What Does MicroRNA Expression Say about Human Preimplantation Blastocysts: A Descriptive Analytical Study

by

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I, Arwa Almutlaq confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Embryo quality is critical in in vitro fertilization treatment, significantly influencing the pregnancy success. While preimplantation genetic testing offers a reliable assessment of embryonic chromosomal status, the investigations of the embryo's molecular characteristics remain less implemented. MiRNAs, known for their post-transcription regulatory functions, have emerged as promising markers for genetic disruptions. These small non-coding RNAs found both inside and outside cells and typically exhibit altered profiles in disorders with genetic abnormalities.

In this study, we utilised next-generation sequencing to explore the miRNA expression profile in 122 cryopreserved human blastocysts collected from CRGH, London. The comprehensive miRNA profiling revealed abundant and stable miRNAs expression in blastocysts, with a substantial increase in the levels of miRNAs encoded in key miRNA clusters, such as C19CM and miR-17/92. Functional analysis linked these miRNAs to crucial biological pathways, including protein modification, cell cycle progression, response to low oxygen levels, and apoptosis.

A series of differential miRNAs expression analyses were conducted to identify potential associations between miRNA expression and embryo competence. The findings revealed consistent and significant dysregulation in the miRNA profile in blastocysts with various types of aneuploidies compared to euploid ones. Additionally, differences in miRNA levels were observed among blastocysts at different blastulation days (day5 versus day 6) and between those with varying TE morphology grades.

The miRNA expression profile was also assessed in relation to parental factors known to influence implantation potential and pregnancy outcomes. The results indicated that advanced reproductive age, both maternal and paternal, high ovarian stimulation dosage and impaired sperm parameters are potentially associated with altered miRNA expression in the examined blastocysts. Notably, one miRNA, hsa-miR-184, was consistently upregulated across these investigations. The dysregulated miRNAs in these analyses were commonly involved in cell cycle dynamics, metabolic processes and signalling pathways.

Understanding the molecular differences between good- and poor-quality embryos through miRNA expression could enhance our knowledge of the underlying causes of poor embryonic development and outcomes. Hypothetically, these miRNAs hold promise as biomarkers for evaluating the quality of preimplantation blastocysts, contributing to advancements in reproductive treatment.

Impact Statement

The research conducted on miRNA expression profile in human blastocysts has significant implications for gaining a comprehensive understanding of the preimplantation embryo competence. It provided a broad spectrum of information, shedding light on the genetic network of developing embryos and highlighting the significant involvement of regulatory miRNAs in the highly dynamic gene expression environment of the blastocyst. The observed alterations in miRNA expression profiles in factors associated with low-quality blastocysts provided a solid foundation for understanding the potential underlying causes of implantation failure and early pregnancy loss, ultimately contributing to enhance the success rates of reproductive treatments.

Furthermore, the potential utility of the identified miRNAs with significant expression changes as biomarkers for embryo quality, whether through invasive methods or non-invasive analysis of miRNAs secreted into the culture media, present a promising avenue to enhance the approaches for evaluating embryo quality prior to transfer. This approach could improve current practice in embryo selection, offering more efficient and non-subjective means to assess the embryo competence, thereby increasing the odds of successful pregnancies.

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Writing this section of the thesis was the most challenging task, as standard dictionary words fall short in expressing my profound gratitude to the many people who have been a great support throughout this journey. Foremost among them is my beloved daughter, Leen, who was a great companion and my sole family in London.

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For always being there and lighting my passion, my warmest thanks go to my family and friends. Their emotional support and encouragement provided strength needed to persevere.

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Abbreviations

Abbreviations commonly found in this thesis include:

aCGH	Array Comparative Genomic Hybridization
COS	Controlled Ovarian Stimulation
Ct	Cycle threshold
DNA	Deoxyribonucleic Acid
FC	Fold Change
FDR	False Discovery Rate
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
GnRHa	Gonadotropin-Releasing Hormone Agonist
GV	Germinal Vesicle oocyte
hCG	human Chorionic Gonadotropin
ICM	Inner Cell Mass
ICSI	Intracytoplasmic Sperm Injection
IMSI	Intracytoplasmic Morphologically Selected Sperm Injection
IUI	Intrauterine Insemination
IVF	In Vitro Fertilization
LH	Luteinizing Hormone
MI	Metaphase I oocyte meiosis
MII	Metaphase II oocyte meiosis
miRISC	microRNA-Induced Silencing Complex
miRNA	micro–Ribonucleic Acid
MSR	Mild Stimulation Regime
NGS	Next Generation Sequencing
OHS	Ovarian Hyperstimulation
PCA	Principal Component Analysis
PCOS	Polycystic Ovary Syndrome
PGT	Preimplantation Genetic Testing
PGT-A	Preimplantation Genetic Testing for aneuploid
PGT-M	Preimplantation Genetic Testing for Monogenic Disorders
PGT-SR	Preimplantation Genetic Testing for Structural Rearrangement
qPCR	quantitative Polymerase Chain Reaction
RISC	RNA Induced Silencing Complex
RNA	Ribonucleic Acid
TE	Trophectoderm
UTR	Untranslated Region

Chapter 1 Background and Literature Review

- **Infertility and Assisted Reproductive Technology**

1.1 History and Definition of Infertility

In early human history, the continuation of humankind and its survival was a profound concern. The woman's ability to conceive received substantial attention, representing a matter of social distress. For those facing difficulties in successfully achieving a pregnancy, their situation was perceived as a source of personal shame and social disapproval (Johnston, 1963).

The inability to conceive continued to be a major aspect affecting the quality of life of many couples these days. However, knowledge of the underlying reasons for infertility and the social boundaries in admitting the reproductive issues has been changed and improved.

The World Health Organization (WHO) defines infertility as a" malfunction in the male or female reproductive tract, causing a failure in achieving a pregnancy after a year or more of unprotected sexual intercourse". Recent statistics reveal that infertility affects around one in six individuals within their normal reproductive age (Njagi et al., 2023). Indeed, the introduction of in vitro fertilisation (IVF) into reproductive assessment was a milestone in infertility treatment, providing valuable solutions that have helped many couples to conceive and deepened our understanding of fecundity.

1.2 Assisted Reproductive Technology in Reproductive Treatment

The underlying reasons of infertility are diverse, primarily including abnormalities in the female reproductive tract and male factors related to sperm production and

quality. The pathological causes are varied, however, inflammation, hormonal disorders, tube obstructions and genetic abnormalities are among the most common. Additionally, many couples seeking reproductive assessment suffer from unexplained infertility.

Reproductive treatment begins with a thorough medical history review and a comprehensive physical examination of the patients. The typical assisted reproductive technology (ART) treatment involves a range of medical procedures including controlled ovarian stimulation (COS) and induction of oocyte triggers via hormonal treatment to access an acceptable number of mature oocytes, followed by the surgical removal of eggs from the females' body, and in vitro fertilising them with the good-quality sperm chosen after semen analysis (Girsh, 2021). The fertilised cells that developed normally and reached day 3 to day 6 stage of development, depending on the clinic's protocols, are returned to the uterus or frozen for future use. The number of transferred embryos differs from case to case and clinic to another, with a general preference for singletons.

During IVF treatment, the natural menstrual cycle is suppressed, and the entire processes of follicular development, stimulation, and maturation, is controlled using exogenous hormones. A typical IVF cycle begins with downregulation of natural hormones, commonly achieved through administration of gonadotropin releasing hormone (GnRH) agonists or antagonists, that eventually leads to reduction/blocking of the GnRH receptors. Both approaches lead to the suppression of Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH) production by the pituitary gland. Following this suppression, the patient undergoes a course of follicular stimulation with exogenous gonadotropins (FSH) for a specific period, which is determined based on the patients' characteristics and previous reproductive history (Alyasin et al., 2016).

When the bundle of stimulated follicles reaches the appropriate size, a trigger medication is given to the patient 36 to 38 hours before the oocyte retrieval procedure (Coccia et al., 2004). The primary purpose of the trigger medication is to ensure that oocytes have reached the appropriate stage of maturity for collection. However, it is commonly observed that some oocytes are retrieved at immature

stages, such as GV and MI stages. In such cases, these oocytes are cultured until they reach MII stage, which is the optimal stage for insemination.

The medication used to trigger oocyte maturation are in two types: gonadotropin-releasing hormone agonist (GnRHa) and human chorionic gonadotropin (hCG). These triggers function in different mechanisms. GnRHa causes a temporary surge of the natural LH, while hCG mimic the action of LH, which is responsible for the final maturation of the egg. The choice between the two methods remains controversial. GnRH is generally administrated when there is a risk of ovarian hyperstimulation (OHS) but it may not be the optimal choice for fresh transfer (Youssef et al., 2014). A combined trigger, also called double or dual trigger, which includes both GnRHa and hCG, was considered for better oocyte maturation and pregnancy odds (Lin et al., 2013).

At the day of egg collection, the male partner provides a semen sample, in which semen analysis is conducted to evaluate the quality of sperm both macroscopically by checking the colour, volume and viscosity, and microscopically, by assessing sperm parameters, such as morphology, motility, concentration and count (Baskaran et al., 2021). The selection of the best quality sperm for insemination constitutes the second essential half of the treatment. Thereafter, the mature oocyte is typically inseminated through either in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (Goldberg et al., 2007).

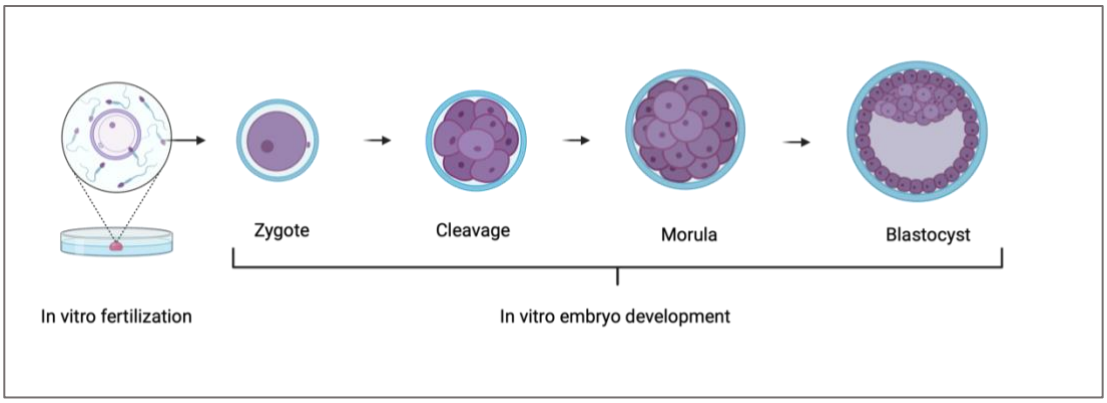
Three main fertilization approaches are employed in the reproductive treatment: intrauterine insemination (IUI), IVF and ICSI. Selection of the most suitable approach usually depends on the diagnosis and the history of infertility. IUI is the less invasive procedure, involving the insertion of sperm sample inside a woman's womb to facilitate fertilization, communally used in mild infertility cases. In cases of more pronounced infertility, IVF and ICSI are utilized. IVF allows for a natural penetration of egg by the sperm provided in the culture media, while ICSI involves a direct injection of a single sperm into an egg using a microneedle (Girsh, 2021).

- **Embryo Quality and Selection to Transfer**

1.3 Fertilization and Embryo Competence

After fertilization, the embryo undergoes critical developmental processes that significantly influence its quality and implantation potential outcomes. Preimplantation development involves four key stages: the zygote, cleavage divisions, morula, and blastocyst formation (Figure 1-1). Throughout these stages, the embryo experiences essential transitions, such as embryonic genome activation and the formation of early embryonic lineages. The morphokinetic properties of the embryo during these stages, assessed microscopically either through traditional methods or by time-lapse imaging, serves as predictors of the embryo's developmental potential. In IVF practice, this evaluation is crucial in determining the embryo fate whether to be transferred, frozen, or discarded.

Figure 1-1: Preimplantation embryo developmental stages

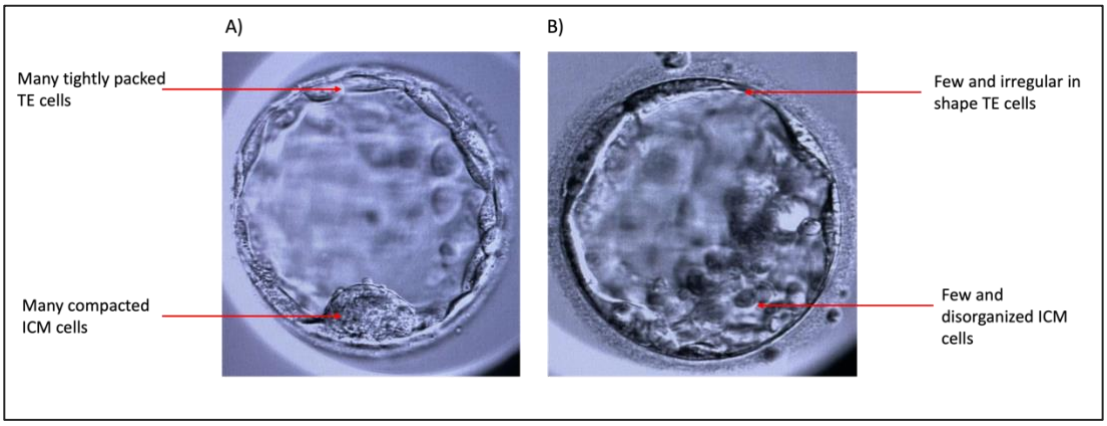


Developmental stages of preimplantation embryos, including zygote, cleavage divisions, morula, and blastocyst formation

One of the most crucial criteria for embryos selection is their morphology at the day of transfer. The assessment of blastocyst morphology follows well-established grading systems, like the Gardner grading system (Gardner and Balaban, 2016). This system assesses three primary components of the blastocyst: the enlargement of the blastocyst fluid cavity and the development of the two cell components, inner cell mass (ICM) and trophectoderm (TE) (Gardner D. K., 2007). The blastocoel cavity expansion is graded on a scale from 1 to 6, with a grade of 6 representing a hatched blastocyst characterized by a large cavity and a thinning zona pellucida lining shell.

The ICM and TE components are evaluated separately based on the cell compaction, size, and distribution. Higher grade (A) indicates good morphology with many compacted cells, while lower grades (C) denote poorer morphology, characterized by fewer, larger cells (Figure 1-2).

Figure 1-2: Blastocyst morphology grading based on the Gardner grading system



The figure shows: A) An unhatched blastocyst graded 4AA based on morphology, with a large, tightly packed ICM and numerous cohesive TE cells. B) A blastocyst graded 3CC, displaying few large, irregular ICM and loosely arranged TE cells.

Investigations into ongoing pregnancy and live birth rates for embryos with varying ICM and TE morphology scores in euploid blastocysts revealed better outcomes of good and average-graded blastocysts compared to the poor-graded ones, for both ICM and TE (Nazem et al., 2019). However, it appears that the ICM quality is a better predictor of final pregnancy outcomes (Ai et al., 2021). Moreover, while some studies have indicated correlations between morphological characteristics and gene expression of blastocysts, the research in this area is still limited in scope (Wells et al., 2005, Chousal et al., 2024).

Reaching the blastocysts stage is a crucial milestone in preimplantation development, indicating good developmental potential of the embryo. It is also the stage where the genetic tests are conducted. In vitro-cultured embryos may exhibit varying rates of development, with the blastocyst stage typically achieved on day 5 of fertilization in some cases, while in others, it may occur on day 6. In rare cases, blastulation may be delayed until day 7, often associated with abnormal development. Several studies have compared the implantation rate and pregnancy outcomes between blastocysts developed on day 5 and day 6, consistently showing a preference for day 5

blastocysts in achieving successful implantation and clinical pregnancy, whether fresh or vitrified (Bourdon et al., 2019, Li et al., 2020). Therefore, it is the usual preference to transfer, or freeze, the embryos that reached blastocysts at day 5 compared to embryos at other developmental stages.

Further quality assessments are conducted when the embryo reaches the blastocyst stage, to additionally ensure good embryo quality. These assessments are usually performed upon clinician or patient request, and typically involve testing of the blastocyst's deoxyribonucleic acid (DNA) after isolation of 5 to 6 trophoblast cells from the outer layer of the embryo, trophectoderm. The genetic testing of the embryo is known as preimplantation genetic testing, which involve three main types, Preimplantation Genetic Testing for Monogenic disorders (PGT-M), Preimplantation Genetic Testing for Aneuploidy (PGT-A), and Preimplantation Genetic Testing for Structural Rearrangements (PGT-SR) (Parikh et al., 2018). The PGT tests emerged as additional tests, promising to help exclude embryos with lower chances of implantation and select those without genetic abnormalities, thereby aiming for optimal pregnancy outcomes.

1.3.1 Preimplantation Genetic Testing for Aneuploidy

Aneuploidy is often considered the major cause of miscarriage (van den Berg et al., 2012). Chromosomal abnormalities are very common in early human embryos, arising during meiotic divisions of the gonad cells and/or mitotic divisions of the preimplantation embryos. Aneuploidy can occur through different mechanisms including chromosomes/chromatids non-disjunction, anaphase lag, endoreplication and uniparental disomy (Taylor et al., 2014a). The meiotic aneuploidies originated during oocyte meiosis I (MI) and/or meiosis II (MII) have long been considered the prime cause of female infertility (Fragouli et al., 2010). Chromosomal errors can also arise post-zygotically in embryos, with chromosomal mosaicism and segmental abnormalities frequently observed during the cleavage stages (Delhanty et al., 1993, Vanneste et al., 2009). Notably, different types of chromosomal imbalances, including trisomies, monosomies, as well as whole, segmental, and mosaic aneuploidies, have been observed in early human embryos (Clouston et al., 1997).

To optimize the benefits of in vitro fertilization (IVF) treatment, PGT-A, previously known as preimplantation genetic screening (PGS), has been adopted. This test aims to increase implantation and pregnancy rates by eliminating transfer of aneuploid embryos (Verlinsky and Kuliev, 1996). Initially, aneuploidy testing involved examining the chromosomal status of the oocyte by analysing its polar bodies (Verlinsky et al., 1998). The next method involved collecting an embryonic biopsy of 1 to 2 blastomeres at the cleavage stage (day 3-4) to aid in embryo selection for transfer. This method was used for approximately 10 years before transitioning to a biopsy of 5-6 trophoblasts from the outer layer of blastocyst embryo at day 5-6 (Huang et al., 2013). The shift from blastomere to trophoblast biopsy in clinical practice was prompted by the high level of mosaicism observed in human embryonic cleavage-stage cells and the limited number of cells available for testing, which affected clinical diagnosis (Dokras et al., 1990, Delhanty et al., 1997).

In this approach, the DNA extracted from biopsied TE cells are utilized to investigate the aneuploidy status of embryos in PGT-A. Over the years, three methods have been employed to examine chromosome number and structure in the embryos: fluorescence in situ hybridization (FISH), limited to testing a few chromosomes, array CGH (Comparative Genomic Hybridization) and sequencing (Verlinsky and Kuliev, 1996, Alfarawati et al., 2011, Handyside and Ogilvie, 1999, Zheng et al., 2015). While aneuploidy testing of TE biopsied cells demonstrated high compatibility with the overall embryo aneuploidy status, this is only true for whole chromosomal aneuploidy, not for segmental or mosaic cases (Orvieto et al., 2016, Capalbo et al., 2016a, Huang et al., 2017, Victor et al., 2019). Therefore, according to the latest recommendation update from the Human Fertilization and Embryology Authority (HFEA), PGT-A can reduce the chance of miscarriage but does not necessarily increase the chance of having a baby.

- **MicroRNA: Discovery, Biogenesis, and Function**

1.4 miRNA Discovery

MiRNA was first observed in Caenorhabditis elegans (C. elegans) in early 1990's, where it was recognized for its regulatory function on *lin-4* gene and its impact on the expression of the LIN-14 protein (Lee et al., 1993, Wightman et al., 1993). MiRNAs are described as non-coding genes that produce small antisense RNAs, which bind to mRNA target and block its function. Their regulatory mechanism operates through a complementary sequence to the 3′ untranslated region (UTR) of its mRNA target, allowing the miRNA to bind and prevent translation (Bartel, 2009, Krol et al., 2010). The insights provided by this discovery into the control of non-coding RNAs on gene expression, and the mechanism of interaction with other gene transcripts, has opened the door to a revolutionary era in molecular biology. Following this discovery, extensive research has been conducted to understand miRNA molecular and structural features.

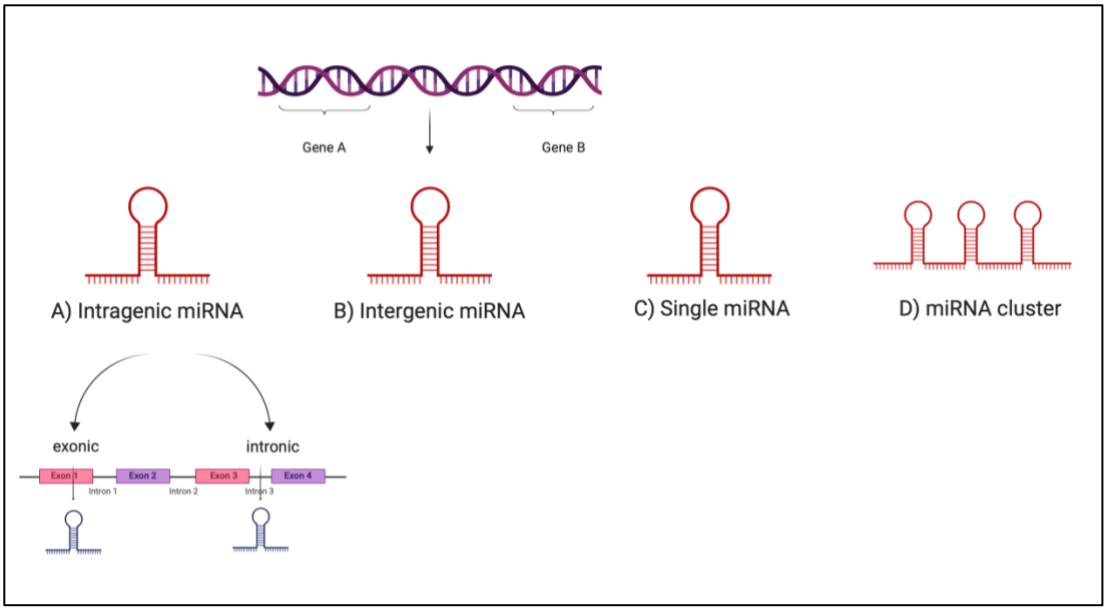
1.5 miRNA Biogenesis and Biological Role

The production of miRNAs necessitates a spatio-temporal control, in which cell/tissue type and stage of cell development determine what miRNAs to be expressed. Moreover, transcriptional factors (TFs), such as TP53, c-Myc and E2F, play a role in controlling the transcription process of miRNA coding genes (Xi et al., 2006, Tarasov et al., 2007, Brosh et al., 2008, O'Donnell et al., 2005). Notably, TFs and miRNAs often participate in co-regulatory loops, exerting control over gene expression at both transcriptional and post-transcriptional levels (Zhang et al., 2015a). Furthermore, miRNA expression process can be modulated by epigenetic mechanisms, like DNA methylation at the promoter regions of miRNA coding genes (Lujambio et al., 2007, Brueckner et al., 2007, Lodygin et al., 2008).

MiRNAs have different genomic roots; most of them are encoded in introns and few in exons (Rodriguez et al., 2004). A large proportion of human miRNA genes occur in clusters (Altuvia et al., 2005), in which a single cluster can comprise two or more adjacent miRNAs, which are usually transcribed together in the same orientation

(Kabekkodu et al., 2018). However, miRNA loci can also be found as single units that are transcribed individually (Figure 1-3).

Figure 1-3: miRNA transcriptional origin and genomic roots



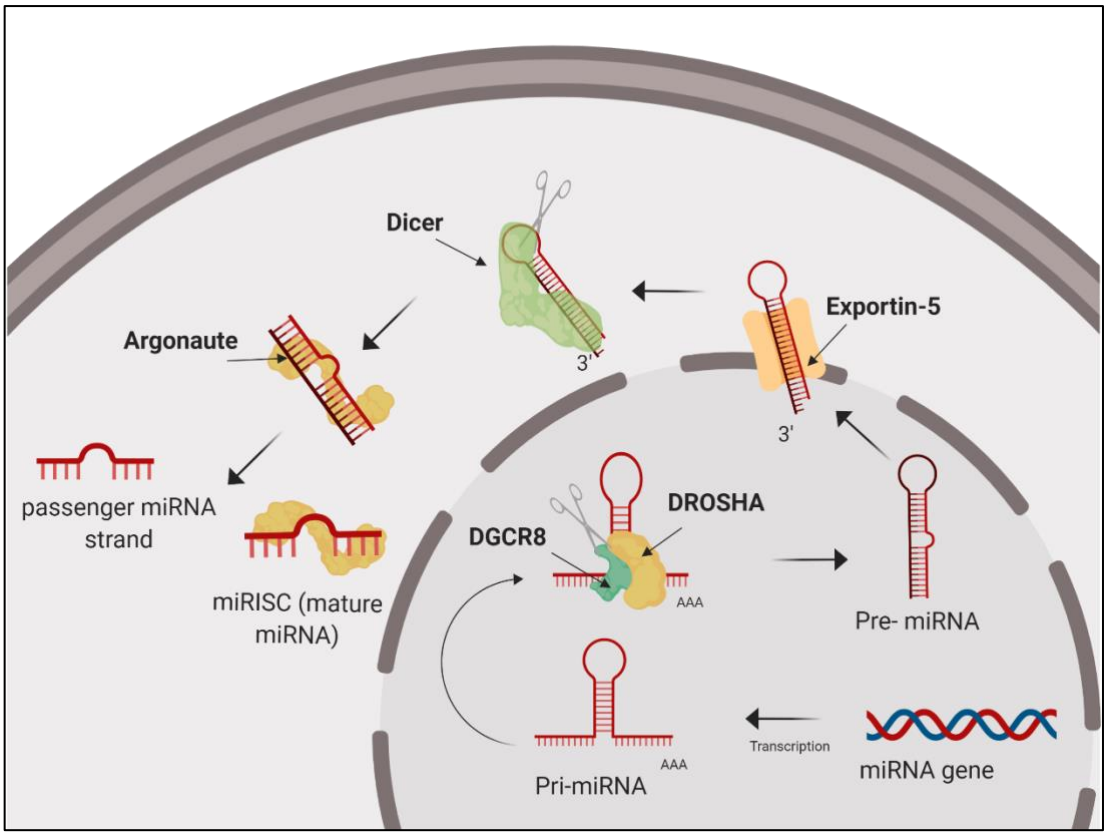
The figure illustrates the genomic origins of miRNA: A) Intragenic: miRNA is transcribed from the protein-coding region; it can be encoded from within an intron or an exon. B) Intergenic: miRNA is transcribed from non-coding region. C) Singly transcribed miRNA gene. D) Adjacent miRNA genes transcribed together (miRNA cluster). (BioRender.com).

1.5.1 Biogenesis Process

The mechanism of miRNA biogenesis has two pathways, canonical/non-canonical, occurring in multiple phases. The vast majority of miRNA genes are processed via the canonical lane (See Figure 1-4), which necessitates the cleavage act of two miRNA biogenesis proteins Drosha Ribonuclease III (DROSHA) and Dicer1 ribonuclease III (DICER1). miRNAs are first transcribed by RNA II, III polymerase enzymes (Lee et al., 2004, Borchert et al., 2006), which yields primary miRNA (pri-miRNA), a long RNA element -over 1K bp- with an embedded hairpin structure that encompasses the miRNA sequence and single strand fragments in the 3′ and 5′ ends (Creugny et al., 2018). Following transcription, the endonuclease protein DROSHA form a microprocessor complex with its cofactor DiGeorge Syndrome Critical Region8 (DGCR8) and cleaves the pri-miRNA transcript to produce a ~ 65-120 nucleotide hairpin structure precursor miRNA (pre-miRNA) (Landthaler et al., 2004,

Denli et al., 2004, Gregory et al., 2004, Han et al., 2004, Lee et al., 2003). This product is recognized by Exportin-5 (XPO5), a nuclear export protein that forms a complex with the pre-miRNA molecule and exports it to the cytoplasm (See Figure 1-4) (Bohnsack et al., 2004, Lund et al., 2004, Zeng and Cullen, 2004).

Figure 1-4: miRNA biogenesis process



The miRNA gene is transcribed to pri-miRNA. The RNA binding protein component of DGCR8 binds the pri-miRNA strand, then DROSHA cleaves it by cutting the 3´ and 5´ strands of the hairpin-shaped miRNA leaving a precursor miRNA (pre-miRNA) with 3′ protruding end (Zhang et al., 2004, Han et al., 2004). Exportin-5 carries pre-miRNA to the cytoplasm where DICER1 continues the miRNA cleavage. Argonaute proteins splits the double stranded miRNA producing a small non-coding single stranded mature miRNA gene. (BioRender.com).

Maturation of miRNA continues in the cytoplasm, where pre-miRNA is cleaved by the cytoplasmic endonuclease DICER1 (Lee et al., 2006, Ketting et al., 2001). The second cleavage process, results in removing the terminal loop and generating a 21-25 nucleotide miRNA duplex (Zhang et al., 2004). The double stranded miRNA is then loaded into Argonaute (AGO) proteins forming a complex called pre-RNA-induced silencing complex (pre-RISCs) (Kawamata et al., 2009). Only one strand, the guide strand, remains loaded into AGO protein, while the other passenger strand

is released and degraded (See Figure 1-4) (Okamura et al., 2004, Yoda et al., 2010). The main functional components of miRISC (miRNA and RISC) are AGO proteins, which mediate the sequence-specific binding of miRNA to mRNA. In humans, four forms of Ago family (AGO1-AGO2-AGO3-AGO4) are involved in miRNA maturation process (Liu et al., 2004, Meister et al., 2004, Yoda et al., 2010). They identify and firmly bind the 5′ phosphate terminus of guide miRNA strand, which contain the complementary sequence to the target mRNA (Parker et al., 2005, Ma et al., 2005, Boland et al., 2011).

While the proteins involved in the canonical pathway are fundamental for biosynthesis of the majority of miRNAs, around 1% of mature miRNAs are produced independintly of these proteins via non canonical pathway. For example, DROSHA cleavage process is bypassed in the production of miR-320, which is recognised and exported by Exportin-5 directly after transcription (Xie et al., 2013). Likewise, miR-451 maturation does not require DICER1 for cleavage, but instead its pre-miRNA is cleaved by AGO proteins catalytic activity (Cheloufi et al., 2010).

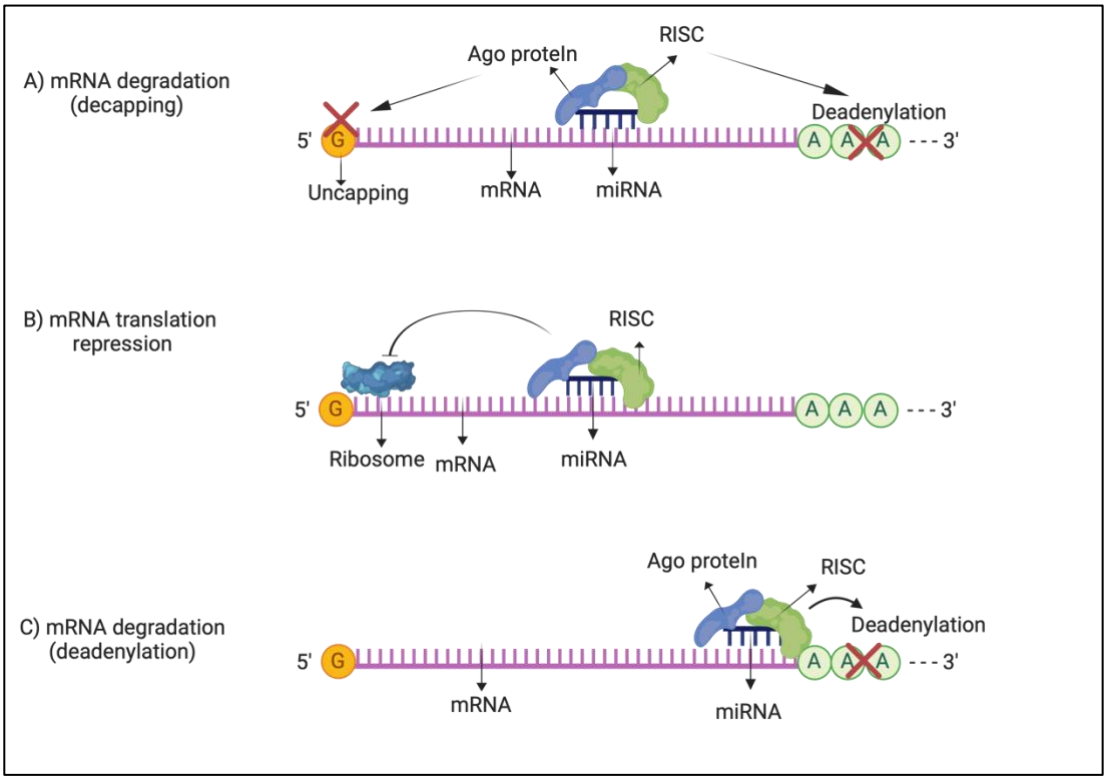
1.5.2 Mechanism of miRNA-mRNA Interaction

The regulatory mechanism of RNA molecules in modulating gene expression was initially elucidated through the discovery of the RNA interference (RNAi), which plays a crucial role in gene suppression (Fire et al., 1998). It was hypothesized that the interference process was not just a simple RNA-RNA binding, but also the work of catalytic actions, which were confirmed later by the discovery of RNA induced silencing complex (RISC) (Fire et al., 1998, Weiss and Ito, 2017). Although the miRNA seed sequence, a short nucleotide sequence complementary to the target RNA, is responsible for target recognition, it is the protein - protein interaction that mediates the silencing mechanism. Via RISC, miRNA can prompt two mechanisms of gene silencing: translational repression and/or deadenylation (Wu et al., 2006). In translational inhibition, miRISC repress the expression of mRNA with or without degradation, whereas deadenylation leads to mRNA decay (Wightman et al., 1993, Bagga et al., 2005).

Several studies on different species suggested various models of target repression (Figure 1-5) (Wang et al., 2006, Mathonnet et al., 2007, Thermann and Hentze, 2007,

Wakiyama et al., 2007). Some suggested that miRNA interference occurs at the initiation step of mRNA translation (Pillai et al., 2005, Humphreys et al., 2005), whereas others revealed that repression can happen at some point after translational initiation (Figure 1-5 (B)) (Petersen et al., 2006, Lytle et al., 2007). Biological and structural experiments revealed the key role of AGO and its accompanying proteins in accuratly predicting AGO–mRNA associations sites and in inducing mRNA repression through different mechanismems (Figure 1-5) (Pillai et al., 2004, Li et al., 2014).

Figure 1-5: Mechanisms of miRNA targeting and blocking mRNA

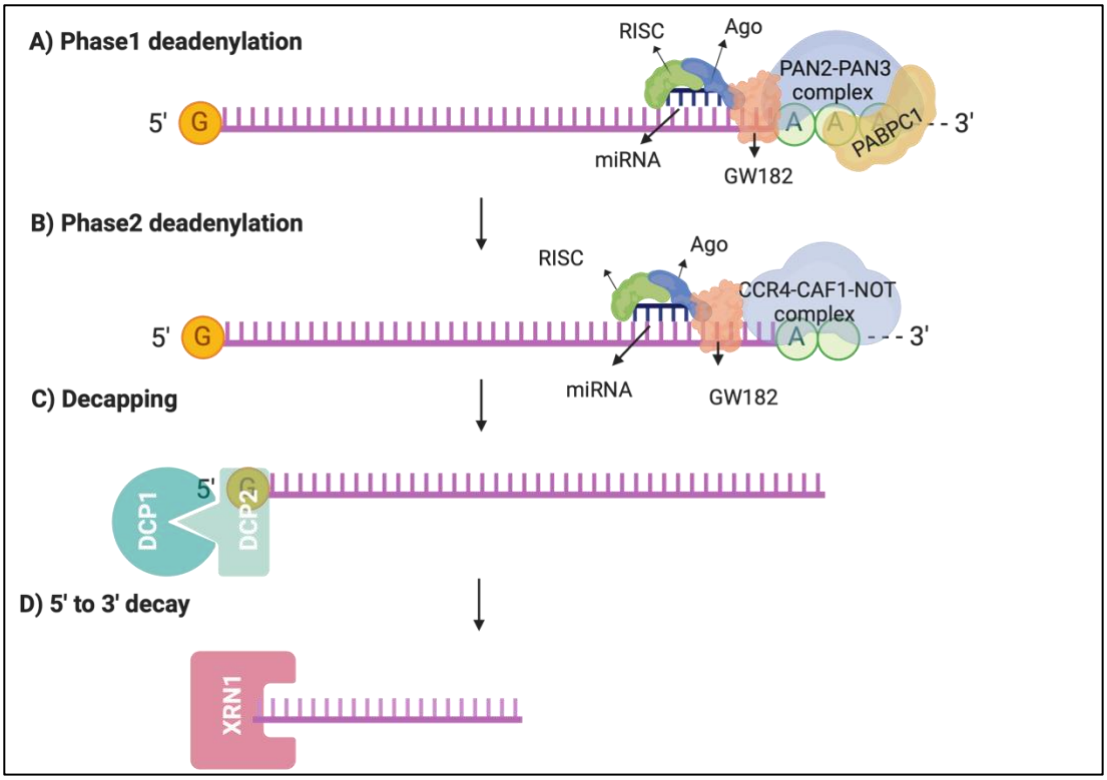


The figure shows various mechanisms of mRNA blocking by miRNA. A) AGO protein competes with a CAP binding protein which prevent translation and RISC disturbs the integrity of mRNA by removing the poly-A tail structure. B) miRISC complex including AGO proteins blocks translation of mRNA. C) miRISC directly decays mRNA without translation repression by deadenylating the poly-A tail. (BioRender.com).

The regulatory role of miRNAs on gene expression is not limited to control protein production by translation repression but extends to induce a significant reduction in mRNA abundance through mRNA decay in some cases. MiRNA mediates degradation through destabilization of mRNA structure by removing the poly(A) tail (Figure 1-6 (A and B)), which contributes strongly to mRNA stability and translation

intiation (Wu et al., 2006). The decay process of mRNA continues by removing the cap structure at the 5`end (Figure 1-6 (C and D)) (Behm-Ansmant et al., 2006).

Figure 1-6: mRNA degradation mechanisms



The illustration shows mRNA deadenylation, decapping and decay by miRNA. A) Deadenylation of mRNA targeted by miRNA starts with shortening of the poly(A) tail by the PAN2-PAN3 complex. B) The second catalytic enzyme complex (CCR4-CAF1-NOT) continuous the deadenylation process. C) DCP1, DCP2 decay complex removes the cap structure at the 5`end. D) XRN1 digests the targeted mRNA. (BioRender.com).

1.6 miRNA localisation

The localisation of miRNAs both within and outside the cell is crucial for their function. Typically, miRNAs are expressed in the cytoplasm, where they associate with AGO proteins to form RISC complex. This complex binds to and regulates the expression of their target mRNAs. Additionally, studies have shown that miRNAs are also present in the nucleus, where they act as modulators of gene transcription and chromatin structure (Makarova et al., 2016). miRNAs are not confined to intracellular locations; they are frequently released into the circulation and found in body fluids and the extracellular space. Packaged in extracellular vesicles, miRNAs are often found in exosomes, small extracellular vesicles that are released by cells,

which play a role in cell-to-cell communicating and signalling (Zhang et al., 2015b, Makarova et al., 2016).

1.7 Review of Literature on the Role of miRNA in Reproductive Health

miRNAs are crucial regulators of gene expression, with 2,654 mature miRNAs discovered in humans according to the miRBase Release 22, March 2018 (Manchester, 2018, Kozomara et al., 2019). Computational and experimental studies estimate that over two-thirds of protein-coding genes are regulated by miRNAs (Friedman et al., 2009). These small RNA molecules play pivotal roles in essential cellular processes, such as proliferation, differentiation and apoptosis (Xu et al., 2003, Brennecke et al., 2003). Importantly, a single miRNA can regulate multiple genes, and reciprocally, one mRNA gene can be targeted by several miRNAs. While most miRNAs act post-transcriptionally by inhibiting translation, some can interact with gene promoters to inhibit the mRNA transcription (Kim et al., 2008).

With a growing body of evidence highlighting the influence of miRNA in various biological processes, their regulatory role on gene expression has gained increased attention in gamete maturation and embryonic development. The utilization of miRNA in this field has helped identify underlying causes of reproductive failure and pregnancy abnormalities. For instance, experiments involving knockout of *DICER1*, a key enzyme in miRNA biogenesis, resulted in embryo lethality (Bernstein et al., 2003). Moreover, mutations in this gene have been linked to infertility in mice (Otsuka et al., 2008). In gamete cells, miRNAs have a key role in the development and maturation of oocytes and have shown significant alterations in morphologically abnormal sperm (Liu et al., 2016, Danis and Samplaski, 2019).

Dynamic changes in the protein levels observed during oocyte maturation indicate significant transcriptional regulation. In the same context, distinct miRNA expression levels were detected at different stages of normal oocyte development, with some acting as important mediators during follicular development (McBride et al., 2012, Abd El Naby et al., 2013, Gilchrist et al., 2016). At later stages of maturation, as the oocyte becomes transcriptionally inactive, gene regulation is maintained at the post-transcriptional level with the assistance of non-coding RNAs (Suh and Blelloch,

2011, Tscherner et al., 2019). The dysregulation of miRNA expression in the ovarian follicular environment has been linked to oocyte aging and reproductive issues, such as polycystic ovary syndrome (PCOS) (Diez-Fraile et al., 2014, Liu et al., 2015, Xu et al., 2015, Battaglia et al., 2016).

Moreover, miRNAs play a significant role in mediating the communication between embryonic cells and the endometrium. Previous studies have highlighted the significance of miRNAs in regulating several signalling pathways, particularly those involved in embryo-endometrium communication, demonstrating their strong involvement in such interaction (Gross et al., 2017). Notably, the miRNAs within the endometrial lining are well-established regulators of the endometrial receptivity (Chen et al., 2016, Zhou and Dimitriadis, 2020, Akbar et al., 2020, Shekibi et al., 2022). Abnormal expression of miRNAs involved in the embryo-endometrial dialogue found to be associated with implantation failure (Cuman et al., 2015, Rosenbluth et al., 2014). On the embryonic side of this interplay, miRNAs are also pivotal in placenta development, with abnormal expression linked to several placental abnormalities, including preeclampsia (Mouillet et al., 2011, Kumar et al., 2013, Lv et al., 2019, Li and Xu, 2020).

miRNAs are also prominently expressed in various testicular cells and play a crucial role in regulating spermatogenesis (Khawar et al., 2019). Aberrations in *DICER1* expression within these cells adversely affect sperm maturation, leading to spermatogenic failure (Romero et al., 2011, Bjorkgren and Sipila, 2015). The depletion of specific miRNAs, such as miR-34b/c, in spermatocytes causes impaired maturation of spermatozoa (Comazzetto et al., 2014). In fact, several studies have demonstrated a link between miRNA expression and various sperm defects, such as asthenozoospermia and oligoasthenozoospermia (Comazzetto et al., 2014, Abu-Halima et al., 2013, Wu et al., 2013). Interestingly, the sperm-born miRNAs showed a broader impact, influencing early embryo development and potentially affecting offspring well-being (Khawar et al., 2019, Alves et al., 2020).

Notably, previous studies have revealed association between gamete's gene expression patterns and pregnancy outcomes (Ouandaogo et al., 2012, Uyar et al., 2013, Tomic et al., 2022, Silva et al., 2022, Llavanera et al., 2022). However, the

potential influence of altered gene expression profiles, particularly miRNAs, on the resulting embryo remains understudied.

The current research on miRNAs in embryos primarily focuses on their expression in placenta and embryonic stem cells (ESC), highlighting their roles in processes like implantation and cell differentiation (Galliano and Pellicer, 2014, Paul et al., 2019, Mouillet et al., 2011, Ran et al., 2017, Zhang et al., 2019b). Research indicates a high abundance of miRNAs in early developing embryos, which were also found extracellularly in the blastocoel fluid and diffused into the culture media (Battaglia et al., 2019, Russell et al., 2020). However, only a limited number of studies have assessed the direct correlation between blastocyst miRNAs and embryo competence, with most research discussing the association between miRNA expression and the aneuploidy status in preimplantation embryos (Rosenbluth et al., 2013, Rosenbluth et al., 2014, McCallie et al., 2014, Esmaeilivand et al., 2022). The results of these studies consistently revealed changes in miRNA profiles in aneuploid embryos, despite variations in the extraction sites within embryos, such as blastocysts, blastocoel fluid and the culture media (Almutlaq et al., 2024).

As a potential non-invasive biomarker for preimplantation embryos, miRNA expression in the culture media have garnered considerable attention and has been widely studied for its corelation with embryo developmental competence and pregnancy outcome (Kropp et al., 2014, Cuman et al., 2015, Borges et al., 2016, Capalbo et al., 2016b, Abu-Halima et al., 2017, Cimadomo et al., 2019, Abu-Halima et al., 2020, Acuna-Gonzalez et al., 2021, Fang et al., 2021, Wang et al., 2021, Timofeeva et al., 2021, Kamijo et al., 2022). Evidence indicates different miRNA expression profiles at various embryo developmental stages, revealing the dynamic nature of miRNA expression during early development (Timofeeva et al., 2019, Paloviita et al., 2021, Esmaeilivand et al., 2024, McCallie et al., 2014, Esmaeilivand et al., 2022). Beyond their correlation with aneuploidy status, changes in miRNA expression in the spent media have also been observed in relation to other aspects of preimplantation embryo quality, such as embryonic morphokinetics and blastocyst morphological criteria (Rosenbluth et al., 2013, Sanchez-Ribas et al., 2019, Timofeeva et al., 2020, Berkhout et al., 2020, Coticchio et al., 2021, Esmaeilivand et al., 2024, McCallie et al., 2014, Esmaeilivand et al., 2022). Studies addressing the correlation between human preimplantation embryo quality and miRNA expression

are summarized in (Table 1-1). While the link between miRNA expression and embryo competence is well-established, a notable gap remains in comprehensively investigating the miRNAs expressed in preimplantation blastocysts and their involvement in compromised quality.

Table 1-1: Overview of Literature on miRNA Expression in Human Embryos

Reference	**extraction site**	**Aim**	**Stage**	**Method**
(Battaglia et al., 2019)	BF	Profiling of miRNA in BF	Blastocyst	Array based real time PCR (TaqMan Low-Density Arrays-(TLDA)
(Russell et al., 2020)	Blastocyst	Profiling of miRNA in the CM	Blastocyst	Sequencing and qPCR
(Rosenbluth et al., 2013)	Blastocyst	Aneuploidy-gender	Blastocyst	Array based real time PCR (TLDA)
(Esmaeilivand et al., 2024)	TE	Aneuploidy	Blastocyst	qPCR
(McCallie et al., 2014)	Blastocysts	Aneuploidy-maternal age	Blastocyst	Array based real time PCR (TLDA)
(Esmaeilivand et al., 2022)	BF	Aneuploidy	Blastocyst	q PCR
(Rosenbluth et al., 2014)	CM	Profiling/aneuploid	Blastocyst	Array based real time PCR (TLDA)
(Sanchez-Ribas et al., 2019)	CM	Profiling/aneuploid	Day 3	Sequencing and qPCR
(Kamijo et al., 2022)	CM	Pregnancy outcome	Blastocyst	qPCR
(Kropp et al., 2014)	CM	Developed/arrested	Blastocyst	qPCR
(Acuna-Gonzalez et al., 2021)	CM	Pregnancy outcome	Blastocyst	RT-PCR
(Fang et al., 2021)	CM	Pregnancy outcome	Blastocyst	Sequencing and quantitative PCR
(Wang et al., 2021)	CM	Pregnancy outcome	Blastocyst	Sequencing and quantitative PCR
(Borges et al., 2016)	CM	Implantation potential	Day 3/blastocyst	qPCR

(Cimadomo et al., 2019)	CM	Implantation potential	Blastocyst	Array based real time PCR (TLDA)
(Cuman et al., 2015)	CM	Pregnancy outcome	Blastocyst	qPCR
(Capalbo et al., 2016b)	CM	Pregnancy outcome	Blastocyst	Array based real time PCR (TLDA)
(Abu-Halima et al., 2017)	CM	Pregnancy outcome	Blastocyst	Microarray
(Timofeeva et al., 2021)	CM	Pregnancy outcome	Blastocyst	Sequencing and qPCR
(Abu-Halima et al., 2020)	CM	Development competent and pregnancy outcome	Day3	PCR and qPCR
(Paloviita et al., 2021)	Oocyte and embryo developmental stages	Developmental stage	reached blastocyst/ arrest in morula	Sequencing
(Timofeeva et al., 2019)	CM	Morphology/developmental stage	Morula/blastocyst	Sequencing and qPCR
(Timofeeva et al., 2020)	CM	Morphology	Morula	Sequencing and qPCR
(Berkhout et al., 2020)	CM	Morphology/fragmentation	Day 3/blastocyst	PCR and Sequencing
(Coticchio et al., 2021)	CM	Morphology	Blastocyst	qPCR

Several aspects related to embryo quality can be better understood through comprehensive studies of its transcriptomic profile. For instance, miRNA may serve as a valuable tool for evaluating the consequences of chromosomal abnormalities in preimplantation embryos. Studies in mouse models have suggested self-correction mechanism for mosaic aneuploidies, indicating a full developmental potential of mosaic embryos (Barbash-Hazan et al., 2009, Bazrgar et al., 2013, Bolton et al., 2016). In humans, the high percentage of mosaicism in blastomeres (the cleavage stage-cells) that decreased downstream in the blastocyst stage also suggest a self-correction response (Vanneste et al., 2009). Given the miRNAs' role in reprograming critical activities such as DNA repair and replication, cell cycle regulation, and

apoptosis, they likely contribute to repairing or arresting cells with chromosomal abnormalities (Zore et al., 2019, Wenzel and Singh, 2018, Weiss et al., 2022, Singla et al., 2020, Golubnitschaja, 2007, Cortez, 2019).

While miRNAs have been frequently studied in the culture media of preimplantation embryos, a comprehensive understanding of their role in determining the embryo quality is still greatly needed (Ciaudo et al., 2009, Hayder et al., 2018). Given the unique characteristics of miRNAs, particularly their size and stability, analysing their expression in relation to known embryonic and parental factors could provide valuable insights into the genetic status of lower-quality embryos. This approach could enhance the prospect for employing gene expression testing in preimplantation embryos, with the added advantage of non-invasive collection of miRNAs from the culture media (Jung et al., 2010, Capalbo et al., 2016b).

- **Next Generation Sequencing**

1.8 Utilizing Next-Generation Sequencing for miRNA Expression: Advantages and Insights

Since the inception of the Human Genome Project, sequencing approaches have undergone remarkable advancement. Among these, high throughput next-generation sequencing (NGS) has emerged as a transformative tool in genomics research, revolutionizing our approach to explore complex gene interactions and biological processes.

Traditionally, gene expression studies have relied on certain primary techniques: quantitative real time polymerase chain reaction (qPCR), array-based qPCR , microarray, and NGS. The distinct advantage of NGS lies in its ability to produce robust results with high of sensitivity and accuracy, while providing full coverage of all genes present in the samples, both known or unknown (Tam et al., 2014). The decreasing cost, along with the time efficiency of NGS making it prevalent in clinical and research settings.

Molecular and genetic testing have gained growing influence in the field of reproductive health and treatment, providing valuable insights into various aspects of

reproductive health by uncovering underlining causes and factors contributing to reproductive failure (Holt-Kentwell et al., 2022, Eshre et al., 2023). Given the susceptibility of reproductive cells and developing embryos to chromosomal errors and genetic abnormalities, the implementation of such tests in this field was crucial (Vidal et al., 2001, Magli et al., 2001, Fragouli et al., 2011). In the context of ART, several molecular tests have been already implemented and proven beneficial in terms of their ability to aid, assess and improve reproductive outcomes (Viotti, 2020, Samura et al., 2023). Among them are the endometrium receptivity array (ERA), hormonal receptors expression analysis, sperm DNA fragmentation analysis, and various types of preimplantation genetic testing, including PGT-A, PGT-M, and preimplantation genetic testing for structural rearrangement (PGT-SR) (Chen et al., 2021). These tests have enabled clinicians and embryologists to make well-informed decisions regarding treatment plans, implantation timing, and embryo selection, ultimately contributing to more personalized reproductive care.

DNA sequencing has become an integral part of IVF treatment, particularly through PGT-A, which offers higher resolution and reduces the risk of misinterpreting mosaicism. Just as DNA sequencing has significantly improved embryo quality assessment, the application of RNA sequencing, specifically miRNA expression analysis, holds the potential to further enhance this evaluation. While embryonic miRNA sequencing is increasingly being employed, most investigations have concentrated on their expression in the culture media. Despite this, the clinical application of miRNA sequencing and differential expression in reproductive treatments remains limited (Calin and Croce, 2006, Salim et al., 2017).

Understanding miRNA expression through sequencing is crucial for determining embryo quality, as it provides insights into the molecular mechanisms that influence developmental competence and implantation potential. Among various gene expression approaches, sequencing represents a significant advancement in miRNA profiling allowing identification of thousands of miRNAs in a single experiment. Its high capacity allows for the capture of diverse RNAs, both coding and non-coding, encompassing well-established and novel entities (Keller et al., 2011). This capability is particularly important as it facilitates the detection of subtle changes in miRNA expression patterns that might be overlooked by less sensitive approaches.

- # Aims and hypotheses

In this research, we aim to investigate the insights provided by miRNA expression regarding human blastocyst quality and developmental status, along with the potential influence of parental factors on the miRNA profile in blastocysts. Our central hypothesis posits that the miRNA expression profile correlates with established embryo quality indicators. Therefore, the primary objective is to explore the associations between common embryonic quality metrics and paternal features, routinely assessed in IVF clinics, and miRNA expression profile in the blastocysts. This study employs a series of precisely designed experiments and comprehensive analyses. The specific objectives and methodologies of each are detailed as follows:

Hypothesis 1: The methods used to extract and sequence miRNAs in blastocysts are expected to be both sensitive and accurate.

Aim 1: Validation of the methods and establishment of research approach.

Study design and approach 1: Assessment of the extraction method and conduct a pilot study for miRNA sequencing. This involved optimising the miRNA extraction protocol to obtain samples of good quality/quantity for miRNA quantification. Subsequently, sequencing miRNAs in a small group of euploid and aneuploid blastocysts using NGS, and identification of differentially expressed miRNAs in the aneuploid group. This pilot study involves 12 samples, allowing an examination of the sequencing capability to generate reliable results for such samples. Follow up with a validation experiment using qPCR to confirm the accuracy of miRNA sequencing. Additionally, to eliminate the potential impact of aneuploidy on the miRNA biogenesis process, the association between aneuploidy and gene copy number of miRNA biogenesis genes was investigated using an external dataset of mRNA expression in human blastocysts, provided by Xuhui Sun.

Hypothesis 2: Abundant expression of miRNAs in blastocysts along with the high expression of specific miRNAs, is proposed to play an important role in embryo development

Aim 2: Explore the complete miRNA landscape in human blastocysts.

Study design and approach 2: Utilize miRNA sequencing results from 122 blastocysts to investigate the general features of miRNAs in human blastocysts. This

includes assessing the total number of expressed miRNAs, analysing the top 100 most expressed miRNAs, and exploring their involvement in biological pathways.

Hypothesis 3: The miRNA expression profile is proposed to vary between i) euploid and aneuploid blastocysts, ii) blastocysts formed on day 5 and those formed on day 6, and iii) blastocysts with good morphological grades compared to those with poor morphology.

Aim 3: Identify the potential change in miRNA expression profile in relation to three embryonic parameters impacting the pregnancy outcomes: aneuploidy status, morphology and the day embryo reaches the blastocyst stage. Conduct further computational analysis, including investigating of their gene targets and the biological pathways they are involved in.

Study design and approach 3: Analyse 122 sequenced blastocysts, categorizing samples based on aneuploidy status, day of blastocyst development, and morphology grades. Identify the differentially expressed miRNAs in each analysis and explore their potential contribution to cellular processes through computational analysis of their pathways and gene targets.

Hypothesis 4: The miRNA expression profile in blastocysts is hypothesized to be intricately linked to the quality of the gametes.

Aim 4: Investigate whether maternal and paternal factors influence the miRNA expression profile in the resulting embryo.

Study design and approach 4: Utilize the same miRNA sequencing data to investigate potential change in miRNA expression profile in relation to gametes quality or factors that impact their quality including treatment protocol, parental age and sperm parameters.

Hypothesis 5: The identified differentially expressed miRNAs in blastocysts are deemed valid and consistent with previous investigations, and they are expected to show coherence in their impact on RNA targets.

Aim 5: Use multiple methods to validate differentially expressed miRNAs identified in relation to aneuploidy.

Study design and approach 5: Conduct a systematic review to identify previous literature on the miRNA and aneuploidy association and compare their results to the present study findings. Additionally, use an external dataset of mRNA expression in human blastocysts, provided by Xuhui Sun, to validate the dysregulation found in miRNAs in aneuploid blastocysts by examining the expression levels of their target mRNAs in the same cohort of samples.

Chapter 2 Methodology and Protocols

- **Methodology**

The main aim of this research was to obtain insights into the role of miRNA in determining embryo quality. The primary objectives can be summarised in three research questions: 1) What insight does miRNA expression provide about the developmental dynamics of human blastocyst, including their highly active biological pathways? 2) Does the miRNA profile reflect blastocyst quality and developmental status? 3) Do parental factors influence the miRNA profile in blastocysts?

Both miRNAs and blastocysts possess unique characteristics that make them highly suitable for assessing the quality of preimplantation embryos. MiRNAs, known for their presence in circulation and encapsulation within exosomes outside cells, serve as informative biomarkers accessible from bodily fluids. The blastocyst stage, pivotal in embryonic development, represents a critical milestone where optimal development signifies potential for successful implantation and further growth. Therefore, by investigating miRNA in blastocysts, researchers can gain valuable insights into embryo health and viability, enhancing our ability to assess and optimize reproductive outcomes. However, due to the small number of previous studies investigating miRNA in blastocysts, as well as the intricate characteristics of developing embryos, which can influence miRNA expression in various ways, it was important to establish a robust protocol to extract and measure the miRNA in blastocysts, ensuring good integrity and detectability of miRNA levels.

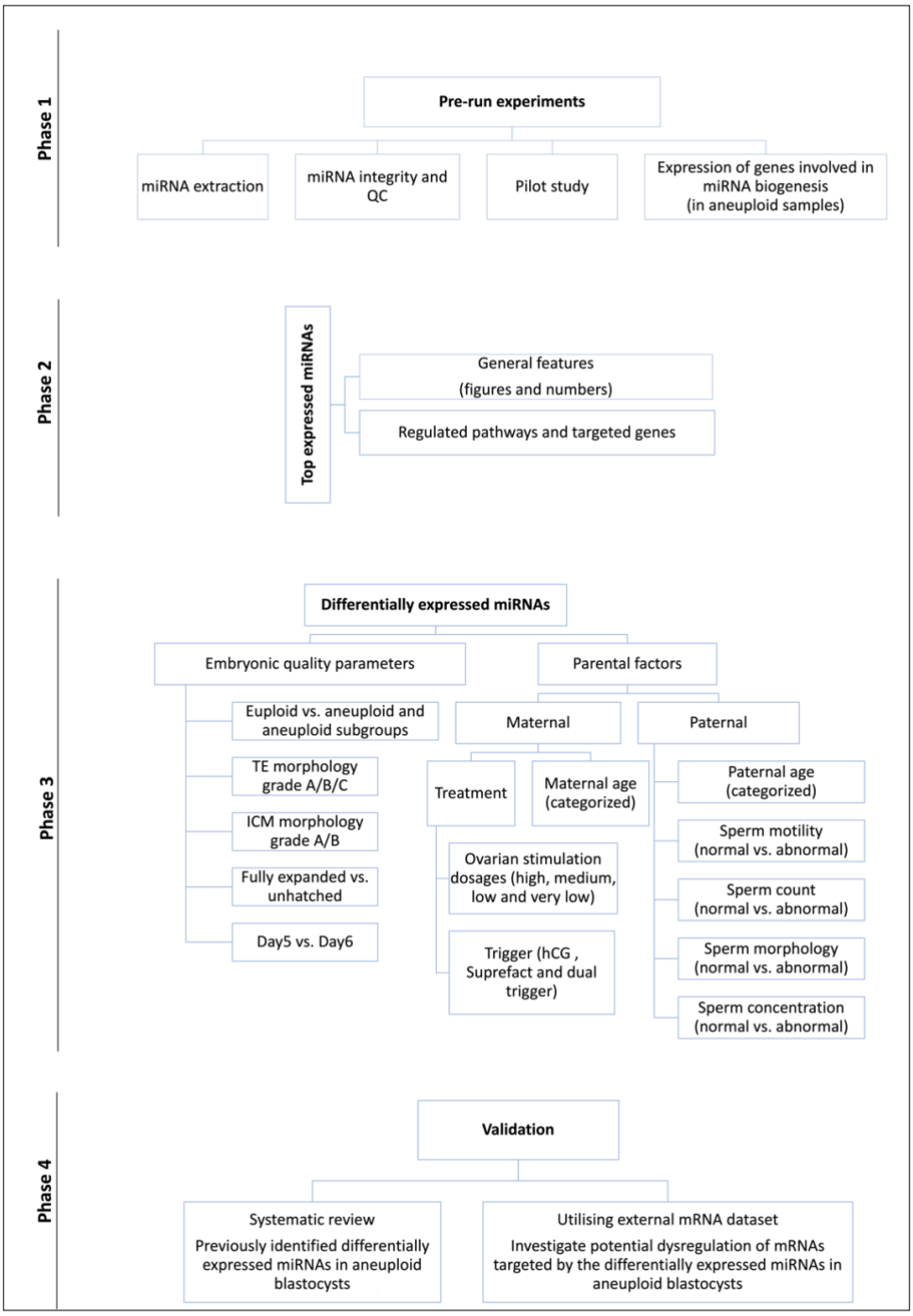
2.1 Study Design and Methodological Approach

A comprehensive observational, analytical, descriptive case-control study was designed to scrutinize the miRNA profile in a population of human preimplantation

embryos at the blastocyst stage, employing high throughput NGS technology. While NGS and microarray are commonly used in similar investigations for gene expression and profiling, the choice of sequencing in this study was intentional. miRNA sequencing was selected for its ability to detect all mature miRNAs present in human blastocysts, coupled with its cost and time effectiveness compared to alternative approaches (Tam et al., 2014).

The study proceeded in four main phases (Figure 2-1). In Phase 1, validation processes were undertaken to establish the implemented techniques, ensuring accuracy and validity of the results. This involved testing miRNA extraction and expression levels in blastocysts, confirming that the copy number of genes involved in miRNA biogenesis machinery was not influenced by aneuploidy in their chromosomes. Lastly, a pilot study was conducted, sequencing miRNAs in a small number of blastocysts to ensure overall quality and reliability of the miRNA sequencing and the sequencing results were validated by qPCR.

Figure 2-1: Research workflow and main phases



The research was conducted in four main phases. Phase 1 included validating the extraction procedure, ensuring the abundance of extracted miRNAs, a pilot study to generate preliminary sequencing data, and assessing the quality of miRNA sequencing, followed by the validation of sequencing results through PCR. In Phase 2, the miRNA landscape in human blastocysts was profiled, and highly expressed miRNAs along with their targeted pathways were determined. Phase 3 investigated the differentially expressed miRNAs in a series of comparisons that explored changes in miRNA expression across various blastocyst and gametes biological statuses, including aneuploidy status, time taken to reach the blastocyst stage, blastocyst morphology, ovarian stimulation dosages, trigger signifying oocyte maturation, parental age, and sperm quality. Phase4, involves validating the miRNA results.

It is important to mention that the analyses conducted throughout this project were motivated by two sets of data: the main data produced for miRNA expression analysis and an external dataset that includes mRNA expression in human blastocysts. The latter was prepared and provided by Xuhui Sun and was used for additional validation experiments, specifically when investigating genes involved in miRNA biogenesis, and for the co-regulation analysis of mRNA and miRNA in aneuploid blastocysts.

Subsequent phases were performed on sequencing data from both first (preliminary), and second (main), sequencing runs. In Phase 2, the miRNA profile was analysed in a group of blastocysts in order to identify the top expressed ones, aiming to gain an overview of the common active or regulated biological pathways during early embryonic development. Throughout Phase 3, the main hypothesis was tested, asserting that miRNA expression differs with different blastocyst parameters. The first analysis interrogated whether the miRNA profile exhibited an altered expression pattern with different embryonic quality parameters, including aneuploidy status (euploid versus aneuploid and aneuploid subgroups), the day of blastocyst formation (day 5 versus day 6) and different morphology scores of inner cell mass, trophectoderm and the expansion status of the blastocyst. The same set of samples was investigated for the possible influence of parental factors on miRNA levels in the blastocysts. Factors with a potential effect on gamete quality, such as the ovarian stimulation dosage and oocyte maturation trigger of the female patient, age of both parents at the day of providing their samples, and sperm parameters were considered for miRNA differential expression analysis (Figure 2-1). The final phase, Phase 4, involves validating the miRNA results using various methods, including a systematic review of the existing literature on the differentially expressed miRNAs in aneuploid blastocysts. Additionally, an external mRNA dataset was utilised to confirm the dysregulation of mRNAs in aneuploid blastocysts targeted by the differentially expressed miRNAs identified in the present investigation.

2.2 Sample Size, Collection, and Significance

The standard protocol for gene expression studies typically requires three biological replicates, all derived from the same cell line. However, given the inherent diversity

in human embryos and the need to account for the internal biological differences between families, the use of three replicates is considered insufficient and could generate biased results. Consequently, a deep exploration for a statistically validated method to calculate sample size for gene expression in such diverse conditions was undertaken, with an aim to determine the optimal cut-off for the statistical measures, namely the p-value and the expression fold difference.

In the context of differential mRNA expression, several approaches have been suggested for sample size determination (Hart et al., 2013, Ching et al., 2014, Li and Shyr, 2016, Bi and Liu, 2016, Li et al., 2019, Su et al., 2020). However, none have specifically addressed the unique features of small RNAs, including miRNAs. We could not apply these available approaches due to substantial difference between total number of miRNAs and mRNAs expressed per sample, making mRNAs more susceptible to false discovery compared to the more precise results observed with miRNAs.

Therefore, a common approach employed by researchers to identifying significantly differentially expressed genes was followed. This method involves ranking genes based on the false discovery rate p-value (FDR) and subsequently applying a fold change (FC) cut-off. In this study, statistically significant values stated set as FDR $\leq$ 0.1 and FC $\leq$ 1.5.

