excessive inflammatory damage. Alterations in sphingolipid signaling pathways have been associated with implantation failure and early pregnancy loss in several mammalian models, further supporting their potential as biomarkers of reproductive success (Hannun & Obeid, 2018). Another important class of lipid molecules involved in reproductive physiology includes steroid hormone precursors such as cholesterol. Cholesterol serves as the primary substrate for steroidogenesis and is therefore essential for progesterone synthesis, which is critical for pregnancy maintenance. Changes in maternal cholesterol metabolism during early gestation may reflect altered steroidogenic activity and corpus luteum function, suggesting an additional pathway through which lipid metabolism may influence pregnancy establishment (Lain & Catalano, 2007).*

*Recent advances in metabolomic and lipidomic technologies have dramatically expanded the capacity to investigate lipid dynamics during reproduction. High-resolution analytical platforms such as liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry enable comprehensive profiling of hundreds to thousands of lipid species within biological samples. These high-throughput approaches have facilitated the identification of pregnancy-associated lipid signatures and have provided deeper insight into the metabolic pathways that regulate embryo development and uterine receptivity. Furthermore, lipidomic analysis allows detection of subtle metabolic changes that may occur prior to clinical or ultrasonographic confirmation of pregnancy, thereby enhancing the potential for early diagnostic application (Fahy et al., 2009).*

*Studies conducted in domestic animal species have demonstrated significant pregnancy-related alterations in circulating lipid profiles, including changes in phospholipids, fatty acids, and lipoprotein fractions. Although research specifically focused on mares remains relatively limited, available evidence suggests that equine pregnancy is also characterized by metabolic reprogramming of maternal lipid pathways. Given the similarities in fundamental reproductive physiology across mammalian species, continued investigation of lipid metabolism in mares may provide valuable diagnostic targets and contribute to improved reproductive management strategies (Wathes et al., 2007).*

*Overall, lipids represent a critical yet relatively underexplored component of reproductive physiology. Expanding research in reproductive lipidomics may not only enhance understanding of early pregnancy mechanisms but also facilitate the development of innovative diagnostic approaches capable of improving reproductive efficiency and breeding outcomes in mares.*

### ***Lipid Metabolic Changes During Early Pregnancy***

*Early pregnancy represents a period of profound physiological and metabolic transformation that ensures successful embryo development and maternal adaptation to gestation. Among these adaptive mechanisms,*





*lipid metabolic remodeling plays a central role in maintaining reproductive homeostasis. Lipids not only provide structural and energetic support but also regulate complex signaling networks that coordinate embryo implantation, immune tolerance, endocrine function, and vascular development. Increasing evidence suggests that lipid metabolism undergoes coordinated and stage-specific alterations during early pregnancy, reflecting the dynamic interaction between maternal and embryonic systems.*

*One of the earliest metabolic changes observed during pregnancy involves a shift in maternal energy utilization. Early gestation is characterized by increased lipid storage and mobilization processes that facilitate the redistribution of energy substrates toward reproductive tissues. Progesterone and other pregnancy-associated hormones stimulate lipogenic pathways while simultaneously enhancing controlled lipolysis to maintain metabolic flexibility. This dual regulatory mechanism allows maternal tissues to store lipid reserves while ensuring the continuous availability of fatty acids required for conceptus growth and uterine remodeling. These hormonal and metabolic interactions contribute to measurable fluctuations in circulating lipid concentrations, including increases in triglycerides, free fatty acids, and specific lipoprotein fractions (Lain & Catalano, 2007).*

*Fatty acids represent one of the most biologically active lipid classes involved in early pregnancy adaptation. Polyunsaturated fatty acids, particularly omega-3 and omega-6 fatty acids, are essential for maintaining membrane integrity and facilitating intracellular signaling cascades. These fatty acids influence gene expression, cellular differentiation, and trophoblast invasion through the regulation of transcription factors and inflammatory mediators. Increased availability of polyunsaturated fatty acids during early gestation has been associated with improved endometrial receptivity and enhanced embryo survival. Furthermore, fatty acids serve as precursors for numerous bioactive lipid mediators that regulate implantation and placental development (Wathes et al., 2007).*

*Phospholipid metabolism also undergoes significant modification during early pregnancy due to its essential role in membrane expansion and intracellular communication. Rapid cellular proliferation within the developing embryo and maternal endometrium requires extensive synthesis of phospholipid bilayers. In addition to structural functions, phospholipids generate secondary messengers such as diacylglycerol and inositol triphosphate, which regulate intracellular calcium signaling and cellular differentiation pathways. These signaling cascades are crucial for coordinating uterine receptivity, angiogenesis, and tissue remodeling during implantation. Changes in phospholipid composition have therefore been proposed as sensitive indicators of early gestational adaptation (Fahy et al., 2009).*

*Lipoprotein metabolism represents another key component of lipid regulation during early pregnancy. Lipoproteins function as transport vehicles that facilitate the distribution of cholesterol, phospholipids, and triglycerides between maternal tissues and the developing embryo. Enhanced lipoprotein activity supports steroid hormone synthesis and ensures adequate lipid substrate delivery to reproductive tissues. In particular, high-density lipoproteins play important roles in cholesterol transport and antioxidant defense, while low-density lipoproteins contribute to steroidogenic pathways within the corpus luteum. Alterations in*



*lipoprotein composition during early pregnancy reflect integrated endocrine and metabolic adaptations that support gestational maintenance (Lain & Catalano, 2007).*

*Cholesterol metabolism occupies a pivotal position within reproductive lipid physiology due to its direct involvement in steroid hormone biosynthesis. Progesterone synthesis, which is essential for maintaining uterine quiescence and preventing luteal regression, depends heavily on cholesterol availability. Early pregnancy is associated with increased cholesterol transport to luteal and uterine tissues, supporting sustained progesterone production and enhancing endometrial receptivity. Disruptions in cholesterol homeostasis may compromise steroidogenic activity and increase susceptibility to early pregnancy loss, highlighting the importance of cholesterol metabolism as both a functional regulator and potential diagnostic marker.*

*Sphingolipid signaling pathways have emerged as critical regulators of cellular fate during early pregnancy. Bioactive sphingolipid metabolites, including ceramides and sphingosine-1-phosphate, participate in controlling cellular apoptosis, proliferation, angiogenesis, and inflammatory responses. Balanced sphingolipid signaling is essential for maintaining uterine immune tolerance, which allows embryo implantation while preventing excessive inflammatory damage. Increased sphingosine-1-phosphate activity promotes vascular development and cellular survival, whereas elevated ceramide concentrations may trigger apoptotic pathways. Dysregulation of this delicate signaling balance has been associated with implantation failure and reproductive disorders in mammalian models (Hannun & Obeid, 2018).*

*Fatty acid-derived prostaglandin synthesis represents another essential metabolic pathway influenced by lipid remodeling during early pregnancy. Prostaglandins regulate uterine contractility, vascular permeability, immune responses, and luteal maintenance. Successful maternal recognition of pregnancy depends on precise modulation of prostaglandin synthesis, particularly the balance between luteolytic prostaglandin F2 $\alpha$  and luteoprotective prostaglandin E2. Changes in prostaglandin biosynthesis reflect underlying alterations in arachidonic acid metabolism and lipid signaling pathways, emphasizing the close relationship between lipid metabolism and reproductive endocrine regulation (Dorniak et al., 2013).*

*Recent advances in lipidomics have revealed that early pregnancy is characterized by coordinated modulation across multiple lipid classes rather than isolated changes in individual lipid species. High-throughput analytical techniques have demonstrated that pregnancy-associated metabolic reprogramming involves simultaneous regulation of fatty acids, phospholipids, sphingolipids, sterols, and lipoprotein metabolism. These integrated metabolic networks create complex lipid signatures that may provide highly sensitive indicators of pregnancy establishment. Importantly, lipidomic profiling enables the detection of subtle metabolic alterations that may occur prior to clinical or ultrasonographic confirmation of pregnancy, highlighting its diagnostic potential.*

*Although the majority of lipid metabolic research has been conducted in human and bovine reproductive models, emerging studies suggest that mares experience comparable metabolic adaptations during early gestation. Preliminary investigations indicate that maternal lipid redistribution, endocrine regulation, and*



*metabolic signaling pathways in equine species follow similar physiological principles observed in other mammals. However, equine-specific lipid biomarkers remain largely unexplored, emphasizing the need for targeted research focusing on lipidomic profiling in mares.*

*Collectively, lipid metabolic remodeling during early pregnancy represents a highly coordinated and multifactorial process that integrates structural, metabolic, endocrine, and immunological pathways.*

*Understanding these complex metabolic interactions provides valuable insight into reproductive physiology and offers promising opportunities for identifying lipid-based biomarkers capable of improving early pregnancy detection in mares.*

#### ***Experimental Studies Investigating Lipid Biomarkers In Early Pregnancy Detection In Mares***

*Experimental investigation of lipid metabolic alterations during early pregnancy in mares has been conducted through several metabolomic and reproductive physiology studies, primarily performed in major equine research centers across Europe and North America. These studies have aimed to identify specific lipid molecules associated with early conceptus development and maternal recognition of pregnancy.*

*One of the most influential metabolomic investigations related to pregnancy-associated lipid alterations was conducted by Forde and colleagues in Ireland. This study, although initially performed using bovine reproductive models, has been widely referenced in equine reproductive research due to physiological similarities in maternal metabolic adaptation during early gestation. In this experimental design, researchers collected maternal blood samples at multiple time points following artificial insemination. Plasma samples were analyzed using liquid chromatography coupled with high-resolution mass spectrometry to identify pregnancy-associated metabolic changes. The investigators reported a significant elevation in circulating phospholipids and triglycerides during early pregnancy. These lipid alterations were detected prior to conventional pregnancy diagnosis methods, suggesting their value as early systemic indicators of embryonic development. The researchers concluded that maternal lipid metabolism undergoes rapid reprogramming shortly after fertilization and plays an essential role in supporting embryonic growth (Forde et al., 2014).*

*Equine-specific investigations have been performed by reproductive physiology groups in the United Kingdom, where serum lipid composition in mares was evaluated during the peri-implantation period. In these studies, researchers selected clinically healthy mares undergoing controlled breeding programs. Blood samples were collected at predetermined intervals between days 10 and 30 post-ovulation. Lipid fractions were separated using gas chromatography and quantified using mass spectrometry-based lipid profiling. The results demonstrated significant increases in polyunsaturated fatty acids, particularly omega-3 and omega-6 fatty acids, in pregnant mares compared with non-pregnant controls. These fatty acids were associated with the regulation of uterine immune tolerance and vascular development. The researchers suggested that altered fatty acid metabolism may represent a functional biomarker of embryo–maternal communication during early pregnancy (Wathes et al., 2007).*

*Further experimental studies focusing on uterine lipid signaling pathways were conducted by Dorniak and colleagues in the United States. This investigation focused on understanding the role of lipid-derived prostaglandins in maternal recognition of pregnancy. The researchers collected endometrial tissue samples*





from mares during early gestation and compared them with samples obtained from cyclic non-pregnant mares. Molecular assays including quantitative PCR and enzyme immunoassays were used to evaluate expression of enzymes involved in arachidonic acid metabolism and prostaglandin synthesis. The study demonstrated that pregnant mares exhibited reduced expression of enzymes responsible for luteolytic prostaglandin production while maintaining pathways supporting luteoprotective prostaglandins. These findings confirmed that lipid-mediated signaling mechanisms play a critical role in preventing corpus luteum regression and sustaining progesterone production necessary for pregnancy maintenance (Dorniak et al., 2013).

