CIRCULAR RNA AS INDICATORS FOR STILLBIRTH AND PLACENTAL AGEING

By
Susan Nasimiyu Babikha
2237939

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ABSTRACT

Placental ageing is associated with the gestation period and leads to cellular and molecular changes that promote fetal independence as the placental function is reduced towards labour. However, premature ageing can compromise pregnancy outcomes, affecting fetal growth and development. Premature placental ageing has been associated with stillbirth. Research on the use of circular RNAs (circRNAs) in other species as indicators of cellular ageing and senescence is emerging, with their stability and regulatory functionality in samples making them promising candidates for this purpose.

My study aimed to quantify circRNA in blood samples collected from mothers who experienced unexplained fetal deaths compared with those who went on to have healthy pregnancies, to determine the biomarker potential of a panel of circRNAs for stillbirth screening. As *in vitro* studies are required to determine the biological mechanism involved with circRNAs in placental ageing, and Dr Arthurs is currently culturing placental organoids which have been genetically edited to overexpress candidate circRNAs, my project also aimed to optimise the process of *in situ* localisation of antigens in placental organoids via fluorescence microscopy.

Elevated levels of candidate circRNAs were found in maternal blood samples from women who experienced unexplained stillbirths compared to controls, suggests that a diagnostic threshold could be established for early intervention. This approach could enhance prenatal care, improve pregnancy outcomes, promote fetal health and development monitoring, and support better-informed pregnancy-related decisions. Furthermore, the immunofluorescence protocol for *in situ* localisation was successfully optimised, allowing for further investigation into the biological mechanisms underpinning placental ageing pathology due to circRNA accumulation.

DECLARATION

I certify that this thesis:

1. Does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. And the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. To the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signed

Date.....30/10/2024.....

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8-OHdG-…….. 8-hydroxy-2'-deoxyguanosine

DAPI ……4′,6-diamidino-2-phenylindole

IPC-…….. induced pluripotent cells

cfRNA-……Circulating free RNA

PCR-…….….Polymerase Chain Reaction

Eci RNA-……Exon Circular RNA

PEG 10-…….Paternally Expression Gene 10

PSG1-……...Pregnancy Specific Glycoprotein 1

ROS: ……...Reactive Oxygen Species

ANKRD52: …….Ankyrin Repeat Domain 52

IRES…….. Internal Ribosome Entry Site

LAMP: …….Lysosomal-associated Membrane Protein

LC3B: …….Microtubule-associated protein 1 light chain 3B

LMIC: …….Low- and Middle-Income Country

HIC: ……High-Income Country

PBS: …….Phosphate-Buffered Saline

DDR: ……DNA Damage Response

CHAPTER 1: LITERATURE REVIEW

I. Circular RNA

Circular RNAs are continuous RNA that are covalently closed loop without a 5'-3' polarity or a polyadenylated tail (Ebbesen K et al., 2018; Li H *et al*., 2015; Li H *et al*., 2016: Blandino *et al*.,2021) Initial discovery of this RNA was in the early 1970s as plant pathogenic viroids, by use of an electron microscope(Liu et al., 2023). Due to limited knowledge on the RNA at that time, circRNA was considered a "scrambled exon." Through recent scientific development of high throughput technology, sequencing, and bioinformatics, these naturally occurring genetic material have been identified in a range of eukaryotic organisms(Blandino *et al*.,2021;Xia *et al*.,2020:Ashwal-Fluss *et al*.,2014.)

Further research after identification revealed their peculiarity. This was attributed to their formation, morphology, and physiological characteristics(Verduci *et al.*, 2021; Yuxiang Bai *et al.*, 2018; Ma *et al.*, 2020).

1.1 Biogenesis of Circular RNA

Contrary to the linear counterparts, which are a result of transcription, circRNAs are formed by back splicing. This involves joining the 5' splice site with the 3' splice site of an upstream intron in a back splicing reaction(Andreeva K *et al*., 2015; Ashwal-Fluss *et al*.,2014;Ebbesen K *et al*.,2017.)

Figure 1 :Image removed due to copyright restrictions

1.1.2 Functions of circRNA

The Majority of circRNA have been classified as non-coding for proteins. The most extensively characterised function is as microRNA sponges, which is where sequester miRNAs and hence regulate their activity. By binding to mRNA, they prevent translation, making them useless(Andreeva K *et al*., 2015; Ashwal-Fluss *et al*.,2014;Ebbesen K *et al*.,2017.)

Another function is as a transcriptional regulator. It was proved that knocking down intronic circRNAs led to reduced expression of parental genes, resulting in their influence on cell survival and growth. One such circRNA from the gene ANKRD52 accumulated to its sites of transcription, where it is associated with elongation of Pol II machinery and acted as positive regulator of Pol II transcription(Oudejans, C *et al*.,2021:Flenady *et al.*, 2020).).

Notably, despite their established ability to restrict translation, recent research has shown that circRNAs are able to make proteins, especially if it holds ribosome Entry site, IRES. This finding challenges the first identification that circRNAs are strictly non-coding as they indeed result in protein formation. However, this seems only applicable for a small subset of circRNAs tail (Ebbesen K *et al*., 2018; Li H *et al*., 2015; Li *H et al*., 2016: Blandino *et al*.,2021).

Some studies have shown that circRNA can up the regulation of ROS production hence causing DNA strands to break. Also ability of circRNA to act as sponges for miRNA, this can downregulate genes necessary for repair and cause damage to DNA (Ebbesen K *et al*.,2017.)

2. The placenta

This placenta is a highly specialized organ exists solely to support and develop early life throughout the gestation period. It acts as the maternal-fetal interface, providing nutrition, metabolism, growth, and gestational adaptation for the mother(Gude, Roberts, Kalionis, King., 2004L Cindrova-Davies & Sferruzi-Perri, 2023).

Placental development begins as soon as implantation occurs, about a week after ovulation during the pilot stages of embryonic growth. Initially, the zygote divides into two cell types: the trophoblast, which forms the outer layer, and the embryoblast, which forms the embryo(Kazma ,J *et al*.2020;Cindrova -Davies,T *et al*.,2022). As the embryo implants into

the uterus, the trophoblast transforms into the chorionic epithelium and, along with the embryonic mesenchyme, forms the villous chorion(Burton, G,J *et al.,* 2023). Over time, blood vessels develop within the chorionic villi, providing an extensive circulatory system between the embryo and the placenta. The spiral arteries in the uterine lining undergo significant changes to accommodate the increased blood flow to the fetus(Olimajaovna,F *et al* 2020:Kazma *et al.*, 2020).).

During this period, the trophoblast invades the uterine lining, forming an intervillous space filled with maternal blood. By the eighth week, the cotyledons, the structural units of the placenta, are formed(Zur R,L *et al*.,2020).