2.2.1 Study Participants

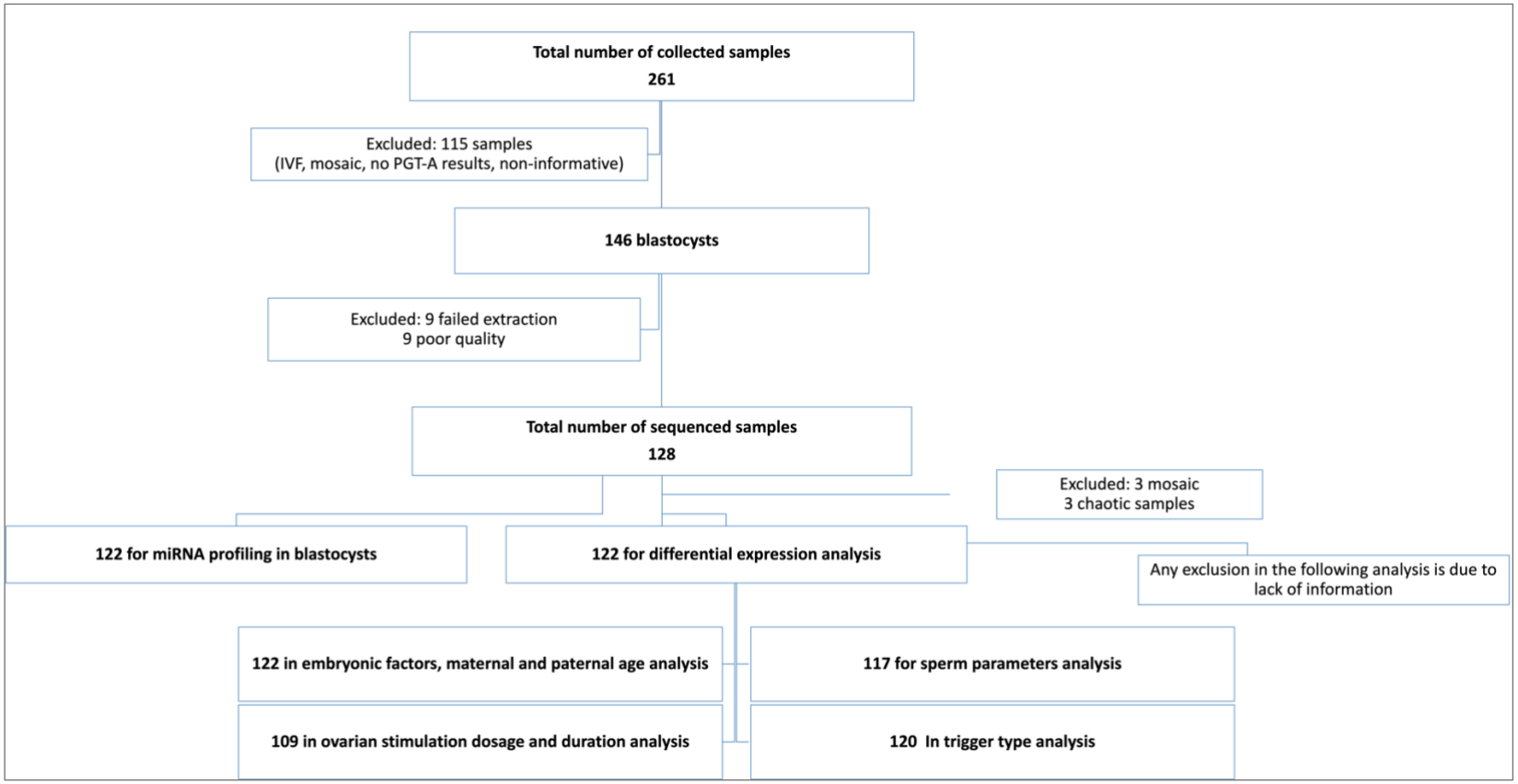
Vitrified human embryos at blastocyst stage were collected from The Centre for Reproductive and Genetic Health (CRGH), London. A group of surplus un-transferred frozen embryos, donated for research purposes, came from couples who provided informed consent. This study received HFEA licensing (centre number: 0245, licence reference number: R01130), ethical approval from the NHS Research Ethics Committee (licence reference number: 10/H0709/26).

During the study period, a total of 261 samples were collected (Figure 2-2). The samples were individually thawed in batches by the embryologist at the IVF unit (CRGH) and then placed in individual tubes of lysis buffer. Lysis buffer is used to break down cell membranes and release nucleic acids from cells. Following thawing,

the samples were promptly transported from the IVF unit to the research laboratory for further processing.

Several blastocysts were excluded from the main analysis for various reasons, such as embryos produced by IVF rather than ICSI which increases the possibility of sperm contamination, and non-informative samples with ambiguous PGT-A results. However, these samples were used for the initial validation of the methods. Only ICSI-produced blastocysts with reported PGT-A results were included in the miRNA sequencing, which comprise of 146 blastocysts. During sample preparation, 18 blastocysts were lost due to failed extraction or poor quality. The remaining 128 blastocysts proceeded to sequencing. Six of them were removed for their undefined aneuploidy status, leaving 122 blastocysts donated from 48 couples to be analysed.

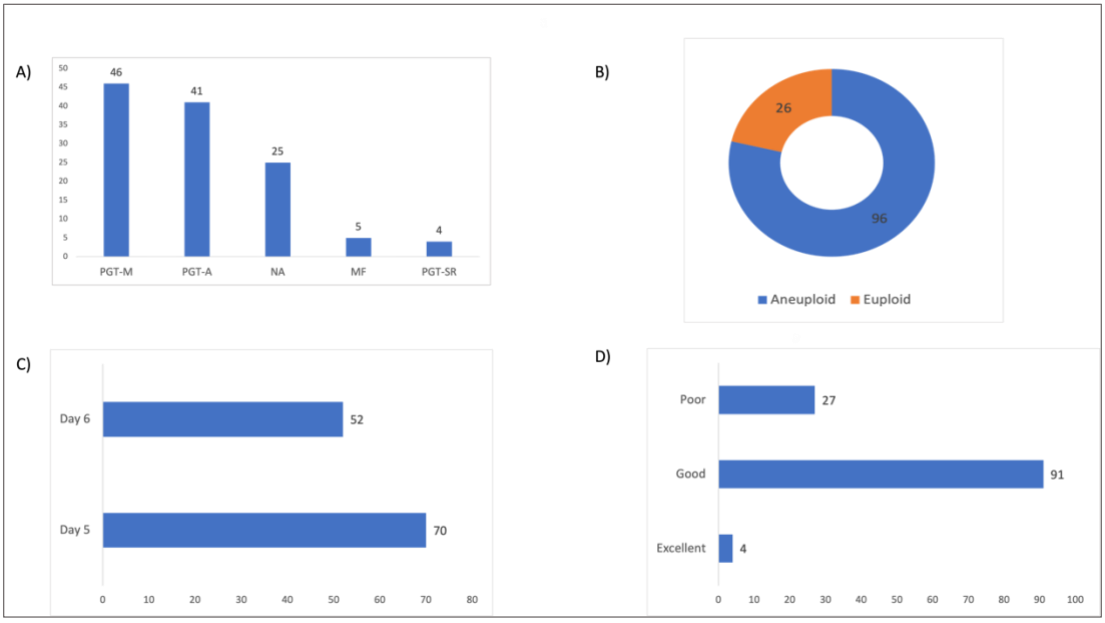
Figure 2-2: Sample inclusion and exclusion process throughout the study



Initially, a total of 261 blastocysts were collected. Subsequently, only blastocysts produced through (ICSI) with informative (PGT-A) results were included. Further exclusions occurred post-sequencing, where all samples exhibiting poor sequencing quality are having ambiguous aneuploid results were omitted from subsequent miRNA differential expression analysis (including 122 blastocysts). Samples lacking information on the investigated factor were excluded from that specific analysis.

These blastocysts had a mean maternal age of 37 years, ranging from 25 to 43 years. Paternal age ranged from 29 to 60 years, with a mean age of 41 years. The PGT of these samples was requested for various reasons, most commonly for parental mutations and advanced maternal age (AMA). According to the PGT-A reports of the blastocysts investigated, 26 were euploid and 96 were aneuploid (Figure 2-3). Although all the samples were at blastocyst stage, some have reached this milestone on day 5 and some others did on day 6. Notably, all blastocysts exhibited good to excellent ICM morphology grades and displayed a range from excellent to poor TE morphology. The majority of blastocysts were fully expanded, with very few remaining unhatched.

Figure 2-3: Sample characteristics and distribution



The figure illustrates various criteria for the investigated samples, including: A) Indications for PGT, showing that mutations and aneuploidy were the most frequent reasons for requesting this test. B) Distribution of aneuploidy status, with a high prevalence of aneuploid blastocysts. C) A slightly higher number of samples reaching the blastocyst stage on day5 compared to day 6. D) Distribution of blastocysts with different TE morphology grades, with the majority having good morphology.

2.3 Phase1: Protocol Validation

The initial phase of the study was conducted for the purpose of establishing the optimum experimental settings for the main investigations (Figure 2-1- Phase 1). The challenge posed by the low yield of investigated genes, considering that miRNAs constitute only around 0.01% of the total RNA in a sample, approximately ~ 250 ng

of the total RNA in a human blastocyst (Peltier and Latham, 2008, Li et al., 2017b, Hardy et al., 1989). Therefore, it was essential to assess the miRNA integrity in the samples before proceeding to sequencing.

2.3.1 miRNA Integrity Assessment

Several experiments were conducted to ensure good quality of miRNAs and to guarantee their sufficient concentration in the samples. All these experiments were performed on a group of blastocysts that were not suitable for differential expression analysis, either because they were IVF produced embryos or lacked the information needed for this investigation.

Initially, attempts were made to assess the integrity of genetic material from blastocysts using a sensitive NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to measure the approximate levels of DNA and RNA in the blastocysts. However, no results were detected even after several runs of sample concentration. Due to the low concentration of the genetic material in blastocysts, it was not possible to evaluate its quality before amplification.

Assessing the quality control (QC) became feasible only after amplification. The sequencing library used allows for assessing the miRNA quality after library amplification prior to sequencing. The blastocyst nucleic acid quality was evaluated using the Agilent Bioanalyzer and Agilent TapeStation (Agilent Technologies, Santa Clara, CA, USA).

2.3.2 miRNA Extraction and Purification

Validation of the applied techniques was essential to ensure the robustness and consistency of the miRNA results. Commercial RNA extraction kits are typically designed for extracting nucleic acid from single cells, tissues, or plasma. However, no such kit was specifically created to suit a sample that is more than a single cell but not a complete tissue nor a body fluid. Given these circumstances and being the first study to employ sequencing for miRNA expression in human blastocysts, finding the right extraction kit presented a challenge. The aim was to find a kit capable of eluting a representative amount of miRNA from such unique samples.

After an extensive search for an appropriate extraction protocol, AllPrep® DNA/RNA Micro Kit (QIAGEN, Crawley, UK) was chosen for this task. To confirm the efficiency of the extraction and assure the capture of miRNAs from individual blastocysts, four purified samples were tested for expression of two small RNAs, miR-103a and U6, using qPCR (the results are presented in section 3.2.1). This step aimed to confirm that successful purification of miRNA had been achieved and to validate the reverse transcription and amplification procedures. The successful detection of miRNA amplificants in blastocysts confirmed the readiness to proceed to the miRNA sequencing analysis phases (Figure 2-1- Phase 2).

2.3.3 Pilot Study

One of the main challenges in human reproductive-related projects is the limited availability and accessibility of the samples. Due to the vulnerability of the samples and high cost of NGS, as well as to prevent delay in the research progress, we conducted a pilot study and generate preliminary data to ensure the applicability of the approach before the main sequencing run.

For the pilot study, miRNAs extracted from 12 blastocysts were sequenced. These samples included blastocysts with various aneuploidy statuses, involving complex aneuploid, defined as multiple chromosomal abnormalities, mosaic, and single aneuploid, all of which were compared to euploid blastocysts for miRNA differential expression analysis. The successful outcomes of these experiments permitted the progression to the main miRNA sequencing run (Figure 2-1-Phase 1).

To validate the sequencing results, three miRNAs with varying expression levels were selected for reanalysing in six blastocysts, three aneuploid and three euploid, using qPCR. Differential expression analysis on the PCR results was performed and compared to the sequencing findings.

2.3.4 Assessing the Potential Impact of Aneuploidy on Genes Involved in miRNA Biogenesis

Since aneuploidy is a common feature in preimplantation embryos, it was the primary factor inspected in this study. Before addressing the relationship between

miRNA expression and numerical chromosomal defects, it was important to confirm that aneuploidy has no confounding effect on the expression of genes involved in the miRNA biogenesis process.

For this experiment, descriptive information and mRNA expression data, from a different set but same cohort of blastocysts, provided by Xuhui Sun, were analysed. The PGT-A reports of these blastocysts showed that some of the samples comprised defects in chromosomes that encode key genes involved in miRNA biogenesis and maturity. Using the provided data, our analysis aimed to determine potential correlation between aneuploidies in Chromosome 5, Chromosome 22, Chromosome 6, and Chromosome 14, and the gene expression levels of miRNA production genes: *DROSHA*, *DGCR8*, *XPO5* and *DICER1*, which are located on these chromosomes, respectively. Regression analysis was employed to assess these correlations.

2.4 Phase2: miRNA Profiling

2.4.1 miRNA Expression via High-Throughput Sequencing

MiRNA sequencing was performed in two lanes; the first lane aimed to generate preliminary data from 12 blastocysts, as previously descried in the pilot study. Results from this step confirmed the suitability of the approach and allowed continuing with 125 samples in a second track. Nine samples from this patch were excluded due to poor quality according to the pre-sequencing QC results. It is important to note that the excluded samples were from different families and processed in separate extraction batches, aiming to eliminate potential human and environmental errors.

RNA was extracted from each blastocyst, and aliquots were prepared for sequencing. These aliquots were processed using QIAseq miRNA Library Kit (QIAGEN, Crawley, UK). Although the samples were included in the same library, they were sequenced individually using the NextSeq^{TM} 500 platform (Illumina, San Diego, CA, USA). While running the samples in two batches, it was not possible to apply the exact sequencing settings, especially regarding the number of reads, due to differences in the total number of samples loaded per library. Nevertheless, the number of reads provided was ~11 and ~6.5 million reads per sample in the first and

the second run, respectively. These sequencing depths fall within the recommended range for miRNA sequencing, as outlined in the QIAGEN handbook.

The final analysis involved combined results of the two sequencing runs, encompassing 128 sequenced blastocysts. However, throughout this analysis, sequencing data of six samples were removed for having ambiguous PGT-A results or reported as mosaic blastocysts. Although mosaicism was initially considered as one of the aneuploidy statuses for inclusion, these embryos were later excluded due to their limited number in this cohort of samples. A total of 122 blastocysts remained for the subsequent miRNA differential expression analyses.

The initial analysis of miRNA sequencing data aimed to offer a general overview of miRNA profile in human blastocysts, highlighting the numbers and potential biological function of highly expressed miRNAs. However, the primary focus of the study drew on analysing the miRNA differential expression among various blastocysts groups, as detailed in the following phase. The miRNA expression data for the same set of blastocysts were used in all the subsequent analyses, and the grouping was performed according to the parameter investigated.

2.5 Phase3: Differential Expression Analysis of miRNAs

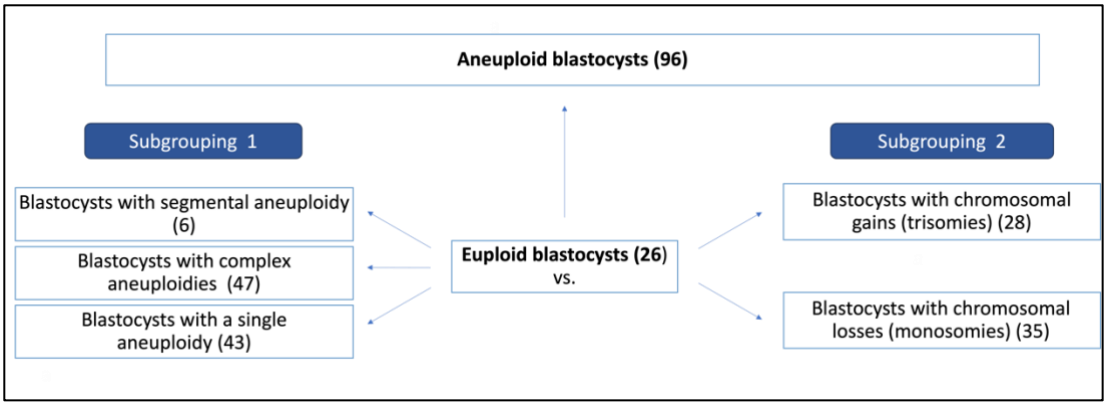
2.5.1 Methodological Approach for Sample Categorisation for Differential miRNA Expression Analyses

2.5.1.1 Ploidy Status

The initial differential expression analysis compared euploid and aneuploid blastocyst groups. Given the diverse nature of aneuploidy affecting various chromosomes in different ways, a detailed exploration was warranted. Consequently, miRNA expression levels were further investigated across different aneuploid groups. Samples with abnormal chromosome complement were categorized based on the number of chromosomes affected: single (one chromosome affected), complex (two or more aneuploidies) and segmental (partial chromosomal aberrations). Additionally, subgrouping was done based on chromosomal losses and gains, with each group having two or more monosomies or trisomies. Each group was then

compared to the euploid blastocysts group for differential miRNA expression analysis (Figure 2-4).

Figure 2-4: Grouping of aneuploid samples for miRNA differential expression analysis



The figure illustrates the Categorisation of blastocysts according to various aneuploidy types, with the numbers within each category representing the respective sample counts. Subgrouping of aneuploid samples includes segmental, complex, single aneuploidy, and chromosomal losses and gains.

To ensure reliability of the differentially expressed miRNAs identified through all comparisons, a sensitivity test involving repeated measurements was performed. This test involved conducting the differential expression analysis multiple times while controlling for potential confounding factors (Thabane et al., 2013). When investigating the embryo quality factors, the miRNA differential expression analysis was performed twice, with one accounting for the day of blastocyst formation.

2.5.1.2 Day of Blastocysts Formation

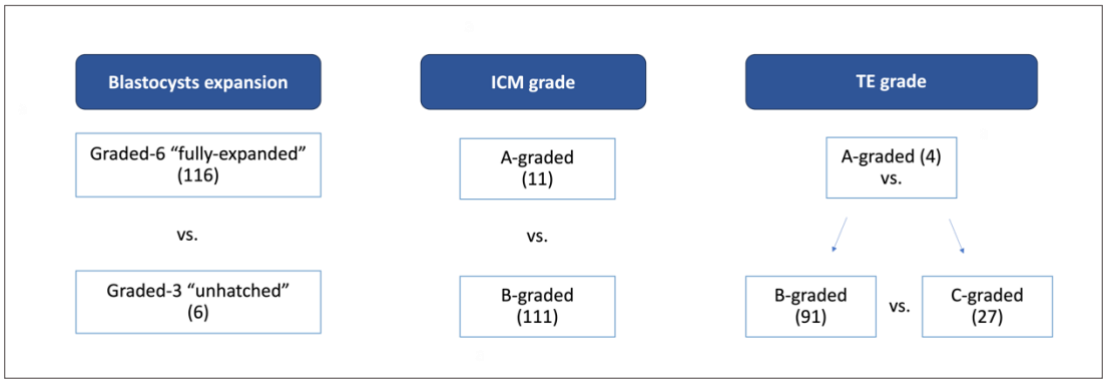
To investigate the potential impact of developmental delay on embryonic quality and explore the molecular factors contributing to higher implantation potential of day 5 blastocysts, a comparison of miRNA profiles was conducted between 70 samples of day 5 blastocysts and 52 samples of day 6 blastocysts. Given the known higher incidence of aneuploidy in day 6 blastocysts (Kort et al., 2015), another analysis was performed while accounting for aneuploidy.

2.5.1.3 Embryonic Morphology

Several investigations of the miRNA levels were carried out on blastocysts with diverse morphological grades. These investigations include comparisons between hatched and unhatched blastocysts, A and B ICM-graded blastocysts, and blastocysts

with A, B and C TE grades, according to the Gardner grading system (Figure 2-5). It is crucial to highlight that the majority of embryos included in the study were fully expanded with a good to fair ICM grades. This resulted in a small number of samples in the opposing groups, which introduces susceptibility to statistical errors in certain comparisons. To avoid the potential confounding effect of aneuploidy, we controlled for aneuploidy in this analysis (Majumdar et al., 2017).

Figure 2-5: Blastocysts distribution according to their morphology



The figure illustrates the distribution of blastocysts based on their expansion scores, ICM grade and TE grades.

2.5.1.4 Parental Factors

Through investigating the parental factors, the primary concern was to assess the potential impact of reproductive cells quality on the competence of resulting embryos. On the maternal side, miRNA expression was examined concerning maternal age, known for its correlation to aneuploidy, the dose of exogenous hormones provided for ovarian stimulation, and the type of trigger used for oocyte maturation (Gurbuz et al., 2016, Bosch et al., 2016, Verdyck et al., 2023)

For maternal age, blastocysts were divided into three groups, namely A: encompassing blastocysts obtained from women aged 34 years or younger, B: women in their mid-thirties (aged between 35-40 years), and C: women aged 40 years or older.

Regarding ovarian stimulation dose, blastocysts were categorized into four groups based on the dosage administrated to the female patient: high stimulation dose (Merional / Fostimon 225 IU/ 225 IU or Merional / Fostimon 150 IU/ 225 IU), medium stimulation dose (Merional / Fostimon 75 IU /150IU or Merional / Fostimon

150 IU/150 IU), low stimulation dose (Merional / Fostimon 75 IU / 75 IU), and very low stimulation dose, used in the mild stimulation regime (MSR) treatment plan.

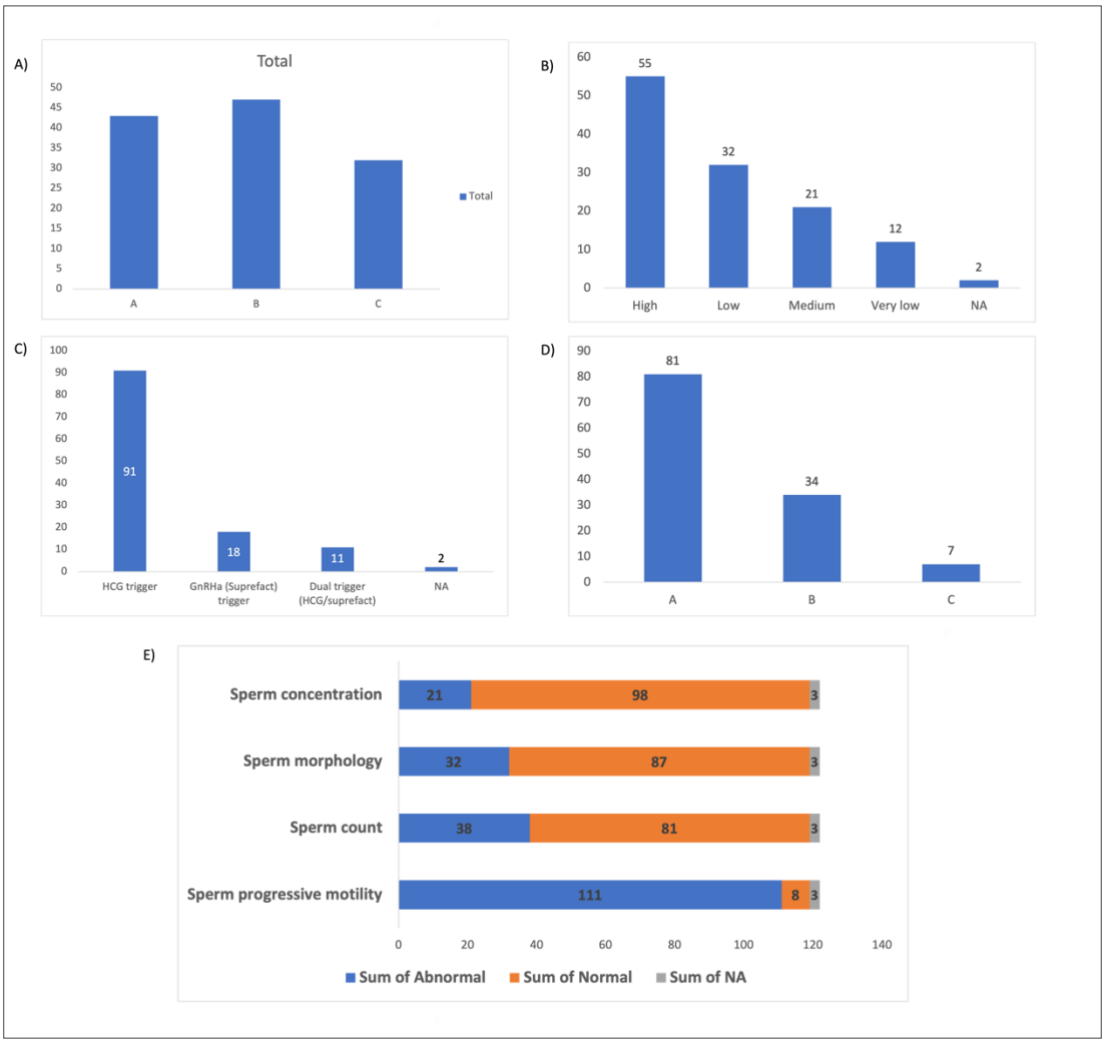
To investigate the potential influence of the trigger type administrated for oocyte maturation during the IVF cycle, the blastocysts were divided into three groups: one obtained from the hCG-tiggered oocytes, receiving either Gonasi or Pregnyl; another obtained from oocytes that had received the GnRHa (Suprefact) trigger, and the third group developed from oocytes triggered with the dual trigger, receiving both hCG and GnRHa.

For paternal factors, the investigated parameters included age and primary sperm characteristics. Similar to maternal age, the blastocysts were categorized based on the male partner's age; group A comprised blastocysts from men younger than 40 years, group B from men in their forties, and group C from men aged 50 years or older.

Following the WHO guide for semen analysis, the sperm characteristics were categorized as normal if the grade given by the embryologist at CRGH falls within the normal range or abnormal if not (World Health, 2010). Four parameters related to sperm quality were considered for miRNA expression: morphology (percentage of normal forms), count (total number of sperm per ejaculate), concentration (number of sperm per ml) and the sperm progressive motility (actively moving sperm).

Notably, the number of samples used in each comparison varied according to the availability of the required information for each test. The disruption of blastocysts across the different parental factors also varied, as illustrated in the (Figure 2-6).

Figure 2-6: Distribution of blastocysts according to investigated parental factors



The figure depicts the distribution of blastocysts across different groups based on parental factors. A) The samples were equally distributed across the three maternal age groups, A, B and C. B) Distribution of blastocysts according to the ovarian stimulation dose showing a significant number of females received a high dose. C) In the majority of cases, the oocytes were triggered using hCG trigger. D) Distribution of blastocysts according to parental age shows the majority of male patients are younger than 40 years. E) Number of blastocysts obtained from normal versus abnormal sperm status.

Of note, the intricate complex nature of the relationships between parental factors and miRNA expression in blastocysts necessitates establishing a robust methodological approach to ensure the validity of the final results. Initially, a regression analysis was conducted to identify potential confounding factors that may interact with maternal and paternal factors under investigation (Table 2-1) (results are shown in Appendix1). These factors were subsequently controlled for in the miRNA differential expression analyses.

Table 2-1: The accounted for factors for parental miRNA differential expression investigations

Factors investigated	**Parameters controlled for in differential expression analysis**
Maternal age	Paternal age, trigger, sperm count
Ovarian stimulation dose	Paternal age, sperm morphology, TE morphology
Trigger type	Maternal age, paternal age, PGT indication
Paternal age	Maternal age, trigger, ovarian stimulation dose, sperm morphology
Sperm motility	Aneuploidy
Sperm morphology	TE morphology, ovarian stimulation dose, paternal age, sperm concentration
Sperm count	Maternal age, sperm concentration
Sperm concentration	Sperm morphology, sperm count

Since the analysis tool (DESeq2) used for miRNA differential expression does not allow controlling for more than one factor per run, the analysis of each parameter was conducted two to four times, depending on the number of identified potential confounding parameters. The consistency of the finding across the repeated measurements, known as a sensitivity test, increased the reliability of the results (Thabane et al., 2013).

2.6 Phase 4: Validation of Differentially Expressed miRNAs in Aneuploid Blastocysts

2.6.1 Systematic Search of Literature

Early in this project, a preliminary search was conducted to explore the literature addressing the correlation between miRNA and embryonic quality parameters. The overview search revealed that this topic is underexplored, with only a limited number of studies investigating the relationship between miRNA and chromosomal abnormalities in blastocysts. Notably, none of these studies considered miRNA expression in the context of poor versus good blastocyst morphology or comparing between day 5 versus day 6 developed blastocysts.

To gather and extract data examining miRNA expression in aneuploid blastocysts, a systematic review was conducted. This review has not only established the foundational knowledge for the present study but also addressed the limitations of the previous research trails. Additionally, it provided insights into the frequently reported miRNAs with potential association to aneuploidy, thereby confirming the ones identified in the present study.

The systematic review involved searching four databases, namely EMBASE, Medline, Web of Science and Cochrane, using keywords such as miRNA, aneuploidy and blastocyst, along with their alternative terms. The search results were processed and filtered following PRISMA guidelines. The review specifically focused on preimplantation embryos at the blastocyst stage and included only English transcripts. For a detailed study design, refer to Appendix2.

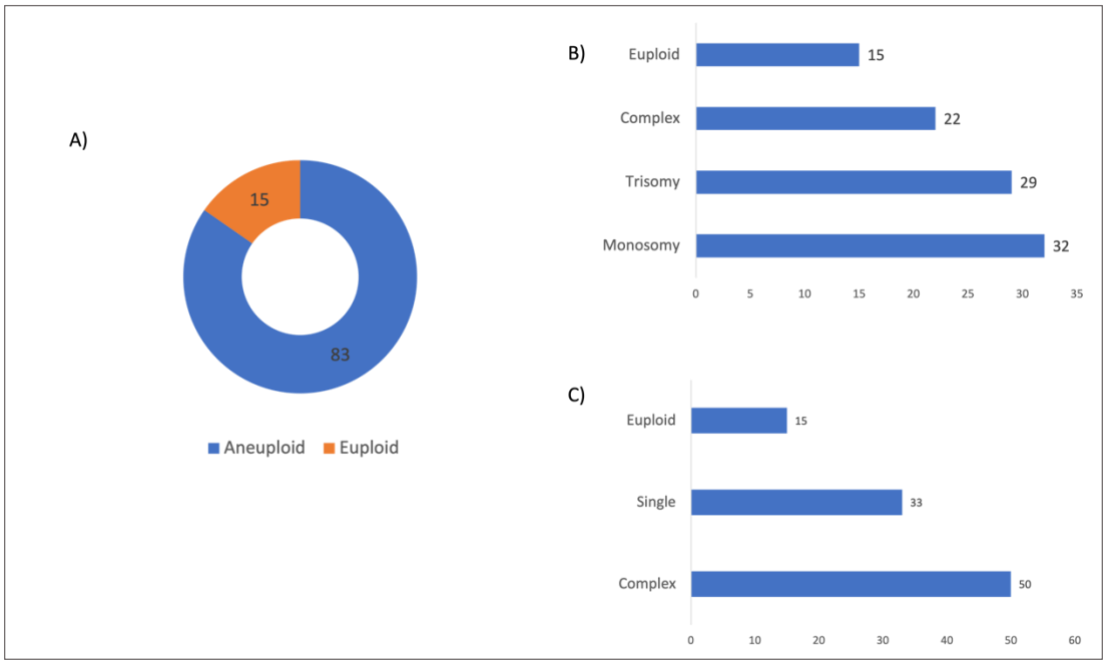
By comparing the differentially expressed miRNAs identified in prior studies with those found in the current analysis, consistency with the existing literature was ensured, providing robust validation of the findings.

2.6.2 mRNA Targets of the Differentially Expressed miRNAs in Aneuploid Blastocysts

An external set of mRNA data, comprising unnormalized gene expression reads from 100 human blastocysts, was provided by Xuhui Sun, along with their PGT-A results. This dataset was utilised for two main purposes: first, to investigating the expression of genes involved in miRNA biogenesis, and secondly, to explore the relationship between changes in miRNA expression and potential alterations in their mRNA targets (through co-regulation analysis). For this, the identified differentially expressed mRNAs in the aneuploid blastocysts compared to euploid blastocysts, were linked to the differentially expressed miRNA in the same comparison.

Similar to the miRNA analysis, multiple comparisons were performed to identify dysregulated genes in both overall aneuploid blastocysts and specific aneuploid subgroups (single, complex, loss and gain) (Figure 2-7). The analysis of segmental defects, or partial aneuploidies was not applicable in this analysis due to the absence of such abnormalities in the provided samples.

Figure 2-7: Categorisation of blastocysts by ploidy status for mRNA differential expression analysis



The figure illustrates the distribution of blastocysts investigated for mRNA differential expression. A) Prevalence of aneuploidy among the blastocysts. B) and C) Subgrouping of aneuploid blastocysts based on different types of aneuploidies, showing the predominance of chromosomal losses and complex (multiple) aneuploidies across samples.

Subsequently, the genes identified from the mRNA analyses were correlated with the differentially expressed miRNAs in the aneuploid blastocysts. This confirmatory step adds support to the altered functionality of the differentially expressed miRNAs in aneuploid blastocyst, demonstrating their potential association with changes in the expression of their target genes.

2.7 Data Collection

A wide variety of methods were employed for data collection and processing in this study, incorporating four primary types of data:

1. Descriptive Data:

Descriptive data, including samples characteristics and demographics, were gathered from CRGH databases and processed using Microsoft Excel. This information, detailed in Appendix3, include the egg collection date, biopsy date, type of IVF

procedure, PGT diagnosis, PGT indications, gene mutation site (if available), along with the mutated gene, embryo morphology, maternal age, and paternal age.

Furthermore, comprehensive baseline information was extracted, including embryonic parameters such as aneuploidy status, blastocyst formation day, and morphology grades. The dataset also included parental factors, such as treatment details (ovarian stimulation dosage and the type of trigger used), along with the sperm parameters assessed by the embryologist (see Appendix4 for details). This information was accessed and compiled from the patient records via the CRGH patient database system.

2. Raw sequencing data:

The first line of sequencing data, called raw data or source data, represent a massive amount of information requires computational processing and cleaning to derive meaningful results. The data were produced in FASTQ format, required bioinformatics pipelines to be processed. Details about the processing methods and tools used are further explained in the data analysis section.

3. Numerical data:

Numerical data represent the neat and processed version of sequencing data, suitable for statistical tests. This includes tables of identified genes with their expression numbers of each blastocyst. The cleaned data were generated and stored in a (.csv) format to be used in subsequent analyses.

4. Statistical data:

This type of data comprises the outcomes of differential expression analysis, encompassing differentially expressed genes. Key metrics for interpreting the results include the significance of the observed change (fold change, FC) determined by the false discovery rate (FDR) p-value. These values were extracted from the platforms utilized for this analysis in (.csv) format and represented in the thesis as they are.

- **Protocols**

2.8 Sample Collection and Storing

For sample collection, single use aliquots containing 350 µl of lysis buffer (20 µl of 2 M Dithiothreitol (=0.0062g DTT) per 1ml RLT buffer Plus) were prepared and aliquoted in sterile microfuge tube (QIAGEN, 2019 and 2020). At the day of collection, the embryologist at CRGH thawed each embryo gradually to prevent crystal formation, then transferred it to the collection tube with lysis buffer. The mixture was vortexed for 1 minute to enable homogeneity. After thawing, the samples were transferred to a biological substance labelled transport box with ice bags and transported to the PGT laboratory at UCL, where they were stored at -80° for later use.

2.9 Extraction and Purification of the Genetic Materials

Prior to extraction, frozen lysed embryos were thawed at 37°C for 5 minutes. Total RNA was then extracted from each sample using the AllPrep® DNA/RNA Micro Kit (QIAGEN, 2019 and 2020). The extraction was performed in batches of 6 to 7 samples per run, with RNA being individually extracted and aliquoted separately for each blastocyst. The processes of extraction and purification were conducted following the manufacturer's protocols described in the kit handbook titled: Simultaneous Purification of Genomic DNA and Total RNA from Animal and Human Cells (pages 24-26) and Purification of Total RNA Containing Small RNAs from Cells (pages 51-52) for DNA/total RNA and total RNA containing miRNAs, respectively (QIAGEN, 2019).

Two main principles were applied in these procedures: filtering and washing the genetic material. Two filter columns were provided from manufacture for this purpose, a DNA spin column, which is used first to capture gDNA and let through all RNAs, and another RNA spin column that filters total RNA from the DNA flow-through. Before RNA purification, the DNA flow-through was treated with ethanol to ensure appropriate binding conditions for RNA. Several washes with different alcohol buffers, provided by the company, were applied to both columns. The total

RNA eluted for each sample was 14µl. Lastly, to prevent RNA degradation, 0.5µl RNasin® Plus RNase Inhibitor (Promega, 2019) was added to the final product of total RNA. The extracted samples were then kept at -80°C for future use.

2.10 Quantitative Real Time PCR for Gene Expression

Real time qPCR was employed in two scenarios for various purposes during this study. Initially, it was performed to ensure that abundant miRNAs were extracted from blastocysts for gene expression. Subsequently, it was used to validate the miRNA sequencing results generated in the pilot study, confirming their accuracy. The extracted RNA underwent two steps: reverse transcription into complementary DNA (cDNA), and then amplification by qPCR. Detailed information about the PCR procedures and the cycling programme is provided in the subsequent sections.

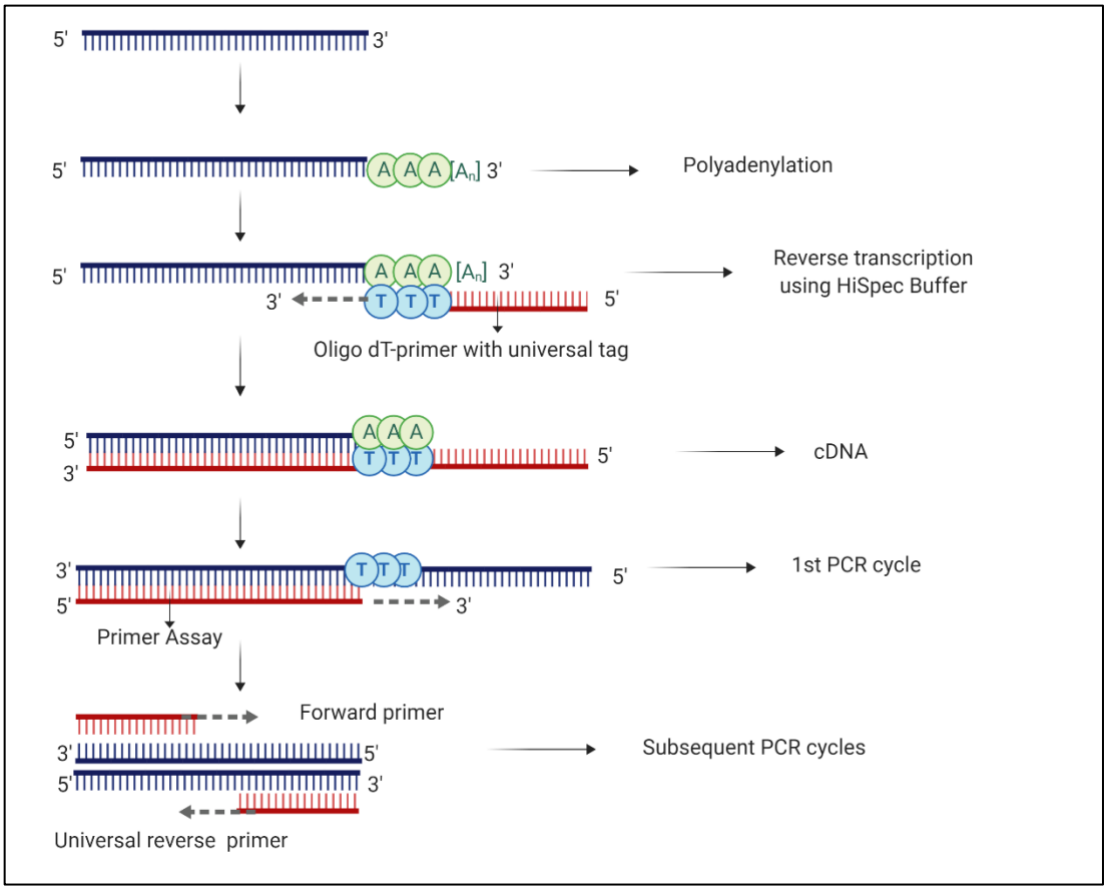
2.10.1 cDNA Synthesis

For miRNA reverse transcription, 6µl of the total RNA containing miRNA was aliquoted and reverse transcribed using miScript II RT Kit and following the protocol: *Reverse Transcription for Quantitative, Real-Time PCR* on kit handbook (pages 18-21) (QIAGEN, 2019). Samples were thawed on ice and added to the reverse transcription mix, which was prepared as illustrated in (Table 2-2). Each reaction has a total volume of 20µl, 14µl reverse transcription master mix and 6µl RNA. The synthesised cDNA was then diluted in 200 µl RNase-free water and stored at -80°C. The actual process of cDNA synthesis is depicted in (Figure 2-8).

Table 2-2: Reverse transcription reaction (miScript)

Component	**1 Reaction**
5x miScript HiSpec Buffer	4 µL
10x miScript Nucleics Mix (dNTPs)	2 µL
miScript Reverse Transcriptase Mix	2 µL
RNase free H2O	6 µL
Template RNA	6 µL
Total reaction	20 µL

Figure 2-8: miRNA reverse transcription process



The miRNA reverse transcription process started with adding poly(A) tale to the mature miRNA. The cDNA synthesis is produced with help of oligo-dT primer. The cDNA is then amplified by PCR. This figure is adapted from (QIAGEN, Crawley, UK).

2.10.2 Real-time qPCR for miRNA Gene Expression

Amplification of the selected miRNAs with an internal reference gene (U6[1]) was carried out using miScript SYBR® Green PCR Kit following the protocol: *Real-Time PCR for Detection of Precursor miRNA* on kit handbook (pages 23-27) (QIAGEN, 2019) and the PCR amplifier LightCycler® Nano (Roche Molecular Diagnostics, Indianapolis, IN). Two microliters of the cDNA from each sample were added to 8 µL master mix which contained: SYBR Green, primer assays, and

[1] U6 or RNU6 is a small non-coding RNA (snRNA) that is widely used as a control gene to normalize miRNA expression level. This gene is expressed stably across the sample.

RNase-free water (Table 2-3). Three miRNAs were selected for validating the sequencing results, and their primers, provided by QIAGEN (Crawley, UK), are listed in (Table 2-4).

Table 2-3: miRNA qPCR preparation reaction (miScript)

Component	**1 Reaction**
SYBR Green PCR Master mix	5 µL
Universal primer	1 µL
Primer assay	1 µL
RNase free H2O	2 µL
Template cDNA	1 µL
Total reaction	10 µL

Table 2-4: Sequences of miRNA primer assays used in the validation of sequencing results

MiRNA	**Primer sequence**
hsa-miR-16-5p	5’ UAGCAGCACGUAAAUAUUGGCG
hsa-miR-5583-5p	5 ‘AAACUAAUAUACCCAUAUUCUG
hsa-miR-625-3p	5’ GACUAUAGAACUUUCCCCCUCA

The reaction tubes were then loaded into LightCycler® Nano (Roche Molecular Diagnostics, Indianapolis, IN) and the amplification cycle was programmed according to recommendation by the manufacturer, as detailed in (Table 2-5).

Table 2-5: PCR cycle programme for miRNA expession

Step	**Temperature**	**Duration**	**Cycles**
UDG* activation	50 C˚	2 minutes	Hold
DNA polymerase	95 C˚	2 minutes	Hold
Denaturation	95 C˚	15 Seconds	40
Annealing	60 C˚	1 minutes	

* Uracil-DNA glycosylase.

2.10.3 Real time PCR Results Analysis

For each gene, the cycle threshold (Ct)[1] was calculated and illustrated in the amplification curve. To normalize this read, the Ct of a reference gene in the same sample was provided and then ΔCt was calculated following the equation:
ΔCt = Ct (target gene) - Ct (reference gene).

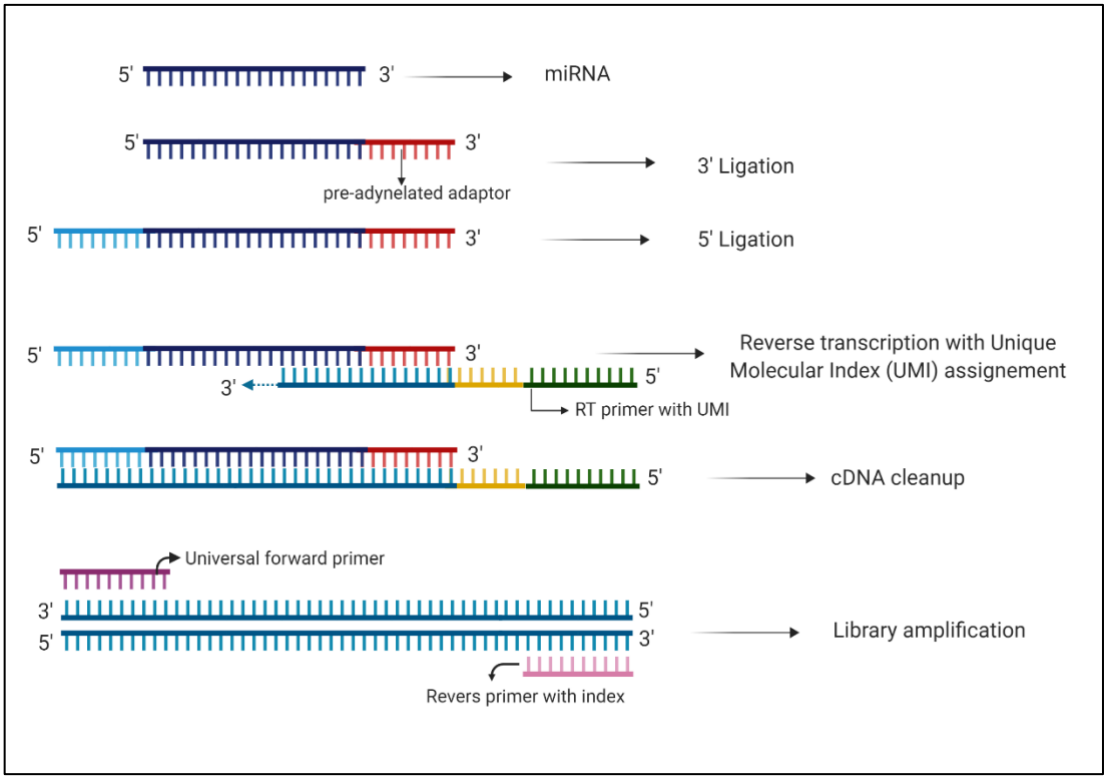
2.11 miRNA Sequencing Processing

Aliquoted RNAs containing miRNAs were sequenced using the NextSeq™ 500 platform (Illumina, San Diego, CA, USA). This experiment was conducted at University College London Genomics (UCLG) laboratory using QIAseq miRNA Library Kit (QIAGEN, 2019 and 2021) in accordance with the protocol handbook: *QIAseq miRNA Library Kit Handbook* (pages 20-43). Following the manufacturer recommendations, the targets were single end sequenced with more than 5 million reads per samples and have a 75bp read length.

The unique structure of miRNA, possessing both 3' and 5' tails, allowed specific ligation of the adaptors to the two ends, which reduces the possibility contamination from other RNAs. This also enables universal reverse transcription of all mature miRNAs and discovery of the novel ones. After adapter ligation, cDNA was synthesised for all detected miRNAs and the library was amplified (See Figure 2-9). Because the investigated embryos consisted of only a relatively small number of cells, the guideline recommendations for low sample input were followed.

[1] The number of amplification cycles required for the fluorescence signal in the sample to exceed the baseline threshold level, which is calculated based in the increase in dye concentration (SYBR Green in this case).

Figure 2-9: Library amplification process for miRNA sequencing

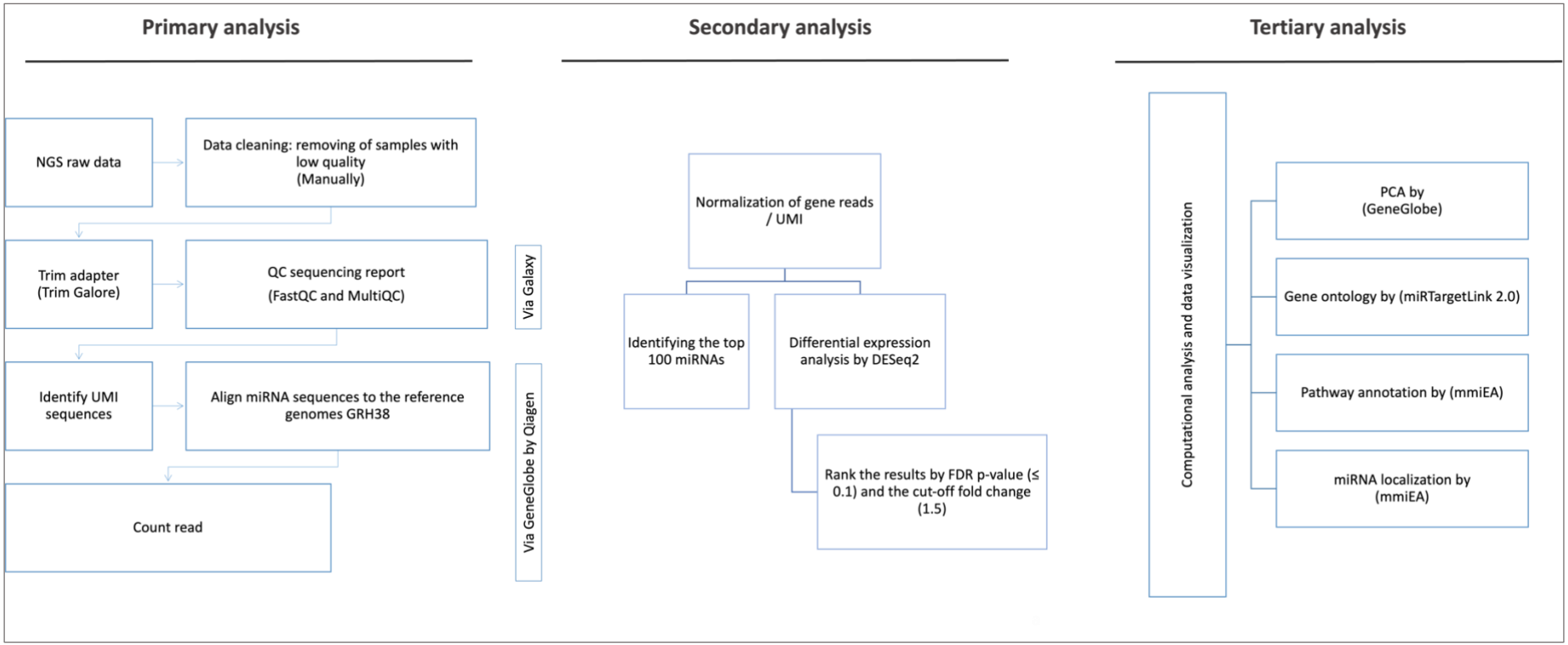


Two pre-designed adaptors are ligated to the miRNAs in the sample. The cDNA is then synthesised with help of reverse transcription primer labelled with a UMI. After cDNA cleaning-up, the library amplification occurs with help of a universal primer and a reverse primer with index. This figure is adapted from (QIAGEN, Crawley, UK).

2.12 Sequencing Expression Analysis

The process of miRNA sequencing analysis involves three main phases: primary, secondary and tertiary (see Figure 2-10). The initial phase focuses on cleaning raw sequencing data and assessing the sequence quality. During the secondary phase, gene reads are normalized, aligned to genome references, and differentially expressed genes are identified. The tertiary phase involves converting numerical data into biological insights by identifying pathways and target genes associated with differentially expressed miRNAs.

Figure 2-10: Workflow of miRNA sequencing data analysis



The figure illustrates the miRNA sequencing data analysis workflow, divided into three phases: primary, secondary, and tertiary. Primary analysis includes data cleaning, quality control, sequence identification, alignment to a reference genome, and read counting, performed using GeneGlobe and Galaxy software. Secondary analysis involves read normalization and identification of differentially expressed miRNAs using DESeq2. Tertiary analysis encompasses miRNA-gene target interaction networks, pathway enrichment, miRNA localization, and principal component analysis (PCA).

2.12.1 miRNA Sequencing Data Processing: Primary Analysis

The raw sequencing data, produced in FASTQ format, underwent a hierarchal comprehensive processing. Initially, sequences were cleaned up by trimming adaptors, removing low-quality bases, and discarding short reads using Trim Galore pipeline. Subsequently, sequencing quality assessment was conducted individually for each sample using FastQC, and collectively for all samples by MultiQC tool. Among various quality metrics assessed, the Phred score was evaluated. This score measures the accuracy of base calling in sequencing and serves as the most representative and crucial indicator of sequencing quality. The utilised pipelines were accessed through Galaxy Project Europe (Martin, 2011, Blankenberg et al., 2011).

Sequences that passed quality assessment underwent alignment to multiple RNA references, named miRBase mature, miRBase hairpin, piRNA, tRNA, rRNA, mRNA and other RNAs to identify the RNAs presents in the samples. Unmapped sequences were subsequently aligned to the human Genome Reference (GRCh38) and other different mammalian reference genomes, such as mouse Genome Reference (GRCm38) and rat Genome (RGSC Rnor_6.0) to identify the possible novel miRNAs. The identified sequences were generated as non-characterized mappable read and were not analysed during the course of this project but are available for future studies. These processes were completed by GeneGlobe analysis service by QIAGEN.

The expression level of each identified miRNA quantified in two forms: read counts and UMI counts. UMI abbreviates unique molecular identifier, representing a small sequence added to the library before amplification and incorporated into individual DNA or RNA molecules to enable accurate identification of amplified products. Preferably, UMI counts were utilised for differential expression analyses due to its ability to accurately quantify gene expression, correct for PCR duplicates, and account for technical noise, especially in low-input samples.

2.12.2 miRNA Expression Analysis: Secondary Analysis

Normalization of gene counts is a crucial step that controls variation between samples and therefore minimising the risk of false results. The bioinformatic pipeline DESeq2 was employed for this assay. DESeq2 is advantageous for adjusting differences in the library size (sequencing depth) and composition (gene expression factors like different tissues), producing reliable gene reads. The key concept in DESeq2's normalization process involves using logs and medians to eliminate variances between samples, smooth the effect of outliers and generate scaling factor around housekeeping genes (Figure 2-10– Secondary analysis).

Sequencing data, by nature, requires a specific distribution called negative binomial distribution[1], to model the gene numbers and enable running statistical tests on them. This model is used for discrete data that has a higher variance count to the mean count. DESeq2 utilise this model, effectively minimising differences in gene expression between samples.

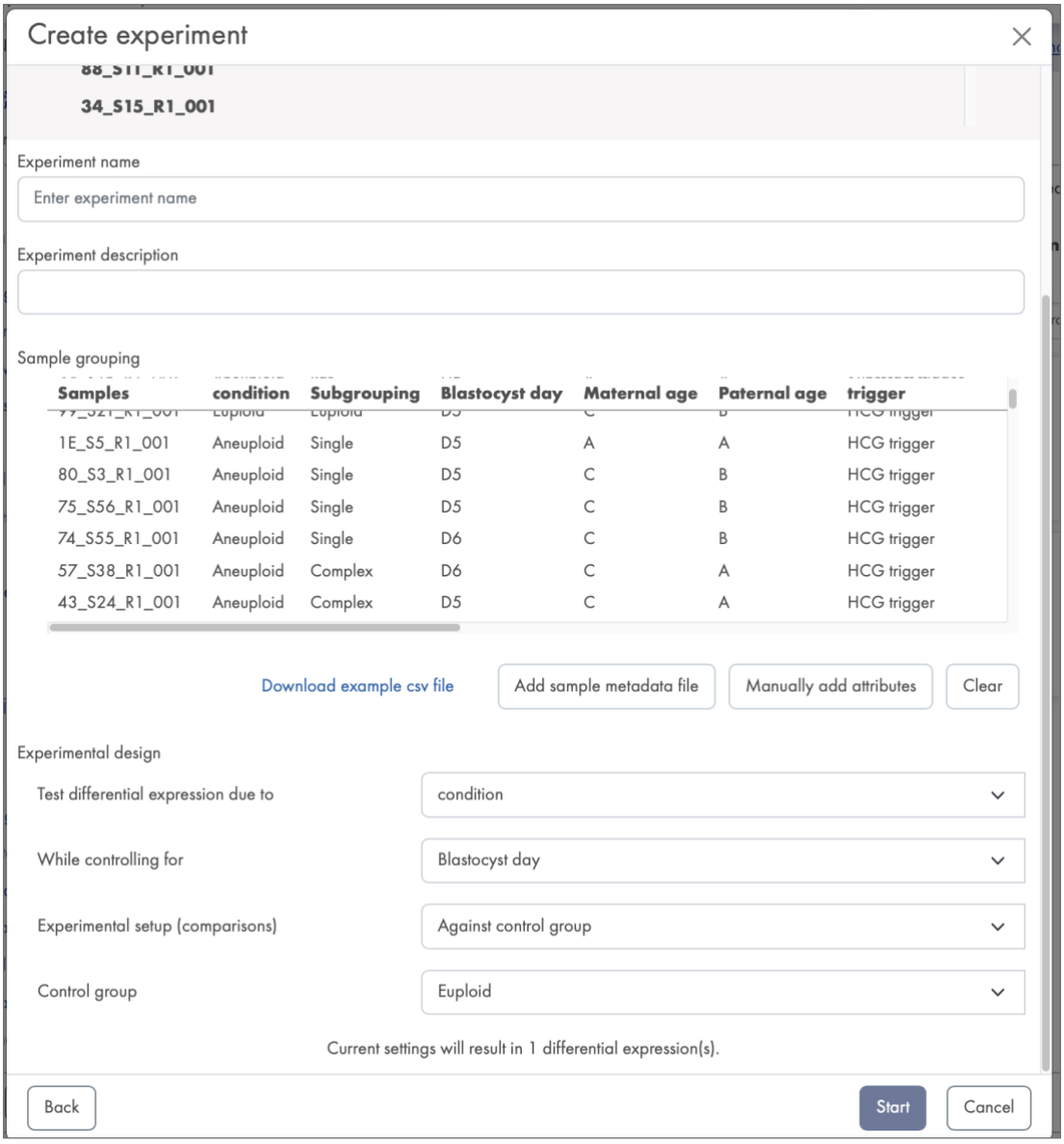
Both miRNA and mRNA gene expression data were normalized using DESeq2 through GeneGlobe and/or RStudio. Normalization is a default step when running DESeq2 for differential expression. Before processing to differential expression analysis, the normalized miRNA reads were extracted and initially used to identify the top 100 miRNAs expressed in blastocysts. The average of normalized UMI counts for all samples was measured and sorted from largest to smallest, with the top 100 expressed genes selected.

The second command in DESeq2 executed the identification of differentially expressed genes between the assigned groups. All factors under investigation were provided in a dataset in (.csv) format, uploaded to GeneGlobe platform. Selecting the factor of interest and choosing the control and test groups for comparison were established through the platform (as shown in Figure 2-11). Remarkably, the platform allowed for controlling of one factor per run, enabling to account for

[1] The negative binomial distribution is the number of failures required to achieve success for a specific number of times by using probability and success rate.

possible confounding factors during this analysis. All investigated groups are stated in (Phase 3 - 2.5).

Figure 2-11: Configuration for differential expression analysis of miRNAs

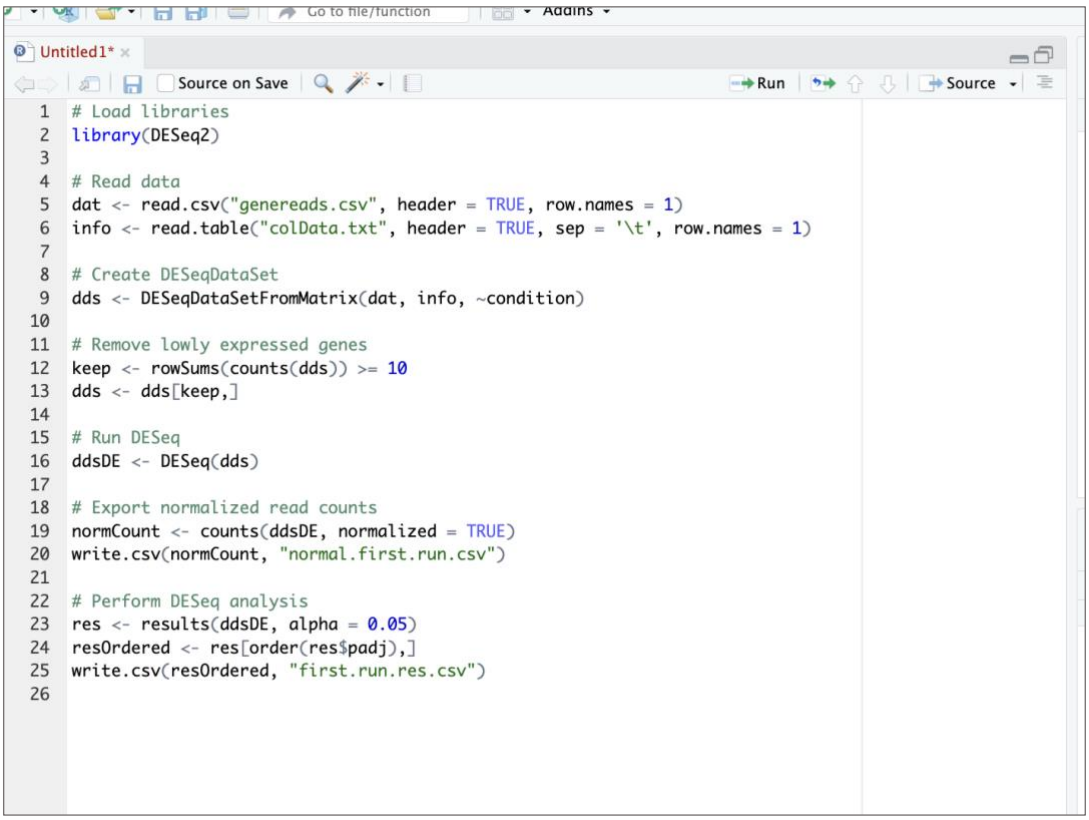


The figure illustrates the GeneGlobe analysis dashboards, by QIAGEN, where the setting for differential expression analysis are configured.

For mRNA differential expression, RStudio was utilised employing the DESeq2 package, and providing two files: one incorporating samples names and categories (the assigned groups) labelled as “colData”, and another containing samples names and their gene expression reads labelled “genereads”, as illustrated in the provided R script (Figure 2-12). Prior to calculating the differentially expressed mRNAs, the low expressed genes with less than 10 reads were eliminated. This analysis was

performed several times, initially comparing the mRNA expression profile between aneuploid to euploid blastocysts, followed by comparisons of each aneuploid subgroup to euploid blastocysts.

Figure 2-12: RStudio script for differential expression analysis of mRNA in RStudio



The figure depicts the RStudio command page where the DESeq2 tool was employed for the differential expression analysis of mRNA. The necessary data were loaded, and DESeq2 was executed to normalize the gene reads, and identify the differentially expressed mRNAs.

The statistical test utilised in DESeq2 to assess the difference between the two investigated groups is known as "Wald test", a hypothesis test that compares the change in the log-fold of gene expression between two groups to a null distribution. Differentially expressed miRNAs were considered significant when the log-fold change (FC) has a cut-off $\leq$ -1.5 or $\geq$1.5, and the FDR p-value was $\leq$ 0.1. While the FC cut-off remained consistent for mRNA reads, the FDR p-value was set to $\leq$ 0.05, considering the higher risk of false positive identification in mRNA genes.

2.12.3 Functional Analyses: Tertiary Analysis

Ultimately, the differentially expressed miRNAs underwent a comprehensive series of analyses to maximize the knowledge derived from these genes. At first, a principal component analysis (PCA) was conducted on the miRNA expression data to investigate potential sample clustering, accomplished through GeneGlobe analysis platform (QIAGEN, Crawley, UK).

The functional analysis of the identified miRNAs, including both those highly expressed in blastocysts and those differentially expressed between blastocyst groups when investigating each factor, was carried out using multiple tools. The miRTargetLink 2.0 platform was utilised to identify targeted genes, with the analysis specifically configured to select experimentally validated genes (Kern et al., 2021). In the collective analysis of a group of miRNAs, results were optimized by minimizing the number of identified targets. This was achieved through the selection of shared target option to identify genes targeted by a higher number of miRNAs.

Using miEAA 2.0 podium, computational pathway analysis was performed on the miRNAs identified throughout different analyses, simultaneously investigating several datasets, including GO biological processes, Reactome, and Wikipathways, (Ashburner et al., 2000, Pico et al., 2008, Fabregat et al., 2017, Kern et al., 2020). Only pathways that were significantly involved, with FDR p-value of 0.05 and a minimum of two hits, were included.

Further aspects of the identified miRNAs, such as chromosomal location and miRNA localization (inside the cells or in exosomes) were of explored. These investigations were also conducted using the miEAA 2.0 platform (Kern et al., 2020), which provides access to several miRNA analysis tools such as miRbase (used to find the miRNA chromosomal location) and RNALocate which (used for miRNA localization) (Zhang et al., 2017, Kozomara et al., 2019, Cui et al., 2022).

For mRNA analysis, we utilised a dedicated mRNA platform, g:Profiler, for pathway enrichment analysis (Reimand et al., 2007). The platform allows for scrutinized various datasets, showing the most significant pathways with highlighting the specific genes involved. Additionally, for target ontology, we applied the same

platform used in miRNA analysis, miRPathDB v2.0, to identify miRNA target genes (Wong and Wang, 2015, Liu and Wang, 2019).

2.13 Data Visualisation

Several tools were employed for data visualisation for different purposes. Statistical Package for the Social Sciences (SPSS) and Excel were used to illustrate the descriptive figures and tables and statistical plots. For gene expression analysis, some figures were extracted from the employed software tools or pipelines such as QIAGEN CLC Genomics Workbench (https://digitalinsights.qiagen.com), Galaxy Project, miRTargetLink 2.0, miRPathDB v2.0, and miEAA 2.0, while some other figures were designed, created or extracted from Morpheus (https://software.broadinstitute.org/morpheus/) and RStudio (Giardine et al., 2005, Kern et al., 2020, Kehl et al., 2020, Kern et al., 2021). In addition, we used BioRender to create biological diagrams and illustrations (BioRender.com.).