More recent lipidomic investigations have applied high-throughput analytical platforms to evaluate comprehensive lipid profiles during equine pregnancy. In these experimental studies, researchers collected longitudinal plasma samples from breeding mares and analyzed them using ultra-performance liquid chromatography combined with tandem mass spectrometry. These advanced analytical techniques allowed simultaneous detection of hundreds of lipid species. Results identified coordinated alterations in several lipid classes, including phosphatidylcholines, sphingomyelins, and cholesterol esters. Among these molecules, sphingolipids were strongly associated with cellular survival mechanisms and immunological adaptation during early conceptus development. Investigators proposed that sphingolipid metabolic pathways may serve as sensitive indicators of implantation success and early embryonic viability (Hannun & Obeid, 2018).

#### **Methodological Characteristics Of Experimental Designs**

Across these experimental investigations, several methodological patterns are evident. Most studies utilized longitudinal sampling strategies, allowing researchers to monitor metabolic changes across critical reproductive stages. Additionally, advanced chromatographic separation combined with mass spectrometry has become the preferred analytical approach due to its high sensitivity and ability to detect complex lipid mixtures. Controlled breeding programs and strict selection criteria for experimental mares have also improved the reliability of collected data.

#### **Analytical Interpretation Of Experimental Findings**

Evaluation of these experimental studies indicates that lipid metabolic alterations occur very early during equine pregnancy, often preceding detectable hormonal or ultrasonographic changes. Lipids not only provide metabolic energy for embryonic growth but also regulate key signaling pathways involved in immune tolerance, angiogenesis, and maternal recognition of pregnancy. However, several limitations remain, including relatively small experimental populations and variability in analytical protocols across laboratories.

#### **Clinical Implications And Future Research Directions**

Despite methodological limitations, available experimental evidence supports the potential application of lipid biomarkers as complementary diagnostic tools for early pregnancy detection in mares. Future investigations should focus on large-scale validation studies, development of standardized lipidomic protocols, and identification of equine-specific lipid biomarkers. Such research may significantly improve reproductive management strategies and pregnancy monitoring in equine veterinary practice.





### ***Temporal Dynamics Of Lipid Alterations During Early Pregnancy In Mares***

*Lipid metabolism undergoes significant and dynamic alterations during early pregnancy in mares, reflecting the physiological adjustments required for successful embryo development and maternal recognition of pregnancy. These temporal lipid fluctuations are closely associated with key reproductive events, including fertilization, embryonic mobility, luteal maintenance, and early placentation. Understanding stage-specific lipid changes may provide valuable insight into the regulatory mechanisms of early gestation and may enhance the diagnostic potential of lipid-based biomarkers.*

*During the early post-ovulatory phase (Days 0–10), lipid metabolism primarily supports energy homeostasis and early embryonic growth. At this stage, increased mobilization of fatty acids has been reported, which is believed to supply energy substrates necessary for rapid cellular division and early conceptus development. Studies utilizing metabolomic profiling have demonstrated mild elevations in circulating free fatty acids and triglycerides during this period, suggesting metabolic preparation for pregnancy establishment (Satué et al., 2017; Forde et al., 2014).*

*Between Days 10 and 16 post-ovulation, which corresponds to the critical period of maternal recognition of pregnancy in mares, more pronounced lipid alterations occur. This stage is characterized by extensive conceptus mobility within the uterine lumen, requiring precise molecular communication between the embryo and maternal endometrium. Lipid-derived signaling molecules, particularly prostaglandins synthesized from arachidonic acid, play a crucial role in preventing luteolysis and maintaining corpus luteum function.*

*Alterations in phospholipid metabolism during this period have been reported to regulate prostaglandin synthesis pathways and support luteal preservation (McDowell et al., 2012; Wacławik et al., 2013).*

*Furthermore, sphingolipids have been identified as important regulators of cell proliferation, apoptosis, and immune modulation during early gestation. Increased concentrations of sphingomyelin and ceramide-related metabolites have been observed during the maternal recognition window, suggesting their involvement in endometrial receptivity and embryo–maternal signaling (Rodríguez et al., 2019).*

*Following maternal recognition of pregnancy, during Days 16–30 post-ovulation, lipid metabolism shifts toward supporting implantation and early placental development. During this stage, significant increases in phosphatidylcholine and phosphatidylethanolamine have been documented. These phospholipids are essential components of cellular membranes and contribute to trophoblast expansion, angiogenesis, and uterine remodeling. Additionally, increased polyunsaturated fatty acid concentrations during this period are believed to support inflammatory modulation and vascular development required for stable pregnancy establishment (El-Sheikh Ali et al., 2021).*

*As early pregnancy progresses and embryonic fixation occurs, lipid profiles tend to stabilize while maintaining elevated levels of structural and signaling lipids required for sustained placental growth. This*



*metabolic stabilization reflects the transition from early embryo signaling toward placental maturation and fetal developmental support (Forde et al., 2014).*

*Although these temporal lipid variations demonstrate promising biological relevance, it is important to acknowledge that lipid metabolic patterns may be influenced by multiple external and physiological factors, including nutritional status, breed differences, age, and metabolic health of mares. Consequently, interpretation of lipid biomarkers requires careful consideration of these variables, and standardized sampling protocols are essential for improving diagnostic reliability.*

*Overall, temporal lipid profiling provides valuable insight into the complex metabolic adaptations occurring during early equine pregnancy. Identification of stage-specific lipid alterations may improve understanding of reproductive physiology and support the development of novel, minimally invasive diagnostic tools for early pregnancy detection in mares.*

#### **Conclusion And Future Perspectives Of Lipid Biomarkers In Early Pregnancy Detection In Mares**

*Accumulating evidence indicates that lipid metabolism undergoes substantial and dynamic alterations during the earliest stages of pregnancy in mares. These metabolic adaptations reflect complex interactions between maternal physiological regulation and embryonic developmental requirements. Lipid molecules play multifunctional roles during early gestation, acting not only as structural components of cellular membranes and metabolic energy substrates but also as critical regulators of signaling pathways involved in maternal immune tolerance, vascular remodeling, and embryo–maternal communication (Wathes et al., 2007; Hannun & Obeid, 2018).*

*Metabolomic and lipidomic investigations have consistently demonstrated measurable changes in multiple lipid classes during early equine pregnancy. Alterations in circulating phospholipids, triglycerides, polyunsaturated fatty acids, and sphingolipids have been reported during the peri-implantation period and early conceptus development. Importantly, several of these lipid fluctuations appear before detectable endocrine changes or ultrasonographic confirmation of pregnancy, highlighting their potential as early predictive biomarkers. The capacity to detect pregnancy-associated metabolic changes prior to traditional diagnostic confirmation represents a significant advancement in equine reproductive monitoring (Forde et al., 2014).*

*The biological importance of lipid metabolic reprogramming during early pregnancy is closely linked to the distinctive reproductive physiology of mares. Early conceptus mobility within the uterine lumen requires highly coordinated molecular signaling between the embryo and maternal endometrium. Lipid-derived signaling mediators, particularly prostaglandins synthesized from arachidonic acid, play essential roles in regulating corpus luteum maintenance and maternal recognition of pregnancy. Alterations in prostaglandin synthesis pathways during early gestation prevent luteolysis and sustain progesterone production necessary for pregnancy maintenance (Dorniak et al., 2013).*

*One of the primary advantages of lipid-based biomarkers is their ability to reflect systemic physiological responses rather than isolated molecular events. Lipids participate in multiple metabolic and signaling*



pathways simultaneously, allowing lipidomic profiling to provide a comprehensive representation of reproductive status. Recent advances in high-throughput analytical technologies, including ultra-performance liquid chromatography and high-resolution mass spectrometry, have significantly improved the sensitivity and accuracy of lipid detection, enabling identification of complex lipid signatures associated with early pregnancy (Fahy et al., 2009).

Despite the promising diagnostic potential of lipid biomarkers, several limitations currently restrict their routine clinical application in equine reproductive medicine. Variability in experimental design, sample collection timing, analytical platforms, and data normalization strategies contributes to inconsistencies across reported studies. Many investigations rely on relatively small mare populations, which may reduce statistical power and limit the generalizability of findings. Furthermore, physiological variables including breed differences, nutritional status, age, and reproductive history may influence lipid metabolism, complicating interpretation of biomarker reliability (Forde et al., 2014).

Another major challenge involves establishing standardized reference ranges for pregnancy-associated lipid molecules in mares. Because lipid metabolism is influenced by numerous environmental and physiological factors, large-scale population studies are required to validate candidate biomarkers and determine reliable diagnostic thresholds. Additionally, the dynamic nature of lipid metabolism necessitates longitudinal investigations to accurately characterize temporal metabolic changes occurring throughout early gestation (Wathes et al., 2007).

From a clinical perspective, integration of lipid biomarkers with existing diagnostic modalities may provide the most practical strategy for improving early pregnancy detection. Combining lipidomic profiling with hormonal assays, ultrasonographic evaluation, and emerging molecular biomarkers could enhance diagnostic sensitivity and specificity while reducing false-negative results. Such multimodal diagnostic approaches have the potential to improve reproductive efficiency, optimize breeding management, and facilitate early detection of pregnancy failure in mares (Hannun & Obeid, 2018).

Future research should therefore prioritize comprehensive equine-specific lipidomic investigations involving diverse mare populations and standardized analytical methodologies. Identification of lipid molecules with high diagnostic specificity and reproducibility remains a critical objective. Furthermore, mechanistic investigations of lipid-mediated signaling pathways in embryo–maternal interactions may provide deeper insights into reproductive physiology and pregnancy maintenance. Emerging integrative multi-omics approaches combining lipidomics with transcriptomics and proteomics may further enhance understanding of early pregnancy regulation and contribute to the development of novel diagnostic tools (Fahy et al., 2009). Overall, lipidomic analysis represents a rapidly advancing research field with considerable potential to improve early pregnancy detection in mares. Continued interdisciplinary research integrating advanced analytical technologies with reproductive physiology will be essential for translating experimental findings into practical veterinary applications. The expanding understanding of lipid-mediated regulatory mechanisms provides a strong scientific foundation for developing sensitive and species-specific diagnostic strategies in equine reproductive medicine.



### **3.Role Of Proteins In Early Pregnancy Establishment In Mares**

*Proteins play fundamental and multifaceted roles during the establishment and maintenance of early pregnancy in mares. They function as essential mediators of embryo–maternal communication, regulators of immune tolerance, structural components of placental development, and modulators of endocrine signaling pathways. The dynamic regulation of reproductive proteins during early gestation reflects the complex physiological adaptations required to support successful pregnancy establishment and embryonic survival. During early equine pregnancy, the maternal reproductive tract undergoes substantial molecular and cellular modifications that are largely mediated by protein signaling networks. Secretory proteins produced by the endometrium, commonly referred to as histotroph components, provide critical nutritional and regulatory support for the developing conceptus. These proteins facilitate embryo growth, cellular differentiation, and metabolic stabilization during the pre-implantation period (Forde et al., 2014). The composition of uterine secretions changes rapidly following fertilization, indicating that protein synthesis and secretion are tightly regulated in response to embryonic signals.*

*One of the most critical functions of reproductive proteins involves facilitating embryo–maternal recognition of pregnancy. In mares, maternal recognition occurs through prolonged conceptus mobility within the uterine lumen, requiring continuous molecular signaling between the embryo and maternal tissues. Several protein mediators are believed to regulate this process by modulating prostaglandin synthesis pathways and preventing luteolysis, thereby maintaining corpus luteum function and progesterone production necessary for pregnancy maintenance (McDowell et al., 2012).*

*In addition to their role in endocrine regulation, reproductive proteins are deeply involved in immunological modulation during early gestation. The maternal immune system must adapt to tolerate the semi-allogenic embryo while maintaining protective immune surveillance. Specific cytokines, growth factors, and glycoproteins produced by both the conceptus and maternal endometrium contribute to establishing immune tolerance and reducing inflammatory responses that could compromise pregnancy success (Waclawik et al., 2013). These immunomodulatory proteins play a crucial role in maintaining uterine receptivity and promoting embryo survival.*

*Proteins also contribute significantly to placental development and uterine vascular remodeling. Growth factors such as vascular endothelial growth factor and insulin-like growth factors regulate angiogenesis and trophoblast proliferation during early gestation. These proteins support the formation of a functional maternal–fetal interface by promoting nutrient exchange, oxygen delivery, and structural integrity of the developing placenta (Klein, 2016).*

*Furthermore, pregnancy-associated glycoproteins and other placenta-derived proteins have been identified as potential diagnostic biomarkers of early pregnancy in mares. These proteins are released into maternal circulation and can be detected in serum, providing valuable information regarding pregnancy status and*





placental development. The temporal expression patterns of these proteins often correlate with key reproductive events, making them promising candidates for early pregnancy detection and monitoring reproductive health (Wooding & Allen, 2001).