Throughout pregnancy, the placenta plays a pivotal role in ensuring the survival and healthy development of both the mother and the fetus(see Figure 2). It is vital for metabolic processes, nutritional supplementation, waste exchange and acts as a barrier against harmful agents. Additionally, the placenta produces crucial hormones and chemical substances that regulate homeostasis in the mother-placenta-fetus complex(Zur R,L *et al*.,2020).

A placenta is considered efficient if it performs vital functions during gestation that sustain fetal growth, nourishment and protection. These functions include removal of wastes from fetus blood, exchange of gases from mother to fetus, efficient transfer of nutrients from maternal blood to the fetus, ability to synthesis important hormones and other mediators and protecting fetus from infections, hence supporting a healthy fetal environment(Kazma ,J *et al*.2020;Cindrova -Davies,T *et al.,*2022).

From around 32 weeks, the fetus is considered mature and begins developing independent life support systems, the circulatory system. The need for trophic, hormonal, gaseous exchange, immune, and excretory functions of the placenta decrease, ceasing by 42 weeks, which is considered the end of a successful pregnancy. These structural and physiological processes show placental ageing and an end to its functionality. However, malfunction by both intrinsic or extrinsic factors before 32 weeks significantly affects fetal development and increases the risk of stillbirth(Kwiatkowski *et al*.,2021;Kwiatkowski *et al*.,2023:Tong *et al*.,2020).

Figure 2 Image removed due to copyright restrictions

2.1 Factors affecting placental dysfunction

Loss of function by the placenta can be attributed to several factors, both intrinsic and extrinsic. Maternal factors play a leading role. Both young or old mothers are at an increased risk of poor placental development and eventual dysfunction(Kadjy *et al.*, 2021; Kadjy *et al.*, 2023; Maiti *et al.*, 2022). Underlying conditions such as diabetes, hypertension and auto immune diseases can impair placental functions(Caroll,A *et al*.,2022;Lawn J,E *et al*,2023; Kwiatkowski *et al*.,2021;Kwiatkowski *et al*.,2023:*Tong et al*.,2020)Substance abuse such as alcohol, smoking and drugs cause damage to the placenta. Essential nutrients like folic acid are necessary during pregnancy and lack of it can affect placental development. Maternal exposure to toxins such as heavy metals can further affect fetal development . By making their way across placenta toxic and heavy metals lead to senescence and eventually cell death.(Sober,S *et al*.,2015:Aplin, J,D *et al*.,2020)

High levels of stress including oxygen levels and an increase in cell death, can cause tissue damage and impair placental functions. Oxidative stress diminishes placental antioxidant ability ,resulting in a surged cell death. Chronic inflammation due to an injury or deformation of the placenta and placenta abnormality impair placental function(Sultan R.*et al* 2010:Kwiatkowski *et al*.,2023).

2.2 The Ageing Process in cells and tissues

Ageing is a natural biological process that occurs within cells and tissues. It is characterized by the accumulation of senescent cells within tissues, leading to age-related phenotypes and physiological changes. These include altered metabolic functions, degeneration of structural components, and reduced tissue regeneration and repair. This degeneration affects the behaviour of neighbouring cells and tissues, diminishing the growth of mitotically competent cells(Burton, Poston, Jauniaux., 2006; Kwiatkowski *et al*.,2023)

2.3 Placental ageing

As pregnancy progresses so does the ageing of the placenta. The timing of placental ageing is crucial in deciding the outcome for the fetus. Ageing seen from 36 weeks results in the birth of a fully developed, healthy baby(Gude, Roberts, Kalionis, King., 2004). However, premature placental ageing, occurring well before 20 weeks, disrupts the normal function of placental tissues, generally affecting neighbouring cells and tissues. This breaks down the extracellular matrix and reduces tissue's regenerative ability and is viewed as a terminal state of growth. It can result in intrauterine growth restriction, preterm birth, long-term health conditions in the developing fetus, fetal death, and stillbirth(Burton, D.G 2009: Cox &Redman, C 2017).

Placental ageing occurs at both molecular and morphological level, and it is triggered by both internal and external factors. Hormones play a crucial role in modulating these processes and influence the pace of senescence. Leading causes of premature senescence are telomere dysfunction, DNA damage, epigenomic disruption, strong mitotic signals, oncogenes, and reactive oxygen species. All of which are interrelated, as progression of one lead to another(Kwiatkowski *et al*.,2021:Kwiatkowski *et al*.,2023).

2.4. Telomere Dysfunction in relation to ageing

Telomeres are highly conserved repetitive DNA regions found at the terminal ends of chromosomes. Primarily they guard against chromosomal fusion and disintegration. Naturally, these terminals shorten with each mitotic cycle and are regulated by telomerase enzymes, a reverse transcriptase enzyme that adds telomeric repeats to the end of the chromosome. The added ends provide integrity and stability to the chromosomes. Once they reach a certain shortened length, cell apoptosis and senescence are triggered. Several environmental factors have been found that affect the length of the ends. They include smoking, hypoxia, hypercalcemia, and oxidative stress which hasten the shortening process resulting in preterm shortened length. All other factors, related to ageing are directly or indirect resultant of pre or post telomere shortening(Maiti *et a*l.,2017; Ozawa T, 1997; Ferdako.,2011; Burton, Poston, Jauniaux., 2006).

2.5 Reactive Oxidative Species (ROS) and Oxidative stress

ROS are free radicles having unpaired electrons that are generated during metabolic processes and aerobic respiration within cells. These radicles activate various signalling pathways necessary for cell growth and metabolism. Unfortunately an accumulation of the free radicles causes oxidative damage to mitochondrial DNA (mDNA) and proteins. This translates to a dysfunctional respirational chain, leading to an overproduction of ROS. Significant ROS within cells causes lipid peroxidation, protein damage and alteration and DNA lesions that impair cellular and tissue functions which inevitably starting pathologic pathways. The imbalance between produced ROS and inability of antioxidants to neutralise them results in Oxidative stress. An Overwhelming surge of oxidative stress diminishes placental antioxidant ability resulting in a surged cell death(Maiti *et al*.,2017; Ozawa T, 1997; Ferdako.,2011; Burton, Poston, Jauniaux., 2006).

2.6 DNA damage and ageing

An organism's genetic material decides the expression and turnover rate of cell proliferation, expression and resultant cell death and senescence. However, some predisposed factors may affect this turn of events(Starodubsteva,M et al.,2021). Environmental factors such as exposure to radiation and chemicals, biological error during replication or over exposure to

significant ROS resulting in oxidative stress and an infection by a virus leads to damage to resultant genetic material, DNA(Pole, A et al.,2016:Schaum,N et al,2020).

This process may start a response, DNA damage repair response (DDR) .The damage may be damage strand breaks (DSB) which if unrepaired can be a cause of cell senescence while its accumulation may lead to placental ageing(Maiti et al.,2017; Ozawa T, 1997; Ferdako.,2011; Burton, Poston, Jauniaux., 2006).

