


# The Role and Regulation of Potassium Ion Channels in Endometrium of Women with Recurrent Implantation Failure (RIF)

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# Abstract

Infertility affects 1 in 7 heterosexual couples in the UK. One of the main causes of primary infertility is unexplained infertility (25%), which is linked to recurrent failure of implantation (RIF). Successful embryo implantation is a process which requires both synchronous development and interaction between the hatched blastocyst and endometrium. Endometrial receptivity is key to successful implantation and understanding its molecular determinants may hold important clues for implantation defects. Endometrial cells are exposed to constantly changing microenvironment al factors, typically pH, lipids, oxygen tension, and electrolytes including a six-fold higher potassium (K+) concentration than is found in plasma. These potassium channels are notable for their unique structure and biophysical properties and may play an important role in fertilisation, blastocyst cleavage, modulating endometrial receptivity and embryo implantation.

The hypothesis is that potassium channels of human endometrium are differentially expressed during the window of implantation and potassium channel dysregulation may affect their function and that this may contribute to implantation failure.

Since many immunotherapies for RIF have been proposed, with variable reported success rates, a meta-analysis was carried out to compare these treatments. The rationale for this was that an improved understanding of endometrial potassium channels may help with the future selection and use of treatments. Ten treatments were selected (glucocorticoids, granulocyte colony stimulating factor (GCSF), heparin, Intralipid, intravenous immune globulin (IVIG), leukocyte inhibitory factor (LIF), lymphocyte immune therapy (LIT), peripheral blood mononuclear cells (PBMC) and TNF-α inhibitors) for their effects on live birth rates and miscarriage rates as primary outcomes in women with recurrent implantation failure (RIF), undergoing IVF or via ICSI. Since Intralipid is composed of phospholipids and fatty acids that are proven to

modify potassium channel activity, this treatment was chosen and tested on endometrium to observe its possible effect on potassium channel expression.

Potassium channels are expressed in endometrium but their function in this tissue is not understood. A lab-based study was therefore conducted to examine transepithelial electrical resistance (TEER) to define the ability of endometrial monolayers to regulate the control of the potassium flux and hence understand their role in endometrium.

This physiological approach was complemented with a molecular study by which changes in the expression of select potassium ion channels (KCNK9, KCNK10, KCNK17, and KCNMA1) were tested through the menstrual cycle and in between mid-secretory fertile and mid secretory infertile human endometrium. In addition, treatment effects of intralipid as well as vitamin D and hypoxia (5% O2) were examined. The same study was conducted on decidual tissue since it is composed mainly of stromal cells and any changes in gene expression from endometrium could be caused by the cell population. Flow cytometry was undertaken to detect potassium channel proteins translation at the single cell level in both endometrium and decidual cells.

To understand the epigenetic regulation of the potassium channels expression, DNA methylation profile at the promoter region of the tested channel genes were examined to determine any association between the changes in expression observed in the endometrium with changes in DNA methylation. This was carried out after producing a new protocol for the new EpiQuik™ Tissue Methylated DNA Immunoprecipitation Kit, to work optimally with endometrium and decidual tissue.

The preliminary meta-analysis results presented show some evidence that intralipid, PBMC, heparin and to a lesser extent GCSF may have some beneficial effects. These findings have to be interpreted with extreme caution because there are risks attached to these treatments, some of which are very

serious. Regarding TEER results, TEER was decreased in 5% O2 (n = 6) in comparison with the control (n=8) ($P<0.005$). Also, TEER was decreased in both KCNK9 blocker (n = 6) and KCNMA1 blocker (n =6) in comparison with the control (n=8) ($P<0.001$), while no statistically significant difference was found between the control vs Intralipids and Vitamin D.

For the potassium channels expression, KCNK17 showed significant altered expression ($P <0.05$) in proliferative phase (n = 6) in comparison to secretory phase (n = 10). Also, KCNK17 was significantly downregulated in infertile mid-secretory endometrium (n = 6) in comparison to fertile mid-secretory endometrium (n = 4) ($P <0.05$). When mid-secretory infertile endometrial samples were tested after treating them with 5% O2 (n = 6), Intralipids (n = 7) and Vitamin D (n = 7), none of the channels showed significant altered expression ($P >0.05$) in comparison to the control group (n = 12). Translation to protein for the potassium channels was confirmed by flow cytometry.

A new protocol for the new EpiQuik™ Tissue Methylated DNA Immunoprecipitation Kit was produced and a pilot study was carried out, showing a hypermethylation of the KCNK17 gene in the fertile mid-secretory endometrium.

The findings from this study provided new knowledge on the role of potassium channels in endometrium, especially KCNK17 that is found to contribute to normal and infertile endometrial function. This demonstrates the involvement of potassium channels in the uterine electrolyte transport and intra-uterine fluid amount and composition which is clinically important in endometrial receptivity and embryo adherence and attachment to endometrium.

# Declaration

I declare that this thesis is my own work and is based on research that was undertaken at the Division of Medical Sciences and Graduate Entry Medicine, School of Medicine, University of Nottingham during the period of December 2014 to November 2018.

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# Publications

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# Abbreviations

| | |
|---|---|
| ACTβ | Actin Beta |
| AMP | Adenosine monophosphate |
| ART | Assisted Reproductive Technology |
| ATCC | American Type Culture Collection |
| $BK_{Ca}$ | Large Conductance Calcium Sensitive Potassium Channel |
| BSA | Bovine serum albumin |
| BSA | Bovine serum albumin |
| cDNA | Complementary Deoxyribonucleic acid |
| CT | Cycle threshold |
| CTD | Cytosolic tail domain |
| CYC1 | cytochrome c-1 |
| DAPI | 4',6-diamidino-2-phenylindole |
| DHEA | Dehydroepiandrosterone |
| DMEM | Dulbecco's modified Eagle's medium |
| DNA | Deoxyribonucleic acid |
| DNMTs | DNA methyltransferases |
| dNTP | Deoxyribonucleotide Triphosphate |
| ECM | Extracellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| EECs | endometrial epithelial cells |
| EGF | Epidermal growth factor |
| EGF | epidermal growth factor |
| EPF | Early pregnancy factor |
| ER | Estrogen receptors |
| ER | estrogen receptor |
| ESCs | endometrial stromall cells |
| FAM | 5-carboxyfluorescein |
| FBS | Foetal Bovine Serum |
| FSH | Follicle Stimulating Hormone |

| | |
|---|---|
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| G-CSF | Granulocyte Colony Stimulating Factor |
| GUS | Glucuronidase |
| GUSB | Glucuronidase, beta |
| HB-EGF | Heparin Binding Epidermal Growth Factor |
| HBSS | Hank's Buffered Saline Solution |
| ICM | Inner cell-mass |
| IGF | Insulin-like Growth Factor |
| IMS | Industrial Methylated Spirit |
| IVF | In vitro fertilization |
| IVIG | Intravenous Immune Globulin |
| K+ | Potassium |
| K2P | Two pore domain potassium channels |
| LH | Luteinizing Hormone |
| LIF | Leukocyte Inhibitory Factor |
| LIT | Lymphocyte Immune Therapy |
| LPA | lysophosphatidic acid |
| MIQE | minimum information for publication of quantitative real-time PCR experiments |
| MSD | Membrane spanning domain |
| NAT | No Added Template |
| ncRNA | non-coding RNA |
| ND | NanoDrop® |
| NICE | National Institute for Health and Care Excellence |
| NK | Natural killer |
| NO | nitric oxide |
| PBA | phosphate buffered albumin |
| PBMC | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate Buffered Saline |
| PCOS | Polycys |
| PCR | Polymerase Chain Reaction |
| PFA | Paraformaldehyde |

| | |
|---|---|
| PGD | Pore-gated domain |
| PGK1 | Phosphoglycerate kinase 1 |
| PGs | prostaglandins |
| PIS | Patient information sheet |
| PKG | cGMP-dependent protein kinase |
| PKTHPP | 1-1-6-(biphenyl-4-ylcarbonyl)-5,6,7,8-tetrahydropyrido4,3-d-pyrimidin-4ylpiperidin-4-yl propan-1-one |
| PPIA | Peptidylprolyl isomerase A |
| PUFAs | Poly unsaturated fatty acid |
| RCOG | Royal College of Obstetricians and Gynaecologists |
| RNA | Ribonucleic acid |
| ROS | reactive oxygen species |
| RPM | Revolutions per minute |
| RT | Real-Time |
| SD | Standard deviation |
| TALK | TWIK-related alkaline-sensitive K+ |
| TASK | TWIK related acid sensitive K+ |
| TBE | Tris/Borate/EDTA |
| TEER | Transepithelial electrical resistance |
| TFRC | Transferrin receptor |
| THIK | Tandem pore domain halothane-inhibited K+ channel 1 |
| TOP1 | topoisomerase DNA I |
| TRAAK | TWIK-related arachidonic acid stimulated K+ channel |
| TREK | TWIK-related K+ channel |
| TWIK | The weakly inward rectifying K+ channel |
| UBQ | Ubiquitin |
| UV | Ultraviolet |
| VSD | Voltage sensor domain |
| WHO | World health organisation |

| | |
|---|---|
| YWHAZ | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide |

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# Chapter One: Introduction and Background

The stage of human development since the time of fertilisation until pregnancy establishment is considered one of the most critical and fascinating physiological processes, which is still not well understood. Many factors are involved in this complicated process and deregulation of any of the stages could lead to reproductive failure.

Implantation of the embryo is a prerequisite for human reproduction, and nearly two-thirds of pregnancy losses in humans are believed to be the result of peri-implantation failure (Simón et al., 1998, De los Santos et al., 2003, Sharkey and Smith, 2003), where peri-implantation period starts by the time when blastocyst is free in the uterine cavity and includes the process of blastocyst recognition and attachment, embryo-endometrial interaction and the start of placental formation (Glasser et al., 1987). Implantation remains a poorly understood process. Despite advances in assisted reproduction technologies (ART) in recent years, the live birth rate of ART remains low, mainly due to implantation failure (BenRafael et al., 2012).

In this thesis, we focused on the population of infertile women with implantation failure to understand the microenvironment of the endometrium and the role and regulation of potassium channels in the human endometrium during the window of implantation.

This chapter contains information on the anatomy, physiology, and pathophysiology of the female reproductive system and potassium channels.

# 1 Female reproductive anatomy and physiology

## 1.1 Anatomy and Histology

The female reproductive system consists of two parts: external genitalia and internal genital organs. The external genitalia include the labia minora and majora, mons pubis, clitoris, external urinary meatus, vaginal orifice and vestibule (the vaginal opening), and the hymen. The internal genital organs include the ovaries, fallopian tubes, uterus, and vagina (Symonds et al., 2013).

The ovaries are paired almond-shaped organs approximately 2.5–5 cm long. They have endocrinal and reproductive functions (Archer and Nelson, 2012). The fallopian tube, also called the oviduct, is the structure that transports the ovum from the ovary to the uterus every month and is divided into four parts: the interstitial, isthmus, ampulla (where fertilisation occurs), and infundibulum, which is surrounded by the fringe of the fimbriae and attached to the ovary (Archer and Nelson, 2012).

The non-pregnant uterus is a muscular, pear-shaped, hollow organ. It measures 2.5 × 5 × 7.5 cm and weighs up to 40 g and consists of three parts: body, isthmus, and cervix (Gray and Lewis, 1918). The internal part of the body of the uterus is lined with the endometrium, which consists of a mucus-secreting columnar epithelium surface that undergoes different histological and physiological changes during the different phases of the menstrual cycle (Hacker et al., 2009).

## 1.2 Menstrual cycle

The menstrual cycle is the regular, natural dynamic change during the adult female reproductive period. It occurs in both the ovaries to prepare for follicular development and ovulation and in the endometrium to prepare it for implantation if conception is to occur (Symonds et al., 2013). The average

length of a menstrual cycle is 28 days, ranging between 25 and 32 days (Silberstein and Merriam, 2000).

Through the menstrual cycle, hormonal changes occur during follicular development, ovulation, and post-ovulation. The key reproductive hormones fluctuate simultaneously with follicular development, so to understand follicular development through the cycle, it is important to understand the hormonal changes that underlie the menstrual cycle. The first stage of follicular development is initiated by the release of gonadotrophin-releasing hormone (GnRH) from the median eminence of the hypothalamus, resulting in the release of both follicle-stimulating hormone (FSH) and luteinising hormone (LH) from the pituitary gland (Berek and Novak, 2007, Danforth and Gibbs, 2008, Jones and Lopez, 2013). These hormones initiate the aggregation of stromal cells and theca cell formation (that secretes progesterone and androgen hormones). By the sixth day of the cycle, the dominant follicle is selected and the corona radiata is formed by the adherence of both the inner layer of granulosa cells (that secretes oestrogen and progesterone hormones) and the selected ovum (Berek and Novak, 2007).

Later, the ovum becomes eccentrically placed, and a mature Graafian follicle is formed with the differentiated theca interna (express receptors for luteinizing hormone) and theca externa layers. Around the time of ovulation, the follicle enlarges and moves towards the surface of the ovary, where the ovarian germinal surface becomes thin (Jones and Lopez, 2013). Finally, at the time of ovulation, GnRH surge occurs, resulting in a LH surge which is responsible for the release of follicle, which is also responsible for the reactivation of meiosis I and the sustention of corpus luteum development, If fertilisation occurs, corpus luteum is responsible for the production of progesterone hormone during early pregnancy to maintain pregnancy till the formation of placenta (Symonds et al., 2013).

After ovulation, the corpus luteum is formed, secreting progesterone, reaching its peak seven days after ovulation (Stanfield, 2012). The fate of the corpus

luteum depends on whether implantation occurs or not; in the case of implantation, the corpus luteum survives under the effect of β-human chorionic gonadotropin (βHCG) secreted from the implanted embryo, maintaining the release of progesterone, until it is replaced by the placenta at the tenth week of gestation. If implantation does not occur, the corpus luteum regresses forming the corpus albicans (Symonds et al., 2013).

## 1.3 Endometrium and changes in it during the menstrual cycle

The endometrium is the main subject of my research project, and understanding its physiological and molecular changes is important, especially as it is a highly dynamic tissue that undergoes menstruation, regeneration, and remodelling of the functional layer in response to the cyclic fluctuations of the ovarian hormones that may help with our understanding of implantation.

Histologically, the endometrium consists of a simple columnar epithelium and a thick stromal layer of connective tissues underlying it. The mucosa is invaginated, resulting in a high density of simple tubular uterine glands that extend through the whole stromal thickness (every $mm^2$, contains around 15 glandular openings) to ensure that glandular secretion is abundant (Salamonsen et al., 2016). The stromal cells of the endometrium are embedded in a network of reticular fibres (Jiménez-Ayala and Portillo, 2008). Still, the specific role of the secretory glandular epithelium is poorly understood, especially in its contribution to the total composition of human uterine fluid that provides the optimum microenvironment needed for successful implantation (Salamonsen et al., 2016).

The endometrium consists of two layers. The first deeper thin layer is the basal layer (stratum basalis), which generates the upper functional endometrial layer (Chan et al., 2004). This upper functional layer (stratum functionalis) is the layer that undergoes shedding, proliferation, differentiation, and apoptosis in response to ovarian hormones and their changes through the menstrual cycle

(Aplin et al., 2008). The average length of the menstrual cycle is 28 days, ranging between 25 and 32 days (Silberstein and Merriam, 2000) and is divided into 4 phases (Figure 1), where the following changes of endometrium occurs during the menstrual cycle (Murray et al., 2004):

*Day 1-4:* Menstruation is the phase when shedding of the function layer occurs as a result of vasoconstriction of the arterioles associated with the drop in oestrogen and progesterone, eventually rupturing the walls of weak arteries, where blood pours in to the connective tissue of the stratum functionalis layer. Degenerated cells and blood cells enter the uterine cavity and are lost by the passage through the external os of the cervix and then through the vagina.

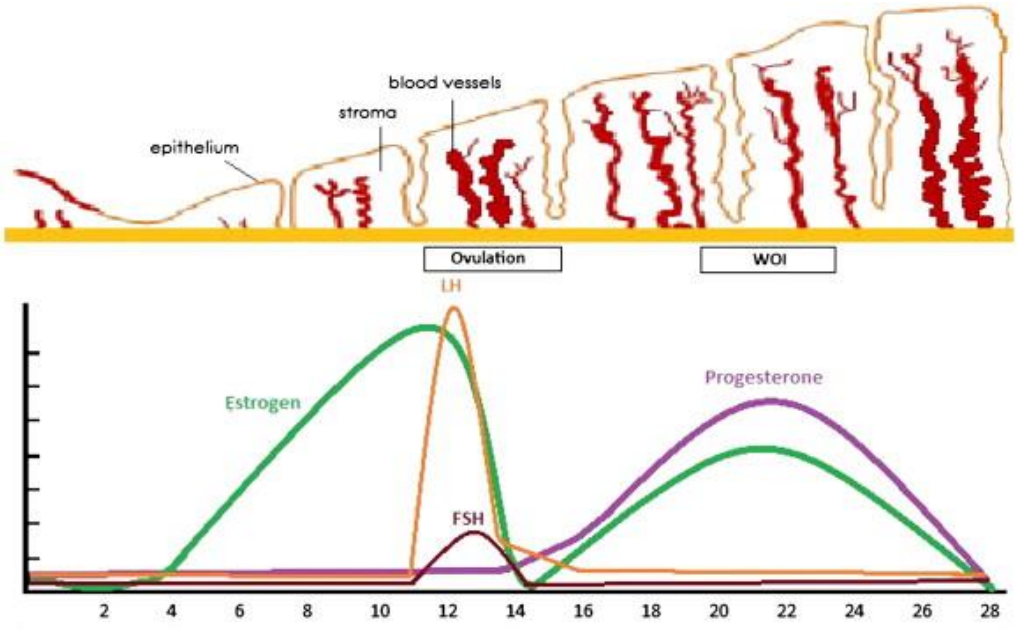
*Day 5-7:* Repair, also named early proliferative phase, is associated with regeneration of the epithelium and remaining glands after menstruation that are narrow, short, and collapsed. This starts on day 5 of the cycle, with significant epithelial cell DNA synthesis and mitotic activity.

*Day 7-15:* Proliferation is the phase of maximal endometrial growth where the glands become elongated, branched, and tortuous, supported by the endometrial stroma. The endometrial thickness reaches 1–4 mm and prominent mucous glands extend to the border of the basilar layer.

*14 days from ovulation:* The secretory phase is divided into early, mid, and late secretory phase. In this phase, there is functional differentiation of the endometrium to provide a healthy environment for implantation and pregnancy (Aplin et al., 2008). During this phase, angiogenesis and functional differentiation of glands occur without further proliferation under the influence of progesterone that eliminates oestrogen-driven mitosis in the glands and stroma (Stanfield, 2012). This is helped by many growth factors including insulin-like growth factor (IGF), mainly epidermal growth factor (EGF) and transforming growth factor β (TGFβ) (Archer and Nelson, 2012). These growth factors play a role in shifting the action of oestrogen throughout the endometrium between both proliferative and secretory phases, with downregulation of oestrogen receptors (ER), giving the upper hand to

progesterone for more differentiation and decidualisation at the end of the secretory phase (Gray et al., 2001).

Endometrial biopsy samples may be dated histologically based on the stage of the menstrual cycle according to Noyes criteria. These criteria are based on 8000 biopsies, modified between 1940 and 1950 (Noyes et al., 1950), and are used clinically in fertility assessments.




| Menstrual | Proliferative | Secretory | | |
|---|---|---|---|---|
| | | ES | MS | LS |
| Desquamation | Cellular proliferation<br><br>Cellular differentiation<br><br>ECM remodelling<br><br>Angiogenesis<br><br>Adhesion<br><br>Ion channels | Metabolism<br><br>Transport<br><br>Proliferation inhibition<br><br>Mitosis inhibition | Metabolism<br><br>Glandular secretion<br><br>Cell differentiation<br><br>Cell communication<br><br>Innate immune response<br><br>Response to stress<br><br>Respond to wounding<br><br>Adhesion<br><br>Proteolysis regulation | ECM degradation<br><br>Inflammatory response<br><br>Apoptosis |

**Figure 1: Endometrial changes in relation to hormonal changes through the menstrual cycle**

(Ruiz-Alonso et al., 2012)

## 1.4 Intrauterine fluid

Uterine luminal fluid has a complex molecular composition, originating from multiple sources of selective transudation from the serum and the influxed peritoneal fluid via the Fallopian tubes (Casslen, 1986). The amount of fluid rages between 5 and 35 mL around the window of implantation, which is the period when the endometrium is receptive for the implantation of the free-lying embryo. (Casslen, 1986). The secretory epithelial glands of the endometrium play a role in the dynamic change in the volume of the intrauterine fluid during the cycle. This process is suggested to occur under the hormonal influence of the menstrual cycle, with a reduction in the fluid volume during the peri-implantation period (Naftalin et al., 2002, Salleh et al., 2005).

The molecular composition of these secretions includes carbohydrates, amino acids, lipids, ions, and protein products, such as cytokines, immunomodulatory factors, enzymes, hormones, growth factors, and transporters (Salamonsen et al., 2016). Studies in 1970 showed that during the menstrual cycle, the concentrations of some ions in the human uterine fluid changes and even differs significantly from the serum concentrations (Clemetson et al., 1973a). The transport of ions creates an electrolyte gradient that influences the water movement, leading to either secretion or absorption across the epithelium and hence controlling the volume during peri-implantation period (Salleh et al., 2005) This reduced volume of fluid during implantation, stabilises the blastocyst in the uterine lumen to achieve close contact with the endometrium, which is believed to be the crucial point during implantation (Salamonsen et al., 2016). Any dysfunction or deregulation of ion channels and their expression can result in disorders in the ionic gradient and fluid transport, resulting in implantation failure (Chan et al., 2013b, Ruan et al., 2014b).

The oxygen concentration changes along the female genital tract, ranging between 2% and 8% (Leese, 1995). Maas et al. reported that oxygen tension is lower during the follicular phase of the menstrual cycle (<1%) and it increases in the oviduct after ovulation (Maas et al., 1976). These fluctuations have been found to be inconsistent amongst studies, and still there are no

clear data on the early pregnancy stage. Especially in the presence of an embryo, the hypothesis becomes complicated, i.e. the oxygen requirement is low when the conceptus resides in the oviduct; then, the oxygen requirement increases dramatically as the blastocyst is formed in the intrauterine cavity (Ng et al., 2017).

## 1.5 Endometrial receptivity

The human endometrium is either hostile or non-receptive (Sharkey and Smith, 2003) during the proliferative phase. During the early and late secretory phases, it is non-receptive. It is only receptive during the mid-secretory (mid luteal) phase 6–10 days post-ovulation (LH+6 to LH+10). At this time, the endometrium receptivity is suitable for implantation and is named the 'window of receptivity' or 'window of implantation' (Aplin et al., 2008). During this period of implantation, the endometrium reaches a thickness of 10–14 mm and both the endometrial epithelium and stroma are rich in lipids and glycogen (Fritz and Speroff, 2012).

Understanding the dynamic endometrial endocrine system can enhance our understanding of the synchronisation of implantation that recruits a large cast of molecular and biochemical players (Fritz and Speroff, 2012). There is a lack of information on the window of receptivity, particularly regarding the correlation between endometrium histology dating and endometrium receptivity markers throughout the endometrium. Also, there is no single reliable marker that appears and then disappears during the window of receptivity. It is believed that a combination of different markers is needed to determine the time of endometrial receptivity (Carson et al., 2000, Chan et al., 2013a).

Receptivity of implantation starts even before the moment of contact between the embryo and endometrium. It starts after fertilisation and prior to implantation, requiring appropriate signals from the embryo. The embryo

sends signals leading to a secretory response from the ovary by secreting the early pregnancy factor (EPF), which is detected in the maternal circulation within two days post-fertilisation (Fritz and Speroff, 2012). EPF is no longer secreted from the ovary and is replaced later by the embryo (Fritz and Speroff, 2012). EPF has immunosuppressive properties and is involved in the maintenance of the foetal allograft (Nahhas and Barenea, 1990). These events are part of the physiological regulation of the maternal recognition of pregnancy (MRP), that involves complex molecular and cellular cross talking at the blastocyst-uterine interface (Smith et al., 2018) and also it is the process that prevents luteal regression (luteolysis), allowing the persistence and continuation of the corpus luteum to secrete progesterone during early pregnancy (Bazer et al., 2013). The human endometrium also expresses the glycoprotein mucin-1 (Muc1), which is a glycoprotein in human endometrium and is abundant at the luminal epithelial surface in the receptive phase (Joschke et al., 2009). Also, it is observed during in vitro culturing 7–8 days after fertilisation where the blastocysts start secreting human chorionic gonadotropin (hCG), which is considered a preimplantation signal, as hCG is delivered in vivo directly from the uterine cavity to the ovary, stimulating the corpus luteum to produce higher levels of progesterone and oestradiol, which play a role in decidualisation associated with implantation (Fritz and Speroff, 2012).

Implantation is a paradox in cell biology whereby two repulsive apical epithelial cell surfaces become adherent to each other and must come together to initiate and activate the window of receptivity. These two surfaces are the endometrial epithelial surface and the embryo trophectoderm; there is modulation of the apicobasal polarity in the luminal epithelium (Aplin et al., 2008). This modulation is initiated by the progesterone-induced formation of pinopodes around day 21 of the cycle. They have smooth apical protrusions that can be detected only by electron microscope. Pinopodes are considered an indicator of uterine receptivity. These help the blastocyst to adhere to the endometrium by absorbing the intrauterine luminal fluid and removal of adhesion inhibiting

mucin, forcing the blastocyst to get in contact with and adhere at the sites of the pinopodes (Bentin-Ley, 2000).

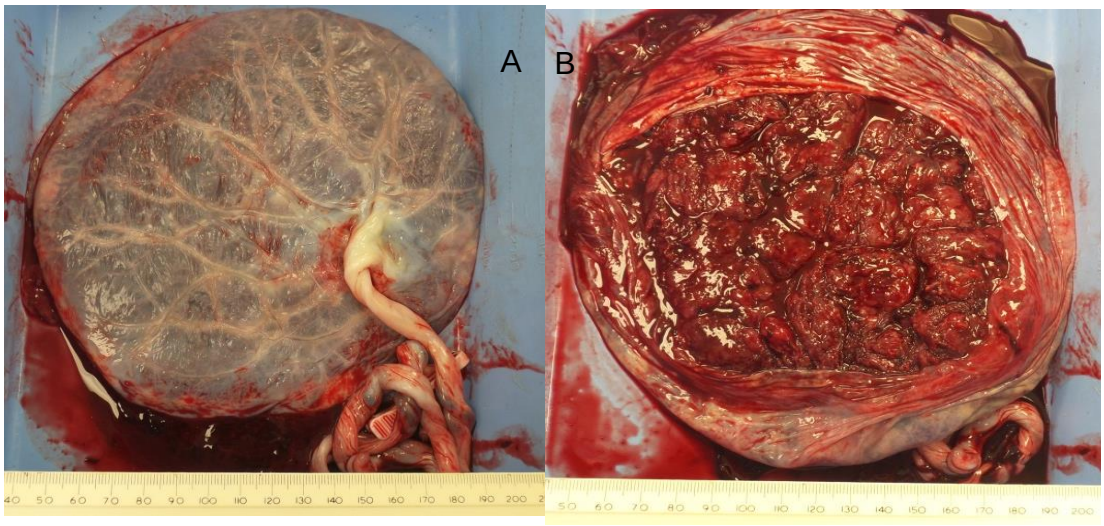
## 1.6 Decidualisation

The decidua is the gravid endometrium and the term is taken from the Latin term *decidere* which means 'fall off' (Gellersen et al., 2007). The decidua is the functional layer of the endometrium during pregnancy. The process of decidualisation starts in the last week of every menstrual cycle and forms the maternal aspect of the maternal-foetal interface. It is the functional and morphologically transformed endometrium during pregnancy, and it is important for the establishment of pregnancy (Aplin, 1996). Endometrial transformation and differentiation starts first in the tissues surrounding spiral arterioles and includes remodelling of the blood vessels and extracellular matrix (ECM), proliferation of uterine natural killer cells (uNK), and stromal fibroblast expansion surrounded by a basement membrane (Loke et al., 1995). The decidualisation process occurs only under the influence of progesterone. It starts in the late secretory phase of every cycle, and at this stage, the endometrium is called predecidual endometrium. The decidualisation process continues only if pregnancy is established with more progesterone secreted (Ashkar et al., 2000), resulting in the formation of three layers of the decidua (Jones and Lopez, 2013). The action of ovarian steroid hormones that controls the endometrial changes depends mainly on their binding to specific receptors, which in turn function as transcription factors for a large number of genes, with evidence of a strong relation between human endometrial histopathology dating and the molecular profiles across different phases of the cycle (Aplin et al., 2008).

If fertilisation occurs during the time of implantation, the decidua plays a role in early embryo survival, as decidual cells close to the invading syncytiotrophoblast degenerate releasing all the glycogen and lipid contents, providing an initial nutrient source with the maternal blood in the lacunae (Kierszenbaum and Tres, 2015). Also, the decidua plays an immune-protective

role through the decidual reaction that includes the production of prostaglandins and immunosuppressive substances that inhibit the action of natural killer (NK) cells at the site of implantation and increase the infiltration of leukocytes that secrete iterleukin-2, preventing embryo rejection by the maternal tissue (Zhang et al., 2012).

The placenta is a foetomaternal organ that consists of foetal parts and maternal parts. (Figure 2) shows the foetal side of the placenta (a) having a smooth and shiny surface provided by the amniotic membrane, which covers it with a number of large veins and arteries converging toward the umbilical cord. The umbilical cord appears to be attached eccentrically. The maternal side of the placenta (b) has a spongy and textured surface divided into cotyledons and lobules by the fissures that contain the remains of septae, extending between the maternal portions and the foetal portions of the placenta.




A: Foetal side B: Maternal side

**Figure 2: Gross picture of placenta**

The decidual regions are named according to their relation to the foetus (Figure 3). The three decidual types are decidua basalis, which is the maternal component of the placenta, decidua capsularis, which is the superficial layer

covering the chorion, and decidua parietalis, which covers the remaining part of the uterine cavity (Kierszenbaum and Tres, 2015). The decidua can be transformed in vitro by culturing endometrial stromal fibroblasts in continuous treatment of isolated oestrogen-primed cells with progesterone over days; also, rapid decidualisation in vitro can occur through the protein kinase A pathway activator cyclic AMP (cAMP) (BARTSCHA and IVELL, 2004).

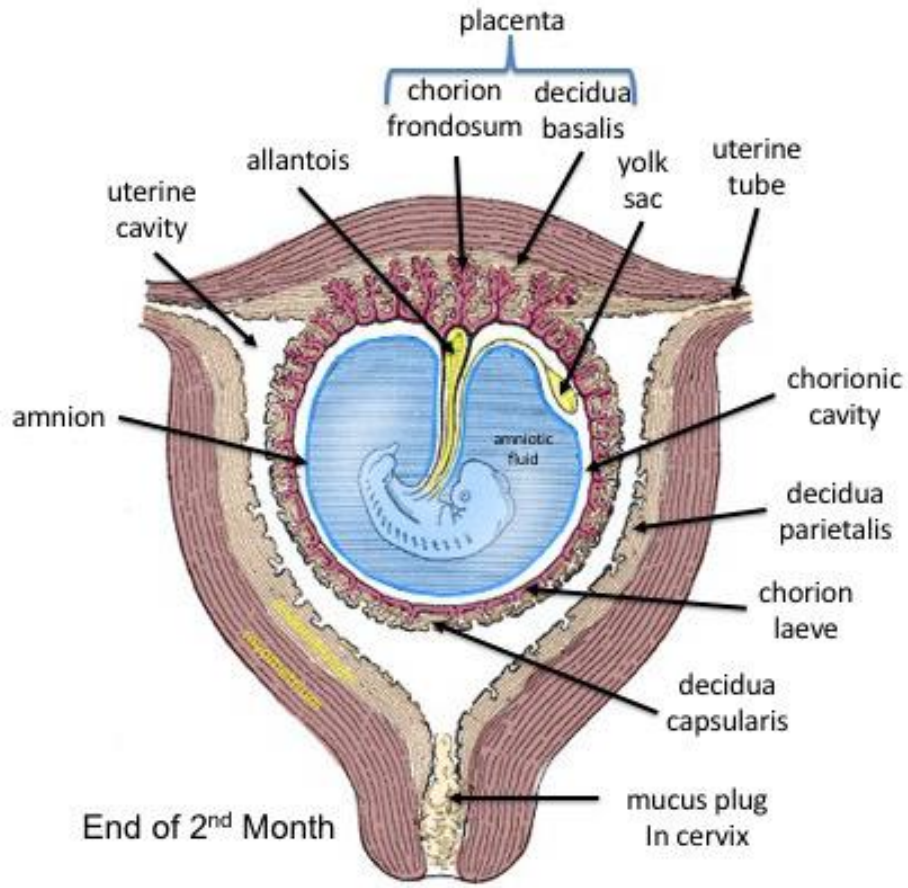



**Figure 3: Decidual region names according to their relation to the foetus**

Picture taken from: Dennis M DePace, PhD [CC BY-SA 4.0 (https://creativecommons.org/licenses/by-sa/4.0)]

## 1.7 Implantation

Implantation is the process when the embryo comes in contact with the endometrium and starts penetrating the epithelial layer and the vascular

system of the mother to form the placenta (Navot et al., 1991). To produce a receptive optimum, i.e. a favourable microenvironment for implantations, a dynamic coordination between the endocrine, cellular, and molecular events is required via paracrine, autocrine, and/or juxtacrine factors (Dey et al., 2004). The process of implantation differs from one species to another, but mammals retain distinctions with the complex strategies used to protect their offspring during development (Carson et al., 2000). Even among mammals, implantation mechanisms differ; the embryo invades the endometrium completely only in apes and humans (Fritz and Speroff, 2012). In all eutherian mammals, during the implantation period, the uterus changes its behaviour when a blastocyst is attached to the endometrium to produce an effective cross-talk communication and initiate implantation (Dey et al., 2004). Implantation in humans is very complex and limited by time, as it starts two to three days after the fertilisation of the ovum and occurs five to seven days after fertilisation. The implantation process includes three phases: apposition, adherence, and invasion.

The blastocyst remains in the uterine luminal fluids and secretions for about one to three days before implantation starts (Fritz and Speroff, 2012) (Figure 4). At this stage, significant absorption and reduction of the intrauterine fluid occurs before the WOI period, leading to narrowing of the uterine cavity (Muggleton-Harris and Johnson, 1976). Uterine secretions contain inorganic ions, albumin, glucose, and urea (Casslen and Nilsson, 1984). Potassium is one of the inorganic ions found in high concentration in the uterine fluid during the mid-luteal phase of the menstrual cycle and it is believed to reduce the membrane potential of the endometrium, facilitating contact between the endometrium and blastocyst, and it could be essential for their adhesion (Clemetson et al., 1972, Clemetson et al., 1973b).

By the time of implantation, the blastocyst differentiates into the inner cell mass (ICM) that later forms the embryo and trophectoderm (TE) that forms the placenta. Both the ICM and TE are essential for implantation, specially the TE that has a primarily role in the distinct stages of implantation that includes

apposition, adhesion, penetration and trophoblast invasion that leads to a controlled manner degradation of maternal tissue, allowing the blastocyst to enter the endometrial lining (Maaike et al., 2008).

The first maternal change to the adherent blastocyst increases the permeability of the capillaries due to the alteration of the heparin-binding epidermal growth factor (HB-EGF) expression in the endometrial surface epithelium (Kierszenbaum and Tres, 2015). Also, the decidualised endometrium mediates cell adhesion by expressing extracellular matrix components such as fibronectin and laminin. This tissue disturbance is associated with an increase in lymphocytes, growth factors, and cytokines. Once the blastocyst comes in very close contact with the endometrium and after the formation of junctional complexes, the embryo can no longer be dislodged from the endometrial surface epithelium (Gellersen et al., 2007).

By the start of the second week post ovulation, the trophoblasts at the site of implantation form masses of syncytiotrophoblast and cytotrophoblast cells, and the invasion of maternal blood vessels begins, and the cytotrophoblast stem cells begin to differentiate and proliferate into the extra-villous trophoblast, which invades the placenta (Aplin et al., 2008).

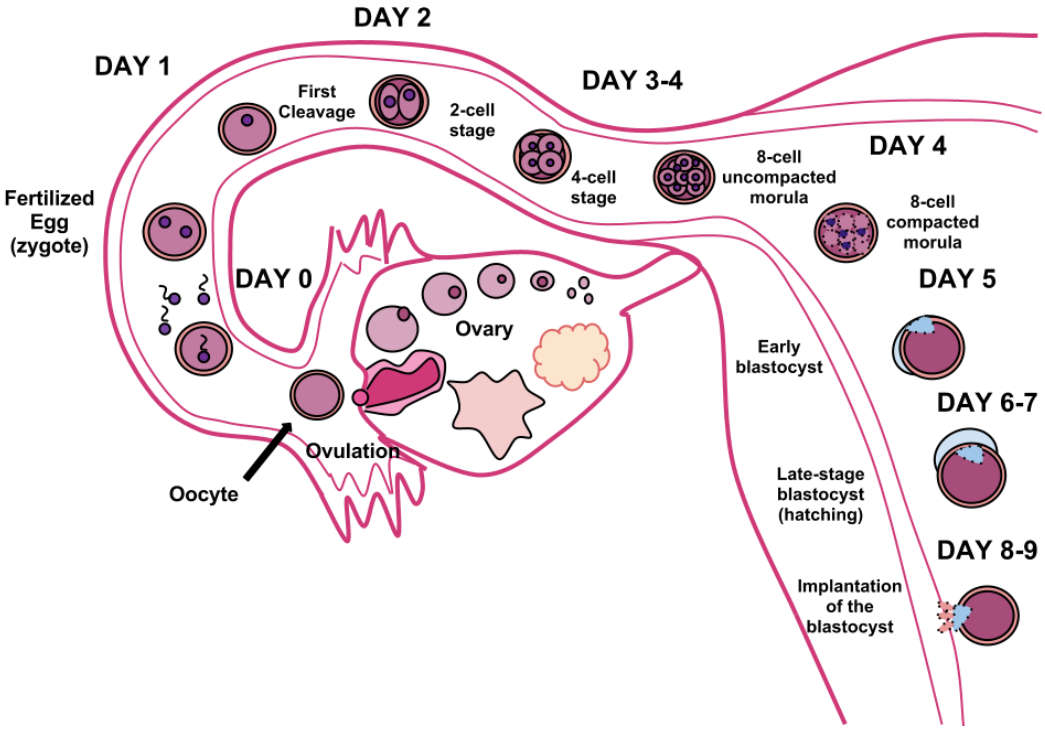



**Figure 4: Stages of embryo development since fertilisation until implantation**

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# 2 Ion channels

Ion channels are protein molecules found in all living cells. The hypothesis of the existence of ion channels was first proposed in the 1950s, while the first experimental evidences emerged in the mid-1970s after the development of the patch clamp technique by Denis Haydon and Erwin Neher. The categorisation of channels started in 1982 after the discovery of the primary structure of the first channel protein (Aidley and Stanfield, 1996).