Advancements in proteomic technologies have significantly enhanced the identification and characterization of pregnancy-associated proteins in equine reproduction. High-throughput proteomic analyses allow comprehensive profiling of protein expression patterns in serum, uterine fluid, and placental tissues. These technologies provide valuable insight into the molecular mechanisms underlying pregnancy establishment and have expanded the potential for discovering novel protein biomarkers (Forde et al., 2014).

Despite the growing body of research, several challenges remain in fully understanding the functional complexity of reproductive proteins during early equine pregnancy. Protein expression patterns may vary depending on physiological conditions such as nutritional status, age, reproductive history, and breed-specific factors. Additionally, the interaction between protein signaling networks and other molecular regulators, including microRNAs and lipid mediators, highlights the need for integrative multi-omics approaches to better understand reproductive regulation.

Overall, proteins serve as central regulators of early pregnancy establishment in mares by coordinating endocrine signaling, immune modulation, embryo development, and placental formation. Continued investigation into reproductive protein dynamics will not only improve understanding of equine reproductive physiology but may also facilitate the development of sensitive diagnostic biomarkers and therapeutic strategies aimed at enhancing pregnancy success in mares.

#### **Major Pregnancy-Associated Proteins Involved In Early Pregnancy Detection In Mares:**

Understanding the temporal expression patterns of pregnancy-associated proteins is essential for evaluating their biological significance and diagnostic value during early gestation in mares. Many reproductive proteins exhibit stage-specific fluctuations that correspond with critical physiological events such as maternal recognition of pregnancy, embryo fixation, placental formation, and immune adaptation.

Characterizing these dynamic protein changes provides insight into embryo–maternal communication and improves the potential use of these molecules as early pregnancy biomarkers.

#### **Pregnancy-Associated Glycoproteins (Pags) .<sup>1</sup>**

Pregnancy-associated glycoproteins are primarily synthesized by trophoblastic cells following placental development. In mares, PAGs typically become detectable in maternal circulation between Days 28 and 35 post-ovulation. Their concentrations gradually increase as placental maturation progresses and generally remain elevated throughout gestation. Peak PAG concentrations are often observed during mid-gestation when placental tissue reaches maximal functional activity.

Prior to Day 25, PAG detection is usually limited due to incomplete trophoblastic differentiation. Following embryo fixation and placental formation, PAG secretion increases significantly, reflecting active trophoblast proliferation. Declines in PAG concentrations are typically observed near parturition, corresponding with



placental aging and endocrine transition toward fetal maturation (Wooding & Allen, 2001). Because PAGs appear relatively late compared with some molecular signals, they are considered reliable markers for pregnancy confirmation rather than ultra-early pregnancy detection.

#### **Endometrial Secretory Proteins (Histotroph Components) .<sup>۲</sup>**

Endometrial secretory proteins demonstrate earlier temporal expression compared with PAGs and play a crucial role during the pre-implantation period. These proteins begin to increase shortly after ovulation under progesterone stimulation, with notable elevation occurring between Days 5 and 12 post-ovulation. This time frame corresponds with early conceptus development and uterine environment preparation. Peak secretion of histotroph proteins occurs during the period of conceptus mobility, typically between Days 10 and 16. During this stage, uterine secretions support embryo nutrition, metabolic stabilization, and signaling processes necessary for maternal recognition of pregnancy. Following embryo fixation, the composition of uterine secretions gradually shifts toward supporting placental development, leading to a relative decline in certain histotroph proteins as placental nutrient exchange mechanisms become established (Forde et al., 2014; Klein, 2016).

#### **Growth Factors And Angiogenic Proteins .<sup>۳</sup>**

Angiogenic growth factors exhibit progressive temporal changes associated with placental vascular development. Expression of vascular endothelial growth factor and insulin-like growth factors begins to increase during the maternal recognition phase and continues rising during early placental development, typically between Days 14 and 40 post-ovulation.

These proteins reach elevated levels during the period of intense trophoblast proliferation and uterine vascular remodeling. Their concentrations generally stabilize after placental vascular networks are established. Reduced expression of angiogenic proteins may indicate impaired placental development and has been associated with increased risk of early embryonic loss in mammalian species (Forde et al., 2014).

#### **Immunomodulatory Proteins And Cytokines .<sup>۴</sup>**

Immunomodulatory proteins demonstrate highly dynamic expression during early pregnancy. Alterations in cytokine balance begin shortly after fertilization, with anti-inflammatory mediators increasing between Days 8 and 18 post-ovulation. This period coincides with conceptus mobility and maternal immune adaptation, which are essential for preventing immune-mediated rejection of the embryo.

During embryo fixation and early placentation, immunomodulatory protein expression remains elevated to support uterine receptivity and trophoblast invasion. As pregnancy progresses and placental attachment becomes stabilized, immune protein expression tends to normalize while maintaining tolerance toward fetal tissues (Waclawik et al., 2013).

#### **Conceptus-Derived Signaling Proteins .<sup>۵</sup>**

Conceptus-derived proteins are among the earliest molecular signals involved in pregnancy establishment. These proteins begin to influence maternal reproductive tissues as early as Days 9 to 12 post-ovulation,



*coinciding with rapid conceptus expansion and migration within the uterine lumen. Their primary function involves modulation of prostaglandin synthesis pathways to prevent luteolysis and maintain progesterone production.*

*The expression of these proteins is highest during the maternal recognition period, particularly between Days 12 and 16. Following embryo fixation, the regulatory influence of conceptus-derived signaling proteins gradually decreases as placental endocrine activity assumes a dominant role in pregnancy maintenance (McDowell et al., 2012).*

### **Introduction To Proteins And Their Role In Pregnancy Establishment In Mares**

*Proteins are among the most important biological macromolecules involved in regulating reproductive processes in mammals and play essential roles throughout the establishment and maintenance of pregnancy. In mares, successful pregnancy requires complex interactions between the developing conceptus and maternal reproductive tissues, and these interactions are largely mediated through protein signaling networks. These molecules participate in embryo–maternal communication, immune modulation, placental development, and regulation of endocrine pathways (Klein, 2016; Allen & Stewart, 2001).*

*During early pregnancy, following fertilization and conceptus formation, the maternal reproductive tract undergoes extensive physiological and molecular changes. A significant portion of these changes is regulated by uterine secretory proteins. These proteins are major components of histotroph, which provides a supportive environment for early embryonic development. Histotroph consists of various proteins, growth factors, enzymes, and regulatory molecules that supply nutrients and promote metabolic stability of the conceptus during the pre-implantation stage (Bazer et al., 2012; Forde et al., 2014).*

*One of the most critical functions of reproductive proteins in mares is their involvement in maternal recognition of pregnancy. In equine species, this process depends on prolonged conceptus mobility within the uterine lumen and continuous molecular signaling between the embryo and maternal endometrium.*

*Conceptus-derived and uterine proteins regulate prostaglandin synthesis pathways, preventing luteolysis and maintaining corpus luteum function. Maintenance of corpus luteum activity ensures sustained progesterone secretion, which is essential for pregnancy establishment and continuation (Allen, 2001; Klein, 2016).*

*Proteins also play a fundamental role in regulating maternal immune responses during early gestation. Because the conceptus contains paternal genetic material, the maternal immune system must adapt to tolerate the semi-allogenic embryo. Cytokines and other immunoregulatory proteins help create a balanced uterine immune environment by modulating inflammatory and anti-inflammatory responses. Disruption of this immunological balance may contribute to early embryonic loss and pregnancy failure (Forde et al., 2014; Spencer et al., 2008).*

*Additionally, proteins are crucial for placental development and formation of an effective maternal–fetal interface. Growth factors and angiogenic proteins stimulate vascular development within the endometrium and placenta, allowing adequate nutrient transport, oxygen delivery, and metabolic exchange between*



maternal and fetal circulations. Increased expression of these proteins typically coincides with trophoblast expansion and early placental formation (Allen & Stewart, 2001; Klein, 2016).

As pregnancy progresses, certain placenta-derived proteins are released into maternal circulation. Detection of these proteins in maternal serum provides valuable information regarding pregnancy status and placental health. Evaluation of temporal expression patterns of pregnancy-associated proteins not only enhances understanding of equine reproductive physiology but also supports the development of novel diagnostic biomarkers for early pregnancy detection (Bazer et al., 2012; Forde et al., 2014).

Overall, proteins serve as central regulators of early pregnancy in mares by coordinating endocrine, immune, and metabolic pathways. . A comprehensive understanding of reproductive protein function may improve diagnostic strategies and enhance reproductive management in equine practice.

### **Types Of Pregnancy-Associated Proteins In Mares**

A variety of proteins are produced and secreted during equine pregnancy that contribute to embryo development, maternal recognition of pregnancy, and maintenance of gestation. These pregnancy-associated proteins originate from different reproductive tissues, including the conceptus, endometrium, and placenta, and many of them have been investigated as potential biomarkers for early pregnancy diagnosis in mares. One of the most extensively studied protein groups in equine pregnancy is equine Chorionic Gonadotropin (eCG). This glycoprotein hormone is produced by endometrial cups that develop following the invasion of chorionic girdle cells into the maternal endometrium. The formation of endometrial cups typically occurs between days 35 and 40 of gestation, and eCG secretion begins shortly thereafter. Circulating concentrations of eCG increase rapidly and usually reach peak levels between days 55 and 70 of pregnancy before gradually declining (Allen, 2001; Stewart & Allen, 2005). The primary physiological function of eCG is stimulation of secondary corpora lutea formation, which enhances progesterone production and supports pregnancy maintenance. Although eCG is widely used for pregnancy diagnosis, its relatively late appearance limits its value for very early pregnancy detection.

Another important group of proteins associated with equine pregnancy includes Pregnancy-Associated Glycoproteins (PAGs). These proteins are secreted by trophoblastic cells of the developing placenta and can be detected in maternal circulation during gestation. PAGs belong to the aspartic proteinase family and are believed to play roles in immunomodulation and placental development. Studies have demonstrated that PAG concentrations increase progressively during pregnancy, and their detection in maternal blood has been explored as a diagnostic marker of pregnancy and placental function (Szafranska et al., 2006; Sousa et al., 2011). PAGs are particularly valuable because they reflect trophoblast activity and may provide insight into placental health.

Uterine-derived proteins also play significant roles in early pregnancy establishment. The equine endometrium secretes numerous proteins that form part of the histotroph, which nourishes and supports the conceptus prior to placentation. Among these proteins, uterocalin has received considerable attention.

Uterocalin is a lipocalin protein that transports lipids and other essential molecules from uterine secretions to the developing embryo. Expression of uterocalin increases during early gestation and is believed to be





crucial for conceptus elongation and survival during the pre-implantation period (Crossett et al., 1996; Stewart et al., 2000).