Results

Chapter 3 Methods Validation and Pilot Study Results: Establishing the Approach

3.1 Introduction and aim

Several previous studies have investigated miRNAs in embryos, particularly focusing on those that diffuse into the culture media, with the aim of identifying potential non-invasive markers to assess in evaluating the preimplantation embryo quality (see Table 1-1) (Kropp et al., 2014, Cuman et al., 2015, Borges et al., 2016, Capalbo et al., 2016b, Abu-Halima et al., 2017, Cimadomo et al., 2019, Abu-Halima et al., 2020, Acuna-Gonzalez et al., 2021, Fang et al., 2021, Wang et al., 2021, Timofeeva et al., 2021, Kamijo et al., 2022). Only a few studies have explored the broader miRNA expression profile within whole blastocysts, investigating its correlation with embryonic developmental competence (Rosenbluth et al., 2013, McCallie et al., 2014). As the lateral ones peaked in the mid-2010s, they primarily relied on array-based qPCR or qPCR for gene expression analysis, as sequencing technologies were not yet widely affordable or applicable. To date, NGS has not been extensively implemented in similar studies of miRNAs in human embryos.

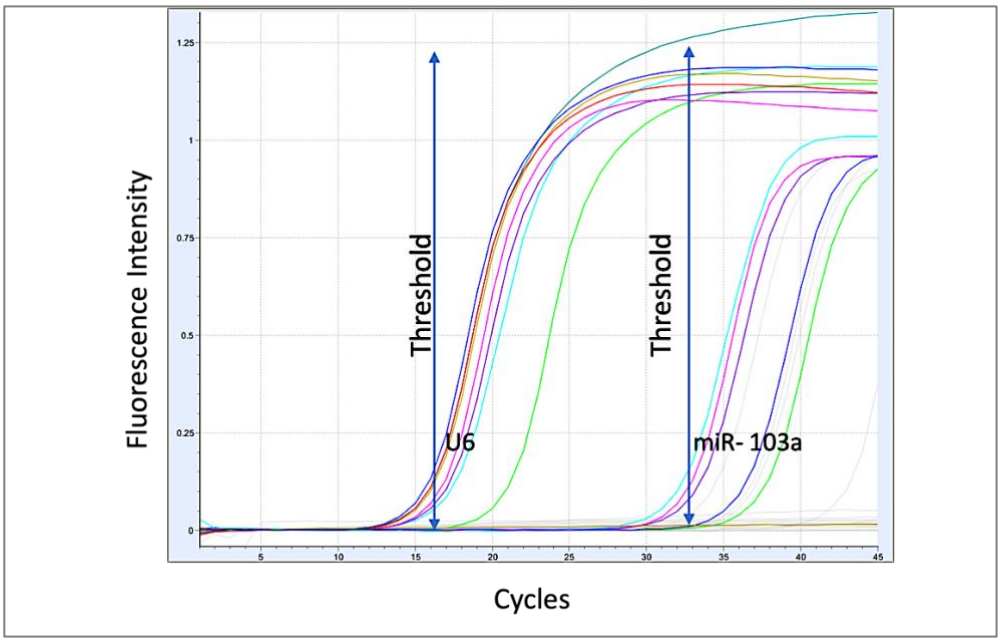
Given the lack of detailed protocols for extracting and sequencing miRNA from blastocysts, and our intention to employ NGS to investigate miRNA profiles in human blastocysts, it was crucial to ensure the suitability of both the biological samples and the chosen methodology. Therefore, before addressing the main objectives of this study, we first aimed to validate the methodologies used and confirm the presence of sufficient genetic material in the blastocysts to ensure that the results would be both valid and conclusive. This validation involved optimizing the purification and extraction protocols and confirming the detectability of miRNAs in the blastocysts. Additionally, due to the high cost of NGS, a preliminary trail was conducted to evaluate the initial results before proceeding with a full sequencing run. This step also provided essential preliminary data to guide the project's direction.

3.2 Results and interpretation

3.2.1 miRNA Isolation and Purification

To estimate the quantity of RNA in a single blastocyst, the extracted nucleic acid was measured using NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Despite several runs of sample concentration, RNA was not detectable, possibly due to the low concentration of nucleic acids in blastocysts. However, after amplifying the RNAs extracted from blastocysts by qPCR, the results demonstrated an adequate yield of miRNA in blastocysts, verifying the functionality of the extraction method (Figure 3-1). It also provided an estimate of the approximate abundance of miRNA in the blastocysts, which was important in designing the subsequent qPCR experiments and determining the required number of amplification cycles.

Figure 3-1: Amplification of small RNAs in blastocysts using qPCR



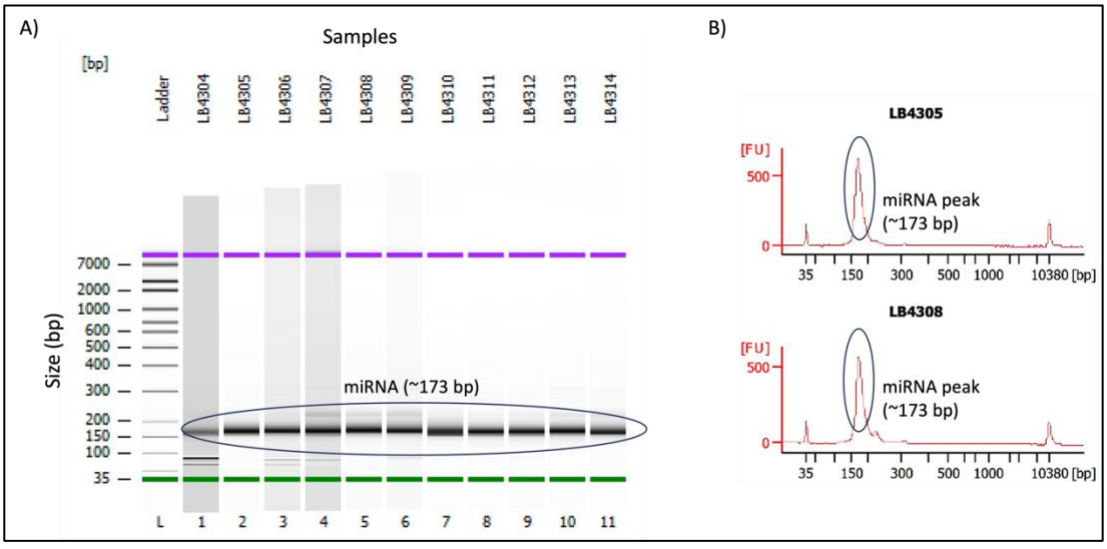
The amplification curves show successful detection of small RNAs, miR-103a and U6, in human blastocysts. The U6 gene, serving as a reference, shows earlier amplification, whereas miR-103a amplifies later, indicating its expression at lower levels. The x-axis shows the cycle number, and the y-axis represents fluorescence intensity. The threshold lines show the points at which fluorescence surpasses background noise, confirming the presence of these small RNAs in the samples.

3.2.2 miRNA Sequencing: A Pilot Study

In this phase of the study, a total of 12 blastocysts, comprising 3 euploid and 9 aneuploid samples, were sequenced. The preliminary data from this experiment aimed to validate the sequencing quality of miRNAs in blastocysts and to identify potential differences in miRNA profiles between euploid and aneuploid groups. These findings provide a foundation for the main sequencing run.

Due to the low concentration of miRNAs in the blastocyst samples, assessing their quality and quantity was feasible only after library amplification. This step, conducted in accordance with the library preparation protocol and using the Agilent Bioanalyzer 2100 (Agilent, 2019) ensured that despite the low input of total RNA, the concentration of amplified miRNAs was sufficient for sequencing (Figure 3-2).

Figure 3-2: Pre-Sequencing Evaluation of miRNA Integrity in Blastocyst Samples (First Sequencing Run)



The figure illustrates the QC results of the samples after library amplification, performed pre-sequencing using Agilent Bioanalyzer 2100. A) Gel Image showing bands of reverse transcribed miRNAs. B) Size distribution of RNA fragments within the library (the expected size for miRNA libraries is approximately 173 bp).

The post-sequencing quality assessment, which focused on sequencing QC, revealed a Phred score greater than 30. This high score indicates excellent base-calling accuracy, confirming that the data is of sufficient quality for further analysis (Figure 3-3).

Figure 3-3: Quality Assessment of Sequencing: Phred Score

The figure shows the mean sequencing quality score (Phred score), with values of ≥ 30 indicating reliable and accurate sequencing results.

Consistent with previous studies that successfully identified and quantified miRNAs from blastocyst sources, our findings also demonstrated good miRNA integrity in human blastocysts (Rosenbluth et al., 2013, McCallie et al., 2014, Esmaeilivand et al., 2022, Esmaeilivand et al., 2024). The applied approach proved successful, as sequencing accurately identified abundant miRNAs. This success paves the way for further gene expression studies using sequencing, which could not only provide comprehensive coverage of expressed genes but also identify novel ones.

3.2.2.1 miRNA Differential Expression Analysis in Euploid versus Aneuploid Blastocysts

The differential expression analysis of miRNAs in blastocysts with varying chromosomal contents was conducted in two phases. First, we analysed differences in the miRNA profiles between aneuploid and euploid blastocysts. Following this, the analysis was extended to subgroup aneuploid blastocysts based on the specific type of chromosomal abnormality, such as complex, single or mosaic aneuploidies, and compared each subgroup to the euploid blastocyst group. his approach aimed to determine whether miRNA dysregulation is consistent across different levels of aneuploidy. The results revealed significant changes in the miRNA expression profile associated with different types of aneuploidies, even with the limited number of blastocysts analysed. The dysregulated miRNAs identified through these analyses

are presented in the tables (Table 3-1, Table 3-2, Fold change values presented as negative indicate downregulation, while values without a sign indicate upregulation

Table 3-3 and Table 3-4), where negative values indicate downregulation and no charge signifies upregulation in the aneuploid samples. Interestingly, all differentially expressed miRNAs were downregulated in the aneuploid blastocysts. However, after subgroups, a few miRNAs showed upregulated, although the majority remained downregulated in the aneuploid samples.

Table 3-1: Differentially expressed miRNAs in aneuploid versus euploid blastocysts

miRNA	**FC**	**p-value**
hsa-miR-125a-5p	-2.15	0.003
hsa-miR-20a-5p	-2.07	0.003
hsa-miR-423-3p	-2.08	0.001
hsa-miR-4793-5p	-2.35	0.0003

Fold change values presented as negative indicate downregulation of the miRNA.

Table 3-2: Differentially expressed miRNAs in complex aneuploid versus euploid blastocysts

miRNA	**FC**	**p-value**
hsa-let-7a-5p	-2.1	0.002
hsa-miR-100-5p	-2.06	0.02
hsa-miR-16-5p	-2.16	0.007
hsa-miR-203a-3p	-2.1	0.003
hsa-miR-3168	-2.1	0.009
hsa-miR-3910	-2.11	0.008
hsa-miR-4793-5p	-2.17	0.006
hsa-miR-512-3p	-2.18	0.005
hsa-miR-518b	-2.09	0.0009
hsa-miR-520f-3p	-2.52	0.003
hsa-miR-520h	-2.14	0.007
hsa-miR-5583-5p	-2.26	0.01
hsa-miR-625-3p	-2.48	0.0001
hsa-miR-662	2.07	0.0008

hsa-miR-6751-5p	2.08	0.013

Fold change values presented as negative indicate downregulation, while values without a sign indicate upregulation

Table 3-3: Differentially expressed miRNAs in single aneuploid versus euploid blastocysts

miRNA	**FC**	**p-value**
hsa-miR-125a-5p	-2.4	0.005
hsa-miR-20a-5p	-2.15	0.011
hsa-miR-219b-3p	2.04	0.009
hsa-miR-323b-3p	-2.21	0.012
hsa-miR-3927-3p	2.04	0.015
hsa-miR-423-3p	-2.36	0.001
hsa-miR-7152-5p	2.13	0.006
hsa-miR-761	-2.15	0.013
hsa-miR-770-5p	2.25	0.0009

Fold change values presented as negative indicate downregulation, while values without a sign indicate upregulation

Table 3-4: Differentially expressed miRNAs in mosaic aneuploid versus euploid blastocysts

miRNA	**FC**	**p-value**
hsa-miR-16-5p	-2.05	N/A
hsa-miR-184	-2.27	N/A
hsa-miR-302a-5p	-2.15	N/A
hsa-miR-371a-5p	-2.04	N/A
hsa-miR-4436b-5p	-2.26	N/A
hsa-miR-4460	2.11	N/A
hsa-miR-4740-3p	-2.02	N/A
hsa-miR-4764-5p	2.22	N/A
hsa-miR-4793-5p	-2.82	N/A
hsa-miR-515-5p	-2.28	N/A
hsa-miR-516a-5p	-2.05	N/A
hsa-miR-520f-3p	-3.3	N/A
hsa-miR-548f-3p	-2.52	N/A
hsa-miR-593-5p	-2.02	N/A
hsa-miR-596	-2.31	N/A

hsa-miR-6731-5p	2.14	N/A
hsa-miR-761	-2.39	N/A

Fold change values presented as negative indicate downregulation, while values without a sign indicate upregulation

In the analysis of mosaic embryos, significant dysregulation of certain miRNAs was observed. However, calculating p-values for these results was not possible because the bioinformatics tool requires a minimum of three samples per group to generate statistical significance, and only two mosaic samples were available.

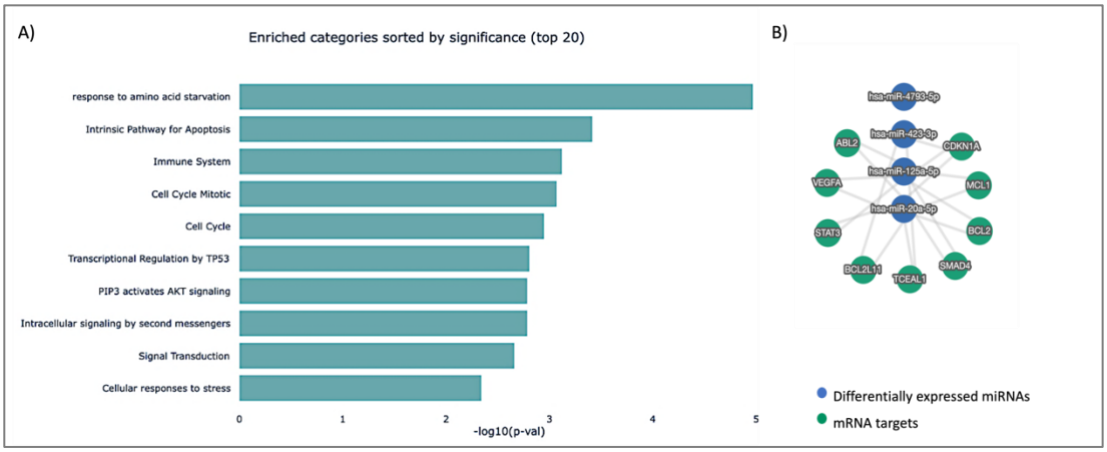
The preliminary miRNA results presented here indicate a potential association between miRNA expression and aneuploidy in blastocysts. While this connection has been proposed in previous studies, our findings particularly align with those of Rosenbluth et al. (2013) and McCallie et al. (2014), which reported the downregulation of hsa-miR-20a-5p and hsa-miR-125a-5p in aneuploid blastocysts (Rosenbluth et al., 2013, McCallie et al., 2014). These miRNAs have been linked to crucial processes in embryo development and implantation (Kim et al., 2016, Capalbo et al., 2016b).

3.2.2.2 Functional Analysis of the Differentially Expressed miRNAs in Aneuploid Blastocyst

Further computationally analysed was performed on the significantly differentially expressed miRNAs in aneuploid blastocysts, including hsa-miR-125a-5p, hsa-miR-20a-5p, hsa-miR-423-3p and hsa-miR-4793-5p, to identify their pathways and mRNA targets. The analysis revealed a strong involvement of these miRNAs in targeting genes related to cell cycle and apoptosis, likely through regulation of p53 downstream processes (Figure 3-4 (A)). Notably, *CDKN1A* emerged as a common target of these downregulated miRNAs in the aneuploid blastocysts (Figure 3-4 (B)). This suggests a potential dysregulation of *CDKN1A* levels in aneuploid embryos, which could lead to alterations in cell cycle progression in the presence of chromosomal abnormalities. The anticipated increase in *CDKN1A* levels in aneuploid blastocysts was later confirmed by the mRNA differential expression analysis comparing aneuploid and euploid blastocysts. The functional consequences

of *CDKN1A* upregulation include inhibiting cell cycle progression and possibly promoting cell death (el-Deiry et al., 1994, Harper et al., 1993).

Figure 3-4: Functional analysis of the significantly differentially expressed miRNAs in the aneuploid blastocysts, including hsa-miR-125a-5p, hsa-miR-20a-5p, hsa-miR-423-3p and hsa-miR-4793-5p



The figure illustrates: A) The biological processes associated with differentially expressed miRNAs in aneuploid blastocysts, highlighting their roles in cell cycle regulation, apoptosis, and signalling pathways. B) The miRNA-gene interaction network, identifying CDKN1A as a common target of three of these miRNAs.

Overall, miRNA sequencing results revealed a high sensitivity in detecting and quantifying miRNAs in human blastocysts. The observed differences in miRNA profiles between blastocysts with different quality highlight the need for further investigations connecting embryo competence to miRNA expression. These insights facilitated the design of the subsequent run by evaluating the feasibility of the experimental design, determining the appropriate sample size and type, and addressing any troubleshooting. Additionally, this step allowed for familiarization with the required bioinformatic tools, the platforms and pipelines needed for analysing and visualizing of the sequencing data. It also provided insights into the potentially altered genes in aneuploid embryos, prompting further exploration in the upcoming run.

3.2.2.3 Validation of The Sequencing Results

To validate the miRNA sequencing results, three miRNAs—miR-16-5p, miR-625-3p, and miR-5583-5p—were selected for reanalysis using PCR. Sequencing showed

these miRNAs to have high, medium, and low expression levels, respectively. Differential expression analysis revealed upregulation of all three miRNAs in aneuploid samples. The qPCR results confirmed the upregulation of miR-16-5p and miR-625-3p in aneuploid blastocysts, consistent with the sequencing data. However, miR-5583-5p, which had low expression, was not detected by PCR.

3.2.3 Investigation of the Potential Association Between Aneuploidy and miRNA Biogenesis Genes Expression levels

To explore the potential association between aneuploidy and miRNA expression, it was essential first to evaluate the impact of aneuploidy on the miRNA biogenesis process. This assessment was necessary to rule out the confounding effects of impaired miRNA synthesis due to chromosomal imbalances in regions containing key genes involved in miRNA biogenesis. For this purpose, we analysed the gene expression read numbers for four critical miRNA synthesis genes: *DROSHA*, *DGCR8*, *DICER1*, and *XPO5* (Table 3-5). We examined whether allele imbalances in these genes would affect their read numbers, using data provided by Xuhui Sun

Table 3-5: Function and chromosomal locations of mRNA genes involved in miRNA biogenesis process.

Stage of miRNA biosynthesis	**Gene**	**Chromosome location**
First cleavage	*DROSHA*	5p13.3
First cleavage	*DGCR8*	22q11.21
Exporting pre-miRNA to cytoplasm	*XPO5*	6p21.1
Second cleavage process	*DICER1*	14q32.13

The analyses in this section aimed to explore the correlation between the expression levels of these genes and both overall aneuploidy and aneuploidy specific to the chromosomes harbouring each gene. In these investigations, we accounted for potential confounding factors, including maternal and paternal age, as well as embryonic morphology. The sample sizes for each analysis are detailed in (Table 3-6).

Table 3-6: Number of samples with aneuploidy in chromosomes containing key miRNA biogenesis genes

Chromosome	**Number of not affected samples**	**Number of affected samples**
All chromosomes	18	82
Chromosome 5	98	2
Chromosome 22	86	14
Chromosome 6	96	4
Chromosome 14	92	8
Chromosome 14 monosomy	96	4
Chromosome 14 trisomy	96	4

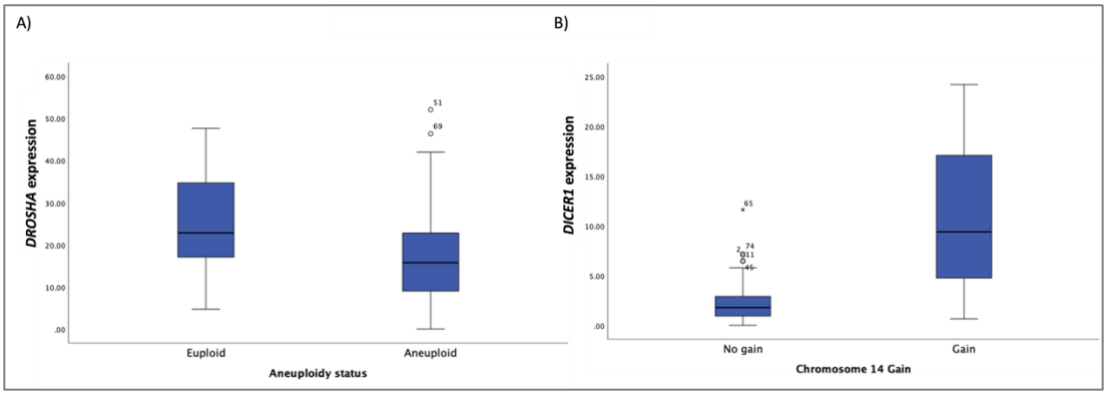
While *DROSHA* demonestrated a significant correlation with aneuploidy, decreasing by six fold in aneuploid embryos with < 0.05 p-value (Table 3-7 and Figure 3-5) its expression was independent of the numerical abnormalities in chromosome 5, where it is located. However, since only two samples with chromosome 5 imbalances were available, additional data are required to draw a definitive conclusion.

Table 3-7: *DROSHA* regression results

Factor	**Size effect**	**p-value**	**95.0% Confidence Interval**	
Aneuploidy	-6.784	.026	-12.737	-.831
Chromosome 5 aneuploidy	8.654	.272	-6.887	24.194
Sample morphology	-1.172	.521	-4.785	2.441
Maternal Age	-.543	.055	-1.098	.012
Paternal Age	.319	.188	-.159	.798

The red highlighted parameters are significant.

Figure 3-5: Difference in miRNA biogenesis genes expression between euploid and aneuploid blastocysts



The box plots illustrate the differences in the mean of A) *DROSHA* expression between euploid and aneuploid blastocysts. B) The in the *DICER1* expression between euploid and blastocysts with gain in chromosome 14, where *DICER1* is located.

Analysis of *DICER1* expression revealed a significant influence of chromosome 14 aneuploidy (Table 3-8). Further analysis, which divided the samples with chromosome 14 aneuploidy into loss and gain subgroups, showed that the gain of chromosome 14 was positively correlated with *DICER1* expression levels (Table 3-9 and Figure 3-5**Error! Reference source not found.**). Conversely, the loss of chromosome 14 did not show a significant correlation with *DICER1* expression, suggesting that this gene may be compensated for when chromosome 14 is lost. In contrast, the analyses of *DGCR8* and *XPO5* levels revealed no significant impact of aneuploidy on their expression (Table 3-10 and

Table 3-11).

Table 3-8: *DICER1* regression results

Factor	**Size effect**	**p-value**	**95.0% Confidence Interval**	
Aneuploidy	-.409	.619	-2.036	1.218
Chromosome 14 aneuploidy	3.771	.001	1.597	5.946
Sample morphology	-.325	.510	-1.300	.651
Maternal Age	.112	.145	-.039	.263
Paternal Age	-.053	.422	-.184	.077

The red highlighted parameters are significantly correlated to DICER1 expression.

Table 3-9: *DICER1* and chromosome 14 aneuploidy regression results

Factor	Size effect	p-value	95.0% Confidence Interval	
(Constant)	1.106	.668	-3.997	6.210
Chromosome 14 Loss	-.938	.484	-3.592	1.715
Chromosome 14 Gain	8.390	.000	5.727	11.053
Sample morphology	-.192	.661	-1.059	.675
Maternal Age	.083	.213	-.048	.215
Paternal Age	-.041	.483	-.156	.074

The red highlighted parameters are significantly correlated to DICER1 expression.

Table 3-10: *DGCR8* regression results

Factor	Size effect	p-value	95.0% Confidence Interval	
Aneuploid	.032	.858	-.320	.384
Chromosome 22 aneuploidy	.206	.277	-.169	.581
Sample morphology	-.085	.428	-.296	.126
Maternal Age	-.011	.524	-.044	.022
Paternal Age	-.001	.916	-.030	.027

Table 3-11: *XPO5* regression results

Factor	Size effect	p-value	95.0% Confidence Interval	
Aneuploid	1.961	.592	-5.278	9.199
Chromosome 6 aneuploidy	-.979	.885	-14.336	12.379
Sample morphology	-.411	.851	-4.752	3.931
Maternal Age	-.515	.132	-1.189	.158
Paternal Age	-.391	.184	-.972	.189

3.2.3.1 Absence of Observed Influence of Aneuploidy on The Expression Level of miRNA Biogenesis Genes

Although miRNAs have been extensively studied in many cancer types, which are also prone to chromosomal defects, the potential impact of aneuploidy on the miRNA production machinery has not been previously explored. In our study, we addressed the possible influence of chromosomal gains and losses on the expression levels of genes involved in miRNA biogenesis, which could confound the overall miRNA expression.

These genes have been long recognized for their roles in several cellular processes within reproductive cells. For instance, DICER1 is essential for chromosome condensation in oocytes, and its absence results in arrest at meiosis I (Murchison et al., 2007). Additionally, germline deficiencies or mutations in of *DICER1*, *DROSHA, DGCR8* and *AGO* can be lethal. Knock-down of these genes, or lack of their proteins, has been shown to severely impact cell differentiation, proliferation, and apoptosis, according to previous mouse studies (Bernstein et al., 2003, Wang et al., 2007, Chong et al., 2010). Moreover, loss of *DROSHA* and *DICER1* is associated with reduced levels of mature miRNAs and the accumulation of precursor miRNAs (Lee et al., 2003, Suarez et al., 2007).

Dysregulation of miRNA biogenesis genes is frequently observed in malignancies, which often exhibit chromosomal abnormalities (Huang et al., 2014). These observations suggest that impaired embryogenesis might be attributed to defects in miRNA production. However, the current analysis investigating the potential impact of aneuploidy on the expression levels of these genes indicated that miRNA biogenesis machinery remains functional in aneuploid embryos. Notably, interesting results from these investigations, such as the downregulation of *DROSHA* in aneuploid blastocysts and the elevation of *DICER1* in embryos with chromosome 14 gain, warrant further investigation in the future.

3.3 Chapter Summary

Blastocysts contain an abundant array of miRNAs that are detectable through both quantitative PCR (qPCR) and next-generation sequencing (NGS). Preliminary sequencing data demonstrate a high quality of miRNA sequencing in human blastocysts. Furthermore, initial findings suggest that aneuploidy in chromosomes harbouring miRNA biogenesis genes does not appear to directly affect the miRNA biogenesis process.

Chapter 4 MicroRNA Profiling in Human Blastocysts: A Novel Sequencing Study

4.1 Introduction and Aim

The continuous advancements in gene expression analysis tools have significantly expanded investigations in reproductive science. Among these developments, NGS stands out as a transformative technology, revolutionizing embryonic selection by enabling the simultaneous testing for multiple genetic defects (Abuli et al., 2016, Garcia-Herrero et al., 2020, Rajcan-Separovic, 2020). Beyond its applications in genetic screening, NGS facilitates specialized and detailed explorations, such as epigenetics and gene expression profiling (Guo et al., 2014, He and Feng, 2022). Applying this high throughput method to investigate miRNA profile in developing embryos could offer significant insights into reproductive health, further enhancing diagnostic and biomarker capabilities.

miRNAs are particularly promising in this context due to their unique characteristics, including their small size, extracellular expression, and stability, which set them apart from other RNA types (Huang, 2017). Most studies on preimplantation embryos have focused on extracellular miRNAs, particularly those found in blastocoel fluid or in the culture media, as a non-invasive approach to embryo assessment (Kropp et al., 2014, Cuman et al., 2015, Borges et al., 2016, Capalbo et al., 2016b, Abu-Halima et al., 2017, Cimadomo et al., 2019, Abu-Halima et al., 2020, Acuna-Gonzalez et al., 2021, Fang et al., 2021, Wang et al., 2021, Timofeeva et al., 2021, Kamijo et al., 2022). While these studies have provided valuable insights, they also have some limitations in fully understanding the miRNA landscape within the blastocyst. By only concentrating on miRNAs found in culture media or blastocoel fluid, important miRNAs within the cells of the blastocyst might be overlooked. Furthermore, extracellular miRNAs could include contributions from non-blastocyst sources, potentially confounding the results, as previously observed (Sanchez-Ribas

et al., 2019). This approach, therefore, offers only a partial view of the miRNA profile. Despite this, comprehensive profiling of miRNAs within the blastocyst, across all its compartments, has not been extensively explored.

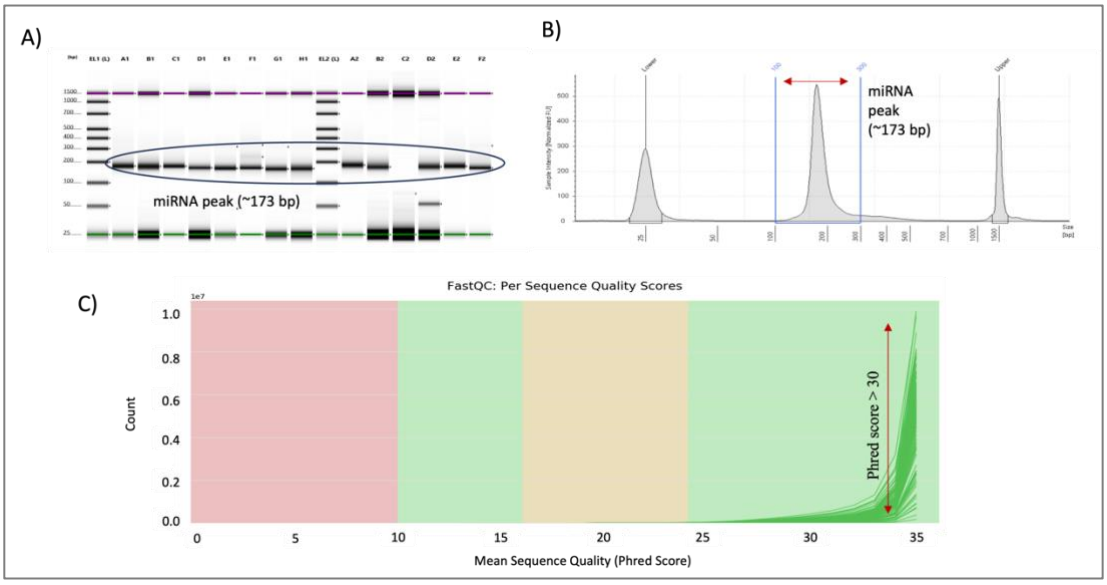
In this phase of the research, the first objective was to verify the quality of miRNA sequencing by assessing and ensuring the integrity of the samples included in the second (main) sequencing run. Following this, we conducted a comprehensive profiling of miRNAs in blastocysts to explore the complete miRNA landscape in human blastocysts. This investigation aimed to identify the most highly expressed miRNAs as and the biological processes they regulate within blastocysts, ultimately elucidating the impact of miRNA expression on embryonic development.

4.2 Results and Interpretation

4.2.1 Sample Integrity and Sequencing Quality

The integrity of the reversed transcribed miRNAs in the blastocysts was evaluated after library amplification but before sequencing, as in the first run (see 2.3.1). The quality reports indicated that the majority of the blastocysts exhibited good miRNA quality and quantity, successfully passing the library pre-sequencing QC (Figure 4-1A and B). Consistent with the quality results from the first analysis, the second track, which included 125 samples, demonstrated uniformly high quality across all samples as assessed using the Agilent TapeStation (Agilent, 2019). The quality traces of all samples are provided in Appendix5. Only 9 blastocysts did not pass the QC in this trial.

Figure 4-1: Pre-sequencing sample QC – second run



The figures illustrate different QC measures. A) Gel image produced by electronic electrophoresis showing the bands of miRNA in 14 samples with two ladders (EL1,EL2). B) The intensity of miRNA library in one sample (~173 bp library was the average size). C) Curve of Mean Sequencing Quality showing Phred score >30 for all samples included.

After cleaning and pre-processing sequencing data, by trimming adaptors and short reads, we performed a quality assessment to evaluate the accuracy of the sequencing data. All samples passed the evaluation without flags for poor sequencing. Especially important was the sequencing quality score, which showed a low probability of base calling error (Phred score above 30), indicating accurate sequencing results (Figure 4-1 C). Despite some quality parameters failing or showing warnings, troubleshooting indicated that the noise was primarily due to the sample's biological features or technology limitations (an example of the QC report via FastQC and MultiQC can be found in Appendix5). The evaluation and interpretation of the quality metrics followed guidelines from Galaxy and Babraham Bioinformatics, collectively affirming the integrity of the samples and the quality of the sequencing data, thus enabling confident progression to subsequent miRNA expression analysis. (Afgan et al., 2018, Batut et al., 2018, Hiltemann et al., 2023, Bioinformatics, 2021a, Bioinformatics, 2021b, Bioinformatics, 2021c, Bioinformatics, 2021d, Bioinformatics, 2021e).

4.2.2 miRNA Abundance in Human Blastocysts

Initially, we conducted a primary analysis of miRNA sequencing data to explore the general characteristics of miRNAs in human blastocysts. The results revealed high expression of miRNAs, with 2,502 miRNAs detected in the blastocysts out of the 2,654 mature miRNAs identified in humans (miRBase release 22) (Table 4-1). A total 128 blastocysts were sequenced in this study, but only 122 samples were included in the subsequent analyses, as six blastocysts were excluded due to chaotic and mosaic aneuploidy. After normalizing the gene reads and removing low-expressed miRNAs, 2,491 miRNAs remained for the upcoming investigations.

Approximately 22 million miRNA reads were captured per blastocyst, resulting in a total of 942 million RNA reads across the 122 samples. Although various types of RNAs were simultaneously identified during the analysis, these are not the focus of this study (Table 4-1). The findings highlight the high abundance of miRNA in preimplantation embryos, emphasizing their significant influence on early embryonic development.

Table 4-1: Sequencing primary analysis - number of reads

Read set	**Number of Reads**
Total reads	942,753,986
UMI defective reads	43,065,023
miRNA Reads	22,150,293
hairpin Reads	28,577
rRNA Reads	26,034,219
mRNA Reads	1,932,954
Not Characterized Mappable Reads	51,364,867

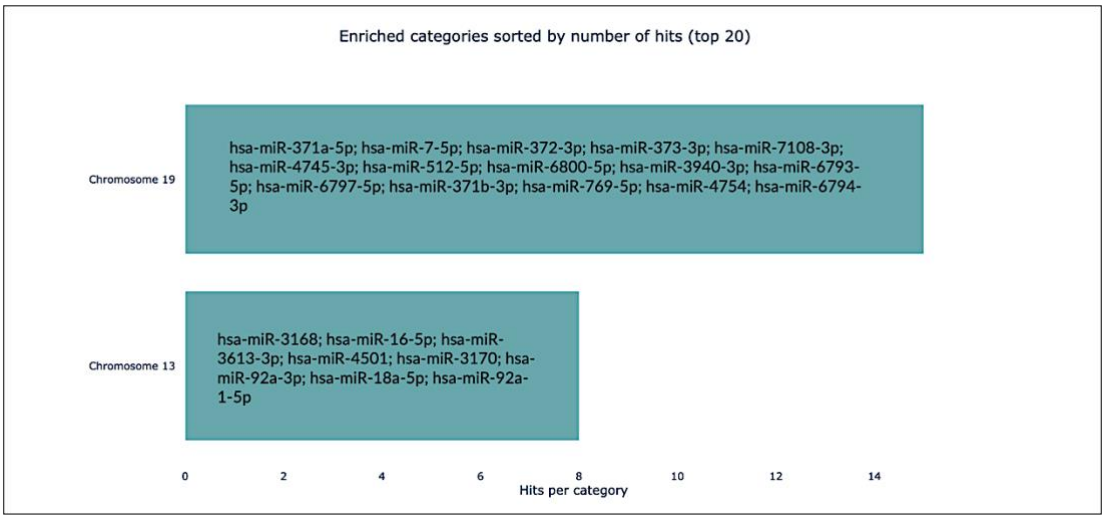
Prior studies on miRNA expression in reproductive cells reported detectable amounts of miRNAs in sperm of various animal species (Selth et al., 2014, Du et al., 2014, Fagerlind et al., 2015, Kasimanickam et al., 2022). In contrast, miRNAs in mammalian oocytes showed low expression and reduced activity (Suh et al., 2010, Ma et al., 2010, Kataruka et al., 2020). However, the miRNA expression levels increase at the eight-cell stage, with even higher concentration in the blastocysts (Berg and Pfeffer, 2018). While our study primarily investigates miRNA expression

in blastocysts, it also confirms ample expression at this embryonic stage which aligns with the previous observations.

4.2.3 Top 100 Most Expressed miRNAs in Blastocysts: Features, Target Genes and Pathways

In this part of the study, we identified the top 100 highly expressed miRNAs after sorting their normalized reads based on average and median values (see Appendix6 for the list). Several analyses were conducted on these miRNAs to provide a comprehensive understanding of their roles in blastocysts. Initially, we examined their chromosomal locations, revealing a notable pattern with significant enrichment of miRNAs encoded on chromosomes 19 and 13. Specifically, 16 highly expressed miRNAs were located on chromosome 19, while 8 were on chromosome 13 (as illustrated in Figure 4-2). Interestingly, some of these miRNAs are members of the well-known 17-92 and C19CM clusters, which are pregnancy-associated clusters (Donker et al., 2012, Bullerdiek and Flor, 2012, Morales-Prieto et al., 2013, Kumar et al., 2013, Xie et al., 2014). Both clusters are particularly known for their roles in trophoblast differentiation (Liang et al., 2023, Kobayashi et al., 2022).

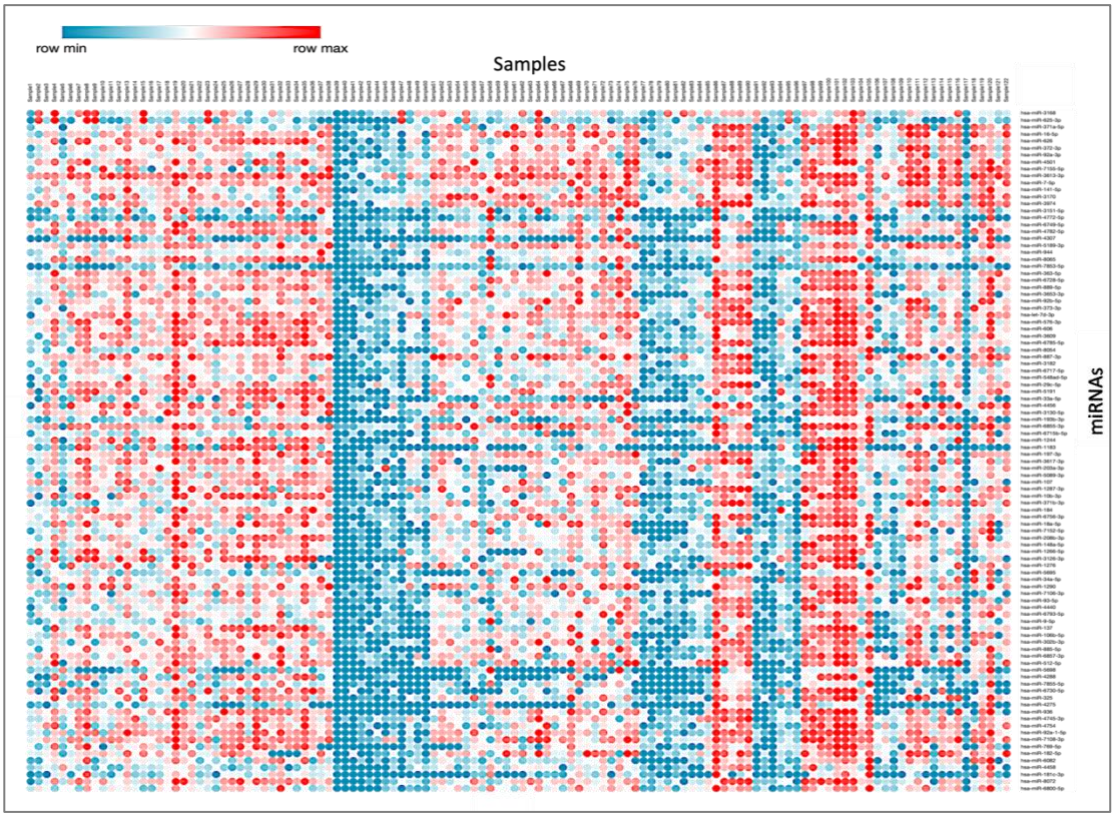
Figure 4-2: Chromosomal location enrichment analysis of the highly expressed miRNAs in human blastocysts



This figure shows the chromosomal location analysis of the top 100 highly expressed miRNAs in human blastocysts, highlighting that a substantial number are encoded on chromosomes 19 and 13.

Additionally, the relative expression between these 100 miRNAs across all samples was investigated, showing elevated expression of certain miRNAs among others, distinct expression pattern of miRNAs across samples as well as a potential clustering of some miRNAs (Figure 4-3). These observations indicate variation in the expression of these genes between the blastocysts, highlighting the need for further investigations to understand the underlying factors contributing to these differences.

Figure 4-3: Relative expression of the highly expressed miRNAs in human blastocysts

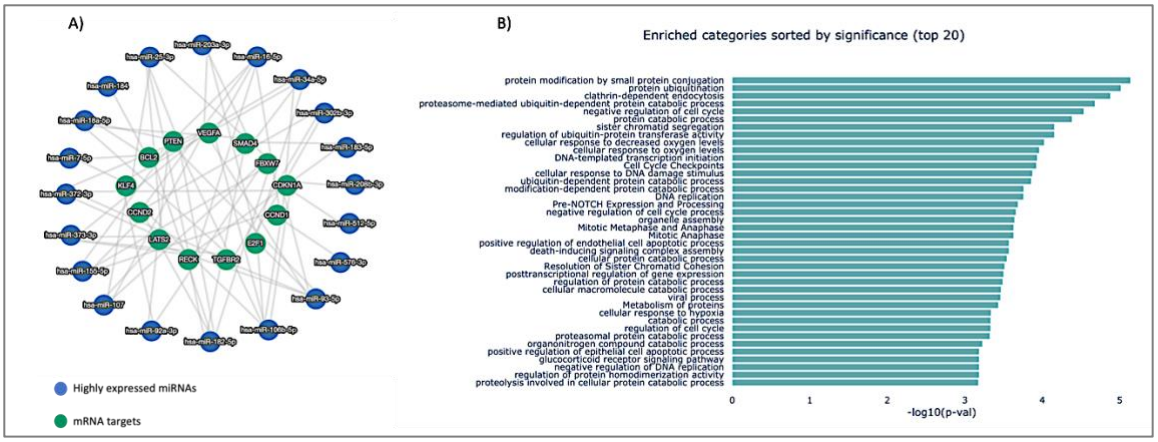


The heatmap illustrates the expression profiles of top-100 miRNAs across 122 human blastocysts. Rows correspond to miRNAs, while columns represent the blastocysts. The color reflects the relative expression level of a particular miRNA within each blastocyst. Blue colors indicate lower expression, whereas red colors indicate higher expression. This figure was generated using Morpheus.

Eventually, as we focused on identifying the potentially highly regulated pathways at the blastocysts stage of development by conducting functional analyses of the selected miRNAs (Figure 4-4 (A)). The results demonstrated a high targeting potential of critical genes, including *VEGFA, SMAD4*, *FBXW7, CDKN1A, CCND1, E2F1, TGFBR2, RECK, LATS2, CCND2, KLF4, BCL2*, and *PTEN*. These genes

serve as key regulators of important cellular processes, such as signalling, cell cycle transitions, and cell growth.

Figure 4-4: Functional analysis of highly expressed miRNAs in human blastocysts

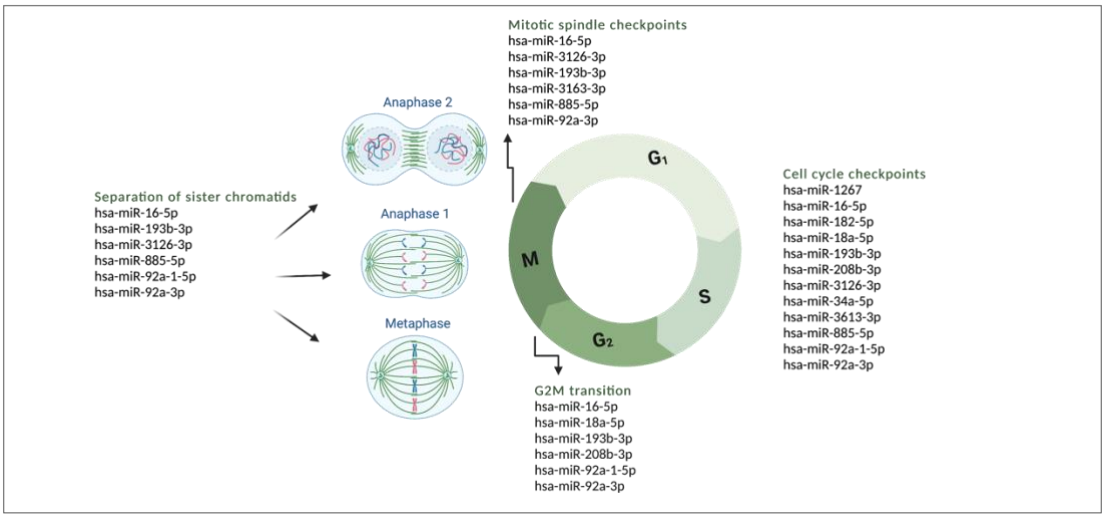


The figure illustrates A) The miRNA-gene target interaction network of the top 100 selected miRNAs in human blastocysts. Blue circles represent highly expressed miRNAs with experimentally validated gene targets, while their commonly regulated genes are shown in green. B) Analysis of the potentially regulated pathways by these miRNAs. The pathways are sorted by significance, showing a significant involvement of these miRNAs in protein processes, cell cycle and checkpoints, responding to low oxygen levels, apoptotic processes.

Additionally, pathway analysis of these miRNAs was conducted, highlighting their strong involvement in numerous cellular processes. Apparently, these miRNAs play a significant role in maintaining protein functional activities by regulating protein modification processes, such as ubiquitination and catabolism (Figure 4-4 (B)). This finding aligns with the well-established importance of ubiquitination in regulating the proliferation and differentiation of embryonic stem cells (Wang et al., 2019).

The findings also highlight substantial involvement of these miRNAs in regulating cell-cycle events. This regulation, particularly during the G1-S transition and the M phases, is evident through targeting of many key genes involved in these transitions (Figure 4-5) (Wang and Blelloch, 2009). Moreover, these miRNAs exert considerable control over mitotic metaphase and anaphase and regulate crucial processes such as sister chromatids segregation and mitotic spindle checkpoints (Fung et al., 2002, Sankaran et al., 2011, Mets et al., 2015).

Figure 4-5: Contribution of highly expressed miRNAs in human blastocysts in various cell cycle phases.



The figure illustrates the highly expressed miRNAs in blastocysts which are involved in regulating different phases of cell cycle and mitotic processes.

Furthermore, cell death mechanisms, including endocytosis and apoptosis, are frequently regulated by the analysed miRNAs, as revealed by pathway analysis. Insights from both pathway annotation and miRNA-gene target interaction highlight their involvement in apoptotic processes, particularly through their targeting of the key apoptotic gene BCL2 (Scherr et al., 2014, Wang et al., 2018).

Given the high mitotic activity in blastocysts, the results indicating that highly expressed miRNAs regulate DNA replication and DNA metabolic processes were intriguing, though not surprising (Tulay and Sengupta, 2016). The observed negative regulation of DNA replication, combined with the suppression of cell cycle processes, suggests potential impairment in cell progression, potentially related to the high prevalence of aneuploidy in our samples.

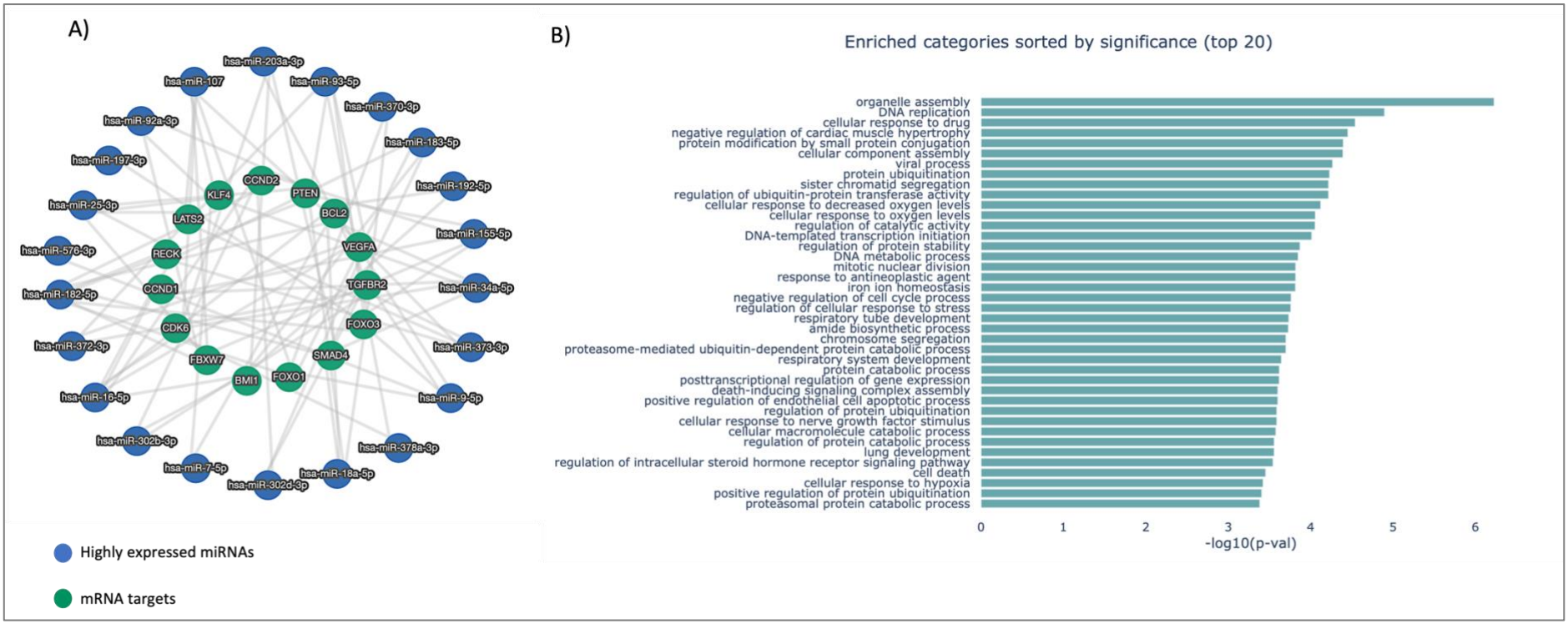
Intriguingly, blastocyst miRNAs are significantly involved in the cellular response to hypoxia, an insufficient supply of oxygen to tissue or organs. This observation suggests that the blastocysts under investigation, and possibly all preimplantation blastocysts, are exposed to low oxygen levels in vitro. It is important to note that the oxygen concentration used in the CRGH laboratory, where the examined samples were prepared, was 5%. This percentage is routinely selected to mimic the low oxygen tension environment in the uterus. Since low oxygen tension is the norm, further investigation is needed to determine whether this response in preimplantation

embryos is specific to those fertilized in vitro or it represents a natural adaptation common to both in vitro and in vivo fertilized embryos.

Another notable finding from the miRNA pathway analysis is the potential regulation of glucocorticoid receptor signalling by blastocyst miRNAs. Glucocorticoids, which are steroid hormones, play a crucial role in immune suppressors, essential for normal decidualization and embryonic implantation by inducing immune tolerance (Korgun, 2012). The observed results of potential embryonic response to glucocorticoids, mediated by signalling receptors, suggests that miRNAs may regulate the embryo's response to maternal immune signals This regulation could be vital in facilitating the implantation process, particularly in the absence of a fully developed embryonic immune system. It has been proposed that endometrial immune cells can detect and help eliminate aneuploid embryos, highlighting their critical role in immune regulation during the window of conception and embryo implantation (Robertson, 2010, Macklon and Brosens, 2014). This notion emphasizes the active immune interaction between the embryo and the maternal uterus.

To characterize the typical miRNA profile in normally developing blastocysts, we conducted an additional analysis focusing only on the 100 most expressed miRNAs in aneuploidy-free embryos (26 euploid blastocysts). Functional analysis of these miRNAs yielded results consistent with those observed in the entire group of blastocysts (Figure 4-6). The miRNA-gene target interaction analysis revealed regulating key controllers of cell cycle progression and proliferation, including *CCND1, CCND2, CDK6, LATS2, FBXW7, VEGFA* and *TGFBR2*, as well as genes involved in cell death and stress response such as *BCL2, FOXO3, FOXO1* and *BMI*, just like the genes identified when analysing the whole set of samples. This consistency in the findings highlights the crucial role these genes play in controlling blastocyst development (Figure 4-6).

Figure 4-6: Functional analysis of the 100 most expressed miRNAs in euploid blastocysts



The figure shows A) miRNA-gene target of highly expressed miRNAs in euploid human blastocysts. Blue circles represent highly expressed miRNAs with experimentally validated gene targets, while commonly regulated genes are shown in green. B) Pathway annotation analysis the potentially regulated pathways by the top-100 expressed miRNAs in 26 euploid blastocysts. The pathways are sorted by significance, showing involvement of these miRNAs in protein modifications, chromosomal segregation, cellular responding to stresses like hypoxia and regulation of cell death.

The pathway annotation analysis also revealed regulation of common pathways between the two groups investigated. However, one pathway was exclusively significant when investigating the euploid blastocysts, the involvement of their miRNAs in regulating steroid hormone receptors. This observation may indicate that euploid embryos are preparing for implantation, with miRNAs mediating the interaction between the embryo and the maternal environment. Moreover, unlike in the previous analysis of the entire cohort of blastocysts, the pathways associated with the cellular responses to DNA damage and the activation of cell cycle checkpoints were not identified in this investigation. Such findings may indicate that chromosomal abnormalities act as stressors in aneuploid blastocysts, triggering the activation of the DNA damage response mechanisms.

In fact, the consistency in the findings of the highly expressed miRNAs in euploid and all other blastocysts was unsurprising, as the samples investigated shared similar general features, including that they were ICSI produced, have good to fair morphology grade, and were all at blastocysts stage. However, what is fundamental in this context and would provide more precise results is to identify the potentially disturbed pathways in aneuploid blastocysts compared to euploid ones by comparing the profile of miRNAs between the two groups. The differential expression analysis of miRNAs will be applied and investigated in the forthcoming chapter. Overall, our findings on miRNAs with abundant expression in blastocysts underscore the prevalent biological processes and corresponding genes essential for normal development and potentially predisposed to abnormal embryonic growth. Detailed pathway annotation illustrations of the selected 100 miRNAs are found in Appendix6.

Previous studies on miRNAs in oocytes and preimplantation embryos have demonstrated their involvement in key processes such as maturation, development, cell cycle regulation, and DNA repair (Tulay et al., 2015, Abd El Naby et al., 2013, Yang et al., 2016). Moreover, miRNAs are key regulators of cell proliferation and differentiation within stem cells and are instrumental in modulating signalling pathways (Gangaraju and Lin, 2009, Avraham and Yarden, 2012). In this study, we provide a broader perspective on the potentially regulated pathways in preimplantation embryos by examining the full miRNA profile in whole blastocysts. Our findings not only align with previous observations but also expand upon them,

offering a more comprehensive understanding of miRNA function during early embryonic development.

The previous investigations of miRNAs in embryos, though limited, have primarily focused on specific miRNA expression differences in relation to factors such as developmental potential and morphology (Kropp et al., 2014, Cuman et al., 2015, Borges et al., 2016, Capalbo et al., 2016b, Abu-Halima et al., 2017, Cimadomo et al., 2019, Abu-Halima et al., 2020, Acuna-Gonzalez et al., 2021, Fang et al., 2021, Wang et al., 2021, Timofeeva et al., 2021, Kamijo et al., 2022). However, comprehensive miRNA expression profiles and their associated biological pathways have rarely been explored in early developing embryos (Battaglia et al., 2019, Russell et al., 2020). In a prior study involving nine blastocysts, 89 miRNAs were identified using TaqMan Array technology. When compared to our results, most of these miRNAs were also present in the blastocysts; however, the use of sequencing in our study enabled the identification of 25 times more miRNAs. Additionally, a study profiling small RNAs secreted into culture media identified the top 20 miRNAs, many of which were also highly expressed in our findings. This alignment not only confirms the expression of the identified miRNAs but also underscores their origin in the blastocyst, positioning both blastocoel fluid and culture media as valuable sources for assessing blastocyst development and quality.

4.3 Chapter Summary

To the best of our knowledge, this study represents the first attempt to employs sequencing for miRNA expression profiling in human blastocysts. We established the application of NGS-based miRNA analysis in human blastocysts, and highlighted the abundance and role of miRNAs in early developing embryos. Initial analyses demonstrated satisfactory quality of miRNA sequences, reflecting the robustness of this approach. Moreover, the consistency of the miRNA results indicates the validity and accuracy of the findings. Perhaps the greatest virtue of miRNAs in this context was their high abundance and integrity in blastocysts, which verified the feasibility and potential of employing molecular techniques in blastocysts to uncover the complicated mechanisms underlying early embryogenesis.

In addition to these roles, our analysis on the highly expressed miRNAs in blastocysts revealed their specific functions within developing blastocysts. These miRNAs commonly contribute to cell cycle transitions, mitotic events, metabolic processes, maintenance of functional protein activity, cellular responses to hypoxia, regulation of hormone receptors, and cell death processes. These findings provide an encompassing panorama of the pathways influenced by miRNAs during blastocyst development and how miRNAs orchestrate key developmental pathways at this stage of development.

Chapter 5 Comparative Analysis of miRNA Differential Expression in Embryos with Varied Quality

5.1 Introduction and Aim

In clinical practice, three primary factors, embryonic morphology, day of blastocyst formation, and aneuploidy status, are commonly used to assess the blastocyst quality and guide decisions regarding embryo transfer. These factors are strongly correlated with implantation potential and pregnancy outcomes, and have been consistently employed in the comparisons between different mechanisms and settings in the IVF treatment. For example, the preference for day 5 on day 6 blastocysts transferer was chosen as day 6 blastocysts are more prone to chromosomal abnormalities (Taylor et al., 2014b).

While these factors have greatly influenced the implantation potential and pregnancy outcomes, they have their limitations. The subjectiveness of the morphology evaluation and the invasiveness of aneuploidy testing are the most drawbacks. While this evaluation is important, pregnancy rates were not significantly enhanced. However, the evaluation of the genetic and metabolic status or biomolecular status in the blastocytes is overlooked. The utilisation of miRNAs as promising biomarkers for various conditions offers a new opportunity to develop more representative indicators of embryonic quality. Given the strong correlation between the aneuploidy status, day of blastulation, blastocyst morphology and the embryo competence, we hypothesized that miRNA profiles would differ between high and the low-quality embryos based on these factors, and therefore could offer a more representative indicator for the genetic status of the embryo.

This hypothesis was formed based on previous studies suggesting that gene expression profiles change in response to chromosomal abnormalities as well as with different morphology scores (Wells et al., 2005, Rosenbluth et al., 2013, McCallie et al., 2014). However, potential differences in miRNA profiles between blastocysts

formed on day 5 versus day 6 have not been fully explored, although miRNA expression is significantly influenced by the developmental stage (Assou et al., 2011).

This chapter presents investigations aimed at identifying differentially expressed miRNAs in low-quality embryos, considering these factors, and explores their contribution to relevant biological pathways.

5.2 Results and Interpretation

5.2.1 Systematic Review Results: Association between Aneuploidy Status and miRNA Expression in Human Blastocysts

A systematic review was conducted to combine the existing literature on the relationship between aneuploidy status and miRNA expression in human blastocysts (Almutlaq et al., 2024). The study design is outlined in Appendix2. This review was a critical component of the thesis, aimed at identifying miRNAs associated with aneuploidies and establishing a foundation for understanding the role of miRNAs in chromosomal abnormalities. The insight gained from this review directly informed the subsequent research presented in this work.

The search yielded 187 records, of which only five studies met the inclusion criteria. These studies specifically investigated changes in miRNAs expression in aneuploid blastocysts compared to those with euploid chromosomal contents. The miRNAs were extracted from various sources, including whole blastocysts, blastocoel fluid and culture media. The extracted miRNAs were analysed using either array-based qPCR or real-time qPCR focused on specific single genes. It is important to note that the sample sizes in these studies were relatively small, ranging from 10 to 28 samples.

In total, sixty-eight differentially expressed miRNAs were identified, with several miRNAs consistently reported as downregulated, namely hsa-miR-19b, hsa-miR-517c, hsa-miR-518e, hsa-miR-522, hsa-miR-92a and hsa-miR-106a. These miRNAs belong to crucial miRNA clusters, such as C19CM, miR-17/92 and miR-106a-363. Aberrant expression of these clusters has been linked to reproductive failure (Liang et al., 2017, Goharitaban et al., 2022). Moreover, these clusters are often referred to as pregnancy-related clusters due to their importance in this context (Morales-Prieto et al., 2013). Functional analysis of these miRNAs demonstrated their involvement in regulating fate-determining pathways such as cell-cycle and cell death.

Overall, this systematic review highlights the limited scope of exciting research on the association between aneuploidies and miRNA expression in human blastocysts,

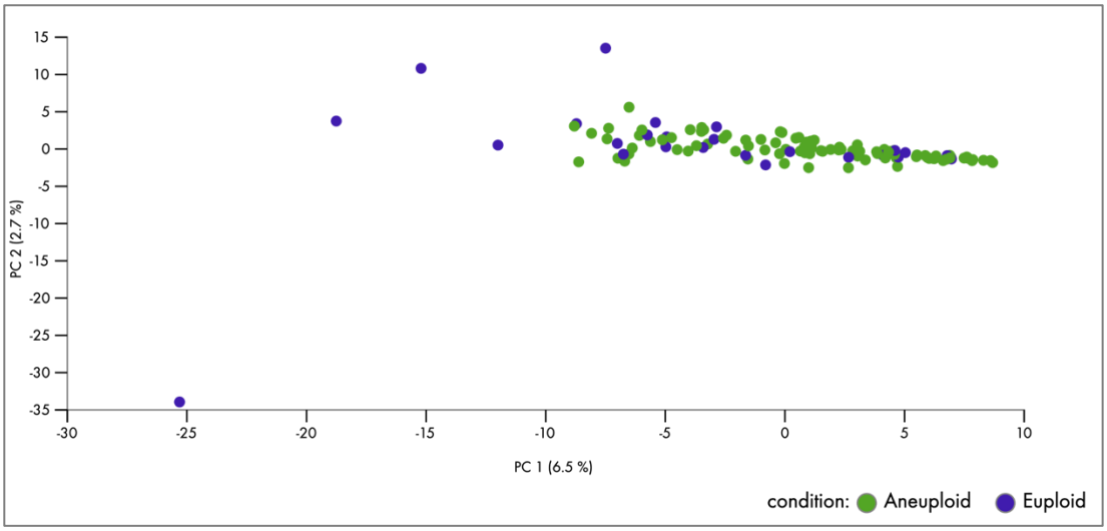
indicating that this relationship remains poorly understood. The findings from this review provided an important reference point for this study, guiding the design and methodological improvements implemented here. For more detailed insights, refer to the published paper (Almutlaq et al., 2024).

In here, we aimed to build on the limitations identified in the previous research by employing several methodological improvements. Firstly, we used high-throughput gene expression technology, NGS, for miRNA expression analysis to comprehensively capture all miRNAs expressed in blastocysts. Furthermore, we expanded the sample size from the small cohorts used in earlier studies to a more substantial cohort of 122 samples. This larger sample size provided more representative dataset of blastocysts, thereby enhancing the precision of the results. Additionally, acknowledging the potential impact of the location and type of chromosomal defects on the findings, we categorised the aneuploid blastocysts into several subgroups. Each subgroup was analysed individually, facilitating for a more detailed examination of specific chromosomal abnormality and their association with miRNA expression.

5.2.2 Analysis of miRNA Expression Profile in Aneuploid Blastocysts

Initially, a principal component analysis (PCA) was performed to determine whether the blastocysts in this study grouped into distinct clusters. The PCA generated two principal components, PC1 and PC2, which accounted for 6.5 % and 2.7% of the total variation, respectively. The relatively low values of PC1 and PC2, along with most samples falling within a narrow range, suggested minimal differences in miRNA expression among the samples (Figure 5-1). This homogeneity could be attributed to the similarity in the type and characteristics of the analysed cohort, consisting of embryos with generally satisfactory quality.

Figure 5-1: Principal component analysis of miRNA expression in euploid and aneuploid blastocysts



The figure illustrates the results of PCA demonstrating no obvious clustering and suggesting a slight disparity in miRNA expression between the samples.