Ion channels are protein molecules containing aqueous pores that control the transfer of specific ions through the cell membrane from one side to another (Hille, 1986). The cell membrane is considered the barrier separating the intracellular contents from the outside, as the cell membrane is composed of two semi-permeable layers of tightly packed phospholipids that do not allow molecules (e.g. amino acids, sugars and charged ions) to pass (Fonseca, 2012). The ion channels are arranged through the membrane, allowing the control of movements of a flow up to 100 million ions per second by switching the gating on and off. This control is needed to keep an electrical potential difference between the extracellular medium and intracellular cytoplasm. The cells use a specific electrochemical gradient into or out of the cell membrane to initiate specific cell signalling and control systems (Zheng and Trudeau, 2015). Therefore, ion channels play an important role in most of the physiological functions, including muscle contractility, cell proliferation, epithelial absorption and secretion, and cell volume regulation (Chambers et al., 2007).

In the endometrium, despite many ion channels being identified (potassium, sodium and calcium) and found to be normally expressed in the endometrium, their role in the regulation of endometrial receptivity and implantation remain poorly understood (Ruan et al., 2014b).

## 2.1 Potassium channels

Potassium channels are fundamental regulators for many physiological processes. They control the waveform frequency and shape of cellular action potential, cell excitability, and hormone and neurotransmitter secretion. They consist of a primary pore-forming core unit often connected to supporting regulatory subunits. In the human genome, potassium channel subunits are encoded by around 70 different genes. Potassium channels are divided into groups and subgroups based on their structural and functional properties. The three main families are the two transmembrane domain (2TM), 4TM, and 6TM families. (Alexander et al., 2011, Alexander et al., 2015, Alexander et al., 2017).

Potassium channels in the endometrium in particular were chosen as the target for this research as they play an important role in regulating the uterine luminal fluid volume, decidualisation, and the expression of genes associated with implantation (Ruan et al., 2014b) and hence play a role in regulating endometrial receptivity and embryo implantation. However, their gating mechanism in the bundle-crossing gate is still unclear (Piechotta et al., 2011).

Clemetson et al. showed that the amount of uterine luminal fluid reduces during the peri-implantation period, facilitating the attachment between the embryo and uterine wall. This reduction of fluid alters the electrolyte concentration resulting in an osmotic gradient within the uterine epithelium. In humans, the potassium concentration and composition in the intrauterine fluid is higher than that in the serum by six folds, which indicates the role of potassium either in fertilisation and blastocyst cleavage or endometrium hostility (Clemetson et al., 1973a, Patel et al., 2013).

The channels are categorised either according to their gating properties or ion selectivity. In recent years, a number of different ion channels such as $Na^{+}$, $Ca^{++}$, and $K^{+}$ channels were found to be expressed in human endometrium (Diaz-Gimeno et al., 2011) and believed to play an important role through different reproductive events, such as cell proliferation, decidualisation,

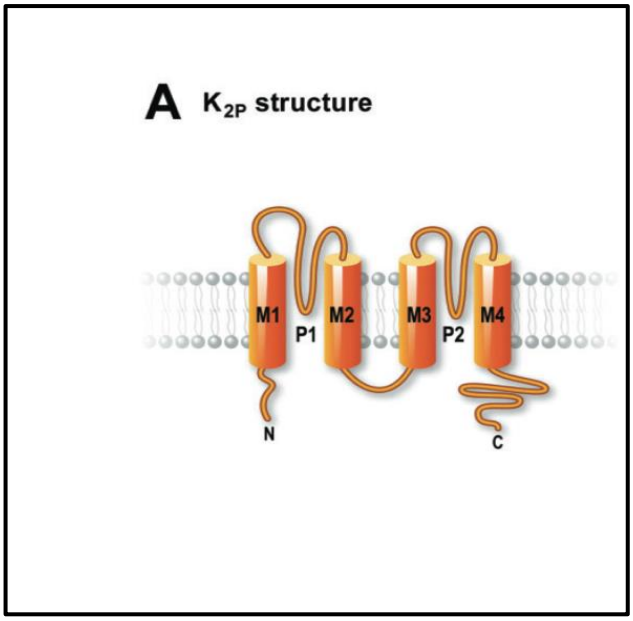
changes in the volume of uterine luminal fluid, and embryo implantation, especially the increase in cDNA levels of $Ca^{++}$ and $K^{+}$ channels during the period of implantation in the human endometrium (Kao et al., 2002). Of note, it is still not clear how the ion channels are involved in the regulation of endometrial receptivity and how impairment of their gene expression can lead to implantation failure (Chan et al., 2013a, Ruan et al., 2014a).

$K^{+}$ channels are divided in four groups: 1) voltage-gated potassium channels ($K_V$); 2) calcium-activated potassium channels ($K_{Ca}$); 3) inward rectifier potassium channels ($K_{ir}$); and 4) 4TM two-pore domain potassium channels (K2Ps) (Zheng and Trudeau, 2015). Our study will focus on both calcium-activated potassium channels and K2Ps.

### 2.1.1 **Two-pore domain potassium channels**

K2Ps are encoded by the *KCNK* genes and are concerned with the generation of leak currents and alteration of many physicochemical stimuli controlling the cell resting membrane potential (Duprat et al., 2007, Patel et al., 2013). They are involved in a variety of cellular functions, including the regulation of neuronal stimulation, contraction of muscles, hormone secretion, and heart rate regulation (Kim et al., 2000). Recent data show that K2Ps could be of crucial significance as they play a role in ion transport, and lipid regulation, and the pH or oxygen tension could threaten the optimum conditions needed for implantation (Patel et al., 2013).

The K2P channels are divided in to six subfamilies (Figure 5) (Patel and Honoré, 2001). These K2P channel superfamily is different from other classical voltage-gated and receptor potassium channels (Piechotta et al., 2011) because they consist of two-pore domains (P1 & P2) in tandem and 4TM segments (M1 to M4). At the molecular level in excitable cells, the walls of the channels are made of P1 and P2, which are considered leakage or background potassium channels (Lesage and Lazdunski, 2000, Lesage and Barhanin, 2011).



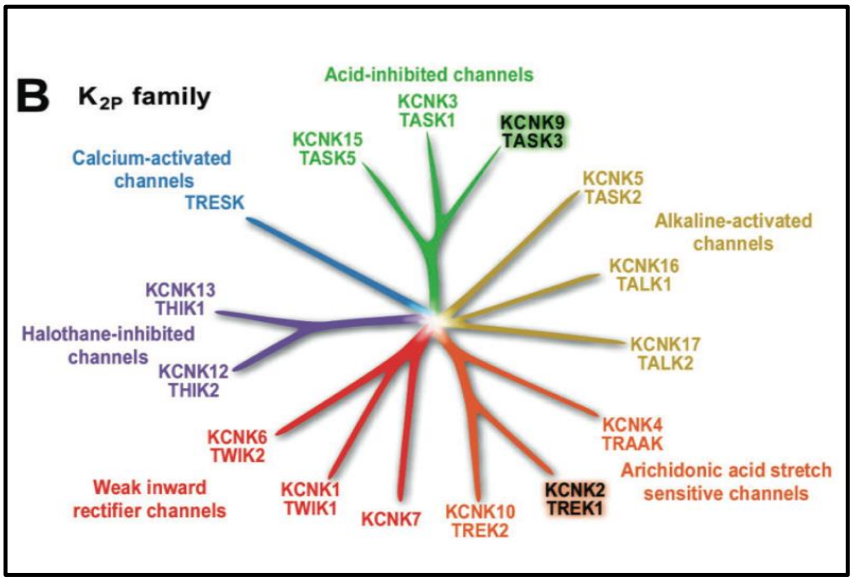




**Figure 5: K2P transmembrane segments and K2P subfamilies**

(Borsotto et al., 2015)

Four potassium channels were chosen to be tested in our thesis. The decision was based on the previous work of our research team in the laboratory, using highly pure preparations of endometrial epithelial cells expressing the epithelial marker intercellular E-cadherin 16. We demonstrated mRNA expression of the K2P channels TWIK-1, TWIK-2, TREK-1, TREK-2, TASK-2, TASK-3, TRAAK, TALK, and THIK in the normal human endometrium of fertile women as well

as their translation to protein. The chosen potassium channels (KCNK9 [TASK3], KCNK10 [TREK2], and KCNK17 [TALK2]) showed clear differential gene expression in the transition from proliferative to secretory phase endometrium, and differential gene expression was observed between fertile endometrium and Ishikawa cells. KCNMA1 ($BK_{Ca}$) was chosen for its important role in myometrial contractility (Khan et al., 2001, Patel, 2009, Patel et al., 2013).

#### *2.1.1.1 KCNK9 (TASK3)*

TWIK-related acid-sensitive potassium (KCNK) channels are a family of K2Ps that are sensitive to extracellular pH, varying from fully opened to fully closed in around 0.5 pH. TASK3 is a new member of the K2P channels. Its amino acid sequence is 54% similar to that of TASK1, but less than 30% similar to that of TASK2 (Kim et al., 2000). They are found in different human tissues, such as in the brain, placenta, pancreas, heart, and kidney. (Zheng and Trudeau, 2015, Bai et al., 2005a, Duprat et al., 2007).

KCNK9 channels are sensitive to pH. The average pH in the human uterine cavity ranges between 6.4 and 6.9 and there is a measurable drop of 0.4 and 0.5 in both proliferative and secretory phases, respectively (Burbank, 2009). This suggests that any alteration of the uterine pH could result in abnormal or altered expression or function of ion channels in the endometrium which may be a factor in impaired endometrial receptivity and/or implantation failure.

KCNK9 is activated by volatile anaesthetics (halothane, isoflurane) (Feliciangeli et al., 2015) and inhibited by copper, zinc, ruthenium red, and acid pHo (Feliciangeli et al., 2015), anandamide (Alexander et al., 2011), barium, lidocaine, bupivacaine (Kim et al., 2000), and PK-THPP (1-1-6-(biphenyl-4-ylcarbonyl)-5,6,7,8-tetrahydropyrido4,3-d-pyrimidin-4ylpiperidin-4-yl propan-1-one) (Cotten, 2013).

#### *2.1.1.2 KCNK10 (TREK2)*

TREK2 is known to have two splice variants (TREK-2b and c) (Gu et al., 2002) as well as links with insulin secretion (Kang et al., 2004). TREK2 channels respond to many chemical and physical stimuli including temperature, where they become active at physiological body temperature and hence contribute to the background potassium conductance and regulate cell excitability (Kang et al., 2005). TREK2 expression has been investigated in the myometrium, but it did not show any mRNA expression (Bai et al., 2005a). It is activated by acid pHo, acid pHi, volatile anaesthetics (halothane, isoflurane, chloroform), mechanical stretch, polyunsaturated fatty acid (PUFA), lipoprotein (LP), temperature (Feliciangeli et al., 2015) and inhibited by alkaline, pHo, and fluoxetine (Feliciangeli et al., 2015).

#### *2.1.1.3 KCNK17 (TALK2)*

The TWIK-related alkaline-sensitive $K^+$ (TALK) channels are a family of K2Ps that are sensitive to extracellular pH. TALK2 is sensitive to external pH changes where an increase in current is seen from pH 8.9 to 10.2. They are identified in different human tissues such as the placenta, pancreas, brain, and heart. TALK2 (Patel et al., 2013, Zheng and Trudeau, 2015) is activated by cell swelling, alkaline pH, quinine, isoflurane (Zheng and Trudeau, 2015), nitric oxide (NO) and reactive oxygen species (ROS). There is no specific highly selective TALK2 blocker, but it can be inhibited by cell shrinking, barium, halothane, quinidine, and chloroform (Zheng and Trudeau, 2015).

### 2.1.2 **Calcium-activated potassium channel: KCNMA1 ($BK_{Ca}$)**

KCNMA1 ($BK_{Ca}$), also called BK, KCa1.1, or MaxiK channel, are voltage-, large conductance-, $Ca^{+2}$-activated $K^+$ channels with a highly conserved selectivity filter within the potassium channel superfamily. They were first discovered in the 1980s, and in the 1990s, a single gene encoding the alpha subunit of the $BK_{Ca}$ channels was discovered in mice and named *KCNMA1* (Wang and Sigworth, 2009). Each channel has seven transmembrane segments and an extracellular *N*-terminus. The channels consist of two essential units, one part

for voltage sensing and other for Ca2+-sensing (Bentzen et al., 2014). KCNMA1 channels are involved in many excitable and non-excitable cellular functions (Ghatta et al., 2006).

$BK_{Ca}$ channels are activated in response to an increase in intracellular $Ca^{+2}$ concentration and depolarisation of the cellular membrane. The channels depend on both voltage and $Ca^{+2}$ concentration, whereby the macroscopic conductance increases with voltage, with more shifts towards more negative voltages as the $Ca^{+2}$ concentration increases (Zhang et al., 2012). Also, voltage alone can activate the $BK_{Ca}$ channels in the absence of $Ca^{+2}$ binding when the voltage sensors are at resting phase and the voltage is highly positive. Many other factors activate the $BK_{Ca}$ channels, such as proton $H^{+}$, intracellular $Mg^{+2}$, haeme, alcohol, and $PIP_2$ (Phosphatidylinositol 4,5-bisphosphate) (Cui et al., 2009, Lee and Cui, 2010).

$BK_{Ca}$ channels play an important role in many physiological stimuli and more research is needed to understand the regulation and modification of $BK_{Ca}$ channels (e.g. phosphorylation and methylation), as this will help understand their tissue-specific functions (Zheng and Trudeau, 2015)

KCNMA1 channels are inhibited and blocked by paxilline, charybdotoxin, iberiotoxin, tetraethylammonium (Alexander et al., 2017), and barium, which block the four ion-binding sites in the $BK_{Ca}$ channel ion conduction pathway. These four binding sites respond to the inner ion position of the internal sites of the selectivity filters. Blockage is prominent only at high $Ca^{+2}$ concentrations of more than 100 µM and at voltage higher than +50 mV. Also, $Mg^{+2}$ has the ability to block or activate the $BK_{Ca}$ channels, with unclear and complex mechanisms (Yang et al., 2008, Zhang et al., 2011).

# 3 Epigenetics and the regulation of menstrual cycle

## 3.1 Background

The inheritance of genetic information carried in the DNA has been a fundamentally important area of research for 70 years, with the aim of understanding the human genome including the discipline of epigenetics (Hallgrimsson and Hall, 2011). In 1942, Conrad Waddington defined ‘epigenetics’ as ‘a branch of biology which studies the causal interactions between genes and their products which brings phenotype into being’ (Armstrong, 2014). During gametogenesis and early embryo development, the genome undergoes numerous stages of epigenetic programming which are believed to be due to epigenetic modification (Pinborg et al., 2016).

Gene expression is regulated epigenetically through:

- DNA methylation
- Histone modification
- A non-coding RNA (ncRNA), including miRNA, siRNA, piRNA, and lncRNA (Choudhuri et al., 2010)

The knowledge on this process is modified through time, with more research being conducted and published and new technology being introduced. Now, the most accepted definition affirms that epigenetic traits are reversible changes in gene function (turn on/off) due to mitotically and/or meiotically changes in the chromosomes without any changes in the DNA sequence, resulting in a stably inherited phenotype (Berger et al., 2009, Munro et al., 2010, Hallgrimsson and Hall, 2011, Armstrong, 2014).

Genomic imprinting is known to be an epigenetic phenomenon and a wide range of environmental factors such as nutrition, chemicals, and toxins can affect the maintenance of epigenetic patterning and gene transcription in the long-term (Jirtle and Skinner, 2007). Environmentally triggered phenotypes are found to be linked with DNA methylation and chromatin alterations and can act

at any stage of the gene-protein pathway for RNA, DNA, and protein during pre-transcription, post-transcription, or post-translation (Feil and Fraga, 2012).

## 3.2 Epigenetic regulation of the menstrual cycle

A study conducted on the global acetylation levels in the endometrium showed that any epigenetic changes are accompanied with differentiation of stem cells in different lineages and could be involved in the process of regeneration of the endometrium (Acton, 2012), where histone acetylation plays an important role during angiogenesis in the pathway of vascular endothelial growth factor (Acton, 2012).

Endometrial histone modification (Figure 6) and DNA methylation are found to be linked with transcription activity that was observed to be changing during the different phases of the menstrual cycle. This strengthens the suggestions and hypotheses of epigenetic involvement in the regulation of endometrial gene expression through the different phases of the menstrual cycle, where any abnormal epigenetic modifications may be associated with failure of implantation, contributing to two-thirds of all ART failure cases (Horcajadas et al., 2007, Munro et al., 2010).

Poor decidualisation, implantation, and placentation have been associated with cases of pre-eclampsia, intra-uterine growth restriction, and preterm labour in humans, and this could be due to failure of regulation of epigenetic modification (Wang and Dey, 2006). Recent data suggests that any modulation of K+ ion currents may regulate cellular function in the endometrial cells. Thus, if the ‘fingerprint’ of a potassium ion channel is clearly identified by association with phases of the menstrual cycle, and the trigger for these phenotypic switches is discovered, then it could be possible to inhibit or facilitate implantation by manipulating ion channel activity (Ruan et al., 2014b).

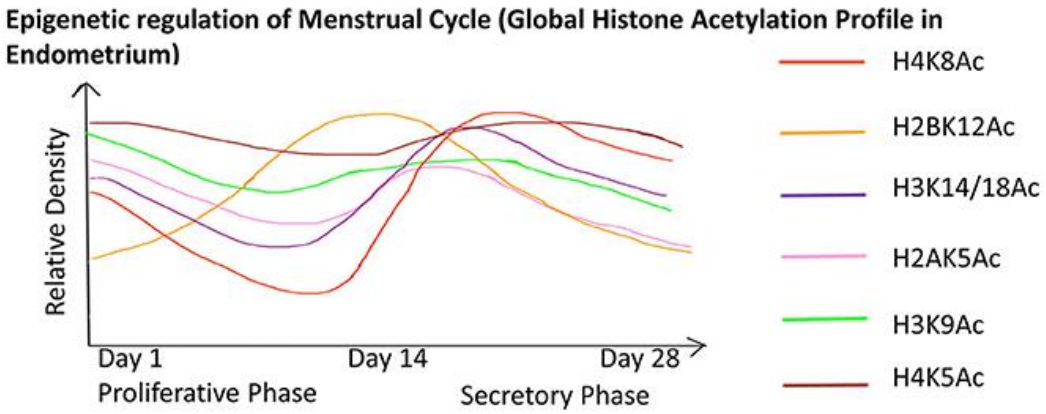



**Figure 6: Histone acetylation profile in endometrium through the menstrual cycle**

(Kanherkar et al., 2014)

In this thesis, we will be testing for the DNA methylation of potassium channels, as it plays an important role in the regulation of gene expression, influences apoptotic cell death, and maintains cellular homeostasis in the functional layer of the endometrium (Caplakova et al., 2016). DNA methylation in vertebrates is characterised by an additional methyl group in the C5 position of cytosine (Kurdyukov and Bullock, 2016). In humans, most of the DNA methylation takes place in cytosine that precedes the guanine nucleotide (CpG dinucleotide) (Caplakova et al., 2016). Moreover, 5-methylcytosine plays a significant role in switching the genes on/off (Li and Liu, 2011). This affects 70–80% of CpGs in the human genome (Bock, 2012).

# 4 Pathophysiology of female human reproduction

## 4.1 Infertility

### 4.1.1 Introduction

Infertility affects 1 in 7 heterosexual couples in the UK. It is defined as failure to conceive among women of reproductive age after one year of unprotected vaginal sexual intercourse, in the absence of any known cause of infertility (Gurunath et al., 2011, NICE, 2013). There has been a small increase in the prevalence of fertility problems in recent years, with a greater proportion of couples now seeking treatment (NICE, 2013). Infertility is considered one of the most significant stressors in life. Among approximately 25% of all infertile couples, the cause of infertility is unknown (Ray et al., 2012).

There are many causes of infertility, and primary and secondary infertility have different prevalence rates. In the UK, the main causes of primary infertility are unexplained infertility (25%), ovulatory factors (25%), male factors (30%), tubal factors (20%), and uterine factors (10%). In about 40% of clinically presented cases, disorders are found among both the female and male partners (NICE, 2013). Nowadays, 85–90% cases of infertility are diagnosed, and 50–60% are treated successfully (Jones and Lopez, 2013).

### 4.1.2 Recurrent implantation failure RIF

From the evidence and expert opinions, there is no universal definition for the terminology of recurrent implantation failure. The two most recently accepted definitions of RIF were published in 2014: the first was presented by Coughlan et al., who defined RIF as the ‘failure to achieve pregnancy after 3 IVF cycles (where at least 4 good quality embryos were transferred)’ (Coughlan et al., 2014). The second definition was proposed in a systematic review by Polanski et al., where RIF was defined as ‘failure of at least 2 consecutive cycles of IVF/ICSI or FER where the cumulative number of transferred embryos was no less than 4 cleavage stage embryos and no less than two blastocysts (good

quality embryos)' (Polanski et al., 2014). In this thesis, we considered the definition of Polanski et al. as our reference for RIF.

### 4.1.3 **Treatments used in cases of recurrent implantation failure (RIF)**

Advances in reproductive medicine have made possible what once seemed impossible, as many emerging treatments (vitamin D, immunotherapy, growth hormones, dehydroepiandrosterone [DHEA]) are reported to have increased the pregnancy rates, but there is a lack of evidence on their efficiency, target cells, and mode of action at the molecular level. In this thesis, we examined some of these treatments to determine their effects on potassium channels in infertile human endometrium.

#### *4.1.3.1 Immunotherapies*

In women with recurrent implantation failure following assisted reproduction, the rationale behind the use of immunotherapy interventions is to address poor uterine receptivity and increase implantation rates, as reported in evidence from basic research and clinical data. In brief, many factors contribute to implantation failure, including cellular adhesion molecules such as immunoglobulins that facilitate adhesion between cells and in cytokines networks and prostaglandins (Benschop et al., 2012).

All these factors work in harmony with other locally acting growth factors to modulate the immune response of the endometrium to be receptive to the blastocyst (Achache and Revel, 2006). Potassium channels are involved in immunological mechanism, by providing molecular tools to elucidate the involvement of voltage and calcium activated potassium channels in T-cell volume regulation and activation, this explains why T-cell activation can be blocked by potassium channel blockers (Cahalan et al., 2001, Chandy et al., 1984)

Over 20 Randomised controlled trials (RCTs) tested the effect of immune therapies on different outcomes (e.g. live birth rate, implantation rate, ongoing pregnancy rate, and miscarriage rate) for women with a history of failed

implantation (Brinsden et al., 2003, Brinsden et al., 2009, Urman et al., 2009, Colicchia et al., 2011, Makrigiannakis, 2011, Kim et al., 2011, Scarpellini and Sbracia, 2011, Cambiaghi and Leao, 2012, Pergolini et al., 2013, Scarpellini and Sbracia, 2013, El-Khayat and El Sadek, 2015, Hamdi et al., 2015, Aleyasin et al., 2016, Gamaleldin et al., 2016, Davari-tanha et al., 2016, Miraj et al., 2015, Madkour et al., 2016, Obidniak et al., 2016, Yu et al., 2016). These treatments include glucocorticoids, intravenous immune globulin (IVIG), lymphocyte immune therapy (LIT), administration of peripheral blood mononuclear cells (PBMC), r-hLIF, granulocyte-colony stimulating factor (G-CSF), anti-TNF-α therapy, and intralipid therapy. Although considered to act as an anticoagulant agent, heparin is also administered to some patients with RIF and has some immunomodulatory effects (Nelson and Greer, 2008).

*Glucocorticoids* have been used in a variety of different protocols, as summarised in a recent Cochrane review (Boomsma and Macklon, 2008). Glucocorticoids cause suppression of endometrial NK cells. Glucocorticoids may also play a role in HCG secretion and trophoblastic proliferation. Various protocols have been described including oral prednisone, methyl prednisone, and dexamethasone. Also, parenteral form of hydrocortisone is administered at a dose of 100 mg (Michael and Papageorghiou, 2008).

*IVIG* is an intravenous immunoglobulin used to treat recurrent pregnancy loss, as described in various studies (Clark et al., 2006). It acts through the suppression of Th1, which in turn causes down-regulation of natural killer cells and minimises their cytotoxic effect on the embryo during implantation (Daya et al., 1999). IVIG is given before conception at a dose of 0.2 or 0.4 g/kg (Clark et al., 2006).

*LIT* is administered with allogeneic paternal lymphocyte immunisation and has shown some success in various studies. It causes Th1 and Th2 modulation. (Carp et al., 1994, Kuhn et al., 1993).

In *PBMC* trials, PBMCs are injected in the uterine cavity before embryo transfer (Nakayama et al., 2002, Yoshioka et al., 2006). This induces functional endometrial changes and enhances implantation (Yoshioka et al., 2006).

*Leukemia Inhibitory Factor LIF* (emfilermin) is administered subcutaneously at a dose of 150 µg twice daily for 7 days starting before embryo transfer (Brinsden et al., 2009). It controls endometrial cell differentiation to promote implantation of the blastocyst (Chen et al., 2000). Many studies were done on mice, as the LIF knockout mice have shown that many of endometrial cell differentiation are not apparent during ordinary development and that they may be due to the result of introducing a LIF expression. Also in mice studies LIF were found to maintain of the totipotency of embryonic stem cells and induced pluripotent stem cells (Nicola and Babon, 2016).

*G-CSF* (filgrastim, a recombinant GCSF) is a cytokine, which enhances proliferation of granulocytes. This may have an influence on trophoblastic proliferation (McCracken et al., 1999). Recombinant G-CSF is given at a dose of 100000 I.U/kg/day to be started at day 6 post ovulation (Scarpellini and Sbracia, 2009) or 10 million IU every 3 days (Würfel et al., 2010).

*TNF-α inhibitors*, either in the form of adalimumab (Winger et al., 2009) or etanercept (Jerzak et al., 2010), are injected subcutaneously in combination with other interventions (Winger et al., 2009). TNF-α inhibitors suppress the action of Th1 cytokines, and can have a beneficial effect on recurrent implantation failure. It is administered subcutaneously in many forms (Clark, 2010).

*Heparin* is best prescribed at a dose of 20–40 mg. It is better administered with aspirin (Qublan et al., 2008). It is notable that all of the heparin treatment modalities have often been investigated in co-administration with aspirin or in combination with each other and also used as luteal phase support.

*Intralipids* has recently gained popularity in the treatment of recurrent implantation failure. It is believed that intralipid therapy suppresses NK cells,

where fatty acids activate peroxisome receptors expressed by NK cells (Roussev et al., 2008). However, there is no solid evidence that supports the use of intralipid therapy as routine for patients undergoing IVF cycles (Granato et al., 2000).

A systematic review will be conducted to generate a meta-analysis and compare the effect of these immunotherapies on live birth rates and miscarriage rates as primary outcomes and one of these treatments will be chosen and tested on endometrium to observe its effect on potassium channels.

#### *4.1.3.2 Vitamin D*

Many studies reported that the prevalence of vitamin D insufficiency has doubled from 1994 to 2004 among women of reproductive age (Bodnar et al., 2007b). Vitamin D has been the focus of many research fields, since it was found to be associated with autoimmune diseases, diabetes, increased risk of cancer, and adverse cardiovascular events (Lerchbaum and Obermayer-Pietsch, 2012). Previous studies involving mice and the recent clinical studies suggest its important role in ovulation (low levels of vitamin D increase the levels of obesity and menstrual disturbance in PCOS patients), endometriosis implantation, local immunological embryo-protection, and embryo development (Halloran and Deluca, 1980, Yoshizawa et al., 1997, Vigano et al., 2006, Bodnar et al., 2007a, Ozkan et al., 2010, Rudick et al., 2010, Rudick et al., 2011, Grzechocinska et al., 2013, Aflatoonian et al., 2014, Dabrowski et al., 2015, Voulgaris et al., 2017, Lopes et al., 2017). Despite the presence of numerous publications, its influence on reproductive health remains unclear (Dabrowski et al., 2015).

Vitamin D is an important regulator of calcium and phosphorus in human tissues; therefore, it plays a significant role in tissue differentiation and cellular growth (Panda et al., 2001). Vitamin D is metabolised by the liver to form 25-hydroxyvitamin D (25[OH]D) through the 25-hydroxylase enzyme (CYP2R1). In renal distal tubules, 25-hydroxyvitamin D (25[OH]D) is converted to the

active form 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) (the form used in this thesis) through the action of the enzyme cytochrome P450 1ahydroxylase (CYP27B1). In addition to the liver, CYP27B1 is also found in the testis and dermal fibroblasts (Lerchbaum and Obermayer-Pietsch, 2012).

Vitamin D exerts its action via the VDR receptor, which is a nuclear hormone receptor. VDR receptor is found in ovarian tissue, endometrium, fallopian tubes, placenta, and testis (Voulgaris et al., 2017, Parikh et al., 2010). It has been reported that potassium channels couple with 1,25(OH)2 vitamin D3 rapid response and secretion in immature Sertoli cells (Menegaz et al., 2016).

#### *4.1.3.3 Oxygen concentration*

As mentioned before, oxygen concentration fluctuates within the female reproductive tract and also changes through the menstrual cycle. Elevated and non-physiological oxygen levels lead to the formation of reactive oxygen species (ROS) that have a negative impact on embryo development (Ng et al., 2017). Many potassium channels (voltage-dependent K channels, maxiK channels and TASK-like channels) were found to be affected by hypoxia and hypoxia-induced signals; hence, it is believed that potassium channels play an important role in sensing any reduction in oxygen concentration (Heitzmann and Warth, 2008). In our study, the effect of 5% oxygen concentration will be explores, since it is considered the average physiological oxygen concentration along the female genital tract, which ranges between 2% and 8% (Leese, 1995). Also, it is reported in most recent studies that 5% oxygen concentration is the most favourable and optimum oxygen tension needed for utilisation of nutrients during mammalian embryo development in the preimplantation stage (Gardner, 1998, Wale and Gardner, 2012, Gardner and Wale, 2013).

## Hypothesis

Potassium channels of human endometrium are differentially expressed during the window of implantation. Modulation/dysregulation of these channels due to changes in the intrauterine microenvironment may alter conditions essential to implantation.

## Aims and objectives

1) To determine whether immunotherapy results in improved live birth rates in women with a history of recurrent implantation failure who are undergoing assisted reproduction, through conducting a Cochrane systematic review to generate a meta-analysis.

2) To determine altered expression of potassium channels (KCNK9, KCNK10, KCNK17 and KCNMA1) within the endometrium during different phases of menstrual cycle and in endometrium from fertile and infertile women.

3) To test the effect of intralipids (from systematic review), low oxygen concentration and vitamin D on the molecular expression of potassium ion channels in the human endometrium and decidua and also the effect of these treatments and other specific potassium channel inhibitors on net ion transport in cultured endometrial monolayers by measuring transepithelial electrical resistance (TEER).

4) To identify protein expression and translation at the single cell level (endometrium and decidua) for the potassium channels (KCNK9, KCNK10, KCNK17 and KCNMA1), using flow cyctometry.

5) To produce a protocol for the new EpiQuik™ Tissue Methylated DNA Immunoprecipitation commercial Kit, to work optimally with endometrium and decidual tissue to investigate the DNA methylation profile at the promoter region of potassium channels genes (KCNK9, KCNK10, KCNK17 and KCNMA1), to determine any association between the changes in expression observed in the endometrium with changes in DNA methylation.

## Working plan

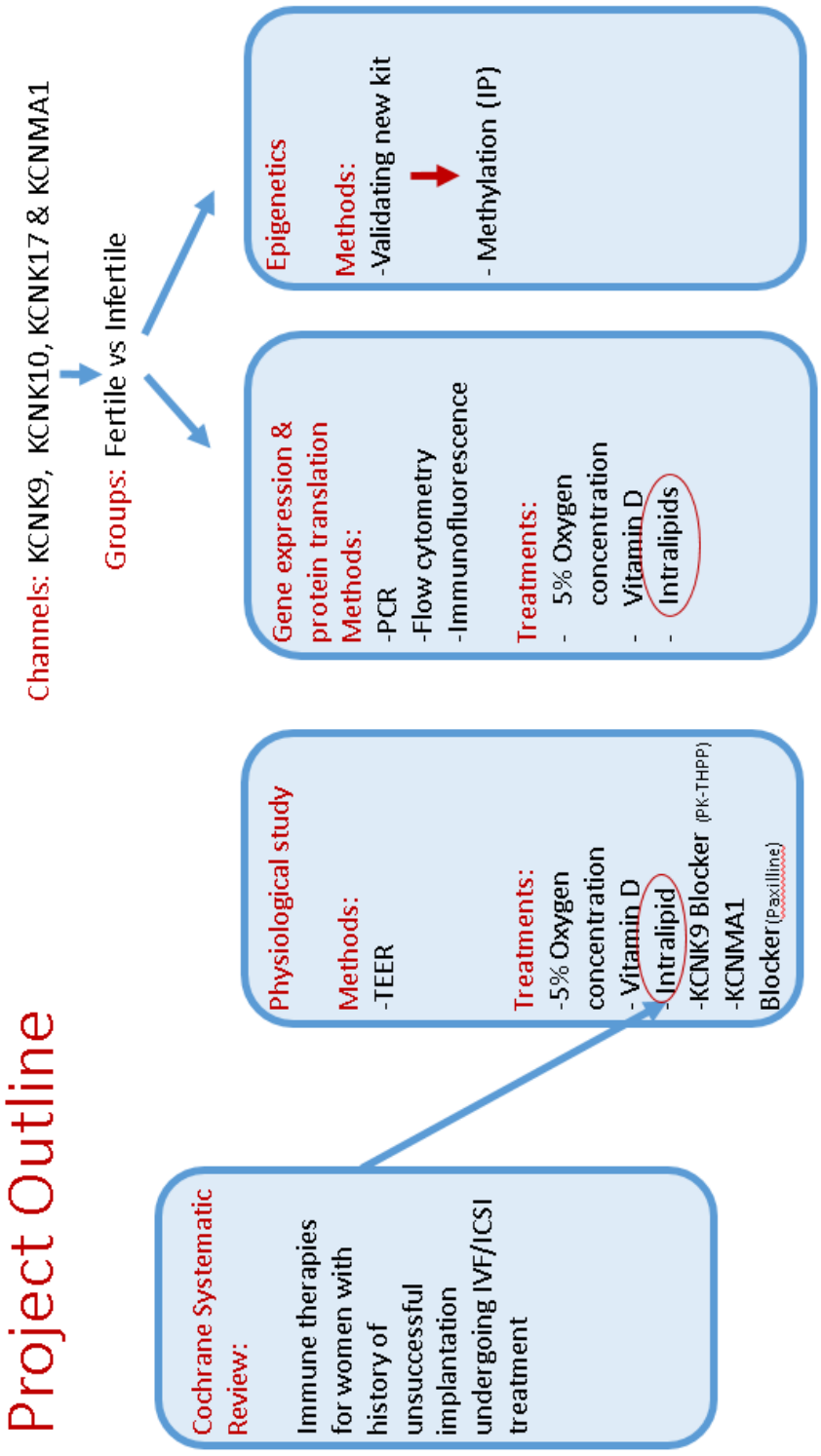



**Figure 7: Project outline for the four result chapters**

# Chapter Two: Materials and Methods

# 1 Tissue collection

## 1.1 Endometrial samples collection

Ethical approval for our study was granted by the Derbyshire Local Research Committee ID: 17022 with study IRAS project ID: 218026. Written, informed consent was obtained prior to recruitment of patients at the Royal Derby Hospital for endometrial biopsy (Appendix 1-2) by a research team member.

**Inclusion criteria:** Non-pregnant females, aged 18–45 years old with a regular menstrual cycle ranging between 25 and 36 days who were able to give informed consent. Study participants were divided into two groups:

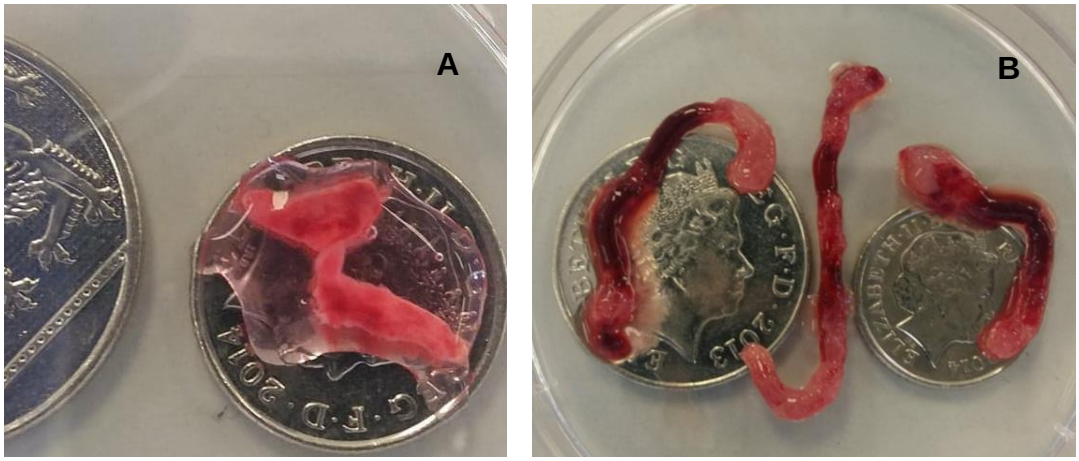
**Group A:** Control group (fertile) females, undergoing surgery or gynaecological investigation for non-fertility related conditions such as pelvic pain, hysterectomy, urinary incontinence; women with proven fertility undergoing laparoscopic sterilisation who have not used any hormonal contraception or intrauterine contraceptive device for three months prior to the study.

**Group B:** Women diagnosed with primary infertility and/or recurrent implantation failure (RIF). Patients were recruited from the fertility clinic at Royal Derby Hospital, where endometrial scratching was performed using a pipelle (Wallach Endocell®).

Samples were taken from the infertile women who were going for an IVF/ICSI cycles. Samples were collected during the mid-secretory phase that was decided to be the day (mid-luteal) of the GnRH agonist injection (the start of the long protocol that includes a period of down regulation followed by daily exogenous FSH stimulating injections before egg collection).

**Exclusion criteria:** Women over 45 years; women with fibroids, uterine anomalies, cancer, and amenorrhoea; women who were unable to give consent.

The samples from the theatre/clinic were collected in a 20-mL universal tube filled with 10 mL Dulbecco's Modified Eagle's medium (DMEM) (Gibco™) (low glucose, pyruvate, HEPES), supplemented with 1% streptomycin/penicillin. The samples were dispatched to the laboratory within 10 min of collection. Samples were washed with Hank balanced salt solution (HBSS) (Gibco™) (no calcium, no magnesium) to remove blood and depending on the amount of tissue obtained (Figure 8**Error! Reference source not found.**), the subsequent technique was planned: Samples were either a) snap-frozen in liquid nitrogen and stored at -80°C until ready to use or used immediately, b) cultured fresh, or c) cut and weighed into 60–70-mg pieces of tissue using a microbalance to be treated and incubated.




**(A) Proliferative phase** **(B) Secretory phase endometrial samples**

**Figure 8: Endometrial biopsy taken by pipelle**

## 1.2 Decidual sample collection

Ethical approval for our study was granted by the Derbyshire Local Research Committee (REC Reference No. 09/H0401/90). Written, informed consent was obtained prior to recruitment of patients at the Royal Derby Hospital for placenta samples (Appendix 3-4) by a research team member. After caesarean section (CS), the placentae were checked by a midwife and then

transported immediately to the laboratory where procedures were initiated within 15 min to ensure freshness of the samples.