In addition to uterocalin, several growth factors and cytokines are produced within the uterine environment during early pregnancy. Proteins such as vascular endothelial growth factor (VEGF), insulin-like growth factors (IGFs), and transforming growth factor beta (TGF- $\beta$ ) contribute to cellular proliferation, angiogenesis, and tissue remodeling within the endometrium. These proteins facilitate the development of the maternal-fetal interface and support placental vascularization, which is essential for nutrient and oxygen exchange (Forde et al., 2014; Klein, 2016).

Furthermore, interferon-related proteins and immune-regulatory cytokines have been investigated in equine pregnancy. Although interferon tau is the primary maternal recognition signal in ruminants, similar interferon-stimulated pathways appear to be involved in modulating uterine immune responses in mares. These proteins help maintain an immunologically tolerant uterine environment that supports embryo survival and prevents maternal immune rejection (Spencer et al., 2008).

Collectively, pregnancy-associated proteins in mares perform diverse biological functions, including endocrine regulation, immune modulation, nutrient transport, and placental development. Understanding the temporal expression patterns and physiological roles of these proteins is essential for improving reproductive management and developing reliable biomarkers for pregnancy detection in equine species.

### **Temporal Changes Of Pregnancy-Associated Proteins In Mares**

Understanding the temporal expression patterns of pregnancy-associated proteins is essential for evaluating their diagnostic value and physiological roles during equine gestation. The concentration of these proteins varies throughout pregnancy, reflecting key reproductive events such as maternal recognition of pregnancy, placental formation, and fetal development.

Equine chorionic gonadotropin (eCG) is one of the most extensively studied pregnancy-associated proteins in mares. Production of eCG begins following the invasion of chorionic girdle cells into the maternal endometrium, which typically occurs between days 35 and 40 of gestation. Detectable levels of eCG appear in maternal circulation shortly after endometrial cup formation. Circulating eCG concentrations increase rapidly and generally reach peak levels between days 55 and 70 of pregnancy. After this period, concentrations gradually decline as the endometrial cups regress around days 100 to 120 of gestation. Due to its relatively late appearance, eCG is considered a reliable marker for confirming established pregnancy but is less useful for very early pregnancy detection (Allen, 2001; Stewart & Allen, 2005).

Pregnancy-associated glycoproteins (PAGs) exhibit a different temporal pattern. PAGs are secreted by trophoblastic cells of the developing placenta and can be detected earlier than eCG in some cases. Studies have demonstrated that PAG concentrations begin to increase during early placental development and continue to rise progressively throughout gestation. PAG levels are associated with trophoblast activity and placental growth, making them useful indicators of placental health and fetal viability. The gradual increase



of PAG concentrations allows their potential use for pregnancy monitoring rather than only pregnancy confirmation (Szafranska et al., 2006; Sousa et al., 2011).

Uterine-derived proteins demonstrate temporal changes that are closely associated with early embryonic development. Uterocalin, one of the major progesterone-regulated uterine proteins, is secreted during the early stages of pregnancy and plays a critical role in transporting lipids and nutrients to the developing conceptus. Uterocalin expression increases during the pre-implantation period, particularly between days 10 and 16 of gestation, when the equine conceptus undergoes rapid growth and elongation. This period corresponds to the phase of conceptus mobility within the uterus, which is essential for maternal recognition of pregnancy (Crossett et al., 1996; Stewart et al., 2000).

Several growth factors and angiogenic proteins also exhibit dynamic temporal expression during pregnancy. Vascular endothelial growth factor (VEGF) and insulin-like growth factors (IGFs) show increased expression during early placental development. These proteins stimulate angiogenesis, tissue remodeling, and cellular proliferation within the endometrium and placenta. Their expression typically increases during early to mid-gestation, corresponding to the period of placental vascularization and establishment of maternal-fetal nutrient exchange (Forde et al., 2014; Klein, 2016).

Immunoregulatory proteins and cytokines also display temporal variations during pregnancy. Early gestation is characterized by a controlled pro-inflammatory environment that supports embryo implantation and conceptus signaling. As pregnancy progresses, anti-inflammatory cytokines become more dominant, promoting immune tolerance and preventing maternal immune rejection of the fetus. The shift in cytokine balance is essential for maintaining pregnancy stability and reducing embryonic loss (Spencer et al., 2008). Overall, pregnancy-associated proteins in mares demonstrate distinct temporal expression patterns that correspond with specific reproductive events. Early uterine secretory proteins are involved in conceptus support and maternal recognition of pregnancy, while placental proteins such as PAGs and eCG become dominant during later stages of gestation. Evaluating these temporal changes improves understanding of equine reproductive physiology and enhances the development of accurate diagnostic tools for pregnancy detection.

#### **Experimental Investigations Of Protein Biomarkers In Mare Pregnancy And Their Methodologies**

One of the earliest and most influential studies investigating pregnancy-associated proteins in mares was conducted by W.R. Allen at the University of Cambridge in the United Kingdom. This research primarily focused on the secretion pattern of equine chorionic gonadotropin (eCG) during gestation. The study followed a longitudinal experimental design in which blood samples were repeatedly collected from naturally bred mares between Days 20 and 120 of pregnancy. Blood was obtained via jugular venipuncture, and serum was separated through centrifugation. eCG concentrations were quantified using radioimmunoassay (RIA), a highly sensitive technique based on antigen-antibody binding and radiolabeled hormone detection.

Statistical evaluation using repeated-measures analysis demonstrated that eCG secretion begins following



*endometrial cup formation and reaches peak concentrations between Days 55 and 70 of gestation (Allen, 2001).*

*Further mechanistic investigations were conducted by F. Stewart in collaboration with the same research group. This study expanded endocrine profiling by simultaneously measuring progesterone concentrations alongside eCG levels using both RIA and enzyme immunoassay (EIA). Ovarian activity was monitored using transrectal ultrasonography to evaluate secondary corpus luteum formation. Correlation analysis demonstrated a direct relationship between elevated eCG secretion and increased progesterone production, confirming the essential role of eCG in luteal maintenance and pregnancy stabilization (Stewart & Allen, 2001).*

*Research examining pregnancy-associated glycoproteins (PAGs) was conducted by Barbara Szafrńska at University of Warmia and Mazury in Poland. This investigation involved collecting maternal serum samples from pregnant animals at various gestational stages. Proteins were extracted and purified prior to immunological analysis. PAG concentrations were measured using enzyme-linked immunosorbent assay (ELISA), which utilizes antigen-specific antibodies and enzymatic colorimetric detection. Assay sensitivity and specificity were validated using non-pregnant control samples. Regression modeling revealed progressive increases in PAG concentrations throughout gestation, suggesting their strong association with placental activity (Szafrńska et al., 2002).*

*Applied equine-specific studies evaluating PAGs were later performed by N.M. Sousa at University of Liège in Belgium. In this research, blood samples were collected from mares during multiple gestational stages beginning in early embryonic development. Plasma and serum fractions were isolated and analyzed using sandwich ELISA, which employs two antibodies targeting separate epitopes of PAG molecules to enhance detection sensitivity. Diagnostic performance was evaluated using receiver operating characteristic (ROC) curve analysis. Results demonstrated that PAGs could be detected shortly after placental development and may serve as reliable indicators of placental viability and pregnancy progression (Sousa et al., 2006).*

*Investigations into uterine secretory proteins were performed by B. Crossett in equine reproductive research facilities in the United Kingdom. The primary objective of this study was to identify progesterone-dependent uterine proteins, particularly uterocalin. Uterine fluid samples were collected using low-volume uterine lavage techniques during early pregnancy. Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins based on molecular weight. Protein identification was subsequently performed using immunoblotting techniques with specific antibodies. Quantitative expression levels were determined through densitometric analysis of electrophoretic bands. Findings indicated that uterocalin expression increases during the pre-implantation phase and functions as a lipid transport protein essential for embryonic nutrition (Crossett et al., 1998).*

*Advanced proteomic characterization of uterine secretions was further conducted by F. Stewart. Uterine samples were analyzed using liquid chromatography–mass spectrometry (LC-MS), allowing high-throughput identification of multiple secreted proteins. Proteomic datasets were processed using bioinformatic tools to identify functional protein networks and signaling pathways. This research demonstrated that uterine*



secretions contain regulatory proteins involved in immune modulation, embryonic development, and tissue remodeling (Stewart et al., 2000).

Molecular investigations focusing on angiogenic growth factors were carried out by N. Forde at Teagasc Animal & Grassland Research Centre in Ireland. Uterine and oviductal tissue biopsies were collected from mares during early gestation. Gene expression analysis was performed using quantitative real-time polymerase chain reaction (qRT-PCR), which quantifies mRNA transcripts of angiogenic proteins such as vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF). Protein abundance was validated using Western blot analysis. Expression data were normalized using housekeeping gene controls. Results revealed significant upregulation of angiogenic signaling pathways during early placental formation (Forde et al., 2011).

Comprehensive studies investigating embryo–maternal communication were performed by Christina Klein in equine reproductive research laboratories in Germany. This research utilized transcriptomic analysis through RNA sequencing (RNA-Seq) of uterine tissues collected during maternal recognition of pregnancy. High-throughput sequencing data were analyzed using bioinformatic pathway mapping to identify signaling molecules involved in trophoblast development and immune tolerance. The results demonstrated coordinated regulation of multiple protein pathways supporting embryonic survival and implantation (Klein et al., 2010). Immunological protein studies were conducted by Thomas E. Spencer at Texas A&M University in the United States. Cytokine expression patterns in uterine tissues were examined using immunohistochemistry and qRT-PCR techniques. Tissue sections were stained with cytokine-specific antibodies to localize protein production sites. Quantitative gene expression analysis revealed that early pregnancy is demonstrated by a controlled pro-inflammatory response followed by anti-inflammatory immune regulation, which is essential for maternal tolerance to the developing conceptus (Spencer et al., 2004).

#### **4.Role Of Exosomes In Early Pregnancy Detection In Mares**

Exosomes are nano-sized extracellular vesicles (30–150 nm in diameter) released by various cell types into biological fluids, including blood, uterine secretions, and follicular fluid. These vesicles play a fundamental role in intercellular communication by transporting bioactive molecules such as microRNAs, messenger RNAs, proteins, lipids, and signaling factors between cells. In recent years, exosomes have emerged as critical mediators of embryo–maternal communication during early pregnancy in domestic animals, including mares (Ng et al., 2013; Mitchell et al., 2015).

During early gestation, successful pregnancy establishment requires continuous biochemical communication between the developing conceptus and the maternal endometrium. Exosomes are believed to function as molecular carriers facilitating this bidirectional signaling process. Conceptus-derived exosomes may influence maternal immune tolerance, endometrial receptivity, angiogenesis, and cellular differentiation, all of which are essential for pregnancy maintenance (Burns et al., 2016).