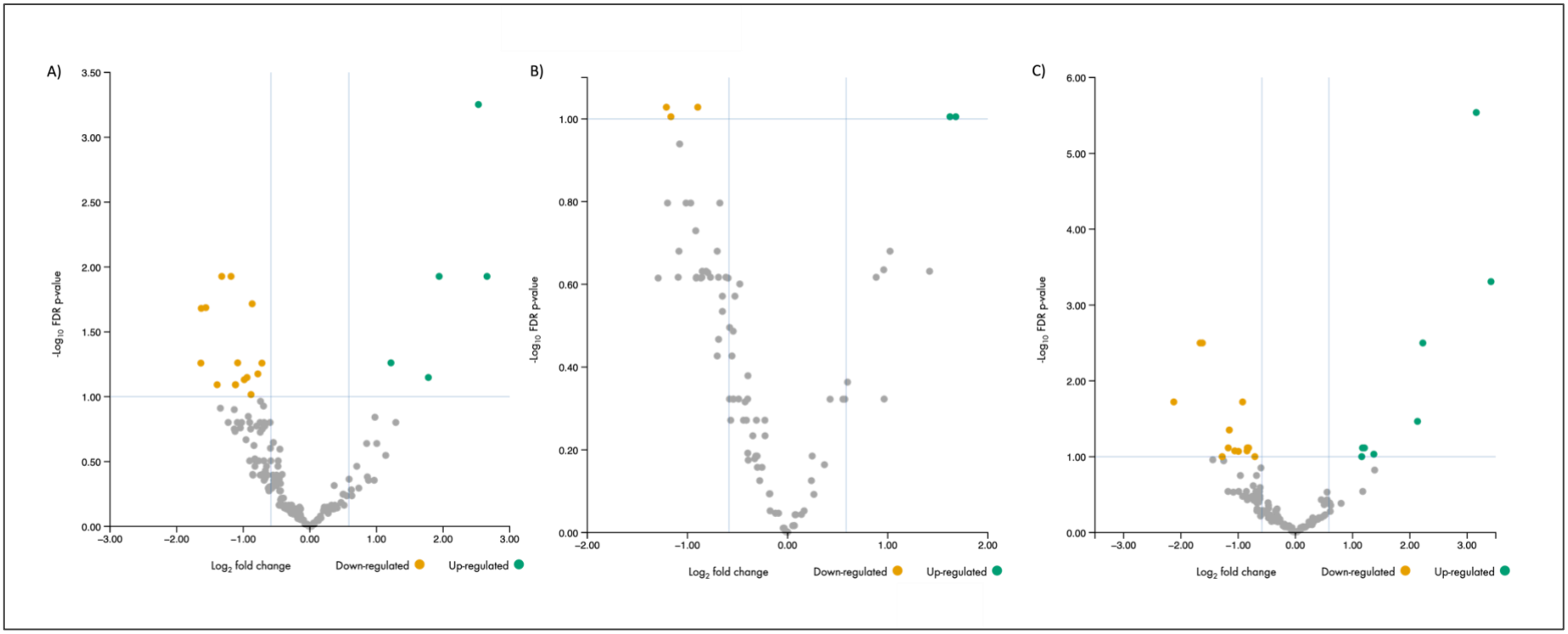
Although no distinct clustering was observed, the PCA plot revealed some interesting insights. Unexpectedly, aneuploid samples showed some degree of grouping, whereas euploid were spread out (Figure 5-1). Five euploid samples appear to fall outside the range. Upon reviewing their data, no distinguishing features were identified, except that they were PGT-M cases with a history of hemoglobinopathies mutations. Although these findings might be outliers, no indications of human or technical errors were found during samples preparation or analysis, leaving the possibility of an internal difference with unknown cause.

The differential expression analysis served as the central component of this study, enabling the determination of differences in miRNA profiles between blastocyst groups and the extent of these variations. The initial analysis identified changes in miRNA profile between euploid and aneuploid blastocysts, revealing a remarkable difference in the expression of 20 miRNAs out of the total identified 2,491 miRNAs in the human blastocysts. Most of the differentially expressed genes exhibited downregulation in the aneuploid group (see

Figure 5-2 (A) and

Table 5-1). However, the upregulated miRNAs, though fewer in number, demonstrated greater significance, showing more than 3-fold increase in the aneuploid blastocysts (Table 5-2).

Figure 5-2: Differentially expressed miRNAs in aneuploid and aneuploid subgroups compared to euploid blastocysts: Volcano plots



The volcano plots illustrate: A) The differentially expressed miRNAs in aneuploid blastocysts compared to euploid. B) The differentially expressed miRNAs in blastocysts with a single aneuploidy compared to euploid blastocysts. C) Significant number of dysregulated miRNAs in samples with two or more chromosomal abnormalities compared to euploid blastocysts. The significantly downregulated miRNAs are presented in yellow while the those upregulated are showed in green.

Table 5-1: Downregulated miRNAs in aneuploid versus euploid blastocysts

Names	**Fold change**	**FDR p-value**
hsa-miR-512-5p	-3.10	0.05
hsa-miR-520a-5p	-3.096	0.02
hsa-miR-498-5p	-2.95	0.02
hsa-miR-2110	-2.62	0.08
hsa-miR-146b-5p	-2.50	0.01
hsa-miR-126-3p	-2.27	0.01
hsa-miR-519b-3p	-2.16	0.08
hsa-miR-576-5p	-2.16	0.08
hsa-miR-103a-3p	-2.11	0.05
hsa-miR-191-5p	-1.97	0.07
hsa-miR-512-3p	-1.92	0.07
hsa-miR-1323	-1.84	0.09
hsa-miR-92a-3p	-1.82	0.01
hsa-miR-378a-3p	-1.71	0.06
hsa-miR-7-5p	-1.64	0.05

Table 5-2: Upregulated miRNAs in aneuploid versus euploid blastocysts

Names	**Fold change**	**FDR p-value**
hsa-miR-206	6.31	0.01
hsa-miR-184	5.77	0.0005
hsa-let-7c-5p	3.83	0.01
hsa-miR-3168	3.43	0.07
hsa-let-7b-5p	2.32	0.05

When analysing a complex and multidimensional factor such as aneuploidy, it is crucial to highlight the challenges inherent in investigating this factor, which involves various sources of variation. These differences include the specific chromosomal location, with 24 possibilities including 22 pairs of autosomal chromosomes and 2 sex chromosomes, as well as the diverse forms of chromosomal defects that may arise. Exploring aneuploidy based on the chromosomal location posed significant challenges due to the variability in affected chromosomes across samples. However, classifying aneuploidy into predefined types seemed to be more sensible (Licciardi et al., 2018). Therefore, the subsequent analyses of miRNA profiles included blastocysts with single chromosomal defects, complex

chromosomal abnormalities, partial loss or gain, whole chromosomal loss and whole chromosomal gain. Each subgroup was compared to 26 euploid blastocysts.

5.2.2.1 Single Aneuploidy

Samples with a single chromosomal defect displayed the fewest differences in miRNA profiles compared to other subtypes. Only four miRNAs demonstrated notable changes in their expression levels, most of which were previously identified in the initial analysis of all aneuploid samples (

Figure 5-2 (B)). The miRNAs hsa-miR-192-5p, hsa-miR-126-3p and hsa-miR-7-5p were downregulated, showing approximately twofold decrease in expression, while hsa-let-7c-5p exhibited significant elevation in samples with single-chromosome aneuploidy (Table 5-3). The discrepancy in the number of dysregulated miRNAs between the volcano plot (in

Figure 5-2 (B)) and the Table 5-3 is attributed to the sensitivity test results. The volcano plot depicted five differentially expressed miRNAs, while the table reported only four, as one miRNA was not significantly dysregulated when accounting for the day of blastocysts formation in this analysis.

Table 5-3: The FDR p-value and FC of the differentially expressed miRNAs in blastocysts comprise a single numerical chromosomal defect

Name	**Fold change**	**FDR p-value**
hsa-miR-192-5p	-2.31	0.09
hsa-miR-126-3p	-2.24	0.09
hsa-miR-7-5p	-1.86	0.09
hsa-let-7c-5p	3.20	0.09

5.2.2.2 Complex Aneuploidy

On the contrary, blastocysts with two or more aneuploidies displayed a more pronounced alteration in their miRNA profiles. A total of 17 miRNAs exhibited significant change in their expression, with seven up-regulated and sixteen down-regulated (

Figure 5-2 (C) and Table 5-4). This observation suggests a positive correlation between the number of affected chromosomes and the level of change in the miRNA expression profile. Similar to the single aneuploidy group, most of the identified miRNAs in this subgroup were also detected in the initial analysis involving all aneuploid samples.

Table 5-4: The FDR p-value and FC of the differentially expressed miRNAs in blastocysts with two or more numerical chromosomal defects

Name	**Fold change**	**FDR p-value**
hsa-miR-498-5p	-4.22	0.02
hsa-miR-103a-3p	-3.030	0.003
hsa-miR-146b-5p	-2.90	0.006
hsa-miR-425-5p	-2.50	0.04
hsa-miR-126-3p	-2.33	0.04
hsa-miR-191-5p	-2.12	0.05
hsa-miR-1323	-1.90	0.09
hsa-miR-515-5p	-1.85	0.04
hsa-miR-93-5p	-1.84	0.023
hsa-miR-92a-3p	-1.76	0.02
hsa-miR-378a-3p	-1.67	0.09
hsa-let-7b-5p	2.36	0.07
hsa-miR-320d	2.39	0.07
hsa-miR-99b-5p	2.67	0.08
hsa-miR-3168	3.45	0.09
hsa-let-7c-5p	5.68	0.001
hsa-miR-206	14.90	6.93858E-05

5.2.2.3 Segmental Aneuploidy

Partial or segmental deletions and duplications may appear less detrimental to preimplantation development, but they carry significant implications for embryonic fate and are commonly associated with developmental disabilities post-birth (Watson et al., 2014, Fragouli et al., 2017, Goldenberg, 2018, Zore et al., 2019). In our study, blastocysts with segmental chromosomal defects exhibited increased levels of four miRNAs: hsa-miR-203a-3p, hsa-miR-27b-3p, hsa-let-7b-5p, and hsa-miR-520a-3p, compared to euploid blastocysts (Table 5-5). Since these miRNAs showed

differential expression only with segmental aneuploidies but not with any other type of aneuploidies, this may indicate that segmental chromosomal abnormalities trigger different cellular mechanisms. However, these results require further investigation due to the limited number of samples in this analysis.

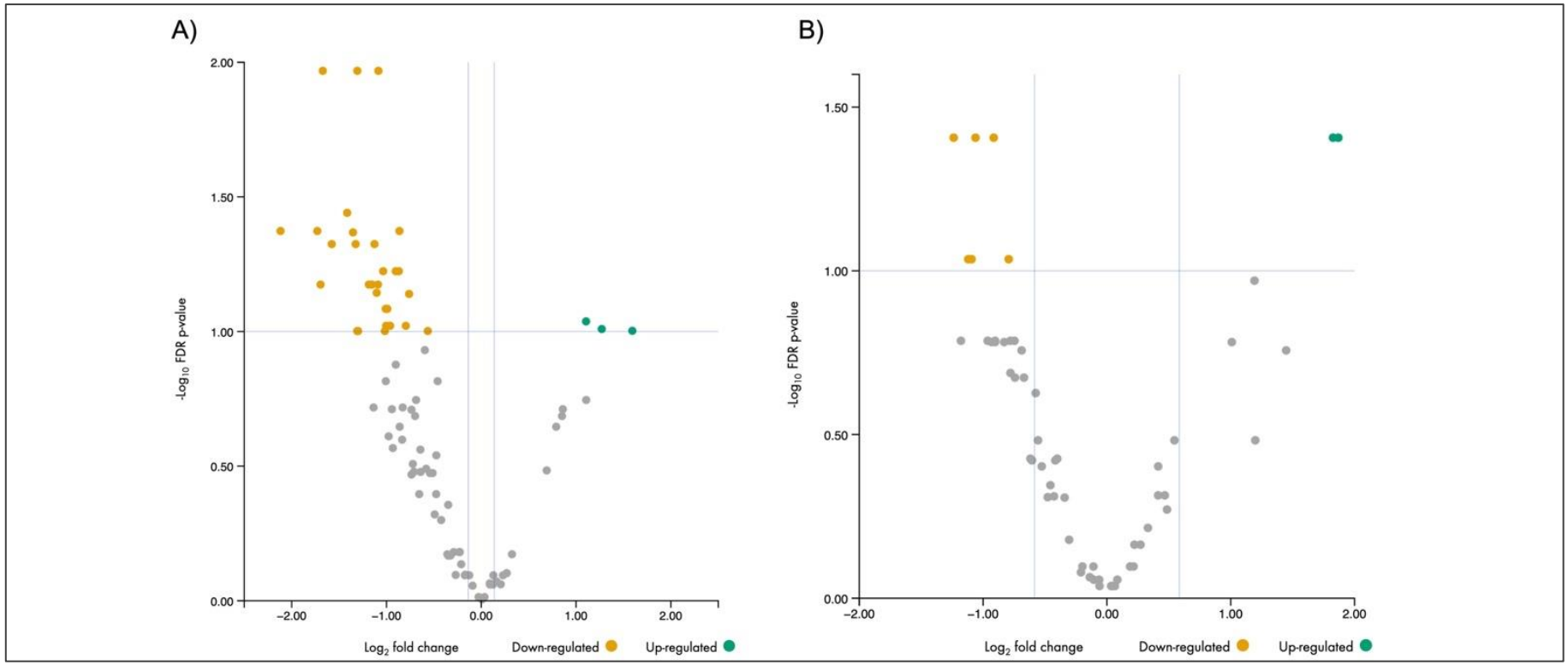
Table 5-5: The FDR p-value and FC of the differentially expressed miRNAs in blastocysts with segmental chromosomal defects

Name	**Fold change**	**FDR p-value**
hsa-miR-203a-3p	10.98	0.005
hsa-miR-27b-3p	7.81	0.05
hsa-let-7b-5p	6.44	0.07
hsa-miR-520a-3p	5.20	0.09

5.2.2.4 Monosomy and Trisomy

Significant alterations of the miRNA expression profile were also observed in both monosomic and trisomic blastocysts compared to euploid ones (Table 5-6 and Table 5-7). In monosomic blastocysts, twenty-seven miRNAs exhibited significant change in expression, with the majority of them being downregulated (Figure 5-3 (A)). However, fewer changes in miRNA profile were observed in the blastocysts with chromosomal gains, as only seven differentially expressed miRNAs were identified (Figure 5-3 (B)). These findings may indicate that chromosomal losses have a more pronounced effect on the genetic status of the embryo than gains. Notably, some miRNAs were commonly dysregulated in the two aneuploidy groups, such as hsa-miR-92a-3p, hsa-miR-7-5p, hsa-miR-378a-3p, hsa-miR-516a-5p and hsa-miR-192-5p. This overlap suggests that certain pathways are impacted by aneuploidy, regardless of whether the change involves a chromosomal loss or gain.

Figure 5-3: miRNAs expression alterations in blastocyst with monosomies and trisomies: Volcano plot analysis



The volcano plots display differentially expressed miRNAs in (A) blastocysts with chromosomal losses and (B) blastocysts with chromosomal gains, both compared to euploid blastocysts. A high number of miRNAs showed significant changes in expression in the blastocysts with monosomies.

Table 5-6: The FDR p-value and FC of the differentially expressed miRNAs in blastocysts with chromosomal monosomies

Name	Fold change	FDR p-value
hsa-let-7b-5p	2.15	0.09
hsa-miR-371a-5p	-1.69	0.07
hsa-miR-7-5p	-1.81	0.04
hsa-miR-378a-3p	-1.82	0.05
hsa-miR-93-5p	-1.86	0.05
hsa-miR-9-5p	-1.94	0.09
hsa-miR-516a-5p	-1.98	0.08
hsa-miR-373-3p	-2.04	0.05
hsa-miR-92a-3p	-2.12	0.01
hsa-miR-1323	-2.12	0.06
hsa-miR-103a-3p	-2.14	0.07
hsa-miR-192-5p	-2.18	0.04
hsa-miR-423-3p	-2.22	0.06
hsa-miR-629-5p	-2.27	0.06
hsa-miR-1283	-2.46	0.09
hsa-miR-515-5p	-2.47	0.01
hsa-miR-126-3p	-2.50	0.04
hsa-miR-191-5p	-2.55	0.04
hsa-miR-512-3p	-2.66	0.03
hsa-miR-125a-5p	-2.98	0.04
hsa-miR-146b-5p	-3.18	0.01
hsa-miR-302a-5p	-3.23	0.06
hsa-miR-20a-5p	-3.31	0.04
hsa-miR-518a-3p	-4.33	0.04

Table 5-7:The FDR p-value and FC of the differentially expressed miRNAs in blastocysts with chromosomal trisomies

Name	Fold change	FDR p-value
hsa-let-7c-5p	3.54	0.03
hsa-miR-92a-3p	-1.73	0.09
hsa-miR-7-5p	-1.88	0.03
hsa-miR-378a-3p	-2.08	0.03
hsa-miR-516a-5p	-2.13	0.09
hsa-miR-519c-5p	-2.17	0.09
hsa-miR-192-5p	-2.35	0.03

5.2.3 Dysregulation of miRNA Expression Profile in Aneuploid Blastocysts

The correlation between miRNA expression and chromosomal faults has been previously investigated in different ways. One earlier study has explored this potential link in different human cell lines that were intentionally engineered to have chromosomal gain (Durrbaum et al., 2018). The results exhibited significant upregulation of miR-10a-5p in the majority of the examined cells, and this increase was suggested to serve as a protective adaption to starvation stress and prevent protein translation. In contrast to this finding, miR-10a-5p did not exhibit significant change in the aneuploid blastocysts. This discrepancy could be attributed to the different nature of the examined cells, as constructed aneuploidies in cell lines probably differ from the naturally occurring ones.

Importantly, previous studies on human blastocysts have identified a group of miRNAs with a potential link to chromosomal abnormalities (Rosenbluth et al., 2013, Rosenbluth et al., 2014, McCallie et al., 2014, McCallie B., 2015, Esmaeilivand et al., 2022). Although these studies are limited in number and constrained by small sample sizes, they demonstrate a notable level of consistency in their findings. The miRNAs previously associated with aneuploidy, along with those that align with our results, are presented in Appendix8. Among them, hsa-miR-146b-5p, hsa-miR-191-5p, hsa-miR-92a-3p and hsa-miR-93-5p showed similar expression patterns in our analysis. Consistent with Rosenbluth et al., 2013, hsa-miR-146b-5p, hsa-miR-92a-3p and hsa-miR-93-5p were significantly downregulated in our analysis of aneuploid samples. Furthermore, Rosenbluth et al. reported downregulation of hsa-miR-20a, hsa-miR-26b-5p, hsa-miR-373, and hsa-miR-518a-3p, which corroborates our observations in blastocysts exhibiting complex aneuploidy.

Our observations in blastocysts with aneuploidy showed a contrast to previous reports that found upregulation of hsa-miR-191 in culture media associated with aneuploidy (Rosenbluth et al., 2014, Acuna-Gonzalez et al., 2021). In our analysis, however, hsa-miR-191 consistently exhibited a two-fold reduction across most aneuploid groups. One potential explanation for this discrepancy lay on the fact that there might be a difference in miRNA expression patterns between culture media and whole blastocysts. Previous studies have suggested that miRNAs secreted from the

TE are more likely to be found in the culture media (Capalbo et al., 2016b), meaning that the miRNA profile in the culture media may not fully represent the miRNA landscape within the entire blastocyst.

In addition, miRNAs resealed into the blastocoel fluid have been investigated for their potential relationship to the aneuploidy status in embryos. A previous study found an elevated expression of hsa-miR-20a-5p in the fluid obtained from aneuploid blastocysts (Esmaeilivand et al., 2022). However, both our findings and another earlier study consistently showed downregulation of this miRNA within aneuploid blastocysts (Rosenbluth et al., 2013). This inconsistency between intracellular and the extracellular miRNA expression pattern has been noted before, suggesting that miRNAs may display different expression levels inside cells compared to the extracellular vesicles released by these cells (Valadi et al., 2007, Zhang et al., 2015b).

When considering the technology used for miRNA profiling, it is essential to note that these previous studies employed array-based qPCR, which limited detection to a predefined set of miRNAs that could hybridize to the chip. In contrast, the sequencing approach applied in this study allows for the identification of all miRNAs expressed in the blastocysts. This broader detection capability means that our findings may include additional miRNAs potentially linked to aneuploidy, which were previously undetected due to the limitations of the technologies.

5.2.4 miRNA Expression Changes Correspond to the Type and Extent of Aneuploidy in Aneuploid Blastocyst

Given the significant variation in the types of numerical aneuploidies that can arise during embryonic development, our investigation included various forms of aneuploidy to ensure a comprehensive analysis of miRNA expression in relation to different chromosomal defects (Hassold and Hunt, 2001). Notably, the number of dysregulated miRNAs varied across each aneuploid subgroup. For example, blastocysts with monosomies exhibited the most significant change in miRNA profiles, suggesting that chromosomal loss has the greatest impact on miRNA expression, and eventually on the cellular processes.

Blastocysts with chromosomal gains exhibited fewer alterations in their miRNA profiles compared to those with other types of aneuploidies. This supports the view that chromosomal gains may cause less cellular disruption than chromosomal losses. It is generally accepted that monosomic embryos have a low likelihood of survival, while embryos with chromosomal gains are more likely to persist and, in some cases, result in live births (Shahbazi et al., 2020). However, embryonic survival also depends on the specific chromosome affected, as certain trisomies can be detrimental, while some others are less harmful (Hassold and Hunt, 2001). This implies that distinct gene expression profiles lead to different cellular responses to various types of chromosomal defects, as previously proposed (Licciardi et al., 2018).

Changes in miRNA expression associated with segmental aneuploidies have been noticed previously (Guo et al., 1996). This was also observed in our findings, as blastocysts with segmental aneuploidies exhibited a unique set of upregulated miRNAs, different from those identified in other aneuploidy groups. These miRNAs target genes with crucial roles in cell cycle transition, such as *CCNA2, CCND1*, and *CDK6*, according to computational gene ontology analysis (Schultz et al., 2008, Johnson et al., 2007, Li et al., 2017a). The upregulation of these miRNAs likely leads to downregulation of their target genes, suggesting that cells with partial chromosomal abnormalities may be attempting to delay cell cycle progression. In contrast, blastocysts with whole chromosomal defects and more complex aneuploidies exhibited decreased levels of miRNAs that target genes contributing to cell arrest and activating intrinsic apoptosis in response to DNA damage, such as *CDKN1A, CCNE1*, and *BCL2*. Together, these findings may indicate that complete chromosomal aneuploidy is more likely to induce cell apoptosis, while segmental aneuploidy may have a lesser effect on cellular outcomes.

5.2.5 Chromosomal Location and Cellular Localization Analyses of the Differentially Expressed miRNAs in Aneuploid Blastocysts

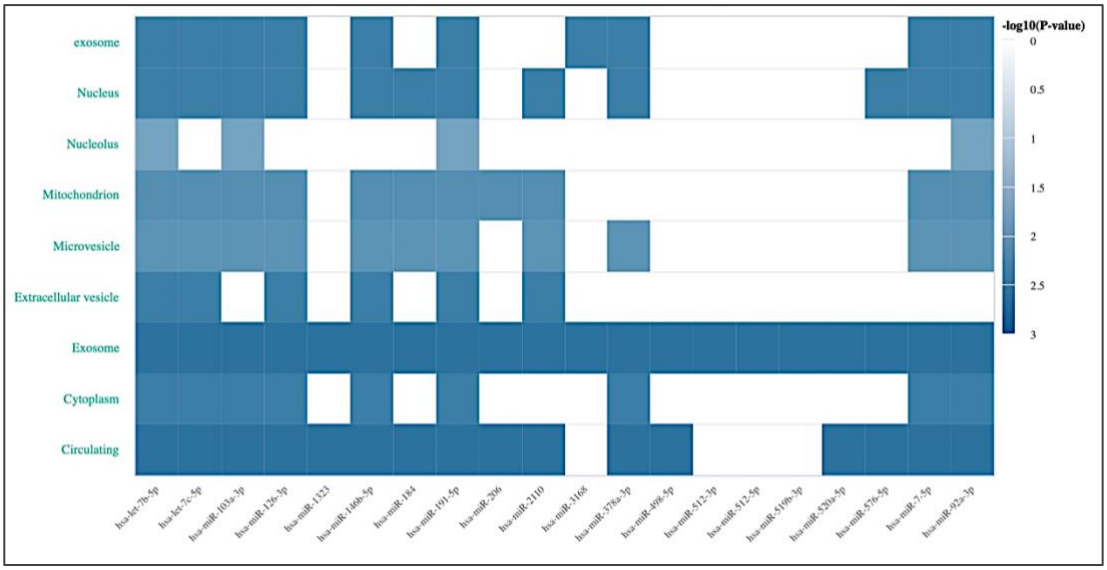
When investigating the chromosomal location of the dysregulated miRNAs, a remarkable proportion of them were found to be encoded on chromosome 19 (Table 5-8). Approximately 35% of the differentially expressed miRNAs belong to the chromosome 19 miRNA cluster (C19MC). Notably, members of both C19CM and miR-371~373 miRNA clusters were significantly suppressed in aneuploid samples. Further analysis of the chromosomal location of differentially expressed miRNAs in aneuploidy subgroups revealed significant changes in chromosome 19 miRNAs in blastocysts with chromosomal loss (Table 5-8).

Table 5-8: Chromosomal location analysis of dysregulated miRNAs in aneuploid blastocysts

Samples	**Number of identified miRNAs**	**Type of analysis**	**Genomic Location**	**P-adjusted value**	**miRNAs**
Aneuploid blastocysts	20	Chromosomal location (miRBase)	Chromosome 19	8.79e-4	hsa-miR-512-5p; hsa-miR-520a-5p; hsa-miR-498-5p; hsa-miR-519b-3p; hsa-miR-512-3p; hsa-miR-1323; hsa-miR-7-5p
Monosomic blastocysts	24	Chromosomal location (miRBase)	Chromosome 19	1.12e-5	hsa-miR-371a-5p; hsa-miR-7-5p; hsa-miR-516a-5p; hsa-miR-373-3p; hsa-miR-1323; hsa-miR-1283; hsa-miR-515-5p; hsa-miR-512-3p; hsa-miR-125a-5p; hsa-miR-518a-3p

Given that differentially expressed miRNAs have the potential to serve as indicative biomarkers for aneuploidy, we also explored their cellular localization to determine whether they could be secreted into the extracellular space and potentially diffused into the culture media. The analysis revealed that the majority of the identified genes were previously found in exosomes, which increases the likelihood of their release to the embryo extracellular spaces (Figure 5-4). Both chromosomal location and miRNA localization analyses were conducted using the miEAA 2.0 platform.

Figure 5-4: miRNA localization analysis of the differentially expressed miRNAs in aneuploid blastocysts



The heat map illustrates the localization of differentially expressed miRNAs in aneuploid samples. It indicates that these miRNAs are typically detected in the circulation and are known to be secreted within exosomes.

5.2.6 Co-regulation Analysis of miRNAs and Their mRNA Targets in Aneuploid Blastocyst

An additional analysis was conducted using external mRNA expression dataset to investigate the direct interaction between differentially expressed miRNAs and their mRNA targets in aneuploid blastocysts, The mRNA expression dataset, provided by Xuhui Sun, was derived from a different set of blastocysts taken from the same cohort as previously mentioned.

The differential expression analysis of mRNAs was initially conducted between euploid and all aneuploid blastocysts, and subsequently extended to include comparisons with aneuploid subgroups, similar to those in miRNA differential expression investigations. Remarkably, the findings across all analyses revealed a significant increase in mRNA abundance, with all differentially expressed genes being upregulated in the aneuploid blastocysts. While the list of identified genes varied across different aneuploidy groups, some mRNAs consistently showed elevated expression in the majority of aneuploid blastocysts, including *MDM2*, *TIMM50*, *TOB1*, *CDKN1A*, *ID1*, *RANBP3* and *PHLDA3*.

Attempts were made to explore the relationship between aneuploidy-associated mRNAs and miRNAs using computational miRNA-gene target interaction network tools. A convincing miRNA/mRNA association was noticed, suggesting that some dysregulated miRNAs significantly impact the expression level of their target genes. Previous experimental evidence, supported by gene ontology computational analysis, indicated that several upregulated mRNAs in aneuploid blastocysts are common targets of downregulated miRNAs found in the same blastocyst group. The individual association between differentially expressed mRNAs and their miRNA regulators in each aneuploid group are detailed in (Table 5-10, Table 5-11, Table 5-12, Table 5-13 and Table 5-13).

Table 5-9: Co-regulation analysis results of mRNA and miRNA in aneuploid blastocysts

Gene	**log2 Fold Change**	**Adjusted p-vale**	**Downregulated miRNAs**	**Upregulated miRNAs**
TIMM50	1.81	0.02	hsa-miR-7-5p	hsa-let-7b-5p
CDKN1A	3.87	0.001	hsa-miR-512-5p hsa-miR-519b-3p hsa-miR-576-5p	hsa-let-7b-5p hsa-let-7c-5p
MDM2	1.56	0.0096	hsa-miR-92a-3p	
TOB1	2.31	0.03	hsa-miR-92a-3p	
RRAD	2.01	0.034		hsa-let-7b-5p hsa-let-7c-5p

It is important to note that miRNA-mediated control of mRNA expression is not only achieved through the degradation of target mRNAs. When miRNAs are expressed at low levels, their target mRNAs may remain unregulated, leading to an uncontrolled abundance of these genes. Therefore, the findings from miRNAs analyses in aneuploid blastocysts has predicted an increase in mRNA levels, which was later confirmed by mRNA expression results.

Interestingly, the aneuploid blastocysts exhibited significant upregulation of *CDKN1A* and *MDM2*, both of which directly interact with the tumour suppressor *TP53*. Although the expression of *TP53* gene itself did not show any changes in aneuploid blastocysts, these downstream genes were consistently elevated (Table 5-9). The upregulation of *CDKN1A* is generally mediated by *TP53* in response to DNA damage and other stress signals (el-Deiry et al., 1994, el-Deiry et al., 1993). Additionally, *MDM2* operates in a negative feedback loop to regulate *TP53*, with its elevation indicating increased activation of *TP53* (Moll and Petrenko, 2003). Since we rely only on gene expression data, further investigation is needed to assess TP53 protein levels, as gene expression does not necessarily correlate with protein expression (Vogel and Marcotte, 2012).

5.2.6.1 Chromosomal Gains and Losses

An intriguing observation from both the miRNA and mRNA analyses is the distinct impact of different types of aneuploidies on the transcriptome profile. The mRNA differential expression analysis revealed significant changes in mRNA levels in blastocysts with chromosomal losses, while those with chromosomal gains showed minimal changes in their mRNA profiles, similar to the miRNA findings (Table 5-10 and Table 5-11).

Table 5-10: Co-regulation analysis results of mRNA and miRNA in blastocysts with monosomies

Name	log2 Fold Change	Adjusted p-vale	Downregulated miRNAs	Upregulated miRNAs
CEP85L	3.41	0.01	hsa-miR-192-5p	
TOB1	2.34	0.03	hsa-miR-25-3p hsa-miR-26b-5p hsa-miR-92a-3p	
MDM2	1.55	0.01	hsa-miR-25-3p hsa-miR-93-5p hsa-miR-26b-5p hsa-miR-92a-3p hsa-miR-20a-5p	
PHLDA3	2.61	0.006	hsa-miR-371a-5p	
BIK	3.60	0.01	hsa-miR-9-5p	
CDKN1A	4.13	0.0001	hsa-miR-93-5p hsa-miR-423-3p hsa-miR-125a-5p hsa-miR-20a-5p	hsa-let-7b-5p
RRAD	2.01	0.02		hsa-let-7b-5p

Table 5-11: Co-regulation analysis results of mRNA and miRNA in trisomic blastocysts

Name	log2 Fold Change	Adjusted p-vale	Downregulated miRNAs	Upregulated miRNAs
ID1	2.95	0.04	hsa-miR-192-5p	hsa-let-7c-5p
RANBP3	3.33	0.04	hsa-miR-192-5p	
TIMM50	2.23	0.01	hsa-miR-7-5p	
FXN	2.44	0.03		hsa-let-7c-5p

5.2.6.2 Single Aneuploidy

In blastocysts with a single chromosomal abnormality, the two downregulated miRNAs, hsa-miR-192-5p and hsa-miR-7-5p, were found to be regulators of several upregulated mRNAs within the same group (as shown in Table 5-12). Notably, most of the identified mRNAs in this comparison were specific to the single aneuploidy group and were not present in other comparisons.

Table 5-12: Co-regulation analysis results of mRNA and miRNA in blastocysts with one chromosomal aneuploidy

Name	**log2 Fold Change**	**Adjusted p-vale**	**Downregulated miRNAs**	**Upregulated miRNAs**
BRD3	1.51	0.004	hsa-miR-192-5p	
UBE2K	1.54	0.003	hsa-miR-192-5p	
MTERF3	1.56	0.0008	hsa-miR-192-5p	
GINM1	2.03	0.003	hsa-miR-192-5p	
POLA2	2.08	0.02	hsa-miR-192-5p	
ID1	3.65	0.002	hsa-miR-192-5p	hsa-let-7c-5p
RANBP3	3.93	0.002	hsa-miR-192-5p	
SNAPIN	1.53	0.0007	hsa-miR-7-5p	
MPDU1	1.54	0.02	hsa-miR-7-5p	
CANT1	1.73	0.01	hsa-miR-7-5p	
DBNL	1.78	0.03	hsa-miR-7-5p	
MFSD9	1.86	0.01	hsa-miR-7-5p	
RNF114	1.87	0.001	hsa-miR-7-5p	
DUS1L	2.22	0.03	hsa-miR-7-5p	
BCKDK	2.26	0.02	hsa-miR-7-5p	
RPS19BP1	2.29	0.001	hsa-miR-7-5p	
MAP2K2	2.54	0.04	hsa-miR-7-5p	
TIMM50	2.60	1.30E-05	hsa-miR-7-5p	
DUSP23	3.35	0.009	hsa-miR-7-5p	
TMEM134	4.77	0.0007	hsa-miR-7-5p	
PMAIP1	1.52	0.001		hsa-let-7c-5p
PTGES2	1.73	0.01		hsa-let-7c-5p
RRAD	1.93	0.01		hsa-let-7c-5p
ZNF581	1.94	0.001		hsa-let-7c-5p
FXN	2.89	0.001		hsa-let-7c-5p
CDKN1A	3.58	0.001		hsa-let-7c-5p
MRPL12	4.09	0.04		hsa-let-7c-5p

5.2.6.3 Complex Aneuploidy

Blastocysts with complex aneuploidies exhibited a substantial number of differentially expressed mRNAs, which was anticipated given to the presence of multiple chromosomal abnormalities in this group. Among the differentially expressed mRNAs, twenty-nine showed significant changes, and seven were

potential targets of the dysregulated miRNAs in the same group. Of these, only *MDM2*, and *CDKN1A* exhibited concordant results with the downregulated miRNAs in the complex aneuploid group (see Table 5-13).

Table 5-13: Co-regulation analysis results of mRNA and miRNA in blastocysts with two or more chromosomes affected

Name	**log2 Fold Change**	**Adjusted p-vale**	**Downregulated miRNAs**	**Upregulated miRNAs**
FXN	1.87	0.04		hsa-let-7b-5p hsa-let-7c-5p
MDM2	1.82	3.86E-05	hsa-miR-425-5p hsa-miR-93-5p hsa-miR-92a-3p	hsa-let-7c-5p
CDKN1A	3.91	0.001	hsa-miR-93-5p	hsa-let-7b-5p hsa-let-7c-5p
MBD3L2B	5.53	0.0005		hsa-let-7c-5p
TAF11L12	5.00	0.001		hsa-let-7c-5p
RRAD	2.25	0.03		hsa-let-7b-5p hsa-let-7c-5p
MDK	2.07	0.02		hsa-miR-320d

A noteworthy observation from this analysis was the expression pattern of the let-7 family members, hsa-let-7b-5p and hsa-let-7c-5p, which showed overexpression in almost all types of aneuploidies, while their targeted mRNAs were also upregulated. Although miRNA do not always have a direct influence on its targets, this expression pattern highlights the exceptional regulatory role that let-7 miRNAs may play. Typically, genes of this family act as tumour suppressors by controlling the overexpression of oncogenes (Zhang et al., 2007). However, in certain contexts, let-7 miRNAs can also exhibit oncogenic activity by targeting tumour suppressor genes (Zhang et al., 2007). In aneuploid blastocysts, the role and consequence of the overexpression of hsa-let-7b-5p and hsa-let-7c-5p remains uncertain.

Notably, the upregulated miRNAs in the aneuploid group did not correspond to matching targets when the results of differentially expressed miRNAs and mRNAs were combined. This can be attributed to the notion that miRNA influence on their targets is not always straightforward (O'Brien et al., 2018). It is also important to note that during the normalization process, genes with low counts were removed,

which may have resulted in the loss of downregulated mRNAs.

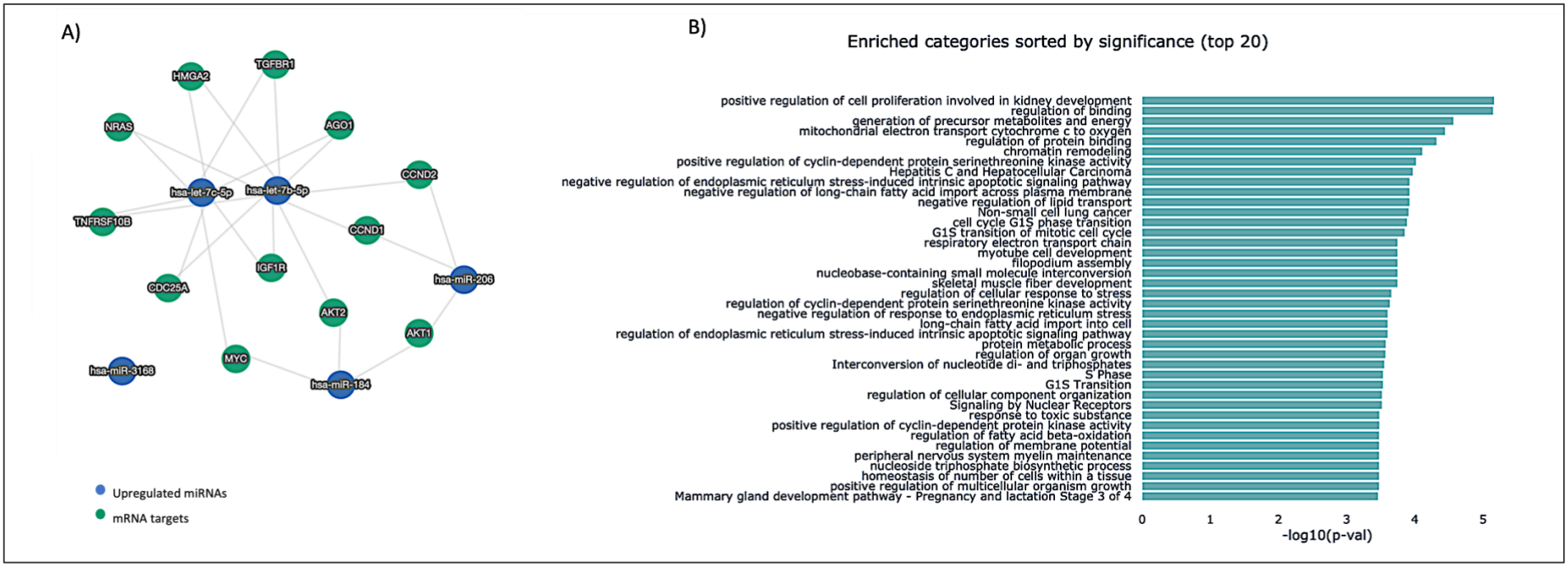
5.2.7 Functional Analysis of Frequently Differentially Expressed miRNAs in Aneuploid Blastocysts

Although the mechanisms underlying the occurrence and physiological outcomes of numerical chromosomal abnormalities are well established, the cellular response towards these faults in terms of recognition and counteraction remains poorly investigated. This knowledge gap may be referred to the fact that aneuploidy is often considered as a secondary feature of a disease or a cause of a syndrome, rather than being studied as a distinct condition. The present findings of differential miRNA expression in embryos with various levels and types of chromosomal abnormalities could enhance our understanding of the molecular changes that occur in response to aneuploidy.

To achieve a comprehensive understanding of the biological processes involved, pathway enrichment analysis was conducted on the dysregulated miRNAs within each aneuploid group. While these analyses yielded substantial data, assembling and interpreting them was challenging (see Appendix7 for detailed results). Therefore, focus was directed toward analysing the results from the aneuploid group, which encompasses blastocysts with all types of chromosomal defects, compared to euploid. This group includes dysregulated miRNAs from all aneuploid groups, making it a representative sample for aneuploidy-related pathways.

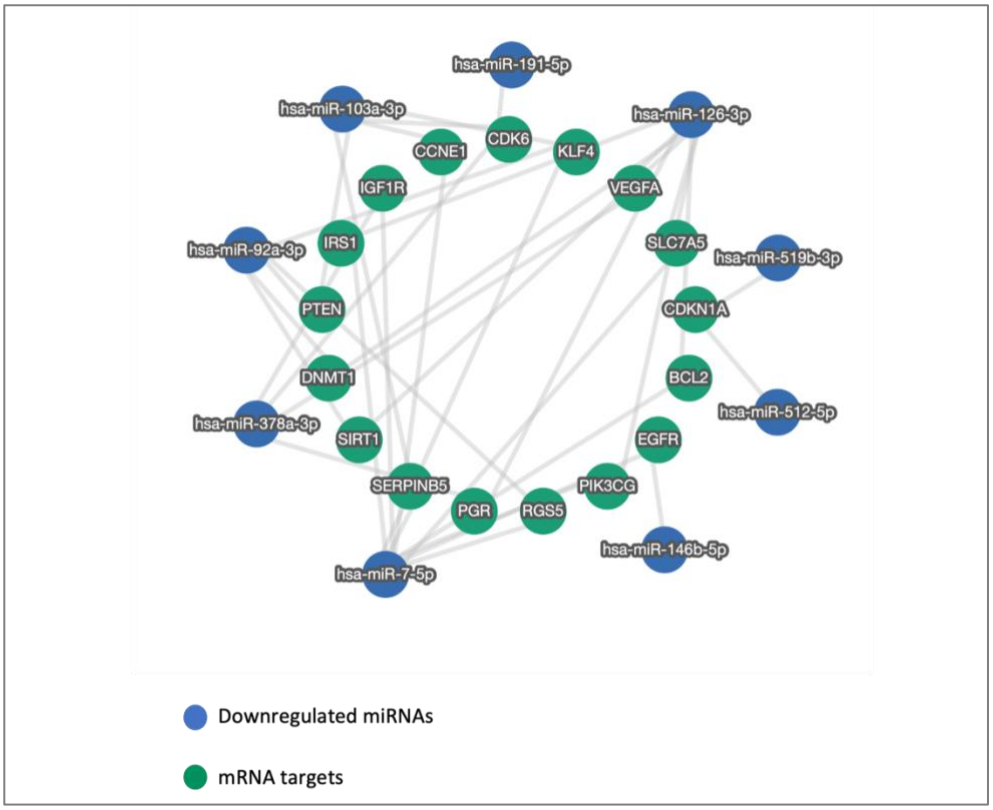
The initial analysis was conducted separately on upregulated and downregulated miRNAs, based on the assumption that these two groups would influence distinct biological pathways. The results showed that overexpressed miRNAs are primarily involved in regulating cell cycle progression and metabolic processes. Gene ontology analysis of downregulated miRNAs indicated that their target genes are key regulators of cell cycle progression and apoptosis, however the pathway annotation analysis did not yield significant findings, likely due to a lack of prior investigation (see Figure 5-5 and Figure 5-6).

Figure 5-5: Functional analysis of the upregulated miRNAs in aneuploid blastocysts



The figure illustrates: A) The miRNA-gene target interaction network of upregulated miRNAs in aneuploid blastocysts compered to euploid. B) Bar graph of the enriched biological pathways targeted by upregulated miRNAs in aneuploid blastocysts. The pathways are sorted by significance, showing involvement of these miRNAs in cell proliferation, cell cycle, cellular responding to signalling and stresses and regulation of apoptotic processes.

Figure 5-6: Gene target interaction network of the downregulated miRNAs in aneuploid blastocysts and the potentially involved biological pathways



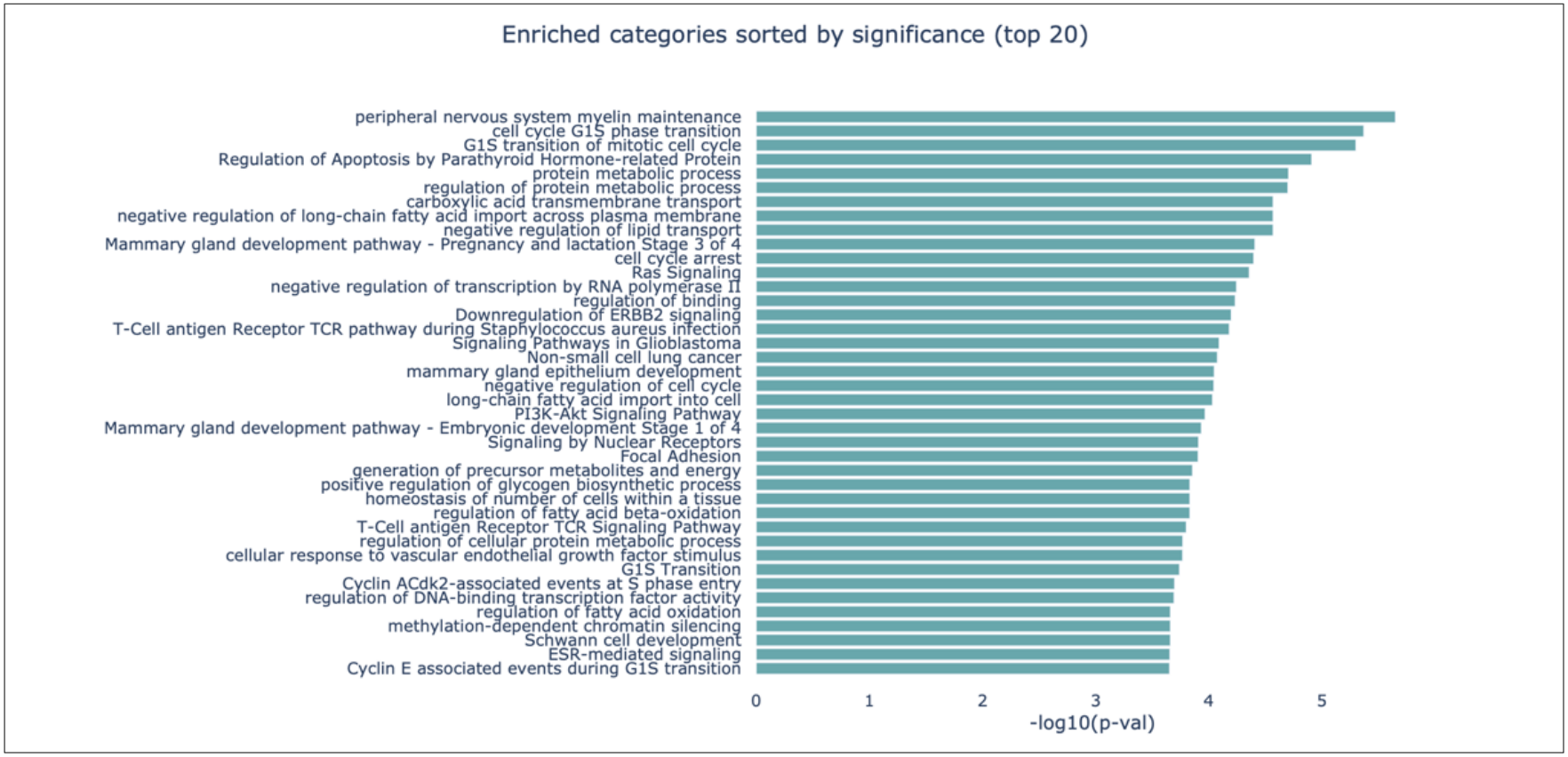
The figure shows the miRNA-target interaction of downregulated miRNAs in aneuploid blastocysts.

A comprehensive functional analysis of all differentially expressed miRNAs, without separating them into upregulated and downregulated subgroups, was conducted. Pathway enrichment analysis was performed using multiple databases, including Reactome, Wikipathway, and GO Biological Pathways, to identify commonly dysregulated pathways in aneuploid samples. The analysis revealed a significant involvement of these miRNAs in regulating the cell cycle, protein metabolic processes, apoptosis, and responses to signalling and stress, as illustrated in (Figure 5-7).

The alteration of these pathways in aneuploid samples is interesting yet not unexpected, as chromosomal abnormalities are well-known cellular stressors. Aneuploidy disrupts the normal balance of gene expression, which the cell senses as a deviation from normal conditions (Licciardi et al., 2018). Under such stress, the affected cells often try to stop or slow their cell cycle progression to prevent further faults. Furthermore, chromosomal abnormalities are typically recognized as a

biological stress, which normally results in activating programmed cell death (apoptosis) to maintain the integrity of the organism (Kerr et al., 1972, Garribba and Santaguida, 2022). The dysregulation of protein metabolic processes suggests that aneuploidy may also affect normal protein synthesis and turnover, likely due to imbalances in gene dosage. Overall, these results indicate that aneuploidy triggers widespread disruptions in cellular homeostasis and critical processes.

Figure 5-7: Pathway enriched analysis of the dysregulated miRNAs in aneuploid blastocysts



This bar graph presents the top 20 enriched biological categories associated with dysregulated miRNAs in aneuploid blastocysts, ranked by statistical significance (-log10 of p-value). Key pathways include cell cycle regulation (G1/S phase transition), protein metabolic processes, regulation of apoptosis, and signalling pathways such as Ras and PI3K-Akt. These results highlight the involvement of miRNAs in critical processes related to cell proliferation, differentiation, and metabolic regulation.

Additional database-specific analyses were conducted using four databases: Reactome, KEGG, Wikipathway, and GO biological pathways, individually. This approach enabled the identification of pathways uniquely detected in each database, offering a more comprehensive understanding of the role of these miRNAs across various contexts. Of note, the differentially expressed miRNAs exhibited a strong involvement in regulating many signalling pathways and were commonly dysregulated in various cancer types (figures are shown in Appendix7).

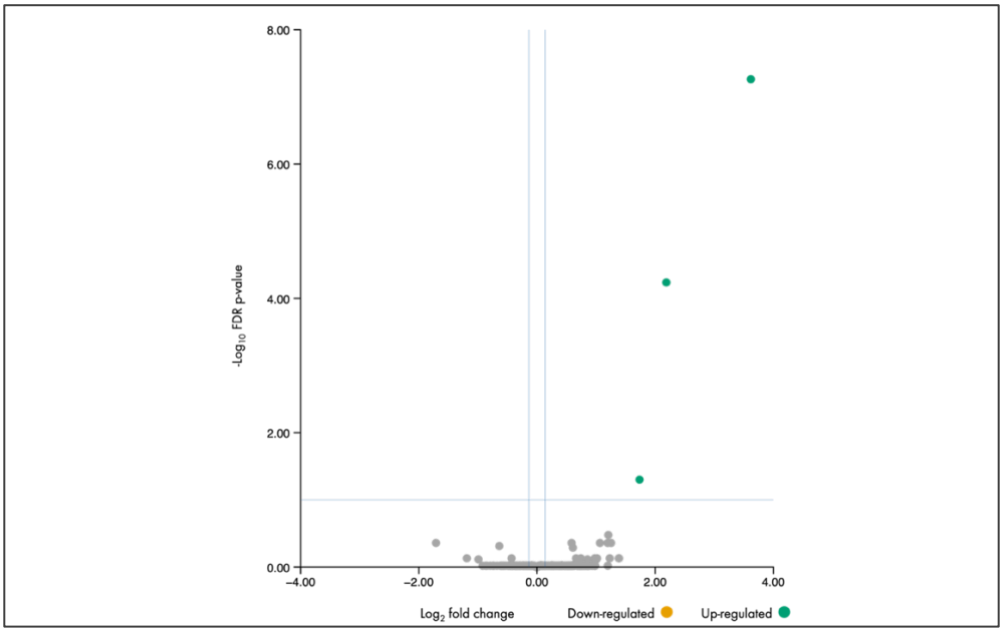
Moreover, additional pathway analyses were conducted on the miRNAs identified in the aneuploidy subgroups. The results revealed quite similar findings of the implicated pathways in all aneuploid blastocysts analysis. This observation suggests that different types of aneuploidies may trigger similar cellular processes. However, this extended analysis allowed detection of additional potentially involved pathways, such as the contribution of these miRNAs in the development of reproductive structures, and their role in regulating several cellular processes including proliferation, differentiation, adhesion, movement, and death (details found Appendix7).

One of the key observations noted during the course of this analysis is that many of the differentially expressed miRNAs have not been extensively investigated for their biological functions. Only a few were previously validated for targeting their complement mRNAs and their involvement in specific pathways. Therefore, it is important to note that all the differentially expressed miRNAs were analysed, and the limited representation of miRNAs in the pathway figures was not because of oversight, but rather reflects the lack of biological data.

5.2.8 Analysis of miRNA Expression Profile in Blastocysts with Varying Blastulation Days

Failure to reach the blastocyst stage reflects poor embryo quality, while a delay in blastulation also signals compromised developmental potential. In the current IVF practice, blastocysts are transferred or frozen on the day of blastulation, which typically occurs on day 5 or day 6 post-fertilization. Given the varied outcomes reported when transferring day 5 versus day 6 blastocysts, it is evident that these blastocysts are not entirely equivalent. In this study, we compared the miRNA expression between embryos that reached the blastocyst stage on day 5 and those that did so on day 6. The overall trend observed showed an elevation in miRNA profiles in the day 6 blastocyst group, with three specific miRNAs significantly upregulated (Figure 5-8). These findings indicate non-equivalence in miRNA expression between embryos reaching blastulation on day 5 versus day 6, which may explain the differences in pregnancy and implantation outcomes between these two groups (Bourdon et al., 2019).

Figure 5-8: Differentially expressed miRNAs in embryos that reached blastocyst at day 6 compared to those did at day 5



The volcano plot displays an elevation of three miRNAs in day 6 blastocysts compared to day 5. The significantly upregulated miRNAs are depicted in green.

Table 5-14: Differentially expressed miRNAs in day 6 versus day 5 blastocysts with aneuploidy adjusted

Name	Fold change	FDR p-value
hsa-miR-206	12.27	5.448E-08
hsa-miR-184	4.55	0.00005
hsa-miR-205-5p	3.32	0.04

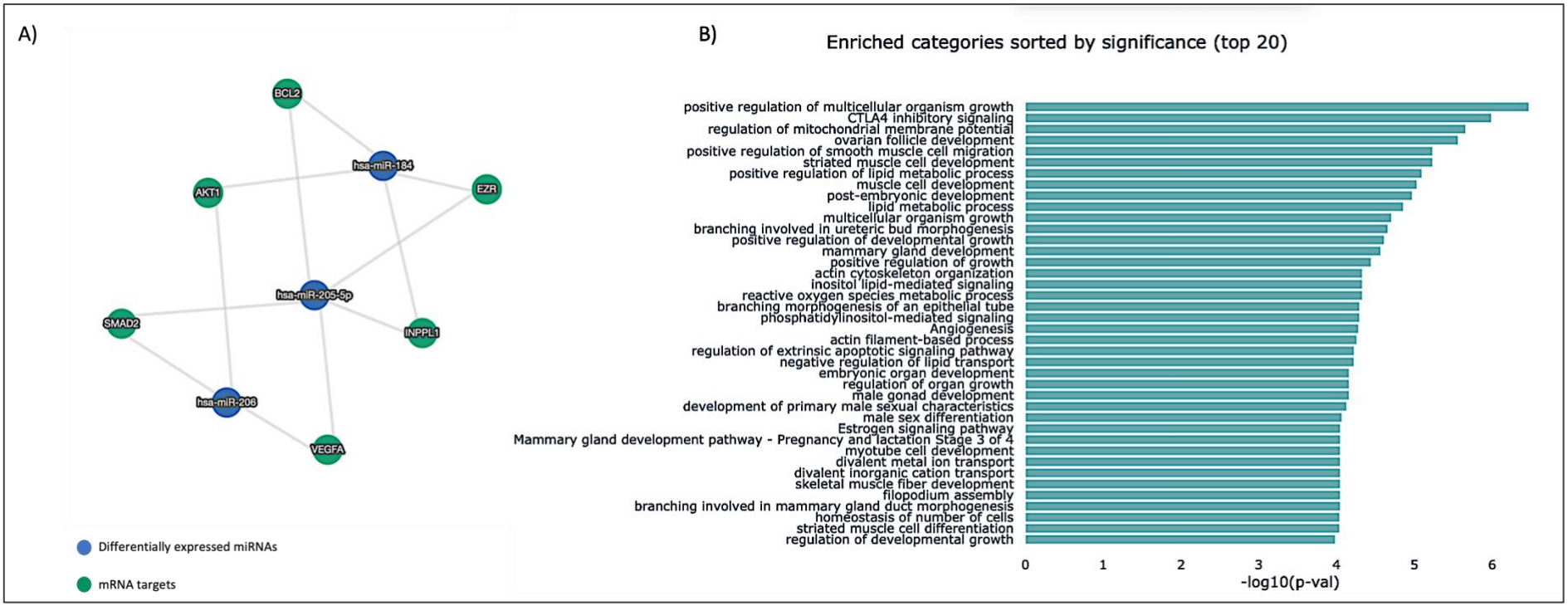
We further investigated the target genes of these upregulated miRNAs in day 6 blastocysts using the miRNA- target interaction analysis. The findings revealed several key insights, including that these miRNAs have a strong contribution in regulating cell growth and cell adhesion by targeting genes such as *VEGFA, EZR* and *INPPL1* (Figure 5-9 (A)). They also target genes involved in cell death regulation, such as the antiapoptotic *BCL2*. Additionally, they are implicated in the control of transcription by targeting genes that activate essential transcription factors, such as the *AKT1* family and transcriptional modulator like *SMAD2*.

When investigated for their biological pathways, the dysregulated miRNAs in this analysis showed significant involvement in various aspects of development, particularly pertaining to the formation of reproductive organs and embryonic growth (as shown in Figure 5-9 (B)). It particularly shows regulating of morphogenesis and cellular structure formation across different developmental stages, including post-embryonic development, as well as controlling cell growth and proliferation. These findings support the hypothesis of a potential correlation between the miRNA profile and the embryo's growth rate. Furthermore, the analysis revealed their contribution to the CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) inhibitory signalling pathway, which modulates excessive immune responses and maintains immune system balance. Interestingly, lipid metabolic processes and the regulation of reactive oxygen species emerged as noteworthy pathways, indicating substantial regulation of metabolic processes and energy production potentially disrupted in embryos with delayed blastulation.

The miRNAs with increased levels in day 6 blastocyst have also demonstrated significant elevation in aneuploid blastocysts. This suggests potential disruption in their expression when the embryo’s competence is compromised. Taken together,

these findings suggest an association between miRNAs expression and the rate of embryonic development.

Figure 5-9: Functional analysis of the dysregulated miRNAs in embryos with different blastocysts formation days (day 6 versus day 5)



The figure demonstrates: A) The mRNA targets of differentially expressed miRNAs in day 6 blastocyst compared to day 5 blastocyst. B) The top 20 enriched biological pathways in embryos that reached the blastocyst stage on day 6 compared to those that reached the blastocyst stage on day 5. The pathways are sorted by significance, highlighting the involvement of these miRNAs in various aspects of development, particularly in the development of reproductive organs, embryonic growth, and metabolic processes.

5.2.9 Variations in miRNA Expression Profiles in Embryos Reaching Blastocyst Stage at Day 5 Versus Day 6

Previous observations suggest that embryos reaching the blastocyst stage at day 6 may experience slower development than those in day 5, though the underlying causes of this delay remain unclear (Yerushalmi et al., 2021). Additionally, chromosomal abnormalities are more frequently observed in day 6 blastocysts than in those reaching the blastocyst stage on day 5 (Taylor et al., 2014b). Given these differences in quality, investigating the gene expression profiles of these two groups may help explain the underlying reasons of delayed blastulation and the lower quality often associated with late-stage embryos.

Our analysis of differentially expressed miRNAs revealed elevated levels in day 6 blastocysts compared to the day 5 group. Although only a few miRNAs showed significant changes, those that did were involved in key pathways regulating post-embryonic development and growth, suggesting that late blastulation may lead to altered embryonic development. Notably, no previous studies have explored gene expression differences between these two types of blastocysts, making our findings a valuable contribution to understanding the molecular mechanisms involved. However, previous research has linked delayed blastocyst development to factors affecting oocyte quality, such as vitrification and elevated progesterone levels on the trigger day (De Gheselle et al., 2020, Villanacci et al., 2023).

5.2.10 Analysis of miRNA Expression in Blastocysts Based on Morphological Grades

Exploring the miRNA profiles of blastocysts with varying morphology grades can provide valuable insights into the molecular mechanisms driving these morphological differences. This analysis specifically focused on three key morphological parameters: blastocyst expansion, ICM, and TE morphology.

5.2.10.1 Hatched versus Unhatched Blastocysts

The comparative analysis of miRNA expression between hatched and unhatched blastocysts revealed a significant increase of hsa-miR-223-3p level (FC = 5.29, FDR = 0.0003) in the unhatched blastocysts. This miRNA is contributing in regulation of immune signalling (Yuan et al., 2021). Therefore, its upregulation in this scenario indicates a potential difference in immune signalling between hatched and unhatched blastocysts. However, the identification of a single differentially expressed miRNA in this analysis suggests no substantial difference in the miRNA profile between blastocysts with different expansion scores. It is important to mention that the small number of unhatched blastocysts included in the analysis may have contributed to potential false-negative results in this comparison.

5.2.10.2 Blastocysts with ICM Grades A versus B

All the blastocysts collected in this study had good to fair ICM morphology, as only these blastocysts were permitted for freezing. Consequently, the analysis of miRNA profiles in relation to the ICM grade was conducted specifically between the available ICM-graded A and B blastocysts. The results indicated no significant difference in the miRNA expression between the two groups, suggesting that blastocyst with A and B-graded ICM cells have relatively similar quality. Again, it is important to acknowledge the limited number of A-graded blastocysts in this comparison.

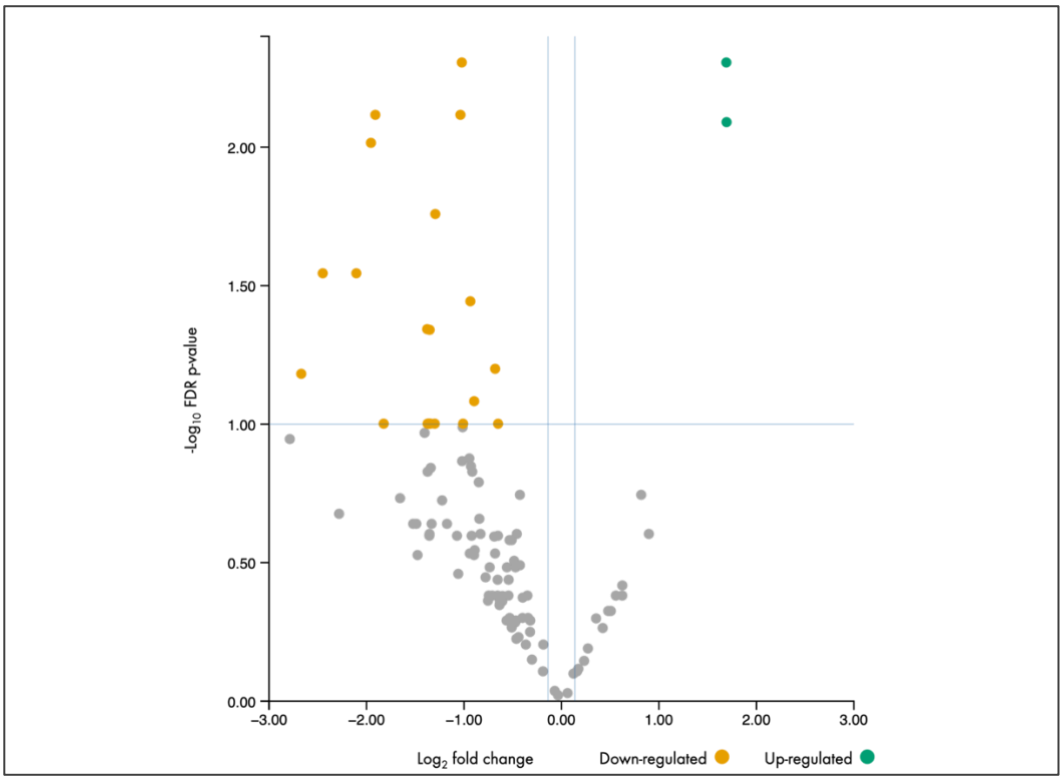
5.2.10.3 Blastocysts with Varying TE Grades

In order to explore the miRNA profiles across different TE morphology grades, the blastocysts were divided into three groups: A, B and C, based on the TE score assigned by the embryologist (as detailed in 2.5.1.3). When comparing A-graded to the B- graded TE cells, no significant changes in miRNA profile was detected. However, a remarkable decrease of two-fold in hsa-miR-371a-5p (with FDR p-value of 0.06) was observed when comparing the good quality A-graded TE cells to the poor-quality C-graded TE cells.

The most notable miRNA findings in this analysis emerged from the comparison between blastocysts with fair TE morphology (grade B) and those with poor TE morphology (grade C) (Figure 5-10). The differentially expressed miRNAs are

displayed in Table 5-15, showing a marked overall reduction in miRNA levels in C-graded TE cells. Many of these miRNAs showed decreased expression in other quality-related comparisons, including those involving aneuploid blastocysts.

Figure 5-10: A volcano plot of the differentially expressed miRNAs in C-graded TE versus B-graded TE blastocysts



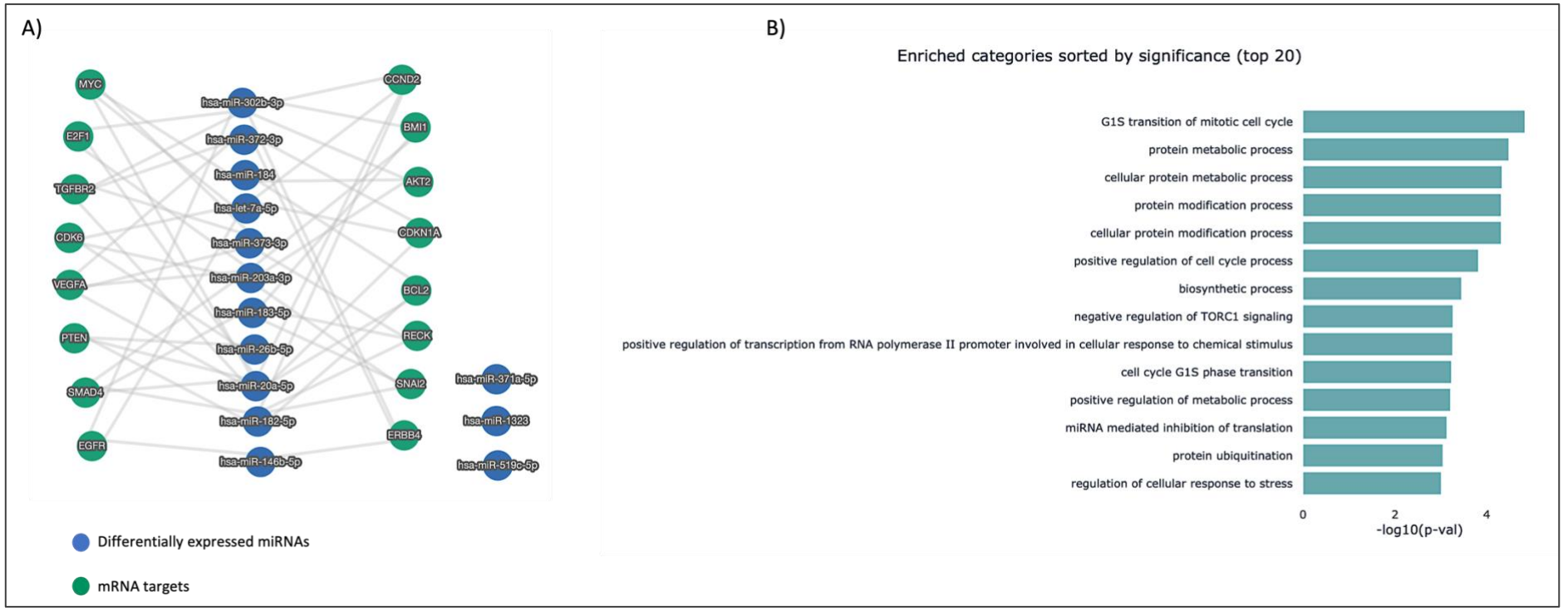
The volcano plot reveals a significant reduction in miRNA levels, shown in yellow, with only two miRNAs upregulated, marked in green, in blastocysts with TE cells graded as C compared to those graded as B.