2.7 Evidence based tissue ageing using known biomarkers

Various immunohistochemical techniques have been developed to detect cell and tissue aging. Recent studies by Maiti et al.,2017 proposed 8-hydroxydeoxyguanosine (8OHdG) as a biomarker for DNA/RNA oxidation. This biomarker had been used to detect for cell death that resulted in premature ageing of brain samples collected from Alzheimer's patients(Blandino et al.,2021; Dube et al.,2019.)

. When applied to placental tissues, immunohistochemistry tests for 8OHdG revealed a significant increase in DNA/RNA oxidation in the nuclei of cells from late-term and stillbirth placentas.

Figure 3 Image removed due to copyright restrictions

Earlier studies had also confirmed that proteins and damaged mitochondria can be recycled in autophagosomes through autophagosome fusion with lysosomes having proteolytic enzymes. Accumulation of abnormal proteins by inhibition of autophagosome function has been

thought to play a crucial role in cellular ageing, particularly in the brain. This was proved in studies involving Alzheimer's disease, which was characterized by the accumulation of tau and amyloid proteins. LAMP2 was used as a biomarker to analyze the distribution of lysosomes in the placenta by immunohistochemistry. It showed lysosomes positioned on the apical surface of early-term placental syncytiotrophoblasts, while in stillbirth and late-term placentas, lysosomes moved to the perinuclear and the basal surface. Alternatively, inhibition of autophagosome function also leads to an increase in autophagosome size. Detection of autophagosome functionality in immunohistochemistry using an antibody against LC3B revealed an increase in the size of the autophagosomes. Double-labelled fluorescence immunostaining showed that larger autophagosomes also had 4HNE, a product of lipid oxidation(Cox & Redman, 2017; Pole *et al.,* 2016; Schaum *et al*., 2020; Schumacher *et al.,* 2021; Starodubsteva,M, 2011; Zhang *et al*., 2020).

An increase in DNA oxidation proved free radical damage that led to lipid oxidation. Lipid oxidation had been previously seen in brain cells with Alzheimer's disease. Immunohistochemistry revealed a similar increase in 4HNE staining in syncytiotrophoblasts observed in placentas associated with stillbirth (Zhang *et al*., 2020: Kwiatkowski *et al*.,2023)

3. Stillbirth

According to the World Health Organization (WHO), stillbirth is defined as the delivery of a baby showing no signs of life after a specified gestational age. In Australia(AIHW., 2024), this period is 20 weeks' or more, with a stillborn fetus weighing >400g(Saleem,S., *et al.,*2018: Froen,J *.,et al.,*2011).

Globally very few countries have reliable preterm birth prevalence data or records. While those recorded are attached to and relatable to maternal health issues, poor lifestyle choices, environmental factors, maternal age, and pregnancy complications (Flenady *et al.*, 2020). The WHO estimates an annual 13 million stillbirths (Hug, L., *et al.,*2016: Kelly,K *et al.,*2021*:* This tragedy occurs every 16 seconds and affects 1 in 200 women (Tsakidiris *et at*., 2022). It is more prevalent within low and middle income than the middle- and high-income countries (Giakoumelou, S *et al.,*2016: Burden, C., *et al.,2016)*. However, of late, there has been a steady increase among the middle- and high-income countries neo nata. It is due to the recent increase that neo natal deaths made it into the Millennium Development Goals them (Smith *et al.*, 2022; Vicky *et al.*, 2016; Murphy *et al.*, 2017.)

Research has shown that stillbirths can be devastating to multiple parties, affected women and their partners, the extended families, friends, and further direct impact the country's economy. This dynamic is considered complex and unique as it causes prolonged grief in comparison to death of a child. To an extent, it leads to separation of couples while to others it leads to a downward spiral by developing anxiety, depression, post-traumatic stress, and suicide (Maiti *et al*.,2022).

3.1 Pathology of stillbirth

Scientific and geographical data highlight several factors have been attributed to high stillbirth rates. In LMIC, Low- and Middle-Income Countries, a lack of formal education, low socio-economic status, poor traditions and customs, tendencies, and inability to make prompt decision surrounding medical care all attribute to poor pregnancy outcomes. Other factors such as maternal age, inability to detect and act on danger signs, delayed hospital visits or care, lack of access to communal care and poor nutrition are high on the list. (Smith *et al.*, 2022; Vicky *et al.*, 2016; Murphy *et al.*, 2017.)

In High Income Countries, factors surrounding fetal death and stillbirth are pegged on maternal age, health, and lifestyle. Advanced or exceptionally low maternal ages have been associated with high birth risks. Preexisting medical conditions such as diabetes, hypertension, obesity, and auto immune diseases increase the risks of stillbirths as they affect both placental function and fetal growth. Inadequate and late initiation of prenatal visits can delay detection and management of arising complications (Giakoumelou, S *et al.,*2016: Burden, C., *et al.,2016*) Smoking and substance use have so far been linked to high rates of obstetric factors such include IUGR and Inter Uterine Growth Restriction while instigating placental inefficiency (Zur, R.L.et al.,2020)

While known risk factors for stillbirth have been identified, many cases remain unexplained. Current diagnostic methods include ultrasounds, cardiotocography for fetal heart rate patterns, genetic screening, and maternal health assessment via blood samples. However, there is still a need to understand unexplained stillbirths and to be able to predict stillbirth risk *in utero(*Sober,S.,*et al*., 2015: Kelly,K., *et al.,*2021:Sutan .R., *et al.,2020.)*

3.2 Circular RNA as Potential biomarkers for placental ageing.

Recent studies report the presence of circRNAs in extracellular vesicles, such as exosomes, which are ideal candidates for new biomarkers. The abundance and diversity of circRNAs in human blood are documented in exoRBase, however this database neglects to mention any data relating to pregnancy. By understanding exosome mechanisms, scientists can develop strategies to target diseased or malfunctioning cells. Given that the human placenta readily secretes extracellular vesicles into maternal circulation, this provides a potential screening tool. In this study, we aim to determine how circRNA malfunction predicts placental function and, ultimately, fetal growth and development(Lai et al., 2022).

4. Organoids

Organoids are self-organized 3D tissues derived from stem cells (pluripotent, fetal, or adult) that imitate the normal functional, structural, and biological capabilities of an organ. The cells that form organoids come from induced pluripotent stem cells or tissue-resident stem cells, which include normal, differentiated, and cancer cells. These cells can multiply into various cell types when grown in a controlled microenvironment (see Figure 4) that regulates their overall capabilities, function, and development(Tang et al., 2022).

For decades, organoid technology has been used to study basic cellular biological processes and to answer fundamental biological questions, such as those related to cellular metabolism and toxicology. This was primarily due to the dependence on stem cell technology, which was traditionally grown in 2D cultures and animal models. Using available monolayer techniques, cells would grow and multiply, but they lacked the cellular complexity and necessary developmental features and functionalities, restricting their use in modern science(Servant et al., 2021).