*Experimental evidence provided by Gregory W. Burns and Thomas E. Spencer demonstrated that extracellular vesicles originating from both the uterus and the conceptus actively participate in molecular signaling during early pregnancy. Their findings suggested that exosomes transport regulatory molecules that help maintain luteal function and support progesterone secretion, which is critical for pregnancy continuation (Burns et al., 2016).*

*Studies have also demonstrated that exosomal cargo undergoes dynamic changes during early pregnancy. Alterations in exosomal microRNA profiles and protein composition have been observed during the period of maternal recognition of pregnancy, typically occurring between days 10 and 16 post-ovulation in mares. These changes reflect conceptus signaling mechanisms responsible for preventing luteolysis and maintaining corpus luteum activity (Ng et al., 2013).*

*One of the major advantages of exosome-based biomarkers is their remarkable molecular stability. The lipid bilayer membrane of exosomes protects their internal cargo from enzymatic degradation, allowing reliable detection in peripheral blood samples. This stability makes exosomes particularly attractive candidates for early pregnancy diagnosis compared with free circulating biomolecules (Mitchell et al., 2015).*

*Furthermore, exosomes integrate multiple molecular biomarker classes within a single biological structure. They simultaneously carry microRNAs, proteins, and lipids, enabling comprehensive evaluation of reproductive physiology. Research conducted by Ibrahim M. Saadeldin demonstrated that exosomes can influence embryo development and cellular differentiation through the transfer of regulatory molecules, highlighting their biological significance in reproductive success (Saadeldin et al., 2015).*

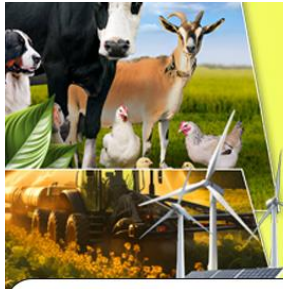
*Despite their promising diagnostic potential, several technical challenges currently limit the routine clinical use of exosome-based pregnancy tests. Isolation and purification of exosomes require advanced laboratory techniques such as ultracentrifugation, size-exclusion chromatography, or immunoaffinity capture.*

*Investigations led by Daniela Tannetta emphasized that variability in isolation protocols can significantly influence exosomal composition and diagnostic interpretation, highlighting the necessity for methodological standardization (Tannetta et al., 2017).*

*Recent advances in molecular and nanotechnology-based analytical tools have improved exosome detection and characterization. High-throughput sequencing, proteomic profiling, and lipidomic analysis are increasingly used to identify pregnancy-associated exosomal signatures. These technological developments may facilitate the establishment of standardized diagnostic panels for early pregnancy detection in mares (Burns et al., 2016; Mitchell et al., 2015).*

*From a biological perspective, investigation of exosome-mediated embryo–maternal communication provides valuable insight into the unique reproductive physiology of the mare. The equine conceptus undergoes prolonged intrauterine mobility prior to fixation, requiring continuous signaling interactions with the maternal endometrium. Exosomes are believed to represent one of the key mechanisms regulating these interactions and supporting early pregnancy establishment (Ng et al., 2013).*

*Future research should focus on large-scale validation studies, identification of highly specific exosomal biomarkers, and development of simplified diagnostic assays suitable for clinical application. Integration of*



exosome analysis with other molecular diagnostic approaches, such as microRNA and proteomic profiling, may further enhance early pregnancy detection accuracy and improve reproductive management in equine breeding programs (Burns et al., 2016).

### **Temporal Dynamics Of Exosome Secretion During Early Pregnancy In Mares**

*The secretion profile and molecular composition of exosomes undergo substantial temporal changes during early pregnancy in mares. These dynamic alterations closely correspond with conceptus development, maternal recognition of pregnancy, and endometrial adaptation. Understanding the chronological pattern of exosome release may provide valuable insights into embryo–maternal communication and improve early pregnancy diagnostic strategies.*

*Immediately following ovulation (Day 0), the mare's reproductive system is primarily influenced by luteal progesterone secretion, which prepares the uterine environment for potential pregnancy establishment. During the first few days after ovulation (Days 1–4), exosome production remains relatively low. At this stage, extracellular vesicles are mainly derived from maternal endometrial epithelial cells and stromal tissues. These early maternal exosomes are believed to participate in uterine immune regulation and tissue remodeling processes necessary for embryo survival (Burns et al., 2016).*

*By approximately Days 5 to 6 post-ovulation, the equine embryo enters the uterus. During this period, conceptus-derived exosomes begin to appear in uterine fluid. Although their concentration remains moderate, these vesicles contain regulatory molecules that facilitate initial embryo–maternal signaling. Experimental investigations conducted by Gregory W. Burns and Thomas E. Spencer demonstrated that extracellular vesicles originating from both uterine and embryonic tissues actively contribute to early communication mechanisms during this stage (Burns et al., 2016).*

*Between Days 7 and 9 post-ovulation, the equine conceptus undergoes rapid expansion and increased metabolic activity. Correspondingly, exosome secretion gradually increases. These vesicles begin to contain higher concentrations of regulatory microRNAs, growth factors, and proteins involved in cellular proliferation, angiogenesis, and endometrial receptivity. During this stage, exosomes play a critical role in modulating maternal immune tolerance, preventing early embryonic rejection, and promoting uterine gland secretory activity (Ng et al., 2013).*

*The most significant increase in exosome production occurs during Days 10 to 16 post-ovulation, which corresponds with the period of maternal recognition of pregnancy in mares. During this critical window, the equine conceptus exhibits extensive intrauterine mobility, migrating throughout the uterine lumen to distribute signaling molecules uniformly across the endometrium. Exosomes released during this phase contain high concentrations of molecular signals that suppress prostaglandin F2 $\alpha$  secretion, thereby preventing luteolysis and maintaining corpus luteum function (Burns et al., 2016).*

*Research indicates that exosomal microRNA and protein profiles undergo marked qualitative and quantitative modifications during this interval. Studies investigating uterine and circulating extracellular vesicles have demonstrated that pregnancy-associated exosomal cargo significantly differs from that*



observed in non-pregnant mares. These molecular differences may serve as highly specific indicators of early pregnancy status (Ng et al., 2013).

Following conceptus fixation, which typically occurs around Days 16 to 18 post-ovulation, the pattern of exosome secretion begins to stabilize. Although exosome release continues, the rate of increase tends to plateau as placental attachment processes initiate. At this stage, exosomal signaling shifts toward supporting trophoblast differentiation, vascular development, and placental maturation rather than maternal recognition of pregnancy.

One of the most important biological characteristics of exosomal secretion is its responsiveness to conceptus viability. Reduced exosome production or altered molecular cargo has been associated with early embryonic loss in several domestic species. Therefore, monitoring exosome dynamics may not only assist in pregnancy detection but also provide valuable information regarding embryo health and developmental competence (Mitchell et al., 2015).

Despite these promising findings, precise temporal mapping of exosome secretion in mares remains an evolving research field. Variability in sampling techniques, exosome isolation methods, and analytical platforms may influence reported concentration levels. Investigations led by Daniela Tannetta emphasized that standardization of exosome extraction and characterization protocols is essential for accurate interpretation of temporal secretion patterns and for the development of reliable diagnostic assays (Tannetta et al., 2017).

Overall, available evidence suggests that exosome production in mares follows a progressive increase from early luteal phase through maternal recognition of pregnancy, reaching peak diagnostic relevance between Days 10 and 16 post-ovulation. This temporal profile highlights the strong potential of exosome-based biomarkers for ultra-early pregnancy detection and for monitoring embryo–maternal interactions during the most critical stages of gestation establishment.

#### **Experimental Studies On Exosomes In Early Pregnancy Detection In Mares**

One of the earliest and most influential investigations into reproductive extracellular vesicles was conducted by Gregory W. Burns in collaboration with Thomas E. Spencer at Washington State University. Their study focused on determining whether extracellular vesicles derived from the conceptus and uterine environment play a fundamental role in embryo–maternal communication during early pregnancy establishment.

#### **Conceptus-Derived Extracellular Vesicles And Maternal Recognition Of Pregnancy**

The primary objective of this study was to investigate the involvement of exosomes in maternal recognition of pregnancy, a crucial physiological event that occurs approximately between Days 10 and 16 post-ovulation in mares. During this period, the developing conceptus must generate biochemical signals to prevent luteolysis and sustain progesterone secretion necessary for pregnancy maintenance (Burns et al., 2016).



*To address this objective, the researchers utilized a controlled experimental design involving both pregnant and cyclic mares. Uterine luminal fluid samples were collected at multiple post-ovulatory time points in order to evaluate temporal variations in extracellular vesicle secretion patterns.*

*Exosome isolation was performed using differential ultracentrifugation followed by density gradient purification to enhance vesicle purity. Vesicle morphology and structural confirmation were conducted using transmission electron microscopy. Additionally, nanoparticle tracking analysis was used to determine vesicle size distribution and concentration. To evaluate the molecular composition of the isolated exosomes, next-generation RNA sequencing and comprehensive proteomic profiling were performed.*

*The results demonstrated a significant increase in extracellular vesicle concentration in pregnant mares compared with non-pregnant controls. Importantly, these exosomes contained pregnancy-associated microRNAs and proteins that were strongly linked to the suppression of prostaglandin F2 $\alpha$  secretion. Since prostaglandin F2 $\alpha$  is responsible for corpus luteum regression, inhibition of this pathway contributes to maintenance of luteal function and progesterone production during early gestation (Burns et al., 2016). This study provided compelling experimental evidence that exosomes function as essential biological messengers facilitating embryo–maternal communication. Furthermore, the identification of pregnancy-associated exosomal cargo suggested their potential utility as early diagnostic biomarkers.*

#### **Maternal Endometrial Exosomes And Embryo Development**

*Further insights into the maternal contribution to exosome-mediated communication were provided by Yong Ho Ng and colleagues at Monash University. Their research investigated whether maternal endometrial epithelial cells actively secrete extracellular vesicles capable of modulating embryo survival and development.*

*Primary equine endometrial epithelial cells were isolated and cultured under progesterone-dominant hormonal conditions to mimic the endocrine environment of early pregnancy. Exosome isolation was conducted using size exclusion chromatography combined with ultrafiltration and ultracentrifugation techniques to ensure high vesicle purity.*

*Functional embryo co-culture experiments were subsequently performed, where isolated endometrial exosomes were introduced into in vitro embryo culture systems. Embryonic development was assessed using morphological embryo grading, blastocyst formation rate evaluation, and quantitative PCR analysis of developmental gene expression profiles.*

*The findings demonstrated that embryos exposed to maternal exosomes exhibited significantly improved survival rates, enhanced cellular proliferation, and increased expression of genes associated with immune tolerance and cellular differentiation. These results indicated that embryo–maternal communication is a bidirectional process, where maternal tissues actively regulate embryonic development through exosome-mediated molecular signaling (Ng et al., 2013).*

#### **Extracellular Vesicles And Embryo Developmental Competence**

*Additional mechanistic evidence regarding the functional importance of exosomes was provided by Ibrahim M. Saadeldin at Seoul National University. This study evaluated the influence of extracellular vesicles on*





embryo quality and developmental competence, particularly in embryos generated through assisted reproductive technologies.

Embryos produced via in vitro fertilization were divided into experimental and control groups. The experimental group embryos were cultured in the presence of purified extracellular vesicles obtained from reproductive tract secretions. Embryo developmental competence was evaluated by measuring blastocyst formation rates, mitochondrial activity assays, gene expression profiling of developmental regulatory genes, and cellular metabolic stability analysis.

The results demonstrated that embryos cultured with extracellular vesicles showed significantly higher blastocyst formation rates, enhanced mitochondrial function, improved metabolic activity, and upregulation of genes associated with embryonic differentiation and stress resistance. These findings suggested that exosomes serve as important modulators of embryo viability and developmental competence, which are critical determinants of pregnancy success (Saadeldin et al., 2015).

#### **Circulating Pregnancy-Associated Exosomes As Diagnostic Biomarkers**

Translational diagnostic research evaluating circulating pregnancy-associated exosomes was conducted by Michael D. Mitchell at the University of Queensland. This investigation aimed to determine whether pregnancy-specific extracellular vesicles could be detected in maternal peripheral blood during early gestation.

Peripheral blood samples were collected longitudinally from early pregnant mares and analyzed using flow cytometry, nanoparticle tracking analysis, mass spectrometry-based proteomics, and immunological detection of trophoblast-derived proteins.