**Patient inclusion criteria**: Women (18–45 years old) undergoing either emergency or elective CS for non-labouring and labouring myometrium.

**Exclusion criteria:** Women with preeclampsia, intrauterine growth restriction, pregnancy-induced hypertension, or gestational diabetes.

The chorio-decidua was gently peeled from the maternal side of the decidua parietalis of the placenta using serrated-tip forceps (Figure 9). The sample (up to 1 g) was then washed in 8–10-mL HBSS (no calcium, no magnesium), where it was either used fresh for tissue culture or cut to be treated and incubated or snap-frozen in liquid nitrogen and stored at -80°C.

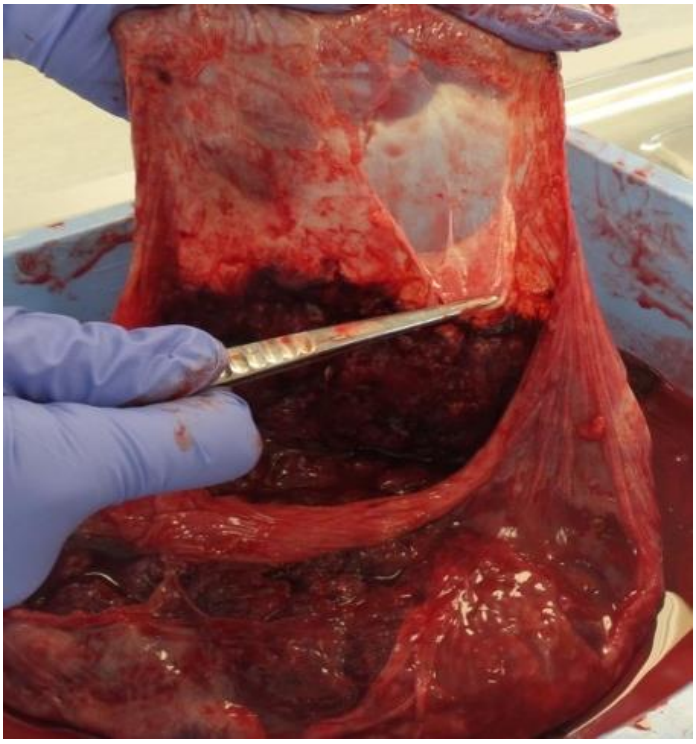
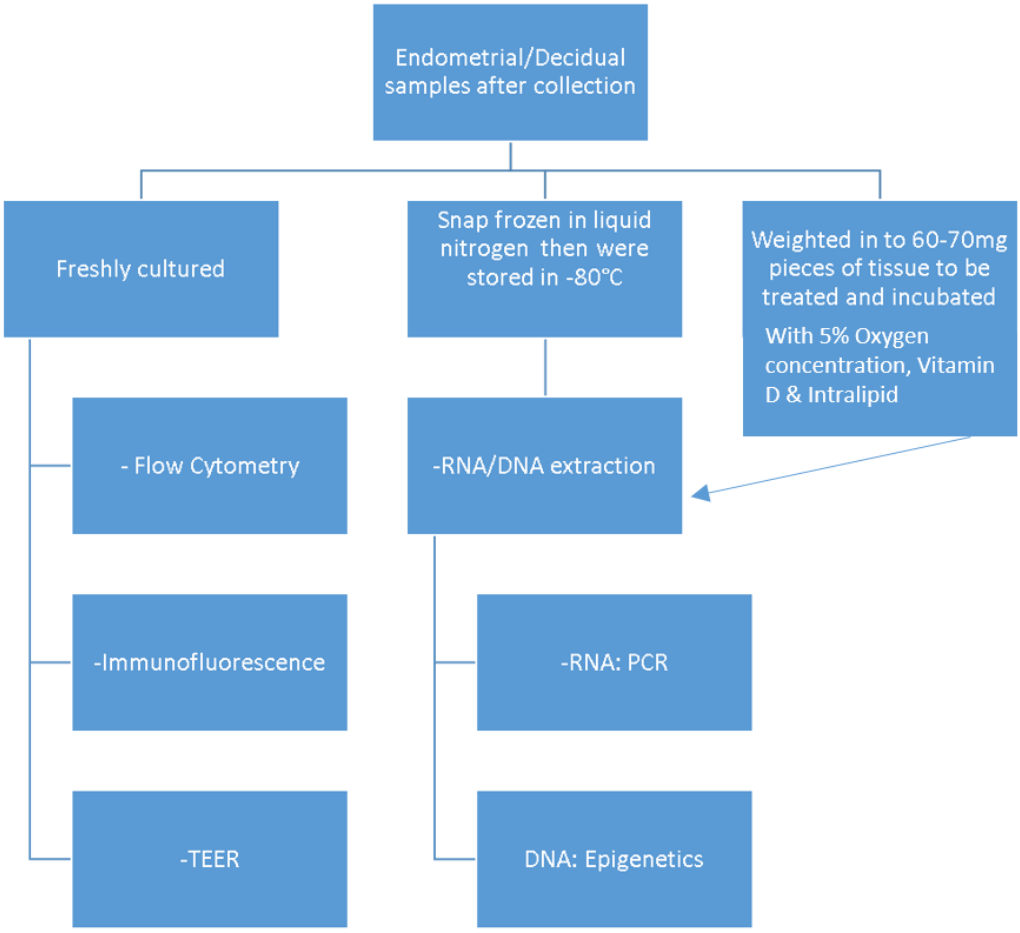


**Figure 9: Separation of chorio-decidua of placenta**




**Flowchart 1:** Flowchart showing how endometrial/decidual samples were taken to next steps after collection

# 2 Treatment of tissue samples

After weighing the samples to obtain 60–70-mg pieces (
Figure **10**), the tissue was shredded to increase the surface area for the treatments, using a size-22 scalpel and forceps in a 60-mm sterile Petri dish (Greiner CELLSTAR®). The tissue (60–70 mg) was then placed in a 24-well culture plate. Each well contained 1 mL of DMEM media supplemented with 10% FBS and antibiotics P/S ± treatment (Table 1). These treatments were added to test their effect on potassium channel gene expression in further experiments.

After 24 h of incubation, the samples and medium were transferred to 1.5-mL Eppendorf tubes and centrifuged at 1400 × g for 60 s. The supernatant was discarded and the pelleted tissue was used for RNA extraction.

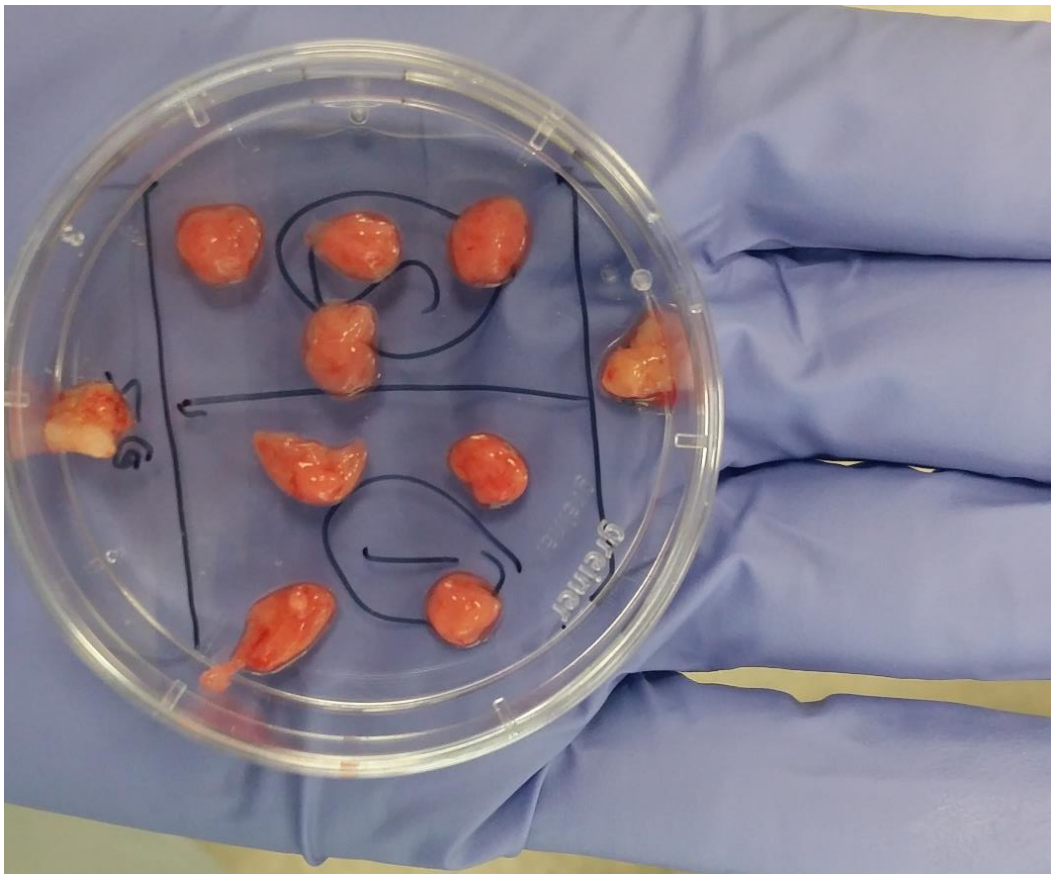


**Figure 10: Endometrial samples cut and weighted into 60–70-mg pieces**

| Treatment | Condition |
|---|---|
| Control | Enriched DMEM only + Normal incubation conditions<br>(37°C, 5% $CO_2$) |
| Low oxygen concentration | Enriched DMEM only + Tri-Gas incubator with 5% oxygen concentration, 37°C |
| Vitamin D<br>(Sigma-Aldrich; Appendix 5) | Enriched DMEM + Vitamin D (2.5 µg/kg) + Normal incubation conditions |
| Intralipid<br>(Sigma-Aldrich; Appendix 6) | Enriched DMEM + 20% Intralipid (12.5 mL/kg) + Normal incubation conditions |

**Table 1: shows the treatments applied to the endometrial/decidual samples and their incubation conditions**

# 3 Tissue culture

Endometrial tissue was cultured as a preparatory step for flow cytometry, immunofluorescence, and Transepithelial electrical resistance (TEER) experiments (Flowchart 1). Before the start of tissue culture, DMEM was enriched with 10% FBS and 5-mL 100 U antibiotic (P/S) from a 500 mL bottle. The HBSS was also supplemented with 5ml Hepes buffer (Gibco™) and 5ml of 100 U antibiotic (P/S).

After washing the fresh endometrial/decidual samples in HBSS solution to remove blood clots, the tissue was finely minced in a petri dish using a disposable scalpel and forceps; the sample was dissected to increase surface area for collagenase to work upon. Mincing was done directly in 10 mL of 0.2% collagenase (100 mg collagenase powder [Sigma-Aldrich] in 50 mL of HBSS (Figure 11).

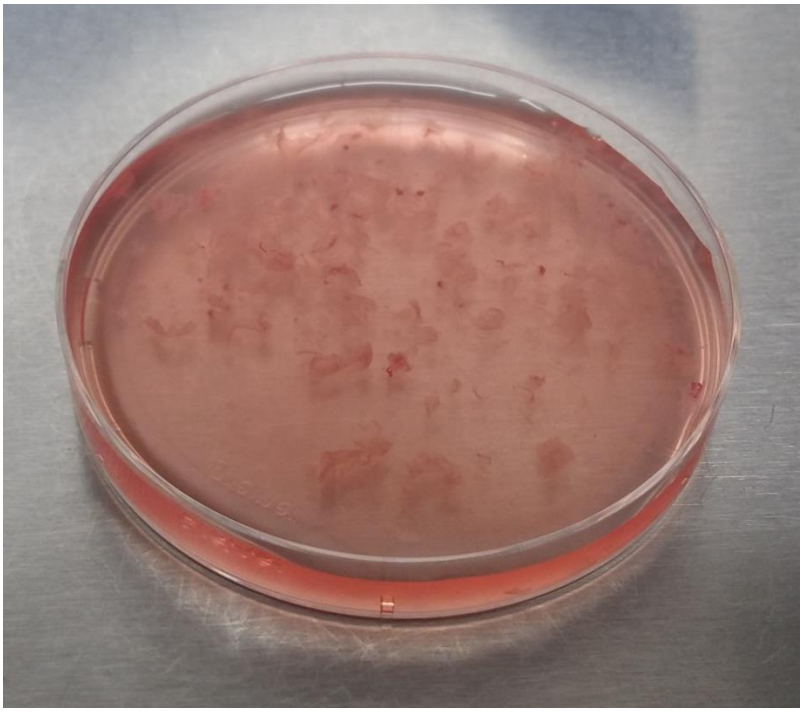


**Figure 11: Tissue after mincing in collagenase**

After mincing in collagenase, both minced tissue and collagenase were placed in a 20-mL tube and incubated at 37°C in a water bath for 15 min, at which

point the sample was checked and mixed again by gently pipetting up and down several times until a homogenous, opaque solution was obtained. The sample was incubated for another 15 min in the water bath. After incubation, the sample was put in 50-mL Falcon tube and centrifuged for 15 min at 500 *g* at room temperature. The supernatant was removed after centrifugation and the remaining pellet was washed free of collagenase twice using HBSS. After washing with HBSS, the supernatant was removed and 10 mL enriched DMEM was added and mixed well with the cells to be divided between two T25 (Nunc™) sterile culture flasks, labelled clearly and incubated in a humidified incubator at 37°C: 5% $CO_2$ and atmospheric oxygen. This step was considered Day 0.

On the following day (day 1 of culture), the culture containing remaining tissue fragments and red blood cells was aspirated. Cells were washed with 5 mL HBSS; then, 5 mL enriched DMEM was added. The flask was incubated and the same steps (washing of cells and medium changing) were repeated day after day until the cells were confluent and covering around 80–90% of the base of the flask bed bottom (Figure 12) and then the cells were passaged using trypsin.

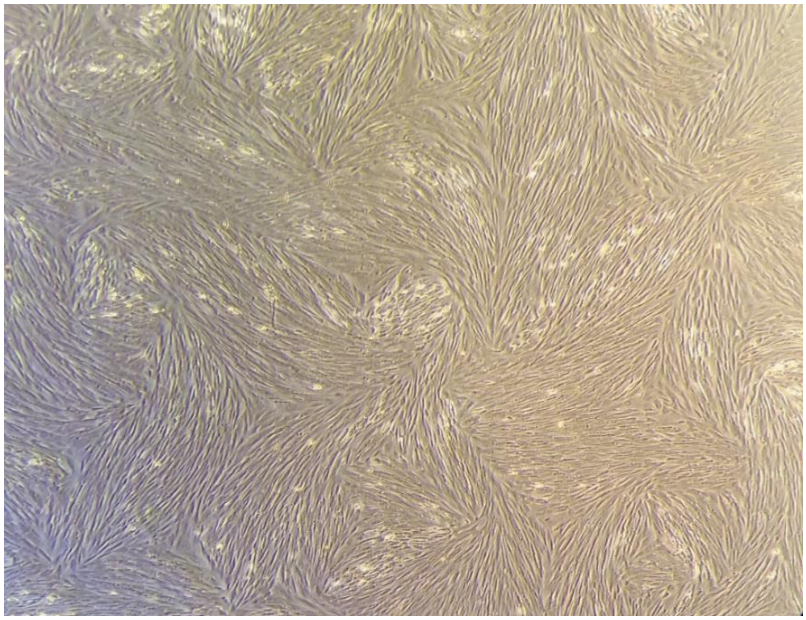


**Figure 12: Endometrial cell culture reaching 100% confluence, giving spindle-shape appearance**

On the day of splitting the cells by trypsin, the medium was aspirated from the flask. The cells were rinsed with two washes of HBSS. About 3–5 mL of trypsin/EDTA 0.05% (Gibco™) was added to cover the cells prior to being left for a maximum of 2 min. The flask was then observed under the microscope as cells started to appear rounded. The flask was then tapped gently against the palm to observe and confirm the detachment of cells from the flask. Immediately after the cells were released, 3–5 mL of FBS was added to neutralise the actions of trypsin. The cells were then transferred to a 15-mL centrifuge tube and topped up with HBSS to be centrifuged at 500 *g* for 5 min at room temperature. The cells were washed twice with HBSS and the supernatant was discarded.

The remaining pellet was resuspended in 1 mL of enriched DMEM; 10 µL of the cell suspension was loaded onto a counting slide and the cell number was recorded using an automated cell counter (Bio-Rad TC10™). The suspension was divided into three T25 flasks reaching a splitting ratio of 1:3 at end of each passage. Then, 4.5 L of enriched DMEM was added to each flask. The new flasks were labelled clearly, where the seeding day was passage one (P-1/ D-0).

# 4 RNA/ DNA extraction

AllPrep DNA/RNA Mini Kit (QIAGEN) was used for RNA and DNA extraction from endometrial/decidual samples per the manufacturer's instructions (Figure 13).

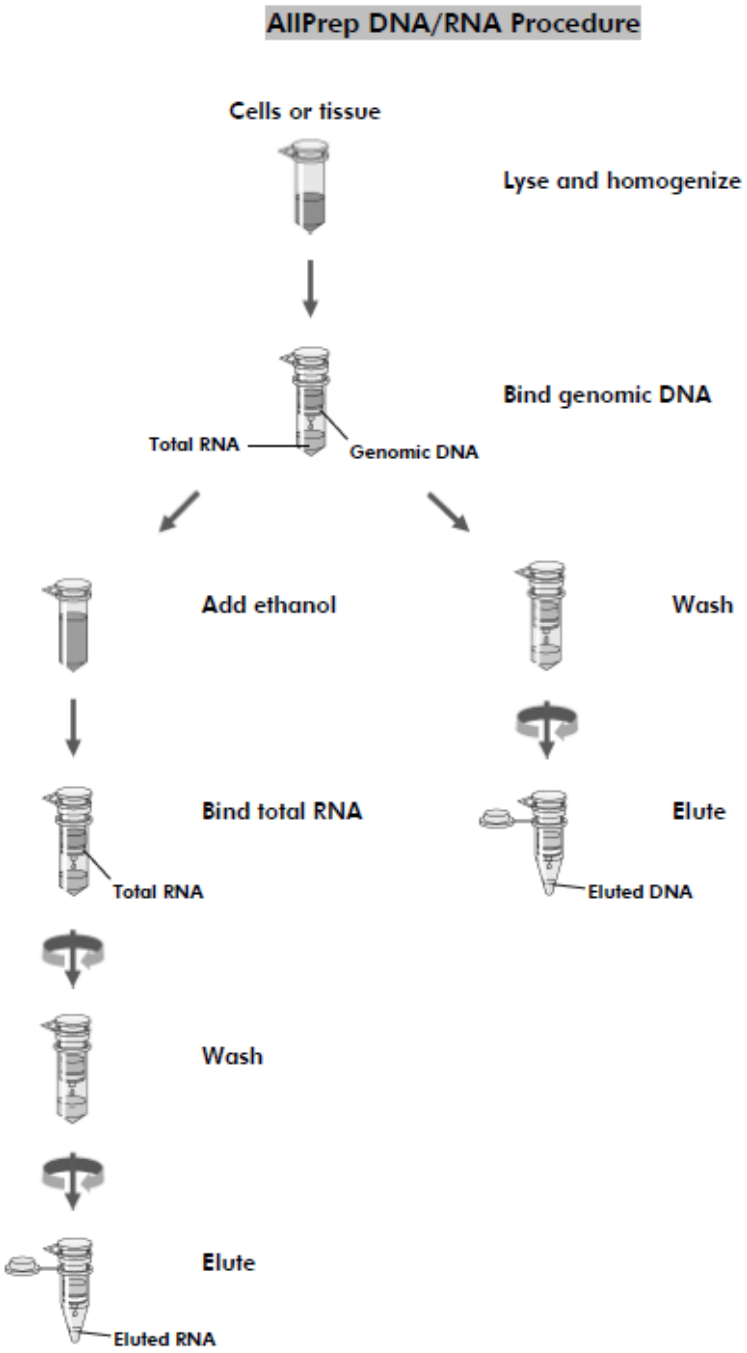



**Figure 13: ALLPrep DNA/RNA mini kit protocol provided by Qiagen**

First, 50–60 mg of tissue was weighed and moved to FastPrep-24™ 5Gl using matrix D in 2 mL tubes (MP Biomedicals, LLC). Then, 650 µL of lysis buffer (supplemented with β-mercaptoethanol) was added to the tissue. The tissue was homogenised using a Fastprep-24® homogeniser (MP Biomedicals) at a speed of 6 m/s for 40 s. The homogenised tissue was left to settle down for 1 min since foam was formed on top of the sample (Figure 14); it was then centrifuged for 1 min at a maximum speed of 14000 × *g*.



**Figure 14: Homogenised tissue using beads.**

The homogenised lysate was transferred carefully to the ALLPrep DNAspin column that was placed in a 2-mL collection tube. The lid was closed and the tube centrifuged for 1 min at 8000 × *g*. The flow-throw was used for RNA extraction. Meanwhile, the ALLPrep DNA spin column was placed on ice for DNA purification later.

**For RNA extraction**: One volume of 70% ethanol was added to the flow-throw and mixed well. Then, 650 µL of the mix was transferred with any precipitate to the RNeasy spin column placed in a 2-mL collection tube that was centrifuged for 15 s at 8000 × *g* and the flow-throw was discarded. Next, 700 µL of RW1 buffer was added to the RNeasy spin column. The lid was closed and the tube was centrifuged for 15 s at 8000× *g*. The flow-through was discarded. Then, 500 µL of RPE buffer was added to the RNeasy spin column and centrifuged for 15 s at 8000 × *g*. The flow-through was discarded. Again, another 500 µL of RPE buffer was added to the RNeasy spin column and centrifuged for 2 min at 8000 × *g*. The RNeasy spin column was placed in a new 1.5-mL collection tube. Next, 57 µL (2 µL for nano-drop, 5µL for RANA integrity, and 50µl for cDNA synthesis) Ambion® Nuclease-free water was directly added to the membrane of the spin column and centrifuged for 1 min at 8000 × *g* for RNA elution.

**For DNA purification:** First, 500 µL of AW1 buffer was added to the AllPrep DNA spin column (that was kept on ice after homogenising) to wash the spin column membrane. The lid was closed and the tube was centrifuged for 15 s at 8000 × *g*. The flow-through was discarded and 500 µL of AW2 buffer was added to the AllPrep DNA spin column followed by centrifugation for 2 min at high speed. The AllPrep DNA spin column was placed in a new 1.5-mL collection tube and 102 µL (2 µL for nano-drop and 100 µL for epigenetic experiment) of EB buffer was added directly to the membrane of the spin column. The lid was closed, and the tube was incubated at room temperature (15–25°C) for 1 min and then centrifuged for 1 min at 8000 × *g* for DNA elution.

The purity and concentration of the extracted RNA and DNA were checked using 2µl of the RNA/DNA solution on the Thermo Scientific™ NanoDrop® ND-1000 spectrophotometer. The RNA/DNA samples were then stored at -80°C.

# 5 RNA integrity

RNA integrity was assessed by agarose gel electrophoresis. To prepare the gel, first, the Tris/Borate/EDTA (TBE) (10×) solution was prepared by dissolving 53 g of Triz base plus 27.5 g boric acid plus 3.5 g EDTA to 500 mL distilled water, which was then mixed for 5 min. The pH was checked and adjusted to 8. The TBE ×10 stock was ready and every 100 mL of the stock was diluted in 900 mL of distilled water to obtain 1× solution that was used in gel preparation and as a running buffer.

For preparation of 1% gels, 1 g of agarose powder was dissolved in 100 mL buffer solution and placed in a microwave for 2 min at high power with caution to avoid boiling. The flask was taken from the microwave to the fume hood, cooled, and mixed through swirling; then, 2 µL of ethidium bromide (EtBr; 1 mg/mL) solution was added to intercalate with double stranded DNA and fluoresce under ultraviolet light. This step was undertaken in the fume hood, as EtBr is toxic. A 10-well separation comb was placed in the horizontal mini-gel electrophoresis system tray and around 80–100 mL of agarose solution was poured in the tray until it covered the wells, leaving the gel to solidify for an hour to form a gel block of 3–4-mm thickness.

Once the gel set, the comb was removed slowly from the solidified gel and the buffer solution was poured to cover the gel. For sample preparation, 5 µL of RNA sample was mixed with 2 µL loading dye and 5 µL of nuclease-free water for a total volume of 12 µL. This was loaded in the wells, with slow dispensing of the contents. Then, 1 kb of DNA ladder (@NorgenBiotek) was loaded as size standard.

Positive and negative electrodes were connected to the tank and the electrophoresis was set at a constant voltage of 90 V for 50 min. The gel was removed from the tray and RNA migration was visualised on the imaging system ChemiDoc (BioRad ChemiDoc MP, imaging systems, UK) using UV light. The relative intensity of the 28S and 18S fractions was used as an index of RNA integrity, whereby smeared bands or a 28S:18S band intensity ratio

below 2:1 indicated poor RNA quality and presence of degradation (Aranda et al., 2012).

# 6 First strand synthesis (cDNA)

For each reverse transcriptase (RT) reaction, a final volume of 20 µL was used with an RNA concentration of 120 ng/µL. Each RT reaction was done in duplicate by checking for any genomic contamination in the RNA sample and other reaction components, as one reaction contained reverse transcriptase enzyme (RT+) and the other did not as it was replaced by nuclease-free water instead (RT-). All procedures were done on ice. After cleaning the bench with 70% IMS, all reactions components were thawed on ice and all tubes were clearly labelled.

The RNA was denatured to eliminate any secondary structures in solution. The calculated amount of RNA, nuclease-free water, and random primers were added together and mixed well by pipetting up and down, followed by incubation for 10 min at 70°C on a heating block. The denatured samples of RNA were transferred immediately to ice. Every 12 µL of denatured RNA was loaded in the PCR polystyrene tubes with 2 µL of 10× buffer, 0.8 µL dNTP, 1 µL of RNAse inhibitor, 3.2 µL of nuclease-free water, and 1-µL of reverse transcriptase. The PCR tubes containing the samples were loaded onto the PCR machine and the reverse transcriptase program was run under the conditions of 25°C for 10 min, at 37°C for 2 h, and then at 85°C for 5 min. The cDNA was used immediately from PCR or stored at -20°C to be used later.

# 7 Quantitative real-time PCR

TaqMan™ assays with FAM-labelled probes were used for this study. Taqman was chosen as it has higher specificity and reproducibility than SYBR Green dye that may generate false-positive signals (ThermoFisher, 2108).

A layout plan for the 96-wells PCR plate was prepared after deciding the number of samples and the number of reference genes used. Every PCR reaction was done in triplicates for the RT+ samples and once for the RT- samples. Also, a No Added Template (NAT) control was included. The NAT sample contained all the reagents except RT+ or RT- cDNA to act as a negative control for determining any contamination in the reagents used.

Every replicate was carried out with a final volume of 20 µL, using 2µL of cDNA, 0.7 µL of Taqman assay, 10 µL of TaqMan™ Gene Expression Master Mix (Applied Biosystems™), and 7.3 µL of nuclease-free water. The 96-well plate was covered with optical covering slips with caution to prevent air bubble formation under the cover. The plate was loaded on the real-time PCR machine and the conditions were set for standard FAM dye, where the cycle parameters were 95°C for 10 min, followed by 50 cycles of 95°C for 15 s, 50°C for 30 s and 72°C for 15 s. Cycle threshold (CT) values were analysed using the BIO-RAD CFX Manager 3.0 (File version 3.0.1216.6723) and GenEx software version GenEx 6.1.0.751.

## 7.1 Choosing housekeeping reference genes

For the endometrium, PCR was done to compare the expression of 6 different reference genes on 10 endometrial tissue samples (Table 2). The reference genes used were as follows:

| Reference genes for endometrium | Gene name | UniGene |
|---|---|---|
| *ACTβ* | Actin, beta | Hs.520640 |
| *CYC1* | Cytochrome C1 | Hs.289271 |
| *HPRT1* | Hypoxanthine Phosphoribosyltransferase 1 | Hs.412707 |
| *PPIA* | Peptidylprolyl isomerase A | Hs.356331 |
| *TOP1* | DNA topoisomerase type I | Hs.472737 |
| *YWHAZ* | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide | Hs.492407 |

**Table 2: Reference genes used for endometrium**

For the decidua, PCR was done to compare the expression of 10 different reference genes on 12 decidual tissue samples. The reference genes used were as follows:

| Reference genes for decidua | Gene name | UniGene |
|---|---|---|
| *18s* | Human 18S ribosomal RNA | N/A |
| *ACTβ* | Actin, beta | Hs.520640 |
| *B2M* | Beta-2-microglobulin | Hs.534255 |
| *GAPDH* | Glyceraldehyde-3-phosphate dehydrogenase | Hs.544577 |
| *GUSB* | Glucuronidase, beta | Hs.255230 |
| *HPRT1* | Hypoxanthine Phosphoribosyltransferase 1 | Hs.412707 |
| *PGK1* | Phosphoglycerate kinase 1 | Hs.78771 |
| *PPIA* | Peptidylprolyl isomerase A | Hs.356331 |
| *TFRC* | Transferrin receptor | Hs.529618 |
| *YWHAZ* | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide | Hs.492407 |

**Table 3: Reference genes used for decidua**

In real-time PCR, the basic principle to quantitate a gene assay is the quantitation cycle (Cq), which is defined as the cycle when the fluorescence is detected and is the main result of the PCR. Lower Cq values show that the initial copy numbers of the target genes are higher. Data analysis was done using the GenEX software, where triplicate Cq values were obtained after qPCR. A 5-point standard curve was generated for the chosen best 3 reference

genes and 4 four target genes (KCNK9, KCNK10, KCNK17, and KCNMA1) by making 1 in 10 serial dilutions over a concentration of 120 ng/µL to 0.012 ng/µL, which showed the efficiency of the genes to be tested.

Determination of amplification efficiency is crucial for the accurate interpretation of data. Ideally, the molecule number of the target gene sequence should be doubled after each replication cycle, which corresponds to 100% efficiency for amplification. Typically, the desired range for amplification efficiency is 80–110%, which can be calculated using the equation (10^ (-1/x)-1) *100 where x is the gradient of the line. The chosen reference genes were corrected to the efficiencies calculated from standard curves for each gene, normalised to the reference genes and treatment expression to control group and $\log_2$.

# 8 Flow cytometry

This experiment was carried out on both endometrial and decidual cells by the end of the second passage either immediately after splitting the cells or after thawing cells that were previously frozen at this stage.

## 8.1 Preparation of cells

In the case of cell culture, after the end of the second passage, cells were trypsinised and washed twice in HBSS. Cell pellets were suspended in 1 mL of enriched medium, counted and tested for viability using trypan blue. For this, 10 µL of cell suspension was mixed with 10 µL of 0.4% trypan blue and incubated for 10 min at room temperature. Then, 10 µL of this mix was loaded on a chamber of a cell-counting slide. An automated cell counter was used to determine the number of viable cells. Cell viability was checked using Trypan blue, which is a cell stain used to estimate the number of dead cells in a viable population of cells; live cells will remain free of dye as they have active transmembrane pumps that extrude the dye from the cells whilst dead cells will be completely blue since they have a compromised, non-intact membrane (ThermoFisher, 2018).

For freezing of cells, 100 µL of DMSO was added to every 900 µL of cell suspension (~$2x10^6$ cells) and mixed well; this mixture was placed in 1.8-mL cryovials and slowly frozen at -80 for 24–48 h and then transferred to LN2 for long-term storage.

## 8.2 Flow cytometry staining

Before starting the flow cytometry experiment, phosphate-buffered albumin (PBA) was prepared by mixing 500 mL of phosphate-buffered saline (PBS) with 16.5 mL 30% bovine serum albumin (BSA) and 2.5 mL 20% sodium azide to preserve it. The PBA was then sterile-filtered and stored at 4°C.

Cells were suspended gently in PBA ($0.5 \times 10^6$ per 5 mL 'FACS tube') to avoid shearing of cells leading to release of DNA, which can cause clumping of cells. Cells were washed twice with 1 mL of PBA. For every sample, 4 tubes were labelled with the names of antibodies used (KCNK9, KCNK10, KCNK17, and KCNMA1); one tube was labelled as 'Cells only' and one as '2$^{ry}$ antibodies only'.

Anti-rabbit primary antibodies (Abcam plc.) were added at a dilution of 1:50 (6 µL antibody + 294 µL of PBA) for the antibodies with a concentration of 1 mg/mL (KCNK10, KCNK17, and KCNMA1) and at a dilution of 2:50 for antibodies with a concentration of 0.5 mg/mL (KCNK9). Cells were mixed by pipetting up and down several times and then cells were incubated for 30–120 min at room temperature. After incubation, the cells were washed 3 times with 500 mL PBA and centrifuged at 300 × *g* for 5 min using a swing out rotor.

Anti-rabbit FITC secondary antibody (company) was added at a dilution of 1:50 (6 µL antibody + 294 µL PBA) as the concentration set by manufacturer was 1 mg at 2 mg/mL. Cells were incubated in the dark for 2 h at room temperature. After incubation, the cells were washed 3 times with 500 mL of PBA, centrifuged at 300 × *g* for 5 min.

For fixation, 500 mL of 4% formaldehyde was added. Cells were incubated for 15 min in the dark and then washed twice using 1 mL PBA. After centrifugation, cell pellets were suspended in 500 mL of 0.5% formaldehyde (diluted in PBS) and stored at 4°C until processing with the Cytomics FC 500 (Beckman Coulter) machine that detects FITC fluorochromes at an emission bandwidth of 514–542 nm using an emission filter of 528/28. FC data were analysed using the software Kalusa® version 1.5a.

# 9 Immunofluorescence staining

The Immunofluorescence technique was used to visualise the location of potassium channels on the endometrial and decidual cells. This technique was a validation step for both flow cytometry and TEER experiments to confirm that both cell populations (stromal and epithelial cells) were present in the sample after the end of the first passage (for TEER) and the end of second passage (for flow cytometry).

Cells were first cultured on round 13-mm coverslips in a 24-well plate until they reached a confluence of around 70%. Immunofluorescence staining was done while coverslips were still in the 24-well plate and it involved two main steps:

## 9.1 Cell fixing

The medium was aspirated from the wells followed by 2 washes with 1 mL PBS at room temperature for 5 min while shaking. The cells were fixed inside the wells with 250 µL 4% previously prepared paraformaldehyde (PFA) for 10 min at room temperature. Another three washes were done with 1 mL PBS for 5 min each at room temperature with shaking. The cells were then incubated for 5 min at room temperature in 500 µL of 0.5% Igepal prepared in PBS. The wells were again washed 3 times with 1 mL PBS for 5 min with shaking. Following washing, fixed cells were incubated in 500 µL of 3% BSA/1% glycine/PBS for 15 min at room temperature. After incubation, the wells were again washed with 1 mL PBS for 5 min with shaking.

## 9.2 Immunofluorescence staining

To stop any non-specific binding of the primary anti-body used, the cells were first blocked with 250 µL 20% goat serum/PBS for 30 min at room temperature, with shaking. The block serum was aspirated and 250 µL of primary antibody was added (dilution of 1:50 for the antibodies of concentration of 1 mg/mL and dilution of 2:50 for antibodies of concentration of 0.5 mg/mL) to the wells. The

plate was incubated with shaking overnight at 4°C with antibodies as shown in (Table 4):

| Targeted channel | 1ry antibody | 2ry antibody |
|---|---|---|
| Vimentin (positive control for stromal cells) | Vimentin Anti-Mouse | FITC Anti-Mouse |
| Cytokeratin (positive control for epithelial cells) | Cytokeratin Anti-Mouse | FITC Anti-Mouse |
| Negative control | No antibody | FITC Anti-Mouse only |
| Cells only | No antibody | No antibody |

**Table 4: 1$^{ry}$ and 2$^{ry}$ antibodies used for positive and negative controls**

The next day, the primary antibody was removed from the wells and the wells were washed with 1 mL PBS for 5 min with shaking at room temperature. Specific fluorescent green secondary antibodies were added. This step was done under minimal light exposure and the plate was covered with foil paper and incubated for 30 min at room temperature with shaking. After incubation, the secondary antibody was aspirated and the wells were washed 4 times with 1 mL PBS for 5 min with shaking while covered in foil. Mounting was done using 5 µL PBS with a clean slide on top. Cover slips were fixed on the slides using nail polish to avoid evaporation of the PBS and cell drying. Visualisation of the cells was done under confocal microscopy (Zeiss LSM

880), controlled with the Carl Zeiss ‘Zen’ software, version 2. The images were analysed using Fiji-64-bit software.

# 10 Epigenetic chromatin

EpiQuik™ Tissue Methylated DNA Immunoprecipitation Kit provided by Epigentek Group Inc. was used per the manufacturer's instructions, unless otherwise stated (Figure 15). Before starting some materials required but not supplied as variable temperature water bath, vortex mixer, desktop centrifuge (up to 14,000 rpm), Dounce homogeniser, sonicator, orbital shaker, TE buffer (pH 8.0), and ethanol (96–100%).

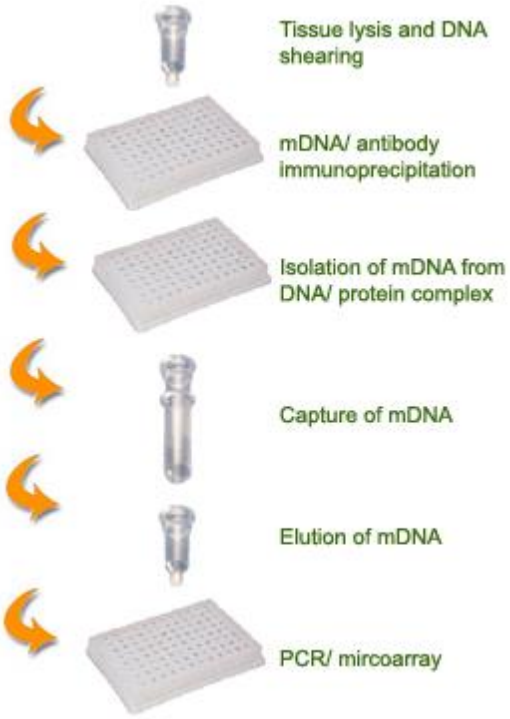



**Figure 15: Schematic Procedure for using the EpiQuik™ Tissue Methylated DNA Immunoprecipitation Kit**

Before starting the experiment, 90% ethanol, 70% ethanol, and 1× TE buffer pH8 solutions were prepared and shaken well before use.

## 10.1 Antibody binding to the assay plate

The number of strip wells required were determined and left in the plate frame. Strip wells were washed once with 150 µL of CP1 (wash buffer). Then, 100 µL of CP2 (antibody buffer) was added to each well and antibodies were added

at a volume of 1 µL with normal mouse IgG as the negative control and 0.5-1 µL of anti-5-methylcytosine as the targeted sample, as provided in the kit. Strip wells were covered with parafilm and incubated at room temperature for 60 min. During these 60 min, cell extracts were prepared as follows:

## 10.2 **Tissue disaggregation**

The decidual/endometrial tissue sample was placed on a 100-mm plate. Unwanted fat, blood, and necrotic tissue were removed from the sample. The sample was weighed and cut it into small pieces of around 1–2 $mm^3$ with a scalpel. Tissue pieces were transferred to a Dounce homogeniser. Nest, 1 mL of the homogenising buffer was added for every 200 mg of tissue and tissue pieces were disaggregated by 10–30 strokes. The homogenised mixture was transferred to a 15 mL conical tube and was centrifuged at 3000 rpm for 5 min at 4°C. The mixture was transferred to a 2-mL vial and was centrifuged again at 5000 rpm for 5 min at 4°C. The supernatant was removed and transferred to the lysis and DNA shearing steps.