Once formed, organoids can be analyzed using various techniques, such as microscopy. Due to ethical concerns regarding human or embryo-based research related to health and moral issues, it was necessary to continue research while supporting life and maintaining the continuity of embryos. This further underscores the importance of organoid technology. Organoid technology offers practical alternatives to embryonic stem cell studies and scientific development(Huang et al., 2024). The collection of cells for organoid-based studies is less invasive than traditional embryonic cell collection procedures. This justifies the

preference for organoid use and expedites ethical approval by prioritizing human dignity and welfare. Despite their promising capabilities, organoids face limitations, such as an incomplete representation of structural cellular components, including immunological components(Deloria et al., 2020).

Currently, advancements in bioengineering, involving the use of synthetic hydrogels, microfluidics, and bioreactors, aim to improve precision models by mimicking the physical and biological cues of real tissues(Deloria et al., 2020). By overcoming current limitations and improving control over the media and organoid development in general, this technology paves the way for biomedical research, such as regenerative medicine for organs(Bian et al., 2021)

Figure 4 Image removed due to copyright restrictions

Organoid imaging is a challenging task for a number of reasons. Different organoids require different culture media and methods hence develop to different densities. So, while one protocol may work on one organoid, it may differ on another. The 3d structure of organoids also locates at a different depth within the media making focus and difficult. Morphological

differences also play a crucial role in how it can be imaged. Thorough images require sensitive artifacts(Bian et al., 2021).

CHAPTER 2: MATERIALS AND METHODS

2.1 Microscopy Sample preparation

Briefly, prior to placement, Dr Arthurs isolated trophoblast stem cells (TSCs) from early gestation placental tissue and grew them to confluence prior to genetically editing using a CRISPR-Cas9 plasmid. Successfully transfected cells were then grown in a placental organoid growth-stimulating media(Pal & Pal, 2022). Once fully formed, organoids were harvested and preserved in HistoGel (Thermo Fisher Scientific) before being fixed in 10% formalin for 25 mins, then stored in phosphate-buffered saline (PBS) at 4°C(Denti et al., 2020).

2.1.1 Sample preparation , Dewaxing and Rehydration

Each sample was dehydrated through an ethanol gradient and cleared overnight in chloroform. Samples were then embedded into paraffin wax. Formalin-fixed paraffin-embedded (FFPE) blocks were sectioned (4 μm) using a Thermo Scientific HM325 microtome, then sections were floated in a warm water bath at 37°C and then transferred to Knittel StarFrost Advanced Adhesive microscope slides (ProSciTech). Slides were appropriately labelled, then dried at 37°C for 48 hours.

Slides were transferred to a fume hood for processing (see Figure 4). Slides were sequentially were deparaffinised using xylene (2x 4 mins) and rehydrated using descending alcohol concentrations (2 mins each, 100-70% EtOH) and de-ionised water (2 mins). Finally, slides were transferred to the bend and washed in PBS (3x 5 mins(Denti et al., 2020)).

2.1.2 Heat-induced antigen retrieval

On the bench, slides were immersed in 600 mM sodium citrate (pH 6, 10 mM), then heated in a microwave at P10 setting for 1 minute and 50 s, followed by P1 setting for 10 mins(Pal & Pal, 2022). Finally, the slides were dried in an oven at 37°C for 30 mins(Denti et al., 2020).

2.1.3 Enzymatic Antigen Retrieval: Modification of the study

Following two unsuccessful attempts, the protocol was modified. Proteolytic enzyme retrieval was suggested to cater to the delicate nature of the small (~300μm diameter)

organoids. The enzymes selected were trypsin (0.05%) and pronase (2X, 3.5 units/mg). On a single slide containing three serial sections, trypsin was added to the first section, pronase to the second and PBS as a control to the third section. The samples were placed in a sealed container and heated in an oven at 37°C for 10 mins(Pal & Pal, 2022).