Table 5-15:Differentially expressed miRNAs in C-graded TE versus B-graded TE blastocysts

Name	**Fold change**	**FDR p-value**
hsa-let-7a-5p	2.96	0.004
hsa-miR-1323	-3.21	0.009
hsa-miR-146b-5p	-2.78	0.04
hsa-miR-182-5p	-1.73	0.01
hsa-miR-183-5p	-1.58	0.09
hsa-miR-184	2.79	0.03
hsa-miR-203a-3p	3.78	0.001
hsa-miR-20a-5p	-3.29	0.05
hsa-miR-26b-5p	-3.99	0.005
hsa-miR-302b-3p	-2.02	0.009
hsa-miR-371a-5p	-2.01	0.004
hsa-miR-372-3p	-1.77	0.009
hsa-miR-373-3p	-2.37	0.009
hsa-miR-519c-5p	-2.32	0.05

Gene target analysis of differentially expressed miRNAs in blastocysts with poor morphology highlighted their role in regulating cell proliferation and survival by targeting key genes involved in the cell cycle and growth. The pathway annotation analysis further revealed their involvement in the regulation cell cycle events, protein phosphorylation and metabolic activity (Figure 5-11). Furthermore, the negative regulation of TORC1 (Target of Rapamycin Complex 1), a protein complex involved in regulating critical pathways such as cell growth and metabolism, suggests that blastocysts with poor trophectoderm morphology may experience disrupted development (Wullschleger et al., 2006). Such disruption could affect their normal appearance and functionality.

Figure 5-11: Pathway enriched analysis of the differentially expressed miRNAs in C-graded TE versus B-graded TE blastocysts



The figure illustrates A) B) potentially altered pathways in embryos with C-grade versus B-grade TE morphology. The pathways are sorted by significance, highlighting the strong involvement of these miRNAs in protein modification and metabolic processes, and cell-cycle regulation.

Notably, hsa-miR-371a-5p exhibited significant downregulation in the C-graded blastocysts when compared to both A and B-graded ICM groups. This may indicate association between this miRNA and the morphological appearance of the blastocyst. While hsa-miR-371a-5p has not been extensively studied previously, miRNA databases indicate its potential involvement in regulating stem cell pluripotency (Tang et al., 2016).

5.2.11 Blastocysts With Varying Morphological Appearance Differ in the Gene Expression Pattern

When investigating euploid blastocysts across varying maternal age groups, those with good morphology displayed the highest implantation rate compared to other morphology groups (Awadalla et al., 2021). However, an alternative study has shown that poor-quality blastocysts were also capable of viable implantation (Majumdar et al., 2017). These findings suggest that while blastocysts morphology can be influenced by the aneuploidy status, there might be other factors contributing to poor morphology of euploid samples.

While extensive research has been conducted on embryo morphology and morphokinetics, limited attention was given to the gene expression profiles across different morphological groups. A previous study proposed a potential causal relationship between poor morphology and altered gene expression in preimplantation embryos. Specifically, the study showed that day 3 embryos with certain forms of abnormal morphologies exhibited changes in the expression of genes related to cell fragmentation with the deregulation of TP53 level (Wells et al., 2005).

Consistent with earlier observations, findings in this study revealed a significant difference in miRNA profiles among blastocysts with different TE grades. The identified miRNAs were primarily linked to the regulation of protein metabolic processes, hinting at possible disruptions in cellular health within lower-quality blastocysts. While these results provide foundational data on the association between blastocyst metabolism and appearance, more comprehensive investigations are certainly warranted to understand the underlying causes of poor morphology in blastocysts.

5.3 Chapter Summary

In summary, the findings of differentially expressed miRNAs and mRNAs across various aneuploid groups highlight the profound impact of aneuploidy on the gene expression profile of blastocysts. These results align with the miRNA and aneuploidy association evidenced through the systematic review conducted in this study. The in-depth analysis revealed significant changes in miRNA profiles associated with different types and levels of numerical chromosomal abnormalities, supporting this correlation between miRNA expression and aneuploidy. Notably, genes targeted by these miRNAs, such as *CDKN1A*, exhibited consistent dysregulation across various aneuploidies, suggesting that aneuploidy may be linked to specific cellular pathways.

Additionally, differences in miRNA expression were observed between embryos reaching the blastocyst stage on day 5 versus day 6, indicating potential disruptions in key developmental pathways in slower-developing embryos. Moreover, the significant differences in miRNA profiles between poor and fair morphology blastocysts underscores importance of TE morphology in assessing embryo development and quality. Overall, low-quality blastocysts displayed notable alterations in their miRNA profiles, with these miRNAs playing crucial roles in regulating essential cellular processes. These changes may impact crucial functions such as embryo-endometrium communication, and blastocyst implantation.

Chapter 6 Differential miRNA Expression in Blastocysts: Influence of Parental Factors

6.1 Introduction and Aim

Traditionally, assessing the quality of oocytes and sperm has relied primarily on their physical appearance, supplemented by evaluating surrounding cells, such as cumulus cells, which introduces a degree of subjectivity (World Health, 2010, Liu et al., 2016, Ouandaogo et al., 2012, Dell'Aversana et al., 2021, Sharma et al., 2015, Halvaei et al., 2020). The transcriptomes of these cells have been extensively studied and linked to various reproductive issues, including PCOS, advanced reproductive age, spermatogenic impairment and infertility (Abu-Halima et al., 2020, Uppangala et al., 2016, Sharma et al., 2015, Yang et al., 2022, Vegetti et al., 2000, Tomic et al., 2022, Abu-Halima et al., 2013, Fragouli et al., 2010, Liu et al., 2016, Hawkins and Matzuk, 2010, Yang et al., 2016, Liu et al., 2015). While gene expression studies on gamete cells can provide useful insights, they result in the loss of the final product necessary for assessing treatment outcomes, such as blastocyst formation, implantation, and pregnancy. However, the blastocyst, as the product of fertilized gametes, offers a valuable alternative and serves as a reliable indicator of gamete quality.

The competence of the gamete cells is influenced by numerous biological and environmental factors. In the context of IVF, several variables are particularly noteworthy, such as the effect of exogenous hormones through ovarian stimulation and oocyte maturation triggers, which have shown consistent impact on both oocyte and preimplantation embryo quality (Bosch et al., 2016, Ezoe et al., 2014, Ertzeid and Storeng, 2001, Santos et al., 2010, Machtinger et al., 2023, Gurbuz et al., 2016, Villanacci et al., 2023). Not to mention the great influence of age, the maternal and to less extent the paternal age, on the reproductive cells, which could pass through to affect the resulting embryo (Sharma et al., 2015, Gunes et al., 2016, du Fosse et al., 2020, Mikwar et al., 2020, Verdyck et al., 2023, Charalambous et al., 2023).

Moreover, while many studies have investigated the correlation between infertility and poor sperm parameters, limited research have focused on the downstream effect of sperm quality on the resulting embryo after fertilisation (Bashiri et al., 2021).

This chapter explores the potential influence of these factors on gamete quality by investigating miRNA expression in blastocysts. Our study is the first to examine how variables like age, hormone treatments, and sperm quality impact miRNA profiles in blastocysts, providing novel insights into the molecular mechanisms that govern preimplantation embryo development.

6.2 Results and Interpretation

6.2.1 Differential Expression Analysis of miRNAs in Blastocysts Under Different Ovarian Stimulation Doses

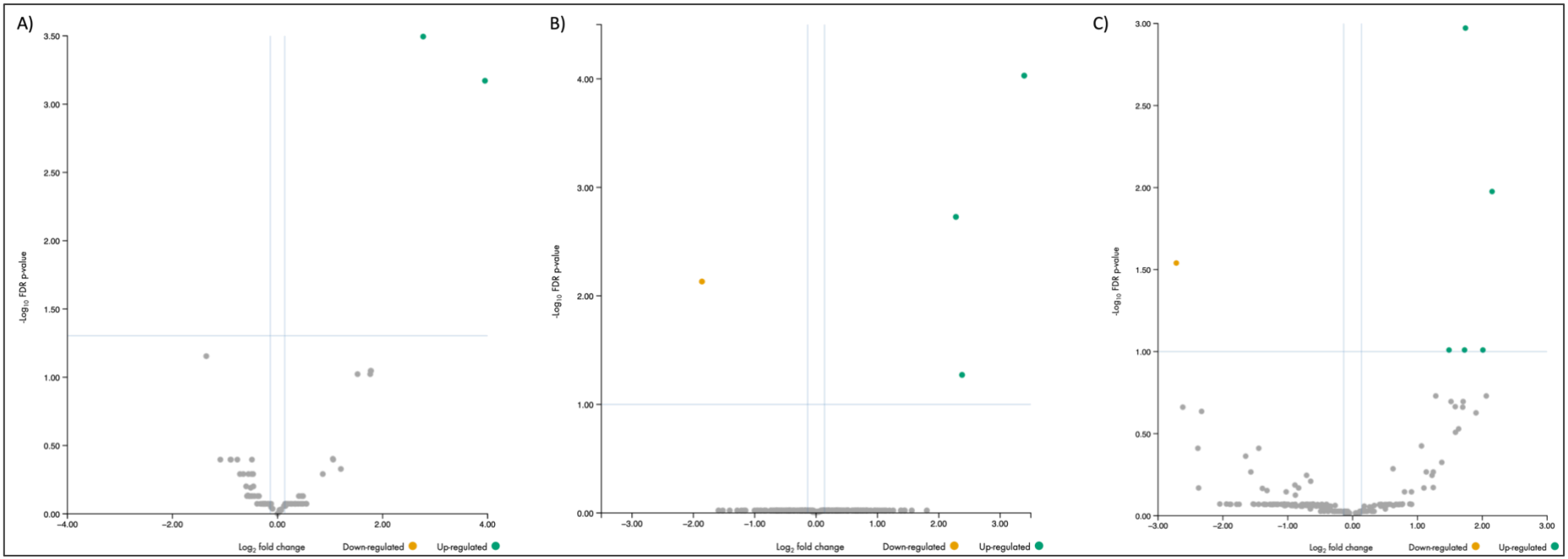
In this part of the study, we analysed the miRNA profile in blastocysts originating from oocytes exposed to varying ovarian stimulation dosages, with the aim to see whether miRNA expression can be influenced by the dose of hormone treatment. Blastocysts were classified into four groups according to the dosage medication administered to the female patient: high stimulation dose, medium stimulation dose, low stimulation dose, and very low stimulation dose. Since ovarian stimulation dosage is often influenced by factors, such as female age, the potential confounders in this analysis were identified and controlled for. This approach resulted in multiple analyses, only consistent findings are considered significant and reported (as detailed in 2.5.1.4).

The observed miRNA expression patterns across these groups revealed significant variations in blastocysts derived from oocytes stimulated with a high-dose medication compared to other stimulation dosage groups (medium and low) (

Figure 6-1 (A and B). The differentially expressed miRNAs in these comparisons are presented in Table 6-1 and

Table 6-2, respectively. Remarkably, hsa-miR-184 and hsa-miR-206 exhibited constant upregulation in the blastocysts from high-dose group compared to those from medium and low-dose groups. When comparing the blastocysts from high-dose stimulation group to the ones obtained from oocytes with minimal stimulation (very low dose group), hsa-miR-184 expression exhibited elevation with a more than six-fold increase (FDR p-value = 0.02) (Figure 6-1 (C)).

Figure 6-1: Volcano plot of the differentially expressed miRNAs in blastocysts derived from oocytes subjected to various ovarian stimulation dosages



The volcano plot displays A) Elevation of two miRNAs in high dose stimulation blastocyst group compared to those from medium stimulation dose group. B) Differentially expressed miRNAs between high dose stimulation blastocyst group and low stimulation dose group. The extra downregulated gene in this figure was not found after controlling confounding factors. C) Dysregulated miRNAs in blastocysts with high dose stimulation compared to those with very low stimulation dose group. Although several miRNAs were detected, only has-miR-184 remained to be significantly upregulated after controlling confounding factors.

Table 6-1: Differentially expressed miRNAs in high-dose stimulation group versus medium-dose stimulation group blastocysts

Name	Fold change	FDR p-value
hsa-miR-184	6.8	0.0003
hsa-miR-206	15.4	0.0006

Table 6-2: Differentially expressed miRNAs in high-dose stimulation group versus low-dose stimulation group blastocysts

Name	Fold change	FDR p-value
hsa-miR-184	4.8	0.001
hsa-miR-203a-3p	-3.6	0.007
hsa-miR-206	5.2	0.05

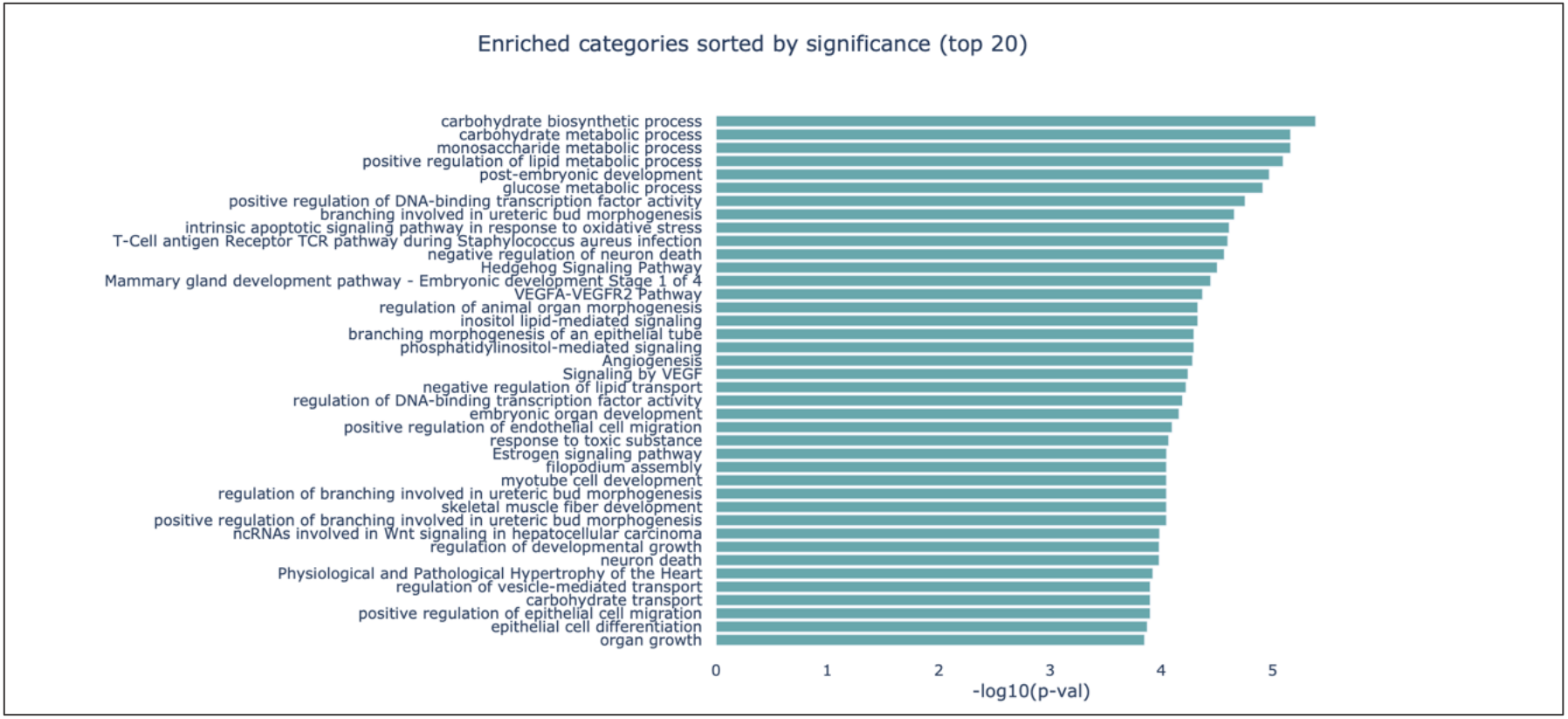
Interestingly, the miRNA profiles in blastocysts derived from oocytes stimulated with the medium, low, and very low doses did not show substantial differences when compared to each other. However, certain findings merit consideration, like the consistent upregulation of hsa-miR-203a-3p in blastocysts from low-dose group compared to those from high and medium dose groups.

Collectively, these findings highlight the potential impact of stimulation dosages on the expression of specific miRNAs in developing blastocysts, particularly hsa-miR-184, hsa-miR-206 and hsa-miR-203a-3p. While previous studies have established correlations between stimulation dose and embryo quality, the observed changes in the expression of these specific miRNAs, known for their involvement in regulating genes crucial for implantation, offer novel insights and pave the way for further exploration into more refined treatment protocols (Roberts et al., 2005, Santos et al., 2010, Chen et al., 2012, Movaghar and Askarian, 2012, Zhang et al., 2019a).

The most significant alterations in the miRNA profile were observed in the group exposed to a high dose of ovarian stimulation. Functional analysis of the associated miRNAs demonstrated their critical role in regulating early embryonic development processes, including organ growth and angiogenesis. Additionally, these miRNAs are involved in carbohydrate and lipid metabolic processes and contribute to crucial

implantation-related signalling pathways, such as estrogen signalling. The three dysregulated miRNAs also play a role in responding to oxidative stress by regulating intrinsic apoptotic signalling (Figure 6-2). Pathway analysis of theses miRNAs suggests that high ovarian stimulation doses may impact the embryonic development, possibly by introducing stress and modulation of endometrium-embryo signalling crosstalk.

Figure 6-2: Pathway enriched analysis of the differentially expressed miRNAs in blastocysts obtained from oocyte stimulated using high ovarian stimulation dose compared to the those received lower dosages



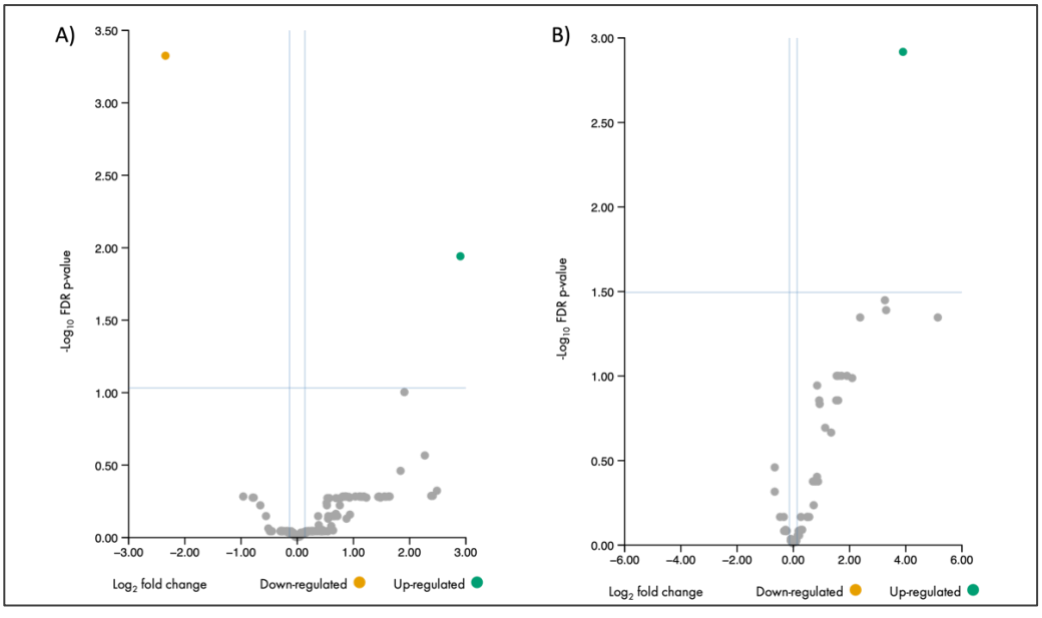
The pathway enrichment analysis illustrates the potentially regulated pathways by the differentially expressed miRNAs in blastocysts subjected to high-dose stimulation compared to the medium and low-dose stimulation groups. This includes carbohydrate and lipid metabolic processes and estrogen signalling.

6.2.2 Differential Expression Analysis of miRNAs in Blastocysts Across Different Types of Oocyte Maturation Triggers

A subsequent analysis of miRNA expression was conducted on blastocysts categorized based on the trigger medication administrated for oocyte maturation during the treatment cycle. Three groups of blastocysts were compared: the hCG-tiggered group, the GnRHa (Suprefact)-triggered group, and the dual-triggered group, receiving both hCG and GnRHa.

The miRNA differential expression results revealed an overexpression of hsa-miR-3168 and downregulation of hsa-miR-203a-3p in blastocysts derived from hCG-triggered oocytes compared to the GnRHa group (Figure 6-3 (A) and Table 6-3). However, the most significant difference was the upregulation of hsa-miR-184, with more than 14-fold increase (FDR p-value = 0.001) in hCG triggered group compared to dual-triggered derived group (Figure 6-3 (B))

Figure 6-3: Differentially expressed miRNAs in blastocysts from oocytes subjected to hCG-trigger compared to those that received GnRHa-trigger and dual trigger: volcano plots



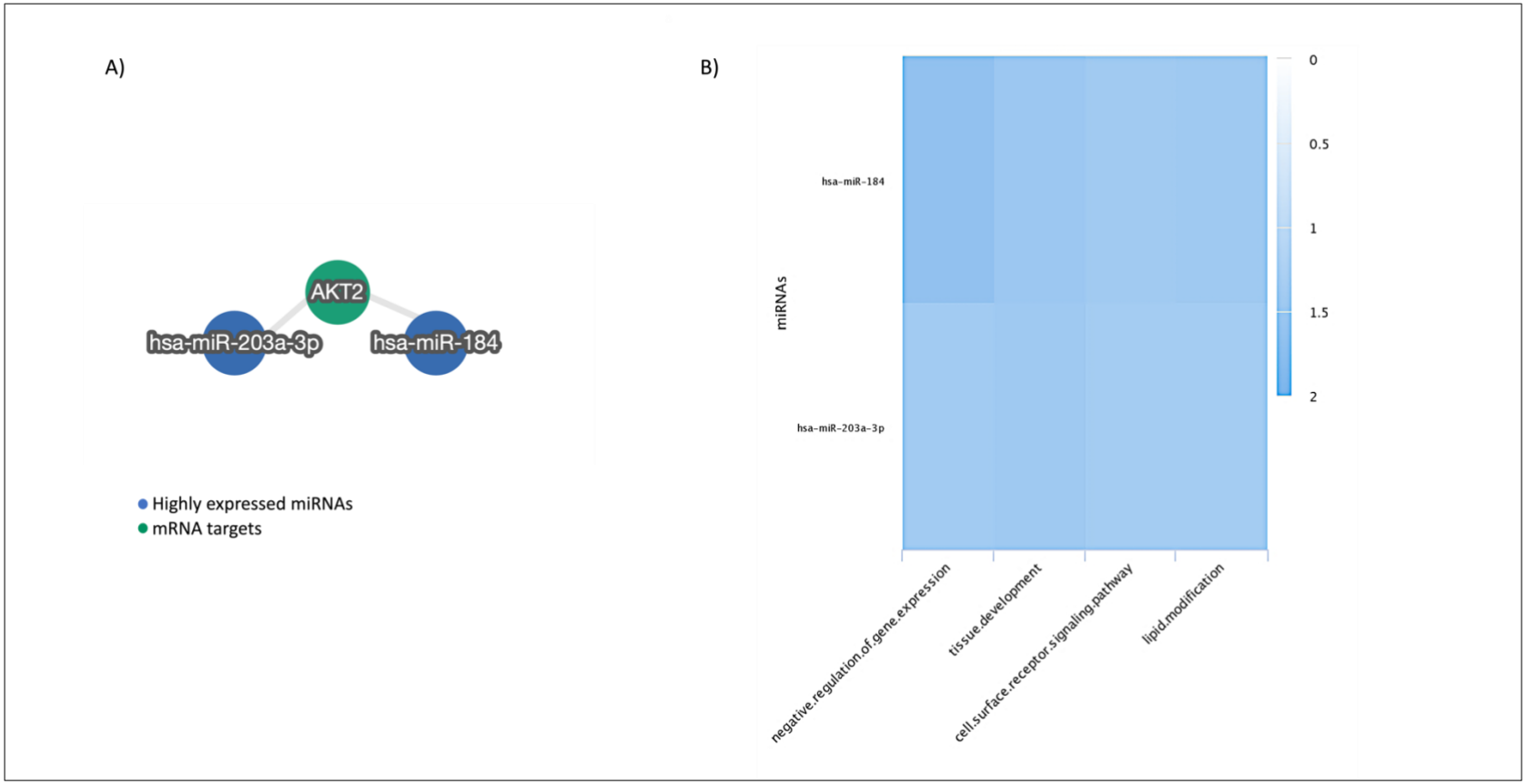
The volcano plots show A) Two miRNAs were significantly changed, the upregulated hsa-miR-3168 is presented in green while the downregulated hsa-miR-203a-3p is shown in yellow. The volcano plot depicts the differentially expressed miRNAs in hCG-triggered group versus dual-triggered group blastocysts. One miRNA, hsa-miR-184, was significantly upregulated and presented in green.

Table 6-3: Differentially expressed miRNAs in hCG-triggered group versus GnRHa-triggered group blastocysts

Name	Fold change	FDR p-value
hsa-miR-203a-3p	-5.07	0.0004
hsa-miR-3168	7.4	0.01

The dysregulated miRNAs were further analysed for potential target genes, revealing that hsa-miR-203a-3p and hsa-miR-184 have a shared target *AKT2*. *AKT2,* a serine/threonine-protein kinase gene, regulates diverse cellular processes, including signalling, metabolism and growth (Figure 6-4 (A)) (Hers et al., 2011). Additionally, pathway analysis was conducted on these miRNAs, showing their involvement in regulating important cellular processes such as gene expression, signalling and tissue development (Figure 6-4 (B)). The hsa-miR-3168 did not show significant results in the functional analysis.

Figure 6-4: miRNA-gene target and pathway annotation analyses of the dysregulated miRNAs in blastocysts derived from oocytes triggered by various types of triggers



The figure illustrates A) the gene targeted by the dysregulated miRNAs in hCG triggered blastocyst group compared to GnRHa and dual-triggered group blastocysts. B) Pathway annotation of these miRNAs revealed their involvement in crucial developmental pathways, such as regulating gene expression and contribution in tissue development.

6.2.3 Potential Influence of Oocyte Treatment on the Embryo Quality

While infertility treatments often rely on ovarian stimulation to produce a sufficient number of oocytes for fertilization, several studies have highlighted the potential adverse effect of exogenous hormones on pregnancy outcomes (Bourgain and Devroey, 2003, Devroey et al., 2004, Bosch et al., 2016). Previous animal studies have demonstrated that ovarian stimulation can perturb the normal balance of pregnancy-related hormones (Miller and Armstrong, 1981, Miller and Armstrong, 1982, Ertzeid and Storeng, 1992). Additionally, a higher incidence of chromosomal abnormalities was evident in oocytes and embryos following ovarian stimulation (Roberts et al., 2005, Santos et al., 2010). These hormones have also been shown to affect pregnancy outcomes by altering the expression of steroid receptors, leading to failed embryo implantation and impaired decidualization (Ezoe et al., 2014).

The present analysis of miRNA expression among blastocysts exposed to varying doses of ovarian stimulation revealed significant differences, particularly in response to higher stimulation doses. The frequently altered miRNAs, such as hsa-miR-206 and hsa-miR-184, are known regulators of genes crucial for embryo development. For instance, hsa-miR-206 targets the Estrogen Receptor 1 (*ESR1),* which plays a key role in regulating implantation from both maternal and embryonic sides (Chen et al., 2012, Logsdon et al., 2023). Similarly, hsa-miR-203a-3p regulates the expression of Cadherin 1 (*CDH1),* an important implantation-related gene (Babb and Marrs, 2004). Supporting these findings, a previous study reported altered *CDH1* expression in blastocysts from patients subjected to high stimulation doses (Movaghar and Askarian, 2012).

Another essential gene expressed during the window of implantation and influences the uterine receptivity is Leukemia Inhibitory Factor (*LIF)*. *LIF* plays an important role in mediating the interactions between invading trophoblasts and maternal decidual cells. This regulatory action occurs through *LIF* binding to its receptor, LIF Receptor Subunit Alpha (*LIFR*), which is highly expressed in human blastocysts (Charnock-Jones et al., 1994, Aghajanova, 2004). Intriguingly, *LIFR* is also a common target of the miRNAs hsa-miR-203a-3p and hsa-miR-184, both of which were consistently associated with the oocyte treatment.

Together, it is evident that the dysregulated miRNAs identified in our analysis play a key role in regulating genes associated with implantation. However, further research is needed to fully understand the potential impact of oocyte treatment on blastocysts quality and its subsequent implantation potential. Our findings suggest that higher stimulation doses may enhance *LIFR* expression while reducing *ESR1* levels in blastocysts. Nevertheless, it remains unclear whether these blastocysts exposed to these conditions can progress normally and achieve successful implantation, warranting additional investigation.

Additionally, miRNAs are known for their role in oocyte maturation, regulating functions within the oocyte and its surrounding cells. In a mouse model, granulosa cells showed altered levels of specific miRNAs following hCG treatment, suggesting an impact of this treatment on the miRNA expression within these cells (Hawkins and Matzuk, 2010). The influence of miRNA expression in human cumulus cells extended beyond oocyte developmental potential, also impacting blastocyst formation (Bartolucci et al., 2020, Dehghan et al., 2021).

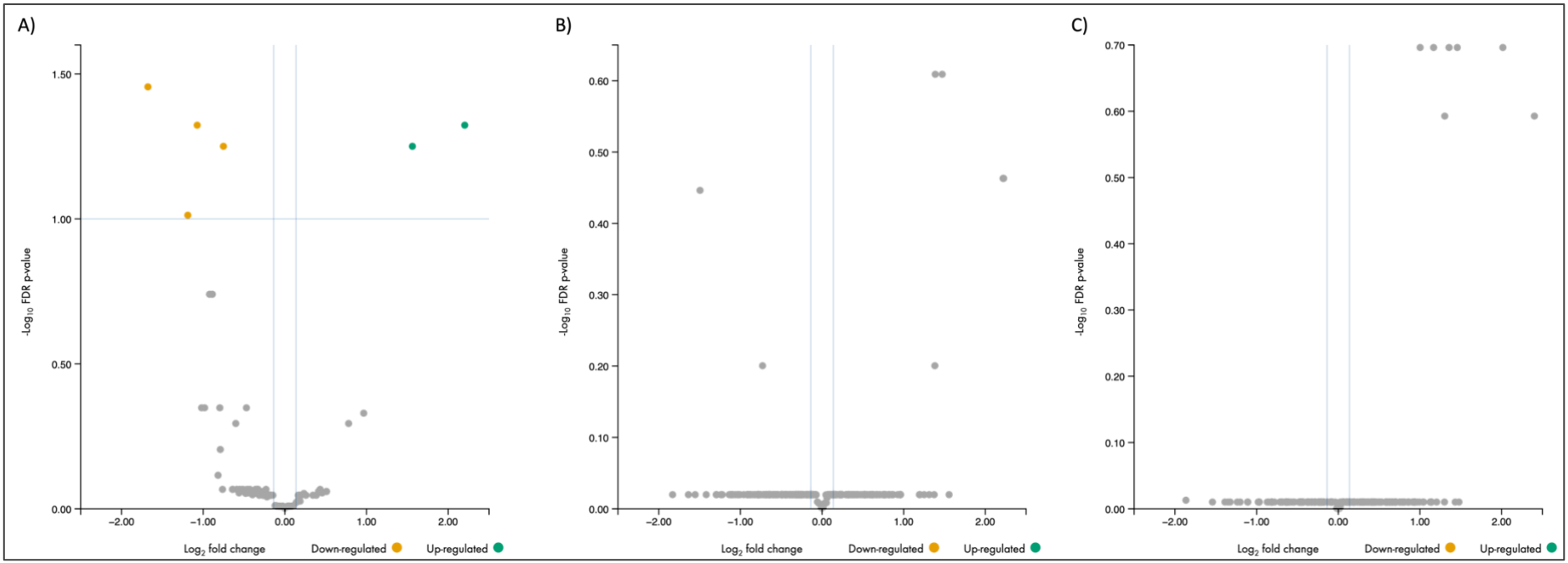
Of note, two miRNAs hsa-miR-184 and has-miR-203a, consistently exhibited dysregulation in blastocysts not only under different stimulation doses but also with varying oocyte triggers. The frequent upregulation of miR-184 in blastocysts with high stimulation doses, as well as those triggered with hCG, is noteworthy. This miRNA plays an important role in oogenesis and early embryonic development (Iovino et al., 2009). Elevated levels of miR-184 have been linked to spontaneous abortion, as it promotes apoptosis and inhibits trophoblast cell proliferation (Zhang et al., 2019a).

Moreover, the significant change in miR-203a expression in response to the administrated oocyte treatment has been previously reported, with this miRNA in follicular fluid showing dysregulation in patients subjected to hCG compared to those who received GnRHa (Machtinger et al., 2023). Hsa-miR-3168 also showed elevated levels in hCG group compared to dual triggers in our analysis. Although this miRNA has not been extensively studied, previous research has shown its overexpression in conditions involving DNA damage and cytokine-mediated responses (Abramowicz et al., 2020).

6.2.4 Maternal Age-Related miRNA Expression Variation in Blastocysts

Analyses of miRNA expression were conducted on blastocysts across three maternal age groups: A, B, and C (as detailed in the section 2.5.1) with inter-group comparison. The most significant differences were observed between groups A and B, representing blastocysts obtained from women younger than 34 and those aged between 35 and 40 years. Four miRNAs, namely hsa-miR-20, hsa-miR-184, hsa-miR-26a-5p and has-miR-92a-3p, showed significant variation between these age groups (Figure 6-5 (A) and Table 6-4).

Figure 6-5: Differentially expressed miRNAs in blastocysts derived from women with different maternal ages



The volcano plots illustrate miRNA expression differences across different age groups. A) Shows the differential miRNA expression between women younger than 34 years and those aged 35 to 40 years. Of the six miRNAs identified as significantly dysregulated, only four remained significant after adjusting for confounding factors. B) and C) Show no significant differences in miRNA expression profiles between blastocysts grouped into A and B (from women under 40 years old) compared to group C (blastocysts from women aged 40 and older).

Table 6-4: Differentially expressed miRNAs in B-grouped blastocysts compared to A-grouped blastocysts based on maternal age

Name	Fold change	FDR p-value
hsa-miR-206	4.16	0.05
hsa-miR-184	2.95	0.06
hsa-miR-26a-5p	-2.1	0.05
hsa-miR-92a-3p	-1.68	0.06

Unexpectedly, comparisons involving the more advanced age group (over 40 years) showed the least change in miRNA profile. Although some differences were observed between blastocysts from women under 40 (A and B groups) and those above 40 (C group), the statistical significance of these variances fell below the established threshold, as presented in (Figure 5-6 (B) and (C)). Given the well-documented influence of maternal age on overall embryo quality, these findings were surprising and warrant further investigation.

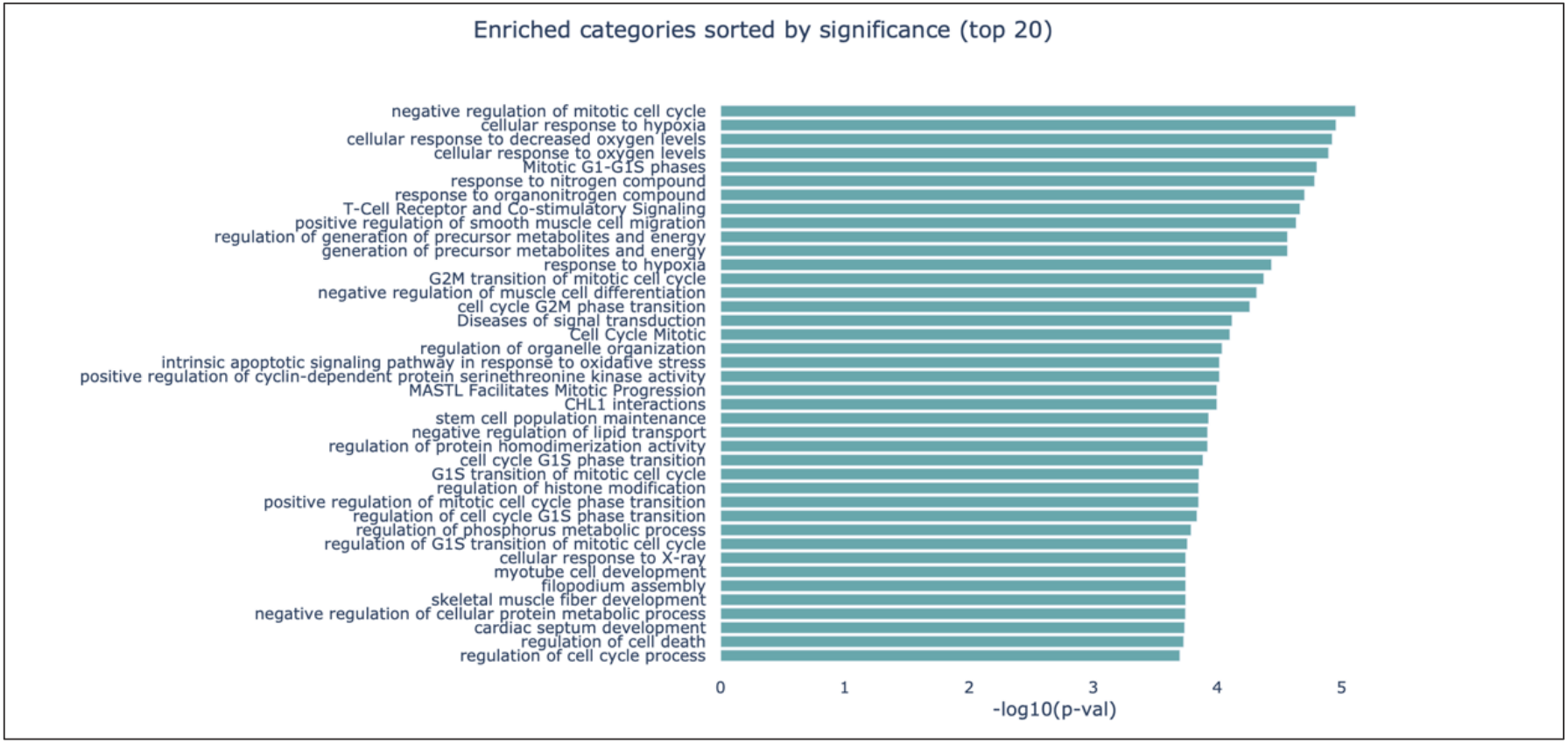
A noteworthy observation emerged regarding hsa-miR-26a-5p, which showed distinct expression patterns across these comparisons. This miRNA exhibited significant downregulation in B-grouped blastocysts compared to those in A group and, to a lesser extent, when compared to C-grouped blastocysts. This result suggests that embryos from middle-aged women exhibited the lowest level of hsa-miR-26a-5p.

Since some of the dysregulated miRNAs in this analysis, particularly hsa-miR-206, hsa-miR-184 and hsa-miR-92a-3p, were also found in aneuploidy analysis, we questioned whether aneuploidy was a confounding in this investigation, especially given its correlation with advanced maternal age. To address this, we conducted an additional analysis controlling for aneuploidy. The results showed that significant findings persisted only for hsa-miR-26a-5. Although the other identified miRNAs remained differentially expressed, they lost statistical significance after controlling for aneuploidy.

However, we continued to performe functional analysis on all the differentially expressed miRNAs in B-grouped blastocysts compared to A-grouped blastocysts. Results showed significant implications of these miRNAs in the regulation of mitotic processes and responding to low oxygen levels. Moreover, the analysis highlighted their contribution to metabolic processes and the regulation of energy generation (as shown in Figure 6-6).

Contrary to prospects, maternal age showed the least impact on the miRNA profile in blastocysts among the other invistigated influences. However, the changes observed of few miRNAs in relation to the maternal age in the current study, along with findings from previous research, suggest a potential association between miRNA expression in embryos with the age of the mother (McCallie et al., 2014).

Figure 6-6: Pathway enriched analysis of the differentially expressed miRNAs in B-grouped blastocysts compared to A-grouped blastocysts based on maternal age



The figure illustrates the potentially affected pathways influenced by the dysregulated miRNAs in blastocysts obtained from women aged between 35 and 40 years (B-grouped) compared to those from women younger than 34 (A-grouped). The findings showed involvement of these miRNAs in mitotic processes, response to low oxygen levels, contribution to metabolic processes, and regulation of energy generation.

6.2.5 Paternal Age-Related miRNA Expression Variation in Blastocysts

Considering the potential adverse impact of advanced paternal age on blastocyst formation and fertility treatment outcomes, we hypothesised differences in miRNA expression patterns in blastocysts obtained from male patients in different age groups. This hypothesis was tested after categorized the blastocysts into A, B and C base on the paternal age at the time of sample collection (as previously explained in 2.5.1.4).

The miRNA expression analysis revealed subtle alterations of miRNA profiles of blastocysts in relation to paternal age. Notably, these changes were evident only between the two youngest groups, A and B, wherein the latter exhibited upregulation of two miRNAs, hsa-miR-342-3p and hsa-miR-183-5p (Table 6-5). Surprisingly, blastocysts from patients over 50 years old (group C) exhibited no significant differences in miRNA expression compared to those in groups A and B. An intriguing finding of this study is the parallel miRNA expression patterns observed in both maternal and paternal age comparisons, where significant changes were identified between the younger age groups (A and B), while no alterations were detected relative to the advanced reproductive age (C group).

As limited number of differentially expressed miRNAs in paternal age-related comparisons was found, conducting enriched functional analyses of these miRNAs was not feasible.

Table 6-5: Differentially expressed miRNAs in B-grouped blastocysts compared to A-grouped blastocysts based on the paternal age

Name	Fold change	FDR p-value
hsa-miR-342-3p	3.37	0.0002
hsa-miR-183-5p	2.04	0.003

6.2.6 Impact of Parental Age on miRNA Expression Profiles in Blastocysts

Oocyte quality is closely linked to maternal age, with advanced maternal age being associated with a higher incidence of aneuploidy, reflecting the effects of aging on the chromosomal and genetic integrity of oocytes. Previous studies have highlighted the influence of maternal age on miRNA profiles, revealing significant differences in miRNA expression between oocytes from older and younger women (Salas-Huetos et al., 2019).

In blastocysts, the expression pattern of miRNAs was also linked to the maternal age. An earlier study revealed exclusive expression of 11 miRNAs in euploid samples obtained from patients in their forties compared to those from young oocyte donors, and significant changes in the expression of other 42 miRNAs between the two groups (McCallie et al., 2014). Consistent with this variation, our findings revealed dysregulation in the expression of four miRNAs in blastocysts obtained from females with reproductive age of less than 35 and those aged from 35 to 40. Only hsa-miR-184 was consistently upregulated with advanced age in both our study and the earlier research.

Another intriguing result was the downregulation of hsa-miR-26a-5p in blastocysts from middle-aged group women when compared to those from both younger and older groups. This miRNA plays an important role in embryonic early development by targeting genes essential for trophoblast attachment and proliferation (Szuszkiewicz et al., 2022). Upregulation of this miRNA has been observed in preeclampsia patients, a condition that is thought to be more severe in women with extreme ages, younger than 25 and older than 45 (Wu et al., 2012, Choi et al., 2013, Chang et al., 2023).

Additionally, hsa-miR-92a-3p also exhibited upregulation in blastocysts derived from women over 35 compared to those from younger women. Although these two miRNAs have not been previously linked to the maternal age, their expression, along with miR-342-3p, has been associated with pregnancy complications such as implantation failure and preeclampsia (Choi et al., 2013, Juarez-Barber et al., 2023). Further investigation is needed to explore the potential association between

overexpression of these miRNAs, embryonic development and physiology of preeclampsia in relation to the maternal age.

Although there is no consensus on the exact definition or threshold for advanced paternal age in the reproductive health, several studies have established a strong association between increased paternal age and genetic abnormalities affecting the sperm competence. These include DNA fragmentation, abnormal gene expression regulation through methylation or epigenetic modifications, chromosomal defects, and alterations in hormone production and spermatogenesis in the reproductive tract (Sharma et al., 2015, Gunes et al., 2016, Halvaei et al., 2020, Kaltsas et al., 2023). Additionally, advanced paternal age has been linked to pregnancy complications, such as spontaneous miscarriage and a wide array of conditions such as preterm birth, congenital diseases and mental disorders (Janeczko et al., 2020, du Fosse et al., 2020).

To date, no studies have directly linked paternal age with embryo quality. Our analysis presents preliminary findings suggesting a potential association, with upregulation of two miRNAs, hsa-miR-342-3p and hsa-miR-183-5p, in the blastocysts from males in their forties compared to those aged 50 or older. These miRNAs have been associated with pregnancy-related abnormalities, including preeclampsia, and implantation failure (Suo et al., 2020, Mo et al., 2022).

Remarkably, hsa-miR-342-3p showed a distinct association with both paternal age and, to a lesser extent, maternal age. This miRNA targets DNA Methyltransferase 1 (*DNMT1)*, which may hint to a potential impact of parental age on the epigenetic machinery during early development (Xiong et al., 2022).

6.2.7 Analysis of miRNA Expression in Blastocysts Based on Sperm Quality Parameters

The potential impact of sperm quality on blastocyst quality, and by extension, the miRNA expression profile in blastocysts, was explored considering four routinely examined sperm parameters: morphology, count, concentration, and progressive motility. The classification of these factors into normal and abnormal was sorted

according to the WHO guideline (as described in 2.5.1.4) and was assessed by the embryologists at CRGH.

6.2.7.1 Sperm Concentration

Blastocysts from patients with low sperm concentration exhibited upregulation in hsa-miR-183-5p and hsa-miR-342-3p, as shown in (Table 6-6). These miRNAs were also overexpressed in blastocysts from patients of advanced paternal reproductive age, hinting at a potential link between age and sperm concentration. This finding aligns with previous research, which demonstrated that advanced paternal age is associated with reduced sperm concentration and increased DNA fragmentation (Halvaei et al., 2020). Intriguingly, upon further analysis of sperm concentration while controlling for paternal age, the statistical significance of these miRNAs disappeared.

Table 6-6: Differentially expressed miRNAs in blastocysts derived from sperm with normal concentration versus blastocysts derived from abnormal concentration

Name	Fold change	FDR p-value
hsa-miR-183-5p	2.07	0.01
hsa-miR-342-3p	2.94	0.01

6.2.7.2 Sperm Motility

In the analysis concerning progressive motility, which is defined as the forward movement of sperm in a straight line, blastocysts were categorized into normal (above 30%) and abnormal (below 30%) based on the percentage of progressive motility assessed by the embryologist. Notably, around 93% of the collected blastocysts derived from sperm with abnormal progressive motility percentage. Moreover, it is worth highlighting that semen analysis is typically conducted before sample processing in ICSI cases, and the motility may have improved after processing. Despite the initial significant difference in miRNA expression observed between blastocysts from normal and abnormal sperm motility, there was a complete absence of this variation when accounting for aneuploidy. These findings suggest that aneuploidy may act as a confounding factor, implying that miRNA expression was more influenced by the aneuploidy status than the sperm motility.

6.2.7.3 Sperm Count

The sperm count per ejaculation appears to have no significant impact on the miRNA profile in blastocysts. The miRNA expression analysis conducted on blastocysts from patients with less and more than 39ml per ejaculate revealed no significant alterations in the miRNA levels.

6.2.7.4 Sperm Morphology

The final investigation involved comparisons between blastocysts obtained from patients with and without sperm morphological defects. Samples were classified based on the morphological score provided by the embryologist, where more than 4% abnormal morphology was classified as defective. A key finding is that this analysis yielded the most significant results among all the other sperm parameters, revealing dysregulation in the expression of five miRNAs (demonstrated in Table 6-7).

Of particular interest was the upregulation of two members of the let-7 miRNA family, hsa-let-7b-5p and hsa-let-7a-5, in blastocysts originated from sperm with abnormal morphology. Earlier findings in this study indicated that these miRNAs have also been linked to aneuploidy and poor TE morphology, which together could suggest a potential interplay between sperm morphology, blastocysts morphology and chromosomal integrity.

Unexpectedly, hsa-miR-206 and hsa-miR-184 showed reduced levels in the group with abnormal sperm morphology. This finding contrasts with their previous results of consistent elevation in low-quality blastocysts, particularly those with aneuploidy and late blastulation. However, the identified change in expression pattern, either upregulation or downregulation, indicate the involvement of these miRNAs in the overall embryo quality.

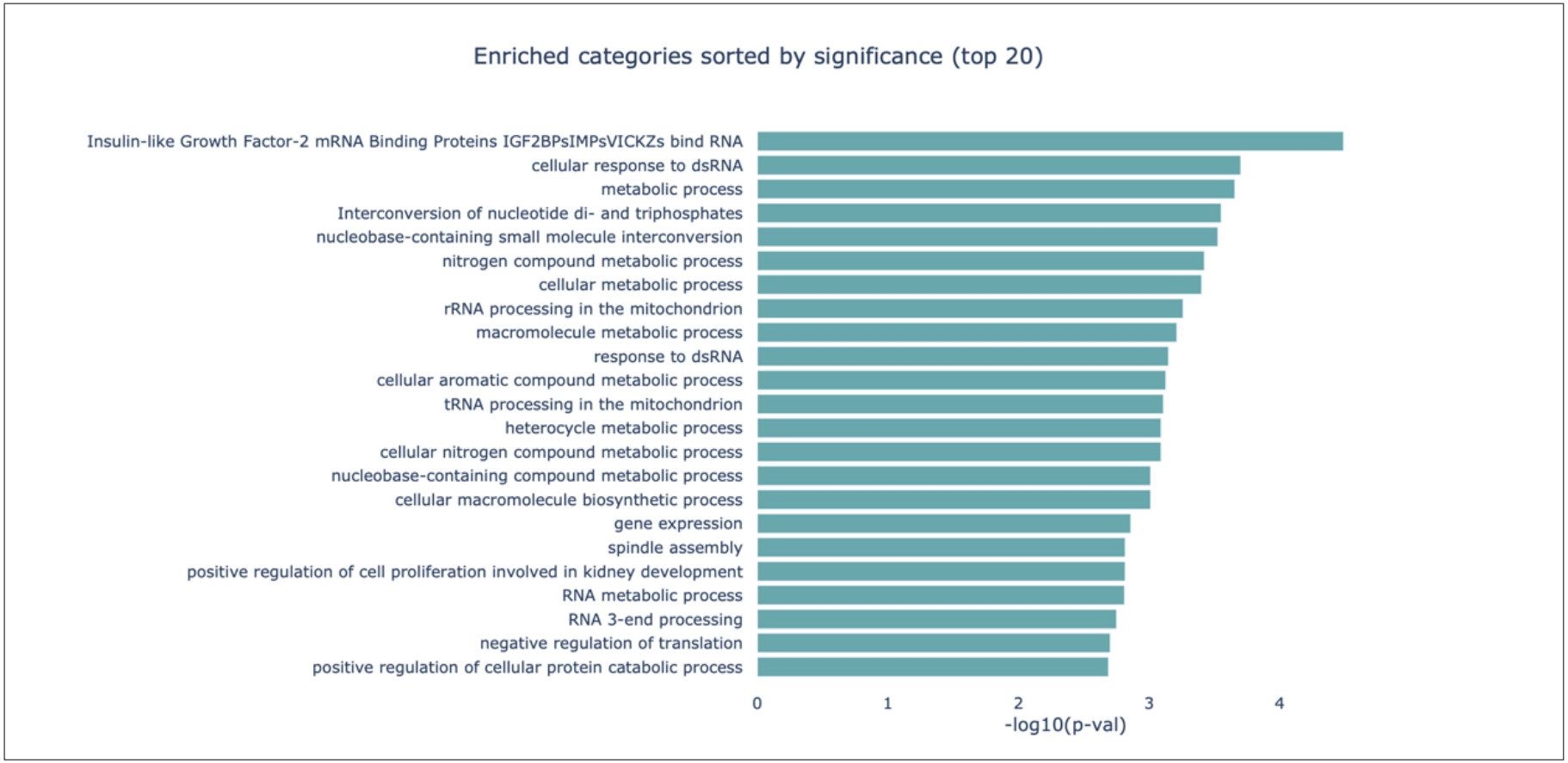
Table 6-7: Differentially expressed miRNAs in blastocysts derived from sperm with normal morphology versus blastocysts derived from abnormal morphology

Name	Fold change	FDR p-value
hsa-let-7b-5p	2.96	0.008
hsa-miR-320b	4.25	0.01
hsa-miR-206	-9.41	0.01
hsa-miR-184	-3.80	0.02
hsa-let-7a-5p	2.44	0.06

Given that the majority of differentially expressed miRNAs were upregulated in blastocysts derived from sperm with lower quality, we focused the functional analysis on these upregulated miRNAs: hsa-miR-183-5p, hsa-miR-342-3p, hsa-let-7b-5p, hsa-miR-320b, and hsa-let-7a-5p. The analysis revealed their strong involvement in various cellular metabolic processes (Figure 6-7). In fact, this association between impaired sperm quality and disrupted metabolism in embryos have been previously noticed (Wu et al., 2023, Pasquariello et al., 2024).

The analysis also pinpointed the predominant function of these miRNAs in regulating RNA binding, processing, and responding to double-stranded RNA (dsRNA). Although miRNA involvement in RNA regulation is a well-established function, these results suggest that miRNAs may play a more complex role in controlling these genes beyond simple complement sequence binding. In this context, an earlier study highlighted the potential role of miRNAs in RNA localization, during early embryonic patterning (Medioni et al., 2012), further supporting the idea that miRNAs may regulate RNAs at multiple levels, such as guiding RNA molecules to specific cellular locations.

Figure 6-7: Pathway annotation analysis of the differentially expressed miRNAs in blastocysts obtained from abnormal versus normal sperm parameters



The figure illustrates the potentially affected pathways influenced by the dysregulated miRNAs in blastocysts obtained from abnormal sperm characteristics, including cellular metabolic processes, RNA binding, regulation of gene expression and spindle assembly.

While some miRNAs consistently displayed dysregulation across the examined factors, others have exhibited specific alterations when investigating gamete parameters or factors that influencing sperm and oocyte quality. For instance, hsa-miR-342-3p and hsa-miR-26a-5p were found to be differentially expressed specifically in relation to parental factors. These observations suggest that the quality of gametes influences the genetic status of the embryo. Although these miRNAs may not be directly associated with chromosomal integrity or the overall competence of early-stage developing embryos, they may play a role in shaping the embryo's molecular landscape and developmental pathways.

6.2.8 Potential Association Between Sperm Quality and miRNA Expression in Blastocysts

Our analysis in relation to sperm characteristics indicated a potential influence of poor sperm concentrations and morphology to the miRNA expression in blastocysts. Interestingly, the differentially expressed miRNAs in blastocysts derived from low sperm concentration, hsa-miR-183-5p and hsa-miR-342-3p, were similar to the those associated with advanced paternal age, indicating that some miRNAs were specifically influenced by the paternal factors. Previous research has shown upregulation of these miRNAs in placenta of patients with preeclampsia, aligning with the established link between paternal factors and placental development (Choi et al., 2013, Suo et al., 2020). Moreover, a previous study in pigs reported a significant upregulation of miR-183-5p levels in correlation with poor sperm characteristics, particularly motility (Shabtaie et al., 2016). Collectively, these findings suggest a broader link between these miRNAs and parental factors.

Earlier research claimed that altered RNA expression in gamete cells could have downstream effects on the resulting embryos (Medioni et al., 2012, Gross et al., 2019). Our findings align with this perspective, demonstrating that blastocysts derived from impaired sperm, particularly those characterized with structural deficiencies, potentially have disrupted metabolic pathways and distinct gene expression profiles. This observation stresses the notion that the impact of sperm quality extends beyond mere microscopic appearance, influencing the genetic

contributions that ultimately shape embryonic development. Specifically, our data showed elevated levels of hsa-let-7a-5p in blastocysts from sperm with abnormal morphology, a miRNA linked to unsuccessful pregnancies when overexpressed in culture media (Abu-Halima et al., 2020). This suggests that altered miRNA expression related to paternal factors may be tied to pregnancy complications and failure.

Our investigations into the potential impact of sperm motility on the miRNA profile of blastocysts initially revealed differences in miRNA expression levels between embryos from motile and non-motile sperm samples. However, this variation disappeared when accounting for aneuploidy, hinting to a potential link between chromosomal abnormalities and sperm motility. Supporting this view, previous studies have pointed at the inverse relationship between progressive sperm motility and aneuploidy (Vegetti et al., 2000, Yang et al., 2022).

These observations may shed light on the potential contribution of the sperm centrioles to the sperm quality and function. The centrioles play a key role constructing the sperm's tail and contribute to assembling sperm aster through the centrosome in human (Avidor-Reiss et al., 2019). They also play a crucial role in facilitating egg fertilization by promoting the fusion of the female and male pronuclei (Simerly et al., 1995, Schatten and Sun, 2010). A previous study with oocytes failing to fertilize due to poor-quality sperm showed the absence of sperm aster formation, which likely indicates centrosome dysfunction (Terada et al., 2004). Additionally, centrioles are involved in cell division by aiding in the positioning of spindles during mitosis (Bobinnec et al., 1998). Altogether, these insights suggest that distributions with sperm centrioles may impair motility and affect the early stages of embryo cell division, which could lead to aneuploidy (Garanina et al., 2019). Although this notion is complex, it holds logical merit and certainly warrants further investigation. Interestingly, the functional analysis of the differentially expressed miRNAs in relation to sperm parameters showed their involvement in regulating spindle assembly, which supports the potential link between sperm parameter-related miRNAs and their downstream impact on embryo competence.

Additionally, several studies have emphasized the role of sperm-borne miRNA, particularly miR-34c, in embryonic development. This miRNA is involved in many

developmental processes, including the regulation of maternal transcriptomes in early development and controlling metabolic and proliferation processes (Cui et al., 2023). The overexpression of this miRNA in teratozoospermic and asthenozoospermic cases has been linked to successful implantation and higher pregnancy rates (Yeh et al., 2022). However, our investigation of this miRNA in blastocysts did not reveal significant results, suggesting that while miR-34c may be important for sperm development, its influence on embryo quality may be limited.

6.3 Chapter Summary

This chapter delves into the potential influence of parental factors, including ovarian stimulation dosage, oocyte maturation protocol, maternal and paternal age, and sperm quality parameters, on the miRNA expression profile in blastocysts. Our findings demonstrated significant changes in miRNA profiles in relation to high ovarian stimulation dose and various oocyte maturation triggers, highlighting the profound impact of treatment on oocyte quality and subsequently embryo development.

Moreover, the data revealed that advanced parental age, both maternal and paternal, may influence on the miRNA profile within blastocysts. While impact of parental age on embryonic quality is well-documented, our study highlights its impact on miRNA expression in blastocysts, with a few miRNAs showing significant association.

Notably, among the sperm parameters investigated, morphology yielded the most significant findings of differentially expressed miRNAs in blastocysts. These results underscore the often-overlooked impact of sperm quality on embryonic gene expression and therefore its overall competence.

In summary, these observations contribute to our understanding of how parental characteristics and gametes features may impact embryonic development and competency. They offer valuable insights into the persistent effect of gamete quality on the resulting embryo, which can inform and improve treatment practice.

Chapter 7 Discussion

7.1 miRNA Expression in Blastocysts

This study provides a thorough examination of miRNA expression in blastocysts, offering valuable insights into the regulatory functions of miRNAs during this critical phase of development. By identifying the biological pathways and molecular mechanisms underlying blastocyst formation and development, this research enhances our understanding on its subsequent events, such as implantation. Additionally, our analyses of differentially expressed miRNAs highlights how their expression is influenced by blastocyst quality, developmental timing, and parental factors.

Our findings, along with previous research, revealed abundant miRNA expression in blastocysts, highlighting the representational role of miRNAs in early embryonic development. This enabled us to further explore the miRNA profile to identify specific markers that can distinguish blastocysts with high developmental potential from those of lower quality.

7.1.1 Influence of Chromosomal Abnormalities

Chromosomal abnormalities, a leading cause of pregnancy loss, were a focal point in our study as we explored their association with miRNA expression profiles in blastocysts. Previous studies have consistently reported changes in miRNA levels linked to chromosomal abnormalities in human blastocysts (Almutlaq et al., 2024). In line with these findings, our analysis revealed substantial differences in the miRNA profiles between euploid and aneuploidy blastocysts. Notably, prior research did not distinguish between different types of chromosomal abnormalities, a critical oversight given that various defects affect cellular processes and blastocyst fate differently. Our investigation accounted for this diversity by conducting several analyses across different types of aneuploidies, allowing us to identify distinct miRNA profiles associated with each abnormality. This approach revealed that

monosomies had the most pronounced effect on miRNA expression, compared to other chromosomal defects.

While the specific results varied across different types of aneuploidies, certain miRNAs were consistently identified in all groups with complete chromosomal abnormalities. Interestingly, blastocysts with segmental aneuploidies exhibited a unique miRNA expression pattern compared to euploid blastocysts, suggesting a distinct cellular response. Although these findings are novel, they align with existing knowledge, as monosomy is known for its detrimental effect on embryos, which is reflected in significant alterations in miRNA profiles (Shahbazi et al., 2020). Additionally, the cellular response to segmental defects, which likely arise pre- or post- mitotically, may differ from that whole aneuploidies caused by mitotic errors (Hintzen et al., 2022, Garribba and Santaguida, 2022).

7.1.2 Developmental Timing and Morphological Differences

Given that abnormal blastocysts can appear morphologically normal and show good developmental progression, we hypothesised that the delay in blastulation and morphological differences might be linked to distinct gene expression profiles. Our miRNA analysis showed that certain developmental pathways are altered in blastocysts developed at day 6 compared to these that reached blastocysts stage at day 5. While previous investigations have not specifically examined miRNA expression between these two types of blastocysts, several studies have documented dynamic changes in gene expression at different embryonic developmental stages (Ciaudo et al., 2009, Assou et al., 2011).

Additionally, our analyses of blastocyst morphology revealed that differences in appearance are linked to variations in genetic status, particularly in TE evaluation, as blastocysts graded C for TE showed significant changes in miRNA profiles compared to those graded B. While the previous research on the correlation between blastocysts gene expression and morphology was limited, the association has been noted (Wells et al., 2005). Our analysis provides a novel perspective by linking these morphological differences in TE cells to alterations in protein metabolic processes, which is consistent with the concept that changes in protein structure and function significantly influences the cellular appearance (Sivakumar and Kurpios, 2018).

7.1.3 Influence of Parental Factors

We extended our analysis to investigate the impact of parental factors on the blastocyst quality. It is well-established that abnormalities in blastocysts often reflect poor quality of parental cells. Unexpectedly, parental age showed the least significant correlation with the miRNA profile in blastocysts. While advanced maternal age is strongly associated with chromosomal abnormalities in oocytes, its influence appears to be more pronounced on the embryonic chromosomal status rather than directly affecting gene expression. However, the type and dosage of exogenous hormones used during IVF treatment exhibited a noteworthy corelation with miRNA expression in blastocyst. While the impact of hormonal treatments on oocyte quality is well-documented, the persistence of these effects in embryos is interesting and warrants deep investigation (Santos et al., 2010, Ezoe et al., 2014, Bosch et al., 2016).

Among the semen parameters examined, sperm morphology had the most significant influence on miRNA expression in the blastocysts. Previous Studies have demonstrated a direct association between sperm morphology and resulting embryo morphology (Parinaud et al., 1993). It also impacts the fertilization and pregnancy outcomes, as injection of morphologically abnormal spermatozoa resulted in lower fertilisation and pregnancy rates (De Vos et al., 2003).

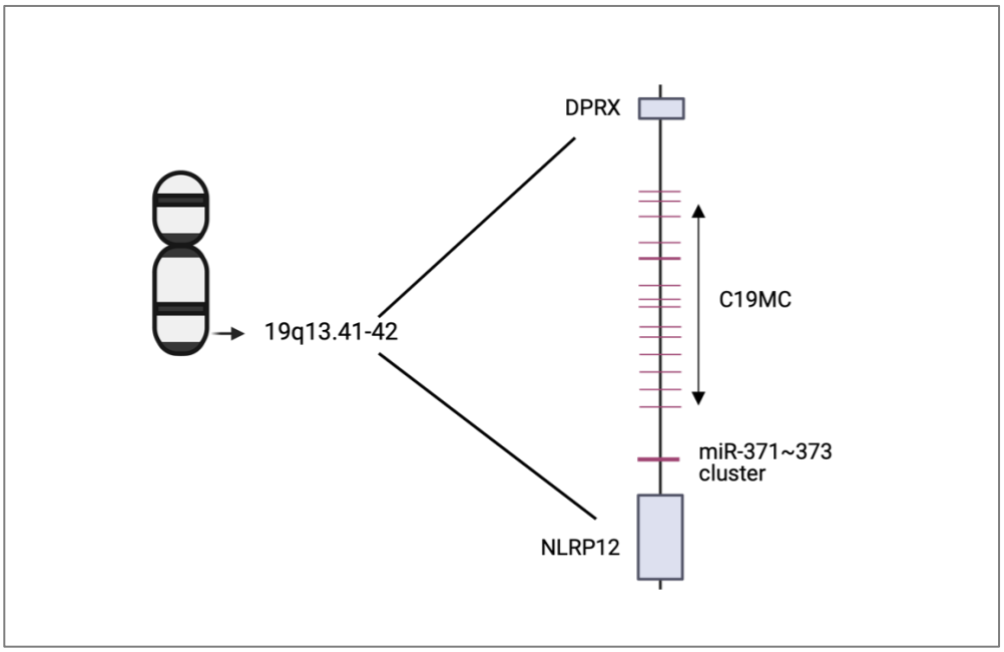
Through these comprehensive analyses, we uncovered several key insights into miRNA expression in blastocysts, many of which were particularly striking. The following sections will explore these findings in greater detail.