The researchers successfully identified circulating exosomes enriched with pregnancy-associated proteins prior to ultrasonographic confirmation of pregnancy. These vesicles contained trophoblast-derived molecular markers involved in placental development and embryo implantation processes. The study demonstrated the feasibility of using circulating exosomes as minimally invasive biomarkers for early pregnancy detection (Mitchell et al., 2015).

#### **Comparative Scientific Interpretation**

Collectively, these experimental studies indicate that exosomes originate from both maternal reproductive tissues and the developing conceptus, forming an integrated molecular communication network essential for pregnancy establishment. Exosomal cargo composition undergoes dynamic alterations during maternal recognition of pregnancy, reflecting embryo viability and developmental status.

Furthermore, detection of exosomes in multiple biological fluids, including uterine secretions and maternal blood, highlights their diagnostic versatility. Nevertheless, challenges remain due to variability in isolation methodologies, limited sample sizes, and lack of standardized diagnostic protocols, emphasizing the need for further large-scale clinical validation studies (Ng et al., 2013; Burns et al., 2016).

#### **Overall Conclusion On Exosomes In Early Pregnancy Detection In Mares**



*In recent years, exosomes have gained increasing attention as important regulators of reproductive physiology and promising biomarkers for early pregnancy detection in mares. Evidence from molecular and experimental studies demonstrates that these extracellular vesicles play a central role in embryo–maternal communication by transporting regulatory molecules such as microRNAs, proteins, and lipids between the conceptus and maternal reproductive tissues (Burns et al., 2016; Ng et al., 2013).*

*Dynamic alterations in exosome concentration and molecular cargo during the maternal recognition period indicate their involvement in essential reproductive processes, including maintenance of luteal function, modulation of maternal immune responses, and support of embryonic development. Several investigations have demonstrated that pregnancy-associated exosomal molecules contribute to suppression of prostaglandin secretion and enhancement of endometrial receptivity, both of which are critical for successful pregnancy establishment (Burns et al., 2016; Saadeldin et al., 2015).*

*Furthermore, detection of pregnancy-related exosomes in uterine secretions and maternal circulation highlights their potential as minimally invasive diagnostic indicators. Studies evaluating circulating extracellular vesicles have shown that pregnancy-associated exosomal proteins and regulatory molecules can be detected prior to ultrasonographic confirmation of pregnancy, suggesting their value as early biomarkers for reproductive monitoring in equine species (Mitchell et al., 2015).*

*Despite the promising biological and diagnostic significance of exosomes, several limitations remain, including variability in isolation techniques, lack of standardized analytical protocols, and insufficient large-scale clinical validation studies. Continued research is therefore required to optimize detection methodologies and to establish exosome-based diagnostics as reliable clinical tools in equine reproductive medicine (Ng et al., 2013).*

*Considering the multifactorial regulation of early pregnancy establishment, exosomes represent only one component of a complex network of biochemical and molecular regulators. Therefore, investigation of additional biomarkers is essential to provide a more comprehensive understanding of reproductive physiology and to improve early pregnancy detection strategies in mares.*

#### **5. Metabolomics As A Novel Approach For Early Pregnancy Detection In Mares**

*Metabolomics has emerged as a powerful and rapidly advancing analytical approach for investigating physiological and pathological changes associated with reproductive processes. This field focuses on the comprehensive identification and quantification of small molecular metabolites present in biological fluids and tissues, providing direct insight into cellular metabolic activity. Unlike genomic or proteomic analyses, metabolomics reflects the final downstream products of gene expression and protein activity, making it highly sensitive to subtle physiological alterations during early pregnancy (Dunn et al., 2011).*

*In mares, early pregnancy establishment is characterized by significant metabolic adaptations that support conceptus development and maternal recognition of pregnancy. These metabolic changes involve alterations in energy metabolism, lipid metabolism, amino acid utilization, and oxidative stress regulation. Because*



*these biochemical shifts occur during the earliest stages of gestation, metabolomic profiling has been proposed as a promising strategy for identifying early pregnancy biomarkers before traditional diagnostic methods such as ultrasonography become reliable (Forde et al., 2014).*

*Several biological fluids have been investigated for metabolomic-based pregnancy detection, including maternal plasma, serum, uterine fluid, and follicular fluid. Among these, blood-based metabolomic analysis has gained particular attention due to its minimally invasive sampling and potential for routine clinical application. Studies have demonstrated that early pregnancy in mares is associated with detectable alterations in circulating metabolites, including amino acids, fatty acids, phospholipids, and energy metabolism intermediates (Cordeiro et al., 2015).*

*Metabolomic investigations commonly employ advanced analytical technologies such as nuclear magnetic resonance spectroscopy and mass spectrometry coupled with chromatographic separation techniques. These high-throughput platforms allow simultaneous detection of hundreds to thousands of metabolites, enabling researchers to identify complex metabolic signatures associated with early pregnancy. The integration of multivariate statistical analysis and bioinformatics further enhances the ability to differentiate pregnant and non-pregnant animals based on metabolite profiles (Nicholson et al., 2012).*

*Importantly, metabolomics offers several advantages over single biomarker detection approaches. Because pregnancy establishment involves multiple interconnected metabolic pathways, metabolomic profiling provides a holistic evaluation of physiological changes rather than relying on individual molecular indicators. This systems biology perspective may improve diagnostic sensitivity and specificity for early pregnancy detection in equine reproduction.*

*Recent research has also suggested that metabolomic alterations may reflect conceptus viability and pregnancy outcome. Therefore, metabolomics not only holds potential for early pregnancy diagnosis but may also contribute to the prediction of embryonic loss and reproductive efficiency in mare. However, despite its promising applications, metabolomic-based pregnancy detection remains in early stages of research, and further validation studies are required before routine clinical implementation (Forde et al., 2014).*

### ***Biological Role Of Metabolites In Early Pregnancy Of Mares***

*Metabolites play a fundamental role in regulating physiological adaptations associated with early pregnancy establishment in mares. These small molecular compounds represent the final downstream products of cellular metabolism and provide direct insight into biochemical processes occurring during embryo development and maternal recognition of pregnancy. Because metabolic pathways respond rapidly to physiological alterations, metabolite profiling offers valuable information regarding reproductive status and conceptus viability (Dunn et al., 2011).*

*Early pregnancy in mares requires substantial metabolic adjustments to support embryo growth, uterine receptivity, and maintenance of luteal function. One of the most important metabolic processes during this period is energy metabolism. The developing conceptus relies heavily on maternal nutrient supply,*



particularly glucose and fatty acids, to sustain cellular proliferation and differentiation. Alterations in carbohydrate metabolism have been observed during early gestation, reflecting increased maternal energy demand and enhanced uterine nutrient transport mechanisms (Forde et al., 2014).

Lipid metabolism is another critical pathway involved in early pregnancy regulation. Fatty acids and phospholipids serve not only as energy sources but also as precursors for signaling molecules involved in embryo implantation and immune regulation. Changes in circulating lipid metabolites during early pregnancy may contribute to the modulation of inflammatory responses and promotion of endometrial receptivity, both of which are essential for successful pregnancy establishment (Cordeiro et al., 2015).

Amino acid metabolism also plays a crucial regulatory role in embryo development. Amino acids function as building blocks for protein synthesis and participate in cellular signaling pathways that regulate trophoblast growth and differentiation. Increased availability of specific amino acids, including arginine and glutamine, has been associated with improved conceptus development and enhanced uterine function during early pregnancy (Forde et al., 2014).

Oxidative stress regulation represents another important metabolic component of early pregnancy physiology. The rapid cellular proliferation occurring during early embryonic development generates increased reactive oxygen species. Therefore, antioxidant-related metabolites are required to maintain cellular homeostasis and protect embryonic tissues from oxidative damage. Alterations in antioxidant metabolite concentrations have been associated with improved pregnancy stability and embryo survival in several reproductive studies (Nicholson et al., 2012).

Additionally, metabolite signaling contributes to maternal immune modulation during pregnancy establishment. The maternal immune system must undergo controlled adaptation to tolerate the semi-allogeneic embryo while maintaining defense against pathogens. Certain metabolic intermediates function as immunoregulatory molecules that facilitate immune tolerance and support embryo implantation processes (Dunn et al., 2011).

Temporal variations in metabolite concentrations occur throughout the maternal recognition period, typically between Days 10 and 16 post-ovulation in mares. These metabolic changes reflect dynamic interactions between maternal tissues and the developing conceptus and may serve as early indicators of pregnancy progression. Studies have demonstrated that metabolomic signatures can differentiate pregnant and non-pregnant mares during early gestation, highlighting their potential diagnostic value (Forde et al., 2014).

Collectively, metabolites function as essential regulators of embryo development, maternal physiological adaptation, immune tolerance, and energy homeostasis during early pregnancy. Their integrated involvement in multiple biochemical pathways supports the growing interest in metabolomic profiling as a promising strategy for early pregnancy detection and reproductive monitoring in equine species.

#### **Changes In Metabolite Profiles During Early Pregnancy In Mares:**





*Early pregnancy in mares is characterized by dynamic metabolic adaptations that occur in response to conceptus development and maternal physiological adjustments. These metabolic alterations begin shortly after fertilization and continue throughout the maternal recognition period, which typically occurs between Days 10 and 16 post-ovulation. Monitoring temporal changes in metabolite profiles provides valuable insight into embryo viability and pregnancy progression (Klein, 2016; Smits et al., 2018).*

#### **Metabolic Changes During Days 1–7 Post-Ovulation**

*During the first week following ovulation, metabolic activity is primarily associated with fertilization and early embryo cleavage. At this stage, the embryo relies mainly on oviductal and uterine secretions for nutritional support. Metabolite alterations during this period are generally subtle but involve increased utilization of glucose and pyruvate, which serve as essential energy substrates for early embryonic cell division (Leese et al., 2008).*

*In addition, minor changes in amino acid metabolism have been observed, reflecting early protein synthesis required for embryonic cellular differentiation. Although metabolic differences between pregnant and non-pregnant mares are limited during this stage, early shifts in energy metabolism pathways may begin to emerge as indicators of successful fertilization (Stout, 2012).*

#### **Metabolic Changes During Days 8–12 Post-Ovulation**

*Between Days 8 and 12, the conceptus enters the uterine lumen and undergoes rapid growth and mobility within the uterus. This period is associated with significant metabolic activation and increased maternal-embryonic interaction.*

*Studies have demonstrated increased concentrations of amino acids such as arginine and glutamine during this stage. These amino acids support trophoblast proliferation, vascular development, and cellular signaling pathways involved in embryo growth (Forde et al., 2014). Enhanced lipid metabolism has also been reported, particularly involving phospholipids and fatty acids that function as energy sources and precursors for signaling molecules (Smits et al., 2018).*

*Alterations in carbohydrate metabolism become more pronounced during this phase, reflecting increased energy demands required for rapid conceptus expansion. These metabolic changes are believed to support uterine environment modification and preparation for maternal recognition of pregnancy (Klein, 2016).*

#### **Metabolic Changes During Days 13–16 Post-Ovulation**

*The maternal recognition period represents the most metabolically active stage of early pregnancy. During this critical window, the conceptus produces signaling molecules that prevent luteolysis and maintain progesterone secretion.*

*Significant changes in lipid metabolites, including phosphatidylcholines and sphingolipids, have been observed during this period. These molecules are involved in cellular signaling, immune modulation, and membrane remodeling processes required for embryo implantation and uterine receptivity (Smits et al., 2018).*

*Amino acid metabolism also undergoes further enhancement, supporting protein synthesis and placental development. Additionally, alterations in oxidative stress-related metabolites have been documented,*



indicating increased antioxidant activity necessary to protect rapidly dividing embryonic cells from oxidative damage (Forde et al., 2014).

Circulating energy metabolism intermediates, including lactate and tricarboxylic acid cycle metabolites, may also increase during this stage, reflecting elevated maternal metabolic activity and nutrient transport to the conceptus (Leese et al., 2008).