## 10.3 **Tissue lysis and DNA shearing**

CP3 (lysis buffer) was added (100 µL/20 mg of tissue) on the disaggregated tissue pellet, which was re-suspended. The solution was transferred to a 1.5 mL vial with a maximum of 500 µL for each vial. This mixture was incubated at room temperature for 10 min. After incubation, the mixture was vortexed, and DNA was sheared by sonication at 4–5 pulses of 15–20 s each at level 2 (medium) using a Branson Microtip probe, followed by 30–40 s rest on ice between each pulse. Then, 5 µL of sonicated cell lysate was removed for agarose gel analysis to be sure that the length of the sheared DNA was between 200–1000 bp. After shearing, the pellet cell debris was centrifuged at 14,000 rpm for 10 min and the clear supernatant was transferred for methylated DNA immunoprecipitation step or was stored at -80°C to be used later.

## 10.4 Methylated DNA immunoprecipitation

The transferred supernatant was diluted with an equal volume of CP4 (ChIP dilution buffer) at 1:1 ratio. Then, 5 µL of the diluted supernatant was removed into a 0.5-mL vial, labelled as ‘input DNA’ and placed on ice.

Regarding the incubated antibody solutions, the solution was removed and strip wells were washed 3 times with 150 µL of CP2 by pipetting in and out. Next, 100 µL of the diluted supernatant (from previous step) was transferred to each strip well. Strip wells were covered with parafilm and incubated at room temperature on an orbital shaker set at 50–100 rpm for 90–120 min. After incubation, the supernatant was removed and the wells were washed 5 times with 150 µL of CP1, followed by 2 min on an orbital shaker (50–100 rpm) between washes. Finally, the wells were washed once with 150 µL of 1X TE buffer for 2 min on the orbital shaker.

## 10.5 Methylated DNA isolation/purification

First, 1 µL of proteinase K was added to each 40 µL of CP5 (DNA release buffer) and mixed. Then, 40 µL of this mix containing the CP5 and proteinase K was added to the samples (including the ‘input DNA’ vial). Sample wells were covered with strip caps and incubated at 65°C in a water bath for 15 min. Next, 40 µL of CP6 (reverse buffer) was added to the samples, mixed by pipetting up and down and then the wells were re-covered with strip caps and incubated at 65°C in a water bath for another 30 min. Also, at the same time, 40 µL of CP6 was added to the vial containing supernatant labelled as ‘input DNA’, followed by mixing and incubation at 65°C for 30 min.

The spin column provided with the kit was placed into a 2-mL collection tube. Next, 150 µL of CP7 (binding Buffer) was added to the samples. Mixed solutions were transferred to the column and centrifuged at 12,000 rpm for 20 s. Then, 200 µL of 70% ethanol was added to the column and centrifuged at 12,000 rpm for 20 s; the column was removed from the collection tube and the flowthrough was discarded. Next, 200 µL of 90% ethanol was added to the

column and centrifuged at 12,000 rpm for 20 s; then, the column was removed from the collection tube and the flowthrough was discarded.

The column was washed again with 200 µL of 90% ethanol at 12,000 rpm for 35 s, and the column was put in a new 1.5-mL vial and 10–20 µL of CP8 (elution buffer) was added directly to the filter in the column followed by centrifugation at 12,000 rpm for 20 s to elute the purified DNA. At this step, the methylated DNA was ready for PCR or storage at -20°C to be used later.

## 10.6 **Primers design**

For PCR, positive methylated human controls (TSH2B promoter primer pair and human ChIP-seq grade H19 imprinting control region primer pair) were purchased ready designed from Diagenode; negative non-methylated controls (β-Actin & GAPDH) and targeted CpG primers were designed using the UCSC genome browser https://genome.ucsc.edu/cgi-bin/hgGateway and primer-blast software https://www.ncbi.nlm.nih.gov/tools/primer-blast/ and purchased from Eurofins (Table 5).

| **PCR Primer** | | **Primer sequence** |
|---|---|---|
| β-Actin | Forward CpG | CGG CCA ACG CCA AAA CT (17mer) |
| | Reverse CpG | TCC CCT CCT TTT GCG AAA A (19mer) |
| GAPDH | Forward CpG | TAC TAG CGG TTT TAC GGG CG (20mer) |
| | Reverse CpG | TCG AAC AGG AGG AGC AGA GAG CGA (24mer) |

| | | |
|---|---|---|
| KCNK9 | Forward CpG | TCG CAA AGT AGA AGG AGC CG (20mer) |
| | Reverse CpG | GGT AAA AGG GGA AGC AGG TT (20mer) |
| KCNK10 | Forward CpG | CTG GCT CTC TCG CCT GAG T (19mer) |
| | Reverse CpG | GAA CAA AAG GCT CAT GGC TGG (21mer) |
| KCNK17 | Forward CpG | GAA GAC TCG GAG TAA AGC GGG (21mer) |
| | Reverse CpG | GAA GAC TCG GAG TAA AGC GGG (21mer) |
| KCNMA1 | Forward CpG | AGG TGG TAA CTC GTT AAT AAT GGA (24mer) |
| | Reverse CpG | GCA GCC GGG GGA GGA (15mer) |

**Table 5: Primer sequence designed for the forward and reverse CpG**

## 10.7 SYBR Green PCR

For PCR, SYBR green dye was used. SYBR dye is able to detect PCR products by binding to double-stranded DNA formed during PCR. It is suitable for detecting the double-stranded DNA sequence amplification without a probe, thus reducing the running cost (ThermoFisher, 2108).

The PCR plate was designed as shown in (Table 6), where each well contained 20 µL of the solution (Table 7). The PCR unit was set at: 95°C for 10 min; 39 cycles of 95°C for 15 s followed by 60°C for 1 min and a melting curve 60°C to 95°C with increments of 0.5.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | |
|---|---|---|---|---|---|---|---|---|
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | ACTB |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | GAPDH |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | H19ICR |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | TSH2B |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | KCNK9 |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | KCNK10 |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | KCNK17 |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | KCNMA1 |

**Table 6: Plate design including all the four targeted genes; 2 positive controls and 2 negative controls**

| | Power SYBR assay | 10X primers mix | $H_2O$ | template | Final Volume in µl |
|---|---|---|---|---|---|
| Each 20µl reaction | 10 | 0.8 | 7.2 | 2 | 20 |

**Table 7: Each well containing 20 µL reaction solution**

# 11 Transepithelial electrical resistance

TEER is a measurement used to evaluate the function of the epithelial cell barrier on the porous permeable membrane. The TEER is measured while passing current through the cells on both transcellular and paracellular paths (Powell, 1981). The Transwells used for the TEER experiments consist of an upper apical chamber and a lower basolateral chamber with a porous semipermeable membrane where cells are seeded on and grow, forming a tight monolayer. In this technique, transport is measured in the direction from upper to lower chamber, crossing the cell monolayer and the porous membrane, and finally, the medium is collected in the lower chamber for quantification of transported materials.

In our study, TEER was measured as a marker of paracellular permeability influenced mainly by epithelial cells. The TEER was measured using an electrode pair (4 mm wide and 1 mm thick) containing a silver/silver chloride knob for measuring voltage and a silver electrode for passing current. The resistance across the cell monolayer was measured in units of Ω, where resistance is inversely proportional to the effective area of the Transwell membrane, which is reported in $cm^2$. Resistivity of the tissue samples was calculated (resistance was normalised to surface area; $\Omega \times cm^2$) by subtracting baseline resistance (Transwell inserts with medium only and without cells) and multiplying by membrane surface area.

## 11.1 Seeding of cells and treatments

Cultured endometrial cells were seeded in a 12-well chamber plate (12-mm Transwell® with a 0.4-µm pore polyester membrane insert; Corning Life Sciences) at a density of $7.5 \times 10^4$ in each well. DMEM (enriched with 10% FBS and antibiotics P/S) was then added (1200 µL in the lower chamber and 600 µL in the upper chamber). The medium was changed every day when checking for cell confluence under a microscope and the resistance across the membranes was recorded. When the cells had reached a confluence of more

than 90 (day 5–6), the treatments were added as follows (no further change of media was done after adding the treatments so as not to wash the treatments off):

- Inserts: no cells
- Control: cells only, no treatment
- 5% Oxygen concentration in Tri-gas incubator
- KCNK9 blocker: 35 µM/well
- KCNMA1 blocker: 1 mM/well
- Vitamin D: 2.5 µg/kg
- Intralipid 20%:12 mL/kg

## 11.2 **Recording TEER measures**

The resistance across the membrane was measured using STX2 electrodes linked to an EVOM2 resistance meter (World Precision Instruments, Hitchin, Hertfordshire, UK).

Before every use, the electrodes were calibrated and sterilised by immersing in ethanol for 15 min followed by air drying for 15 s and rinsing with sterile electrolyte solution (HBSS). The electrode cable was connected to the port on the EVOM volt-ohm meter, where an internal short circuit system was created and maintained.

Readings were taken with the V/ Ω meter set, and the electrodes were vertically and perpendicularly immersed in a well containing the Transwell insert. The short arm of the electrode was placed in the upper apical chamber, while the long arm was placed in the lower basolateral chamber, touching the bottom surface of the well (Figure 16**Figure 16**). Once the EVOM reading was stabilised, the resistance (Ω) was recorded for each well. Four readings were taken per insert for each triplicate group and the average value was recorded.

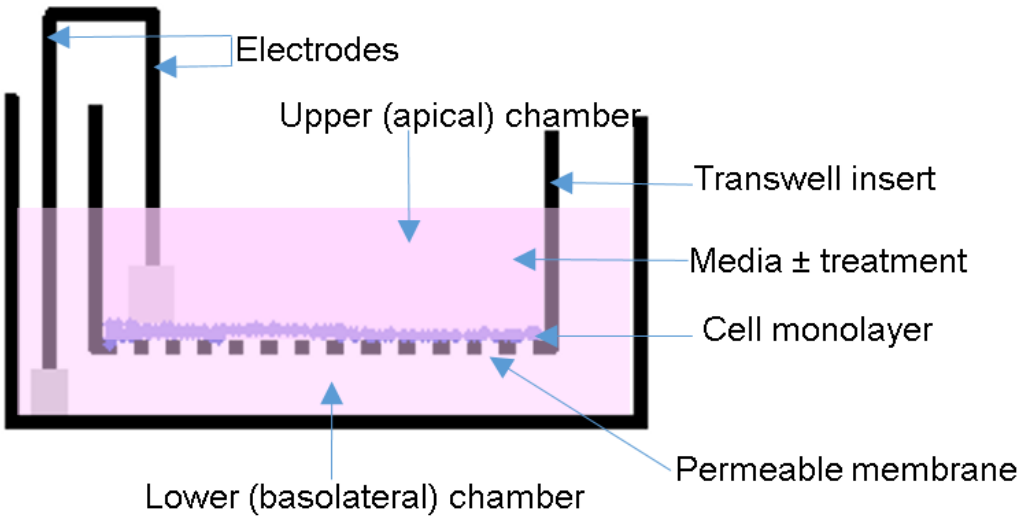



**Figure 16: Position of electrodes while recording TEER**

The TEER was measured at various time points, and measurements were taken at the same time on day 0, 2, 4, 6, 8, and 10 and 2 h after adding the treatments to determine the immediate effect of the treatment.

# 12 Statistics

Results were presented as means ± SD. GraphPad Prism-7 (La Jolla, CA, USA) was used. Data were tested for normality by the Shapiro-Wilks test. A one-way ANOVA test or unpaired *t*-test was carried out for parametric data analysis, while a Kruskal-Wallis test or Mann-Whitney test was carried out for non-parametric data analysis. Bonferroni's multiple comparisons were performed to compare the means between the treatment groups and the controls.

**Chapter Three: Systematic Review and Meta-Analysis:** Immune therapies for women with history of unsuccessful implantation undergoing IVF/ICSI treatment

# 1 Introduction

Many patients are offered new techniques and add-ons that may enhance their in-vitro fertilisation (IVF) results and take-home baby rates. Most of these additional procedures are still not clinically proven (Nardo et al., 2015). Any new techniques in ART should be first clinically tested to prove its safety and efficacy. This is done first through animal experiments and then through controlled clinical trials (National Health Service, 2018)

Any new IVF technology (e.g. immunosuppressive treatments, scratching of endometrium, zona pellucida laser drilling, use of embryo glue) should not be offered as routine workup for patients, unless it is applied to patients matching those who participated in RCTs. After that, a follow-up is necessary to determine long-term safety (Harper et al., 2017).

National Institute for Health and Clinical Excellences costing report stated that annually, an estimated £68m has been spent on IVF by the NHS since 2013. It is believed there are hidden costs of infertility treatment (Howard, 2018) which is considered to be commercialization of IVF (Adamson and Rutherford, 2018).

Many IVF clinics in the UK offer extra add-on treatments and procedures besides the classic IVF treatment. They convince the patients that these additional procedures are beneficial (Datta et al., 2015). Unfortunately, most of these additional procedures are not evidence based and additional studies as well as clinical trials are needed to support their routine use in IVF practice (Spencer et al., 2016).

HEFA suggests some questions that the patients may ask before starting an IVF cycle. These questions might be as follows: Is the procedure recommended by NICE? Is there an RCT that shows the beneficial effect of this treatment? Is there a Cochrane meta-analysis done to help interpret the effectiveness of these treatments on the birth rate? (Heneghan et al., 2016).

Therefore, we decided to collaborate with Cochrane UK and conduct a systematic review to examine these add-on treatments provided by fertility clinics. The objective was to understand their role and determine whether immunotherapy improved the live birth rates in women with a history of recurrent implantation failure who are undergoing assisted reproduction. Another objective was to investigate the effect of some of the treatments in-vitro on potassium channels in human endometrium.

Among the add-on treatments, the one most relevant to our project was ‘intralipid’ treatment. First because of its unsaturated fatty acids component such as arachidonic acid which as a link to potassium channels. Secondly its role in immunomodulation during embryo implantation is not clear (Rutherford, 2017), especially since this treatment was marked red per the traffic light rating system by HFEA in the absence of evidence showing its effectiveness and safety. This rating is to be reviewed in 2020 (Human Fertilisation and Embryology Authority, 2016).

This Cochrane systematic review (chapter) is currently in editorial process and will be published once approved by the reviewing committee.

# 2 Materials & Methods

## 2.1 Eligibility criteria

### 2.1.1 Types of studies

Only randomised clinical trials (RCTs) comparing immune therapies for women with a history of unexplained recurrent implantation failure who were undergoing IVF treatment were included. No cross-over trials were identified. Quasi- and pseudo-randomised trials were excluded.

### 2.1.2 Types of participants

#### *2.1.2.1 Inclusion criteria*

Women with a history of recurrent unexplained failed implantation who were undergoing IVF/ICSI treatment and were receiving a form of immune therapy interventions were included. RIF was defined as 'failure of at least two consecutive cycles of IVF/ICSI or FER where the cumulative number of transferred embryos was no less than four cleavage stage embryos and no less than two blastocysts (good quality embryos)' (Polanski et al., 2014).

#### *2.1.2.2 Exclusion criteria*

- Women with any gynaecological cancer
- Women diagnosed with any autoimmune conditions
- Women with known thrombophilic conditions
- Women undergoing IVF/ICSI treatment using donor eggs

#### *2.1.2.3 Types of interventions*

The following immune therapies were compared with no treatment, placebo, or another immune therapy treatment.

- Aspirin
- Glucocorticoids
- Granulocyte colony stimulating factor (GCSF)
- Heparin
- Intralipid
- Intravenous immune globulin (IVIG)
- Leukocyte inhibitory factor (LIF)
- Lymphocyte immune therapy (LIT)
- Peripheral blood mononuclear cells (PBMC)
- TNF-α inhibitors

## 2.2 Study outcomes

### 2.2.1 Primary outcomes

1) Live birth rate per woman (LBR), which was defined as delivery of a live foetus after 24 weeks of gestation.
2) Miscarriage rate per woman (MR), which was defined as the involuntary loss of a pregnancy before 24 weeks of gestation.

### 2.2.2 Secondary outcomes

1) Clinical pregnancy rate, which was defined as the presence of one or more gestation sacs with positive foetal heart activity observed on ultrasonography at six to eight weeks of gestation.
2) Implantation rate, defined as the number of foetal sacs divided by the number of embryos transferred.
3) Number of embryos transferred.
4) Ongoing pregnancy rate, which was defined as pregnancies continuing after 12 weeks.

### 2.2.3 Adverse outcomes

1) Drug side effects.
2) Ectopic pregnancy, to be confirmed by histology and ultrasonography.
3) Multiple pregnancies.
4) Ovarian hyperstimulation syndrome (OHSS).
5) Preterm delivery, which was defined as spontaneous delivery before 37 weeks of gestation.

## 2.3 Literature search methods

Any published or unpublished randomised controlled trials (RCTs) were included using the following strategies:

### 2.3.1 Electronic searches

The following electronic databases, trial registers, and websites were searched, from inception to 1st September 2017.

1) CINAHL (searched from 1961 to September 2017)
2) Cochrane Central Register of Studies Online (CRSO) (searched September 2017)
3) Cochrane Gynaecology and Fertility Group (CGF) Specialised Register of controlled trials (searched September 2017)
4) DARE database (searched September 2017)
5) Embase (searched from 1980 to September 2017)
6) LILACS (searched September 2017)
7) MEDLINE (searched from 1946 to September 2017)
8) PsycINFO (searched from 1806 to September 2017)
9) PubMed (searched September 2017)

Ongoing and registered trials included from the following platforms:

1) Citation indexes
2) ClinicalTrials.gov, a service of the US National Institutes of Health (Clinical Trials)
3) Current Controlled trials (Current Controlled trials)
4) World Health Organization International Trials Registry Platform search portal (World Health Organisation)
5) Conference abstracts in the ISI web of Knowledge (ISI web of Knowledge)
6) Clinical Study results for clinical trial results of marketed pharmaceuticals (Clinical Study Results)
7) OpenSIGLE database (OpenSIGLE)
8) Google for grey literature (Google scholar for unpublished trials, example: e-thesis)

### 2.3.2 Searching other resources

The reference lists of trials and systematic reviews were retrieved by manual search and personal contact was made with experts in the field to obtain any additional data.

## 2.4 Data collection, selection of included studies, and data extraction

Data collection and analysis was conducted according to the Cochrane Handbook for Systematic Reviews of Interventions (version 5.1.0) (Higgins and Green, 2011).

The titles and abstracts were retrieved by a Cochrane Information Specialist (MS) and were read by SA and HA (first authors). The ones that were irrelevant were deleted and the full text of the remaining trials were retrieved. Two review authors (SA & HA) independently examined the full text articles for compliance with the inclusion criteria. Authors corresponded with trial investigators (HN), if required, to clarify trial eligibility. Disagreements were resolved by consensus

or by discussion with a third author (PC). The selection process is documented in a PRISMA flow chart (Figure 17).

Data were extracted independently by two authors and any disagreement between these authors was resolved by a third author.

## 2.5 Assessment of risk of bias of included studies

The Cochrane risk of bias assessment tool was used to assess the risk of bias in the included trials. Risk of bias assessment was checked for the following: sequence generation; allocation concealment; blinding of participants, providers, and outcome assessors; completeness of outcome data; selective outcome reporting; and other potential sources of bias. These six biases were independently assessed (high, low or unclear risk) by two authors (SA & PC), with any disagreements resolved by consensus or by discussion with a third author (HA).

## 2.6 Measures of treatment effect

For dichotomous data, such as live birth, the number of events in the control and intervention groups of each trial were used to calculate the risk ratio (RR) that was used wherever possible, and the results reported as percentages were converted to event numbers to facilitate this. A Peto OR was used only in the analyses of studies showing no events in either the intervention or control group. Every outcome was presented with a 95% confidence interval after entering the data into the RevMan 5.3 software (Copenhagen: The Nordic Cochrane Centre, The Cochrane collaboration, 2012).

## 2.7 Dealing with missing data

All data were analysed. Attempts were made to obtain missing data by contacting the original investigators. Live birth was assumed not to have occurred in participants with unreported outcomes.

## 2.8 Assessment of heterogeneity

Statistical heterogeneity was examined by inspecting the scatter in the data points within the graphs and the overlap in their confidence intervals. It was assessed using the $I^2$ statistic, where $I^2$ statistically greater than 50% was considered an indicator of substantial heterogeneity (Higgins and Green, 2011).

## 2.9 Data synthesis

The data from primary trials were combined using a fixed-effect model for the interventions. RevMan 5.3 software was used for meta-analyses with the Mantel-Haenzel method. A sensitivity analysis was conducted when there was heterogeneity that could not readily be explained.

## 2.10 Summary of findings table

The summary of findings table was prepared using GRADEpro and Cochrane methods (GRADEpro, 2015, Higgins and Green, 2011). The primary outcomes of live birth and miscarriage rates were reported where possible, along with the main secondary review outcomes (clinical pregnancy rate, implantation rate) and adverse outcomes (ectopic pregnancy, pre-term delivery, drug side effects, OHSS, and multiple pregnancy).

The GRADE criteria were assessed including risk of bias, imprecision, indirectness, consistency of effect, and publication bias. High, moderate, low, or very low judgement were made by two reviewers (SA & PC) working independently, with disagreements resolved by discussion.

# 3 Results

## 3.1 Results of the search

(Flowchart 2) shows the search results. In all, 3,351 articles were retrieved from the database searches and hand-searching of the reference lists of relevant articles. Duplicates were removed. From reference and abstract screening, 27 studies were found to be eligible for inclusion and were selected for full-text reading. Finally, 19 studies were included in the review with three studies selected for their abstracts, later replaced by the full study, and 13 studies were included in the meta-analysis.

## 3.2 Included studies

In all, 19 studies were included (Aleyasin et al., 2016, Brinsden et al., 2003, Brinsden et al., 2009, Cambiaghi and Leao, 2012, Colicchia et al., 2011, Davari-tanha et al., 2016, El-Khayat and El Sadek, 2015, Gamaleldin et al., 2016, Hamdi et al., 2015, Kim et al., 2011, Madkour et al., 2016, Makrigiannakis, 2011, Miraj et al., 2015, Obidniak et al., 2016, Pergolini et al., 2013, Scarpellini and Sbracia, 2011, Scarpellini and Sbracia, 2013, Urman et al., 2009, Yu et al., 2016). Three studies were included in abstract form and have been replaced by full text versions: Davari-Tanha (2016), Hamdi (2015), and Madkour (2016).

## 3.3 Study design and setting

Seventeen studies had a parallel design with 1,748 women in total, while two studies had three groups of patients. Davari-Tanha (2016) and Obidniak (2016) tested G-CSF. Davari-Tanha (2016) had an intervention arm with intrauterine (IU) G-CSF and two control arms with IU saline or passage of a Cook catheter only. Obidniak (2016) used both subcutaneous (SC) and IU GCSF as well as control group that did not receive any intervention.

The following studies were carried out in IVF clinics: Brinsden (2009), Cambiaghi (2012), Colicchia (2011), Madkour (2016), Pergolini (2013), Urman (2009), and Yu (2016). The following studies were carried out in fertility clinics or hospitals: Aleyasin (2016) (2 hospitals and 1 fertility clinic), Davari-Tanha (2016) (2 hospital based departments), Hamdi, 2015 (hospital based fertility clinic), and Miraj (2015) (Research Centre and hospital based fertility clinic). The following studies did not specify the location: Brinsden (2003), El-Khayat (2015), Gamaleldin (2016), Kim (2011), Makrigiannakis (2011), Obidniak (2016), Scarpellini (2011), and Scarpellini (2013).

Studies were conducted in the following countries: Brazil (Cambiaghi, 2012), China (Yu, 2016), Egypt (El-Khayat, 2015), Greece (Makrigiannakis, 2011; Iran Aleyasin, 2016; Davari-Tanha, 2016; Hamdi, 2015; Miraj, 2015), Italy (Colicchia, 2011; Pergolini, 2013; Scarpellini, 2011; Scarpellini, 2013), Morocco (Madkour, 2016), Russia (Obidniak, 2016) South Korea (Kim, 2011), Turkey (Urman, 2009), and the UK (Brinsden et al., 2003; 2009; Gamaleldin, 2016). The locations of the 28 recruitment centres were not specified in the study by Brinsden et al. in 2009 but the authors conducted a pilot study in the UK in 2003 using a similar protocol.

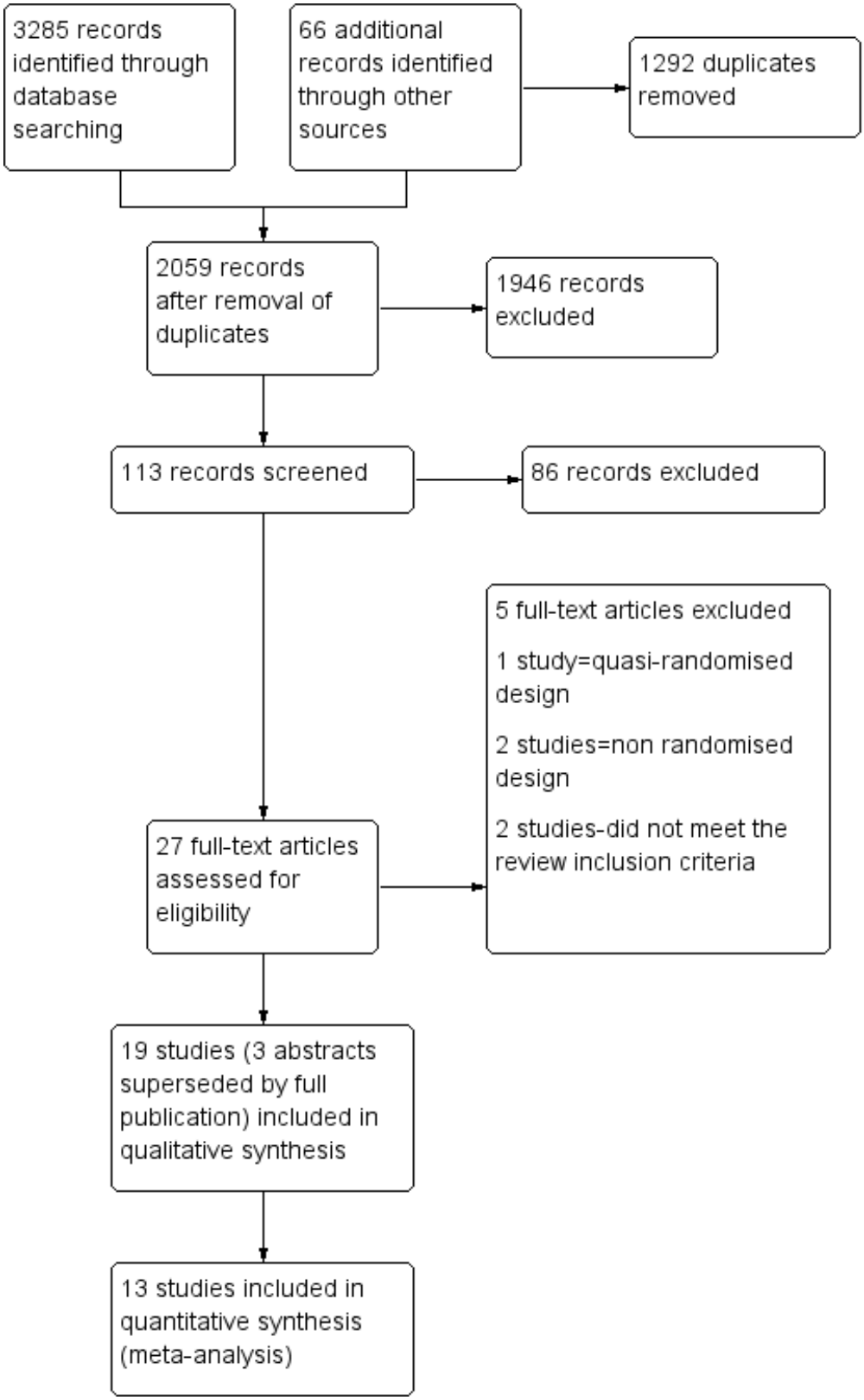



**Figure 17**: PRISMA flow chart showing search results

## 3.4 **Interventions**

(Table 8) shows a summary of the interventions used in the included studies. No RCT studies were found for steroids, IVIG, TNF, LIT, and aspirin only.

| Immunotherapy | Studies |
|---|---|
| GCSF | 8 studies: Aleyasin 2016; Cambiaghi 2012; Davari-Tanha 2016; Kim 2011; Miraj 2015; Obidniak 2016; Scarpellini 2011; Scarpellini 2013 |
| Heparin | 4 studies: Colicchia 2011; Hamdi 2015; Pergolini 2013 (versus aspirin); Urman 2009 |
| PBMC | 3 studies Madkour 2016; Makrigiannakis 2011; Yu 2016. |
| LIF | 2 studies Brinsden 2003; Brinsden 2009 |
| Intralipids | 2 studies El-Khayat 2015; Gamaleldin 2016 |

**Table 8: Interventions used in the included studies**

## 3.5 **Outcomes**

The outcome measures differed across the studies as follows:

Eight studies reported live birth (LBR): Brinsden et al., (2003; 2009), El-Khayat (2015), Gamaleldin (2016), Makrigiannakis (2011), Pergolini (2013), Urman (2009), Yu (2016).

Five studies reported miscarriage rate: Brinsden et al. (2009), Davari-Tanha (2016), El-Khayat (2015), Madkour (2016), Obidniak (2016).

Thirteen studies reported clinical pregnancy: Aleyasin (2016), Brinsden et al. (2003), Brinsden et al. (2009), Cambiaghi (2012), Davari-Tanha (2016), El-Khayat (2015), Gamaleldin (2016), Kim (2011), Madkour (2016), Makrigiannakis (2011), Miraj (2015), Obidniak (2016), Urman (2009).

Eleven studies reported implantation rate: Aleyasin (2016), Cambiaghi (2012), Davari-Tanha (2016), Hamdi (2015), Kim (2011), Madkour (2016), Makrigiannakis (2011), Miraj (2015), Obidniak (20160, Pergolini (2013), Urman (2009).

Only Urman (2009) reported the ongoing pregnancy, multiple pregnancy, and preterm delivery rates. None of the studies reported the number of transferred embryos. Obidniak (2016) was the only one to comment that no patient had reported any side effects. El-Khayat (2015) reported 2 cases of congenital middle ear anomaly in the intralipid group.

## 3.6 Excluded studies

Five studies were excluded from the full text stage. Fawzy's (2014) study was excluded as it had a quasi-randomised design (Fawzy and El-Refaeey, 2014), Makrigiannakis' (2015) and Yoshioka's (2006) studies were excluded as they had a cohort design and were non-randomised (Makrigiannakis et al., 2015, Yoshioka et al., 2006). Two studies included patients with additional clinical conditions, such as at least one case diagnosed with thrombophilia in Qublan's (2008) study and evidence of autoimmune disease as well as RIF in Stephenson's (2000) study (Qublan et al., 2008, Stephenson and Fluker, 2000).

## 3.7 Risk of bias in included studies

Risk of bias assessment was performed (Figure 18) for all included studies (Figure 19) to evaluate their quality, focusing on the following main domains: 1) random sequence generation, 2) allocation concealment, 3) blinding of participants and of outcome assessment, 4) incomplete outcome data, 5) selective reporting, and 6) other potential biases.

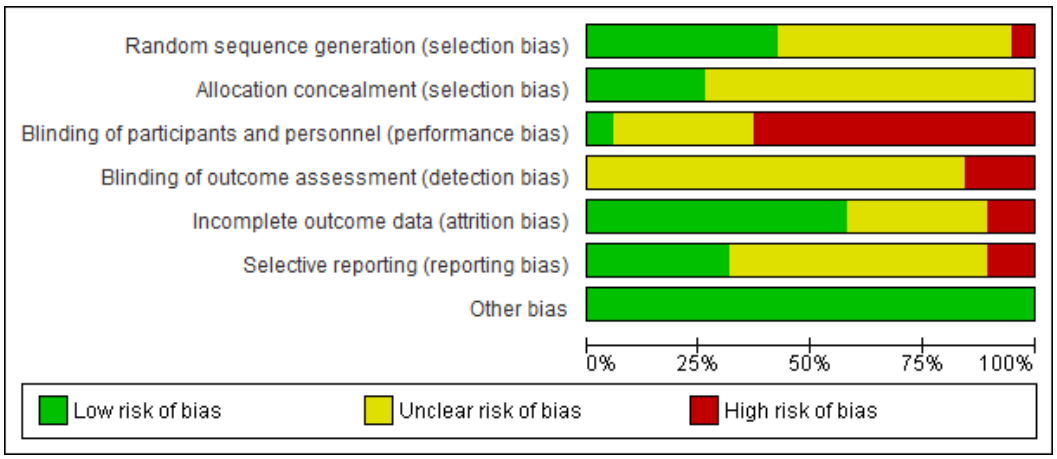



**Figure 18: Risk of bias as percentage across included studies**

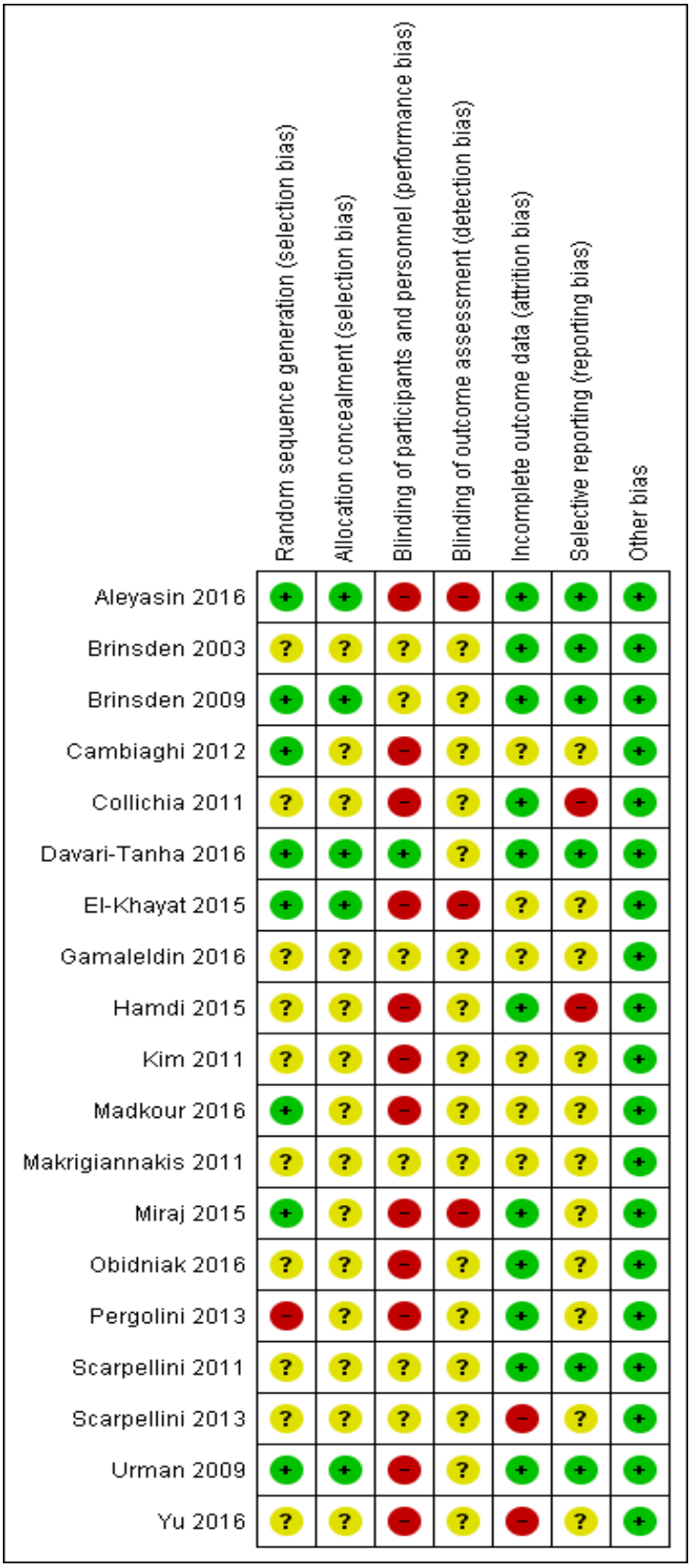



**Figure 19: Risk of bias for all included studies**

## 3.8 Effect of intervention

Each intervention will be presented as a summary by means of a findings table, presenting the main findings of the meta-analysis and risk of bias assessment. This is followed by detailed analysis of each intervention's reported primary and secondary outcomes.

### 3.8.1 Intralipids vs. no treatment

| Intralipid compared to no treatment for women with a history of unsuccessful implantation undergoing IVF/ICSI treatment | | | | | | |
|---|---|---|---|---|---|---|
| Patient or population: Women with a history of unsuccessful implantation undergoing IVF/ICSI treatment<br>Setting: Not specified<br>Intervention: IV Intralipid<br>Comparison: No treatment | | | | | | |
| Outcomes | Anticipated absolute effects* (95% CI) | | Relative effect (95% CI) | № of participants (studies) | Certainty of the evidence (GRADE) | Comments |
| | Risk with no treatment | Risk with Intralipid | | | | |
| Live birth | 197 per 1,000 | 420 per 1,000 (266 to 663) | RR 2.13 (1.35 to 3.36) | 303 (2 RCTs) | ⊕⊕⊕⊖ MODERATE [1] | |
| Miscarriage rate | 69 per 1,000 | 39 per 1,000 (8 to 112) | RR 0.43 (0.12 to 1.63) | 203 (1 RCT) | ⊕⊕⊖⊖ LOW [1 2] | |
| Clinical pregnancy rate | 230 per 1,000 | 437 per 1,000 | RR 1.90 (1.27 to 2.82) | 303 (2 RCTs) | ⊕⊕⊕⊖ MODERATE [1] | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | (292 to 649) | | | | |
| Adverse effects<br><br>Pre-term delivery rate<br><br>Multiple pregnancy rate<br><br>Drug side effects | Not reported by any study | | | | | |
| *__The risk in the intervention group__ (and its 95% confidence interval) is based on the assumed risk in the comparison group and the __relative effect__ of the intervention (and its 95% CI).<br>**CI:** Confidence interval; **RR:** Risk ratio; **OR:** Odds ratio; | | | | | | |
| **GRADE Working Group grades of evidence**<br>**High certainty:** We are very confident that the true effect lies close to that of the estimate of the effect.<br>**Moderate certainty:** We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.<br>**Low certainty:** Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect.<br>**Very low certainty:** We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of the effect. | | | | | | |

**Table 9: Summary of findings for intralipids compared to no treatment**

### *3.8.1.1 Primary outcomes*

#### 3.8.1.1.1 Live birth rate

(Figure 20) shows that both El-Khayat (2015) and Gamaleldin (2016) reported the live birth rate for a total of 303 participants. El-Khayat (2015) reported an RR of 1.6 (CI 1.28–2.13), while Gamaleldin (2016) reported an RR of 1.56 (CI 0.74–3.27). This means intralipid may have improved the live birth rate in those who received this intervention (RR 2.13, 95% CI 1.35–3.36; participants = 303; studies = 2; $I^2$ = 6% moderate certainty evidence).