7.2 Chromosome 19 miRNA Cluster Contributes to Embryonic Quality and Development

Chromosome 19 holds particular significance in pregnancy for encoding genes essential for placental development and serve as key regulators of implantation. A key component of this chromosome is the chromosome 19 miRNA cluster (C19MC), one of the largest human miRNA clusters containing 46 miRNA genes and spanning approximately 100 kb on chromosome 19q13.41 region (Figure 7-1) (Bentwich et al.,

2005, Morales-Prieto et al., 2013). The expression of C19MC is restricted to placenta and embryonic stem cells (Liang et al., 2007, Stadler et al., 2010). Its encoded miRNAs, highly abundant in human trophoblastic cells, are believed to play a role in maintaining stem-like properties of trophoblasts (Donker et al., 2012, Maraghechi et al., 2023).

Figure 7-1: Key miRNA clusters encoded on Chromosome 19



This figure illustrates key miRNA clusters located on Chromosome 19, showing their specific genomic positions and the relative distances between clusters. Each cluster consists of multiple miRNAs known to play crucial roles in embryonic development.

In the present study, significant reduction in C19CM miRNAs was observed in aneuploid blastocysts, particularly hsa-miR-512-3p, hsa-miR-512-5p hsa-miR-519b-3p hsa-miR-520a-5p and hsa-miR-576-5p. Although miRNAs of this cluster exhibited frequent downregulation in different types of aneuploidies, a higher number of them were affected in the group with chromosomal losses. The dysregulation of C19CM members in aneuploid embryos was noted before, strengthening the association between aneuploidy and this cluster (Rosenbluth et al., 2013, McCallie et al., 2014). Playing a critical role in immune modulation and trophoblast invasion, the alterations of these miRNAs in aneuploid embryos may explain their low probability of implantation (Bullerdiek and Flor, 2012, Xie et al., 2014).

In fact, the association between chromosomal abnormalities and the expression of chromosome 19 genes might extend beyond this cluster. An interesting previous study on the transcriptome profile in monosomic and trisomic human blastocysts depicted an overall downregulation in the majority of chromosome 19 genes in both types of aneuploidies, suggesting a potential association between chromosomal numerical abnormalities and the copy number of genes encoded on this chromosome (Licciardi et al., 2018). This pattern of expression was evident through the presented figures, although it was not explicitly explained in the text.

One essential characteristic of C19MC is its maternally imprinted status, meaning that only paternal alleles are expressed while the maternal alleles are silenced (Noguer-Dance et al., 2010). This imprinting pattern indicates the potential involvement of paternal genome in implantation, supported by the well-known role of paternal epigenome in regulating placental gene expression and development (Wang et al., 2013, Denomme et al., 2020). The observed downregulation of C19MC miRNAs in blastocysts with chromosomal abnormalities suggests alterations in the paternally inherited genes in association with aneuploidy, consequently affecting the gene expression profile in these embryos.

Another interesting finding from our analysis was the high abundance of the miR-371~373 cluster, a miRNA cluster also encoded on chromosome 19, among the top-expressed miRNAs in blastocysts. This cluster is located adjacent to C19MC, and its miRNAs are involved in the differentiation of human embryonic stem cells during the early stages of development (Suh et al., 2004). Interestingly, our analysis revealed significant downregulation of these miRNAs in association to aneuploid and low-quality blastocysts. Consistent with these results, reduced levels of miR-371-373 miRNAs were observed in aneuploid blastocysts from previous research (Rosenbluth et al., 2013, McCallie et al., 2014). In view of that, downregulation of these miRNAs in association to aneuploidy and poor morphology may indicate lower opportunities for proper placentation of blastocysts with these features, given their role in placental and embryonic stem cells gene expression regulation.

7.3 Contribution of Other miRNA Pregnancy-Related Clusters to Human Blastocysts Competence

Other significant miRNA clusters were recognized for their contribution to embryo quality. One such cluster is the miR-17-92, also referred to as C13orf25 or OncomiR-1, located on chromosome 13. Mammals have two paralogous clusters of the miR-17-92, namely miR-106a-363 and miR-106b-25, located on chromosome X and chromosome 7, respectively. Previous studies have shown abundant expression of these clusters in embryonic stem cells with significant overexpression compared to differentiated cells (Laurent et al., 2008, Mens and Ghanbari, 2018). Similarly, our findings revealed high expression of recognized members from these clusters in blastocysts, including hsa-miR-18a-5p, hsa-miR-92a-1-5p, hsa-miR-92a-3p, hsa-miR-363-5p, hsa-miR-106b-5p, and hsa-miR-93-5p.

While these clusters are known for their oncogenic role in tumorigenesis, they also play essential roles in normal embryonic stem cells, where they regulate critical embryonic events such as trophoblast differentiation, gastrulation, and embryo **implantation (Foshay and Gallicano, 2009, Kumar et al., 2013, Jevnaker et al., 2011). The knockdown of the miR-17/92 cluster in mice led to impaired spermatogenesis, and in humans, a noteworthy decrease in miR-19b levels was observed in the semen of infertile men, suggesting a potential link between these genes and male fertility (Meng et al., 2015, Xie et al., 2016, Abu-Halima et al., 2022).**

However, our investigations into sperm parameters did not reveal a specific correlation with miRNAs from this cluster. This suggests that the cluster's impact might be more pronounced during the earlier stages of sperm development.

7.4 Potentially Disrupted Pathways in Developing Blastocysts with Suboptimal Quality

The functional analyses of the differentially expressed miRNAs conducted throughout this study revealed interesting association between the investigated quality factors and the potentially impacted pathways. These pathways and their correlation to the embryo development were discussed in here, with an aim to unravel the underlying mechanisms that could influence the embryo overall competence.

First and foremost, it is important to state that the regulatory function of miRNAs operates in a manner that adapts to the encountered physiological or pathological conditions of the cell. For instance, in cancerous cells, miRNAs typically downregulate tumor suppressor genes while promoting oncogenes, leading to unchecked cell growth (Fasoulakis et al., 2020). Similarly, miRNA expression in developing embryos varies depending on the specific physiological context (Paloviita et al., 2021).

Additionally, it is important to acknowledge that our analyses may not capture all the mRNA targeted by the dysregulated miRNAs. Nevertheless, genes and pathways that have been previously investigated were considered, showcasing only miRNAs with well-established significant roles in regulating the associated cellular processes. Many of these pathways are linked to different types of cancers, being the most investigated condition for miRNA change in expression (Lee and Dutta, 2009).

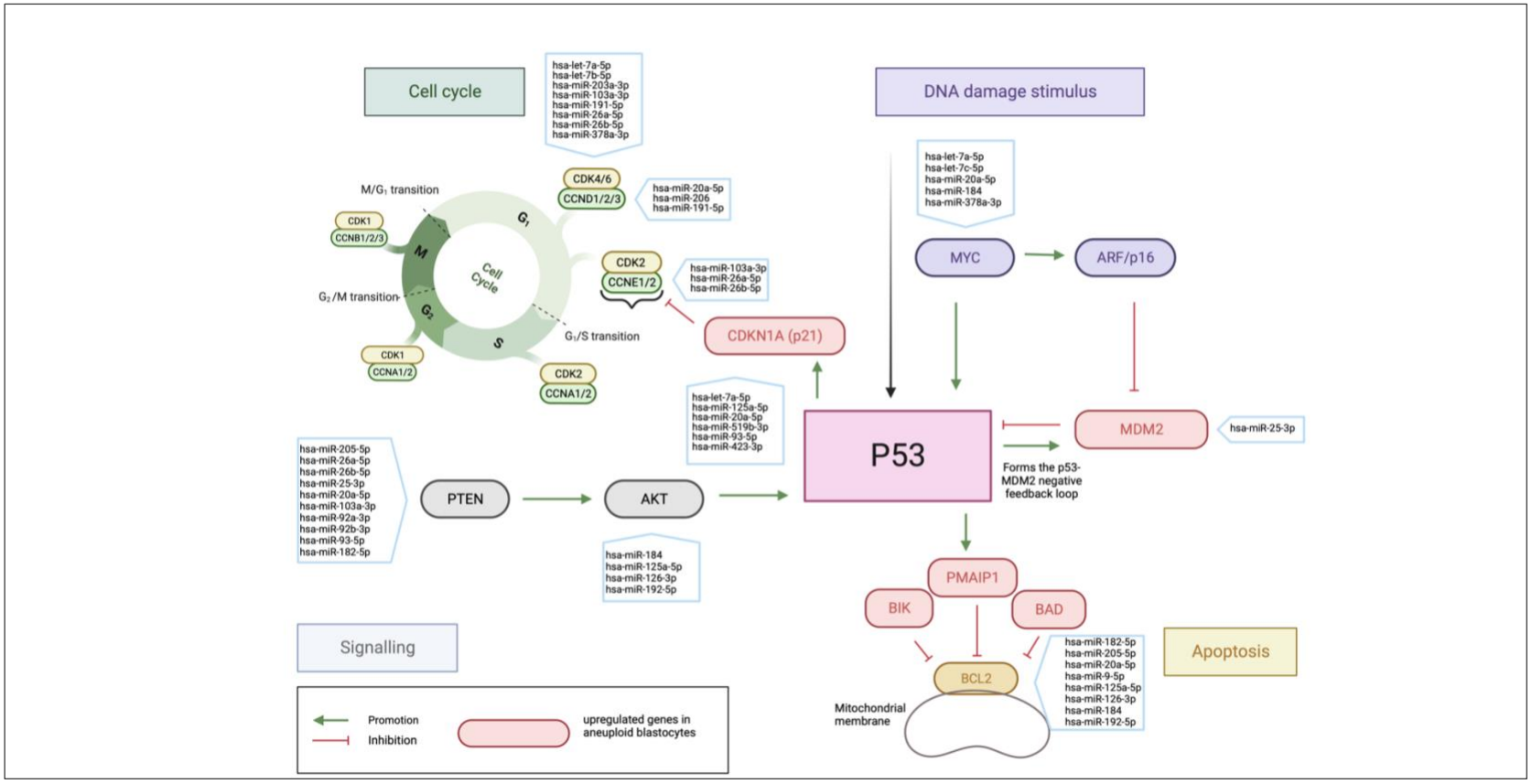
7.4.1 Cell Cycle Dynamics and DNA Damage Response

MiRNA emerged as key players in controlling the cell cycle transitions, particularly enabling rapid cell proliferation in stem cells (Wang et al., 2008). One prominent target of miRNAs in the cell cycle is *CDKN1A*, also known as *P21*, *WAF1/CIP1*. *CDKN1A* operates downstream of p53 and serves a multifunctional role, prominently controlling the G1/S transition by inhibiting CDK/cyclin interaction (Harper et al., 1993, Wang et al., 2008). A noteworthy characteristic of stem cells is their self-renewal capacity, facilitated by a shortened G1 phase compared to somatic cells (Divisato et al., 2020). The functional importance of *CDKN1A* in maintaining

cellular proliferation of ESCs is evident (Orford and Scadden, 2008, Mens and Ghanbari, 2018). However, overexpression of its protein has been observed in many cancer cells, suggesting unfavourable outcomes associated with its excess levels (Marchetti et al., 1996).

In the context of aneuploidy, our investigations showed significant upregulation of *CDKN1A* in aneuploid blastocysts, with many of its complementary miRNAs being downregulated in the same cohort of samples. This overexpression suggests heightened p53 activity in response to aneuploidy, potentially treating it as a cellular stressor. The significant upregulation of *CDKN1A* suggests an interference with cell cycle progression by inhibiting the formation of the CDK2/cyclin E1-E2 complex, thereby preventing the G1/S cell cycle transition and likely resulting in cell arrest (Figure 7-2). In this scenario, genes necessary for the synthesis phase, including RB1 and E2F1, would remain inactivated.

Figure 7-2: Dysregulated mRNAs and miRNAs in aneuploid blastocysts and their contribution to key biological pathways



The figure illustrates the differentially expressed mRNAs and miRNAs in aneuploid blastocysts, and their involvement in regulating crucial pathways that operate downstream p53, including cell cycle progression, DNA damage, PTEN/Akt signalling and apoptosis. Upregulated mRNAs in aneuploid blastocysts are labelled in red, and miRNAs within the blue arrows are dysregulated in aneuploid blastocysts. The figure showcases both the direct and indirect contributions of miRNAs in modulating these essential cellular pathways influenced by p53.

A specially intriguing finding in this study was that *CDKN1A* was not upregulated in blastocysts with chromosomal gains, although it was significantly overexpressed in the other blastocysts aneuploid groups. Such an observation suggests that chromosomal gains may not initiate a damage response in the cell whereas as chromosomal losses do. The identified potential differences in the cell responding to aneuploidy hint at better survival of cells with whole chromosome or segmental trisomies compared to those with whole chromosome or segmental monosomies.

The data derived also suggests an increase expression of *MDM2* in aneuploid blastocysts, accompanied by low expression of its complimentary miRNAs in the same type of samples. Since *MDM2* is a key component in the ATM-dependent DNA damage response, its upregulation in aneuploid blastocysts may indicate that the cell is encountering DNA damage (Maya et al., 2001). Under normal conditions, *MDM2* functions to maintain low levels of p53 (Moll and Petrenko, 2003). However, when p53 undergoes phosphorylation under stress conditions, such as DNA damage, its ubiquitination by *MDM2* is inhibited (Levine and Oren, 2009). Notably, the simultaneous upregulation of both *MDM2* and *CDKN1A* in aneuploid blastocysts indicate that the cell is encountering stress or damage, possibly due to the chromosomal abnormalities (el-Deiry et al., 1993, el-Deiry et al., 1994, Levine and Oren, 2009).

The elevation of Phorbol-12-Myristate-13-Acetate-Induced Protein 1 (*PMAIP1* or *NOXA*), another gene regulated by p53, in aneuploid blastocysts also suggest a potential response to DNA damage, as this gene is known to promote apoptosis (see Figure 7-2) (Roufayel et al., 2022). Overall, the dysregulation of these mRNAs highlight a collective contribution to the p53-mediated DNA damage response. However, it remains to be determined whether or not this damage is a consequence of aneuploidy, as a condition that commonly introduces genomic instability.

Although the differentially expressed mRNAs did not yield significant results of c-*myc* expression, its regulatory miRNAs displayed substantial results in aneuploid blastocysts. For instance, hsa-miR-184, hsa-let-7c-5p and hsa-miR-378a-3p showed consistent dysregulation in the majority of aneuploid groups of blastocysts. These

findings suggest a complex regulatory network influencing cell survival through c-*myc*, which necessitate further investigation.

Although the potential disruption of cell cycle pathways was particularly significant in the context of aneuploidy, other factors investigated also demonstrated possible interference with cell cycle processes. Notably, consistent alterations in miR-17-92 and let-7 family miRNAs, which are critical regulators of cell cycle genes, were associated with low-quality embryos. Overall, the role of miRNAs in regulating various cell cycle events in mammalian embryos has been well established and thoroughly reviewed in previous literature (Tulay and Sengupta, 2016).

7.4.2 Metabolic Processes

Metabolites and metabolic processes in preimplantation embryos are critical factors influencing embryonic development, uterine receptivity, and ultimately the success of reproductive treatment. Previous research has shed light on the impact of carbohydrate, protein and lipid metabolism on both oocyte and developing embryos. One study revealed changes in the concentration of amino acids and fatty acids in the follicular fluid of sows with low reproductive performance when compared to normal ones (Chen et al., 2019). Another investigation reported differential expression of several metabolites in the endometrial fluid obtained from implanted versus non-implanted embryos (Matorras et al., 2020). Research on both human and mouse embryos has revealed abnormalities in protein metabolic processes in those that failed to implant (Liu et al., 2022). Additionally, a previous study highlighted the overexpression of lipid species in blastocysts, indicating active lipid metabolism at this developmental stage (Sudano et al., 2016).

A significant discovery from our study was the elevated expression of blastocysts' miRNAs that play crucial roles in mediating a wide array of metabolic processes involving diverse biochemical molecules. These processes encompass protein modification and metabolism, including ubiquitination and the regulation of protein catabolism, as well as lipid metabolism. Additionally, although less prevalent, carbohydrate metabolism was also implicated. Regulating metabolite utilization during embryonic development reflects varying energy demands at each stage.

The miRNAs associated with protein metabolism showed high expression levels in the overall miRNA profiling conducted in this study. This observation aligns with findings from a previous investigation into the secretome of human and mouse preimplantation embryos, which demonstrated a significant increase in ubiquitin levels towards the blastocyst stage, suggesting active regulation of protein metabolism (Katz-Jaffe et al., 2006). Additionally, a study examining the proteome profile of early mouse embryos revealed a significant shift in protein expression profiles upon reaching the blastocyst stage compared to earlier developmental stages. The highly expressed proteins at the blastocyst stage were primarily involved in metabolic processes and protein modifications (Gao et al., 2017).

Interestingly, both protein and lipid metabolic processes frequently emerged in the pathway annotation analyses of differentially expressed miRNAs identified in low-quality blastocysts. Specifically, in the context of aneuploidy, protein metabolic processes were among the most affected pathways. Consistent with this finding, previous research have established the link between metabolism and the cytogenetic composition of human embryos, showing significant differences in the turnover of certain amino acids between embryos with normal chromosomal complement and those with aneuploidy (Picton et al., 2010). Additionally, defects in protein metabolism, such as inhibition of protein folding, found to be correlated with chromosomal instability (Hintzen et al., 2022).

The involvement of miRNAs in regulating metabolic processes was also notably significant in blastocysts with poor morphology or those obtained from low-quality sperm. In this context, it has been previously shown that the consumption of amino acids and carbohydrates is associated with morphokinetics of in vitro fertilized mouse embryos (Lee et al., 2015). Additionally, previous studies have demonstrated that sperm DNA damage correlates with abnormal metabolite profiles in the culture media of resulting embryos (Uppangala et al., 2016, Souza et al., 2019).

Furthermore, the miRNAs identified as differentially expressed in relation to ovarian dosage were implicated in carbohydrate and lipid metabolism. Previous observations have indicated distinct lipid profiles in blastocysts derived from natural and IVF cycles, indicating that ovarian stimulation may induce changes in blastocyst phospholipid profiles (Berteli et al., 2023). Collectively, these findings highlight the

role of miRNAs in regulating various metabolic processes and imply potential disruptions in metabolite utilization in low-quality blastocysts.

7.4.3 Response to Hypoxia and Other Stresses

Our functional analysis on the top-expressed miRNAs in human blastocysts indicated their involvement in regulating the embryo's hypoxic response. Among them, three miRNAs, namely hsa-miR-106b-5p, hsa-miR-155-5p and hsa-miR-18a-5p, are known regulators of the hypoxia inducible factor 1 subunit alpha (*HIF1A*), a gene recognized for its role in responding to low oxygen tension (Ziello et al., 2007). The blastocysts showing a potential response to hypoxia was not a surprising result, considering that the investigated embryos were cultured under low oxygen concentration (5%). This percentage was determined to mimic the low oxygen tension environment in the uterus. Moreover, culturing stem cells in hypoxic media is a common practice. In fact, adult stem cells, such as hematopoietic stem cells, thrive in approximately 5% oxygen in the bone marrow (Chason et al., 2011). Similarly, culturing human mesenchymal stem cells in low oxygen favors natural processes and may reduce DNA damage and aneuploidy (Estrada et al., 2012). In general, low oxygen levels appear favorable for normal human stem cell growth, including embryonic stem cells (Ezashi et al., 2005, Simon and Keith, 2008).

The developing embryo also requires low oxygen concentrations for natural developmental purposes such as angiogenesis (development of blood vessels) and placental formation (Kapiteijn et al., 2006). However, cells respond to reduced oxygen availability (hypoxia) as part of their survival mechanism. High atmospheric oxygen tension during culture has been associated with the production of reactive oxygen species (ROS) and impaired developmental competence (Jagannathan et al., 2016).

According to the computational pathway analysis conducted during the course of this study, blastocysts responding to low oxygen levels was only significant in association to the maternal age. This observation suggests a potential influence of maternal age on embryonic responses to environmental factors. Although the exact mechanism of hypoxic response in blastocysts is not extensively studied, previous research highlight the implication of known hypoxia inducible factor (*HIF*) and

related proteins in the transcription activation of many genes involved in development of embryo and its adaptation to hypoxia (Dunwoodie, 2009).

Another integrating frequently observed finding is the miRNA controlling cellular responses to various stresses. Analyses of low-quality blastocysts showed possible impact of various types of stresses, such as endoplasmic reticulum stress, or oxidative stress. The potential occurrence of endoplasmic reticulum stress in aneuploid samples, coupled with defects in protein metabolic processes, may indicate abnormalities in protein folding in aneuploid embryos, leading to the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (Xian et al., 2021). Indeed, further investigation is required to determine whether these stresses, particularly hypoxia, originate from environmental factors during culture or result from biological abnormalities within the developing embryo.

7.4.4 Cellular Fate: Apoptosis and Cell Death

Cell death has long been observed and described in preimplantation embryos (Saunders, 1966, Kerr et al., 1972). Apoptosis, a programmed process of cell death regulated by a balance of pro and anti-apoptotic genes, is generally believed to be a normal feature in early development (Saunders, 1966, Hardy, 1997, Levy et al., 2001). Some proposed that apoptosis contributes to amniotic cavitation by destroying inner ectodermal cells and is necessary for trophoblast differentiation in placental formation (Straszewski-Chavez et al., 2005, Huppertz et al., 2006, Agnello, 2015). Conversely, others suggest that an elevated ratio of apoptotic cells may indicate abnormal development (Levy et al., 2001). Consistent with the aforementioned established roles of apoptosis in early development, as well as the widely recognized significant role of miRNA in regulating apoptotic genes, our findings revealed high expression of apoptosis-related miRNAs in blastocysts.

An intriguing finding from our analysis is the significant role of miRNAs in controlling cell death processes within aneuploid blastocysts. Consistent with the miRNA results, the differentially expressed mRNAs in aneuploid blastocysts have shown upregulation of genes associated with controlling cell death, such as apoptotic promoters *PMAIP1*, and *CDKN2A* Interacting Protein N-Terminal Like (CDKN2AIPNL - a paralogous of CDKN2AIP), which mediate cellular senescence

in response to DNA damage. Moreover, the overexpression of *CDKN1A* in aneuploid blastocysts, when interacting with p53, may also indicate the regulation or alteration of apoptosis, as this complex is known to target Bcl-2 proteins (Kim et al., 2017). The *BCL2* Interacting Killer (*BIK*), implicated in inducing apoptosis, was also significantly elevated in aneuploid blastocysts, suggesting active engagement of apoptotic machinery in aneuploid blastocysts (Chinnadurai et al., 2008).

Additionally, aneuploid cells, present in more than 80% of preimplantation cleavage stage and 50% of blastocyst embryos, believed to undergo apoptosis or cellular arrest as means to eliminate cells with chromosomal abnormalities in embryos, a process known as self-correction (Barbash-Hazan et al., 2009, Fragouli et al., 2013). This view is supported by a mosaic mouse model demonstrating the elimination of aneuploid cells through a p53-dependent apoptosis process (Singla et al., 2020). The exact mechanism underlying this phenomenon is uncertain, however, our transcriptome analysis of aneuploid blastocysts suggests that apoptosis is likely to be activated in almost all aneuploidies, possibly mediated by p53.

Two specific miRNAs, hsa-miR-206 and hsa-miR-184, were consistently upregulated in blastocysts from less favourable groups. These miRNAs play a pivotal role in diverse cellular processes by regulating key genes such as c-*myc* and *BCL2*. The interaction between miR-184 and these genes, and the subsequent impact on their respective proteins repeatedly reported with suppression of cell growth through c-*myc* targeting and promotion of the apoptotic activity through regulation of *BCL2* (Zhen et al., 2013, Wang et al., 2018).

It is generally expected to find numerous genes involved in regulating cell cycle expressed in early embryonic development as the embryo undergoes fast division and differentiation. However, identifying genes involved in different mechanisms of cell death at this early developmental stage may shed light on the philosophy that apoptotic genes contribute to other cellular functions. It has been previously found that caspases, known apoptotic proteins, are also required for cell differentiation (Julien and Wells, 2017). A remarkable similarity between caspase-mediated apoptosis and cellular differentiation has been previously noticed in terms of mitochondrial membrane perturbations and DNA fragmentation (Bell and Megeney, 2017). Our results of miRNA expression in blastocysts showed consistent

involvement of the differentially expressed miRNAs in mitochondrial apoptosis (the caspase-mediated intrinsic apoptosis), indicating DNA damage in these blastocysts. This interplay between miRNAs, apoptosis, and cell differentiation highlights the multifaceted role of miRNAs in shaping cellular fate during early embryonic development.

In terms of cell fate, aneuploid embryos typically face challenges in survival, but they do not exhibit behaviours resembling cancer or invasive characteristics within the uterine lining. Interestingly, many of the dysregulated miRNAs in aneuploid embryos demonstrate an opposite direction of expression compared to their counterparts in various cancers. For example, the continuously elevated levels of let-7 family members in aneuploid embryos is opposite to the downregulation of these miRNAs observed in most cancer types (Wang et al., 2012). While aneuploid cancer cells often lead to genomic instability and accumulate errors, aneuploid blastocysts seem to show a contrasting response and compete aneuploidy by activating apoptotic processes (Singla et al., 2020). This observation implies that the pattern of miRNA expression may play a crucial role in determining the cellular fate during early embryonic development.

7.4.5 Hormone and Immune Signalling

While many studies have explored the role of miRNA in implantation, the majority have focused on the maternal aspects, investigating miRNAs function in various endometrial cells and tissues. Conversely, there has been limited interest in the embryonic contribution to this process. The investigation of miRNAs expression in the current study showed their extensive involvement in multiple hormonal and immune signalling pathways, suggesting a heightened receptive capacity of the blastocyst. This insight indicates robust dialog between the endometrium and blastocyst, even before implantation occurs. Although prior research has acknowledged communications between the endometrium and embryos through several hormonal and growth signalling, investigations into the regulation and interaction of these pathways on the embryonic side is relatively limited (Massimiani et al., 2019).

Indeed, implantation failure itself may signify a reaction against an abnormal or compromised embryo, possibly implying an immune response orchestrated by the uterus. Previous studies highlighted the potential link between aberrant miRNA expression and implantation failure, showing differentially expressed miRNAs between receptive and pre-receptive endometrium as well as between implanted and non-implanted blastocysts (Paul et al., 2019). In this context, several miRNAs have been identified as modulators of endometrial receptivity, either by promoting or suppressing decidualization, thereby influencing implantation. One such miRNA is miR-183, which contributes to mediating the estrogenic effects on endometrial receptivity by targeting catenin alpha 2 (*CTNNA2*) (Akbar et al., 2020). The *CTNNA2* contributes in regulating the endometrial receptivity and enhancing implantation though its role in cell-to-cell adhesion. In our analysis, miR-183 exhibited lower expression levels in blastocysts obtained from oocytes treated with a high ovarian stimulation dosage. This dysregulation in response to different hormonal environments may offer insights into the influence of stimulation, not only on endometrial receptivity but also on the embryo's potential to implant.

Furthermore, interleukin signalling was notably significant in the analysis of aneuploid blastocysts, suggesting a shift in cell growth and differentiation, as it may also reflect an immune response. Since embryonic immune cells do not specialize until week 5 of gestation, detecting immune signalling at blastocyst stage is intriguing. Previous research in zebrafish and mouse embryos have shown early indications of innate immunity, with the surface epithelium layer (trophoblast) exhibiting phagocytic activity to clear apoptotic cells from the embryo (Hoijman et al., 2021). Another study has specifically highlighted the importance of interleukin signalling in the trophoblast invasion, impacting both embryonic and endometrial interactions (Guzeloglu-Kayisli et al., 2009).

Our analysis also revealed significant involvement of estrogen signalling and regulation of estrogen receptors, particularly in blastocysts with aneuploidy, delayed growth (day 6), and those obtained from high ovarian stimulation dose cycles. These findings suggest that miRNAs of embryonic origin may regulate estrogen signalling and its interactions with the endometrium, which might be altered under varying conditions of embryo quality. A previous study indicated an accumulatio of estrogen receptor1 (ESR1) in a the trophoblast of hatching blastocyst (Logsdon et al., 2023).

The expression of ESR1 in trophoblast cells suggests a potential response to estrogen signalling, which is crucial for the blastocyst's ability to interact with the endometrium, a key step for successful implantation. Supporting this understanding, our findings demonstrate changes in regulating the estrogen signalling receptors in blastocysts with compromised quality. The dysregulated miRNAs identified in our study may disrupt normal ESR1 signalling pathways, particularly in aneuploid and low-quality embryos, potentially impairing the trophoblast's ability to respond effectively to estrogen and leading to suboptimal interactions with the endometrium.

7.4.6 Involvement of Other Signalling Pathways

The functional analysis of differentially expressed miRNAs in aneuploid blastocysts showed involvement of ERBB tyrosine kinases receptors pathway signalling. These receptors are essential for several cellular processes, including cell growth, differentiation, apoptosis, and immune regulation. Elevated expression of *ERBB1* and E*RBB3* has been observed during decidualization in rabbit, demonstrating their contribution in the embryo-uterus crosstalk (Klonisch et al., 2001). Given the diverse functions of these genes, dysregulated miRNAs may contribute to *ERBB* signalling pathway in various ways, including direct regulation of *ERBB* receptors, modulation of the downstream components, or interreference with interactions involving other signalling pathways.

The dysregulated miRNAs in aneuploid blastocysts have also shown implication in the PI3K/Akt pathway, which is a downstream signalling pathway of *ERBB.* Inhibition of *AKT* in this pathway has been shown to impact the normal composition of blastocyst and delayed hatching (Riley et al., 2005). Furthermore, the PI3K/Akt pathway play a role in the interaction between blastocyst and the endometrium, facilitating the attachment and invasion of the trophoblast into the endometrial lining (Massimiani et al., 2015). Potential disruption of PI3K-Akt signalling in the aneuploid blastocysts, as indicated by the pathway analysis, may explain why aneuploid embryos have lower chances of implantation.

7.5 Clinical Significance

7.5.1 Reducing Implantation Failure

One of the key insights from our findings is the dynamic fluctuations in gene expression within blastocysts, which highlight general trends in gene regulation. Previous transcriptome analyses have explored various aspects of infertility, identifying association between altered gene expression and pregnancy complications, such as preeclampsia, PCOS, and recurrent miscarriages (Enquobahrie et al., 2008, Lv et al., 2019, Craciunas et al., 2021, Cozzolino et al., 2022, Moufarrej et al., 2022). Ongoing research is focusing on using gene expression, particularly miRNA and small non-coding RNAs, to pinpoint informative biomarkers linked to embryo quality and implantation potential, ultimately aiming to improve pregnancy outcomes (Hromadnikova et al., 2023).

In exploring impaired endometrium receptivity, many studies have deeply investigated the role miRNAs play in implantation and implantation failure. Some studies have compared the miRNA expression in culture media from both implanted and non-implanted blastocysts, revealing a downregulation of certain miRNAs, such as miR-20a, miR-30c and miR-142-3p, and an upregulation of others, like miR-661, miR-372 and miR-191, in non-implanted embryos (Rosenbluth et al., 2014, Cuman et al., 2015, Borges et al., 2016, Capalbo et al., 2016b). When accounting for chromosomal defects, miR-20a, miR-30c continued to show differential expression between implanted and non-implanted euploid blastocysts (Capalbo et al., 2016b).

Consistent with previous studies, our analysis revealed alterations in the expression of miR-20a, miR-372 and miR-191, all of which have been found dysregulated in non-implanted embryos. These changes were particularly pronounced in blastocysts with chromosomal abnormalities and poor TE morphology. While miR-142-3p was previously reported as upregulated in the culture media of non-implanted blastocysts, it did not show significant changes in our findings (Borges et al., 2016). Nevertheless, the miRNAs identified, especially those consistently observed in different studies, could serve as predictors of embryos higher implantation potential. Further investigation of these miRNAs could offer deeper insights into the mechanisms behind implantation failure.

7.5.2 Minimizing Aneuploidy

The existing data indicates that over 50% of pregnancy losses are attributed to chromosomal defects (Fritz et al., 2001, Benkhalifa et al., 2005, Niakan et al., 2012). While the current practice employs PGT-A for chromosomal abnormality detection, its limitations are well-recognised, including the invasiveness of the method and that only TE cells are investigated. Notably, the last HFEA recommendation on the PGT-A in 2019 suggest that it lacks strong evidence to be effective at improving the pregnancy chances (HFEA, 2019). However, this recommendation does not eradicate the effect of aneuploidy on the pregnancy outcomes. It might be suggested that improving the aneuploidy testing approaches, using non-invasive biomarkers or finding a more representative markers, would give better view about the embryo overall quality.

In the present study, miRNA patterns showed significant changes in aneuploid samples, aligning with the previous studies of this relationship (Almutlaq et al., 2024). While variations were observed among the differentially expressed miRNAs in distinct aneuploid groups, due to the complexity of this phenomenon, certain miRNAs consistently showed dysregulation across all types of aneuploidies. These findings further validated the strong association between miRNA expression patterns and of chromosomal abnormalities.

7.5.3 Optimizing Reproductive Treatment

Many previous studies have investigated factors that directly impact the competence of embryos. Some have discussed the adverse impact of ovarian stimulation on implantation potential and quality of mouse embryos (Ertzeid and Storeng, 2001). Additionally, it has been demonstrated that manipulating oocytes during sample preparation directly affects the oocyte quality, particularly the meiosis process, morphology, and gene expression (Combelles et al., 2009, Jiang et al., 2023).

Similarly, abnormal sperm parameters have the potential to impact embryonic competence, although research in this area is still limited. Despite selecting sperm with the best features for injection, it was suggested that the overall semen sample

quality influences the developmental competence of the resulting embryo (Piccolomini et al., 2018). Moreover, sperm varying characteristics was linked to different miRNA expression profiles, as detected in a recent research that explored the correlation between sperm morphology and miRNA expression and showed a group of altered miRNAs associated with teratozoospermia (Tomic et al., 2022). On the other hand, the specific laboratory preparation procedures, such as cryopreservation and the exposure to heat stress, were also linked to alterations in the miRNA expression of sperm (Shangguan et al., 2020, Alves et al., 2021, Ezzati et al., 2021).

These studies highlighted the significance of parental cells quality, indicating that alterations in their gene expression profiles, whether stemming from biological or influenced by in vitro manipulation, could impact the treatment outcomes. Supporting this notion, our findings from miRNA expression analyses concerning parental factors showed several miRNAs with altered expression levels associated with oocyte treatment and sperm abnormalities. This suggests that the competence of these blastocysts is intricately tied to the quality of the parental gametes. Consequently, improving practices to produce competent oocytes and collect high-quality sperm is crucial for enhancing the treatment process.

7.5.4 Potential miRNA Biomarkers for Preimplantation Embryo Selection

Accumulating evidence indicates that miRNAs serve as reliable indicators for assessing the quality of blastocysts and predicting pregnancy outcomes (Mutia et al., 2023). This positions them as promising biomarkers for embryo selection before transfer. The extracellular localization of miRNA has drawn attention to the possibility of their diffusion into culture media or their presence in blastocoel fluid, presenting potential alternatives to the invasive trophectoderm biopsy. Furthermore, unlike mRNAs, evidence has shown no correlation between miRNAs levels and copy number status in various cell types, making them excellent markers in population where the chromosomal variations are common (Ramsingh et al., 2013, Veigaard and Kjeldsen, 2014, Durrbaum et al., 2018).

Of the quality factors examined, aneuploidy had the most well-established association with miRNA expression in blastocysts. To further validate this link, we compared our findings with previous studies. Table 7-1 presents a detailed comparative analysis, highlighting miRNAs consistently altered in aneuploid blastocysts across studies. While the earlier studies aggregated all aneuploid samples without subdivision, consideration was given to the results from each aneuploidy subgroup in our study. The miRNAs showing consistent alterations across studies may serve as potential biomarkers for identifying aneuploidy in blastocysts.

Table 7-1: Consistently differentially expressed miRNAs in aneuploid blastocysts: matching results from previous research to current findings

miRNA	Expression in aneuploid blastocysts	Matching with previous
hsa-miR-146b-5p	Downregulated	(Rosenbluth et al., 2013)
hsa-miR-20a-5p	Downregulated	(Rosenbluth et al., 2013)
hsa-miR-26b-5p	Downregulated	(Rosenbluth et al., 2013)
hsa-miR-373-3p	Downregulated	(Rosenbluth et al., 2013)
hsa-miR-518a-3p	Downregulated	(Rosenbluth et al., 2013)
hsa-miR-92a-3p	Downregulated	(Rosenbluth et al., 2013) (McCallie B., 2015)
hsa-miR-93-5p	Downregulated	(Rosenbluth et al., 2013)
hsa-miR-125a-5p	Downregulated	(McCallie et al., 2014)

Additionally, we found the miRNAs linked to various embryonic quality factors and pregnancy complications from previous literature and compared them to our findings. Table 7-2 highlights miRNAs that may act as potential predictive markers of blastocyst competence, taking into account different quality factors and developmental outcomes. Only miRNAs that showed consistent results with our study were included.

Table 7-2: Consistent miRNA pattern alterations associated with low quality or poor pregnancy outcomes: A comparison of results between previous research and current study

miRNA	In the present study	In the previous research	Source of miRNA
miRNA 20a-5p	Downregulated in poor TE morphology, monosomy, and advanced reproductive maternal age.	Downregulated in CM of blastocyst with poor morphology (Coticchio et al., 2021)	Blastocyst
hsa-miR-378a-3p	Downregulated in aneuploid blastocysts	Important in promoting hatching (Pavani et al., 2022)	Blastocyst
hsa-miR-191-5p	Downregulated in aneuploid blastocysts	Upregulated in CM with successful pregnancy (Acuna-Gonzalez et al., 2021) Upregulated in sperm with high-quality embryo rate (Xu et al., 2020)	Culture media
hsa-miR-372-3p	Downregulated in the blastocysts with the poor TE morphology	Upregulated in CM of cleavage and blastocyst embryos with successful pregnancy (Fang et al., 2021)	Culture media
hsa-miR-184	Upregulated in blastocysts with aneuploidy, poor morphology, in day 6 blastocysts compared to day 5, in HCG trigger compared to dual, in advanced reproductive maternal age, in high dose ovarian stimulation compared to low dose	Upregulated in recurrent pregnancy loss cases (Dong et al., 2014, Zhang et al., 2019a, Jairajpuri et al., 2021)	Villus or decidua, mother circulating blood and blastocyst.

Evidently, many previous studies have linked the expression of miRNAs to embryo competence and pregnancy outcomes (Capalbo et al., 2016b, Abu-Halima et al., 2017, Acuna-Gonzalez et al., 2021, Fang et al., 2021). Some have demonstrated the presence of these miRNAs in the culture media, purposing non-invasive markers for embryo quality (Rosenbluth et al., 2014, Zhou and Dimitriadis, 2020, Coticchio et al., 2021, Pavani et al., 2022). For example, Robertson et al., found elevation of hsa-miR-191-5p levels in the culture media from aneuploid embryos. In comparison to our findings, this miRNA showed opposing expression pattern, being downregulated in the aneuploid blastocysts. However, our findings align with the results from a more recent study, which exhibited decreased levels of this miRNA in the culture media with association to pregnancy failure (Acuna-Gonzalez et al., 2021).

Another previous investigation showed downregulation of miRNA 20a-5p in the culture media obtained from poor morphology blastocysts (Coticchio et al., 2021). The potential correlation between the expression of this miRNA in the blastocysts and the blastocysts quality was also evident in our analyses, showing consistent reduced levels across multiple comparisons, especially in blastocysts with poor morphology, chromosomal losses, and advanced reproductive maternal age.

Moreover, miRNA profile was investigated in relation to blastocysts expansion, revealing overexpression of miR-378a-3p in the culture media of hatching blastocysts (Pavani et al., 2022). In our analysis, a significant downregulation of this miRNA in the aneuploid blastocysts was observed. This indication may shed light to a potential link between chromosomal abnormalities and blastocysts expansion, which requires future investigations. Additionally, current research particularly focused on the potential use of miRNA in the culture media to predict the pregnancy results. A study revealed a significant downregulation of miRNA, miR-372-3p in the culture media from unsuccessful pregnancy cases (Fang et al., 2021). In our analysis, the blastocysts with poor morphology exhibited significant downregulation of has-miR-372-3p. When we put these insights together, the dysregulated identified miRNAs in low-quality embryos, as well as those correlated with the implantation and pregnancy potential, we may unveil some of the unknowns in the sector.

Remarkably, hsa-miR-184 miRNA exhibited the most significant dysregulation in expression associated with poor-quality blastocysts in our analyses. While not

extensively studied, prior research has linked elevated levels of this miRNA in decidua and maternal circulation to recurrent pregnancy loss (Zhang et al., 2019a). Consistently, significant high levels of this miRNA were frequently observed during the course of our study, particularly in association with aneuploidy, poor morphology, delayed development, HCG trigger, and high-dose ovarian stimulation. Indeed, further investigations are required to comprehensively study the role of this miRNA in the developing embryos and the impact of its dysregulation on the pregnancy outcomes.

For potential implementation of miRNAs as quality makers, identifying patterns of expression rather than relying on a single miRNA level, could enhance the robustness of utilising them for clinical application. Interestingly, our sequencing results revealed that dysregulated miRNAs in the majority of the analyses often work together, either by targeting the same genes, participating in the same pathways, or continuously displaying similar patterns of expression in embryos with low-quality. In this context, advanced statistical and machine learning tools can be implemented to map miRNAs, along with their gene targets and pathways, to embryo quality outcomes.

It is also worth noting that diagnostic results for any condition typically rely on several biological tests rather than a singular one. In the IVF practice, miRNA biomarkers can be suggested as additional biological tool alongside other assessments, such as patient characteristics, embryo division, the day the embryo reaches the blastocyst stage, and the morphological features of the preimplantation embryo. Evaluating these factors together would provide a comprehensive assessment of the preimplantation embryo quality, contributing to more informed embryo selection.

7.6 Limitations

The utilization of human samples introduces many sources of variance that could confound the results. Due to the broad range of patient characteristics as well as inability to access information of all confounders, the influence of these factors remains challenging to assess. Thus, further investigations using animal samples with controlled variables are suggested to confirm the results generated in this analysis.

Moreover, both miRNAs and preimplantation embryos are susceptible to the influence of various biological and external factors. This includes miRNA haploinsufficiency, the gender of the embryo, and potential differences in laboratory sample preparation procedures such as the incubation, centrifugation, freezing and culturing, which could impact the gene expression of the resulting embryos. Additionally, the embryos may have been exposed to mechanical or technical errors during processing, although we have tried to limit this by collecting samples from one clinic, the thawing was made by two embryologists, and processing by one researcher, though the complete elimination of errors cannot be guaranteed.

Additionally, despite the study's aim to capture and analyse all miRNAs present in blastocysts, uncertainty exists regarding the possibility of capturing miRNAs in blastocoel fluid. Since the study only included vitrified embryos, the blastocoel fluid was replaced with vitrification reagent, and it is unknown if blastocoel-origin miRNAs diffused out during this process or remained. Additionally, although we only include ICSI-produced blastocysts, that underwent denudation, the potential for contamination with cumulus cells exists. Nevertheless, such contamination is considered negligible due to their small quantity in comparison to the larger number of embryonic cells.

Considerably, the analysis of gene expression data, although facilitated by sequencing technology, remains an area for improvement. Challenges encountered during miRNA expression analysis in the current study includes managing a big amount of data, possibly leading to ambiguous results. Although the study aimed to provide a comprehensive overview of the miRNA rather than in-depth exploration of specific miRNAs, many of the identified genes present opportunities for future research. Additionally, the practice of limiting the number of identified genes by

filtering out the low-expressed genes and setting specific analysis criteria, such as the FDR p-value and fold change threshold, may result in the loss of important genes. However, this step was crucial to reduce the risk of false positive results. Enhancing the bioinformatic analysis would contribute to a greater coverage of genes in the results and complete understanding of the genetic aspects in developing embryos.

The study has also faced logistical challenges related to the time and facilities constrictions, conducted within the framework of a PhD programme. The COVID-19 lockdown significantly delayed the project timeline. Although the initial plan was to validate the results of the most significant miRNAs identified using qPCR, the remaining cDNA samples were lost due to a -80°C freezer malfunction. Although the limited accessibility of the blastocysts prevented further validation of the results by PCR, the multiple analyses conducted, relatively large sample size, confirmed target mRNA results, and alignment with previous studies all contribute to the confidence in our findings. Moreover, limited knowledge about many identified miRNAs, their targets and associated biological pathways, posed challenges during the results interpretation. While the sample size of the sequenced samples is relatively large compared to previous studies, the multifactorial relationship between miRNA expression and blastocyst competence, along with the diversity in infertility causes and patients histories, suggests that an even larger number of samples would be needed to draw more statistically significant conclusions.

7.7 Future Work and Conclusion

The study findings provide novel insights into several aspect of blastocysts quality, however; further investigations will pave the way for future advancements in reproductive treatment and contribute to a better understanding of pregnancy failure. For example, exploration of potential novel miRNAs in blastocysts and detection of mutations and polymorphisms in miRNAs that might be linked to adverse pregnancy outcomes are important areas of future research.

Additionally, although our analysis yielded interesting results, the findings were explored to a specific extent due to the availability of samples and the time allocated for completion of the study. On the other hand, the computational analysis of

miRNAs was highly confined and focused which might have led to omitting important results. Therefore, further focused investigations of the identified miRNAs would provide more detailed and conclusive results.

Lastly, tailoring fertility treatments to the individual genetic profiles of patients would enhance both the diagnosis of infertility and the practice of selection the highest quality reproductive cells and embryos. Investigating the miRNA profile in a case-dependent basis may offer a broader understanding of which miRNAs are case-specific and which ones are generally related to the embryo quality.

In conclusion, our research has provided valuable insights into the miRNA expression profile and its association to the quality of preimplantation human various quality parameters, whether related to the embryo or parental cells, highlight the pivotal role of miRNAs in regulating crucial pathways controlling early development, and their significance in reflecting the developmental competence of embryos. Despite the complexity of the investigated factors and the big amount of data generated, this study highlights alterations in specific miRNAs linked to poor-quality embryos, creating opportunities to the potential integration of miRNAs into genetic testing for embryo selection. Moving forward, future work could validate the role of miRNAs in predicting pregnancy outcomes, and further exploration of these miRNAs in culture media holds promise as non-invasive biomarkers for assessing embryo quality prior to transfer. In summary, this thesis advances our understanding of miRNA in human blastocysts and their regulating roles in pathways essential for normal development of blastocysts, establishing a groundwork for ongoing exploration of miRNA in the field of reproductive treatment.

Personal Scientific Contributions During the Ph.D. Journey

During the course of my Ph.D. program, I participated in many activities and events that enriched my studying experience and had a positive impact on my growth as a researcher and educator.

1. Supervisory roles and teaching Experience:

I had the privilege to supervise several master's projects during my Ph.D. journey. I also contributed to the academic community through some teaching responsibilities. It was a grateful opportunity to work with these exceptional students and contribute to their academic journeys.

2. Conferences participations:

In my commitment to academic engagement, my research findings have been presented many times in different practices. Two posters were submitted and presented in the Institute for Women's Health (IfWH) annual meetings 2021 and 2022. I have also presented a piece of my findings in the 38th Annual Meeting of European Society of Human Reproduction and Embryology (ESHRE), Milan, 2022. My work has been accepted for an oral presentation in meeting of the Scottish Human Reproduction and Embryology Group (SHREG), Dundee, 2023 and it was a particular honour to be recognized as a runner-up for the Best Presentation. These activities were a good opportunity to share my research findings with a broader audience and receive valuable feedback.

3. Publications and peer review

I have published a research paper as a second author: Systematic review of mRNA expression in human oocytes: understanding the molecular mechanisms underlying oocyte competence, Journal of Assisted Reproduction, (2023).

I have contributed to the peer review process for multiple manuscripts in the field, providing constructive feedback and evaluation of research quality.

4. 3MT Competition Victory:

I participated in the Three Minute Thesis (3MT) competition, conducted in the IfWH department, several times and had the honour to be selected as a winner in 2020. This competition challenged me to summarise my complex research into a concise three-minute talk with a single-slide presentation, which helped me to simplify and communicate complex ideas effectively.

5. Membership in social societies:

Throughout my Ph.D., I was an active member in different social sociates and clubs. My involvement in these organizations included organising both academic and social events, as well as sharing my research expertise in different domines.

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Appendices

Appendix1

Regression results

Dependent Variable: Maternal age

Parameter	B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Intercept	.047	.915	-.825	.920
Aneuploidy	.217	.244	-.150	.585
Paternal age	.582	<.001	.330	.834
Trigger	.231	.028	.026	.437
Sperm motility	.217	.439	-.337	.771
Sperm morphology	-.260	.142	-.609	.089
Sperm count	.634	.001	.257	1.010
Sperm concentration	-.203	.400	-.678	.272
Day of blastocyst formation	.067	.617	-.198	.331
TE morphology	.141	.362	-.164	.446
Ovarian stimulation dose	-.024	.727	-.160	.112

Dependent Variable: Ovarian stimulation dose

Parameter	B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Intercept	.378	.557	-.894	1.651
Aneuploidy	.081	.766	-.455	.617
Paternal age	.578	.004	.190	.965
Trigger	-.022	.887	-.333	.288
Sperm motility	-.218	.585	-1.008	.571
Sperm morphology	-.595	.017	-1.082	-.109
Sperm count	.280	.341	-.300	.860
Sperm concentration	.213	.537	-.469	.895
Day of blastocyst formation	.344	.069	-.027	.714
TE morphology	.456	.036	.030	.882
Maternal age	-.035	.829	-.356	.286
Indication	-.012	.879	-.172	.148

Dependent Variable: Trigger type

Parameter	B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Intercept	1.941	<.001	1.242	2.641
Aneuploidy	-.218	.195	-.550	.114
Paternal age	-.449	<.001	-.685	-.213
Sperm motility	.183	.461	-.308	.675
Sperm morphology	.176	.262	-.134	.486
Sperm count	-.074	.687	-.437	.289
Sperm concentration	-.142	.510	-.567	.283
Day of blastocyst formation	-.112	.345	-.345	.122
TE morphology	-.101	.461	-.372	.170
Maternal age	.326	<.001	.136	.516
Indication	-.139	.005	-.235	-.043
Ovarian stimulation dose	-.009	.887	-.129	.112

Dependent Variable: Paternal age

Parameter	B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Intercept	1.010	<.001	.434	1.587
Aneuploidy	-.040	.756	-.299	.218
Day of blastocyst formation	-.076	.413	-.261	.108
Maternal age	.283	<.001	.160	.406
Trigger	-.229	.002	-.369	-.089
TE morphology	-.141	.190	-.353	.071
Ovarian stimulation dose	.143	.002	.052	.234
Sperm motility	.141	.472	-.246	.528
Sperm morphology	.333	.006	.096	.570
Sperm count	-.050	.722	-.326	.226
Sperm concentration	.055	.743	-.277	.388

Dependent Variable: Sperm concentration

Parameter	B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Intercept	.023	.897	-.329	.376
Aneuploidy	-.100	.184	-.248	.048
Day of blastocyst formation	.052	.336	-.055	.158
Maternal age	-.033	.400	-.111	.044
Trigger	-.012	.775	-.097	.073
TE morphology	-.020	.753	-.143	.104
Ovarian stimulation dose	.018	.517	-.037	.073
Sperm motility	.033	.769	-.191	.258
Sperm morphology	.281	<.001	.149	.412
Sperm count	.524	<.001	.400	.648
Paternal age	.019	.743	-.093	.130

Dependent Variable: Sperm count

Parameter	B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Intercept	-.019	.928	-.444	.405
Aneuploidy	.162	.074	-.016	.339
Day of blastocyst formation	-.051	.431	-.180	.077
Maternal age	.150	.001	.061	.239
Trigger	-.064	.217	-.165	.038
TE morphology	-.054	.473	-.203	.095
Ovarian stimulation dose	.032	.342	-.034	.098
Sperm motility	.010	.942	-.260	.280
Sperm morphology	-.090	.297	-.261	.080
Paternal age	-.024	.722	-.158	.110
Sperm concentration	.760	<.001	.580	.940

Dependent Variable: Sperm morphology

Parameter	B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Intercept	-.675	.004	-1.134	-.216
Aneuploidy	.186	.067	-.013	.385
Day of blastocyst formation	-.030	.686	-.174	.115
Maternal age	-.078	.142	-.182	.027
Trigger	.072	.217	-.043	.186
TE morphology	.321	<.001	.166	.477
Ovarian stimulation dose	-.089	.017	-.162	-.017
Sperm motility	-.004	.978	-.308	.299
Paternal age	.204	.006	.059	.350
Sperm concentration	.514	<.001	.273	.754
Sperm count	-.114	.297	-.329	.101

Dependent Variable: Sperm motility

Parameter	B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Intercept	.553	<.001	.270	.836
Aneuploidy	.268	<.001	.150	.385
Day of blastocyst formation	-.076	.097	-.167	.014
Maternal age	.026	.439	-.040	.093
Trigger	.037	.320	-.036	.109
TE morphology	.032	.545	-.073	.138
Ovarian stimulation dose	-.013	.590	-.060	.034
Paternal age	.035	.472	-.061	.130
Sperm concentration	.024	.769	-.141	.190
Sperm count	.005	.942	-.132	.142
Sperm morphology	-.002	.978	-.124	.120

Appendix2

Systematic review study design

Research question: Does the expression profile of miRNA change in aneuploid preimplantation blastocysts when compared to euploid?

Defining the question using PICO:

Population: Pre-implantation embryos (blastocysts).

Intervention (exposure): aneuploidy.

Comparison (control): euploid blastocysts.

Outcome: miRNA differential expression analysis results.

Inclusion criteria

Publication type	In primary literature: peer-reviewed journal articles. In grey literature: conference abstracts with informative results, theses and dissertations.
Year of publication	No limit.
Language	English.
Study design	Case control observational studies.
Population	Blastocyst embryos.
Outcome	Differentially expressed miRNAs of blastocyst origin (extracted from whole blastocysts or from trophectoderm (TE) biopsy or found in blastocoel or secreted into the culture media.)

Electronic databases to use:

Excerpta Medica database (Embase), MEDLARS Online (Medline), Web of Science database and Cochrane clinical trials database**.**

Internet sources to search:

U.K. National Research Register and British Library (EThOS).

Type of literature included:

Peer reviewed journal articles, reference lists of eligible studies, conference abstracts, and theses and dissertations

Search strategy using Boolean operators

Database	Keywords
Excerpta Medica database (Embase)	(miRNA*.mp. OR microRNA/ OR microRNA*.mp. OR “micro RNA*”) AND (embryo* OR embryo/ OR preimplantation embryo/ OR blastocyst/ OR blastocyst*.mp.) AND (Aneuploidy/ OR aneuploid*.mp. OR "abnormal karyotype".mp. or chromosome aberration OR trisomy/ OR trisomy.mp. OR monosomy.mp. OR monosomy/).
MEDLARS Online (Medline)	(miRNA*.mp.OR microRNA*.mp. OR MicroRNAs/ OR "micro RNA*".mp) AND (embryo*.mp. OR Blastocyst/ OR "preimplantation embryo*".mp. OR blastocyst*.mp.) AND (Aneuploidy/ or aneuploid*.mp. OR "abnormal karyotype".mp. OR Chromosome Aberrations/ OR Abnormal Karyotype/ OR trisomy.mp. OR Trisomy/ OR monosomy.mp. OR Monosomy/)
Web of Science database	(TS=miRNA* OR TS= microRNA* OR TS= "micro RNA*") AND (TS= aneuploid* OR TS= "chromosome aberration" OR TS="abnormal karyotype" OR TS= trisomy OR TS= Monosomy) AND (TS= embryo* OR TS= "preimplantation embryo*" OR TS= blastocyst*).
Cochrane clinical trials database	((Mesh: [miRNAs] OR miRNA* OR microRNA* OR “micro RNA”) AND (Mesh: [Aneuploidy] OR “Aneuploid” OR “abnormal karyotype” OR “chromosome* aberration” OR ‘Trisomy OR “Monosomy”) AND) Mesh: [Embryonic structure] OR “embryo*” OR “preimplantation embryo*” OR “Blastocyst*))
UK National Research Register	(miRNA) AND (Aneuploidy) AND (Blastocyst OR embryo)
British Library (EThOS)	(miRNA) AND (Aneuploidy) AND (Blastocyst OR embryo)

Excluded studies

Excluded study for eligibility	Publication type	Justification
Differential expression of micro-RNA in day 5 human euploid and aneuploid blastocysts.	Abstract	Repeated. Results were reported in an included study (MicroRNA expression in the human blastocyst).
Human blastocysts exhibit unique microrna profiles in relation to maternal age and chromosome constitution.	Abstract	Repeated. Results were reported in an included study (Human blastocysts exhibit unique microrna profiles in relation to maternal age and chromosome constitution).
MicroRNA in culture media from human blastocysts exhibits a distinct signature that correlates with embryonic chromosomes and IVF outcome.	Abstract	Repeated. Results were reported in an included study (Differential expression of micro-RNA in day 5 human euploid and aneuploid blastocysts).
MicroRNA testing: A novel, non-invasive technique to detect aneuploidy and live birth potential in human embryos.	Abstract	Repeated. Results were reported in an included study (Differential expression of micro-RNA in day 5 human euploid and aneuploid blastocysts).
Correlation between differential expression of microRNA and quality of embryos	Article	Language - Chinese

Appendix3

Participant Demographics

Date of sample collection	analysis number	PGT[1] Indications	Mutation	Target Gene	EC Date	Biopsy Date	Procedure	PGT Diagnosis	Embryo Morphology	Maternal age	Paternal age
27/11/2018	1	Telangiectasia, hereditary haemorrhagic, type 2	paternal mutation, Male: Exon 8, c.1120C>T,p.R374W	ACVRL1	15/06/2018	20/06/2018	ICSI[2]	Affected. NA[3]- euploid based on DNASeq	D5Bφ6B+B+	32.96	38.14
	2	Telangiectasia, hereditary haemorrhagic, type 2	paternal mutation, Male: Exon 8, c.1120C>T,p.R374W	ACVRL1	15/06/2018	20/06/2018	ICSI	Affected. NA- euploid based on DNASeq	D5Bφ6AB+	32.96	38.14
	3	Telangiectasia, hereditary haemorrhagic, type 2	paternal mutation, Male: Exon 8, c.1120C>T,p.R374W	ACVRL1	15/06/2018	21/06/2018	ICSI	Unaffected. Abnormal: +19	D6Bφ6B-B+	32.96	38.14
	4	Telangiectasia, hereditary haemorrhagic, type 2	paternal mutation, Male: Exon 8, c.1120C>T,p.R374W	ACVRL1	25/08/2018	30/08/2018	ICSI	Unaffected. Abnormal: +16	D5Bφ6B+B+	33.16	38.34
08/01/2019	30	Optic atrophy 1	Male: Exon 14, c.1212+1G>A		21/10/2018	26/10/2018	ICSI	Unaffected. Abnormal: +6	D5Bφ6B+B+	34.13	44.61
	5	Optic atrophy 1	Male: Exon 14, c.1212+1G>A		21/10/2018	26/10/2018	ICSI	Unaffected. Abnormal: +14	D5Bφ6B-B-	34.13	44.61
08/01/2019	31	AMA[4]			21/09/2018	27/09/2018	ICSI	Abnormal: +15, -19	D6Bφ6B-C	40.81	37.81
	32	AMA			21/09/2018	27/09/2018	ICSI	Abnormal: -15, -22	D6Bφ6B-C	40.81	37.81

	33	AMA			21/09/2018	27/09/2018	ICSI	Abnormal: +16, -19, -22	D6Bφ6B-B-	40.81	37.81
08/01/2019	6	NA			21/09/2018	26/09/2018	ICSI	Abnormal: -18, +21, -22	D5Bφ6B+B-	40.81	37.81
12/04/2019	34	NA			23/05/2016	29/05/2016	ICSI	Abnormal: part 3, part 20	D6Bφ6AB-	34.89	39.89
15/04/2019	15	Tuberous sclerosis	TSC1		20/06/2016	25/06/2016	ICSI	Unaffected / euploid	D5Bφ6AB-	34.97	39.97
15/04/2019	36	NA			18/09/2018	23/09/2018	ICSI	Abnormal: -1,-10,+16	D5Bφ6B-B-	40.41	40.10
02/05/2019	37	NA			18/03/2019	24/03/2019	ICSI	Complex Abnormal: +19, -21, XXY	D6Bφ6B-B-	34.34	34.33
	35	NA			25/01/2019	24/03/2019	ICSI	Complex Abnormal: -12, -15	D6Bφ6B-C	34.20	34.19
02/05/2019	11	NA			03/04/2019	08/04/2019	ICSI	Complex Abnormal: -10, -11, -22	D5Bφ6B-B-	42.03	38.40
	12	NA			03/04/2019	08/04/2019	ICSI	Abnormal: -13	D5Bφ6B+B+	42.03	38.40
	13	NA			03/04/2019	08/04/2019	ICSI	Complex Abnormal: -4, -11, +17	D5Bφ6B-B-	42.03	38.40
	14	NA			03/04/2019	08/04/2019	ICSI	Abnormal: +18	D6Bφ6B-C	42.03	38.40
02/05/2019	38	NA			30/01/2019	04/02/2019	ICSI	Complex Abnormal: -18, +20, -22	D5Bφ6B+B-	43.01	48.18
	39	NA			30/01/2019	05/02/2019	ICSI	Complex Abnormal: +11, -18	D6Bφ6B-C	43.01	48.18
	40	NA			30/01/2019	05/02/2019	ICSI	Complex Abnormal: -15, -20	D5Bφ6B-B+	43.01	48.18

02/05/2019	41	NA			12/03/2019	18/03/2019	ICSI	Abnormal: -4	D6Bφ6B+B-	42.30	38.06
	42	NA			12/03/2019	18/03/2019	ICSI	Abnormal: -22	D6Bφ6B-B-	42.30	38.06
	43	NA			12/03/2019	17/03/2019	ICSI	Complex Abnormal: +4, -7, -13, -21	D5Bφ6AB+	42.30	38.06
02/05/2019	44	Maternal Mutation	Maternal Mutation: Exon 11, c.5946delT, p.S1982Rfs*22 (aka 6174delT)	BRCA2	31/01/2019	05/02/2019	ICSI	Unaffected. Abnormal: +4, -14	D5Bφ6B+B-	41.30	51.93
	45	Maternal Mutation	Maternal Mutation: Exon 11, c.5946delT, p.S1982Rfs*22 (aka 6174delT)	BRCA2	03/06/2018	05/02/2019	ICSI	Unaffected. Abnormal: dup(6)(q16.1-qter), -14	D5Bφ6B+B+	40.64	51.27
03/05/2019	46	Male factor, previous IVF[5] failure			23/10/2018	28/10/2018	ICSI	Complex Abnormal: +2q, -11p	D5Bφ6B+B-	35.01	37.08
	47	Male factor, previous IVF failure			23/10/2018	28/10/2018	ICSI	Complex Abnormal: +2p, +11q	D5Bφ6B+B-	35.01	37.08
	48	Male factor, previous IVF failure			23/10/2018	28/10/2018	ICSI	Complex Abnormal: +1p, +2q, -11q, -13, Y0	D5Bφ6B+B-	35.01	37.08
03/05/2019	49	previous IVF failure, patient request, recurrent miscarriage			13/01/2019	19/01/2019	IMSI[6]	Abnormal: +18	D6Bφ6B-C	39.51	39.09
	50	previous IVF failure, patient request, recurrent miscarriage			25/10/2018	18/01/2019	IMSI	Abnormal: +8, X0	D5Bφ6AB-	39.30	38.87
03/05/2019	51	AMA			04/12/2018	10/12/2018	ICSI	Abnormal: -19	D6Bφ6B+B+	43.08	60.66

	52	AMA			04/12/2018	10/12/2018	ICSI	Complex Abnormal: -2, +13	D6Bφ6B+B-	43.08	60.66
10/05/2019	53	AMA			25/04/2019	30/04/2019	ICSI	Abnormal: -9	D5Bφ6B-B-	40.77	41.26
	54	AMA			25/04/2019	30/04/2019	ICSI	Abnormal: +16	D5Bφ6B-C	40.77	41.26
	55	AMA			25/04/2019	30/04/2019	ICSI	Complex Abnormal: +14, +16	D5Bφ3B-C	40.77	41.26
	56	AMA			25/04/2019	30/04/2019	ICSI	Complex Abnormal: +9p, +21	D5Bφ3B-B-	40.77	41.26
10/05/2019	28	AMA			18/02/2019	24/02/2019	ICSI	High mosaic aneuploid:+19	D6Bφ6B-B+	42.48	34.26
	57	AMA			18/02/2019	24/02/2019	ICSI	Complex Abnormal: +6, XXY	D6Bφ6B-B+	42.48	34.26
	58	AMA			18/02/2019	24/02/2019	ICSI	Abnormal: -15	D6Bφ6B+B+	42.48	34.26
	59	AMA			18/02/2019	24/02/2019	ICSI	Complex Abnormal: +1, +2, +6, -8q, +20q	D6Bφ6B-C	42.48	34.26
05/07/2019	60	AMA			10/12/2018	16/12/2018	IMSI	Abnormal: -19	D6Bφ6B-C	39.08	45.15
	61	AMA			10/12/2018	15/12/2018	IMSI	Abnormal: -18	D5Bφ6B-B-	39.08	45.15
	62	AMA			10/12/2018	16/12/2018	IMSI	Complex Abnormal: -2, +9, -11q, +15	D6Bφ6B-C	39.08	45.15
	63	AMA			15/12/2018	16/12/2018	IMSI	Abnormal: -22	D6Bφ6B+B-	39.09	45.16
	64	AMA			15/12/2018	16/12/2018	IMSI	Complex Abnormal: -13, -15	D6Bφ6B-C	39.09	45.16