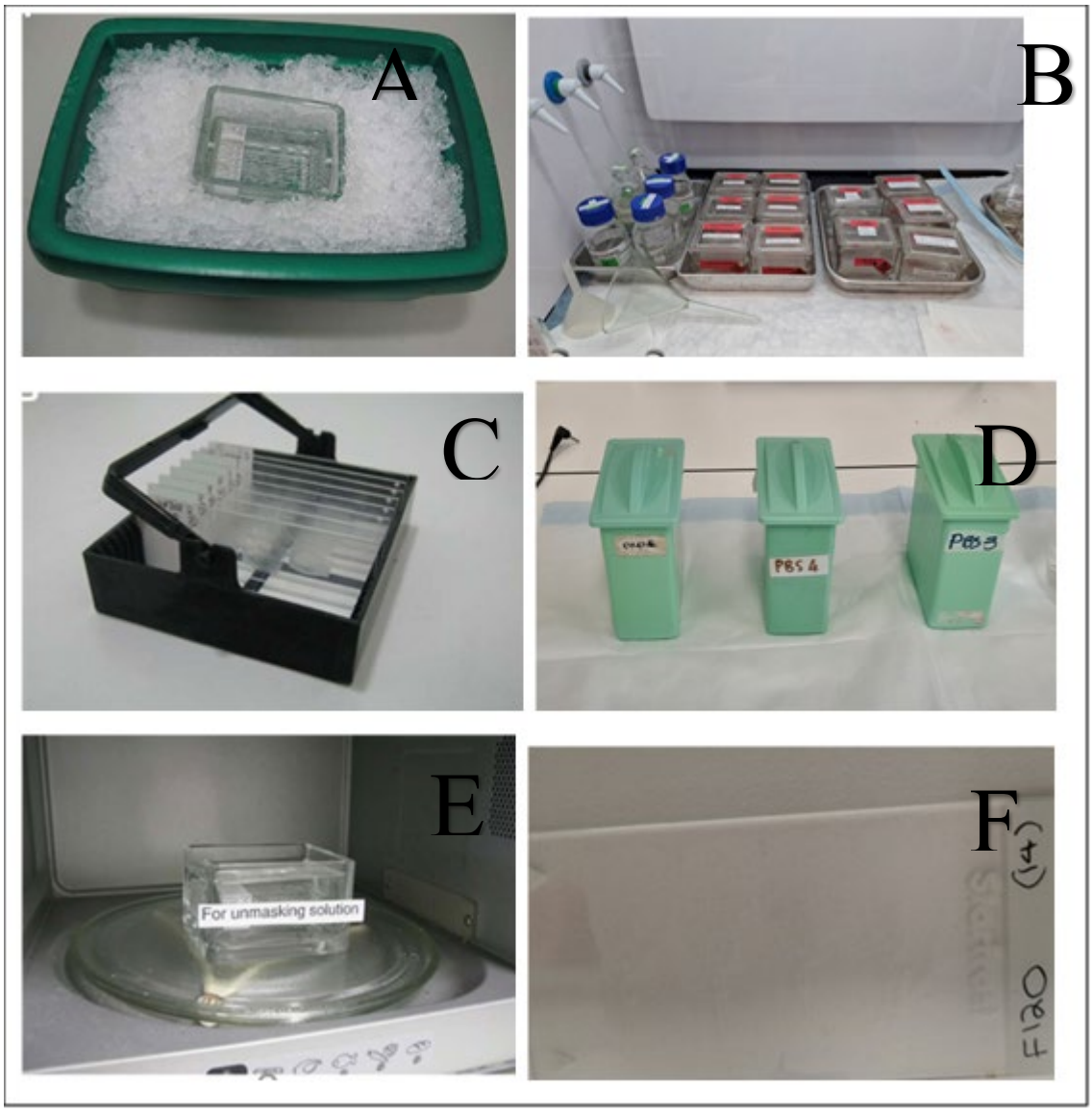


Figure 5*: Sample preparation, for antigen retrieval and staining (**A**) Sample preparation and Dewaxing of ribbons. (**B**) Histological staining boxes cleaned and filled with relevant solution within a fume hood as per arranged order. (**C**) Drying of paraffin slides within rack. (**D**)Washing of slides in PBS. (**E**) Container with slides is microwaved at P10 for 1 Minute 50 Seconds and later at P1 for 10 minutes. (**F**) Antibody and enzyme are introduced to the samples.*

2.1.5 Blocking

Slides were first washed in Phosphate Buffered Saline (PBS) (3x 5 mins) and then blocked in 6% H_2O_2 (in milliQ water) for 15 mins. The slides were washed again in PBS (3x 5 mins), followed by a 15-minute block in normal goat serum(Pal & Pal, 2022).

2.1.6 Application of antibodies

Primary antibodies were added onto sections. The antibodies used were PSG1 (mouse monoclonal; Invitrogen, PA5-110230), PEG10 (rabbit polyclonal; Invitrogen, MA515475), Ki67 (rabbit polyclonal; Abcam, ab16667), Cleaved Caspase 3 (mouse monoclonal; Invitrogen, 43-7800), 8OHdG (mouse monoclonal; Abcam, ab48508) and 4HNE (rabbit polyclonal; BIOSS, BS-6313R). Slides were placed in a humid box, contained and covered with aluminium foil overnight (24 h, 4° C).

The slides were washed with PBS (3x 10 mins) excess PBS was drained off, and lint-free tissue was used to wipe around the sections. Secondary antibodies of goat anti-rabbit and goat anti-mouse (1:1000) were prepared individually and pipetted onto sections with the corresponding primary antibodies (goat anti-mouse, goat anti-rabbit; Abcam), along with DAPI (1:1000, #D95464 Sigma Aldrich) to stain the cell nuclei. Slides were placed into a humid box, kept hydrated and covered with aluminium foil to allow for antibody binding (2 hours, 25° C)(Pal & Pal, 2022).

2.1.7 Mounting and imaging

Slides were washed in excess PBS (3x 10mins), and excess PBS was again wiped off with lint-free tissue. 20µL of buffered glycerol was added, and a glass cover slip was placed on the sections. Excess glycerol was blotted off with blotting filter paper, and transparent nail polish was applied to the edges of the cover slip.

Staining was visualised using the Olympus BX50 fluorescence microscope + Zeiss Axiocam Monochrome camera with Zen Blue Image Capture software. Antibodies were conjugated to fluorophores which excited at 525 nm (CY3), 470 nm (FITC) or 365 nm (UV)(Pal & Pal, 2022).

2.2 Quantification of circular RNAs (circRNAs) in maternal blood

2.2.1 RNA Extraction and Isolation

The Qiagen RNA isolation kit (cat. #217184) was used for RNA extraction. Frozen serum samples (maternal blood samples taken at 20 weeks', n=4 from women who experienced stillbirth, n=4 gestation-matched controls) were collected and thawed on ice. To 50 µL of thawed sample, 250 µL of QIAzol lysis reagent was added, mixed by pipetting, and vortexed for 30 s. The homogenate was incubated on ice for 5 mins. In a fume hood, 50 µL of chloroform was added to the mixture, mixed by pipetting, and centrifuged for 15 mins at 4°C.

The upper aqueous phase was collected into a sterile collection tube, and 450 µL of 100% ethanol was added and mixed by pipetting. Half of the sample was transferred into an RNeasy MinElute spin column in a 2 mL collection tube, then centrifuged for 15 s at >8,000 ×g at 4°C. This process was repeated with the remaining sample.

To the spin column, 700 µL of Buffer RWT was added and centrifuged for 15 s at >8,000 × g at 4°C. The flow-through was discarded. Next, 500 µL of Buffer RPE was added to the spin column, centrifuged for 15 s, and the flow-through was discarded. Another 500 µL of 80% ethanol was added, and the spin column was centrifuged for 2 mins. The flow-through and collection tube were discarded, and the spin column was placed in a new 2 mL collection tube, centrifuged at maximum speed for 5 mins, and the flow-through and collection tube were discarded.

The spin column was then placed in a 1.5 mL collection tube, 14 µL of RNase-free water was added, and the tube was centrifuged at full speed for 1 minute to elute RNA, which was collected for further processing.

The purity and integrity of extracted RNA samples were determined using the Experion™ (BioRad) and samples used had a RIN ≥ 8(Arthurs et al., 2022).

2.2.2 cDNA Synthesis

Synthesis of complementary DNA (cDNA) was conducted beginning with 1 µg of total RNA using the QuantiNova Reverse Transcription Kit (Qiagen) according to the manufacturers protocol(Arthurs et al., 2022).

2.2.3Quantifiction Polymerase Chain Reaction (q PCR)

As stated in Arthurs, et al., qPCR was conducted with SYBR Green (QIAGEN) according to manufacturer's instructions, with YWHAZ and β-actin as housekeeping genes (primer sequences listed in Table 1).

PRIMER SEQUENCES:

Transcript	**Forward primer**	**Reverse primer**	**T_m**
hsa_circ_0009000	TGGCTACAATTCCTGCTCCA	ACATCTGCTGCTTTGTGTGG	59
hsa_circ_0024157	AGAAGTGATATACCATTCTTAAATGTA	GATAAATTACAAAATTACATGGATAA	53
hsa_circ_0061017	CCCATTGGCTGAACTCTTCC	AGCCACTACACTTGTTCTTGA	57
hsa_circ_0036877	TTTGTAAGATGCTGGGTTGGTG	ACTGCATCTGTCACCTCGC	57
hsa_circ_0054624	AGCATGTTAGGCAATGTTGAT	TTTACTGACCGCTGTGACCA	60
hsa_circ_0111277	AGTCGATGGATTCCTCTCAT	AAGTTGTTCATCACTCTGTT	55

Table 1:

Denaturation was performed at 95°C for 10 secs, annealing at melt temp listed for 45 secs, and extension at 72°C for 30 secs, for a total of 50 cycles. qPCR results were analysed using the 2-ΔΔCT method(Arthurs et al., 2022).