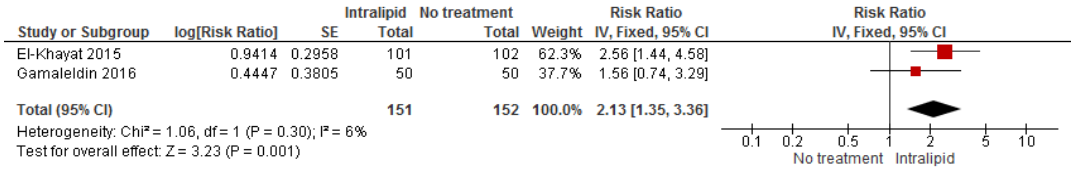



**Figure 20: The effect of intralipid on live birth rate**

#### 3.8.1.1.2 Miscarriage rate

(Figure 21) shows that the miscarriage rate was reported only by El-Khayat (2015). The meta-analysis showed insufficient evidence between the study groups (RR 0.43, CI 0.12–1.63; participants = 203; studies = 1; $I^2$ = 0, low certainty evidence)

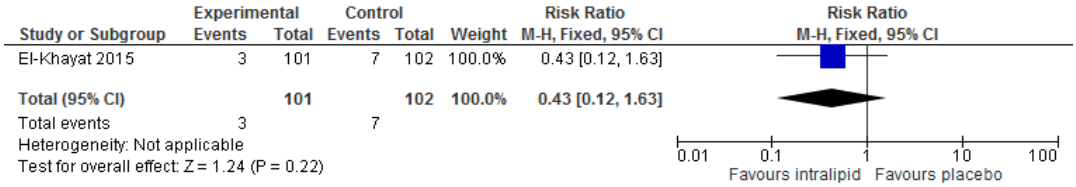



**Figure 21: The effect of intralipid on miscarriage rate**

### *3.8.1.2 Secondary outcomes*

#### 3.8.1.2.1 Clinical pregnancy rate

(Figure 22) shows that the clinical pregnancy rate was reported by El-Khayat (2015) and Gamaleldin (2016). The meta-analysis was performed with the RR

1.90 (CI 1.27–2.82). This interpret that Intralipid may improves the clinical pregnancy (RR 1.90, 95% CI 1.27–2.82; studies = 2; I2 = 28% moderate certainty evidence).

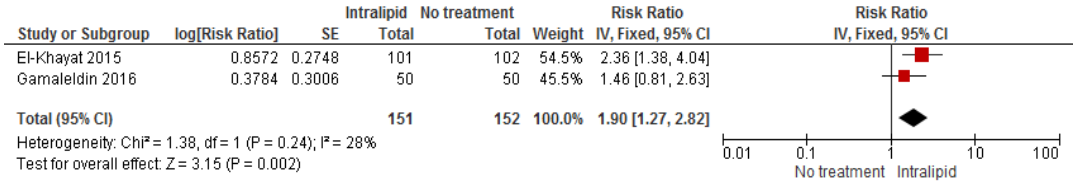



**Figure 22: The effect of Intralipid on clinical pregnancy rate.**

### 3.8.1.2.2 OHSS rate

(Figure 23) shows that OHSS rate was only reported by El-Khayat (2015), where OHSS was classified into mild, moderate. and severe (no details given in the study on how this classification was done). The meta-analysis for all three categories of OHSS showed insufficient evidence between the study groups as the CI crossed the line of no effect:

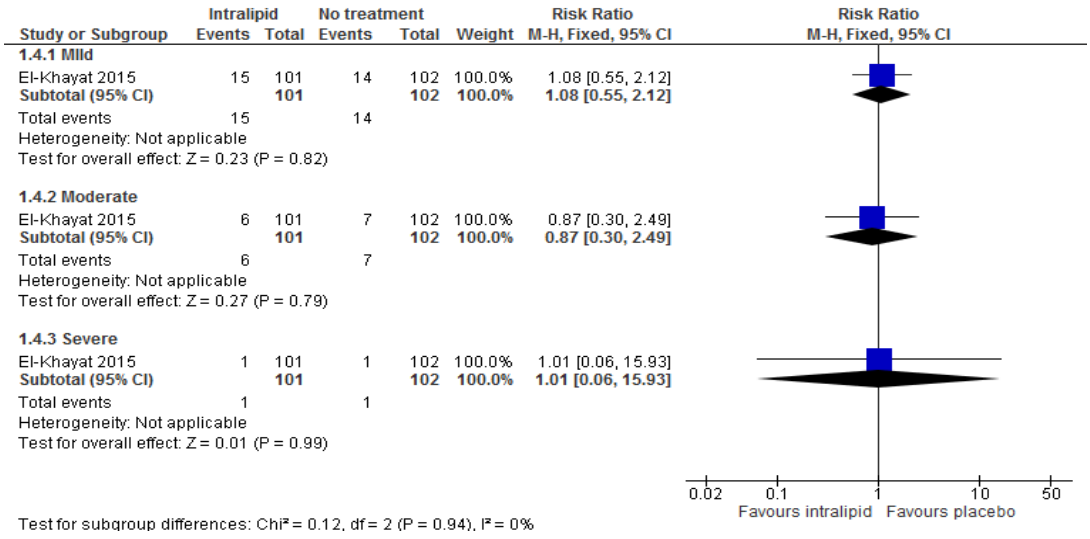



**Figure 23: The effect of intralipid on OHSS rate**

### 3.8.2 **PBMC vs. no treatment**

| PBMC therapy compared to no treatment for women with a history of unsuccessful implantation undergoing IVF/ICSI treatment | | | | | | |
|---|---|---|---|---|---|---|
| Patient or population: Women with a history of unsuccessful implantation undergoing IVF/ICSI treatment<br>Setting: IVF clinic<br>Intervention: PBMC<br><br>Comparison: No treatment | | | | | | |
| Outcomes | Anticipated absolute effects* (95% CI) | | Relative effect (95% CI) | № of participants (studies) | Certainty of the evidence (GRADE) | Comments |
| | Risk with no treatment | Risk with PBMC | | | | |
| Live birth rate | Not reported by any study | | | | | |
| Miscarriage rate | 741 per 1,000 | 148 per 1,000 (59 to 378) | RR 0.20 (0.08 to 0.51) | 54 (1 RCT) | ⨁⊖⊖⊖ VERY LOW [1,2] | |
| Clinical pregnancy rate | 114 per 1,000 | 272 per 1,000 (156 to 474) | RR 2.39 (1.37 to 4.17) | 252 (2 RCTs) | ⨁⨁⨁⊖ MODERATE [3] | |
| Adverse events:<br><br>Pre-term delivery rate<br><br>Multiple pregnancy rate<br><br>Drug side effects | Not reported by any study | | | | | |

**Table 10: Summary of findings for PBMC therapy compared to no treatment**

### *3.8.2.1 Primary outcomes*

#### 3.8.2.1.1 Miscarriage rate

(Figure 24) shows that the miscarriage rate was only reported by Madkour (2016). The meta-analysis showed that no treatment with PBMC probably improved the miscarriage rate (RR 0.20, 95% CI 0.08–0.51; participants = 54; studies = 1, $I^2$ = 0%; very low certainty evidence).

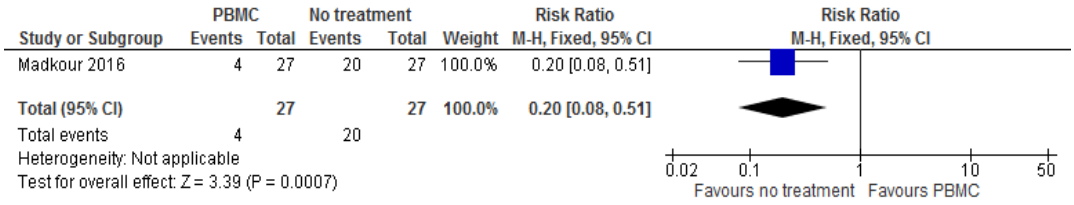



**Figure 24: The effect of PBMC on miscarriage rate**

### *3.8.2.2 Secondary outcomes*

#### 3.8.2.2.1 Clinical pregnancy rate

(Figure 25) shows that the clinical pregnancy rate was reported by Madkour (2016) and Yu (2016). The meta-analysis showed a probability of improvement in favour of PBMC treatment (RR 2.39, 95% CI 1.37–4.17; participants = 252; studies = 2; $I^2$ = 0% moderate certainty evidence).

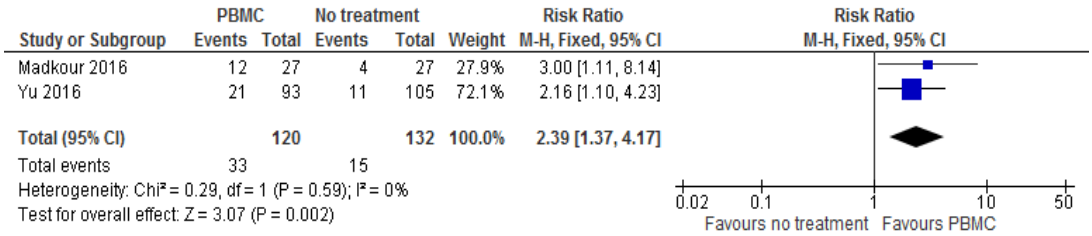



**Figure 25: The effect of PBMC on clinical pregnancy rate**

#### 3.8.2.2.2 Implantation rate

(Figure 26) shows that the implantation rate was reported by Madkour (2016) and Yu (2016). The meta-analysis showed a probable improvement in favour

of PBMC treatment (RR 2.21, 95% CI 1.27–3.83; embryos transferred = 519; studies = 2; $I^2$ = 0% moderate certainty evidence).

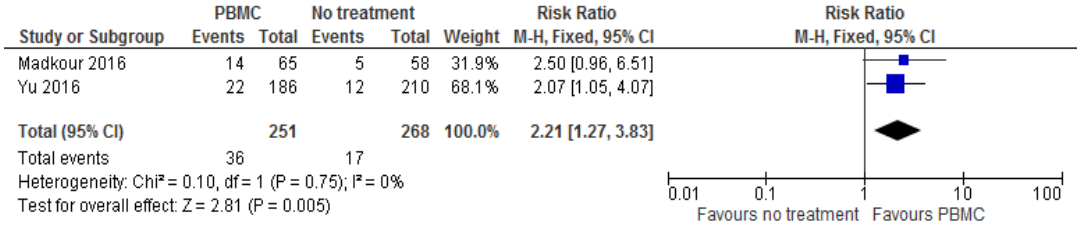



**Figure 26: The effect of PBMC on implantation rate**

### 3.8.3 Heparin vs. no treatment

| Heparin treatment compared to no treatment for women with a history of unsuccessful implantation undergoing IVF/ICSI treatment | | | | | | |
|---|---|---|---|---|---|---|
| Patient or population: Women with a history of unsuccessful implantation undergoing IVF/ICSI treatment<br>Setting: IVF clinic<br>Intervention: Heparin<br>Comparison: No treatment | | | | | | |
| Outcomes | Anticipated absolute effects* (95% CI) | | Relative effect (95% CI) | № of participants (studies) | Certainty of the evidence (GRADE) | Comments |
| | Risk with no treatment | Risk with Heparin | | | | |
| Live birth rate | 267 per 1,000 | 347 per 1,000 (213 to 565) | RR 1.30 (0.80 to 2.12) | 150 (1 RCT) | ⨁⨁⊖⊖ LOW [1] | |
| Miscarriage rate | Not reported by any study | | | | | |
| Clinical pregnancy rate | 387 per 1,000 | 452 per 1,000 (309 to 661) | RR 1.17 (0.80 to 1.71) | 150 (1 RCT) | ⨁⨁⊖⊖ LOW [1] | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Adverse events:<br><br>Pre-term delivery | 80 per 1,000 | 120 per 1,000 (45 to 321) | RR 1.50 (0.56 to 4.01) | 150 (1 RCT) | ⨁⨁⊖⊖ LOW [1] | |
| Adverse events:<br><br>Multiple pregnancy rate | 345 per 1,000 | 352 per 1,000 (179 to 693) | RR 1.02 (0.52 to 2.01) | 63 (1 RCT) | ⨁⨁⊖⊖ LOW [1] | |
| Adverse events:<br><br>Drug side effects | Not reported by any study | | | | | |

**Table 11**: **Summary of findings for heparin treatment compared to no treatment**

### *3.8.3.1 Primary outcomes*

#### 3.8.3.1.1 Live birth rate

(Figure 27) shows that the live birth rate was only reported by Urman (2009). The analysis showed insufficient evidence between the study groups (RR 1.30, 95% CI 0.80–2.12; participants = 150; studies = 1; $I^2$ = 0 low certainty evidence).

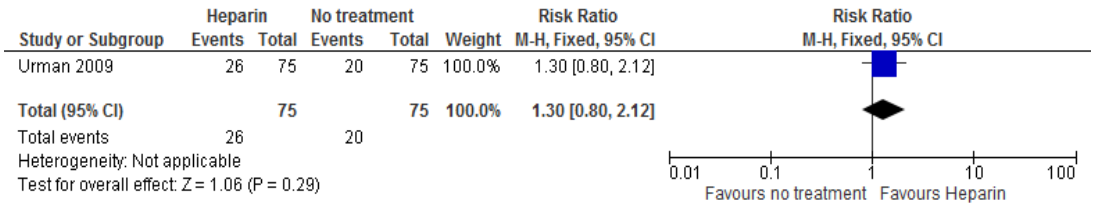



**Figure 27: The effect of heparin on live birth rate**

### *3.8.3.2 Secondary outcomes*

#### 3.8.3.2.1 Ongoing pregnancy rate

(Figure 28) shows that the ongoing pregnancy rate was reported only by Urman (2009). These percentages were converted to event numbers and the RR was calculated. Interpretation of the analysis showed insufficient evidence between the study groups for ongoing pregnancy rate (RR 1.40, 95% CI 0.87–2.25; participants = 150; studies = 1, low certainty evidence).

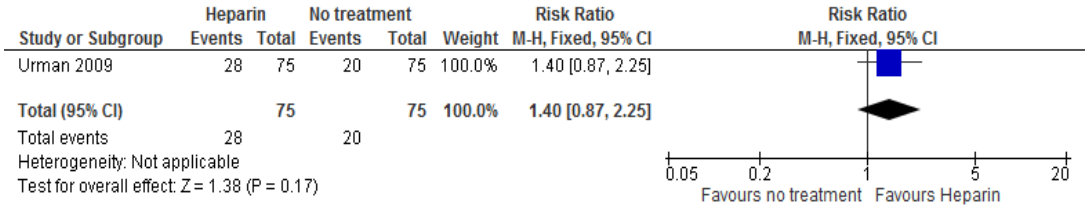



**Figure 28: The effect of heparin on ongoing pregnancy rate**

#### 3.8.3.2.2 Clinical pregnancy rate

(Figure 29) shows that the clinical pregnancy rate was only reported by Urman (2009). The meta-analysis showed insufficient evidence between the study groups for the effect of heparin on the clinical pregnancy rate (RR 1.17, 95% CI 0.80–1.71; participants = 150; studies = 1, moderate certainty evidence).

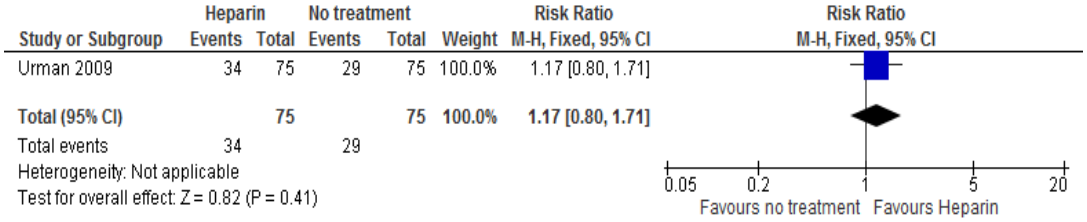



**Figure 29: The effect of heparin on clinical pregnancy rate**

#### 3.8.3.2.3 Implantation rate

The implantation rate was reported in two studies: Hamdi (2015) and Urman (2009). Hamdi (2015) stated that the implantation rate would be reported, but no figures were given in the results section. Therefore, (Figure 30) shows the

meta-analysis was performed with Urman's (2009) data by converting the percentages to event numbers. The analysis showed insufficient evidence between the study groups for the implantation rate with heparin (RR 1.27, 95% CI 0.87–1.86; embryos transferred = 390; studies = 1; $I^2$ = 0%, low certainty evidence).

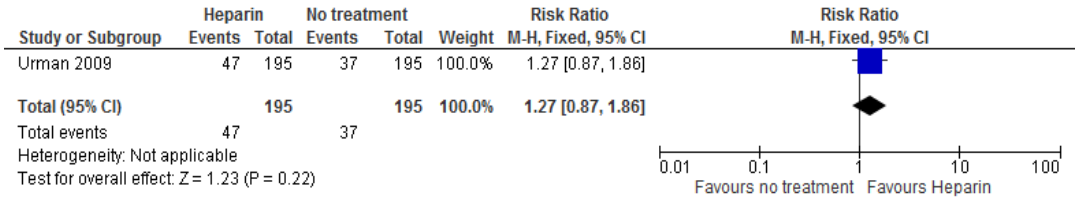



**Figure 30: The effect of heparin on implantation rate**

### 3.8.3.2.4 Ectopic pregnancy rate

(Figure 31) shows that ectopic pregnancy was only reported by Urman (2009). The meta-analysis showed an increase in the ectopic pregnancy rate in those who received heparin (RR 1.50, 95% CI 0.56–4.01; participants = 150; studies = 1, low certainty evidence).

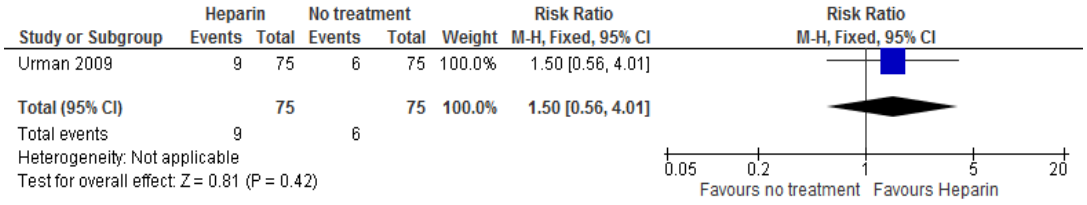



**Figure 31: The effect of heparin on ectopic pregnancy rate**

### 3.8.3.2.5 Multiple pregnancy rate

(Figure 32) shows that the multiple pregnancy rate was only reported by Urman (2009). The analysis showed insufficient evidence between heparin and no treatment groups (RR 1.02, 95% CI 0.52–2.01; participants = 63; studies = 1, low certainty evidence).

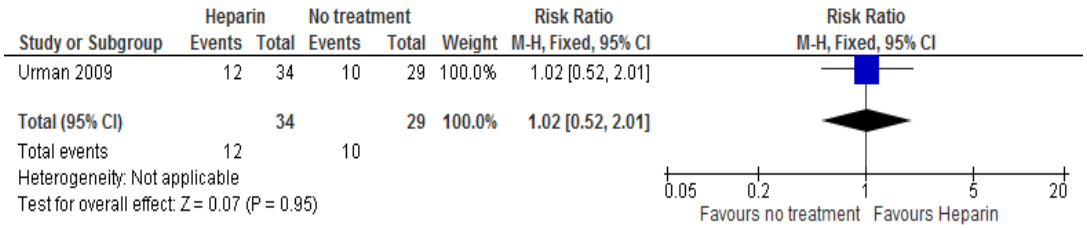



**Figure 32: The effect of heparin on multiple pregnancy rate**

### 3.8.4 **Heparin vs. aspirin**

| Treatment with heparin compared to that with aspirin for women with a history of unsuccessful implantation undergoing IVF/ICSI treatment | | | | | | |
|---|---|---|---|---|---|---|
| Patient or population: Women with a history of unsuccessful implantation undergoing IVF/ICSI treatment<br>Setting: IVF clinic<br>Intervention: Heparin<br>Comparison: Aspirin | | | | | | |
| Outcomes | Anticipated absolute effects* (95% CI) | | Relative effect (95% CI) | № of participants (studies) | Certainty of the evidence (GRADE) | Comments |
| | Risk with Aspirin | Risk with Heparin | | | | |
| Live Birth | 121 per 1,000 | 309 per 1,000 (141 to 673) | RR 2.56 (1.17 to 5.58) | 126 (1 RCT) | ⨁⨁⊖⊖ LOW [1] | |
| Miscarriage rate | Not reported by any study | | | | | |
| Clinical pregnancy rate | Not reported by any study | | | | | |
| Adverse events: | Not reported by any study | | | | | |

| | |
|---|---|
| Pre-term delivery rate<br><br>Multiple pregnancy rate<br><br>Drug side effects | |

**Table 12: Summary of findings for heparin treatment compared to aspirin treatment**

### *3.8.4.1 Primary outcomes*

#### 3.8.4.1.1 Live birth rate

(Figure 33) shows that the live birth rate was only reported by Pergolini (2013). Although slight improvement was seen in the heparin group, the evidence was graded as low certainty (RR 2.56, 95% CI 1.17–5.58; participants = 126; studies = 1; $I^2$ = 0, low certainty evidence).

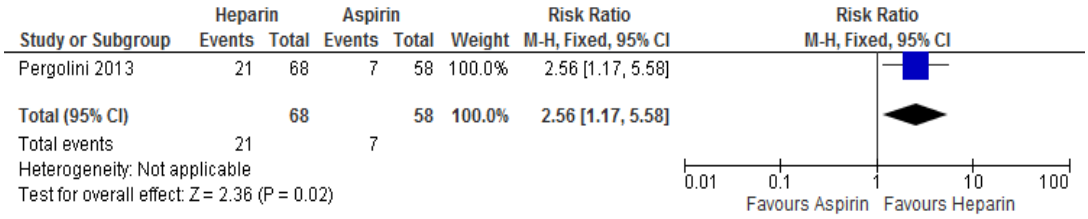



**Figure 33: The effect of heparin vs. aspirin on live birth rate**

## 3.8.5 G-CSF vs. no treatment

| |
|---|
| G-CSF therapy compared to no treatment for women with a history of unsuccessful implantation undergoing IVF/ICSI treatment |
| Patient or population: Women with a history of unsuccessful implantation undergoing IVF/ICSI treatment<br>Setting: Fertility/IVF clinic<br>Intervention: IU or SC G-CSF<br>Comparison: No treatment |

| Outcomes | Anticipated absolute effects* (95% CI) | | Relative effect (95% CI) | № of participants (studies) | Certainty of the evidence (GRADE) | Comments |
|---|---|---|---|---|---|---|
| | Risk with no treatment | Risk with GCSF | | | | |
| Live birth rate | Not reported by any study | | | | | |
| Miscarriage rate | Not reported by any study | | | | | |
| Clinical pregnancy rate | 197 per 1,000 | 366 per 1,000 (261 to 509) | RR 1.86 (1.33 to 2.59) | 363 (5 RCTs) | ⊕⊕⊕⊖ MODERATE [1] | |
| Adverse events:<br><br>Pre term delivery rate<br><br>Multiple pregnancy rate<br><br>Drug side effects | Not reported by any study | | | | | |

**Table 13: Summary of findings for G-CSF compared to no treatment**

### *3.8.5.1 Secondary outcomes*

#### 3.8.5.1.1 Clinical pregnancy rate

(Figure 34) shows a combined meta-analysis (SC and IU G-CSF) of five studies: Aleyasin (2016), Cambiaghi (2012), Davari-Tanha (2016), Miraj (2015), and Obidniak (2016). The analysis showed that G-CSF probably improved the clinical pregnancy rate compared to no treatment (RR 1.86, 95% CI 1.33–2.59; participants = 363; studies = 5; $I^2$ = 26%, moderate certainty evidence).

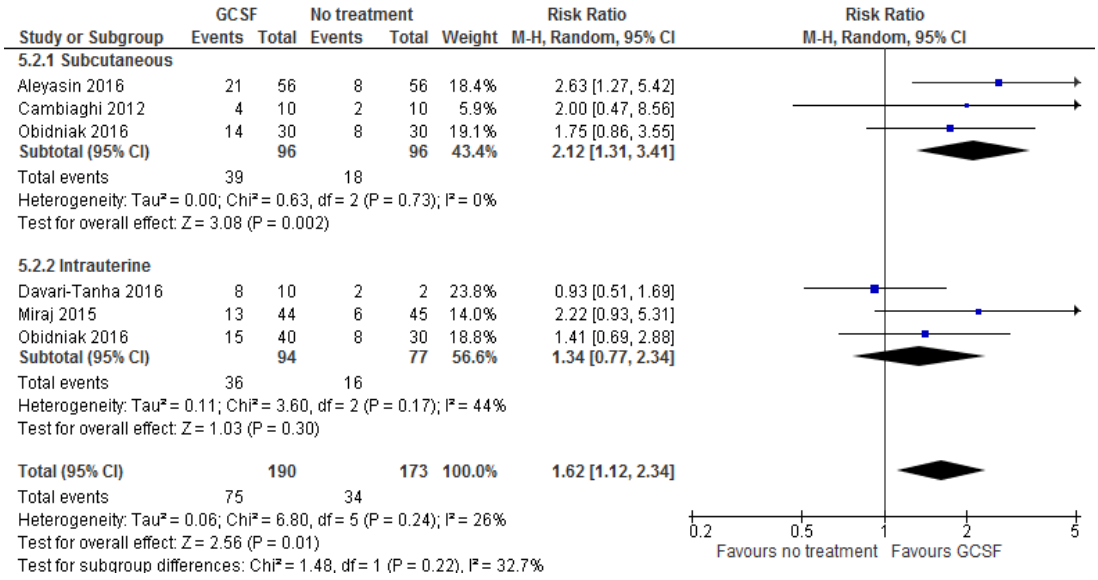



**Figure 34: The effect of G-CSF on clinical pregnancy rate**

### 3.8.5.1.2 Implantation rate

(Figure 35) shows a combined meta-analysis (SC and IU G-CSF) of three studies: Aleyasin (2016), Davari-Tanha (2016), and Obidniak (2016). Interpretation of analysis showed insufficient evidence between study groups (RR 2.05, 95% CI 1.38–3.03; total embryos transferred = 588; studies=3; $I^2$ = 0%, moderate certainty evidence).

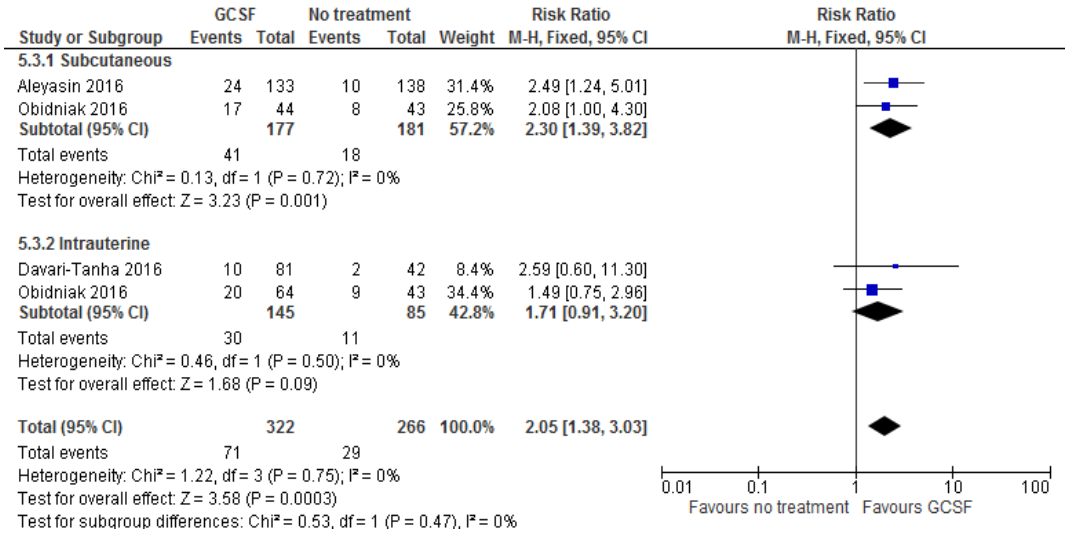



**Figure 35: The effect of G-CSF on implantation rate.**

#### 3.8.5.1.3 Ectopic pregnancy rate

(Figure 36) shows that the ectopic pregnancy rate was reported only by Aleyasin (2016). This study used SC G-SCF with 2 participants who had an ectopic pregnancy in the G-CSF group versus 1 participant in the control group. The meta-analysis showed insufficient evidence between the studied groups with a confidence interval that was very wide (RR 2.00, 95% CI 0.19–21.43; participants = 112; studies = 1, very low certainty evidence).

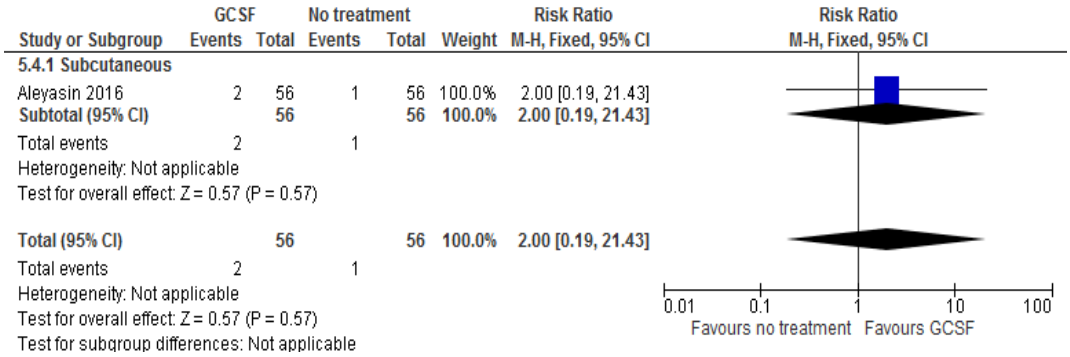



**Figure 36: The effect of G-CSF on ectopic pregnancy rate.**

### 3.8.6 **LIF vs. placebo**

| LIF compared to placebo for women with a history of unsuccessful implantation undergoing IVF/ICSI treatment | | | | | |
|---|---|---|---|---|---|
| Patient or population: Women with a history of unsuccessful implantation undergoing IVF/ICSI treatment<br>Setting: IVF clinic<br>Intervention: LIF<br>Comparison: Placebo | | | | | |
| Outcomes | Anticipated absolute effects* (95% CI) | Relative effect (95% CI) | № of participants (studies) | Certainty of the | Comments |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Risk with placebo | Risk with LIF | | | evidence (GRADE) | |
| Live birth rate | 209 per 1,000 | 162 per 1,000 (82 to 290) | OR 0.73 (0.34 to 1.55) | 208 (2 RCTs) | ⊕⊖⊖⊖ VERY LOW [1] [2] | |
| Miscarriage rate | 64 per 1,000 | 39 per 1,000 (9 to 169) | RR 0.61 (0.14 to 2.64) | 149 (1 RCT) | ⊕⊕⊖⊖ LOW [3] | |
| Clinical pregnancy rate | 299 per 1,000 | 233 per 1,000 (90 to 615) | RR 0.78 (0.30 to 2.06) | 208 (2 RCTs) | ⊕⊖⊖⊖ VERY LOW [1] [2] | |
| Adverse events<br><br>Preterm delivery rate<br><br>Multiple pregnancy rate<br><br>Drug side effects | Not reported by any study | | | | | |
| *The risk in the intervention group (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).<br>CI: Confidence interval; RR: Risk ratio; OR: Odds ratio; | | | | | | |

**Table 14: Summary of findings for LIF compared to placebo**

### *3.8.6.1 Primary outcomes*

#### 3.8.6.1.1 Live birth rate

(Figure 37) shows that live birth rate was reported by Brinsden et al. (2003; 2009). The analysis showed insufficient evidence to determine the difference between the studied groups (RR 1.68, 95% CI 0.06–48.49; participants = 208; studies = 2; $I^2$ = 82%, very low certainty evidence).

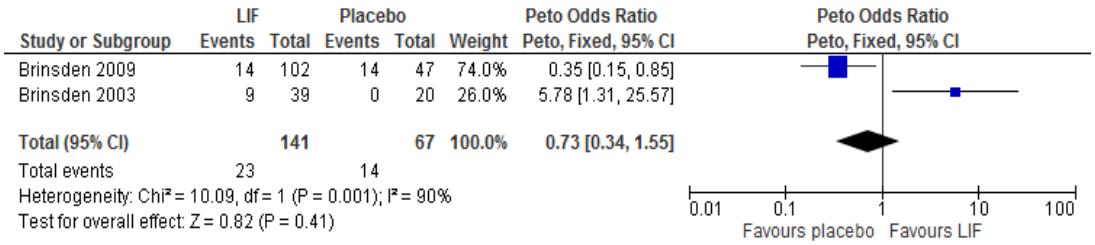



**Figure 37: The effect of LIF on live birth rate**

#### 3.8.6.1.2 Miscarriage rate

(Figure 38) shows that miscarriage rate was reported only by Brinsden et al. (2009). The results of the analysis showed insufficient evidence between the LIF and placebo treatment as the confidence interval crossed the line of no effect (RR 0.61, 95% CI 0.14–2.64; participants = 149; studies = 1) low quality evidence).

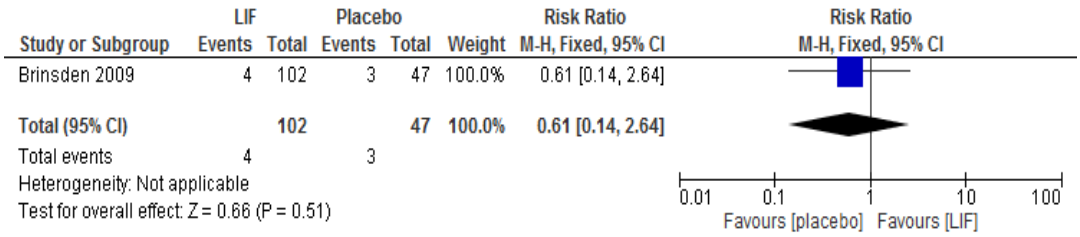



**Figure 38: The effect of LIF on miscarriage rate**

### *3.8.6.2 Secondary outcomes*

#### 3.8.6.2.1 Clinical pregnancy rate

(Figure 39) shows that the clinical pregnancy rate was reported by Brinsden et al. (2003; 2009). The analysis showed insufficient evidence of differences between the study groups (RR 0.78, 95% CI 0.30–2.06; participants = 208; studies = 2; $I^2$ = 65%, very low certainty evidence).

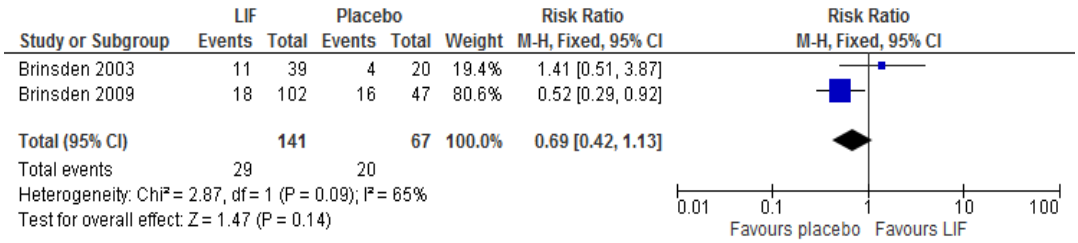



**Figure 39: The effect of LIF on clinical pregnancy rate**

# 4 Discussion

The rationale behind using immunotherapy is to treat ‘abnormal' immune parameters (Seshadri and Sunkara, 2013). Recent studies do not support the use of natural killer cells measurement as a diagnostic test in taking a decision to start immunotherapy in women with adverse reproductive outcomes. However, immunotherapy is still being offered to couples with RIF in the absence of concrete evidence. This literature review is the first attempt to answer a highly important question in reproductive medicine that both clinicians and patients find perplexing; which immunotherapy is beneficial for women with a history of recurrent unexplained implantation failure?

There is concern in the research and clinical fields that due to the lack of strong evidence, IVF clinics may be exploiting patients for profit by offering immune therapies, even when not indicated (Moffett and Shreeve, 2015, Hviid and Macklon, 2017). Many experts have debated that IVF clinics may be offering these interventions to make profits from a desperate population (Templer and Sacks, 2016). Clinics are charging a high fee for these interventions adding an ethical dimension to the whole debate (Datta et al., 2015).

## 4.1 Summary of main results

The main objective of this systematic review was to investigate whether the administration of commonly used immunotherapies affected the live birth rate outcomes for women with unexplained RIF undergoing IVF or ICSI treatment when compared to control or placebo groups. Overall, there is no clear or overwhelming evidence that administration of most of the immunotherapies improves live birth, miscarriage rates, or clinical pregnancy. However, our review reflects that the use of intralipid, G-CSF PBMC, and to some extent heparin as treatments for unexplained RIF may help in improving the outcomes. These results should only be applied to the research setting and further work is needed before offering these treatments in the clinical context.

Furthermore, studies reported very few side effects and clinical risks for these interventions.

At first, the review took the pragmatic approach in the review protocol to include as least two unsuccessful IVF cycles as a definition of RIF, relying on the definition of Polanski et al. (Polanski et al., 2014). Despite the broad search for studies, 19 studies were included, covering most immunotherapies used; however, 11 out of the 19 studies were in conference abstract format. This resulted in limitations with small sample numbers and high heterogeneity. Also, the studies reported different outcomes ranging from chemical pregnancy rate (that showed definition variation between studies) to live birth. The dosage, administration, and duration of treatment also varied across studies. The majority of the studies reported very few side effects and clinical or teratogenic risks for these interventions.

Ultimately the meta-analysis was limited due to the high heterogeneity across studies with some comparisons only using one or two studies leading to questionable interpretations.

Two studies used only intralipid treatment and both showed that the intervention may improve the live birth rate (Gamaleldin 2016; El-Khayat 2015). However, the quality of evidence was shown to be low to moderate and the findings need to be interpreted with caution as both were conference abstracts with a small study population. From a clinical perspective, intralipid has been shown to be harmful for maternal/foetal well-being (Shreeve and Sadek, 2012) and is not fully licensed for use in the IVF setting (Allahbadia, 2015); therefore, intralipids should be used with caution.

PBMC studies showed heterogeneity in their methodology. Yu (2016) used PBMC after it was co-cultured with HCG for 24 hours before intrauterine administration, while Makrigiannakis (2011) cultured the PBMC for 48 hours with CRH and Madkour (2016) incubated the PBMC for 72 hours. This heterogeneity could not allow us to perform a meta-analysis for live birth, but

showed an overall probability in improving implantation and clinical pregnancy rates with moderate-quality evidence.

The use of G-CSF was reported in eight studies, showing high heterogeneity with various drug regimens and administrative routes. Davari-Tanha (2016) used the passage of a cook catheter and administration of IU saline as separate control groups. Although this was a robust method of blinding to reduce any operator bias, this complicated the meta-analysis. Also, Obidniak (2016) compared both IU and SC G-CSF, and this made the analysis highly heterogeneous.

Interestingly, although G-CSF was the most frequently used intervention, none of the studies reported live birth. Both SC and IU G-CSF were found to improve both clinical pregnancy and implantation rates. However, these findings were uncertain as they were extracted from only one study and were deemed to be very low-quality evidence.