	65	AMA			15/12/2018	16/12/2018	IMSI	Complex Abnormal: +15, +18, +19, -20, +21	D6Bφ6B-C	39.09	45.16
05/07/2019	16	Sickle cell	Maternal mutation in: ch.11, c.20A>T	HBB	15/05/2019	20/05/2019	ICSI	Euploid	D5Bφ6B-B-	32.27	31.70
	66	Sickle cell	Maternal mutation in: ch.11, c.20A>T	HBB	15/05/2019	21/05/2019	ICSI	Complex abnormal: +2, -13, -15, -X	D6Bφ6B-B-	32.27	31.70
	17	Sickle cell	Maternal mutation in: ch.11, c.20A>T	HBB	15/05/2019	21/05/2019	ICSI	Euploid	D6Bφ6B-B-	32.27	31.70
	67	Sickle cell	Maternal mutation in: ch.11, c.20A>T	HBB	15/05/2019	21/05/2019	ICSI	Abnormal: -3	D6Bφ6B-B-	32.27	31.70
	68	Sickle cell	Maternal mutation in: ch.11, c.20A>T	HBB	15/05/2019	21/05/2019	ICSI	Abnormal: -15	D6Bφ6B-B-	32.27	31.70
05/07/2019	69	AMA, Male factor			11/06/2019	17/06/2019	ICSI	Abnormal: -13	D6Bφ6B+C	40.32	60.79
	70	AMA, Male factor			25/10/2018	17/06/2019	ICSI	Abnormal: +11	D6Bφ6B-A	39.69	60.17
05/07/2019	29	Paternal mutation	Paternal Mutation: 45,XY,der(13:14)(q10:q10)	UPD14	03/05/2019	08/05/2019	ICSI	dup (11) (q11-qter)	D5Bφ6B+B+	34.68	31.85
	96	Paternal mutation	Paternal Mutation: 45,XY,der(13:14)(q10:q10)	UPD14	03/05/2019	08/05/2019	ICSI	del(12) (q13.13-qter),-13,+21	D5Bφ6B+B+	34.68	31.85
	71	Paternal mutation	Paternal Mutation: 45,XY,der(13:14)(q10:q10)	UPD14	03/05/2019	09/05/2019	ICSI	Abnormal: -13, +16	D6Bφ6B-B	34.68	31.85
	72	Paternal mutation	Paternal Mutation: 45,XY,der(13:14)(q10:q10)	UPD14	03/05/2019	08/05/2019	ICSI	Abnormal: +14	D5Bφ6B+B-	34.68	31.85

05/07/2019	73	NA			19/06/2019	25/06/2019	IMSI	Complex Abnormal: +12, +16	D6Bφ6B-B-	41.22	41.08
	74	NA			19/06/2019	25/06/2019	IMSI	Abnormal: +16	D6Bφ6B-B+	41.22	41.08
	75	NA			19/06/2019	24/06/2019	IMSI	Abnormal: -8	D5Bφ6B+B-	41.22	41.08
11/09/2019	18	Uniparental Disomy	45,XY,der(13:14)(q10;q10)		06/05/2018	11/05/2018	ICSI	Euploid embryo with maternal contribution for chromosome 14	D5Bφ6B+B+	30.39	29.76
11/09/2019	76	NA			18/01/2019	23/01/2019	IMSI	Abnormal: Chaotic	D5Bφ6B-B-	42.40	40.89
	77	NA			18/01/2019	24/01/2019	IMSI	Complex Abnormal: +18, +19	D6Bφ6B-C	42.40	40.89
	78	NA			31/05/2019	05/06/2019	PIMSI	Complex Abnormal: +15, -16, +22	D5Bφ6B+B-	42.77	41.26
	79	NA			18/01/2019	23/01/2019	IMSI	Complex Abnormal: +14, -16, -17	D5Bφ6B-B-	42.40	40.89
	80	NA			03/07/2018	24/01/2019	IMSI	Abnormal: +6	D5Bφ6B+B+	41.86	40.35
04/10/2019	81	Facioscapulohumeral muscular dystrophy	Paternal Mutation: D4Z4 reduced allele	DUX4	29/08/2019	04/09/2019	ICSI	Aneuploid -15	D6Bφ6B-B-	34.39	36.83
	82	Facioscapulohumeral muscular dystrophy	Paternal Mutation: D4Z4 reduced allele	DUX4	29/08/2019	03/09/2019	ICSI	Aneuploid +13, +14	D5Bφ6B-B-	34.39	36.83
	83	Facioscapulohumeral muscular dystrophy	Paternal Mutation: D4Z4 reduced allele	DUX4	29/08/2019	03/09/2019	ICSI	Aneuploid -16	D5Bφ6B-B-	34.39	36.83
	84	Facioscapulohumeral muscular dystrophy	Paternal Mutation: D4Z4 reduced allele	DUX4	29/08/2019	03/09/2019	ICSI	Aneuploid +22	D5Bφ6B-C	34.39	36.83
	85	Facioscapulohumeral muscular dystrophy	Paternal Mutation: D4Z4 reduced allele	DUX4	29/08/2019	04/09/2019	ICSI	Aneuploid -16	D6Bφ6B-C	34.39	36.83

	86	Facioscapulohumeral muscular dystrophy	Paternal Mutation: D4Z4 reduced allele	DUX4	29/08/2019	04/09/2019	ICSI	Aneuploid +16	D6Bφ6B-C	34.39	36.83
	87	Facioscapulohumeral muscular dystrophy	Paternal Mutation: D4Z4 reduced allele	DUX4	29/08/2019	04/09/2019	ICSI	dup(16) (q23.1-qter)	D6Bφ6B-C	34.39	36.83
	88	Facioscapulohumeral muscular dystrophy	Paternal Mutation: D4Z4 reduced allele	DUX4	29/08/2019	03/09/2019	ICSI	Aneuploid -21, -13	D5Bφ6B-B-	34.39	36.83
18/10/2019	89	Maternal mutation	Maternal Mutation: Chr. 13, c.5909C>A	BRCA2	16/07/2019	23/07/2019	ICSI	Aneuploid: -22, -X	D5Bφ6B-C	34.95	39.40
	19	Maternal mutation	Maternal Mutation: Chr. 13, c.5909C>A	BRCA2	16/07/2019	24/07/2019	ICSI	Euploid	D6Bφ6B-B+	34.95	39.40
18/10/2019	90	Translocation	45,XX,der(13;14)(q10;q10)		18/06/2019	22/07/2019	ICSI	Abnormal: +13, +14	D6Bφ6B+B+	30.35	29.01
18/10/2019	91	AMA			22/08/2019	28/08/2019	ICSI	Complex Abnormal: -14, -21	D6Bφ6B-B-	42.96	37.52
07/11/2019	20	Sickle cell	HbAS / HbAC	HBB	04/09/2017	09/09/2017	ICSI	Carrier / Euploid	D5Bφ6AB+	28.11	30.71
	21	Sickle cell	HbAS / HbAC	HBB	04/09/2017	09/09/2017	ICSI	Unaffected / No result	D5Bφ6B-B-	28.11	30.71
12/02/2020	92	Neurofibromatosis Type 1		NF1	01/10/2015	04/11/2015	ICSI	Euploid	D5Bφ6B+B+	36.31	47.43
	93	Neurofibromatosis Type 1		NF1	01/10/2015	04/11/2015	ICSI	Euploid	D5Bφ6B+B+	36.31	47.43
12/02/2020	94	Translocation	46,XX,t(9:18)(q11;q11.1)		17/01/2015	22/01/2015	ICSI	Euploid	D5Bφ6B+B+	38.60	34.44
	95	Translocation	46,XX,t(9:18)(q11;q11.1)		17/01/2015	22/01/2015	ICSI	Euploid	D5Bφ6B+B+	38.60	34.44
14/02/2020	148	Duchenne Muscular Dystrophy			10/10/2016	16/10/2016	ICSI	Euploid	D6Bφ6B-C	36.71	37.46

14/02/2020	97	Translocation	Paternal : 46,XY,t(2;7)(p25.1;q32)		04/12/2017	09/12/2017	ICSI	Euploid	D5Bφ6B-B+	35.25	35.56
	98	Translocation	Paternal : 46,XY,t(2;7)(p25.1;q32)		04/12/2017	10/12/2017	ICSI	Euploid	D6Bφ6B-B+	35.25	35.56
15/09/2020	99	AMA, Previous miscarriage/ implantation failure			28/07/2018	02/08/2018	IVF	Euploid	D5Bφ6B+B-	41.69	45.40
15/09/2020	100	X-Linked Retinoschisis, inheritance: X Linked Recessive	RS1		13/10/2017	05/02/2019	ICSI	Euploid	D6Bφ6B-B-	33.40	37.05
15/09/2020	101	BETA-Thalassemia	c.92+5G>C	HBB	16/05/2019	21/05/2019	ICSI	Abnormal: del(4) (pter-p14)	D5Bφ6B+B-	37.20	39.30
	102	BETA-Thalassemia	c.92+5G>C	HBB	16/05/2019	21/05/2019	ICSI	Euploid	D5Bφ6B+B+	37.20	39.30
15/09/2020	103	AMA, Previous miscarriage/ implantation failure			15/08/2020	21/08/2020	ICSI	Abnormal: +1, -7, -13	D6Bφ6B-C	41.15	40.09
	104	AMA, Previous miscarriage/ implantation failure			15/08/2020	21/08/2020	ICSI	Abnormal: +1, -15, +22, X0	D6Bφ6B+B-	41.15	40.09
15/09/2020	105	AMA			15/08/2020	20/08/2020	ICSI	Abnormal: +9	D5Bφ6B+B-	41.91	38.29
	106	AMA			15/08/2020	20/08/2010	ICSI	Abnormal: -4	D5Bφ6B+C	41.91	38.29
	107	AMA			15/08/2020	20/08/2020	ICSI	Abnormal: +15	D5Bφ6B+B-	41.91	38.29
23/09/2020	108	Neurofibromatosis Type 1	c.2546dupG (de novo) autosomal dominant	NF1	03/04/2015	08/04/2015	ICSI	Euploid	D5Bφ6BB	34.16	31.09

	110	Neurofibromatosis Type 1	c.2546dupG (de novo) autosomal dominant	NF1	03/04/2015	09/04/2015	ICSI	Euploid	D6Bφ6BA	34.16	31.09
	111	Neurofibromatosis Type 1	c.2546dupG (de novo) autosomal dominant	NF1	03/04/2015	09/04/2015	ICSI	Euploid	D6Bφ6BB	34.16	31.09
24/09/2020	112	Myopathy, congenital, bailey-bloch	Maternal Mutation: c.997-1G>T, Paternal Mutation: c.851G>C	Chromosome: 12 Gene: STAC3	06/07/2019	11/07/2019	ICSI	Euploid	D5Bφ6B+B+	25.76	29.12
	113	Myopathy, congenital, bailey-bloch	Maternal Mutation: c.997-1G>T, Paternal Mutation: c.851G>C	Chromosome: 12 Gene: STAC3	06/07/2019	11/07/2019	ICSI	Abnormal: del(22)(q 13.31-qter), -X	D5Bφ6B+B+	25.76	29.12
	115	Myopathy, congenital, bailey-bloch	Maternal Mutation: c.997-1G>T, Paternal Mutation: c.851G>C	Chromosome: 12 Gene: STAC3	06/07/2019	11/07/2019	ICSI	Abnormal: -X	D5Bφ6B+B+	25.76	29.12
	116	Myopathy, congenital, bailey-bloch	Maternal Mutation: c.997-1G>T, Paternal Mutation: c.851G>C	Chromosome: 12 Gene: STAC3	06/07/2019	11/07/2019	ICSI	Abnormal: -X	D5Bφ6AB+	25.76	29.12
	117	Myopathy, congenital, bailey-bloch	Maternal Mutation: c.997-1G>T, Paternal Mutation: c.851G>C	Chromosome: 12 Gene: STAC3	06/07/2019	11/07/2019	ICSI	Abnormal: +15	D5Bφ6B+B-	25.76	29.12
	118	Myopathy, congenital, bailey-bloch	Maternal Mutation: c.997-1G>T, Paternal Mutation: c.851G>C	Chromosome: 12 Gene: STAC3	06/07/2019	11/07/2019	ICSI	Abnormal: del(9)(pter-p 12)	D5Bφ6B+B-	25.76	29.12

25/09/2020	119	Previous miscarriage/ implantation failure			26/07/2019	01/08/2019	ICSI	Complex Abnormal: -5p, -10	D6Bφ6B+C	38.59	45.34
	120	Previous miscarriage/ implantation failure			27/05/2019	01/08/2019	ICSI	Abnormal: -2	D6Bφ6B-B-	38.43	45.18
25/09/2020	139	NA			31/08/2020	05/09/2020	ICSI	Abnormal: +22	D5Bφ6AB	32.42	35.64
25/09/2020	123	AMA			22/10/2019	27/10/2019	ICSI	Abnormal: -2q	D5Bφ3B+B+	38.02	43.55
	124	AMA			22/10/2019	28/10/2019	ICSI	Abnormal: -19	D6Bφ3B-B-	38.02	43.55
	125	AMA			22/10/2019	28/10/2019	ICSI	Abnormal: +8	D6Bφ3B+A	38.02	43.55
25/09/2020	126	Previous miscarriage, implantation failure			11/09/2020	17/09/2020	ICSI	Aneuploid: -22	D6Bφ6B-B-	36.61	36.39
	127	Previous miscarriage/ implantation failure			11/09/2020	16/09/2020	ICSI	Aneuploid: +4p	D5Bφ6AB+	36.61	36.39
25/09/2020	128	AMA			05/09/2018	10/09/2018	ICSI	Complex Abnormal: -7, +11, -15	D5Bφ6B-C	42.17	59.20
05/11/2020	132	AMA			01/03/2014	18/04/2018	IMSI	Euploid	D5Bφ4B+B-	33.04	33.57
05/11/2020	134	Beta-thalassemia		HBB	30/01/2016	05/02/2016	ICSI	Euploid	D6Bφ6AB+	35.66	36.69
	135	Beta-thalassemia		HBB	30/01/2016	04/02/2016	ICSI	Euploid	D5Bφ6B-C	35.66	36.69
	136	Beta-thalassemia		HBB	30/01/2016	04/02/2016	ICSI	Euploid	D5Bφ6B+A	35.66	36.69
	137	Beta-thalassemia		HBB	30/01/2016	04/02/2016	ICSI	Euploid	D5Bφ6B+B+	35.66	36.69

19/11/2020	140	AMA, poor ovarian reserve			27/10/2019	01/11/2019	IMSI	Abnormal: -22	D5Bφ6AB+	40.25	39.60
	141	AMA, poor ovarian reserve			23/09/2019	01/11/2019	IMSI	Complex Abnormal: +19, -20, +21	D5Bφ6B-B-	40.16	39.51
	142	AMA, poor ovarian reserve			27/10/2019	01/11/2019	IMSI	Abnormal: +16	D5Bφ6B+C	40.25	39.60
	143	AMA, poor ovarian reserve			23/09/2019	01/11/2019	IMSI	Complex Abnormal: -4, -10, -21	D5Bφ6B+B-	40.16	39.51
	144	AMA, poor ovarian reserve			17/01/2020	25/02/2020	IMSI	Abnormal: +15	D5Bφ6B-B-	40.47	39.83

1: PGT: Preimplantation genetic testing. 2: ICSI: Intracytoplasmic sperm injection. 3: NA: Not available data. 4: AMA: Advanced maternal age. 5: IVF: In vitro fertilization, 6: IMCI: Intracytoplasmic morphologically-selected sperm injection. Highlighted cases are from frozen oocytes.

Appendix4

Baseline information

Sample	Aneuploidy status	Aneuploid Subgrouping1	Aneuploid Subgrouping2	Maternal age	Paternal age	Trigger	TE morphology	Ovarian stimulation dose	Sperm motility	Sperm morphology	Sperm count	Sperm concentration	PGT indication
100_S22_R1_001	Euploid	Euploid	Euploid	A	A	hCG trigger	B	High	Normal	Normal	Normal	Normal	PGT-M
101_S23_R1_001	Aneuploid	Segmental	Segmental	B	A	Dual trigger (hCG/suprefact)	B	Medium	Abnormal	Normal	Normal	Normal	PGT-M
102_S24_R1_001	Euploid	Euploid	Euploid	B	A	Dual trigger (hCG/suprefact)	B	Medium	Abnormal	Normal	Normal	Normal	PGT-M
103_S25_R1_001	Aneuploid	Complex	Complex	C	B	hCG trigger	C	High	Abnormal	Abnormal	Normal	Normal	PGT-A
104_S26_R1_001	Aneuploid	Complex	Complex	C	B	hCG trigger	B	High	Abnormal	Abnormal	Normal	Normal	PGT-A
105_S27_R1_001	Aneuploid	Single	Gain	C	A	Dual trigger (hCG/suprefact)	B	Medium	Abnormal	Normal	Normal	Normal	PGT-A
106_S28_R1_001	Aneuploid	Single	Loss	C	A	Dual trigger (hCG/suprefact)	C	Medium	Abnormal	Normal	Normal	Normal	PGT-A
107_S29_R1_001	Aneuploid	Single	Gain	C	A	Dual trigger (hCG/suprefact)	B	Medium	Abnormal	Normal	Normal	Normal	PGT-A

109_S30_R1_001	Euploid	Euploid	Euploid	A	A	GnRHa (suprefact) trigger	B	Low	Normal	Normal	Normal	Normal	PGT-M
11_S1_R1_001	Aneuploid	Complex	Loss	C	A	hCG trigger	B	High	Abnormal	Normal	Abnormal	Normal	NA
110_S31_R1_001	Euploid	Euploid	Euploid	A	A	GnRHa (suprefact) trigger	A	Low	Normal	Normal	Normal	Normal	PGT-M
111_S32_R1_001	Euploid	Euploid	Euploid	A	A	GnRHa (suprefact) trigger	B	Low	Normal	Normal	Normal	Normal	PGT-M
112_S33_R1_001	Euploid	Euploid	Euploid	A	A	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
113_S34_R1_001	Aneuploid	Complex	Loss	A	A	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
115_S35_R1_001	Aneuploid	Single	Loss	A	A	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
116_S36_R1_001	Aneuploid	Single	Loss	A	A	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
117_S37_R1_001	Aneuploid	Single	Gain	A	A	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
118_S38_R1_001	Aneuploid	Segmental	Segmental	A	A	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
119_S39_R1_001	Aneuploid	Complex	Loss	B	B	hCG trigger	C	High	Abnormal	Normal	Normal	Normal	PGT-A
12_S2_R1_001	Aneuploid	Single	Loss	C	A	hCG trigger	B	High	Abnormal	Normal	Abnormal	Normal	NA
120_S40_R1_001	Aneuploid	Single	Loss	B	B	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	PGT-A

123_S41_R1_001	Aneuploid	Single	Segmental	B	B	hCG trigger	B	Medium	Abnormal	Normal	Normal	Normal	PGT-A
124_S42_R1_001	Aneuploid	Single	Loss	B	B	hCG trigger	B	Medium	Abnormal	Normal	Normal	Normal	PGT-A
125_S43_R1_001	Aneuploid	Single	Gain	B	B	hCG trigger	A	Medium	Abnormal	Normal	Normal	Normal	PGT-A
126_S44_R1_001	Aneuploid	Single	Loss	B	A	Dual trigger (hCG/suprefact)	B	High	Abnormal	Normal	Normal	Normal	PGT-A
127_S45_R1_001	Aneuploid	Segmental	Segmental	B	A	Dual trigger (hCG/suprefact)	B	High	Abnormal	Normal	Normal	Normal	PGT-A
128_S46_R1_001	Aneuploid	Complex	Complex	C	C	hCG trigger	C	High	Abnormal	Abnormal	Abnormal	Abnormal	PGT-A
13_S3_R1_001	Aneuploid	Complex	Complex	C	A	hCG trigger	B	High	Abnormal	Normal	Abnormal	Normal	NA
132_S47_R1_001	Euploid	Euploid	Euploid	A	A	NA	B	NA	Abnormal	Abnormal	Normal	Normal	NA
134_S48_R1_001	Euploid	Euploid	Euploid	B	A	Dual trigger (hCG/suprefact)	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
135_S49_R1_001	Euploid	Euploid	Euploid	B	A	Dual trigger (hCG/suprefact)	C	Low	Abnormal	Normal	Normal	Normal	PGT-M
136_S50_R1_001	Euploid	Euploid	Euploid	B	A	Dual trigger (hCG/suprefact)	A	Low	Abnormal	Normal	Normal	Normal	PGT-M

137_S51_R1_001	Euploid	Euploid	Euploid	B	A	Dual trigger (hCG/suprefact)	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
139_S52_R1_001	Aneuploid	Single	Gain	A	A	hCG trigger	B	High	Abnormal	Normal	Abnormal	Normal	NA
14_S4_R1_001	Aneuploid	Single	Gain	C	A	hCG trigger	C	High	Abnormal	Normal	Abnormal	Normal	NA
140_S53_R1_001	Aneuploid	Single	Loss	B	A	hCG trigger	B	Very low	Abnormal	Abnormal	Normal	Normal	PGT-A
141_S54_R1_001	Aneuploid	Complex	Complex	B	A	hCG trigger	B	Very low	Abnormal	Normal	Normal	Normal	PGT-A
142_S55_R1_001	Aneuploid	Single	Gain	B	A	hCG trigger	C	Very low	Abnormal	Abnormal	Normal	Normal	PGT-A
143_S56_R1_001	Aneuploid	Complex	Loss	B	A	hCG trigger	B	Very low	Abnormal	Normal	Normal	Normal	PGT-A
144_S57_R1_001	Aneuploid	Single	Gain	B	A	hCG trigger	B	Very low	Abnormal	Normal	Normal	Normal	PGT-A
148_S58_R1_001	Euploid	Euploid	Euploid	B	A	hCG trigger	C	Medium	Abnormal	Normal	Normal	Normal	PGT-M
15_S5_R1_001	Euploid	Euploid	Euploid	A	A	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	PGT-M
16_S6_R1_001	Euploid	Euploid	Euploid	A	A	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	PGT-M
17_S7_R1_001	Euploid	Euploid	Euploid	A	A	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	PGT-M
18_S8_R1_001	Euploid	Euploid	Euploid	A	A	hCG trigger	B	Low	Abnormal	Abnormal	Abnormal	Abnormal	PGT-A
19_S9_R1_001	Euploid	Euploid	Euploid	A	A	hCG trigger	B	High	Normal	Normal	Normal	Normal	PGT-M

1C_S3_R1_001	Aneuploid	Complex	Complex	B	A	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	NA
1D_S4_R1_001	Aneuploid	Single	Gain	A	A	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
1E_S5_R1_001	Aneuploid	Single	Gain	A	A	hCG trigger	B	Medium	Abnormal	Normal	Normal	Normal	PGT-M
1F_S6_R1_001	Euploid	Euploid	Euploid	A	A	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
1G_S7_R1_001	Euploid	Euploid	Euploid	A	A	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
20_S10_R1_001	Euploid	Euploid	Euploid	A	A	hCG trigger	B	Medium	Abnormal	Normal	Normal	Normal	PGT-M
2A_S9_R1_001	Aneuploid	Single	Gain	A	B	hCG trigger	B	Medium	Abnormal	Normal	Abnormal	Abnormal	PGT-M
30_S11_R1_001	Aneuploid	Single	Gain	A	B	hCG trigger	B	Medium	Abnormal	Normal	Abnormal	Abnormal	PGT-M
31_S12_R1_001	Aneuploid	Complex	Complex	B	A	hCG trigger	C	High	Abnormal	Normal	Normal	Normal	PGT-A
32_S13_R1_001	Aneuploid	Complex	Loss	B	A	hCG trigger	C	High	Abnormal	Normal	Normal	Normal	PGT-A
33_S14_R1_001	Aneuploid	Complex	Complex	B	A	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	PGT-A
34_S15_R1_001	Aneuploid	Segmental	Segmental	A	A	GnRHa (suprefact) trigger	B	High	Abnormal	Normal	Abnormal	Abnormal	NA
35_S16_R1_001	Aneuploid	Complex	Loss	A	A	hCG trigger	C	High	Abnormal	Normal	Normal	Normal	NA
36_S17_R1_001	Aneuploid	Complex	Complex	B	B	hCG trigger	B	High	Abnormal	Normal	Abnormal	Normal	NA

37_S18_R1_001	Aneuploid	Complex	Complex	A	A	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	NA
38_S19_R1_001	Aneuploid	Complex	Complex	C	B	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	NA
39_S20_R1_001	Aneuploid	Complex	Complex	C	B	hCG trigger	C	High	Abnormal	Normal	Normal	Normal	NA
40_S21_R1_001	Aneuploid	Complex	Loss	C	B	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	NA
41_S22_R1_001	Aneuploid	Single	Loss	C	A	hCG trigger	B	Very low	Abnormal	Normal	Abnormal	Normal	NA
42_S23_R1_001	Aneuploid	Single	Loss	C	A	hCG trigger	B	Very low	Abnormal	Normal	Abnormal	Normal	NA
43_S24_R1_001	Aneuploid	Complex	Complex	C	A	hCG trigger	B	Very low	Abnormal	Normal	Abnormal	Normal	NA
44_S25_R1_001	Aneuploid	Complex	Complex	C	C	hCG trigger	B	High	Abnormal	Normal	Abnormal	Normal	PGT-M
45_S26_R1_001	Aneuploid	Complex	Loss	B	C	hCG trigger	B	High	Abnormal	Normal	Abnormal	Normal	PGT-M
46_S27_R1_001	Aneuploid	Complex	Complex	B	A	GnRHa (suprefact) trigger	B	Medium	Abnormal	Normal	Abnormal	Normal	MF
47_S28_R1_001	Aneuploid	Complex	Gain	B	A	GnRHa (suprefact) trigger	B	Medium	Abnormal	Normal	Abnormal	Normal	MF
48_S29_R1_001	Aneuploid	Complex	Complex	B	A	GnRHa (suprefact) trigger	B	Medium	Abnormal	Normal	Abnormal	Normal	MF
49_S30_R1_001	Aneuploid	Single	Gain	B	A	hCG trigger	C	High	Abnormal	Abnormal	Abnormal	Normal	PGT-A

50_S31_R1_001	Aneuploid	Complex	Complex	B	A	hCG trigger	B	High	Abnormal	Normal	Abnormal	Abnormal	PGT-A
51_S32_R1_001	Aneuploid	Single	Loss	C	C	hCG trigger	B	High	Abnormal	Normal	Abnormal	Normal	PGT-A
52_S33_R1_001	Aneuploid	Complex	Complex	C	C	hCG trigger	B	High	Abnormal	Normal	Abnormal	Normal	PGT-A
53_S34_R1_001	Aneuploid	Single	Loss	B	B	hCG trigger	B	High	Abnormal	Abnormal	Normal	Normal	PGT-A
54_S35_R1_001	Aneuploid	Single	Gain	B	B	hCG trigger	C	High	Abnormal	Abnormal	Normal	Normal	PGT-A
55_S36_R1_001	Aneuploid	Complex	Gain	B	B	hCG trigger	C	High	Abnormal	Abnormal	Normal	Normal	PGT-A
56_S37_R1_001	Aneuploid	Complex	Gain	B	B	hCG trigger	B	High	Abnormal	Abnormal	Normal	Normal	PGT-A
57_S38_R1_001	Aneuploid	Complex	Gain	C	A	hCG trigger	B	High	Abnormal	Normal	Abnormal	Abnormal	PGT-A
58_S39_R1_001	Aneuploid	Single	Loss	C	A	hCG trigger	B	High	Abnormal	Normal	Abnormal	Abnormal	PGT-A
59_S40_R1_001	Aneuploid	Complex	Complex	C	A	hCG trigger	C	High	Abnormal	Normal	Abnormal	Abnormal	PGT-A
60_S41_R1_001	Aneuploid	Single	Loss	B	B	hCG trigger	C	High	Abnormal	Abnormal	Abnormal	Abnormal	PGT-A
61_S42_R1_001	Aneuploid	Single	Loss	B	B	hCG trigger	B	High	Abnormal	Abnormal	Abnormal	Abnormal	PGT-A
62_S43_R1_001	Aneuploid	Complex	Complex	B	B	hCG trigger	C	High	Abnormal	Abnormal	Abnormal	Abnormal	PGT-A
63_S44_R1_001	Aneuploid	Single	Loss	B	B	hCG trigger	B	High	Abnormal	Abnormal	Abnormal	Abnormal	PGT-A

64_S45_R1_001	Aneuploid	Complex	Loss	B	B	hCG trigger	C	High	Abnormal	Abnormal	Abnormal	Abnormal	PGT-A
65_S46_R1_001	Aneuploid	Complex	Complex	B	B	hCG trigger	C	High	Abnormal	Abnormal	Abnormal	Abnormal	PGT-A
66_S47_R1_001	Aneuploid	Complex	Complex	A	A	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	PGT-M
67_S48_R1_001	Aneuploid	Single	Loss	A	A	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	PGT-M
68_S49_R1_001	Aneuploid	Single	Loss	A	A	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	PGT-M
69_S50_R1_001	Aneuploid	Single	Loss	B	C	hCG trigger	C	High	NA	NA	NA	NA	MF
70_S51_R1_001	Aneuploid	Single	Gain	B	C	hCG trigger	A	Low	NA	NA	NA	NA	MF
71_S52_R1_001	Aneuploid	Complex	Complex	A	A	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
72_S53_R1_001	Aneuploid	Single	Gain	A	A	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
73_S54_R1_001	Aneuploid	Complex	Gain	C	B	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	NA
74_S55_R1_001	Aneuploid	Single	Gain	C	B	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	NA
75_S56_R1_001	Aneuploid	Single	Loss	C	B	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	NA
76_S57_R1_001	Aneuploid	Complex	Complex	C	B	hCG trigger	B	Very low	Abnormal	Abnormal	Abnormal	Abnormal	NA
77_S58_R1_001	Aneuploid	Complex	Gain	C	B	hCG trigger	C	Very low	Abnormal	Abnormal	Abnormal	Abnormal	NA

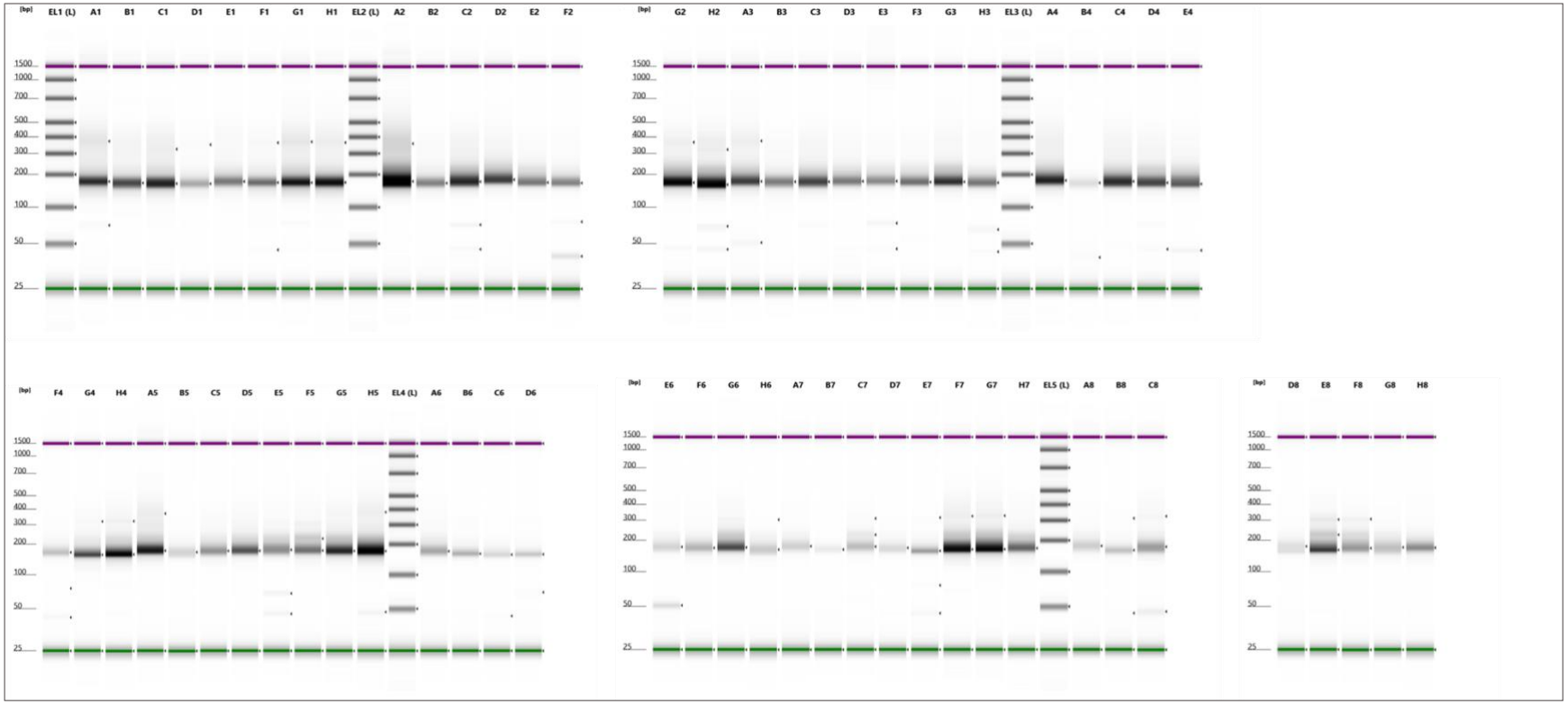
78_S1_R1_001	Aneuploid	Complex	Complex	C	B	hCG trigger	B	Very low	Abnormal	Abnormal	Abnormal	Abnormal	NA
79_S2_R1_001	Aneuploid	Complex	Complex	C	B	hCG trigger	B	Very low	Abnormal	Abnormal	Abnormal	Abnormal	NA
80_S3_R1_001	Aneuploid	Single	Gain	C	B	hCG trigger	B	High	Abnormal	Abnormal	Abnormal	Abnormal	NA
81_S4_R1_001	Aneuploid	Single	Loss	A	A	GnRHa (suprefact) trigger	B	Low	Abnormal	Abnormal	Normal	Normal	PGT-M
82_S5_R1_001	Aneuploid	Complex	Gain	A	A	GnRHa (suprefact) trigger	B	Low	Abnormal	Abnormal	Normal	Normal	PGT-M
83_S6_R1_001	Aneuploid	Single	Loss	A	A	GnRHa (suprefact) trigger	B	Low	Abnormal	Abnormal	Normal	Normal	PGT-M
84_S7_R1_001	Aneuploid	Single	Gain	A	A	GnRHa (suprefact) trigger	C	Low	Abnormal	Abnormal	Normal	Normal	PGT-M
85_S8_R1_001	Aneuploid	Single	Loss	A	A	GnRHa (suprefact) trigger	C	Low	Abnormal	Abnormal	Normal	Normal	PGT-M
86_S9_R1_001	Aneuploid	Single	Gain	A	A	GnRHa (suprefact) trigger	C	Low	Abnormal	Abnormal	Normal	Normal	PGT-M
87_S10_R1_001	Aneuploid	Segmental	Segmental	A	A	GnRHa (suprefact) trigger	C	Low	Abnormal	Abnormal	Normal	Normal	PGT-M

88_S11_R1_001	Aneuploid	Complex	Loss	A	A	GnRHa (suprefact) trigger	B	Low	Abnormal	Abnormal	Normal	Normal	PGT-M
89_S12_R1_001	Aneuploid	Complex	Loss	A	A	hCG trigger	C	High	Normal	Normal	Normal	Normal	PGT-M
90_S13_R1_001	Aneuploid	Complex	Gain	A	A	hCG trigger	B	Medium	NA	NA	NA	NA	PGT-SR
91_S14_R1_001	Aneuploid	Complex	Loss	C	A	GnRHa (suprefact) trigger	B	High	Abnormal	Abnormal	Abnormal	Abnormal	PGT-A
92_S15_R1_001	Euploid	Euploid	Euploid	B	B	GnRHa (suprefact) trigger	B	Medium	Abnormal	Normal	Normal	Normal	PGT-M
93_S16_R1_001	Euploid	Euploid	Euploid	B	B	GnRHa (suprefact) trigger	B	Medium	Abnormal	Normal	Normal	Normal	PGT-M
95_S17_R1_001	Euploid	Euploid	Euploid	B	A	NA	B	NA	Abnormal	Normal	Normal	Normal	PGT-SR
96_S18_R1_001	Aneuploid	Complex	Complex	A	A	hCG trigger	B	Low	Abnormal	Normal	Normal	Normal	PGT-M
97_S19_R1_001	Euploid	Euploid	Euploid	B	A	hCG trigger	B	Medium	Normal	Normal	Normal	Normal	PGT-SR
98_S20_R1_001	Euploid	Euploid	Euploid	B	A	hCG trigger	B	Medium	Normal	Normal	Normal	Normal	PGT-SR
99_S21_R1_001	Euploid	Euploid	Euploid	C	B	hCG trigger	B	High	Abnormal	Normal	Normal	Normal	PGT-A

Appendix5

QC reports

miRNA QC by TapeStation - Patch 1: High Sensitivity D1000 ScreenTape®

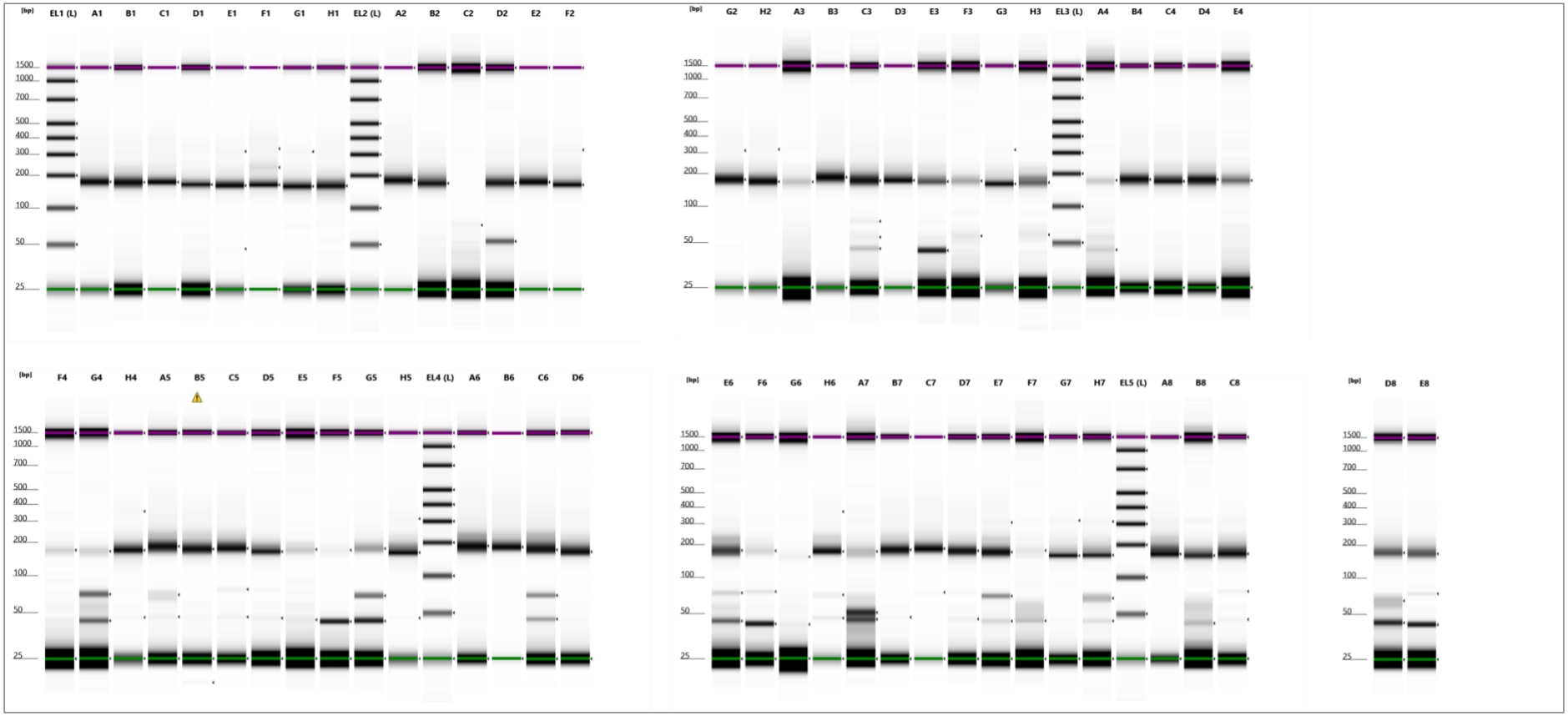


TapeStation Analysis Software 3.2 © Agilent Technologies, Inc. 2019 Generated: 11-May-2021

Patch 1- Trace information

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
EL1	2350	Electronic Ladder		Ladder
A1	1420	11		
B1	1070	12		
C1	1570	13		
D1	385	14		
E1	717	15		
F1	746	16		
G1	1840	17		
H1	1980	18		
EL2	2350	Electronic Ladder		Ladder
A2	3730	19		
B2	597	20		
C2	1660	30		
D2	1220	31		
E2	715	32		
F2	643	33		
G2	2190	34		
H2	2530	35		
A3	1240	36		
B3	678	37		
C3	1190	38		
D3	730	39		
E3	586	40		
F3	746	41		
G3	1330	42		
H3	769	43		
EL3	2350	Electronic Ladder		Ladder
A4	1700	44		
B4	151	45		
C4	1570	46		
D4	1210	47		
E4	1100	48		
F4	300	49		
G4	964	50		
H4	1910	51		
A5	1720	52		
B5	222	53		
C5	578	54		
D5	1000	55		
E5	861	56		
F5	1040	57		
G5	1450	58		
H5	2770	59		
EL4	2350	Electronic Ladder		Ladder
A6	443	60		
B6	284	61		
C6	156	62		
D6	183	63		
E6	321	64		
F6	406	65		
G6	1100	66		
H6	303	67		
A7	226	68		
B7	67.8	69		
C7	419	70		
D7	169	71		
E7	372	72		
F7	2020	73		
G7	2070	74		
H7	1010	75		
EL5	2350	Electronic Ladder		Ladder
A8	253	76		
B8	242	77		
C8	657	78		
D8	121	79		
E8	1440	80		
F8	763	81		
G8	393	82		
H8	598	83		

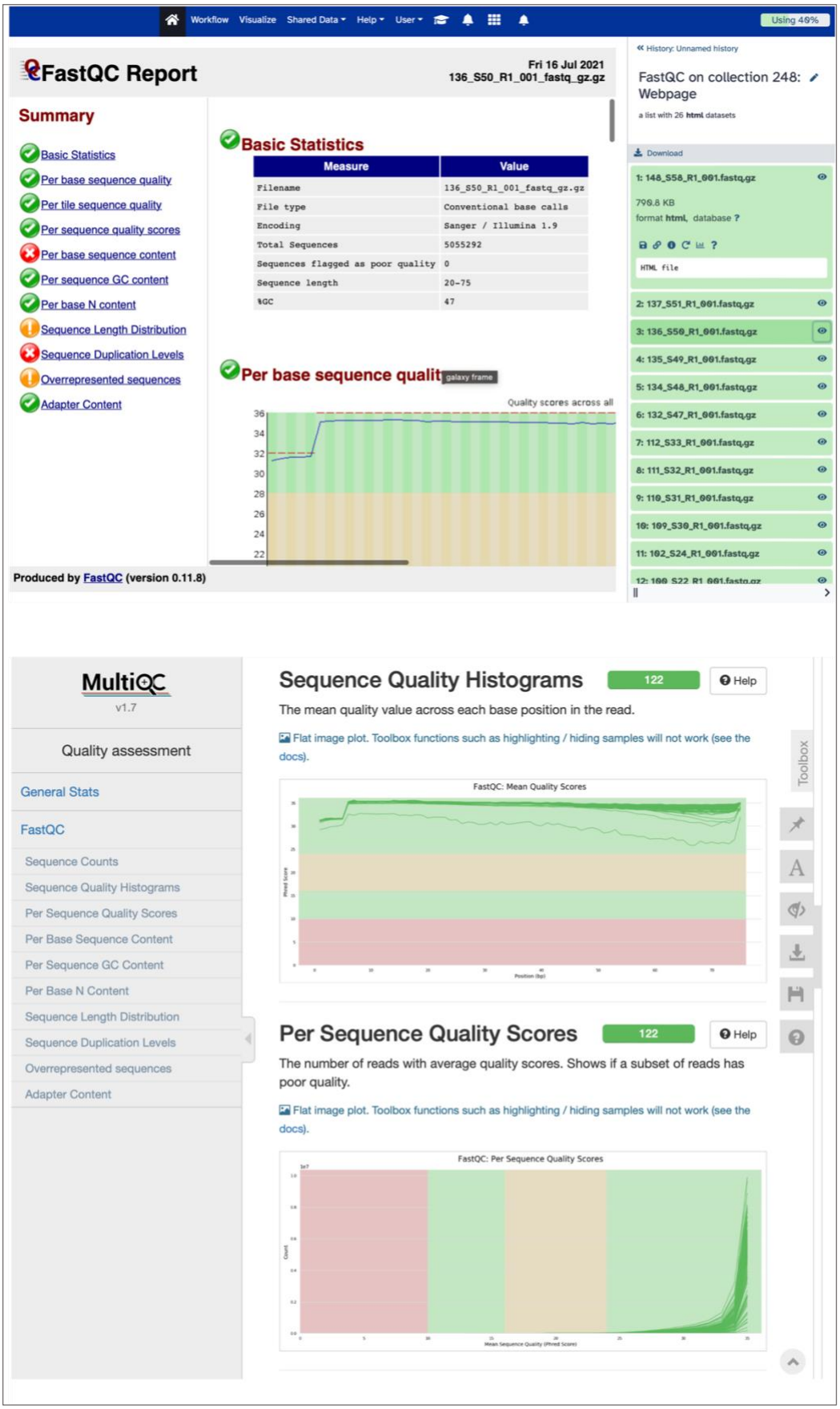
miRNA QC by TapeStation - Patch 2: High Sensitivity D1000 ScreenTape® Page 1



Patch 2 - Trace information

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
EL1	2350	Electronic Ladder		Ladder
A1	693	84		
B1	214	85		
C1	1300	86		
D1	111	87		
E1	602	88		
F1	3360	89		
G1	352	90		
H1	302	91		
EL2	2350	Electronic Ladder		Ladder
A2	1290	92		
B2	91.0	93		
C2	0.703	94		
D2	142	95		
E2	1370	96		
F2	1550	97		
G2	1040	98		
H2	690	99		
A3	9.42	100		
B3	656	101		
C3	162	102		
D3	938	103		
E3	93.5	104		
F3	19.0	105		
G3	357	106		
H3	36.9	107		
EL3	2350	Electronic Ladder		Ladder
A4	14.6	108		
B4	264	109		
C4	120	110		
D4	335	111		
E4	25.1	112		
F4	6.39	113		
G4	54.5	114		
H4	492	115		
A5	310	116		
B5	247	117	⚠	Peak out of Sizing Range
C5	332	118		
D5	106	119		
E5	9.90	120		
F5	60.9	121		
G5	167	122		
H5	582	123		
EL4	2350	Electronic Ladder		Ladder
A6	416	124		
B6	3320	125		
C6	345	126		
D6	244	127		
E6	75.4	128		
F6	117	130		
G6	4.09	131		
H6	1080	132		
A7	129	133		
B7	289	134		
C7	1400	135		
D7	194	136		
E7	246	137		
F7	11.8	138		
G7	171	139		
H7	152	140		
EL5	2350	Electronic Ladder		Ladder
A8	516	141		
B8	53.7	142		
C8	247	143		
D8	132	144		
E8	142	148		

Sequencing QC reports



Sequencing CQ results troubleshooting:

Due to a fragmentation bias, almost all RNA sequencing libraries fail the quality per base sequencing parameter, which is not a problem that affects the expression level. The high duplication level in the results is also common in RNA sequencing libraries, which sequence different RNAs with different starting levels. Therefore, to visualise the lowly expressed RNAs, it is common for the highly expressed transcripts to be over-sequenced, which generates this duplication error. The overrepresented sequencing warning was not existed in all the samples. For the ones that had it, there were no biological hits for the detected sequences. This parameter is commonly triggered in small RNA libraries. The sequence read length warning in this quality check can be ignored as the platform used is expected to have different RNA lengths.

Appendix6

Top 100 miRNAs in all samples

miRNA	Average	miRNA	Median
hsa-miR-3168	205.3228623	hsa-miR-3168	42.71603545
hsa-miR-625-3p	74.5699961	hsa-miR-16-5p	39.38956503
hsa-miR-16-5p	61.71123471	hsa-miR-3613-3p	33.57664603
hsa-miR-371a-5p	61.2407065	hsa-miR-4501	31.49244046
hsa-miR-372-3p	47.88195106	hsa-miR-626	31.16592493
hsa-miR-7155-5p	47.60323765	hsa-miR-3974	28.93351044
hsa-miR-92a-3p	44.85514549	hsa-miR-7155-5p	26.95814134
hsa-miR-626	43.15194004	hsa-miR-371a-5p	26.89206761
hsa-miR-4501	42.19313625	hsa-miR-6749-5p	26.44345398
hsa-miR-3613-3p	41.29791886	hsa-miR-3170	25.64611878
hsa-miR-3170	41.09495856	hsa-miR-7-5p	24.73540356
hsa-miR-141-5p	38.43391786	hsa-miR-5189-3p	24.69797533
hsa-miR-7-5p	37.55052523	hsa-miR-4782-5p	24.57238695
hsa-miR-3974	36.76218802	hsa-miR-372-3p	24.39675067
hsa-miR-3151-5p	35.70262143	hsa-miR-6728-5p	23.25140911
hsa-miR-7853-5p	35.5242635	hsa-let-7d-3p	22.82428075
hsa-miR-4772-5p	35.22044649	hsa-miR-92a-3p	22.36854897
hsa-miR-5189-3p	33.29442563	hsa-miR-889-5p	21.92397049
hsa-miR-944	32.73394886	hsa-miR-92b-5p	21.52913999
hsa-miR-4307	32.52305019	hsa-miR-373-3p	20.69461199
hsa-miR-6749-5p	30.39931151	hsa-miR-944	20.00509703
hsa-miR-4782-5p	29.78559829	hsa-miR-363-5p	19.95982212
hsa-miR-6728-5p	28.41127633	hsa-miR-576-3p	19.89540732
hsa-miR-92b-5p	28.12569907	hsa-miR-6855-3p	19.57287077
hsa-miR-3653-3p	27.82794317	hsa-miR-6785-5p	19.50037856
hsa-miR-8065	27.74685318	hsa-miR-887-3p	19.42597125
hsa-miR-373-3p	27.21244427	hsa-miR-141-5p	18.1633776
hsa-miR-363-5p	25.98989509	hsa-miR-8065	18.06798887
hsa-let-7d-3p	25.53694147	hsa-miR-197-3p	18.02107069
hsa-miR-887-3p	25.51916795	hsa-miR-4456	17.79555411
hsa-miR-889-5p	25.35181571	hsa-miR-606	17.67431664
hsa-miR-4456	23.73858303	hsa-miR-3609	17.36074302
hsa-miR-3609	22.93291558	hsa-miR-29c-5p	16.9200614
hsa-miR-6785-5p	22.76328045	hsa-miR-3182	16.88343954

hsa-miR-548ad-5p	22.73019355	hsa-miR-6756-3p	16.52139464
hsa-miR-606	22.43655221	hsa-miR-5191	15.8830716
hsa-miR-576-3p	22.28065472	hsa-miR-5089-3p	15.59300914
hsa-miR-203a-3p	22.06681541	hsa-miR-1244	15.56343583
hsa-miR-3130-5p	21.64548277	hsa-miR-6717-5p	15.47749043
hsa-miR-6855-3p	21.48143461	hsa-miR-3130-5p	15.22178624
hsa-miR-5191	21.41127754	hsa-miR-4440	14.85074303
hsa-miR-197-3p	21.32758382	hsa-miR-1287-3p	14.65623194
hsa-miR-8054	20.89930919	hsa-miR-148a-5p	14.42753428
hsa-miR-29c-5p	20.76940364	hsa-miR-3617-3p	14.35844389
hsa-miR-371b-3p	20.41854272	hsa-miR-6857-3p	13.8450598
hsa-miR-3182	19.73761064	hsa-miR-203a-3p	13.64210549
hsa-miR-1244	19.65569023	hsa-miR-208b-3p	13.34586005
hsa-miR-1287-3p	19.48623061	hsa-miR-7108-3p	13.2907178
hsa-miR-193b-3p	19.28048253	hsa-miR-18a-5p	13.26684561
hsa-miR-107	19.24599007	hsa-miR-138-2-3p	13.15504193
hsa-miR-6717-5p	19.0162855	hsa-miR-93-5p	13.12016754
hsa-miR-1290	18.8035377	hsa-miR-10b-3p	13.10005594
hsa-miR-34a-5p	18.74441178	hsa-miR-107	12.92242742
hsa-miR-9-5p	18.72897617	hsa-miR-302b-3p	12.91693539
hsa-miR-184	18.45106665	hsa-miR-106b-5p	12.77294072
hsa-miR-5089-3p	18.41300612	hsa-miR-3653-3p	12.68825076
hsa-miR-302b-3p	18.230494	hsa-miR-92a-1-5p	12.42376201
hsa-miR-6756-3p	18.08486305	hsa-miR-4745-3p	12.36709359
hsa-miR-6715b-5p	17.87528819	hsa-miR-8071	12.36077925
hsa-miR-3617-3p	17.86790825	hsa-miR-885-5p	12.2461656
hsa-miR-10b-3p	17.74263591	hsa-miR-1276	12.14628481
hsa-miR-33a-5p	17.51881195	hsa-miR-548ad-5p	12.14433638
hsa-miR-18a-5p	17.51604286	hsa-miR-325	12.08508661
hsa-miR-7152-5p	17.44461424	hsa-miR-512-5p	12.00119011
hsa-miR-208b-3p	17.34287302	hsa-miR-6800-5p	11.97965424
hsa-miR-5695	17.20726498	hsa-miR-3940-3p	11.9519031
hsa-miR-3126-3p	17.19901135	hsa-miR-1290	11.92301831
hsa-miR-1276	17.14586973	hsa-miR-3605-3p	11.89817817
hsa-miR-1909-5p	17.00307223	hsa-miR-664a-5p	11.83638174
hsa-miR-1266-5p	16.98819802	hsa-miR-6793-5p	11.68140208
hsa-miR-512-5p	16.93018032	hsa-miR-6797-5p	11.62067993
hsa-miR-6793-5p	16.84173441	hsa-miR-8072	11.57138284
hsa-miR-1183	16.82753068	hsa-miR-936	11.38609569

hsa-miR-183-5p	16.77930464	hsa-miR-371b-3p	11.36522783
hsa-miR-148a-5p	16.72001245	hsa-miR-6715b-5p	11.35874464
hsa-miR-182-5p	16.66474977	hsa-miR-182-5p	11.35045404
hsa-miR-4440	16.64946252	hsa-miR-184	11.33630337
hsa-miR-885-5p	16.57464267	hsa-miR-4254	11.29078358
hsa-miR-93-5p	16.33032441	hsa-miR-662	11.23198223
hsa-miR-7108-3p	16.04487431	hsa-miR-155-5p	11.11253752
hsa-miR-106b-5p	15.93900822	hsa-miR-34a-5p	11.05058861
hsa-miR-6857-3p	15.67498465	hsa-miR-4290	11.02393609
hsa-miR-325	15.59758327	hsa-miR-1266-5p	11.01842745
hsa-miR-5698	15.44647446	hsa-miR-8054	10.95331121
hsa-miR-515-5p	15.34088872	hsa-miR-6743-3p	10.85638206
hsa-miR-92a-1-5p	15.22864054	hsa-miR-769-5p	10.74826602
hsa-miR-6800-5p	15.04144351	hsa-miR-6752-5p	10.74704229
hsa-miR-4745-3p	14.90395628	hsa-miR-4754	10.74160456
hsa-miR-137	14.8919684	hsa-miR-4655-5p	10.73409112
hsa-miR-155-5p	14.83537963	hsa-miR-6124	10.70087941
hsa-miR-6794-3p	14.77019963	hsa-miR-8069	10.6396297
hsa-miR-7106-3p	14.76560098	hsa-miR-6794-3p	10.62902528
hsa-miR-7855-5p	14.73904093	hsa-miR-4302	10.61797399
hsa-miR-8071	14.68217555	hsa-miR-137	10.58211448
hsa-miR-664a-5p	14.60878685	hsa-miR-3126-3p	10.54334694
hsa-miR-662	14.57942837	hsa-miR-183-5p	10.41022463
hsa-miR-653-3p	14.49159456	hsa-miR-765	10.38861189
hsa-miR-936	14.4883151	hsa-miR-196a-3p	10.26437529
hsa-miR-138-2-3p	14.23577184	hsa-miR-25-3p	10.2622477
hsa-miR-4754	14.13205626	hsa-miR-6721-5p	10.1636875

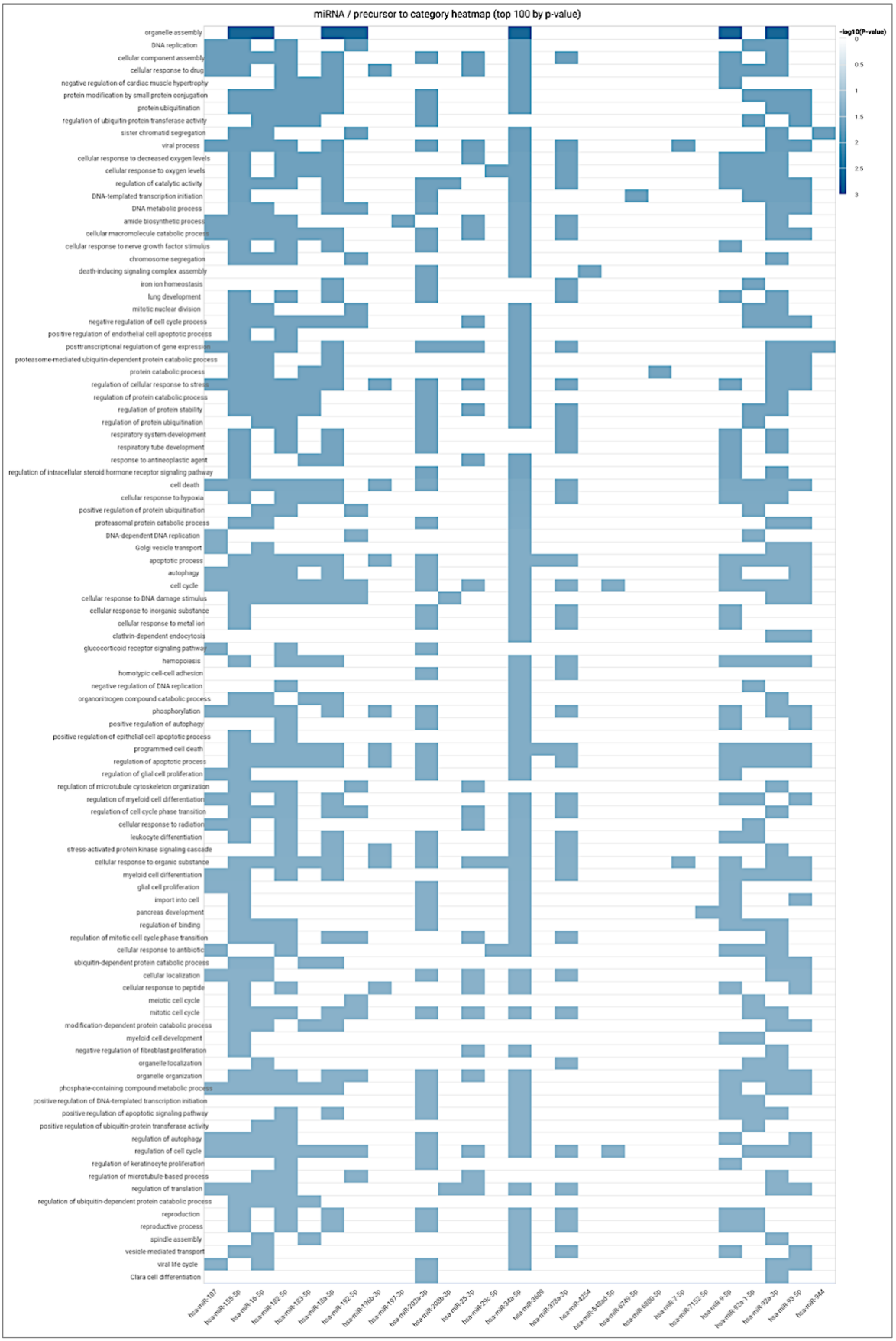

miRNA / precursor to category heatmap (top 100 by p-value)
-log10(P-value)
0
0.5
1
1.5
2
Resolution of Sister Chromatid Cohesion
Pre-NOTCH Expression and Processing
Mitotic Metaphase and Anaphase
Mitotic Anaphase
Metabolism of proteins
Cell Cycle Checkpoints
viral process
ubiquitin-dependent protein catabolic proces
sister chromatid segregation
regulation of ubiquitin-protein transferase activity
regulation of protein homodimerization activity
regulation of protein catabolic process
regulation of cell cycle
proteolysis involved in cellular protein catabolic proces
protein ubiquitination
protein modification by small protein conjugatio
protein catabolic process
proteasome-mediated ubiquitin-dependent protein catabolic pro
proteasomal protein catabolic process
posttranscriptional regulation of gene expressio
positive regulation of epithelial cell apoptotic process
positive regulation of endothelial cell apoptotic process
organonitrogen compound catabolic process
organelle assembly
negative regulation of cell cycle proces
negative regulation of cell cycle
negative regulation of DNA replication
modification-dependent protein catabolic proce
glucocorticoid receptor signaling pathway
death-inducing signaling complex assembly
clathrin-dependent endocytosis
cellular response to oxygen level
cellular response to hypoxia
cellular response to decreased oxygen leve
cellular response to DNA damage stimulu
cellular protein catabolic proces
cellular macromolecule catabolic proces
catabolic process
autophagy
DNA replication
DNA-templated transcription initiation
hsa-miR-1908-5p
hsa-miR-107
hsa-miR-1276
hsa-miR-155-5p
hsa-miR-16-5p
hsa-miR-182-5p
hsa-miR-183-5p
hsa-miR-184
hsa-miR-18a-5p
hsa-miR-203a-3p
hsa-miR-208b-3p
hsa-miR-25-3p
hsa-miR-26c-5p
hsa-miR-3176-3p
hsa-miR-335
hsa-miR-34a-5p
hsa-miR-3613-3p
hsa-miR-363-5p
hsa-miR-3716-3p
hsa-miR-4254
hsa-miR-5481a-5p
hsa-miR-6749-5p
hsa-miR-6752-5p
hsa-miR-6793-5p
hsa-miR-6850-5p
hsa-miR-7-5p
hsa-miR-885-5p
hsa-miR-92a-1-5p
hsa-miR-92a-3p
hsa-miR-93-5p
hsa-miR-944