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Results

Using fluorescence microscopy, the cell nucleus and specific biomarkers of placental types and cellular processes were observed through staining. The diagrams illustrate various distributions and localizations of cells and cellular processes *in situ*. These biomarkers evaluate the presence of stress factors or cellular proliferation within organoids.(Pal & Pal, 2022).

Figures 6 and 7 depict DAPI (blue) staining the nuclei of the organoids. 4HNE appears in red, a marker of lipid peroxidation. 8-OHdG appears in green, a marker of DNA oxidation. In figure 6, Both 4HNE and 8-OHdG co-localise on one side of the organoid. In figure 7 ,they seem to co-localise around the apex of the organoid A merged image displays DAPI with the two antibodies overlaid.

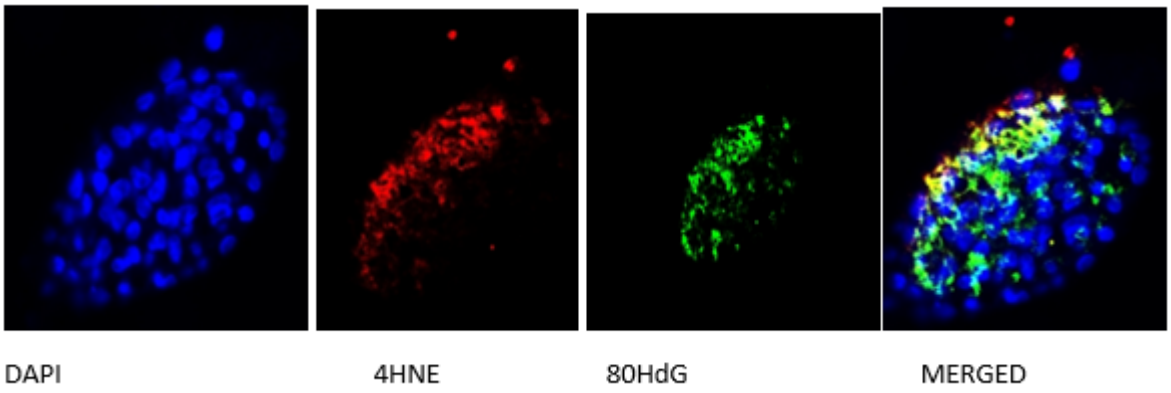


Figure 6: *Successful staining and distribution of markers of cell nuclei (DAPI), lipid peroxidation (4HNE) and oxidative stress (8-OHdG) within one organoid (after 14 days of culture).*

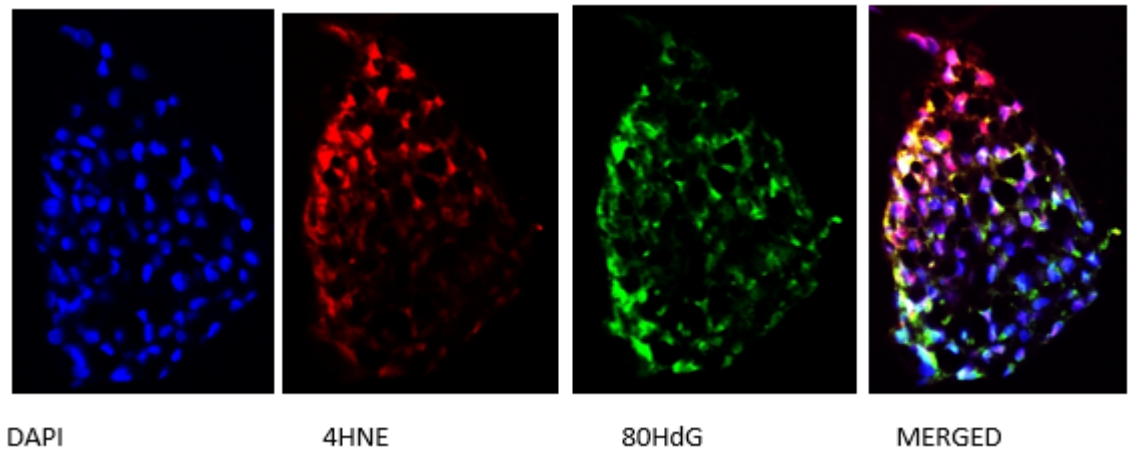


Figure 7: *Successful staining and distribution of markers of cell nuclei (DAPI) lipid peroxidation (4HNE) and oxidative stress (8-OHdG) within one organoid (after 28 days of culture)*