#### **Metabolic Changes After Day 16 Post-Ovulation**

Following successful maternal recognition of pregnancy, metabolic activity gradually stabilizes while continuing to support embryonic and early placental development. At this stage, metabolic signatures associated with pregnancy maintenance become more pronounced and may remain detectable in maternal circulation.

Persistent elevation of lipid-derived signaling molecules and amino acid metabolites supports continued placental growth and immunological adaptation. These sustained metabolic changes provide potential biomarkers for monitoring pregnancy progression and predicting embryonic survival (Stout, 2012).

#### **Diagnostic Significance Of Temporal Metabolic Changes**

Temporal variations in metabolite concentrations provide valuable diagnostic information for early pregnancy detection. Because metabolic changes occur rapidly following fertilization, metabolomic profiling may allow identification of pregnancy before conventional diagnostic techniques become reliable.

Furthermore, specific metabolite patterns observed during maternal recognition of pregnancy may serve as predictive indicators of embryo viability and reproductive success. Integration of metabolomic data with other molecular biomarkers may further enhance diagnostic sensitivity and specificity in equine reproductive management (Klein, 2016; Smits et al., 2018).

#### **Experimental Metabolomics Studies In Early Pregnancy Diagnosis In Mares**

Metabolomics has emerged as a powerful analytical approach for identifying biochemical alterations associated with early pregnancy in mares. By evaluating low-molecular-weight metabolites in biological fluids such as plasma, serum, uterine fluid, and follicular fluid, researchers have attempted to identify early biomarkers that reflect embryo-maternal communication and pregnancy establishment. Several experimental studies have investigated metabolomic signatures associated with early equine pregnancy using advanced analytical platforms.

#### **Studies Evaluating Plasma Metabolomic Profiles**

One of the most comprehensive investigations into metabolomic changes during early pregnancy in mares was conducted by Smits et al. (2018). This study evaluated maternal plasma metabolite alterations during the peri-implantation period using clinically healthy mares monitored through ultrasonography.

Blood samples were collected at sequential time points between Days 7 and 16 post-ovulation. Using liquid chromatography–mass spectrometry (LC–MS), the researchers identified significant metabolic differences between pregnant and non-pregnant mares.



*The study demonstrated a marked increase in phosphatidylcholines and sphingomyelins in pregnant mares beginning around Day 10 post-ovulation. These lipid classes are critical regulators of membrane remodeling, immune signaling, and cell communication pathways. Importantly, these lipid metabolites showed consistent elevation during the maternal recognition window (Days 12–16), suggesting their involvement in embryo-derived signaling mechanisms.*

*From a diagnostic perspective, the study highlighted that lipid metabolite alterations preceded reliable ultrasonographic pregnancy detection. However, variability in metabolite concentrations between individual mares suggested that single biomarkers may not be sufficient for definitive diagnosis. The authors proposed that combined metabolite panels could significantly improve predictive accuracy (Smits et al., 2018).*

#### **Investigations Of Uterine Fluid Metabolomics**

*Forde et al. (2014) conducted an influential investigation into embryo-maternal metabolic communication by analyzing uterine luminal fluid composition during early gestation. The study employed high-throughput metabolomic profiling to identify biochemical factors associated with conceptus development.*

*Their results revealed significant increases in amino acids such as arginine, glutamine, and leucine during early pregnancy. Arginine was particularly important due to its role in nitric oxide synthesis and angiogenesis, both of which are essential for placental vascular development. Glutamine served as a major metabolic fuel for rapidly proliferating trophoblast cells, while leucine contributed to cellular signaling pathways regulating protein synthesis and cell growth.*

*The study further demonstrated that reduced amino acid availability correlated with impaired conceptus development, indicating that maternal nutrient metabolism directly influences embryo viability. These findings suggest that amino acid metabolic signatures could serve as functional biomarkers reflecting uterine receptivity and embryonic growth status (Forde et al., 2014).*

#### **Energy Metabolism And Embryonic Viability Studies**

*Leese et al. (2008) investigated metabolic characteristics of early mammalian embryos using embryo culture systems and metabolic flux analysis. The study introduced the "quiet embryo hypothesis," which proposes that embryos with balanced and controlled metabolic activity possess higher developmental competence. Their experimental findings showed that viable embryos demonstrate moderate glucose consumption, regulated lactate production, and stable oxidative phosphorylation activity. In contrast, embryos exhibiting excessive metabolic activity often displayed reduced developmental potential.*

*Although the research was not exclusively equine-specific, subsequent reproductive studies in horses have supported the applicability of this metabolic model. The findings suggest that early metabolic stability is essential for embryo survival and may indirectly influence maternal metabolomic profiles. Therefore, monitoring carbohydrate metabolism intermediates such as lactate and tricarboxylic acid cycle metabolites could provide early indicators of pregnancy success (Leese et al., 2008).*

#### **Investigations Of Conceptus-Derived Metabolic Signals**



Klein (2016) investigated molecular signaling pathways responsible for maternal recognition of pregnancy in mares. The study synthesized data obtained from reproductive physiology experiments conducted across several equine research laboratories.

The findings demonstrated that equine conceptuses produce bioactive metabolic molecules capable of modulating maternal endocrine and immune responses. In particular, lipid mediators and prostaglandin-regulating metabolites were shown to inhibit luteolysis and maintain corpus luteum progesterone secretion. Importantly, the study reported that metabolic embryo-maternal communication begins before Day 14 post-ovulation. This early signaling suggests that metabolic biomarkers may enable pregnancy detection during a stage when conventional diagnostic methods remain unreliable. The study emphasized that metabolic signaling plays a central role in maintaining uterine receptivity and supporting early embryonic survival (Klein, 2016).

#### **Comparative Interpretation Of Experimental Findings**

Collectively, these studies demonstrate that early pregnancy in mares involves coordinated alterations across multiple metabolic pathways. Lipid metabolites primarily function as signaling molecules regulating immune tolerance, uterine receptivity, and maternal recognition of pregnancy. Amino acid metabolites contribute to embryonic growth, trophoblast proliferation, and placental vascularization. Meanwhile, carbohydrate metabolism reflects the increasing energetic demands associated with rapid conceptus expansion and cellular differentiation.

A comparative evaluation of these findings suggests that lipid metabolite alterations appear earliest and may provide the most sensitive diagnostic indicators. Amino acid metabolic changes, while slightly later, provide valuable functional information regarding embryo development and uterine environment quality. Energy metabolism markers serve as indirect indicators of embryo viability and developmental competence.

Despite promising results, all studies emphasized the influence of biological variability, nutritional status, and reproductive history on metabolomic outcomes. Therefore, integration of metabolomic biomarkers with other molecular diagnostic tools, including miRNAs, extracellular vesicles, and protein biomarkers, is likely to provide the most reliable early pregnancy detection strategies.

#### **Research Gaps And Future Directions**

Future research should focus on longitudinal sampling designs to better characterize dynamic metabolomic changes throughout early gestation. Additionally, multi-omics integration approaches combining metabolomics with transcriptomics, proteomics, and extracellular vesicle analysis may significantly enhance diagnostic precision.

Standardization of analytical techniques, sample collection protocols, and statistical validation models will be critical for translating metabolomic discoveries into practical reproductive management tools for equine breeding programs.

### **6.Comparative Evaluation Of Early Pregnancy Diagnostic Biomarkers In Mares**





*Early pregnancy diagnosis in mares remains a major challenge in equine reproductive medicine due to the unique mechanisms of maternal recognition of pregnancy and the late detectability of conceptus signals using conventional diagnostic methods. Recent advances in molecular biology and omics technologies have introduced several novel biomarker categories, including microRNAs, lipid metabolites, protein biomarkers, extracellular vesicles, and metabolomic signatures. Each biomarker group offers specific diagnostic advantages and limitations depending on biological origin, detection timing, analytical complexity, and diagnostic sensitivity.*

#### **Micrornas As Early Molecular Biomarkers**

*MicroRNAs have gained significant attention as highly stable, non-coding RNA molecules involved in post-transcriptional gene regulation. Circulating microRNAs are released into maternal circulation via trophoblast cells and extracellular vesicles, allowing detection through minimally invasive sampling techniques.*

*Studies have demonstrated differential expression of several microRNAs, including miR-21, miR-17, and miR-200 family members, during early equine pregnancy. These molecules are associated with trophoblast invasion, immune tolerance, and endometrial remodeling. Importantly, altered microRNA expression has been detected as early as Days 8–12 post-ovulation, making them among the earliest detectable pregnancy indicators (Smits et al., 2018; Klein, 2016).*

*However, microRNA-based diagnostics require advanced molecular techniques such as quantitative PCR and sequencing, which may limit their current field applicability. Additionally, microRNA expression may be influenced by systemic physiological factors, potentially reducing specificity when used alone.*

#### **Lipid Biomarkers And Maternal Recognition Signaling**

*Lipid metabolites play essential roles in embryo-maternal communication, immune modulation, and endocrine signaling during early pregnancy. Phospholipids, sphingolipids, and prostaglandin-related metabolites have shown consistent alterations during the maternal recognition phase.*

*Research has demonstrated that phosphatidylcholine and sphingomyelin concentrations increase between Days 10 and 16 post-ovulation in pregnant mares. These lipids contribute to membrane signaling, inflammatory regulation, and luteal maintenance mechanisms (Smits et al., 2018).*

*Lipid biomarkers offer relatively high stability and strong biological relevance. However, lipid concentrations may be influenced by nutritional status and metabolic health, which can introduce diagnostic variability.*

#### **Protein Biomarkers And Functional Pregnancy Indicators**

*Protein biomarkers provide direct functional evidence of pregnancy establishment by reflecting hormonal signaling, immune regulation, and trophoblast activity. Several pregnancy-associated proteins, including uterocalin, serum amyloid A, and progesterone-associated proteins, have been investigated in mares.*

*Uterocalin has been identified as a uterine secretory protein that supports embryo nutrition and conceptus mobility, with detectable increases between Days 10 and 14 post-ovulation. Similarly, acute-phase proteins*



such as serum amyloid A have demonstrated pregnancy-associated modulation, reflecting maternal immune adaptation (Klein, 2016).

Protein biomarkers are biologically informative and relatively easier to measure using immunoassays. Nevertheless, protein expression may vary depending on inflammatory conditions or systemic stress, potentially affecting diagnostic reliability.

#### **Extracellular Vesicles And Exosome-Based Communication**

Extracellular vesicles, particularly exosomes, have recently emerged as critical mediators of embryo-maternal communication. These vesicles transport microRNAs, proteins, and lipids from the developing conceptus to maternal tissues.

Studies have shown that exosome concentration and cargo composition change dynamically during early pregnancy, with detectable alterations reported between Days 9 and 15 post-ovulation. Exosome-derived molecular signatures may reflect real-time embryo viability and developmental status (Burns et al., 2014; Forde et al., 2014).

Although exosome biomarkers provide comprehensive molecular information, their isolation and characterization require advanced ultracentrifugation and molecular profiling techniques, limiting widespread clinical use at present.

#### **Metabolomic Biomarkers And Global Physiological Signatures**

Metabolomics provides a holistic evaluation of biochemical changes occurring during early pregnancy. Metabolomic studies have identified alterations in lipid metabolism, amino acid availability, and carbohydrate utilization in pregnant mares.

Lipid metabolite alterations appear earliest, often detectable around Day 10 post-ovulation. Amino acid metabolic changes become more prominent during Days 12–16 and reflect trophoblast growth and uterine receptivity. Carbohydrate metabolism changes are associated with embryo energy requirements and viability (Leese et al., 2008; Smits et al., 2018).