Top 100 miRNAs in 26 euploid samples

miRNA	Euploid average	miRNA	Euploid median
hsa-miR-3168	113.9078411	hsa-miR-371a-5p	64.28901044
hsa-miR-371a-5p	113.0709123	hsa-miR-16-5p	58.87891682
hsa-miR-16-5p	91.82146175	hsa-miR-7155-5p	48.10814816
hsa-miR-372-3p	82.05884881	hsa-miR-372-3p	46.79198621
hsa-miR-92a-3p	76.6753497	hsa-miR-92a-3p	44.85514549
hsa-miR-7155-5p	65.80268506	hsa-miR-3613-3p	44.05654233
hsa-miR-7-5p	64.60958416	hsa-miR-4501	39.35407396
hsa-miR-4501	50.74716838	hsa-miR-7-5p	37.55052523
hsa-miR-3613-3p	47.90665805	hsa-miR-3168	37.01229759
hsa-miR-3974	46.11086008	hsa-miR-3974	36.86970401
hsa-miR-626	45.14542286	hsa-miR-626	36.54306868
hsa-miR-5189-3p	41.21008199	hsa-miR-141-5p	35.54570445
hsa-miR-141-5p	39.16885081	hsa-miR-3170	34.08779395
hsa-miR-3170	38.10830045	hsa-miR-5189-3p	33.29442563
hsa-miR-373-3p	37.84000989	hsa-miR-6749-5p	28.67882804
hsa-miR-371b-3p	36.16646194	hsa-miR-6728-5p	28.41127633
hsa-miR-5695	35.38713121	hsa-miR-373-3p	27.21244427
hsa-miR-92b-5p	34.91606995	hsa-miR-4456	25.65767902
hsa-miR-193b-3p	34.41300912	hsa-miR-887-3p	25.51916795
hsa-miR-4772-5p	33.13318328	hsa-miR-889-5p	25.40626199
hsa-miR-4782-5p	32.63038725	hsa-let-7d-3p	24.96328597
hsa-miR-6749-5p	32.40956189	hsa-miR-6855-3p	24.79112287
hsa-miR-8054	31.70383621	hsa-miR-4782-5p	24.13325558
hsa-miR-4456	31.61403851	hsa-miR-944	23.93739751
hsa-miR-889-5p	31.37238597	hsa-miR-576-3p	22.28065472
hsa-miR-6728-5p	30.60442535	hsa-miR-8065	22.18945819
hsa-miR-887-3p	29.31733089	hsa-miR-92b-5p	22.02827116
hsa-miR-515-5p	28.4151959	hsa-miR-197-3p	21.22179687
hsa-miR-363-5p	27.87727489	hsa-miR-363-5p	20.51776929
hsa-miR-8065	27.66342815	hsa-miR-371b-3p	20.41854272
hsa-miR-6855-3p	26.30806198	hsa-miR-3609	20.37000042
hsa-miR-6715b-5p	25.87077269	hsa-miR-7108-3p	20.17580647
hsa-miR-944	25.69278903	hsa-miR-3182	20.03165392
hsa-miR-3609	25.21978316	hsa-miR-6785-5p	19.54510968
hsa-miR-93-5p	25.20020151	hsa-miR-6082	19.05922708
hsa-let-7d-3p	25.1158182	hsa-miR-6717-5p	19.0162855

hsa-miR-34a-5p	24.8825965	hsa-miR-1290	18.8035377
hsa-miR-3151-5p	24.4220492	hsa-miR-606	18.59334215
hsa-miR-203a-3p	24.06642846	hsa-miR-148a-5p	17.89551307
hsa-miR-606	23.67667874	hsa-miR-93-5p	17.63965433
hsa-miR-6082	23.66740424	hsa-miR-4440	17.35222258
hsa-miR-6785-5p	23.49539989	hsa-miR-6715b-5p	17.30792734
hsa-miR-6717-5p	23.46390012	hsa-miR-4754	16.8660317
hsa-miR-18a-5p	23.23838915	hsa-miR-6793-5p	16.84173441
hsa-miR-197-3p	23.23291564	hsa-miR-183-5p	16.77930464
hsa-miR-182-5p	23.05562023	hsa-miR-5089-3p	16.7770435
hsa-miR-103b	22.81422163	hsa-miR-7152-5p	16.75295116
hsa-miR-3182	22.45944734	hsa-miR-3130-5p	16.67682369
hsa-miR-378a-3p	22.44084093	hsa-miR-182-5p	16.66474977
hsa-miR-325	22.42994859	hsa-miR-6857-3p	16.6391964
hsa-miR-576-3p	22.22887568	hsa-miR-510-3p	16.22327573
hsa-miR-5191	22.18299693	hsa-miR-3151-5p	16.15455961
hsa-miR-1244	22.0034266	hsa-miR-664a-5p	16.03604939
hsa-miR-3130-5p	21.47667431	hsa-miR-5191	15.89458225
hsa-miR-208b-3p	21.44650997	hsa-miR-6794-3p	15.7344794
hsa-miR-512-5p	21.38125872	hsa-miR-302b-3p	15.7344794
hsa-miR-4440	21.35701481	hsa-miR-6756-3p	15.68661613
hsa-miR-7152-5p	21.25886567	hsa-miR-138-2-3p	15.5512457
hsa-miR-1290	21.22527707	hsa-miR-203a-3p	15.46052906
hsa-miR-7108-3p	20.83416284	hsa-miR-3653-3p	15.46052906
hsa-miR-1287-3p	20.547479	hsa-miR-9-5p	15.44864599
hsa-miR-340-5p	20.37942749	hsa-miR-92a-1-5p	15.44864599
hsa-miR-183-5p	20.37829547	hsa-miR-325	15.32369375
hsa-miR-6800-5p	20.14219063	hsa-miR-512-5p	15.31827645
hsa-miR-6794-3p	20.11049792	hsa-miR-6800-5p	15.04144351
hsa-miR-106b-5p	20.04094223	hsa-miR-4745-3p	14.90395628
hsa-miR-302b-3p	19.99664392	hsa-miR-155-5p	14.83537963
hsa-miR-6857-3p	19.83507036	hsa-miR-7106-3p	14.76560098
hsa-miR-4754	19.73947725	hsa-miR-192-5p	14.44044243
hsa-miR-510-3p	19.57356914	hsa-miR-662	14.43244445
hsa-miR-5089-3p	19.20746542	hsa-miR-1244	14.37755806
hsa-miR-6793-5p	19.14553293	hsa-miR-34a-5p	14.3624385
hsa-miR-625-3p	19.12222051	hsa-miR-18a-5p	14.33444474
hsa-miR-4458	19.10785205	hsa-miR-29c-5p	14.23474676
hsa-miR-9-5p	19.03339303	hsa-miR-515-5p	14.08057894

hsa-miR-146b-5p	18.86458291	hsa-miR-1266-5p	14.0246314
hsa-miR-107	18.83027681	hsa-miR-208b-3p	13.96079263
hsa-miR-4275	18.82197185	hsa-miR-107	13.79132438
hsa-miR-4262	18.67126241	hsa-miR-196b-3p	13.79132438
hsa-miR-6756-3p	18.67024205	hsa-miR-6743-3p	13.77257325
hsa-miR-7106-3p	18.40781375	hsa-miR-302d-3p	13.73333813
hsa-miR-137	18.40488834	hsa-miR-137	13.73137567
hsa-miR-29c-5p	18.37746682	hsa-miR-4254	13.71020376
hsa-miR-148a-5p	18.18114857	hsa-miR-4262	13.69446149
hsa-miR-664a-5p	18.15590144	hsa-miR-3617-3p	13.61833547
hsa-miR-10b-3p	18.14857005	hsa-miR-1287-3p	13.61624573
hsa-miR-25-3p	18.08259855	hsa-miR-3653-5p	13.46837985
hsa-miR-138-2-3p	18.00016414	hsa-miR-769-5p	13.37651282
hsa-miR-4305	17.92321345	hsa-miR-4655-5p	13.26817051
hsa-miR-3653-3p	17.79592982	hsa-miR-25-3p	13.08965597
hsa-miR-92a-1-5p	17.7827795	hsa-miR-8054	12.92364769
hsa-miR-662	17.76151074	hsa-miR-8071	12.91722436
hsa-miR-196b-3p	17.75223336	hsa-miR-3940-3p	12.82555582
hsa-miR-27b-3p	17.7332231	hsa-miR-10b-3p	12.64952377
hsa-miR-3617-3p	17.73118369	hsa-miR-548ad-5p	12.64104138
hsa-miR-5698	17.45016824	hsa-miR-370-3p	12.58758352
hsa-miR-302d-3p	17.42241375	hsa-miR-196a-3p	12.58758352
hsa-miR-155-5p	16.97224423	hsa-miR-378a-3p	12.46712692
hsa-miR-4254	16.90688269	hsa-miR-3675-3p	12.33739266
hsa-miR-370-3p	16.88967194	hsa-miR-6729-3p	12.30316524


miRNA / precursor to category heatmap (top 100 by p-value)
-log10(P-value)
0
0.5
1
1.5
2
2.5
3
organelle assembly
DNA replication
cellular component assembly
cellular response to drug
negative regulation of cardiac muscle hypertrophy
protein modification by small protein conjugation
protein ubiquitination
regulation of ubiquitin-protein transferase activity
sister chromatid segregation
viral process
cellular response to decreased oxygen levels
cellular response to oxygen levels
regulation of catalytic activity
DNA-templated transcription initiation
DNA metabolic process
amide biosynthetic process
cellular macromolecule catabolic process
cellular response to nerve growth factor stimulus
chromosome segregation
death-inducing signaling complex assembly
iron ion homeostasis
lung development
mitotic nuclear division
negative regulation of cell cycle process
positive regulation of endothelial cell apoptotic process
posttranscriptional regulation of gene expression
proteasome-mediated ubiquitin-dependent protein catabolic process
protein catabolic process
regulation of cellular response to stress
regulation of protein catabolic process
regulation of protein stability
regulation of protein ubiquitination
respiratory system development
respiratory tube development
response to antineoplastic agent
regulation of intracellular steroid hormone receptor signaling pathway
cell death
cellular response to hypoxia
positive regulation of protein ubiquitination
proteasomal protein catabolic process
DNA-dependent DNA replication
Golgi vesicle transport
apoptotic process
autophagy
cell cycle
cellular response to DNA damage stimulus
cellular response to inorganic substance
cellular response to metal ion
clathrin-dependent endocytosis
glucocorticoid receptor signaling pathway
hemopoiesis
homotypic cell-cell adhesion
negative regulation of DNA replication
organonitrogen compound catabolic process
phosphorylation
positive regulation of autophagy
positive regulation of epithelial cell apoptotic process
programmed cell death
regulation of apoptotic process
regulation of glial cell proliferation
regulation of microtubule cytoskeleton organization
regulation of myeloid cell differentiation
regulation of cell cycle phase transition
cellular response to radiation
leukocyte differentiation
stress-activated protein kinase signaling cascade
cellular response to organic substance
myeloid cell differentiation
glial cell proliferation
import into cell
pancreas development
regulation of binding
regulation of mitotic cell cycle phase transition
cellular response to antibiotic
ubiquitin-dependent protein catabolic process
cellular localization
cellular response to peptide
meiotic cell cycle
mitotic cell cycle
modification-dependent protein catabolic process
myeloid cell development
negative regulation of fibroblast proliferation
organelle localization
organelle organization
phosphate-containing compound metabolic process
positive regulation of DNA-templated transcription initiation
positive regulation of apoptotic signaling pathway
positive regulation of ubiquitin-protein transferase activity
regulation of autophagy
regulation of cell cycle
regulation of keratinocyte proliferation
regulation of microtubule-based process
regulation of translation
regulation of ubiquitin-dependent protein catabolic process
reproduction
reproductive process
spindle assembly
vesicle-mediated transport
viral life cycle
Clara cell differentiation
hsa-miR-107
hsa-miR-155-5p
hsa-miR-16-5p
hsa-miR-92-5p
hsa-miR-183-5p
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hsa-miR-192-5p
hsa-miR-196b-3p
hsa-miR-197-3p
hsa-miR-203a-3p
hsa-miR-208b-3p
hsa-miR-25-3p
hsa-miR-29c-5p
hsa-miR-34a-5p
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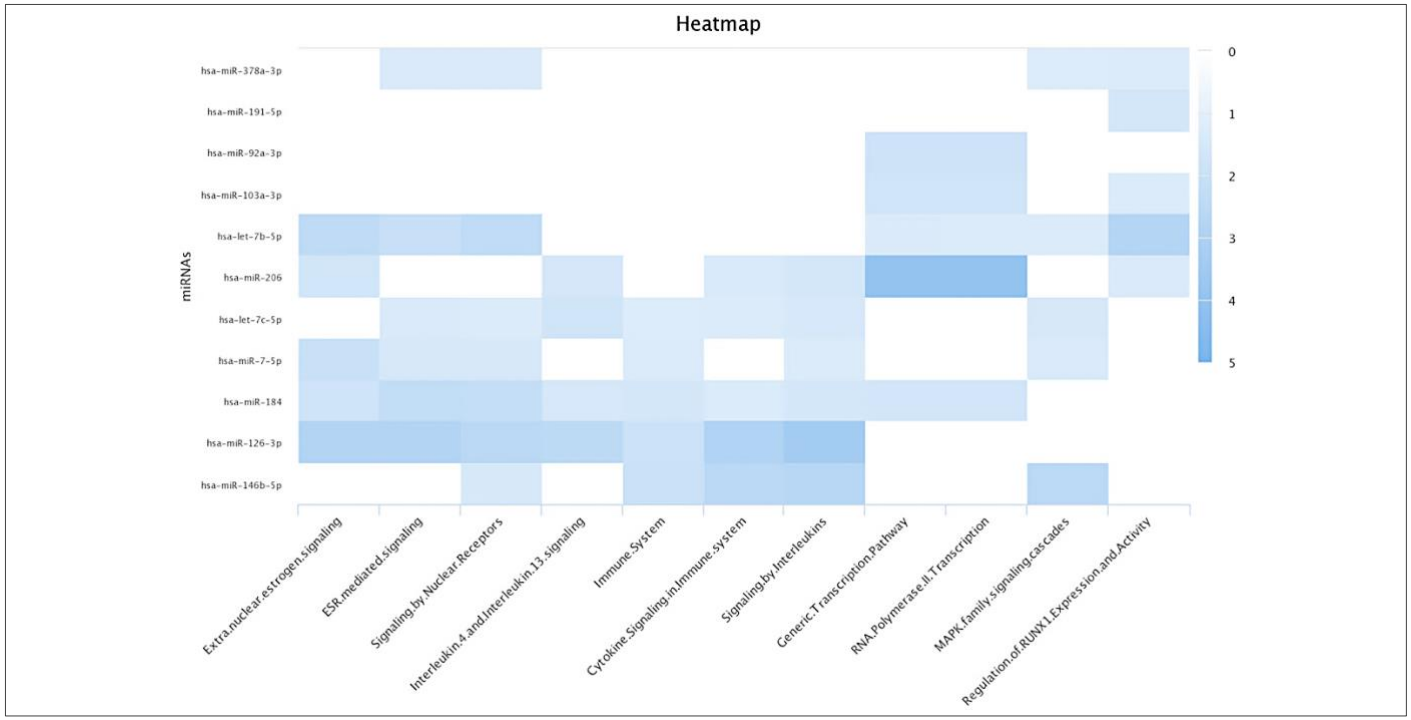
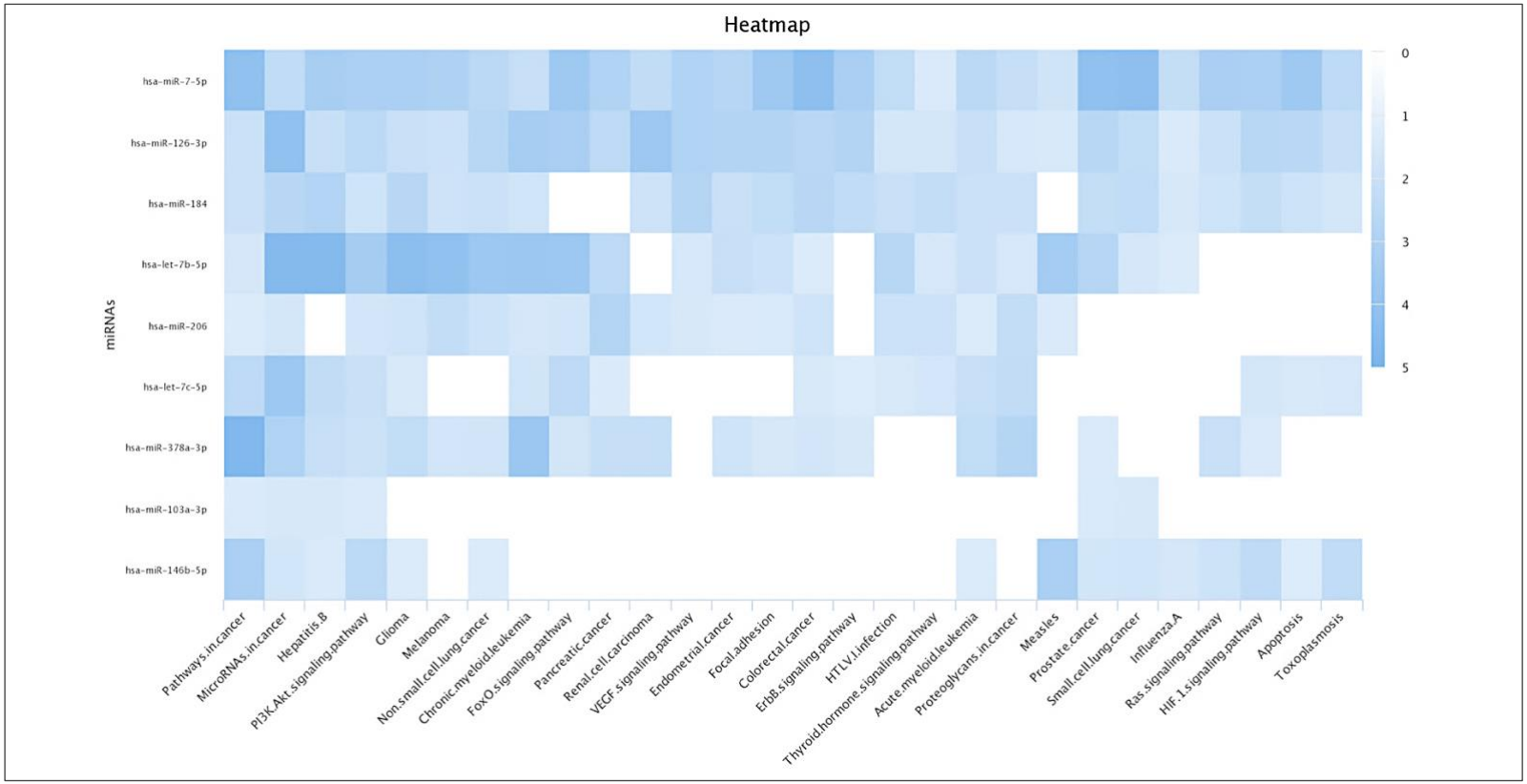
hsa-miR-379a-3p
hsa-miR-4254
hsa-miR-548ad-5p
hsa-miR-6749-5p
hsa-miR-6800-5p
hsa-miR-7-5p
hsa-miR-7152-5p
hsa-miR-9-5p
hsa-miR-92a-1-5p
hsa-miR-92a-3p
hsa-miR-93-5p
hsa-miR-944

Appendix7

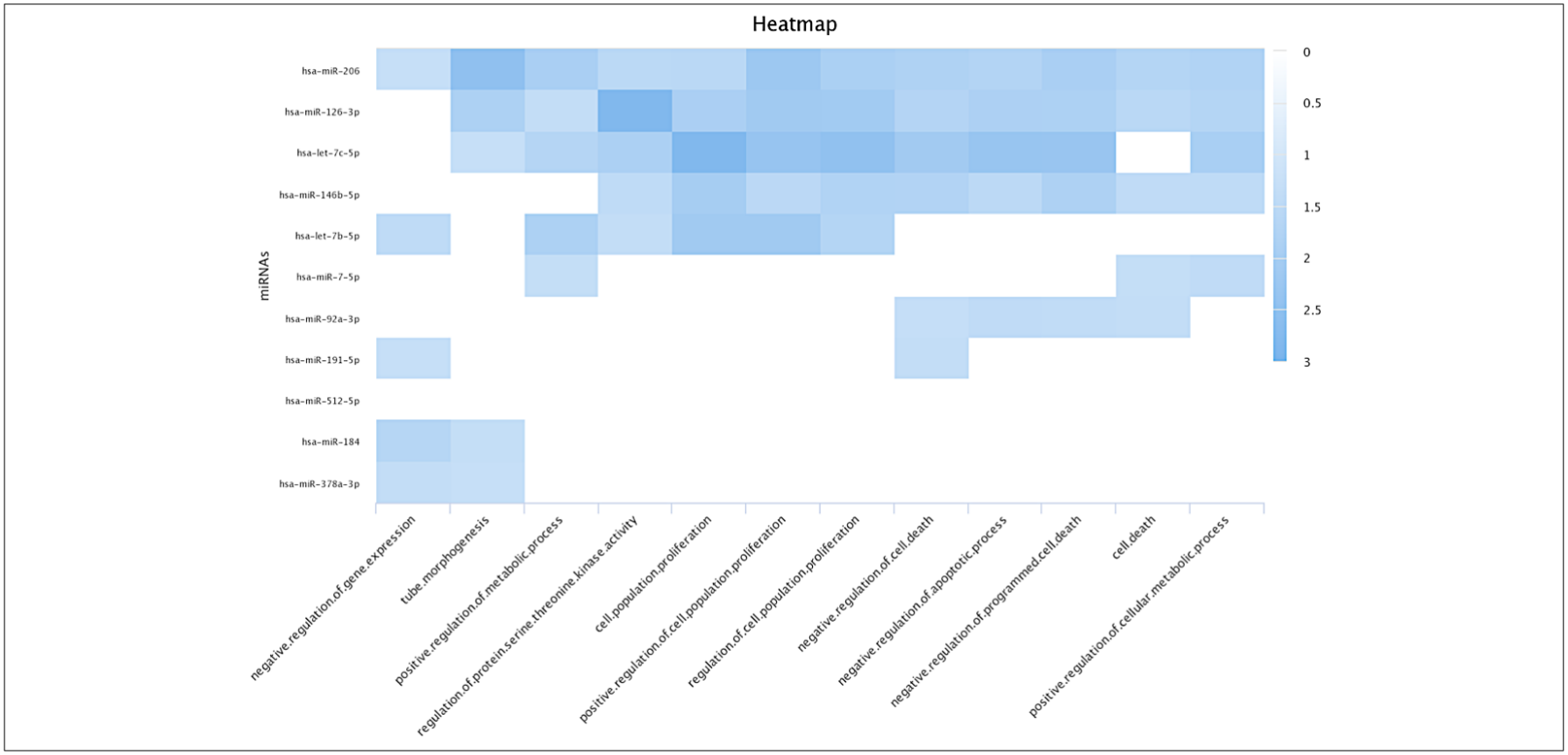
Pathway annotation of aneuploidy and aneuploidy subgroups using Reactome, KEGG, Gene Ontology (GO) biological processes and WikiPathways via miRPathDB v2.0 analysis platform



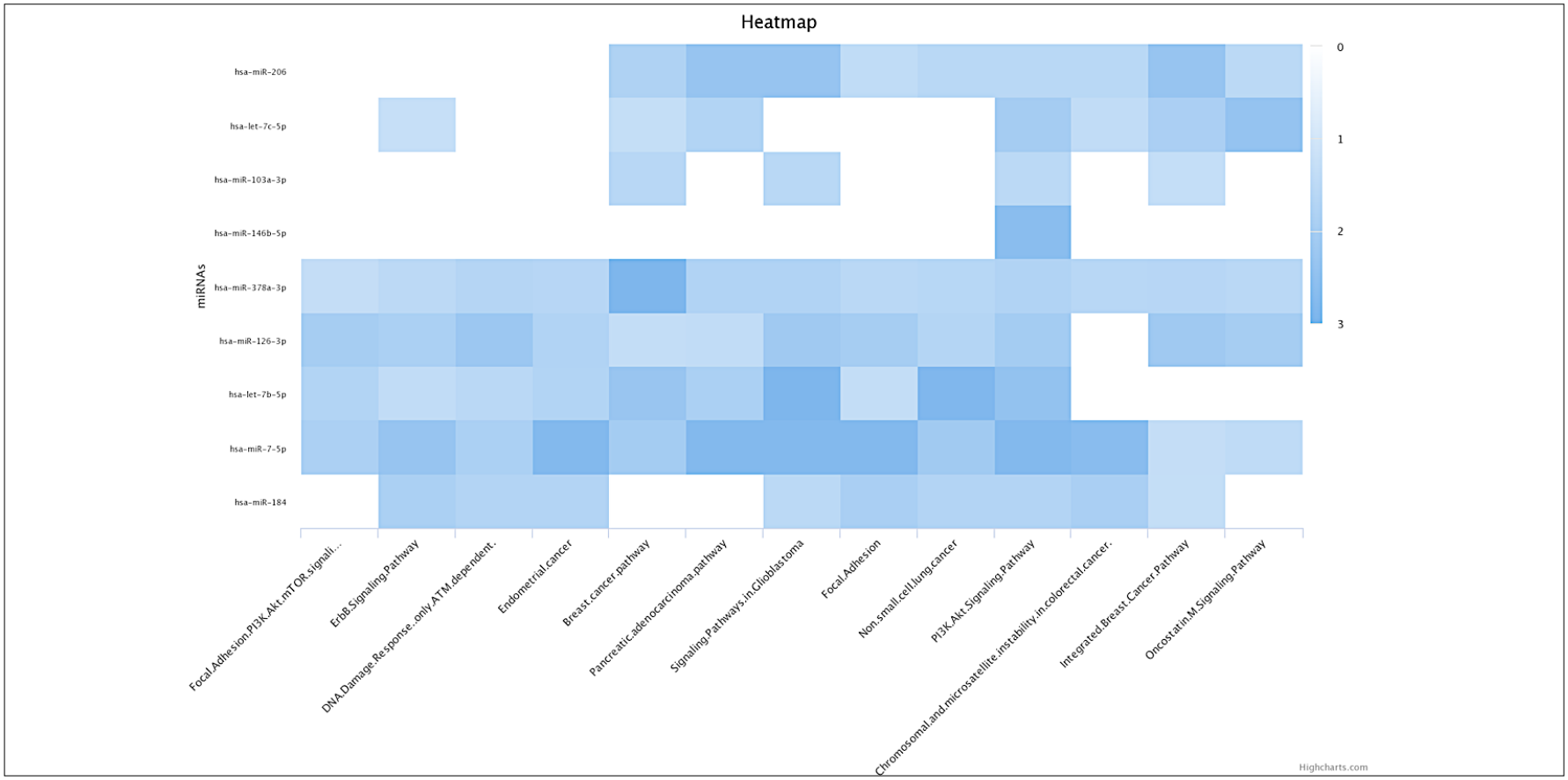
The heatmap illustrates the dysregulated miRNAs in aneuploid blastocysts using Reactome database via miRPathDB v2.0. It reveals significant involvement of these miRNAs in signalling pathways, specifically estorgen interleukin and immune signalling.



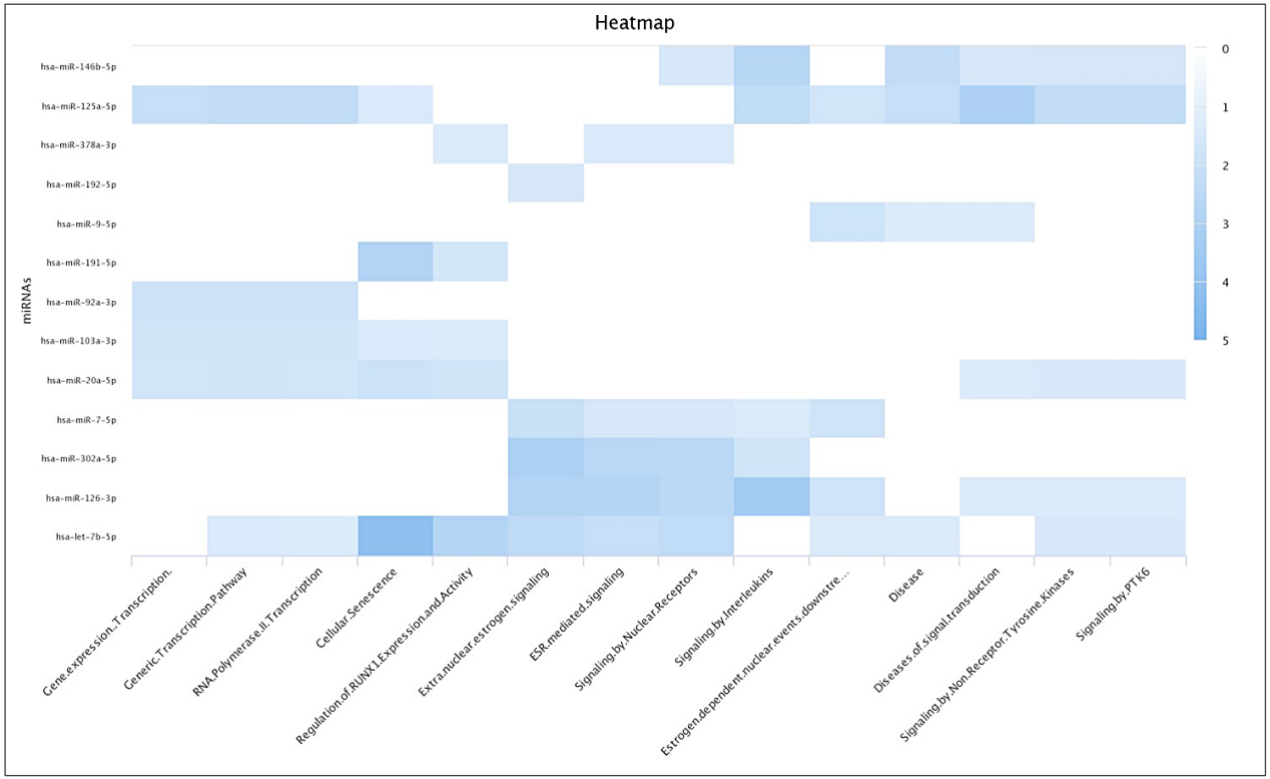
The heatmap illustrates the involvement of dysregulated miRNA in aneuploid blastocysts in various cancer types and cancer-related pathways. The analysis utilised the KEGG database via miRPathDB v2.0.



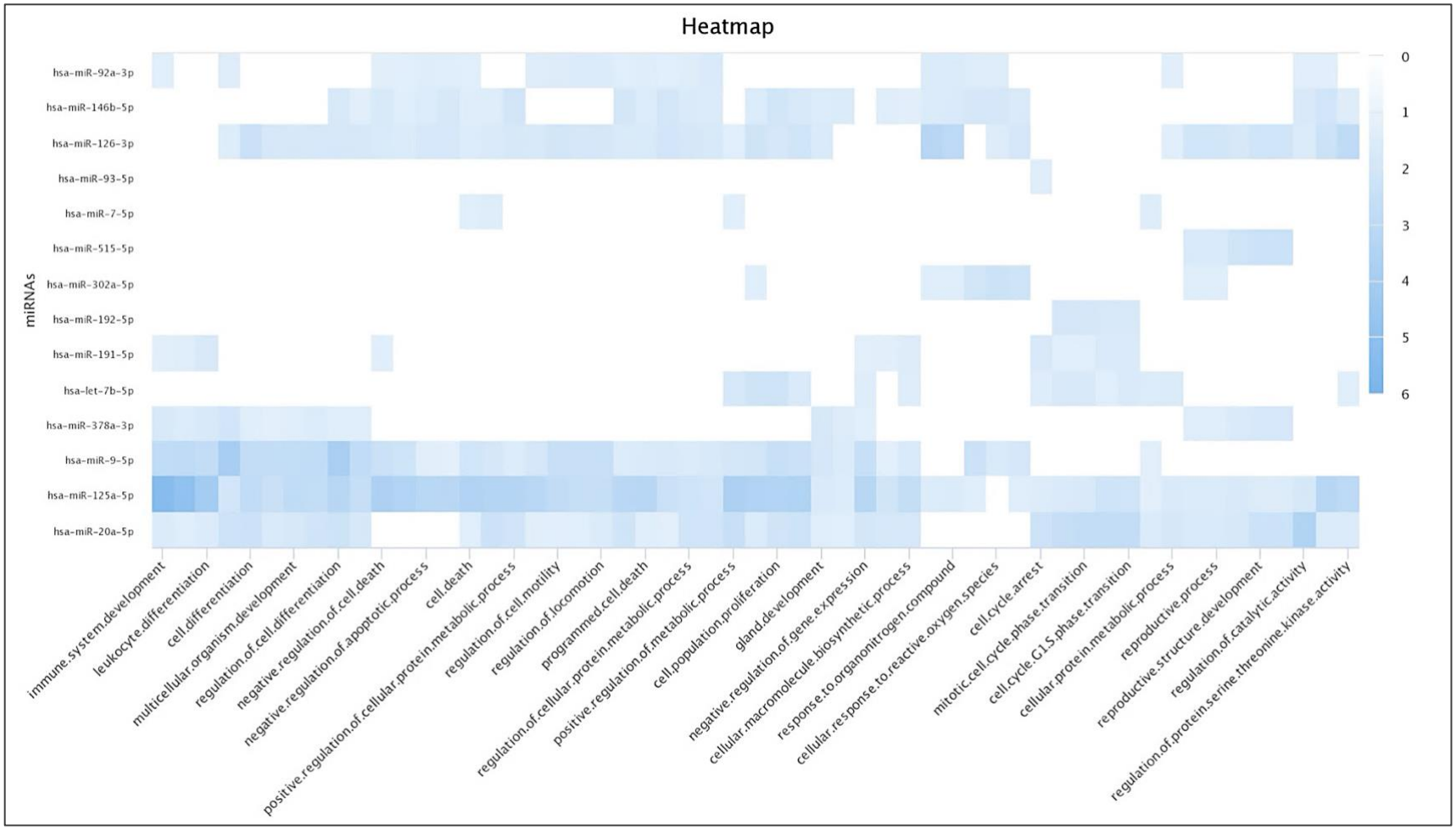
The heatmap of enrichment pathway analysis shows the involvement of dysregulated miRNAs in aneuploid blastocysts in metabolic process, cell proliferation and cell death. This analysis was conducted using Gene ontology (GO) biological processes database via miRPathDB v2.0.



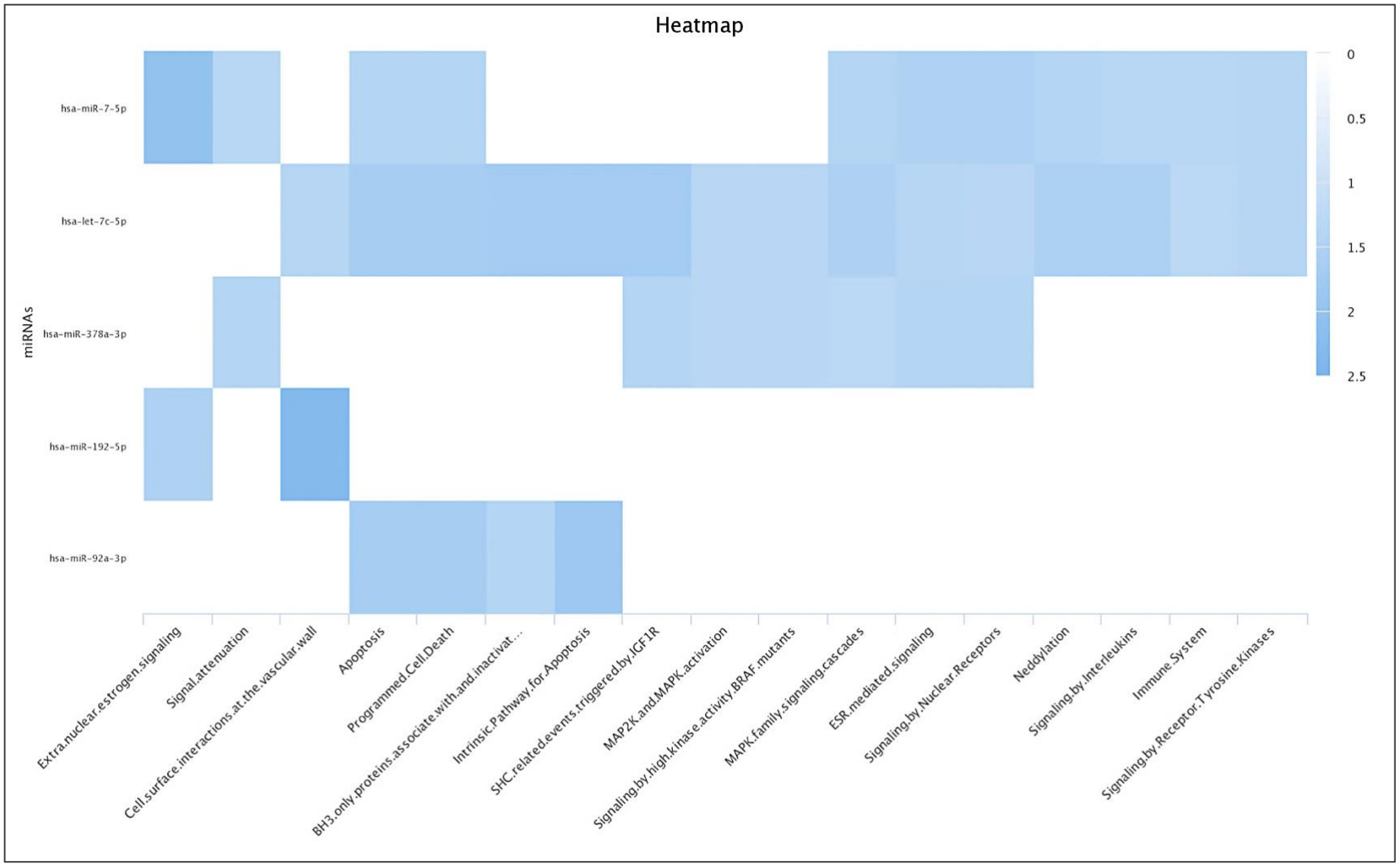
Heatmap of differentially expressed miRNAs in aneuploid blastocysts, depicting their involvement in various signalling pathways according to the WikiPathways database, accessed using miRPathDB v2.0.



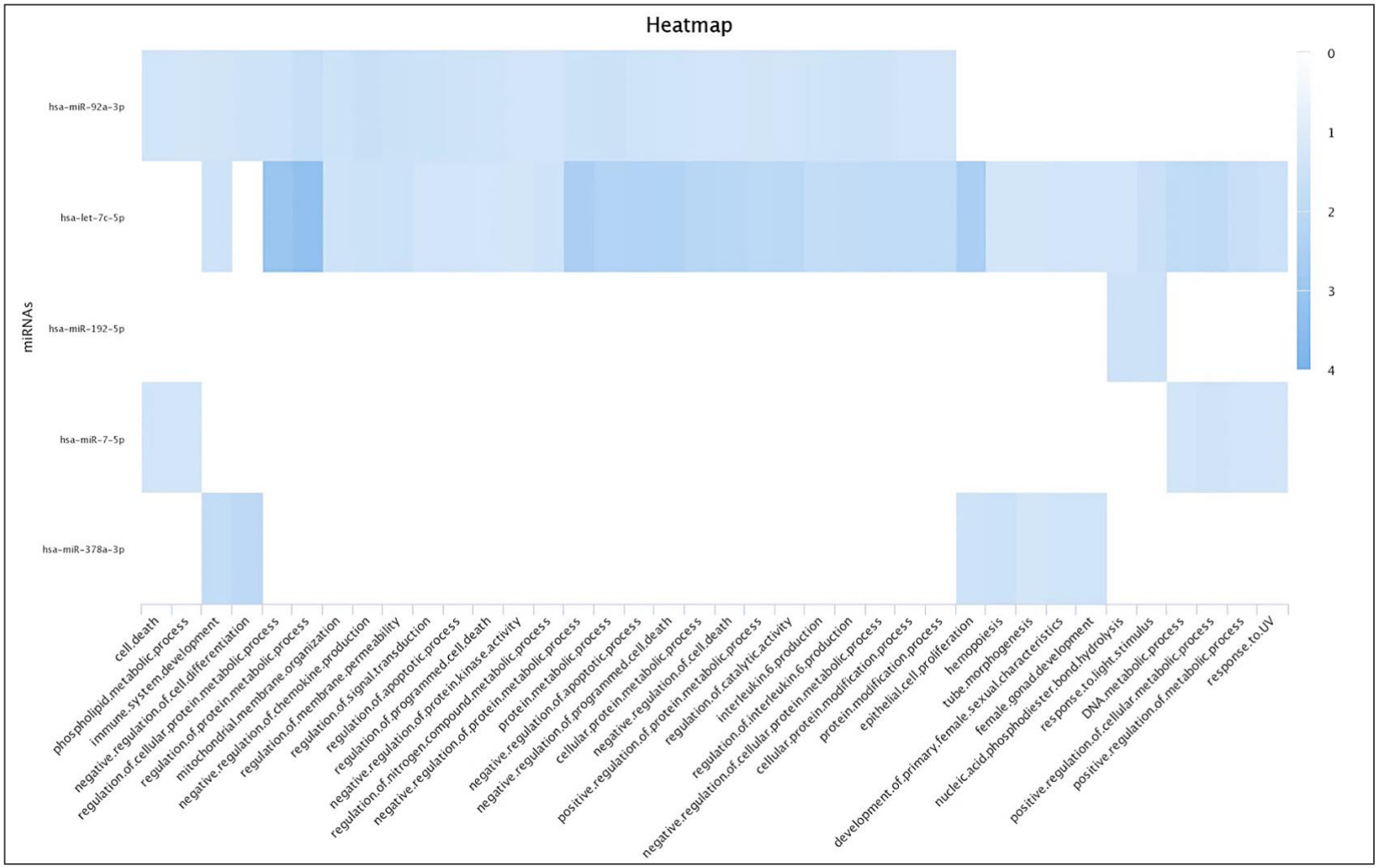
Heat map analysis of pathway annotation for dysregulated miRNAs in blastocysts with chromosomal losses compared to euploid blastocysts, using the Reactome database via miRPathDB v2.0.



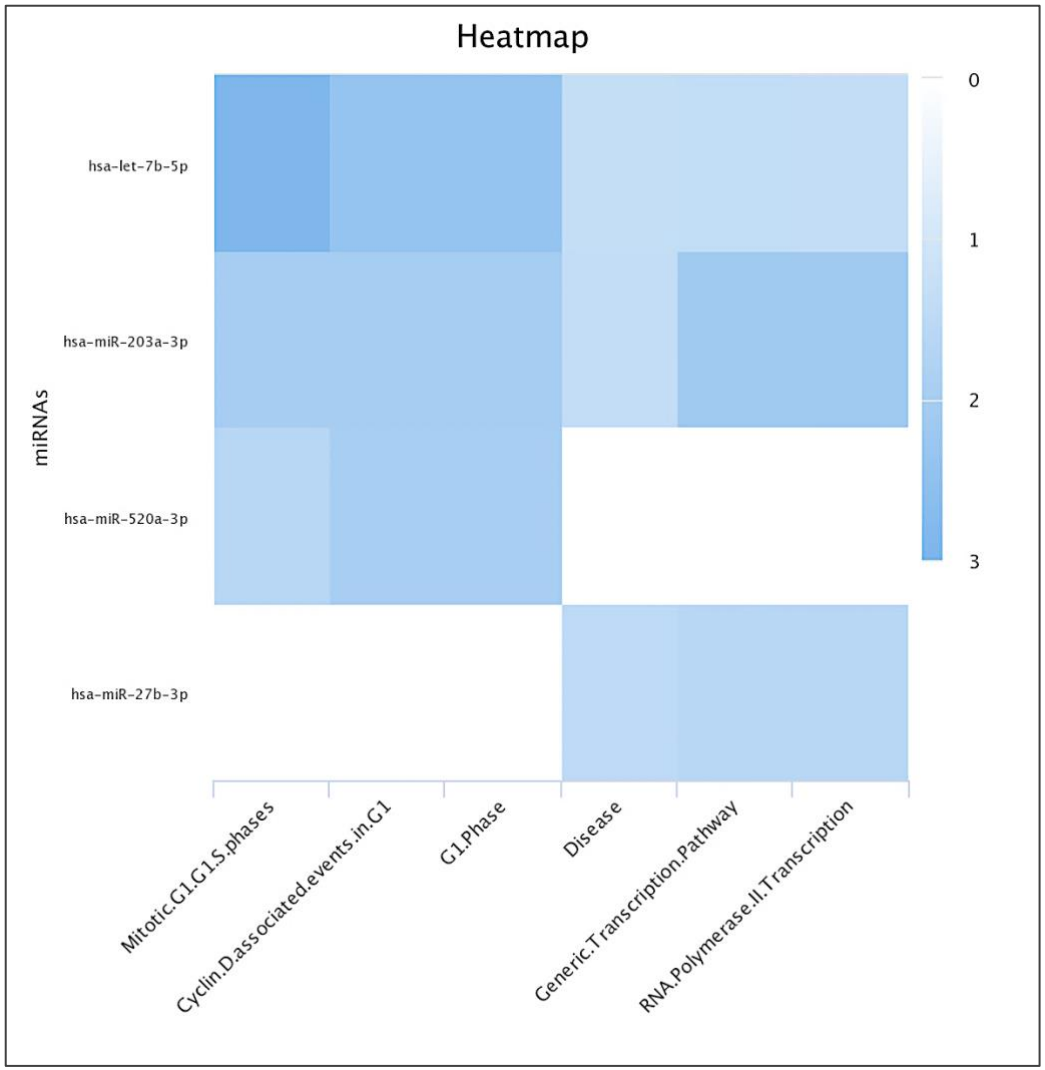
Heat map analysis of pathway annotation for dysregulated miRNAs in blastocysts with chromosomal losses compared to euploid blastocysts, using the GO biological processes via miRPathDB v2.0.



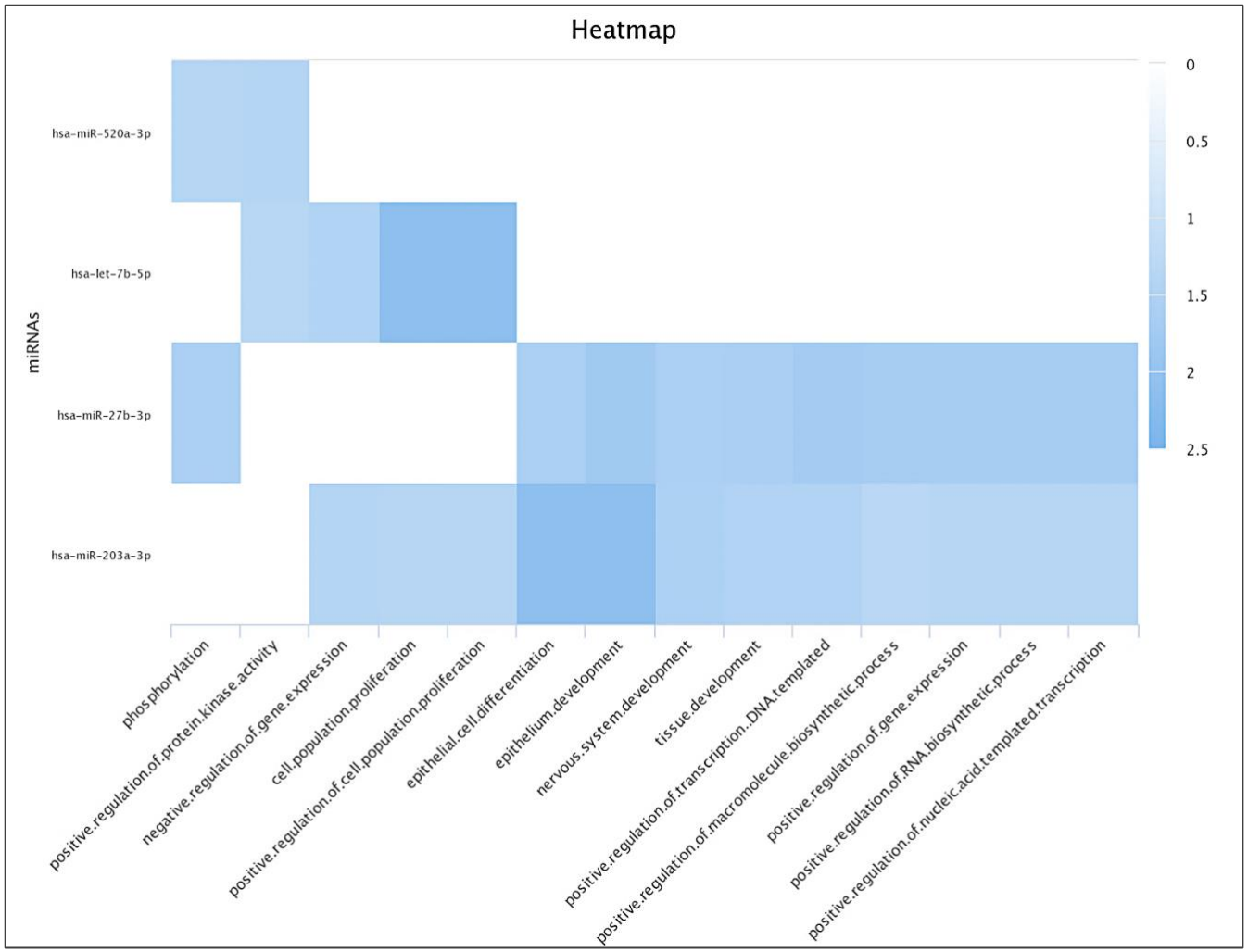
Heat map analysis of pathway annotation for dysregulated miRNAs in blastocysts with chromosomal gains compared to euploid blastocysts, using the Reactome database via miRPathDB v2.0.



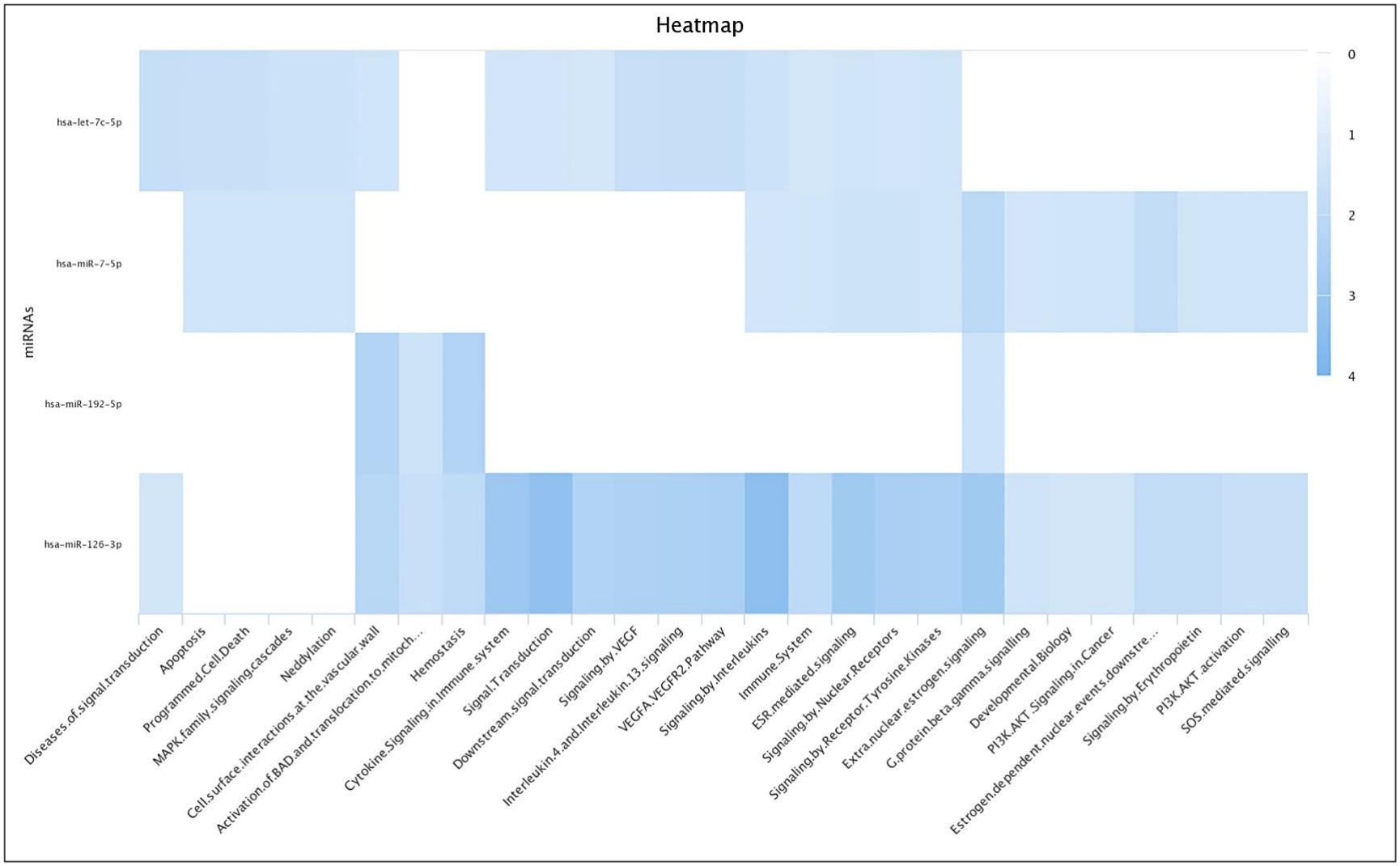
Heat map analysis of pathway annotation for dysregulated miRNAs in blastocysts with chromosomal gains compared to euploid blastocysts, using the GO biological processes via miRPathDB v2.0.



Heat map analysis of pathway annotation for dysregulated miRNAs in blastocysts with segmental chromosomal defects compared to euploid blastocysts, using the Reactome database via miRPathDB v2.0.

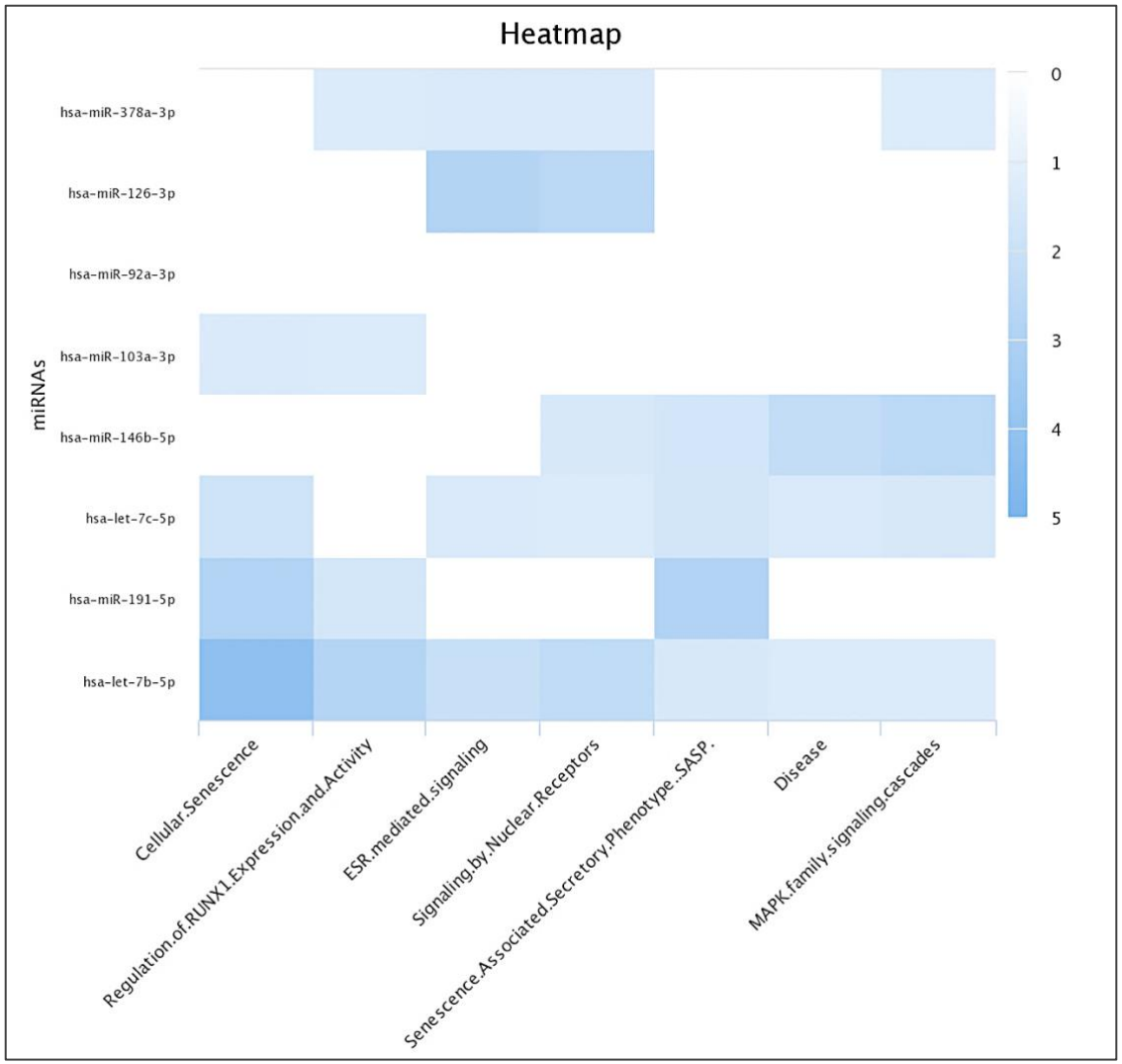


Heat map analysis of pathway annotation for dysregulated miRNAs in blastocysts with partial chromosomal defects compared to euploid blastocysts, using the GO biological processes via miRPathDB v2.0.

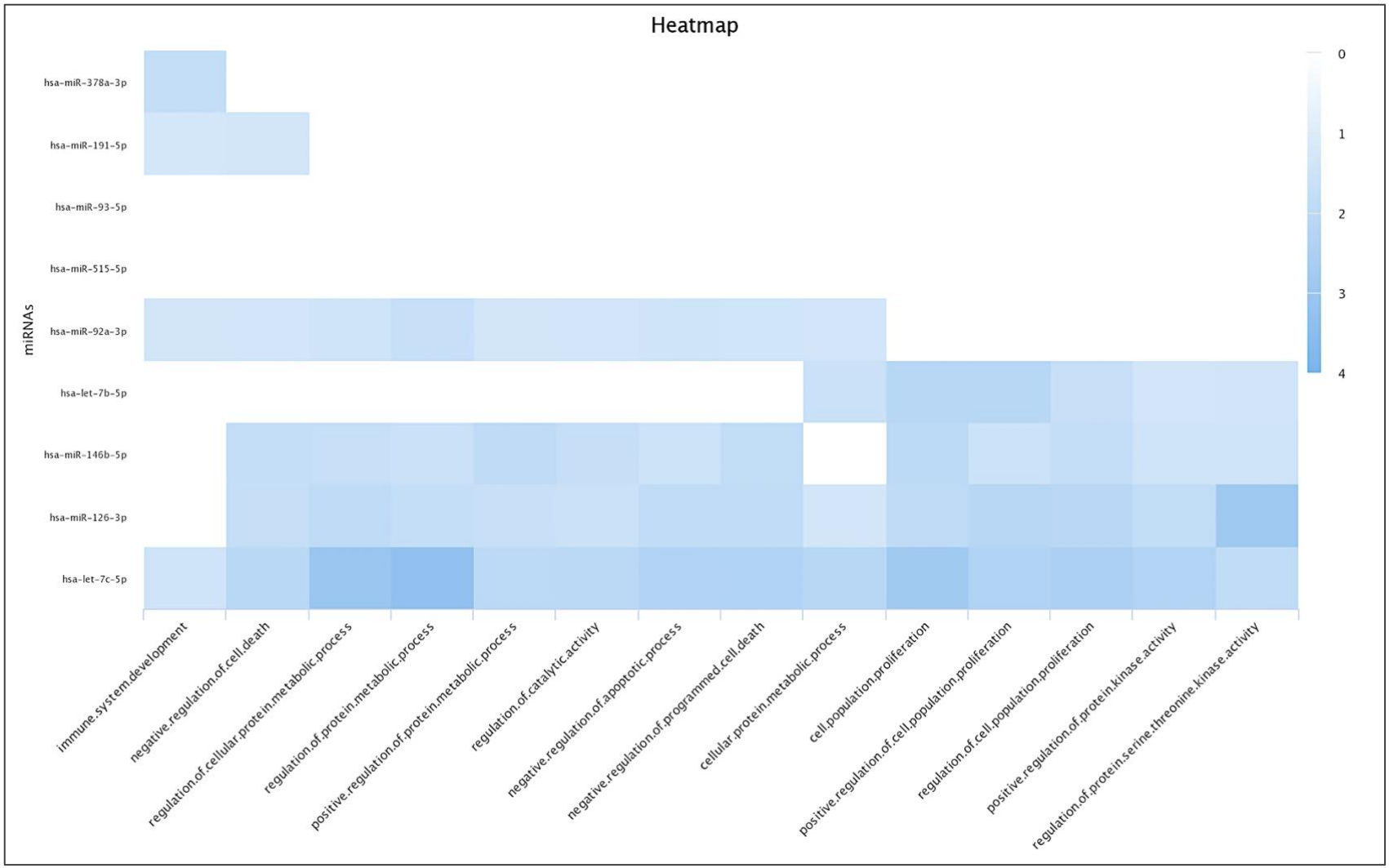


Heat map analysis of pathway annotation for dysregulated miRNAs in blastocysts with single aneuploidy compared to euploid blastocysts, using the Reactome database via miRPathDB v2.0.

Heat map analysis of pathway annotation for dysregulated miRNAs in blastocysts with single aneuploidy compared to euploid blastocysts, using the GO biological processes via miRPathDB v2.0.



Heat map analysis of pathway annotation for dysregulated miRNAs in blastocysts with multiple aneuploidies compared to euploid blastocysts, using the Reactome database via miRPathDB v2.0.



Heat map analysis of pathway annotation for dysregulated miRNAs in blastocysts with multiple aneuploidies compared to euploid blastocysts, using the GO biological processes database via miRPathDB v2.0.

Appendix8

Differentially expressed miRNAs in aneuploid blastocysts from previous research and the present study

Previous studies	differentially expressed miRNAs	miRBase	Regulation in aneuploid to euploid	Fold change as reported	p-value	Aneuploidy subgroup						Matching
						All	Loss	Gain	Single	Complex	Partial	
Rosenbluth et al., 2013	hsa-miR-106a	hsa-miR-106a-5p	Downregulated	1.15	P= <0.05							
	hsa-miR-1276	hsa-miR-1276	Downregulated	6.22	P= <0.05							
	hsa-miR-141	hsa-miR-141-3p	Downregulated	5.49	P= <0.05							
	hsa-miR-146b-5p	hsa-miR-146b-5p	Downregulated	0.61	P= <0.05	Downregulated	Downregulated		Downregulated	Downregulated		Matching
	hsa-miR-148a	hsa-miR-148a-3p	Downregulated	4.19	P= <0.05							
	hsa-miR-155	hsa-miR-155-5p	Downregulated	4.01	P= <0.05							
	hsa-miR-17	hsa-miR-17-5p	Downregulated	1.38	P= <0.05							
	hsa-miR-19a	hsa-miR-19a-3p	Downregulated	1.4	P= <0.05							

	hsa-miR-19b	hsa-miR-19b-3p	Downregulated	0.94	P= <0.05							
	hsa-miR-200c	hsa-miR-200c-3p	Downregulated	1.08	P= <0.05							
	hsa-miR-20a	hsa-miR-20a-5p	Downregulated	1.78	P= <0.05	Downregulated	Downregulated			Downregulated		Matching
	hsa-miR-26b#	hsa-miR-26b-5p	Downregulated	3.07	P= <0.05		Downregulated					Matching
	hsa-miR-27b	hsa-miR-27b-3p	Downregulated	4.29	P= <0.05						Upregulated	Not Matching
	hsa-miR-30b	hsa-miR-30b-5p	Downregulated	1.22	P= <0.05							
	hsa-miR-320	hsa-miR-320	Downregulated	0.48	P= <0.05							
	hsa-miR-339-3p	hsa-miR-339-3p	Downregulated	3.69	P= <0.05							
	hsa-miR-345	hsa-miR-345-5p	Downregulated	1.69	P= <0.05							
	hsa-miR-34b	hsa-miR-34b-3p	Downregulated	5.26	P= <0.05							
	hsa-miR-367	hsa-miR-367-3p	Downregulated	0.9	P= <0.05							

	hsa-miR-371-3p	hsa-miR-371a-3p	Downregulated	0.88	P= <0.05							
	hsa-miR-372	hsa-miR-372-3p	Downregulated	0.67	P= <0.05							
	hsa-miR-373	hsa-miR-373-3p	Downregulated	1.52	P= <0.05	Downregulated	Downregulated					Matching
	hsa-miR-380-5p	hsa-miR-380-5p	Downregulated	3.17	P= <0.05							
	hsa-miR-487b	hsa-miR-487b-3p	Up regulated	−3.35	P= <0.05							
	hsa-miR-509-5p	hsa-miR-509-5p	Downregulated	1.33	P= <0.05							
	hsa-miR-517c	hsa-miR-517c-3p	Downregulated	1.28	P= <0.05							
	hsa-miR-518a-3p	hsa-miR-518a-3p	Downregulated	3.79	P= <0.05		Downregulated					Matching
	hsa-miR-518c	hsa-miR-518c-3p	Downregulated	2.81	P= <0.05							
	hsa-miR-518e	hsa-miR-518e-3p	Downregulated	1.63	P= <0.05							

	hsa-miR-519a	hsa-miR-519a-3p	Downregulated	1.15	P= <0.05							
	hsa-miR-522	hsa-miR-522-3p	Downregulated	2.15	P= <0.05							
	hsa-miR-566	hsa-miR-566	Downregulated	4.08	P= <0.05							
	hsa-miR-590-3p	hsa-miR-590-3p	Downregulated	3.45	P= <0.05							
	hsa-miR-597	hsa-miR-597-5p	Downregulated	4.82	P= <0.05							
	hsa-miR-645	hsa-miR-645	Downregulated	5	P= <0.05							
	hsa-miR-660	hsa-miR-660-5p	Downregulated	2.69	P= <0.05							
	hsa-miR-886-3p	hsa-miR-886-3p	Downregulated	0.64	P= <0.05							
	hsa-miR-92a	hsa-miR-92a-3p	Downregulated	1.73	P= <0.05	Downregulated	Downregulated		Downregulated	Downregulated		Matching
	hsa-miR-93	hsa-miR-93-5p	Downregulated	3.75	P= <0.05	Downregulated	Downregulated		Downregulated	Downregulated		Matching
McCallie et al., 2014	hsa-miR-106b-5p	hsa-miR-106b-5p	Up regulated	2.1	P= <0.05							

	hsa-miR-125a-5p	hsa-miR-125a-5p	Downregulated	<0.5	P= <0.05		Downregulated					Matching
	hsa-miR-146b-5p	hsa-miR-146b-5p	Up regulated	2.61	P= <0.05	Downregulated	Downregulated					Not matching
	hsa-miR-193b	hsa-miR-193b-3p	Up regulated	1.9	P= <0.05							
	hsa-miR-195-5p	hsa-miR-195-5p	Up regulated	3.2	P= <0.05							
	hsa-miR-19b	hsa-miR-19b-3p	Downregulated	0.5	P= <0.05							
	hsa-miR-200c-3p	hsa-miR-200c-3p	Up regulated	1.7	P= <0.05							
	hsa-miR-20b-5p	hsa-miR-20b-5p	Up regulated	2.2	P= <0.05							
	hsa-miR-218-5p	hsa-miR-218-5p	Up regulated	>10	P= <0.05							
	hsa-miR-28-3p	hsa-miR-28-3p	Up regulated	1.5	P= <0.05							
	hsa-miR-302a-3p	hsa-miR-302a-3p	Up regulated	3.8	P= <0.05							

	hsa-miR-302b-3p	hsa-miR-302b-3p	Up regulated	2.7	P= <0.05							
	hsa-miR-302c	hsa-miR-302c-3p	Downregulated	0.5	P= <0.05							
	hsa-miR-30c-5p	hsa-miR-30c-5p	Up regulated	2.4	P= <0.05							
	hsa-miR-320	hsa-miR-320	Up regulated	1.4	P= <0.05							
	hsa-miR-342-3p	hsa-miR-342-3p	Up regulated	1.5	P= <0.05							
	hsa-miR-367-3p	hsa-miR-367-3p	Up regulated	1.6	P= <0.05							
	hsa-miR-371a-3p	hsa-miR-371a-3p	Up regulated	1.5	P= <0.05							
	hsa-miR-372	hsa-miR-372-3p	Up regulated	1.3	P= <0.05							
	hsa-miR-373	hsa-miR-373-3p	Up regulated	2.3	P= <0.05		Downregulated					Not matching
	hsa-miR-374a	hsa-miR-374a-5p	Up regulated	1.4	P= <0.05							
	hsa-miR-374b-5p	hsa-miR-374b-5p	Up regulated	1.9	P= <0.05							

	hsa-miR-381-3p	hsa-miR-381-3p	Up regulated	>10	P= <0.05							
	hsa-miR-454-3p	hsa-miR-454-3p	Up regulated	2.6	P= <0.05							
	hsa-miR-484	hsa-miR-484	Up regulated	1.5	P= <0.05							
	hsa-miR-508-3p	hsa-miR-508-3p	Up regulated	>10	P= <0.05							
	hsa-miR-515-3p	hsa-miR-515-3p	Up regulated	2	P= <0.05							
	hsa-miR-517c	hsa-miR-517c-3p	Downregulated	0.5	P= <0.05							
	hsa-miR-518b	hsa-miR-518b	Downregulated	<0.5	P= <0.05							
	hsa-miR-518e	hsa-miR-518e-3p	Downregulated	0.5	P= <0.05							
	hsa-miR-518f-3p	hsa-miR-518f-3p	Up regulated	>10	P= <0.05							
	hsa-miR-520b-3p	hsa-miR-520b-3p	Up regulated	2.2	P= <0.05							
	hsa-miR-520g	hsa-miR-520g-3p	Downregulated	0.4	P= <0.05							

	hsa-miR-521	hsa-miR-521	Up regulated	3.4	P= <0.05							
	hsa-miR-522	hsa-miR-522-3p	Downregulated	0.7	P= <0.05							
	hsa-miR-628-5p	hsa-miR-628-5p	Up regulated	>10	P= <0.05							
	hsa-miR-886-5p	hsa-miR-886-5p	Downregulated	0.2	P= <0.05							
	hsa-miR-93	hsa-miR-93-5p	Up regulated	1.4	P= <0.05	Downregulated	Downregulated		Downregulated	Downregulated		Not matching
Rosenbluth et al., 2014	hsa-miR-191-5p	hsa-miR-191-5p	Up regulated	4.7	P= 0.031	Downregulated	Downregulated		Downregulated	Downregulated		Not matching
McCallie et al, 2015	hsa-miR-106a	hsa-miR-106a-5p	Downregulated	N/A	P= <0.05							
	hsa-miR-92a	hsa-miR-92a-3p	Downregulated	N/A	P= <0.05	Downregulated	Downregulated		Downregulated	Downregulated		Matching
Esmaeilivand et al, 2022	hsa-miR-661	hsa-miR-661	Up regulated	N/A	P= <0.05							
	hsa-miR-20a	hsa-miR-20a-5p	Up regulated	N/A	P= <0.05	Downregulated	Downregulated			Downregulated		Not matching

Blue label indicates differentially expressed miRNAs with less significance, and red label indicates differentially expressed miRNAs with high significance.