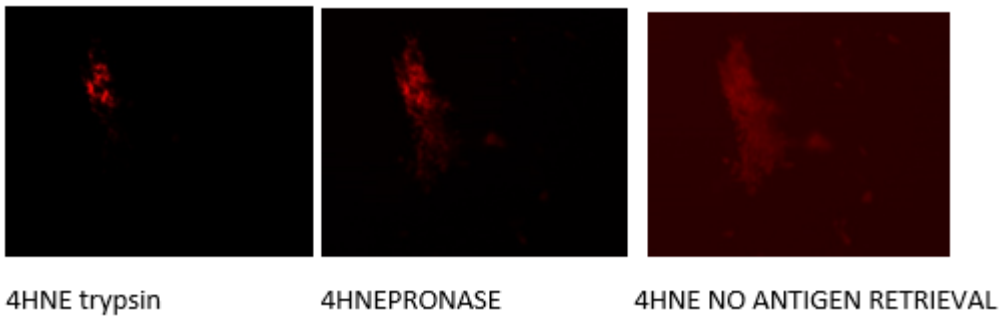


Figure 8: *proteolytic enzyme antigen retrieval optimisation*

Figure 9 displays sucessful antigen retrieval using pronase

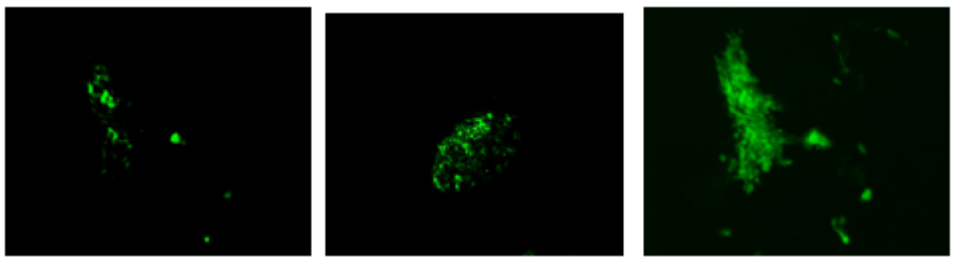
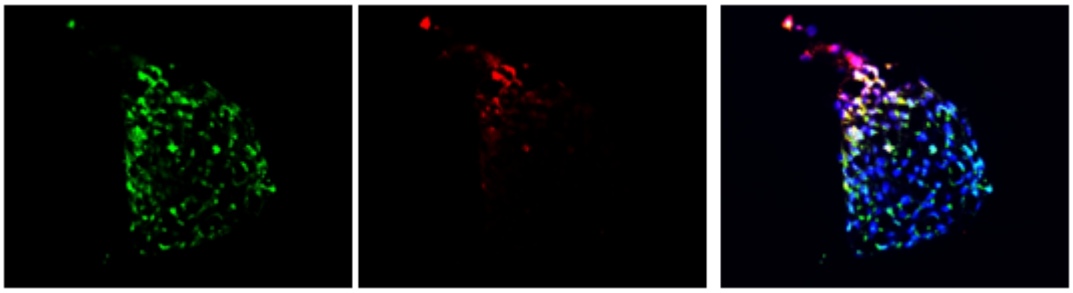


Figure 9*: Successful use of pronase for antigen retrieval within one organoid (after 28 days of culture)*

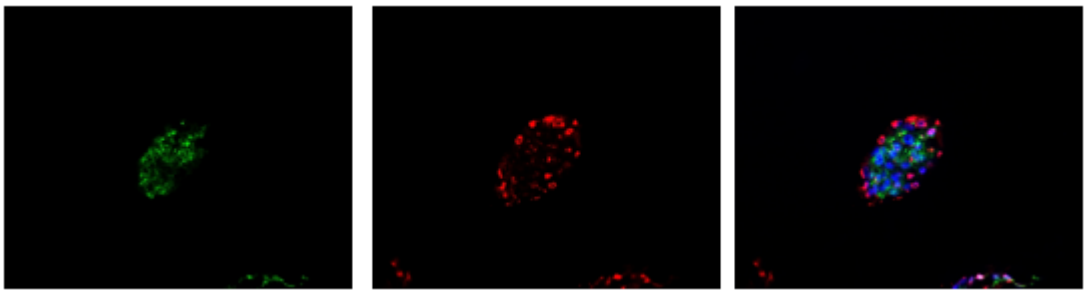
Figure 10 depicts DAPI (blue) staining the nuclei of the organoids. Ki67 appears in green, a marker of cellular proliferation. Cleaved Caspase 3 appears in red, a marker of apoptosis. Ki67 distribution is widespread around the edges of the organoid. Cleaved Caspase 3 is localised to the organoid apex. A merged image displays DAPI with the two antibodies overlaid.



Ki67 Cleaved Caspace 3 Merged

Figure 10: successful staining with DAPI Ki67 and Caspace 3 within organoid .

Figure 11 depicts DAPI (blue) staining the nuclei of the organoids. PSG1 appears in green, a marker of the placental cell syncytiotrophoblast. PEG10 appears in red, a marker of the placental villous trophoblast cells. PSG1 localised to the centre of the organoid, while PEG10 localised to the edges of the organoid. A merged image displays DAPI with the two antibodies overlaid.



PSG 1 PEG 10 Merged

Figure 11: In situ localisation of PEG 10 and PSG1 within cytotrophoblast cells and synciotrophoblast cells

3.2 Fluorescent Optimisation Discussion.

The aim of immunofluorescence was to optimise the fluorescent staining protocol from (Haider et al., 2018) to be suitable for early-gestation, patient-derived gene-edited placental organoids. The major hurdle in this process was ensuring adequate antigen retrieval(Wolf & Dittrich, 1992). As shown in 3.1, no antigen retrieval in these primary antibodies resulted in excess background fluorescence. Initially, we attempted the heat-induced epitope retrieval as recommended in the protocol. However, we observed that the heat retrieval completed destroyed the organoid sections, leaving the microscopy slides completely clear(Denti et al., 2020).

This may be due to the delicate nature of the organoids, which are tiny structure ~300µm in diameter and are hence too small to observe with the naked eye. We therefore used HistoGel to preserve the organoids prior to fixing. As HistoGel is an agar-based medium, its melting point is ~60°C. We anticipate that during the heat-induced epitope retrieval, the slides would have been heated to well over 60°C, hence the HistoGel likely melted and organoids dissolved in the heated buffer(Denti et al., 2020).

To successfully achieve antigen retrieval, heat usage was minimized, and we attempted proteolytic antigen retrieval. Enzymes pronase and trypsin were used(Denti et al., 2020). Both proteolytic enzymes were successful in completing antigen retrieval; this was confirmed by comparing results with a PBS control group, which showed extreme background immunofluorescence. This indicated the importance of antigen retrieval in staining for our chosen antibodies(Haider et al., 2018). We chose trypsin as our enzyme of choice as it produced marginally less background fluorescence(Deloria et al., 2020).

Staining with a primary antibody was complemented by the binding of the secondary antibody, which in turn was conjugated to a specific fluorophore(Haider et al., 2018). The primary antibody recognizes and binds to an antigen due to its specificity and sensitivity, while the secondary antibody binds to the primary antibody, preventing additional conjugates(Wolf & Dittrich, 1992). We used anti-mouse and anti-rabbit antibodies to target specific proteins of interest. While the anti-mouse antibody targeted PEG10, 4HNE and Ki67 biomarkers, the anti-rabbit antibody targeted PSG, 8-OHdG, and cleaved caspase 3. This cross-reaction enhances staining accuracy and visibility(Wolf & Dittrich, 1992).

PEG10 and PSG1 are known markers for villous cytotrophoblasts and syncytiotrophoblast, respectively(D'souza & Shegokar, 2016). This co-staining indicated that villous cytotrophoblasts were present around the outer surface of the organoids, with syncytiotrophoblasts localised to the centre of the organoid(Ha et al., 2010). This morphology is expected according to the Haider protocol. Future experiments will alter the protocol to reverse this morphology, making organoids that better represent human placenta biology(Blois et al., 2014).

The use of protein markers sheds light on the significance of stress factors, relevant growth, and differentiation facilitators within placental organoids. 4HNE, as a biomarker for oxidative stress, was widely present within the tissue(Blandino et al.,2021; Dube et al.,2019). This indicated a compromise of cellular structure within syncytiotrophoblast cells (determined through co-localisation), potentially affecting waste and nutrient exchange between fetus and mother. Visibility of 8-OHdG, a biomarker for DNA damage caused by oxidative stress, was also noted. It was also distributed within the tissue, indicating increased reactive oxygen species (ROS).