The evidence for the use of the LIF was the least robust. Both included studies had the same study group and showed high heterogeneity ($I^2$ statistic = 82%). This caused the quality of evidence to be downgraded and be classified as low. The LIF findings of those studies cannot be generalised to all women with RIF.

One of the most significant findings of this review was that no included study used steroids as a treatment for RIF, despite the presence of two large Cochrane systematic reviews on the use of steroids in women undergoing routine IVF with the inclusion of 14 studies (Boomsma and Macklon, 2008, Boomsma et al., 2012). However, none of these studies were relevant or met our criteria.

## 4.2 Quality of the evidence

Most of the trials (13 studies) did not show the means of blinding and were at high risk of performance and detection bias. The main limitations of the

evidence were poor reporting of study methods and there was a lack of studies investigating the desired outcomes. The evidence for live birth rate was low-to-moderate quality for both intralipid and heparin studies. The evidence for the miscarriage rate was low-to-very low quality and this was influenced by the lack of reporting for this outcome across all trials.

While most outcomes were low-quality evidence, moderate evidence was found for clinical pregnancy and implantation rate in the intralipid, PBMC, and heparin studies. Most of the studies had to be downgraded due to wide confidence intervals and insufficient evidence.

# 5 Conclusion

## 5.1 Implications for practice

Although this review has no definitive conclusion, it highlighted some evidence that intralipid, PBMC, heparin, and G-CSF may be beneficial in some RIF patients undergoing IVF/ICSI. However, these findings have to be interpreted with extreme caution. Scientific and clinical communities have to work together to understand the basic principles involved in the maternal immune system in implantation. Once the physiology is completely understood, then it will be easier to find a clearer definition of the pathological basis of RIF and prescribe the most effective immune therapy.

## 5.2 Implications for research

This review highlighted the heterogeneity between studies despite the intervention used. We found no studies on steroids despite being a very commonly used intervention. This review reflects the urgent need to design and perform adequately powered RCTs, possibly multi-centred, using standardised criteria for defining unexplained RIF to establish a strong evidence base for clinical guidance.

Also it is so important to return to the basic science in order to understand the nature of these treatments, their components and their exact mode of action on the maternal immune system in the IVF setting, this can be applied on different animal models. As the role of Intralipids remains the most unclear and vague (immune-modulator/ nutrient precursor for blastocyst) and there is no direct link between Intralipid and potassium channels. Intralipid was chosen be taken into further molecular research to understand its effect on the role and regulation of potassium channels within human endometrium during window of implantation, focusing on their phospholipids and fatty acids composition that are proved to have a link with potassium channels and may affect the

uterine phospholipids; hence it may affect the biophysical properties of the uterus and the cross-talk between endometrium and blastocyst.

# Chapter Four: Transepithelial Electrical Resistance (TEER) in Cultured Endometrial Monolayer

# 1 Introduction

Intrauterine embryo cross-talk involves many events including embryo recognition by the endometrium, smooth uterine peristaltic movements, reabsorption of the intrauterine fluid, closure of the intrauterine cavity, and orientation of the embryo. Also, many molecular incidences and factors influence this cross-talk, such as lipids, adrenergic and hormone signalling, development of genes, ion and water channels, and embryonic signalling, and play a role in intrauterine embryo distribution (Chen et al., 2013). Given the presence of all these important factors in reproduction, the endometrium has been a key subject of intense research for its important role in women's health (Chen et al., 2016).

In relation to female fertility and embryo implantation, the molecular interactions are considered to be complex processes and play an important role in implantation. These interactions include paracrine, juxta-crine, and autocrine cell-cell and cell-matrix interactions (Dey et al., 2004). In the ovine endometrium, the junctional complexes regulate paracellular trafficking of water, ions, and other molecules (Satterfield et al., 2007). These interactions remains unclear and poorly understood as most of the current information and evidences are based on research involving animals, mainly mouse models, and these models reflect human implantation to a limited extent (Teklenburg and Macklon, 2009, Salker et al., 2010).

The cell layers in the endometrium create a formidable barrier between the upper apical and lower basolateral compartments, proven by the significant transepithelial electrical resistance generated (Matthews et al., 1993). These tight junctions change their architecture and configurations during different physiological and pathological conditions (Winterhager and Kühnel, 1982). Tight junctions are formed and found mainly between epithelial cells of the endometrium, and throughout the menstrual cycle, the epithelial and stromal cell populations change, where in the early secretory phase, they account for 35% and 65% of the cells, respectively, while during mid-secretory phase,

these figures change to 54% and 46%, respectively (Suhorutshenko et al., 2018). Even within the in vitro cell culture, the epithelial cell population, configurations, and regulation change as part of the stromal/epithelial interaction (Arnold et al., 2001).

Transepithelial electrical resistance (TEER) measurement is an appreciated technique used to sensitively quantify the integrity of cell barriers during their growth and differentiation. TEER is a cost-effective tool with high-throughput; it is used to predict the permeability of the endometrium to different drugs (Srinivasan et al., 2015). The technique can also be applied to monitor the movement of ions through different channels within the cell monolayer using Transwell® (Ghaffarian and Muro, 2013). This method has been applied to human and porcine endometrium to show the contribution of certain ions to the cell junction integrity (Chen et al., 2015, Chen et al., 2016, Pemathilaka, 2016).

Given the high IVF success rate for women receiving add-on treatments, in this chapter, we will conduct a physiological study to test and understand the nature of the endometrial cells and how their potassium channels adapt to various treatments (5% oxygen concentration, vitamin D, and intralipids). Also, two specific potassium-channel blockers for the KCNK9 and KCNMA1 channels were examined, as the expression of these channel subtypes is known to be altered between the infertile and fertile groups and also with respect to the secretory phase.

Potassium channels are expressed in endometrium, but their function in this tissue is not understood. Transepithelial electrical resistance (TEER) study was done to define the ability of these endometrial monolayers in culture to regulate and control the potassium flux and hence understand the role of endometrium.

# 2 Materials and Methods

## 2.1 Validation of cell density used for seeding

To achieve a confluent monolayer of cells, it was important to validate the cell density required in the 12-mm Transwell plate to reach the optimum number of cells forming the confluent monolayer. (Figure 40A) shows an over seeded plate that formed clumps of cells within the monolayer of cells, compared to (Figure 40B) with the optimum cell density used ($7.5 \times 10^4$/well) to produce a confluent monolayer of cells.

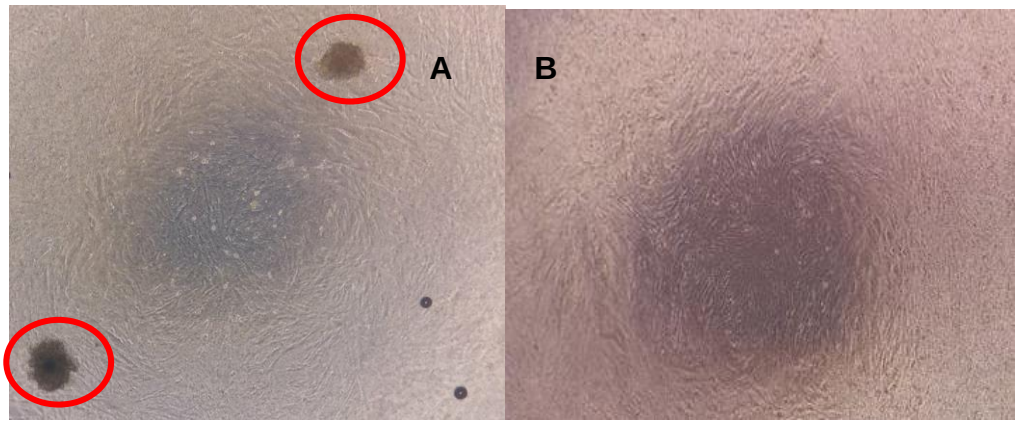



**Figure 40: Transwell monolayer cell culture under different cell densities; magnification of ×10**

## 2.2 Validation of treatments dosage

### 2.2.1 PK-THPP (KCNK9 blocker)

(Figure 41) shows the validation done for the concentration of KCNK9 blocker (PK-THPP) to be used in these experiments. Three concentrations were tested (1 µM, 35 µM, and 350 µM), where every sample was tested in triplicates. One-way ANOVA was done at every time point. The concentration to be used was decided on the basis of statistical analysis of the cell culture day 6 +2 hrs (2 h after adding of treatment) and on cell culture day 7 (next day of adding of treatment). Statistical analysis showed a statistical significant difference (P <

0.005) between the control and 35 µM dose and significant difference (P< 0.001) between the control and the 350 µM dose. Since there was no significant difference between the 35 µM and 350 µM doses, the PK-THPP dose of 35 µM was the concentration of choice.

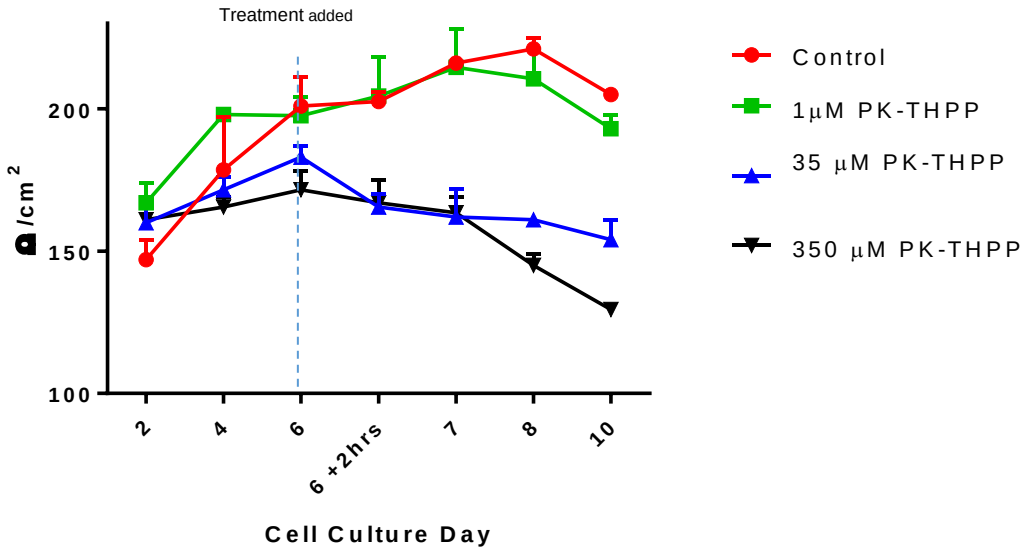



**Figure 41: Validation of the PK-THPP (KCNK9 blocker)**

### 2.2.2 **Paxilline (KCNMA1 blocker)**

(Figure 42) shows the validation done for the KCNMA1 blocker (paxilline) dosage. Two concentrations were used (1 µM and 10 µM) on two endometrial samples, where every sample was tested in triplicates. One-way ANOVA was done at every time point. The concentration to be used was decided on the basis of statistical analysis of the cell culture at day 6 +2 hrs (2 h after adding of treatment) and on cell culture day 7 (next day of adding of treatment). Statistical analysis showed significant difference ($P < 0.001$) between the control and 1 µM dose and significant difference ($P < 0.005$) between the control and the 10 µM dose. Since there was no significant difference between the 1 µM and 10 µM doses, the PK-THPP dose of 1 µM was the concentration of choice.

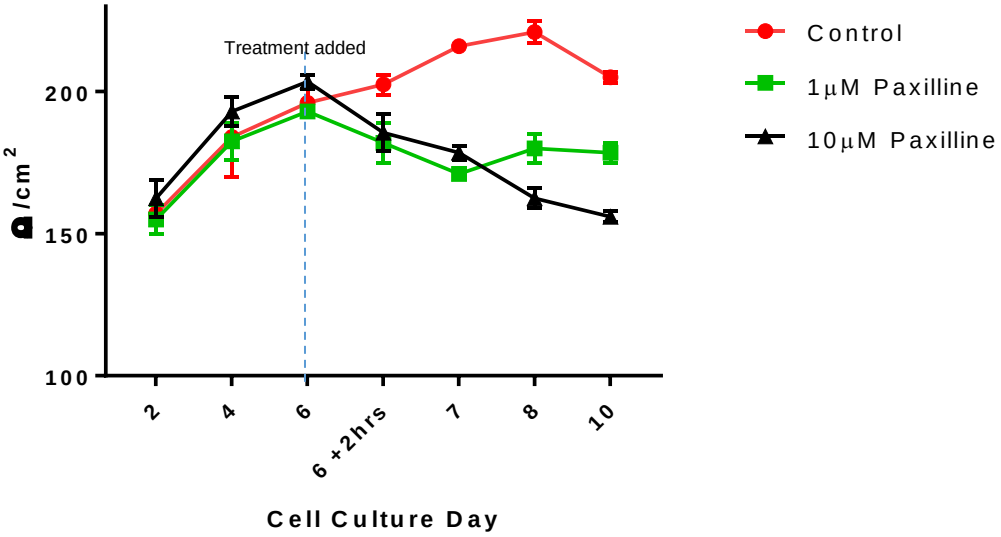



**Figure 42: Validation of the paxilline (KCNMA1 blocker) dosage**

# 3 Results

## Effect of treatments on TEER

(Figure 43-47) shows the effect of each treatment separately on endometrial cell TEER: No treatment control (n = 8), 5% $O_2$ (n = 6), KCNK9 blocker (n = 6), KCNMA1 blocker (n = 6), intralipids (n = 4), and vitamin D (n = 4). Two-way ANOVA was conducted with all treatments in relation to the control to show statistical significant difference for:

1) All time points of cell culture (*)
2) Interaction between the two curves (+)

The effect of KCNK9 blocker on TEER (Figure 43), the TEER value dropped two hours after the addition of the channel blocker and continued falling gradually from day 6 until day 10. The effect of the KCNMA1 blocker (Figure 44) shows an acute drop in TEER immediately after addition of the blocker, where the maximum slope drop is observed in the first 2 hours that continues until day 10. The effect of 5% oxygen concentration (Figure 45) on TEER is found to plateau from the time of incubating the cells in 5% oxygen until day 10, with no decrease in TEER. Intralipids and vitamin D (Figure 46-47) have no effect on TEER, since the values recorded are similar to the TEER values for the control.

(Figure 48) shows a descriptive summary of the effect of different treatments on TEER of endometrial cells in relation to each other.

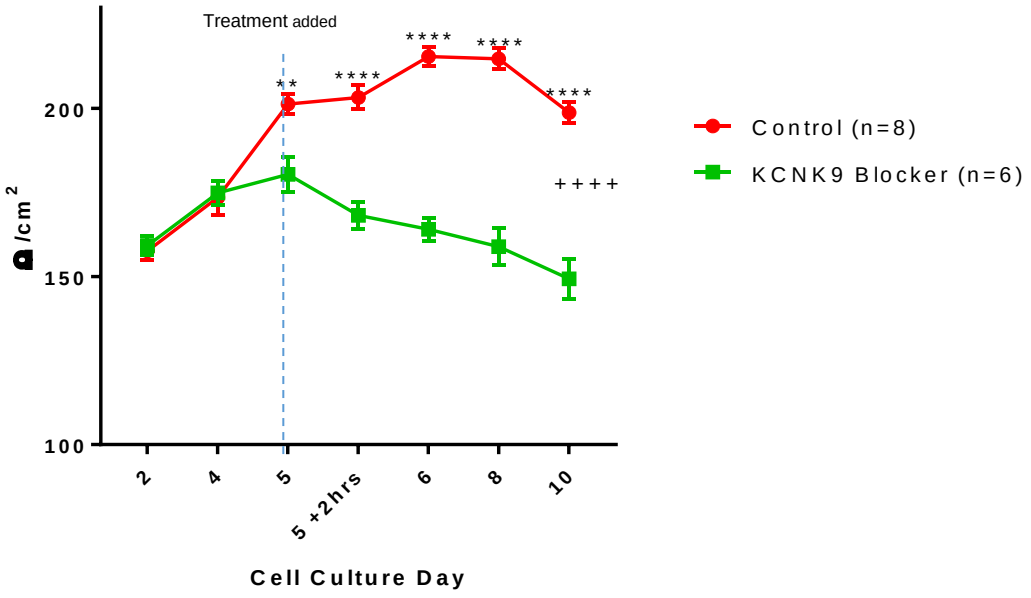



**Figure 43: The effect of KCNK9 blocker on TEER of endometrial cells** Two-way ANOVA was conducted to show statistically significant difference for: All time points of cell culture (*) and Interaction between the two curves (+)

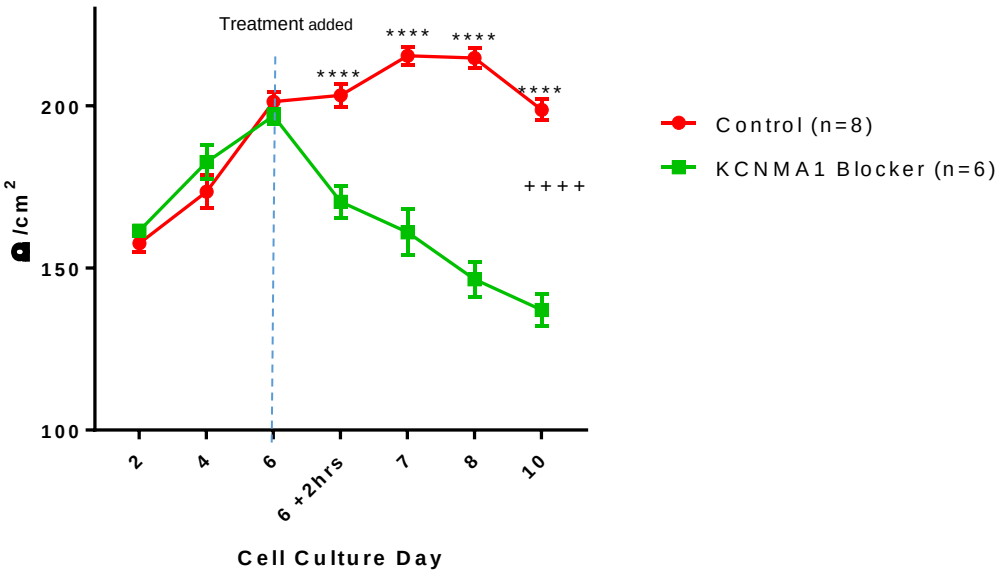



**Figure 44: The effect of KCNMA1 blocker on TEER of endometrial cells** Two-way ANOVA was conducted to show statistically significant difference for: All time points of cell culture (*) and Interaction between the two curves (+)

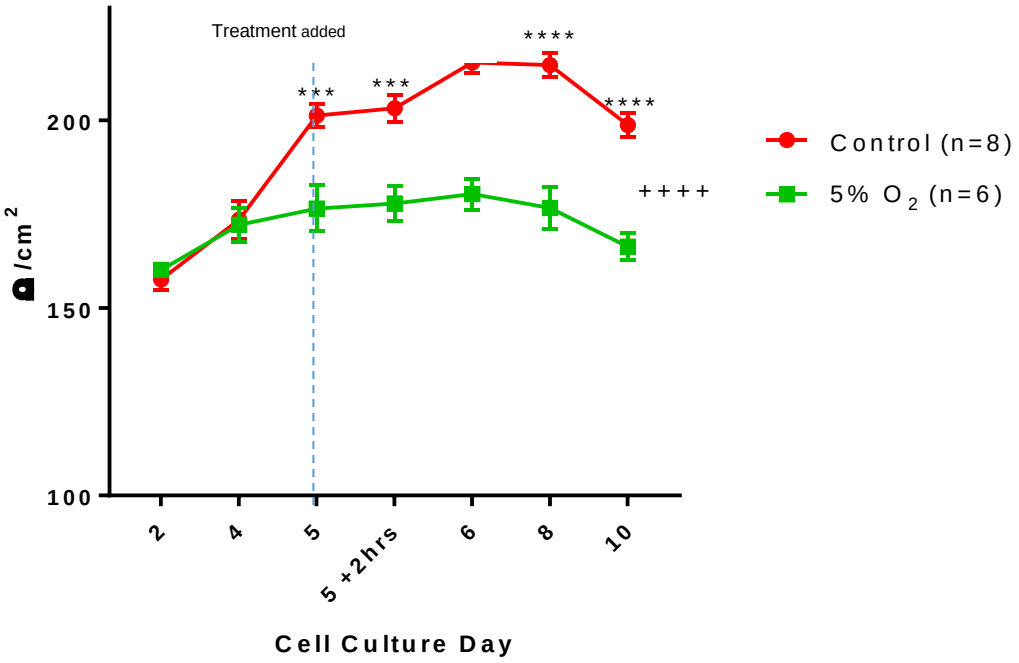



**Figure 45: The effect of 5% oxygen concentration on TEER of endometrial cells** Two-way ANOVA was conducted to show statistically significant difference for: All time points of cell culture (*) and Interaction between the two curves ($^{+}$)

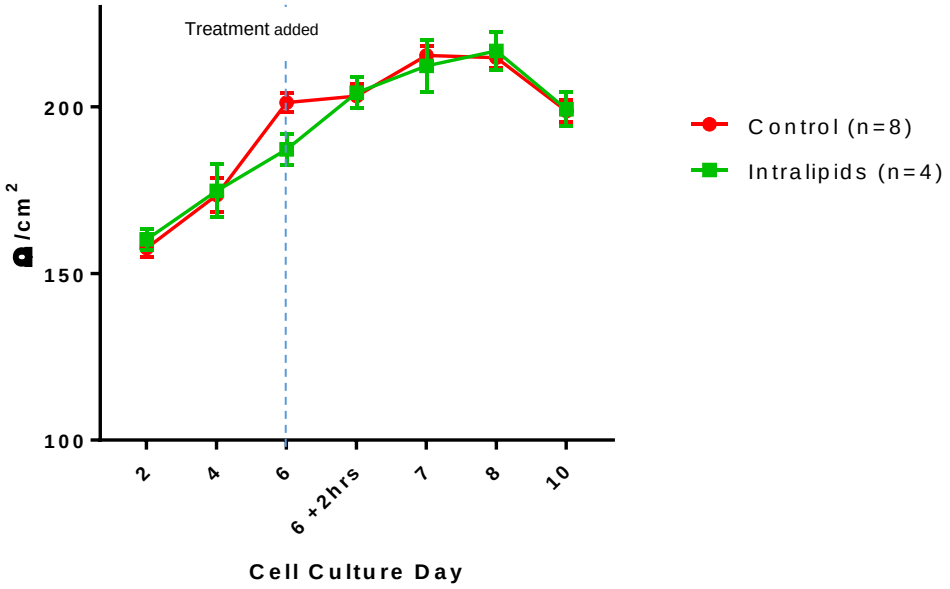



**Figure 46: The effect of intralipids on TEER of endometrial cells** Two-way ANOVA was conducted to show statistically significant difference for: All time points of cell culture (*) and Interaction between the two curves (+)

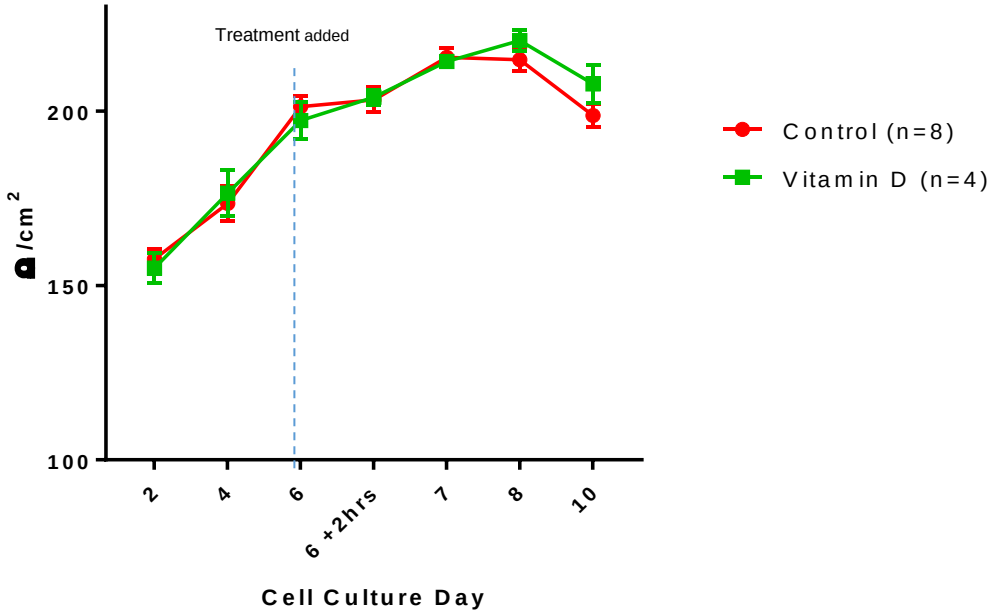



**Figure 47: The effect of vitamin D on TEER of endometrial cells** Two-way ANOVA was conducted to show statistically significant difference for: All time points of cell culture (*) and Interaction between the two curves (+)

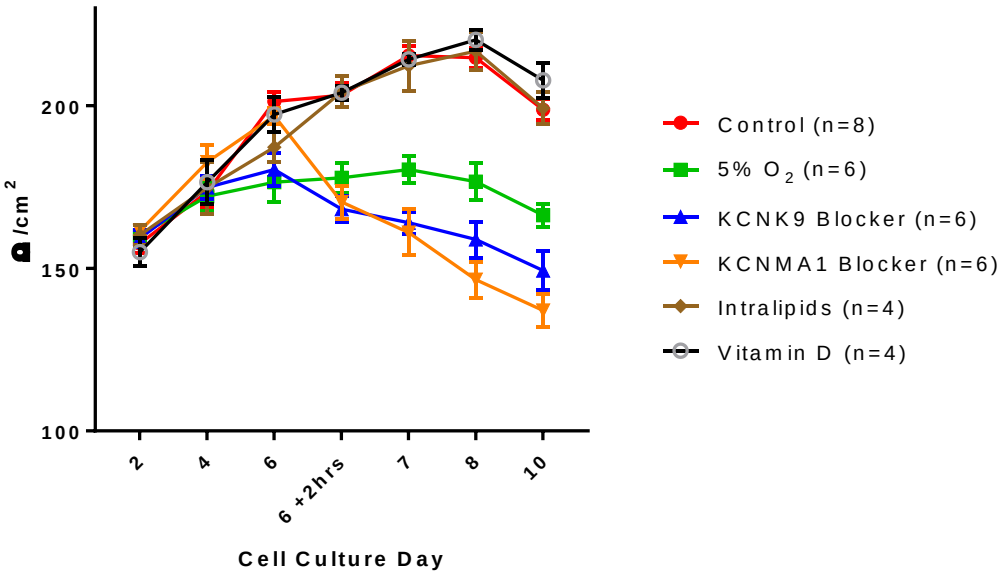



**Figure 48: Descriptive summary of the effect of different treatments on TEER of endometrial cells**

# 4 Discussion

## 4.1 TEER values and cell ratio

Before discussing the effect of treatments on the TEER of endometrial cells, it is important to focus on the TEER results obtained in relation to the results found in other studies and to the results for different cell populations.

TEER is a measurement that is used to evaluate the function of the epithelial cell barrier in the porous permeable membrane. Electrical current resistance is measured by passing continuous current through the cells on both transcellular and paracellular paths (Powell, 1981). The paracellular resistance is a result of the influence of specific tight junction proteins on the cell-cell epithelial resistance. Hence, the quantified TEER results mirror the physical structures, properties (Chen et al., 2015), and the integrity of cell junction dynamics of the cell monolayer (Srinivasan et al., 2015).

The main aim of our study, as mentioned previously, was to mimic the in vivo structure of the endometrium without focusing on the particular role of a specific cell type. Therefore, the cell culture monolayer formed consisted of different endometrial cell populations and we wanted to ensure that both stromal and epithelial cells were present after the end of the passage one of the cell cultures. It is known that the presence of stromal cells affects the integrity of tight junctions within glandular epithelial cells, thus decreasing the TEER value.

Endometrial stromal cells play a key role in the embryo implantation process, controlling the influx of various materials and functions of different immune cells (Gellersen and Brosens, 2014); they are also important when the implanting embryo starts invading the surrounded decidual tight junctions formed between the decidualising cells (Peter Durairaj et al., 2017).

We wanted to retain the stromal cells in the endometrial TEER experiments despite their negative effect on the TEER results, as the presence of the

glandular-stromal complex is important during implantation, since it not only interacts with the embryo directly but also creates a communication mechanism between both cell populations (Teklenburg and Macklon, 2009). It is hypothesised that abnormal cyclic differentiation of endometrial stromal cells into specialized decidual cells is not indispensable for placenta formation in pregnancy but it affects embryo recognition and selection during the implantation period (Salker et al., 2010).

Primary glandular epithelial cells, compared with stromal cells, have limited expansion potential, restricting the size and flexibility of experimental designs using this cell type (Chen et al., 2016). We decided to carry out the TEER experiment after end of passage one to avoid prolong passaging that can lead to loss of epithelial cells. Chan et al. showed that clusters of mature epithelial cells within cell culture that assumed a basic polyhedral shape became apoptotic by day 8–10 in culture and lifted off the plate surface (Chan et al., 2004). This can be confirmed by the findings of Arnold et al., showing the domination of stromal cell population throughout endometrial cell culture (Arnold et al., 2001).

We calculated the resistivity (resistance normalized to area; $\Omega \times cm^2$) of the samples by subtracting background resistance (TEER of Transwell® inserts without cells) and multiplying with the membrane surface area (Hughes et al., 2007) provided by the manufactures (Corning™ Transwell®). Some studies reported the TEER values without subtracting the insert and others reported the TEER value per well without multiplying it with the surface area.

Our endometrial cell TEER values ranged between 160 and 280 $\Omega/cm^2$, as obtained using a 12-mm Corning™ Transwell® with a 0.4 µm-pore polyester membrane insert. These results were similar to those of Chen et al. who tested cryopreservation and recovery of human endometrial epithelial cells. Their TEER values after full cell confluency were reported after 48 hours, ranging between 250 and 400 $\Omega/cm^2$ (including the insert that was around 120 $\Omega/cm^2$ in their study). These values were obtained after seeding of $2 \times 10^5$ cells per

24 wells (Transwell®) on a polyethylene terephthalate-membrane with a 0.33 $cm^2$ filtration area and 1 mm pore (Chen et al., 2016).

Other studies were carried out with the endometrium epithelial cells only (without stromal cells) and gave much higher TEER values, e.g. Matthews et al. cultured human endometrial glandular epithelial cells forming confluent monolayers obtaining a mean maximum TEER value of 1540 ± 52 $\Omega/cm^2$ (Matthews et al., 1993). Also, Chen et al. conducted another study in 2015 but used oviduct epithelial cells, obtaining an average TEER between 500 and 1100 $\Omega/cm^2$, when using 24-well Millicell inserts with a surface area of 0.33 $cm^2$ (Chen et al., 2015).

On other hand, a study by Palmer et al. showed much higher TEER results; the authors were looking at apical small-conductance calcium-activated $K^+$ potassium channels and $Ca^{2+}$-dependent anion secretion in endometrial epithelial cells. The TEER values of the epithelial cell monolayers showed high resistance of ~3000 $\Omega$ /$cm^2$. This study used porcine epithelial endometrial cells (around passage 30) that were seeded on 4.5 $cm^2$ Transwell® filters (Costar, Corning Incorporated, Acton, MA, USA), forming tight junctions of very high transepithelial resistance (Palmer et al., 2008).

The results of Palmer et al. can be explained by the study of Sun Lu et al. that demonstrated the relation between different cell culture passages (early and late) and TEER results, which is linked to the tightness of the cell monolayer junctions, indicating that the more the culturing passages the faster the subpopulation cell growth within the heterogeneous cell line, resulting in a tight monolayer (Lu et al., 1996, Hughes et al., 2007).

In general, TEER values within the formed barriers could range between 150 and 1600 $\Omega/cm^2$, depending on how large the transported substances or solutes were. A TEER value of ≥350 $\Omega/cm^2$ seems to restrict passive diffusion of a relatively large 200-nm monolayer, while a TEER of ≥700 $\Omega/cm^2$ with tighter monolayers allowed the transport of smaller drug delivery systems (Ghaffarian and Muro, 2013).

The TEER values in different studies using the same tissue cells were contingent on the measurement techniques and the type of electrodes used. Also, the results can be affected by other factors such as the surrounding temperature during measurement, medium formulation, number of cell culture passages, and chemical factors that alter the integrity of cell junction by oxidative stress (Srinivasan et al., 2015). It is hard to control all the variables, but the target is to optimise and simplify the conditions as much as possible (Srinivasan et al., 2015).

Furthermore, an optimal Transwell® model could result in different TEER values, depending on the membrane type. Membranes are available in different materials, coatings, surface areas, and pore sizes (ranging between 0.4 and 8 μm). The choice of the optimum membrane depends on various factors such as the size of target substances to be transported and the type and density of the cells grown, as some cells may migrate through pores upon seeding, preventing monolayer formation (Ghaffarian and Muro, 2013). It is also important to choose the optimum coating, as some commercially available membranes are coated with collagen or fibronectin, to improve the attachment of cells. Moreover, the well diameter affects the outcomes, whereby larger diameters (as in 6-well plates) increase paracellular leakiness within the cell monolayer (Ghaffarian and Muro, 2013). All these different factors affect the TEER as shown in the studies using endometrial cells with different Transwell® properties.

## 4.2 Effect of treatments on TEER

Controlling the fluid environment of the uterine cavity is essential for several key reproductive events, including embryo transport, development, and implantation. Many changes are known to occur in the volume and composition of intrauterine fluid throughout the menstrual cycle, but the physiological and molecular mechanisms controlling these are unclear and very poorly understood (Salleh et al., 2005).

Earlier studies on human endometrial epithelial monolayer cultures showed that the potassium concentration is increased in the apical but decreased in the basolateral cells (Matthews et al., 1993). This is a result of the passively secreted potassium into the upper apical compartment (Ruan et al., 2014b). This is consistent with the finding that the potassium level in the uterine lumina is six folds higher than that in the serum (Casslen and Nilsson, 1984) and the apical potassium channels may be responsible for potassium secretion, suggesting the role of the endometrial epithelium in forming and sustaining the distinctive ionic composition of the intrauterine environment (Matthews et al., 1993).

The movement of uterine fluid is controlled and linked to ion composition and movement, as the luminal fluid has a unique ionic composition and in both humans and rats, there is a high $K^+$: $Na^+$ ratio that changes with the hormonal environment (Casslen and Nilsson, 1984, Salleh et al., 2005).

The net transport of potassium takes place in the apical direction, and potassium channels can be coupled functionally to K+-transporting ATPases (Ruan et al., 2014b). These ATPases export Na+ or H+ and take up K+, which is then recycled via K+ channels (Warth, 2003). Therefore, it was important for our study to better understand the functional role of potassium channels within the endometrium using different treatments and potassium channel blockers, as any alteration in the secretory cells that regulate the embryonic microenvironment upon reaching the uterine cavity can alter the endometrial microenvironment and affect embryo implantation, development, and survival (Peter Durairaj et al., 2017). Also, it was reported that both KCNMA1 and KCNK9 channels are involved in the regulation of cell proliferation and apoptosis (Szabo et al., 2010, Patel et al., 2013).

### 4.2.1 **KCNK9 blocker (PK-THPP)**

A number of selective, high-potency KCNK9 blocking compounds are available and have been identified; some have been reportedly used in lab experiments, clinical trials, and preclinical studies (Chokshi et al., 2015). KCNK9 channels

are blocked by anandamide, ruthenium red, acid $pH_0$ (Alexander et al., 2011), barium, lidocaine (Kim et al., 2000), and PK-THPP (Cotten, 2013). In our study, we used PKTHPP (1-1-6-[biphenyl-4-ylcarbonyl]-5,6,7,8-tetrahydropyrido4,3-d-pyrimidin-4ylpiperidin-4-yl propan-1-one). It was proven to be a potent and selective inhibitor of the KCNK9 channel (Coburn et al., 2012).

Cotten (2013) used PKTHPP at a concentration of 0.5 and 5 mg/kg (Cotten, 2013). PKTHPP was dissolved in 2 mg/mL DMSO according to the manufacturer (Sigma) and calculations were done. Three concentrations were tested and according to the results, 35 µM was found to be the optimum concentration to be used. This concentration could be affected by culture media supplementation with foetal bovine serum as blocking of KCNK9 channels is irreversible unless the cells are washed with buffer containing bovine serum albumin (Veale et al., 2007).

The results showed a significant difference in TEER values, which decreased after blocking with PKTHPP. Potassium channels are known to be involved in the regulation of cell proliferation and apoptosis in many cell types and in many pathological conditions including cancer (Jehle et al., 2011). It is important that cell proliferation and cell apoptosis be balanced, as this is crucial for embryo implantation – pregnancy failure in humans is linked to high levels of apoptosis (Ruan et al., 2014b). Several potassium channels such as KCNK9 and KCNMA1 are observed in human endometrial cancer cells, and many studies have observed their roles in cell proliferation and cancer progression (Patel et al., 2013, Ruan et al., 2014b), and potassium blockers suppress colony formation in endometrial cancer cells (Schickling et al., 2011).

The understanding of the role of potassium channels in endometrial cells in relation to implantation and fertility is poor, as most of the studies have been conducted on cancer conditions. Embryo implantation is an invasive and progressive process, similar to that of cancer, with common shared pathways and factors between them (Murray and Lessey, 1999) and hence any observations that involve potassium channels in endometrial cancer

proliferation indicate the possible and similar involvement of potassium channels in the process of embryo implantation (Jehle et al., 2011).

Innamaa et al. found that blocking of potassium channels can inhibit cell proliferation of the K2P superfamily (Innamaa et al., 2013a) and determined the effects of KNCK9 blockers on cell proliferation and apoptosis (Innamaa et al., 2013b). Apoptosis is characterised by a decrease in cell volume and in intracellular potassium concentration (Szabo et al., 2010). Potassium channels are important to stabilise the cell membrane potential at the time of cell stimulation, with mitogenic factors that trigger the entry of calcium into the cytoplasm upon stimulation of cells with a wide variety of mitogenic factors (Yu and Choi, 2000).

### 4.2.2 **KCNMA1 blocker (paxilline)**

KCNMA1 channels are inhibited and blocked by paxilline, charybdotoxin, iberiotoxin, and tetraethylammonium (Alexander et al., 2017). Paxilline, was chosen in our study since it is an indole alkaloid that selectively blocks KCNMA1 activity when used in high concentrations (Ghatta et al., 2006). We decided to optimise the paxilline dose using 1, 10, and 20 µM concentrations, according to two studies: Rose and Yallampalli used paxilline at a concentration of 1 µM (Ross and Yallampalli, 2006) and Li et al. used paxilline at a concentration of 20 µM (Li et al., 2014a).

Since our statistical analysis did not show any significant difference between 1 and 10 µM concentrations, we did not include 20 µM in optimisation, and 1 µM was the concentration of choice. Paxilline (Sigma-Aldrich, Buchs, Switzerland) was dissolved in 100% DMSO to obtain a stock solution and further dilutions were prepared in phenol red-free medium to reach the target concentration (Oeggerli et al., 2012).

Our study observed a high statistically significant difference in TEER results when the KCNMA1 was blocked with paxilline. Oeggerli et al. showed that KCNMA1 may contribute to a high proliferation rate and malignancy, and

pharmacological inhibition by paxilline decreases cell proliferation rate in cancer cell lines by 54% (Oeggerli et al., 2012). Other studies observed the opposite, namely that blocking KCNMA1 by paxilline was ineffective for channel expression (Bortner and Cidlowski, 2014). In general, treatment with potassium channel blockers has been shown to block apoptosis in different cell types (O'Grady and Lee, 2005).