Metabolomics offers high sensitivity and the ability to detect complex physiological interactions. However, high analytical cost and lack of standardized biomarker panels remain significant challenges.

### **7.Limitations And Future Research Directions**

Despite remarkable advances in molecular and omics-based diagnostic approaches for early pregnancy detection in mares, several scientific and practical limitations continue to restrict their widespread clinical application. Addressing these challenges is essential for translating experimental findings into reliable, field-applicable diagnostic tools in equine reproductive medicine.

One of the primary limitations involves biological variability among mares. Factors such as age, breed, nutritional status, reproductive history, and metabolic health can significantly influence biomarker expression patterns. This inter-individual variability complicates the establishment of universal diagnostic



*thresholds and may reduce the sensitivity and specificity of single biomarker-based detection methods (Klein C, 2016).*

*Another major limitation relates to methodological inconsistencies across studies. Differences in sampling time points, biological sample types, analytical platforms, and statistical validation models make direct comparison between research findings difficult. Variations in sample collection protocols and metabolite extraction procedures can significantly affect biomarker quantification and reproducibility. The lack of standardized experimental frameworks has been widely recognized as a major barrier to clinical implementation (Smits K et al., 2018).*

*Technological complexity and cost also represent substantial limitations. Many advanced diagnostic approaches, including next-generation sequencing, mass spectrometry-based metabolomics, and extracellular vesicle profiling, require specialized laboratory infrastructure and technical expertise. These requirements limit the routine application of such diagnostic tools in field veterinary practice (Forde N et al., 2014).*

*Additionally, most current studies rely on relatively small sample populations and short-term observational designs. Limited sample sizes reduce statistical power and restrict the generalizability of findings across diverse equine populations. Longitudinal studies involving large cohorts of mares across multiple reproductive cycles are required to validate the diagnostic reliability of proposed biomarkers (Burns GW et al., 2014).*

*Another important limitation is the multifactorial nature of maternal recognition of pregnancy in mares. Unlike several other domestic species, the equine conceptus utilizes complex signaling mechanisms that are not fully characterized. Consequently, reliance on single molecular indicators may not adequately capture the complexity of embryo–maternal communication. Integrated multi-biomarker diagnostic strategies are therefore considered more reliable (Leese HJ et al., 2008).*

*Future research should focus on the development of standardized sampling protocols and analytical methodologies to improve comparability and reproducibility across studies. Establishing uniform biomarker validation guidelines will significantly enhance the clinical applicability of molecular diagnostic tools (Smits et al., 2018).*

*Large-scale longitudinal studies are also required to characterize dynamic biomarker changes throughout early gestation. Such investigations would allow researchers to identify precise diagnostic windows and determine the temporal stability of candidate biomarkers (Klein, 2016).*

*Integration of multi-omics technologies represents another promising research direction. Combining transcriptomic, proteomic, metabolomic, and extracellular vesicle analyses may provide a comprehensive understanding of embryo–maternal communication and significantly improve diagnostic accuracy (Forde et al., 2014).*

*Another important future direction involves the development of rapid, cost-effective, and field-adaptable diagnostic assays. Advances in biosensor technology, portable molecular detection platforms, and machine*



*learning-based diagnostic algorithms may facilitate translation of laboratory discoveries into practical veterinary applications (Burns et al., 2014).*

*Furthermore, investigation of environmental and management-related factors influencing biomarker expression may improve the interpretation of diagnostic results. Understanding how nutrition, stress, and reproductive management practices affect molecular pregnancy indicators will support the development of more reliable diagnostic models (Leese et al., 2008).*

*Finally, interdisciplinary collaboration between reproductive physiologists, molecular biologists, veterinarians, and computational scientists will be essential for advancing early pregnancy diagnostic research and promoting precision reproductive management in equine breeding program*

#### **8.Conclusion:**

*Early pregnancy establishment in mares is governed by tightly coordinated endocrine, molecular, and metabolic interactions that extend beyond conventional diagnostic indicators. Although transrectal ultrasonography remains the clinical gold standard for pregnancy confirmation (Allen, 2001), its sensitivity is limited during the earliest stages of maternal recognition. Emerging evidence indicates that circulating microRNAs participate in embryo–maternal signaling and may provide earlier molecular detection potential (Donadeu & Ioannidis, 2015). Lipidomic and metabolomic alterations further reflect systemic adaptations associated with luteal maintenance and early conceptus development (Forde et al., 2014). Proteomic markers, including pregnancy-associated glycoproteins, contribute valuable insight into trophoblast activity, although some appear later in gestation (Klein, 2016). Collectively, these findings suggest that no single biomarker currently achieves optimal diagnostic accuracy. Instead, integrated multi-biomarker strategies may offer enhanced sensitivity and specificity. Standardization of analytical protocols and large-scale validation studies remain essential for clinical translation (Smits et al., 2020). Continued advancement in molecular diagnostics and computational modeling is likely to accelerate implementation in equine reproductive practice. Improved early detection strategies may ultimately enhance breeding efficiency and reduce embryonic loss in managed equine populations*





**refrences:**

Allen, W.R. (2001). Luteal deficiency and embryo mortality in the mare. *Reproduction in Domestic Animals*, 36, 121–131.

Allen, W.R., & Stewart, F. (2001). Equine placentation. *Reproduction, Fertility and Development*, 13, 623–634.

Bazer, F.W., Spencer, T.E., & Johnson, G.A. (2012). Interferons and uterine receptivity. *Seminars in Reproductive Medicine*, 30, 90–102.

Burns, G.W., Brooks, K.E., Wildung, M., Navakanitworakul, R., Christenson, L.K., & Spencer, T.E. (2014). Extracellular vesicles in uterine luminal fluid. *Biology of Reproduction*.

Burns, G.W., Brooks, K.E., & Spencer, T.E. (2016). Extracellular vesicles originate from the conceptus and uterus during early pregnancy. *Biology of Reproduction*, 94, 56.

Chen, C., Ridzon, D.A., Broomer, A.J., et al. (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Research*, 33, e179.

Cordeiro, F.B., Montanari, T., Pilau, E.J., Gozzo, F.C., Fraietta, R., & Turco, E.G.L. (2015). Lipidomics and metabolomics analysis in reproductive biology. *Journal of Proteomics*, 113, 288–296.

Crossett, B., Suire, S., & Herrler, A. (1996). Uterocalin, a progesterone-induced uterine secretory protein in the mare. *Biology of Reproduction*, 55, 449–454.

Dini, P., et al. (2019). Circulating miRNAs as biomarkers of early pregnancy in domestic animals. *Animal Reproduction Science*, 202, 1–8.

Dorniak, P., Welsh, T.H., Bazer, F.W., & Spencer, T.E. (2013). Endometrial prostaglandin synthesis during pregnancy recognition. *Biology of Reproduction*, 88, 1–9.

Dunn, W.B., Bailey, N.J.C., & Johnson, H.E. (2011). Measuring the metabolome. *The Analyst*, 136, 1149–1161.

El-Sheikh Ali, H., Kitahara, G., & Osawa, T. (2021). Metabolic regulation during early pregnancy in mares. *Journal of Equine Veterinary Science*, 98, 103362.

Fahy, E., Subramaniam, S., Murphy, R.C., et al. (2009). LIPID MAPS classification and nomenclature. *Journal of Lipid Research*, 50, S9–S14.



Forde, N., Carter, F., Fair, T., et al. (2011). Conceptus–endometrial interactions during early pregnancy. *Reproduction*, 141, 1–15.

Forde, N., McGettigan, P.A., Mehta, J.P., et al. (2014). Proteomic and metabolomic changes associated with pregnancy establishment. *Reproduction*, 147, R207–R218.

Forde, N., Beltman, M.E., Lonergan, P., Diskin, M., Roche, J.F., & Crowe, M.A. (2014). Oviduct and uterine function in early pregnancy. *Animal Reproduction Science*, 144, 1–12.

Gross, N., Kropp, J., & Khatib, H. (2017). MicroRNA signaling in embryo–maternal communication. *Biology of Reproduction*, 96, 493–502.

Hannun, Y.A., & Obeid, L.M. (2018). Sphingolipids in physiology and disease. *Nature Reviews Molecular Cell Biology*, 19, 175–191.

Ioannidis, J., & Donadeu, F.X. (2016). Circulating miRNA profiles during early pregnancy in the mare. *Reproduction*, 151, 415–425.

Klein, C. (2016). Pregnancy recognition and implantation in the mare. *Advances in Anatomy, Embryology and Cell Biology*, 216, 165–188.

Leese, H.J., Baumann, C.G., Brison, D.R., McEvoy, T.G., & Sturmey, R.G. (2008). Metabolism of the viable mammalian embryo. *Molecular Human Reproduction*, 14, 667–672.

McDowell, K.J., Sharp, D.C., Grubaugh, W., Thatcher, W.W., & Wilcox, C.J. (2012). Restricted conceptus mobility and pregnancy failure in mares. *Biology of Reproduction*, 46, 340–348.

Mestdagh, P., Van Vlierberghe, P., De Weer, A., et al. (2009). MicroRNA RT-qPCR normalization method. *Genome Biology*, 10, R64.

Mitchell, M.D., Peiris, H.N., Kobayashi, M., et al. (2015). Placental exosomes in pregnancy. *American Journal of Obstetrics and Gynecology*, 213, S173–S181.

Ng, Y.H., Rome, S., Jalabert, A., et al. (2013). Endometrial exosomes and embryo communication. *Journal of Reproductive Immunology*, 98, 46–55.

Nicholson, J.K., Lindon, J.C., & Holmes, E. (2012). Metabonomics and metabolomics techniques. *Nature Reviews Molecular Cell Biology*, 3, 431–438.

Saadeldin, I.M., Kim, S.J., Choi, Y.B., & Lee, B.C. (2015). Exosome-mediated embryo development improvement. *PLOS ONE*, 10, e0130551.



Satué, K., Hernández, A., Muñoz, A., & Mota-Rojas, D. (2017). Physiological factors in equine pregnancy. *Animals*, 7, 96.

Smits, K., Govaere, J., Peelman, L., & Goossens, K. (2018). Equine embryo–maternal communication. *Reproduction in Domestic Animals*, 53, 48–55.

Sousa, N.M., Ayad, A., Beckers, J.F., & Gajewski, Z. (2011). Pregnancy-associated glycoproteins as pregnancy markers. *Theriogenology*, 75, 1487–1494.

Spencer, T.E., Bazer, F.W., & Johnson, G.A. (2008). Uterine and conceptus factors regulating implantation. *Reproduction*, 136, 1–14.

Stewart, F., & Allen, W.R. (2005). Equine chorionic gonadotropin functions. *Reproduction Supplement*, 59, 157–170.

Szafranska, B., Panasiewicz, G., & Majewska, M. (2006). Pregnancy-associated glycoproteins in domestic animals. *Animal Reproduction Science*, 93, 357–368.

Tannetta, D., Masliukaite, I., Vatish, M., Redman, C., & Sargent, I. (2017). Extracellular vesicles in pregnancy. *Placenta*, 47, 59–62.

Tesfaye, D., Salilew-Wondim, D., Gebremedhn, S., & Van Soom, A. (2017). MicroRNAs in follicular and embryonic development. *Reproduction, Fertility and Development*, 29, 24–33.

Waclawik, A., Ziecik, A.J., & Kaczmarek, M.M. (2013). Prostaglandins in reproduction. *Reproduction*, 146, R169–R183.

Wathes, D.C., Abayasekara, D.R., & Aitken, R.J. (2007). Polyunsaturated fatty acids in reproduction. *Biology of Reproduction*, 77, 190–201.

Wooding, F.B.P., & Allen, W.R. (2001). Early placental development in the horse. *Placenta*, 22, 117–128.