Cleaved Caspase 3 was observed in stained cytotrophoblast cells along the apex of the organoid. Produced by cells under duress, it marks programmed cell death. Its presence potentially indicated the impact of oxidative stress and lipid peroxidation on the cells. While caspase 3 can signify adequate cellular turnover, elevated expression may be detrimental to the placenta. In this scenario, the exclusive expression of cleaved Caspase 3 on the apex of the organoid could be the death of villous cytotrophoblast cells that have attempted differentiation into extravillous trophoblasts. This is a normal phenomenon, but as the organoid media was not altered to support the growth of this cell type it would likely result in cell death(Blois et al., 2014).

3.3 Quantitative analysis of circRNA

The table below (figure 12) presents expression levels of circRNA samples. Among these hsa-circ-0036877 and hsa_circ_ 0111277 showed significant upregulation of circRNA expressions . In contrast hsa_circ_0009000 and hsa_circ_005624 exhibited marginal increase in expression . Hsa_circ_0024157 and hsa_circ_006101017 were deemed insignificant (Arthurs et al., 2022).

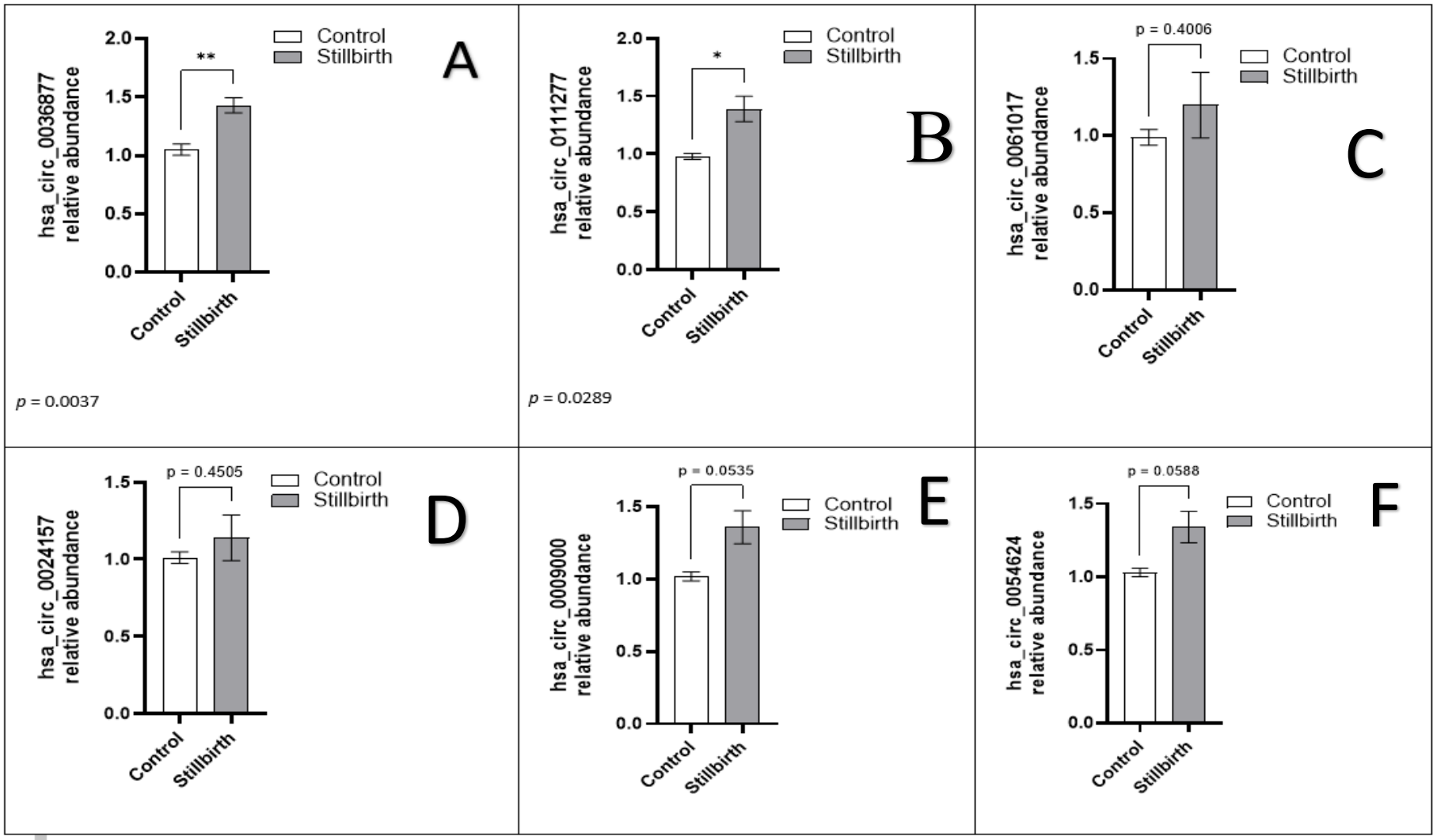


Figure 12 : *Abundance of candidate circRNAs in stillbirth samples relative to healthy gestational age matched controls*

3.4 CircRNA quantification Discussion

This section of the study involved quantification of circRNA. Hsa_circ_0036877 and hsa_circ_ 011127 showed significant differential expression between stillbirth and control groups and these could potentially be used as biomarkers for stillbirth risk.

However, to fully validate this approach, we need a much larger sample size to run additional tests, which could be achieved through future grant funding to recruit more participants and attain statistically significant results. Stillbirth cases in Australia are also challenging to obtain, and excluding explained deaths further limited sample collection. Lastly, this study did not account for fetal sex differences, though there are notable genetic variations between male and female fetuses.

CHAPTER 4: CONCLUSION

We successfully optimized a protocol for immunofluorescent staining, specifically for early-gestation, patient-derived, genetically edited placental organoids(Haider et al., 2018). This will be invaluable for use in future experiments within this project, as it will allow *in situ* localisation of cell types and functions to characterise organoid morphology and function(Wolf & Dittrich, 1992). Importantly, as these organoids were generated to study the effects of gene editing, *in situ* localisation will allow assessment of how specific genes and gene variants affect placental morphology and function *in vitro.* This can inform *in vivo* clinical observations and gene variant associations with disease.

Additionally, we characterised a panel of potentially four circRNAs to be optimised as a biomarker panel for risk of accelerated placental ageing and stillbirth: hsa_circ_00368777, hsa_circ_00111277, hsa_circ_0009000, and hsa_circ_0054624(Zhao et al., 2021). These markers were readily measured in maternal blood, signalling their potential for future screening tools. Based on these results, it would be valuable to acknowledge PCR quantification as a potential diagnostic tool for prenatal care regarding placental ageing, as is currently conducted in other pregnancy conditions(Arthurs et al., 2022).

From our study, the revelation of circRNA as biomarkers for placental ageing in addition to efficient optimization of Immunofluorescent staining of placental organoids may expand on the existing information on placental biology pave way for innovative non-invasive diagnostic approaches and treatment to pregnancy related concerns.

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