Modulation of KCNMA1 channels could play a functional role in fertility in terms of uterine relaxation during implantation. Both ß2- and ß3-receptors form a functional complex with KCNMA1 channels, inducing channel activation and uterine relaxation in response to the activation of ß-receptors (Brainard et al., 2007). Using a KCNMA1 blocker as iberiotoxin eliminates the utero-relaxant effect of ß3-receptor activation in the uterine myometrial tissue (Doheny et al., 2005, Brainard et al., 2007).

The KCNMA1 channel plays an important physiological role in regulating the tone of smooth muscles, as they can cause changes in intracellular calcium and membrane potential, similar to KCNK9 channels, as mentioned above. This makes the KCNMA1 channel an important negative feedback system, resulting in outward hyperpolarizing potassium currents with an increase in intracellular calcium (Bentzen et al., 2014).

In-vivo, other factors are linked to fertility and influence the activity of the KCNMA1 channels, as many studies have reported that endogenous 2-methoxyestradiol and the metabolite of 17ß-estradiol inhibit channel activity by reducing the number of opened and activated channels (Ghatta et al., 2006). Of note, Coiret et al. found that oestrogen increases the rate of cell proliferation in various organs, including the uterus, breast, and prostate, and also activates KCNMA1 (Coiret et al., 2007). Therefore, blocking of the KCNMA1 channel leads to depolarization of membranes, progression of cell cycle, and inhibition of cell proliferation (Oeggerli et al., 2012).

### 4.2.3 **5% $O_2$**

The results show statistically significant difference between the control cells and the cells cultured in 5% $O_2$, but what was observed was a plateau of the curve and not declination (e.g. KCNK9 and KCNMA1 blockers) as if the TEER was frozen at this stage.

Our results are consistent with those of Koto et al. who showed how hypoxia (1% $O_2$) interrupts the neural vasculature barrier's function. This was explained and linked to claudin-5, which is a component of tight junctions between neural endothelial cells, where their movement from the cellular cytoplasm to plasma membrane were correlated with the increase in the TEER (Koto et al., 2007). This can be compared to some potassium channels that are involved in oxygen sensing and are blocked by hypoxia reversibly, suggesting that $O_2$ sensing occurs through a membrane-enclosed mechanism (O'Grady and Lee, 2005).

KCNMA1 channel and the KCNK9 potassium channels functional roles suggest changes in the mitochondrial membrane potential and the volume of its matrix and also regulates the mitochondrial production of reactive oxygen species (ROS) (Szewczyk et al., 2009). Oxidation of potassium channels by ROS is the cause of major destruction of cellular function (Sesti et al., 2010).

Myometrial contractions and vasoconstriction lead to a decrease in oxygen flow, where most of the upper layers of the endometrium become hypoxic in response to progesterone withdrawal and increased production of PGF2. Hypoxia is a powerful inducer of VEGF, which is an angiogenic and vascular permeability factor and a hypoxia-dependent mechanism is presumed to initiate menstruation (Jabbour et al., 2006). These incidences could be similarly happening with infertility and failure of implantation, explaining how oxygen concentration could be playing a role in endometrial receptivity and cell function.

### 4.2.4 **Intralipids**

The results show no statistically significant difference between the control and intralipid-treated cells, although an effect was expected either due to the pH of the intralipids being alkaline or any of the components of the intralipids.

Intralipid formulations are well-developed colloidal drug delivery systems. Lo et al. showed a significant decrease in the efflux of epirubicin across Caco-2 monolayers from the basolateral to apical surface, which could be due to a change in the cell membrane's lipid composition and fluidity, thus regulating the activity of the drug efflux pump proteins (Lo et al., 2001). It was shown by Li et al. that polyunsaturated fatty acids (PUFAs) protect the intestinal barrier in the rat model, where the intestinal mucosal villi and tight junctions were the most intact structures (Li et al., 2014b).

### 4.2.5 **Vitamin D**

Despite the non-significant difference observed in our study between the control and vitamin D-treated cells, 1,25(OH)2 (the active form of vitamin D3 that we used) was found to have an effect on cell differentiation, proliferation, and calcium metabolism, mediated by both genomic and non-genomic action (Bouillon et al., 1995). This was shown by Zanatta et al., who proved that 1,25-D3 receptors are widely distributed and the expressed in the male reproductive tract, reinforcing the key role of vitamin D in active and complete spermatogenesis (Zanatta et al., 2011).

Also 1,25-D3 regulates the activity of ionic channels in the Sertoli cell in the testes, where the activation of ion channels modulates the cells' secretory functions by inducing exocytosis of a fluid rich in ions, proteins, and growth factors critical for male fertility (Menegaz et al., 2016). Studies carried out by Rosso et al. showed the important role of 1,25-D3 stimulation of calcium influx by different ionic channels and how second messengers regulate the activity of these channels through signal transduction pathways (Rosso et al., 2012).

We could have obtained different results with a more significant effect of vitamin D if we had applied the theory of Puzas and Brand (1985), i.e. using a protein-free media as the protein increased the concentration of metabolites in the medium and hence increased the amount of metabolites in the cell compartment (Puzas and Brand, 1985). However, this would have led to a bias since the control and other treatments were applied in a protein-supplemented media.

# 5 Conclusion and future work

Different treatments and conditions were applied to endometrial cells of a mixed population to record the TEER of the cells. Statistical analysis showed high statistical difference ($P < 0.005$) between the control and cells treated with 5% $O_2$, while there was very high statistical difference ($P < 0.001$) between the control and cells treated with KCNK9 blocker and KCNMA1 blocker, but there was no statistically significant difference between the control and cells treated with intralipids and vitamin D.

Future work can focus on different aspects: The TEER of ***endometrial single cell-type*** monolayers can be examined, especially epithelial cells. The endometrium of pregnant ewes showed alterations in its secretory capacity of occludin, a tight junction protein in the uterine epithelia. Also, tight junctions related proteins in the endometrial glands increase during pregnancy (Satterfield et al., 2007). These findings support the hypothesis that progesterone provokes a temporary decrease in tight and adherens junctions in the endometrial luminal epithelium and increases selective serum and tissue fluid levels to facilitate embryo implantation (Satterfield et al., 2007).

Other modifications to our work could involve applying ***co-culture with embryos***. Desai et al. showed how co-culture using a novel human endometrial cell line and Transwell® chambers can facilitate embryo development and differentiation without cell contact (Desai et al., 2004). A co-culture system has been used in different studies. Embryonic signals cause the human endometrial stromal cells to become more sensitive during their differentiation into decidual cells and to respond selectively by inhibiting the secretion of key implantation factors on recognition of low-quality embryos (Brosens et al., 2014, Macklon and Brosens, 2014). Unfortunately, ethical approval remains a barrier to the application of co-culture with human cells, but of all animal species, the baboon is considered the most representative for extrapolation to humans. However, because of technical issues and the physiological differences between different species, the causes of implantation

defects in humans remain elusive. Therefore, studying human embryo implantation using in vitro models is important (Teklenburg and Macklon, 2009).

More ***drugs could be tested*** and treatments could be mixed, especially with most of the publications concerning the decrease in uterine contraction frequency during the secretory phase of ovarian stimulation. Studies have found that during the window of implantation (seven days after administration of HCG), uterine contractility decreases drastically and reaches a nearly inactive state. These findings suggests that relaxation of the uterus during the time blastocyst transfer may assist in embryo implantation (Fanchin et al., 2000, Fanchin et al., 2001). This is supported by Fanchin et al. who observed a decrease in implantation and pregnancy rates when contractile frequency is aggravated on the day of non-cavitating embryo transfer (Fanchin et al., 1998).

# Chapter Five: Gene Expression and Protein Translation of Potassium Channels in Endometrium from Fertile and Infertile Women

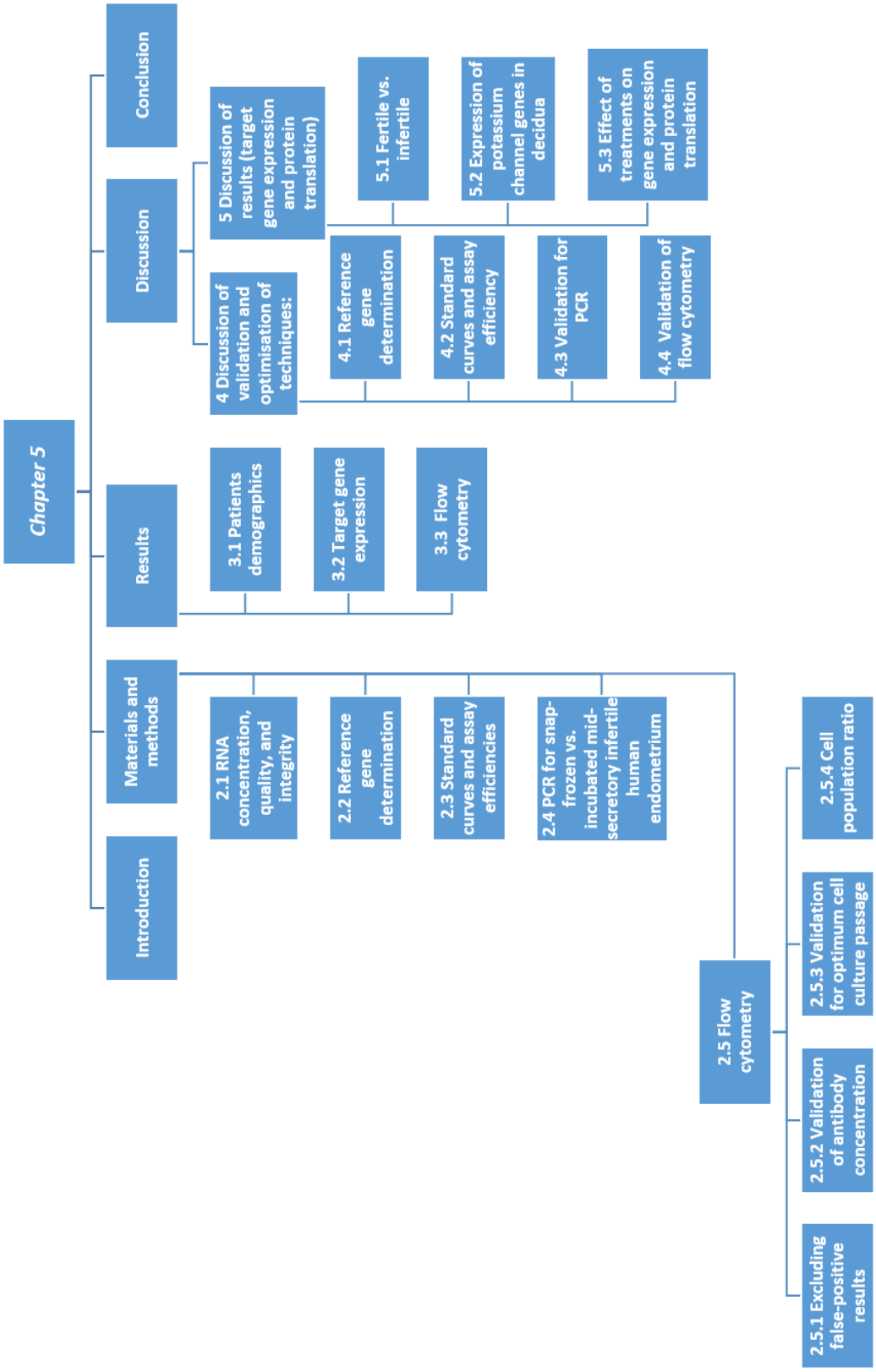
# 1 Introduction

Successful embryo implantation is a process which requires both synchronous development and interaction between the hatched blastocyst and endometrium. Endometrial receptivity is key to successful implantation and understanding its molecular determinants may hold important clues for implantation defects. Endometrial cells are exposed to constantly changing microenvironment factors, typically pH, lipids, oxygen tension, and electrolytes (Ng et al., 2017) including a six-fold higher potassium (K+) concentration than is found in the plasma (Casslen and Nilsson, 1984).

In the endometrium, potassium channels are notable for their unique subunit architecture and biophysical properties, which may be of importance in local homeostasis where slight alterations in micro-environmental physicochemical factors could alter the balance needed for establishing pregnancy.

Until now, a direct relationship between treatments for infertility and their effects on embryo implantation has not been found despite being used clinically worldwide like the evidences suggesting the beneficial role of vitamin D in the hormonal and metabolic factors in endometriosis and how this is associated with the outcomes of IVF (Voulgaris et al., 2017). There is evidence that potassium channels couple with 1,25(OH)2D, i.e. vitamin D3 (Menegaz et al., 2016), but it is still not known if this process is involved in embryo implantation.

In this study, we examined the changes in molecular gene expression of selected potassium ion channels (KCNK9, KCNK10, KCNK17, and KCNMA1) in human fertile and infertile endometrium and how gene expression can be affected by different treatments applied (different oxygen concentration, vitamin D, and intralipids).




**Flowchart 2: Chapter five writing plan**

# 2 Materials and methods

## 2.1 RNA concentration, quality, and integrity

RNA concentration and purity of endometrial samples was assessed using Nanodrop. Samples with purity values of more than 1.8 for the 260/280 ratio and of more than 1 for the 260/230 ratio were included for further processing for RNA integrity.

For endometrial samples (Figure 49), the mean values of RNA concentration of the fertile (n = 13) and infertile snap-frozen endometrial samples (n = 7) were not significantly different (P > 0.05; Mann-Whitney test). The mean RNA concentrations of endometrial samples after 24-h incubation with and without treatments (5% O2, intralipids, vitamin D) between the snap-frozen infertile group and the control, incubated infertile group were significantly different (P < 0.0001).

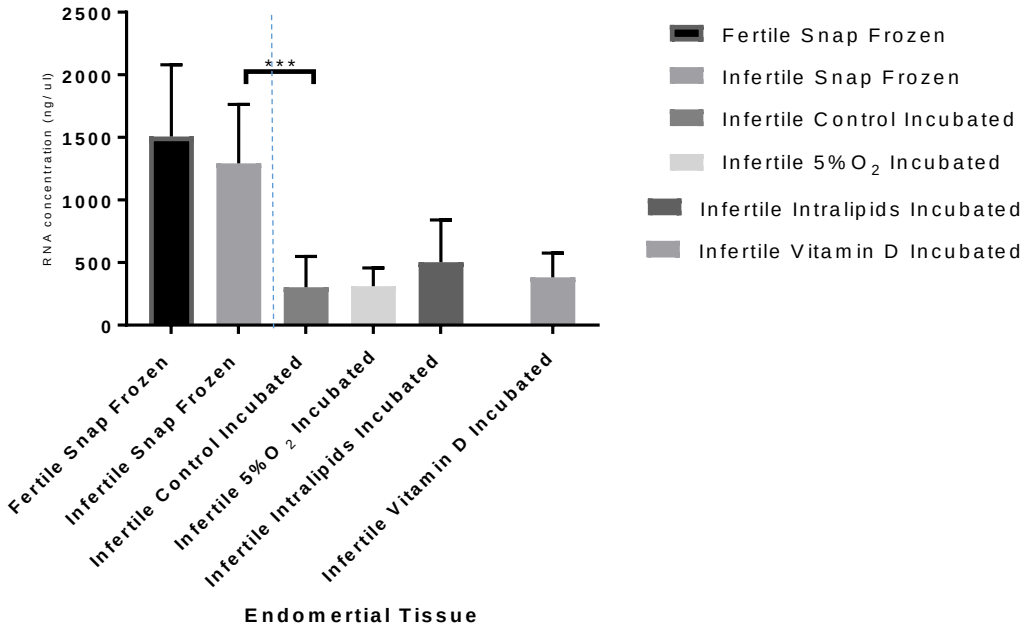



**Figure 49: RNA concentration of endometrial tissues**

RNA integrity was examined by gel electrophoresis using 1% agarose gel. Two bands of 18S and 28S appeared clearly on the gel as shown in (Figure 50):

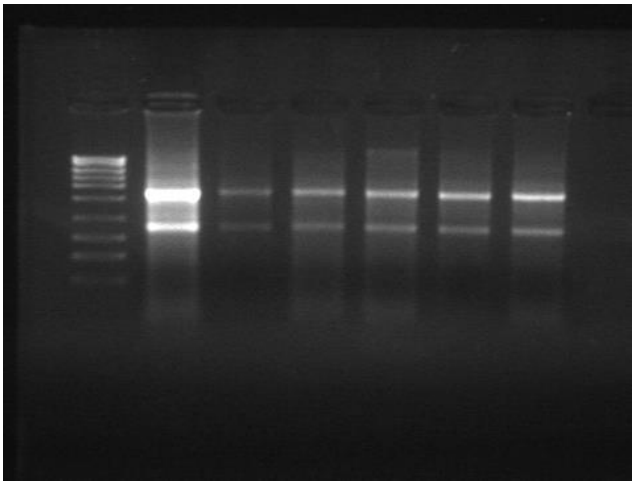


**Figure 50: Gel electrophoresis showing two clear bands of RNA (18S & 28S)**

For the decidua**,** RNA concentrations between 60 and 120 ng/µL were included only if RNA integrity appeared to be intact. PCR values obtained from these samples were normalised against concentration on the GeneX software.

## 2.2 Reference gene determination

### 2.2.1 Endometrium

As mentioned in materials and methods chapter, PCR was done to compare the expression of 6 different housekeeping reference genes (*ACTβ, CYC1, HPRT1, PPIA, TOP1*, and *YWHAZ*) on 10 endometrial tissue samples (without considering sample groups) using Taqman assays. Analysis was done using both Normfinder and Genorm algorithms. Genorm identified *TOP1* and *YWHAZ* as the two most stably expressed reference genes followed by *ACTβ* > *HPRT1* > *CYC1* > *PPIA* (Figure 51), while Normfinder identified *YWHAZ* as a more stable reference gene than *TOP1* > *HPRT1* > *ACTβ* > *CYC1* > *PPIA* (Figure 52). We decided to use three reference genes (*YWHAZ*, *TOP1*, and

*ACTβ*) as this was sufficient for good-quality analysis without compromising on quality.

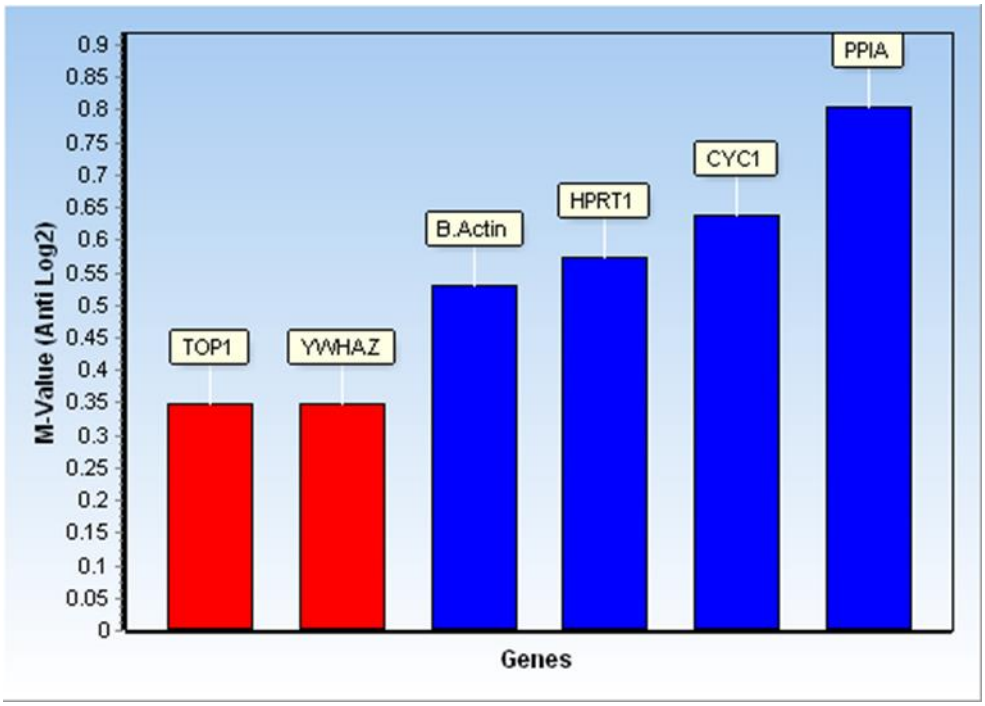



**Figure 51: M-Value from Genorm shows that *TOP1* and *YWHAZ* are the two most stably expressed reference genes with *ACTβ > HPRT1 > CYC1 > PPIA***

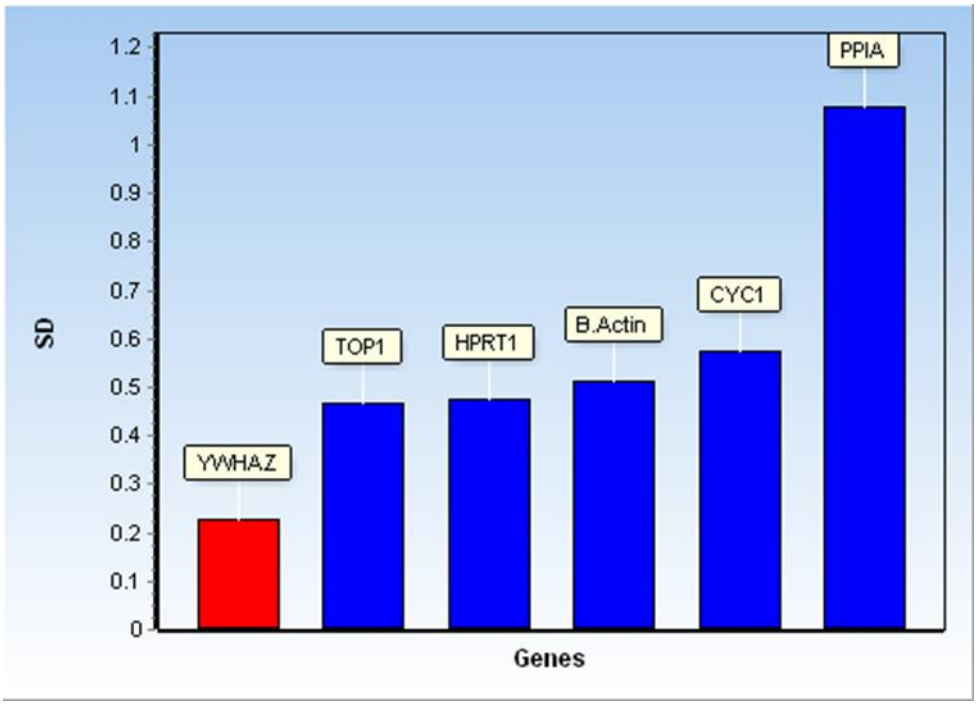



**Figure 52: SD from Normfinder shows that *YWHAZ* is the most stably expressed reference gene**

### 2.2.2 Decidua

PCR was done to compare the expression of 8 different housekeeping reference genes (*ACTβ, B2M, GAPDH, GUSB, HPRT1, PGK1, PPIA,* and *YWHAZ*) on 12 decidual tissue samples. Analysis was done using both Normfinder and Genorm algorithms. Genorm showed that *ACTβ* and *HPRT1* were the two most stably expressed reference genes followed by *YWHAZ* > *PGK1* > *PPIA* > *GUSB* > *B2M* > *GAPDH* (), while Normfinder showed that *HPRT1* was the most stable reference gene followed by *YWHAZ* > *ACTβ* > *PGK1* > *PPIA* > *GUSB* > *B2M* > *GAPDH* (Figure 53). The accumulated SD of the Normfinder indicated that one reference gene would be the optimum number of reference genes (Figure 54), but we decided to use three reference genes (*HPRT1, ACTβ,* and *YWHAZ*) for better quality analysis.

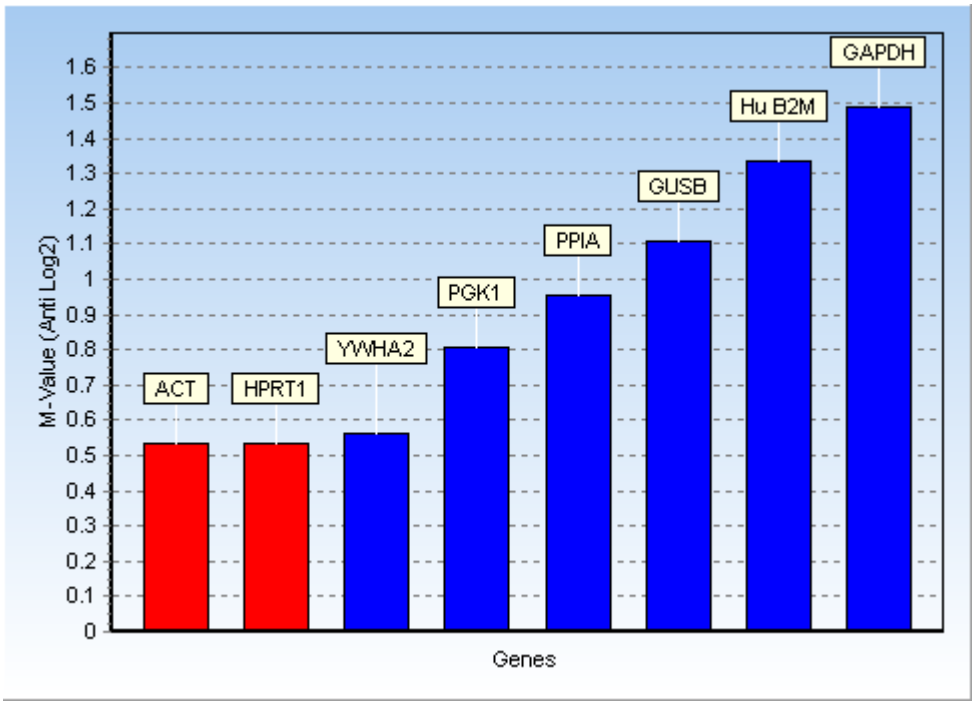



**Figure 53: M-Value from Genorm shows that *ACTβ* and *HPRT1* are the two most stably expressed reference genes with *YWHAZ > PGK1 > PPIA > GUSB > B2M > GAPDH***

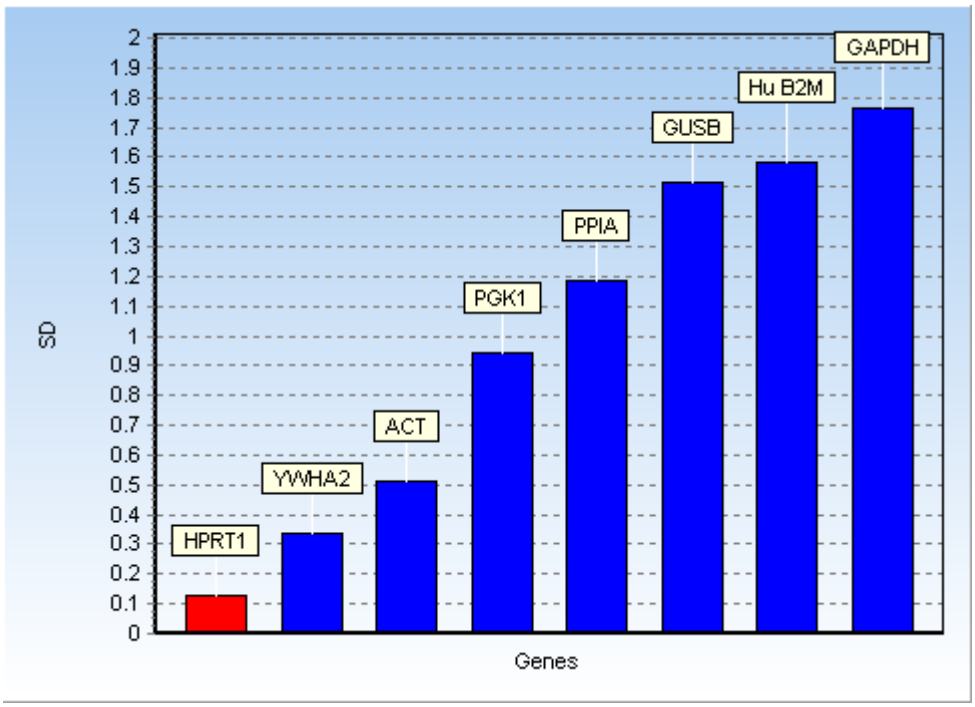



**Figure 54: SD from Normfinder showed that *HPRT1* was the most stably expressed reference gene**

## 2.3 Standard curves and assay efficiencies

### 2.3.1 Endometrium

The selected references genes were tested for assay efficiency. Standard curves were carried out in triplicates for all reference genes on both controls and treated endometrial samples. The three reference genes chosen (*YWHAZ, TOP1, ACTB*) gave an assay efficiency ranging between 85% and 115% after plotting a minimum of 4-point DCT (discrete cosine transform) of the replicates to create the standard curve, with an $R^2$ of more than 0.990 (Figure 55). The calculated efficiency values were used to correct CT values when investigating the relative expression in both controls and treated endometrial PCR experiments (Table 15).

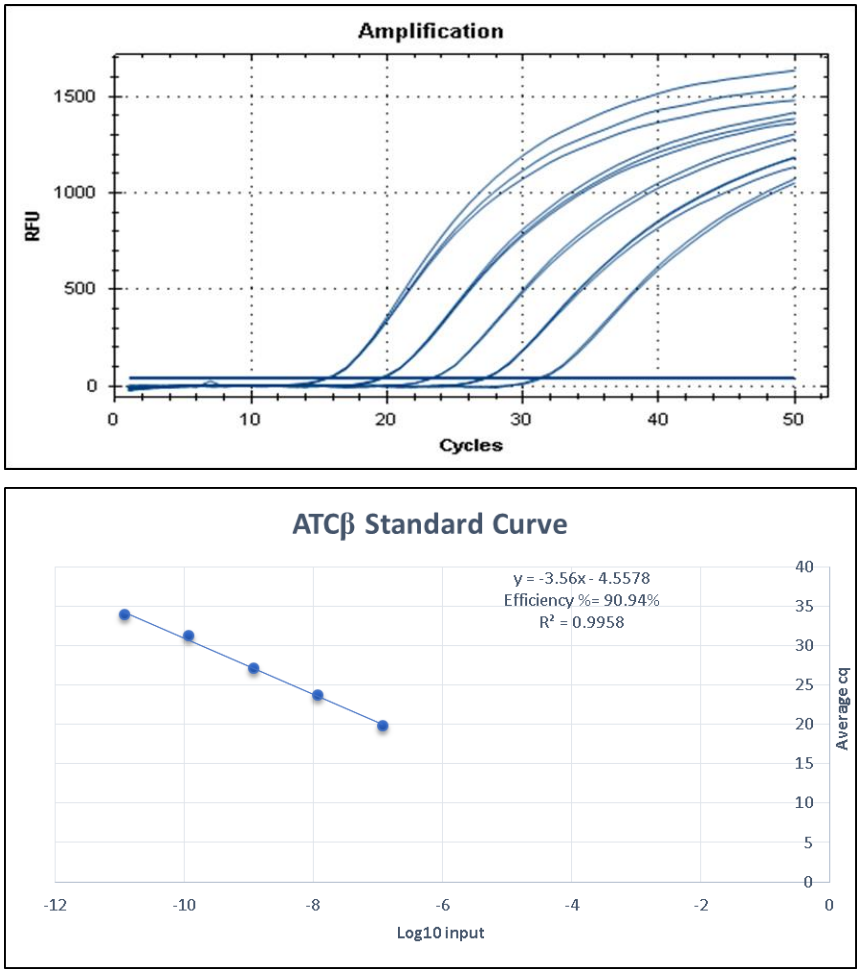



**Figure 55: Standard curve of the reference gene (ACTβ)**

The top panel shows the amplification of the *ACTβ* gene at five different dilutions (10 folds, starting from 120 ng till 0.012 ng). The lower panel shows an efficiency % value of 90.94% and $R^2$ value of 0.996.

| Reference Gene | | Efficiency % |
|---|---|---|
| TOP1 | Control | 90.75 |
| | 5% $O_2$ | 111.6 |
| | Intralipids | 89.69 |
| | Vitamin D | 117.29 |
| YWHAZ | Control | 88.71 |
| | 5% $O_2$ | 90.36 |
| | Intralipids | 110.74 |
| | Vitamin D | 88.36 |
| ACTβ | Control | 90.94 |
| | 5% $O_2$ | 87.30 |
| | Intralipids | 112.14 |
| | Vitamin D | 118.15 |

**Table 15: Assay efficiency of reference genes against control and treated endometrial samples**

### 2.3.2 **Decidua**

The selected references genes were tested for assay efficiency. Standard curves were plotted for all reference genes with both controls and treated

decidual samples. The three reference genes chosen gave an assay efficiency ranging between 85% and 115% after plotting a minimum of 4-point DCT of the replicates to create the standard curve, with a $R^2$ of more than 0.990 (Figure 56). The calculated efficiency values were used to correct CT values when investigating the relative expression in both controls and treated decidual groups (Table 16).

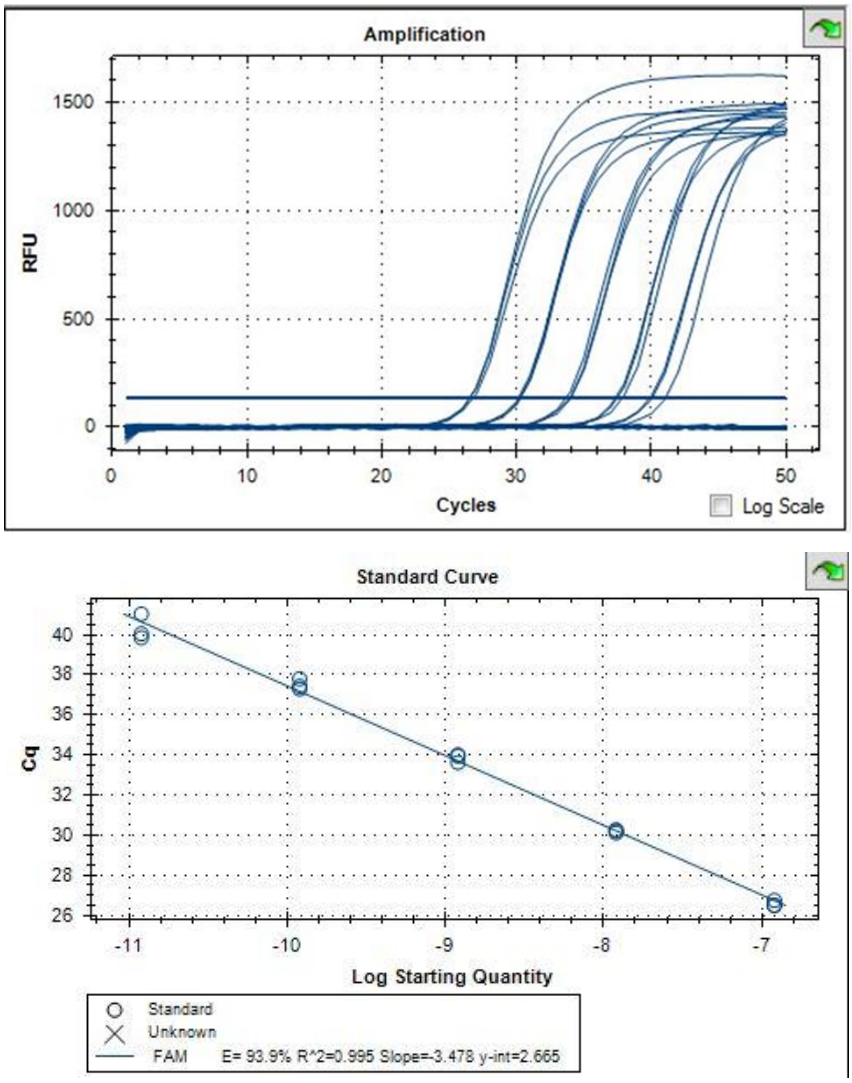



**Figure 56: Standard curve of the reference gene (*HPRT1*), showing an efficiency % value of 93.90% and $R^2$ value of 0.995, indicating a high-quality standard curve**

| Reference Gene | | Efficiency% |
|---|---|---|
| HPRT1 | Control | 93.90 |
| | 5% $O_2$ | 78.94 |
| | Intralipids | 112.90 |
| | Vitamin D | 89.36 |
| ACTβ | Control | 92.56 |
| | 5% $O_2$ | 118.32 |
| | Intralipids | 110.62 |
| | Vitamin D | 99.81 |
| YWHAZ | Control | 90.37 |
| | 5% $O_2$ | 111.35 |
| | Intralipids | 110.30 |
| | Vitamin D | 83.26 |

**Table 16: Assay efficiency of reference genes against control and treated decidual samples**

## 2.4 PCR for snap-frozen vs. incubated mid-secretory infertile human endometrium

It was important to determine whether the incubation process will affect the expression of potassium channels within the infertile group, as this may affect our interpretation while analysing the data after incubation of the samples with the treatments. The $\log_2$ mRNA expressions of *KCNK9, KCNK10, KCNK17*, and *KCNMA1* were tested in infertile MS (n = 5) snap-frozen endometrium vs. infertile MS (n = 7) endometrium incubated for 24 h. All target genes showed

lower expression in incubated MS infertile endometrium. Mann-Whitney test was done for every gene separately, showing no statistically significant difference (P > 0.05) between the groups (Figure 57).

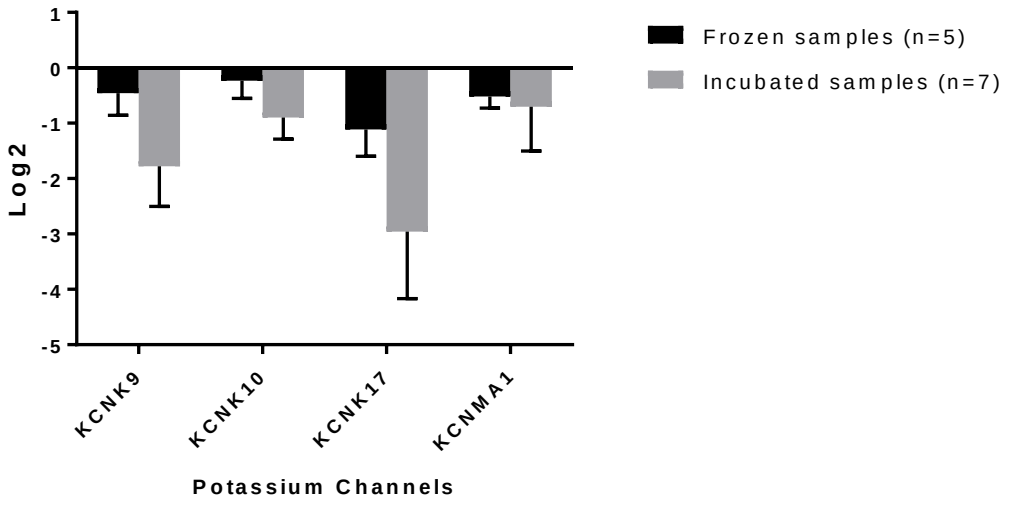
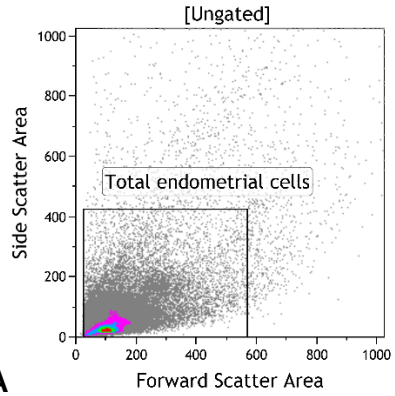
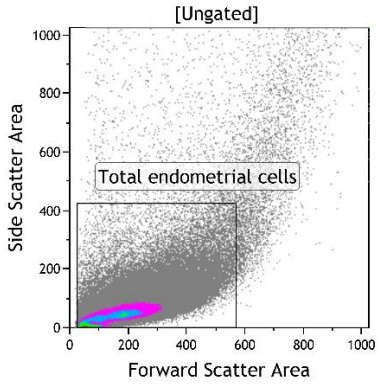
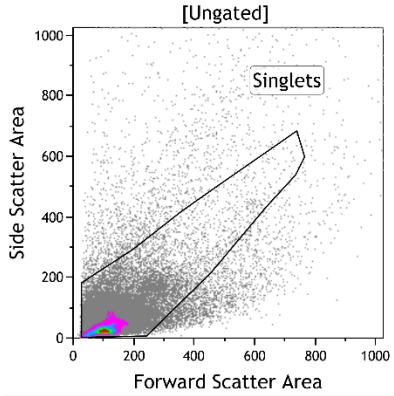
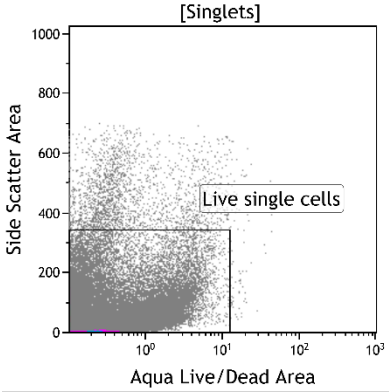
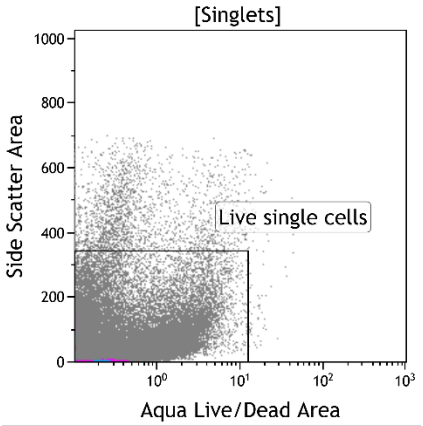
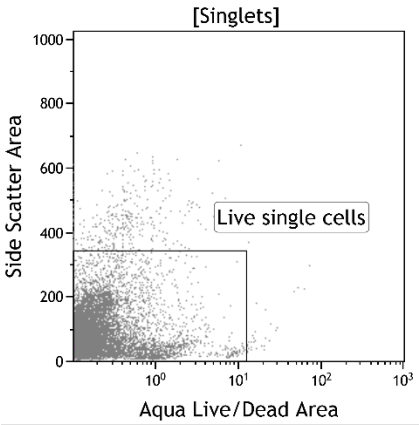



**Figure 57: PCR of snap-frozen vs. incubated MS infertile human endometrium**

## 2.5 Flow cytometry

### 2.5.1 Excluding false-positive results

The results showed that the isolation procedure produced a leukocyte-rich preparation with measurable sub-populations of the differing leukocytes (Figure 58), indicated by the reduction in the number of NK cells in the fresh endometrial sample (start P0 ~ 10,000) and after passaging (end of P0 < 500); this was done to prevent any false-positive results that could rise from the staining of the NK cells with the surface potassium channel antibodies.

| | Fresh Sample (start of P0, P0-D0) | Cultured Sample (end of P0, P0-D7) |
|---|---|---|
| **A** |  |  |
| **B** | <br>**Gate** **%Gated**<br>All 100.00<br>Singlets 97.98 | <br>**Gate** **%Gated**<br>All 100.00<br>Live single cells 99.51 |
| **C** | <br>**Gate** **%Gated**<br>All 100.00<br>Live single cells 99.51 | <br>**Gate** **%Gated**<br>All 100.00<br>Live single cells 99.81 |

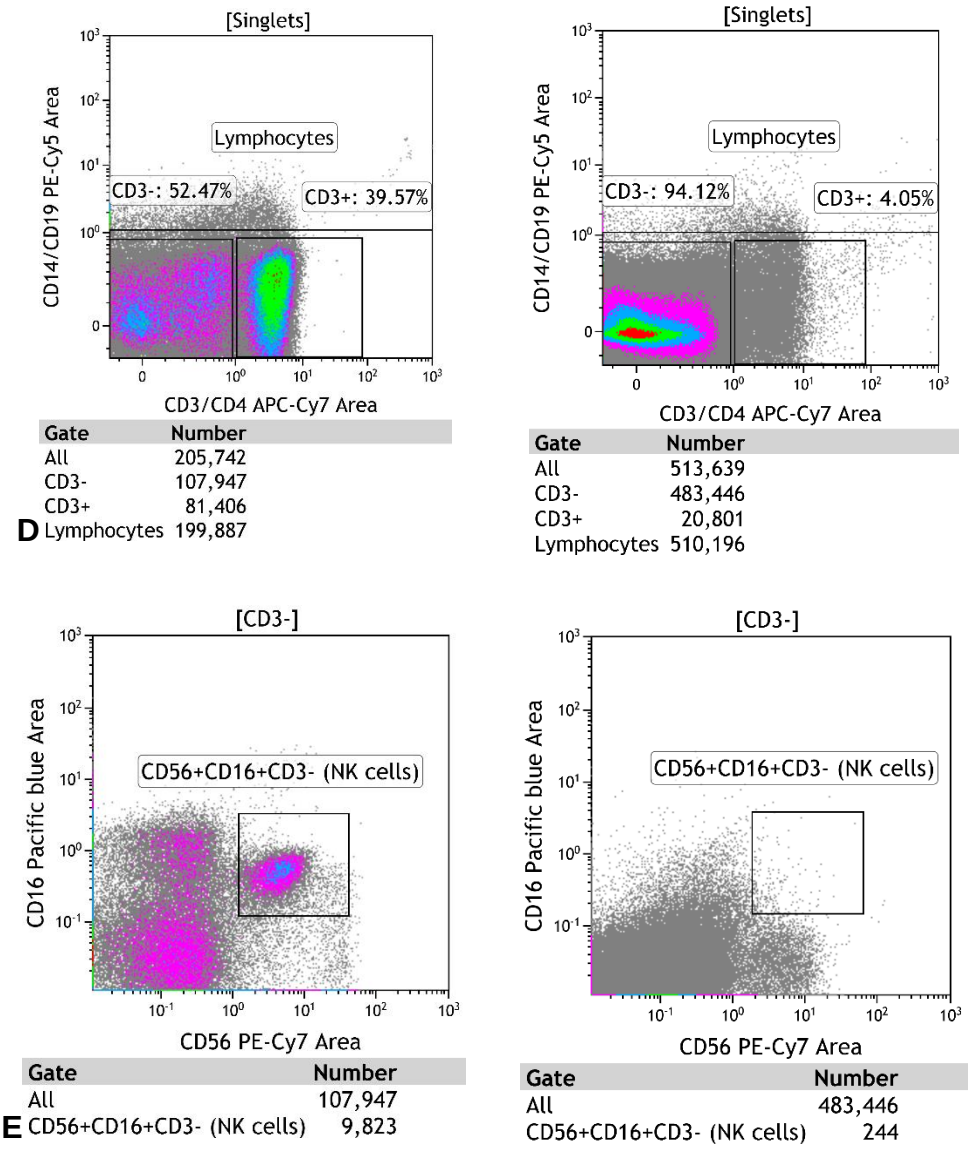
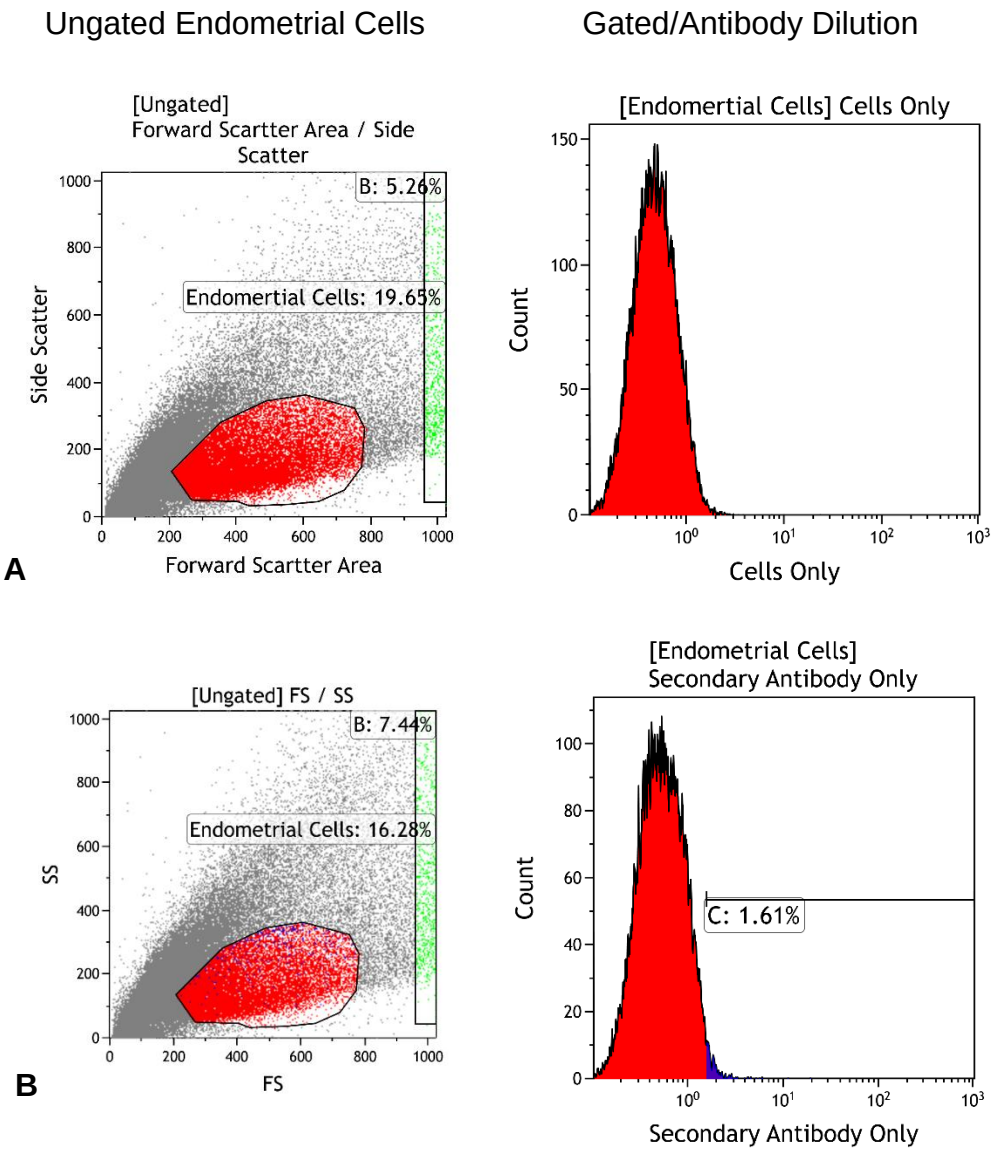



**Figure 58: Reduction of NK cells at the start and end of P0 of endometrial cell culture**

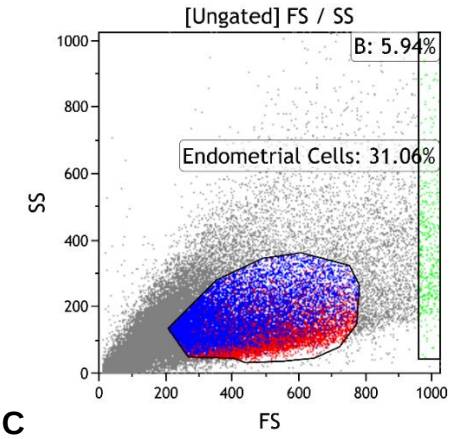
The initial gating defined all the cells present in the sample **(A)**. The single cells (endometrial) were then selected **(B)** and the proportion of live cells were plotted and gated as the cells of interest **(C)**. In order to remove the B cells and monocytes, cells positive for CD14/CD19 were excluded. Various gates for CD56, CD16, CD3, and CD8 were applied to define the immune cell populations **(D)**. The CD56- and CD16-positive cells were classified as NK cells. The NK cells were defined as CD56+/CD3- and CD16+ **(E)**.

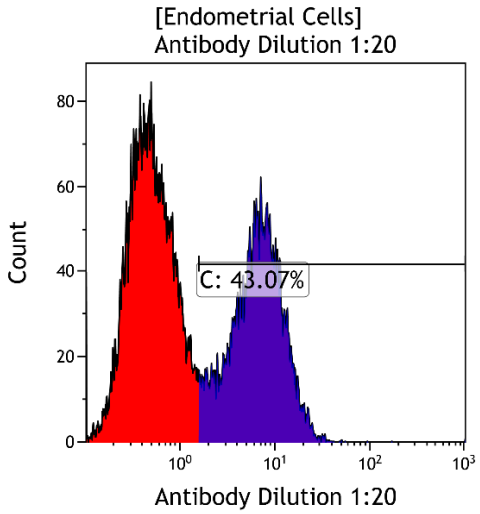
### 2.5.2 **Validation of antibody concentration**

(Figure 59) shows the validation of antibodies concentration used, where 1:50 was chosen to be the dilution of choice to carry on the next experiments.

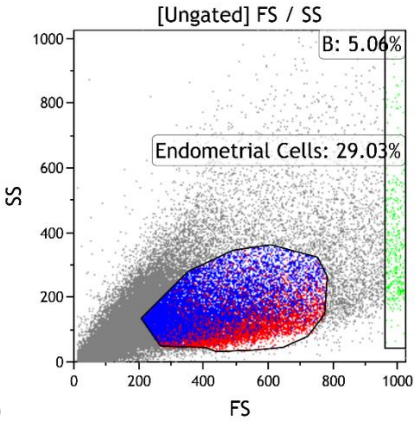


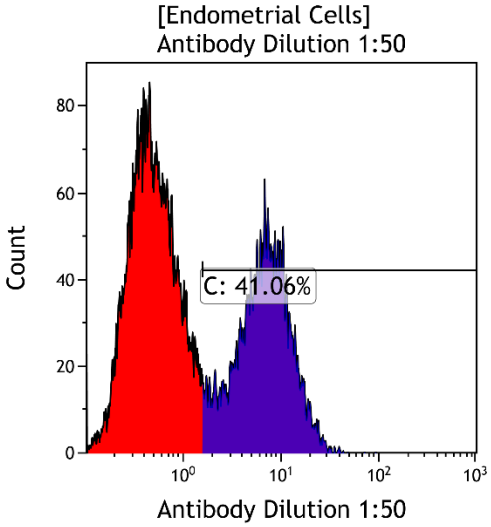
C


[Ungated] FS / SS
B: 5.94%
Endometrial Cells: 31.06%
SS
FS

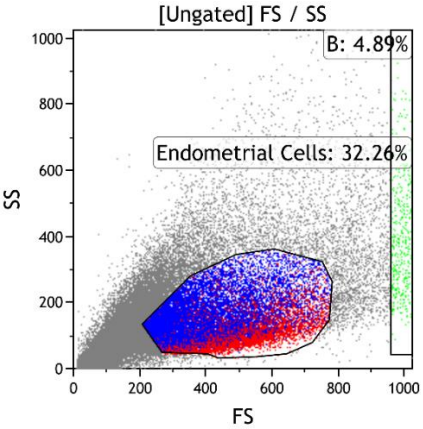

[Endometrial Cells]
Antibody Dilution 1:20
Count
C: 43.07%
Antibody Dilution 1:20

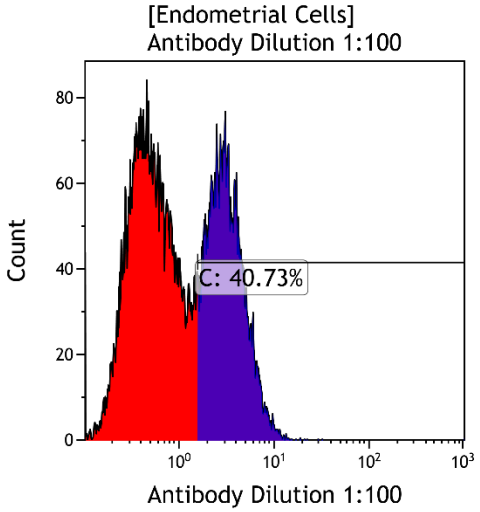
D


[Ungated] FS / SS
B: 5.06%
Endometrial Cells: 29.03%
SS
FS


[Endometrial Cells]
Antibody Dilution 1:50
Count
C: 41.06%
Antibody Dilution 1:50

E


[Ungated] FS / SS
B: 4.89%
Endometrial Cells: 32.26%
SS
FS


[Endometrial Cells]
Antibody Dilution 1:100
Count
C: 40.73%
Antibody Dilution 1:100

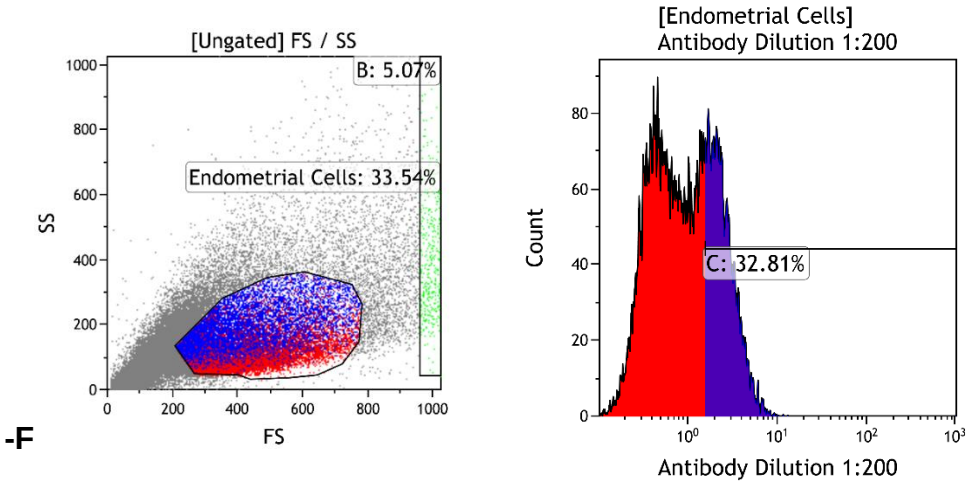



**Figure 59: Validation of different concentrations of antibodies** FC shows the ungated endometrial cells **(Left)** and the gated targeted antibody dilution **(Right).** Four different antibody concentrations were used: **(C)** 1:20, **(D)** 1:50, **(E)** 1:100, and **(F)** 1:200) in addition to **(A)** cells only and **(B)** $2^{ry}$ antibody only. This procedure was applied to two samples (both in duplicates), using the same channel antibody. The % gated cells with positive antibody appeared to be the same in all dilutions, but dilution 1:20 and 1:50 showed to be the best, generating specific staining with the least amount of background.

### 2.5.3 **Validation for optimum cell culture passage**

Endometrial cells were cultured, and at the end of each passage (using all samples from the same patient), cells were examined to determine how passaging affects the potassium channel proteins found on the surface of endometrial cells. (Figure 60) shows the gated percentage of the KCNK17 antibody for the translated potassium channel protein after different cell culture passages (end of P0 - end of P4) on 2 samples (each done in duplicates). A massive decline in the % gated appeared after the end of P2. Using the cells after the end of P2 was the passage of choice reaching the optimum number of cells needed to apply 4 different antibodies without the channel proteins being affected.

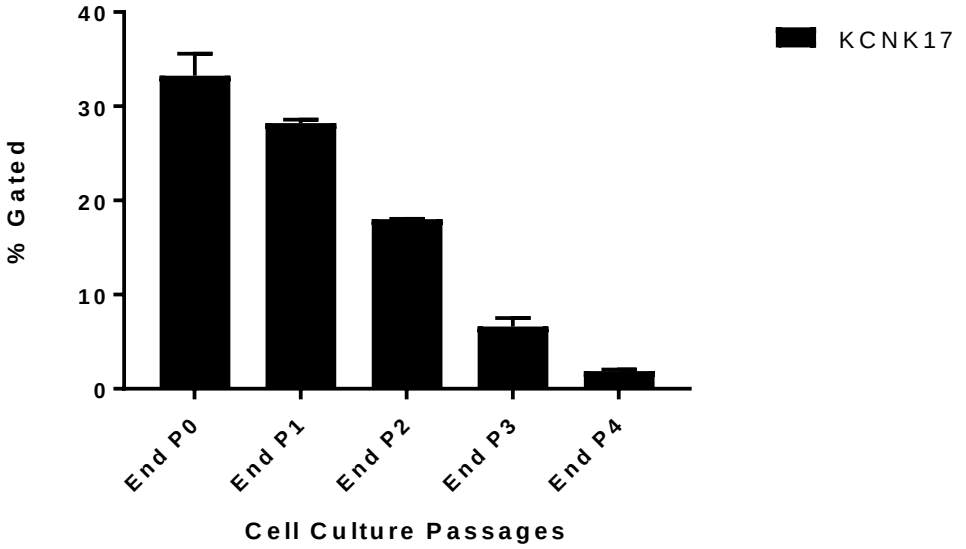
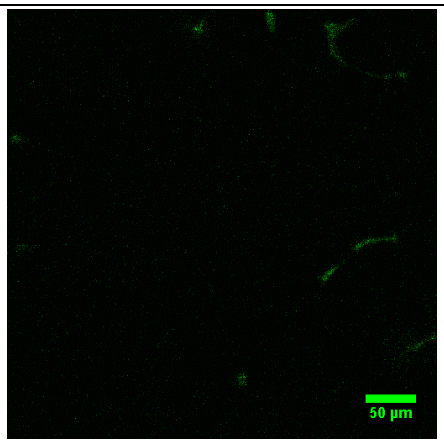
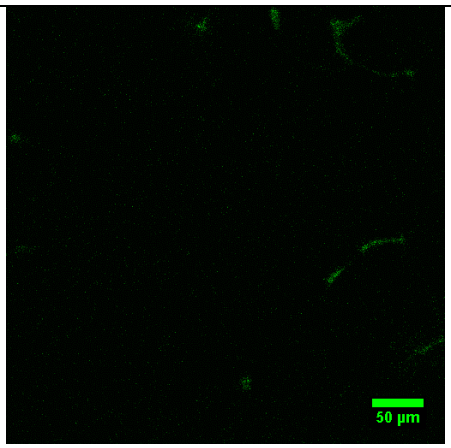
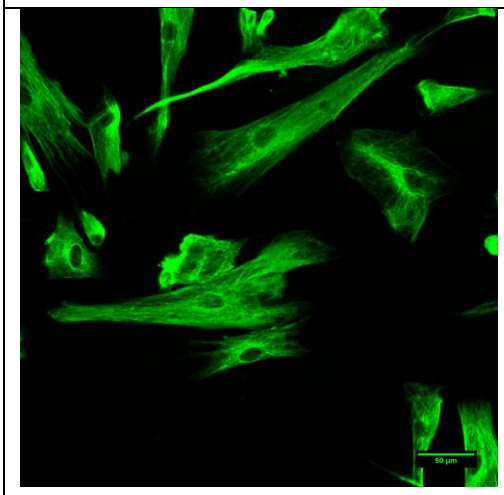
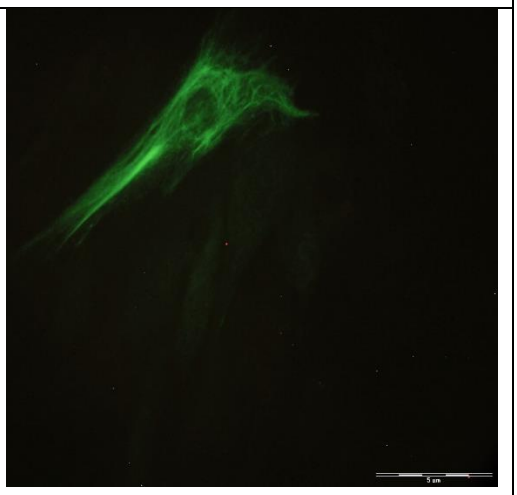



**Figure 60: % gated of KCNK17 antibody after different passages**

### 2.5.4 **Cell population ratio**

Immunofluorescence and flow cytometry were carried out at the end of the first cell culture passage (Figure 61) to detect vimentin (Figure 61A) and cytokeratin (Figure 61B) expression and their % gated. Vimentin is an intermediate filament of the mesenchymal tissue, which is found in various non-epithelial cells (Chang et al., 2002). Positive staining may confirm mesenchymal origin. Cytokeratin belongs to the family of intracytoplasmic structural proteins which are the dominant intermediate proteins of epithelial cells. (Figure 62) shows the unchanged cell population after the end of the second passage in comparison to the end of the first passage. This helped prevent any bias resulting from other variables affecting our results other than the tested factor (treatment).

| Vimentin<br><br>(A) | Cytokeratin<br><br>(B) |
| --- | --- |
| <br>Secondary Antibody Only | <br>Secondary Antibody Only |
| <br>Immunofluorescence Antibody | <br>Immunofluorescence Antibody |

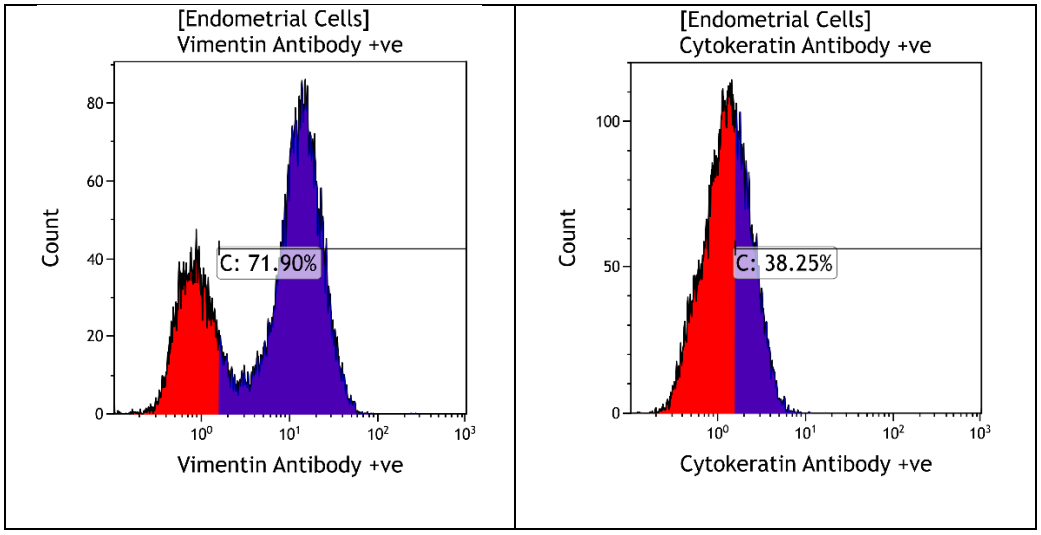



**Figure 61: Vimentin & cytokeratin immunofluorescence staining & % gated FC of endometrial cells**

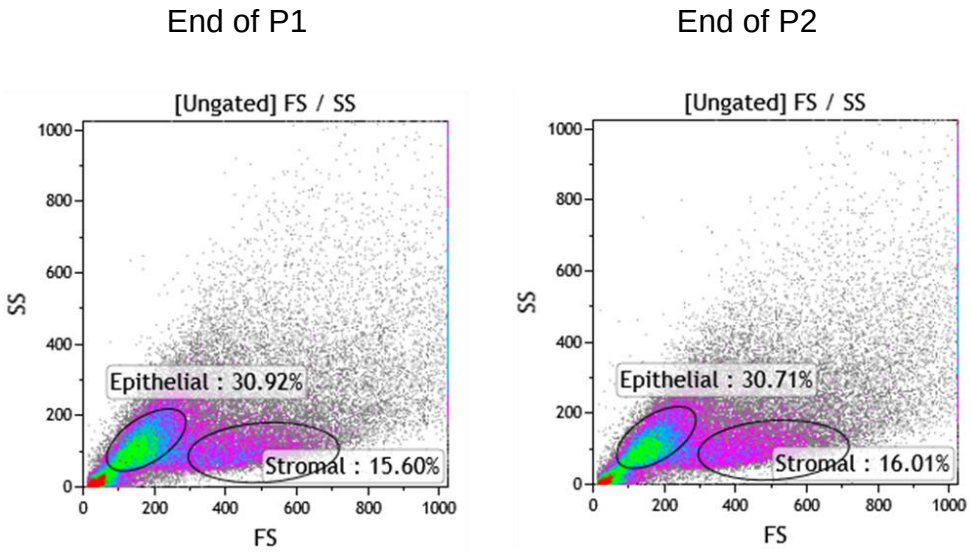



**Figure 62: Assumption of FC for cell populations between passages**

# 3 Results

The main target was to compare the expression of potassium channels between fertile and infertile endometrial samples and also to observe any difference in gene expression after application of different treatments to the infertile samples. The recruitment of women to obtain decidual samples was important to understand the expression of potassium channels into detail, since decidual tissue is similar to endometrial samples (histologically), but with greater stromal cell population that can provide better information on the role of potassium channels.

## 3.1 Patients demographics

**For endometrial samples**

| Parameter | Endometrial samples (n = 41) |
|---|---|
| Age (yrs) | 34.4 ± 1.02 |
| Parity | 1.06 ± 0.35 |
| Total cycle length (days) | 29.62 ± 0.46 |
| Cycle phase | Proliferative phase 6/41 (14.5%) |
| | Secretory phase 25/41 (61%) |
| | Irregular cycle 8/41 (19.5%) |

**Table 17: Demographics of patients recruited for the endometrial samples, values presented as mean ± SEM**

| Parameter | Fertile (mid-secretory) (n = 4) | Infertile (mid-secretory) (n = 15 ) |
|---|---|---|
| Age (yrs) | 32.96 ± 1.39 | 36.80 ± 1.27 |
| Parity | 1.64 ± 0.35 | 0.00 |
| Total cycle length (days) | 27.41 ± 0.39 | 28.93 ± 0.41 |
| Previous failed IVF/ ICSI or FER | 0.00 | 1.80 ± 0.24 |

**Table 18: Demographics of mid-secretory fertile vs mid-secretory infertile patients recruited for the endometrial samples, values presented as mean ± SEM**

On following up the infertile recruited patients, 5/12 (41.67%) were reported to be pregnant (confirmed by 6 wks ultrasound).

### For decidual samples

| Parameters | Decidual samples (n = 23) |
|---|---|
| Age (yrs) | 31.59 ± 4.39 |
| Parity | 1.43 ± 0.29 |
| Gestation at delivery (wks) | 38.92 ± 0.79 |

**Table 19: Demographics of patients recruited for the decidual samples, values are presented as mean ± SEM**

## 3.2 Target gene expression

### Endometrium

### 3.2.1 Proliferative vs. secretory stages of human menstrual cycle

mRNA expressions of KCNK9, KCNK10, KCNK17, and KCNMA1 were determined in fertile proliferative endometrium (n = 6) and fertile secretory endometrium (n = 10). A Mann-Whitney test was conducted for each gene separately. Only KCNK17 showed a statistically significant difference in expression ($P < 0.05$), (Figure 63).

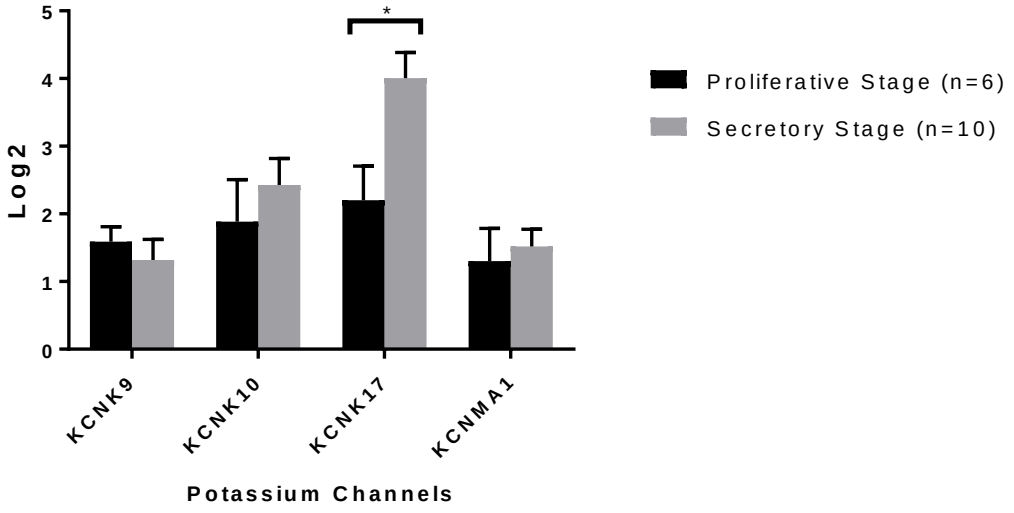



**Figure 63: PCR of proliferative vs. secretory stages of menstrual cycle in human fertile endometrium** A Mann-Whitney test was conducted for each gene separately. Only KCNK17 showed a statistically significant difference in expression ($P < 0.05$)

### 3.2.2 Proliferative and secretory sub-stages of human menstrual cycle

mRNA expressions of KCNK9, KCNK10, KCNK17, and KCNMA1 were determined in fertile early proliferative (EP) endometrium (n = 3), fertile late proliferative (LP) endometrium (n = 3), fertile early secretory (ES) endometrium

(n = 6), and fertile mid-secretory (MS) endometrium (n = 4). KCNK17 levels were significantly different (P < 0.001) between EP and MS endometrium. Also, KCNK17 expression showed statistically significant difference (P < 0.05) between LP and MS endometrium, as well as between ES and MS fertile human endometrium (Figure 64).

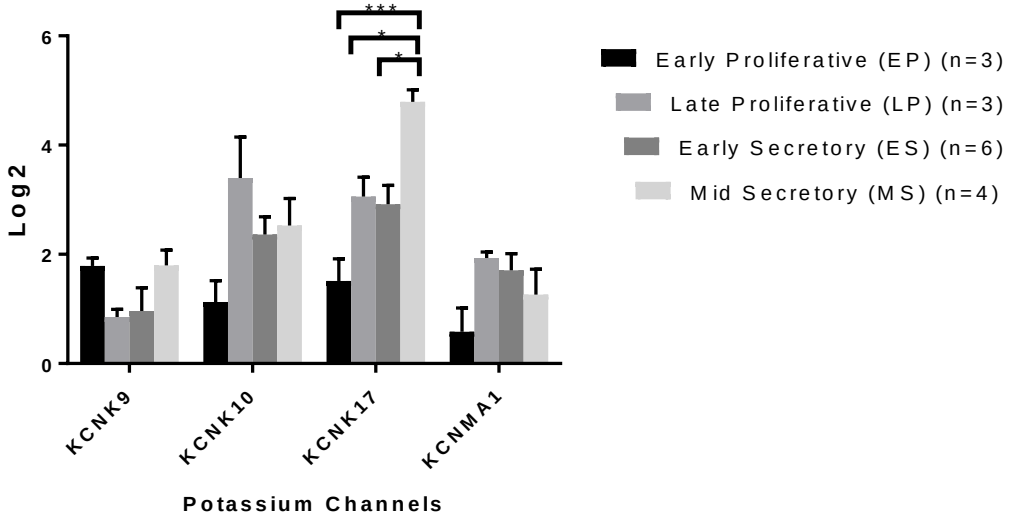



**Figure 64: PCR of proliferative and secretory sub-stages of human menstrual cycle in human fertile endometrium.** Kruskal-Wallis test was conducted on each gene separately, KCNK17 shows significant different (P < 0.001) between EP and MS endometrium. Also, KCNK17 expression showed statistically significant difference (P < 0.05) between LP and MS endometrium, as well as between ES and MS fertile human endometrium

### *3.2.2.1 Fertile vs. infertile human mid-secretory endometrium*

The $log_2$ mRNA expressions of KCNK9, KCNK10, KCNK17, and KCNMA1 were determined in fertile MS (n = 4) snap-frozen endometrium and infertile MS (n = 6) snap-frozen endometrium (Figure 65). All target genes show lower expression in MS infertile endometrium. A Mann-Whitney test was conducted for each gene separately. KCNK17 showed statistically significant difference (P < 0.05) was observed between groups.

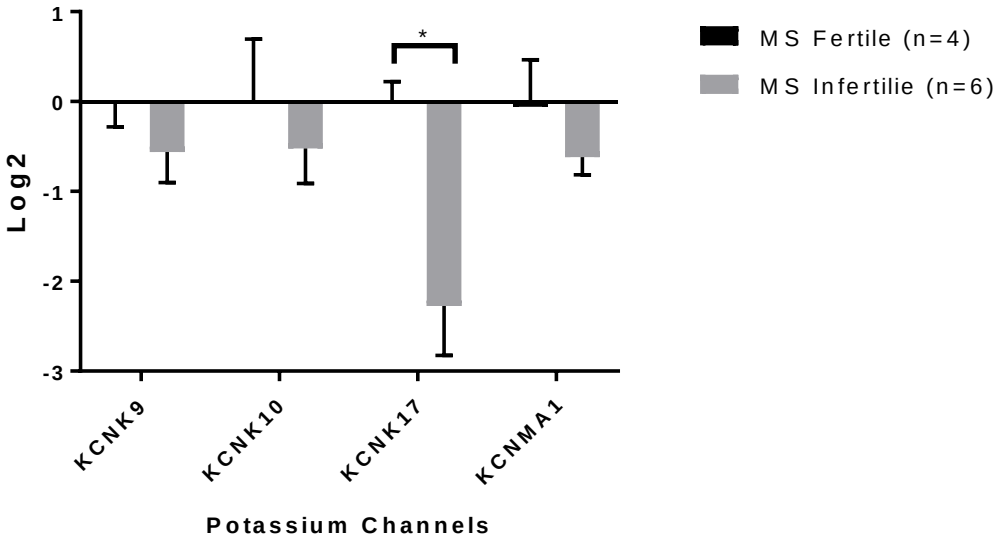



**Figure 65: PCR of MS fertile vs. MS infertile in human endometrium.** A Mann-Whitney test was conducted for each gene separately. KCNK17 showed statistically significant difference ($P < 0.05$) was observed between groups.

### 3.2.3 Effect of different treatments on infertile mid-secretory human endometrium

The $log_2$ mRNA expressions of KCNK9, KCNK10, KCNK17, and KCNMA1 were determined in infertile MS (n = 11; control) endometrium vs. infertile MS endometrium, incubated separately with the following treatments for 24 h: 5% $O_2$ (n = 6), intralipids (n = 7), and vitamin D (n = 7). There was alteration in gene expression (Figure 66). Kruskal-Wallis test was carried out for every gene separately, showing no statistically significant difference ($P > 0.05$) between the control and treated groups (Figure 67).

These results were interesting when compared to the TEER results, as vitamin D and intralipid showed no effect on the TEER, despite having an effect on the potassium channel expression (even if not statistically significant). Also, oxygen concentration had a statistically significant effect on the TEER results, but not on gene expression.

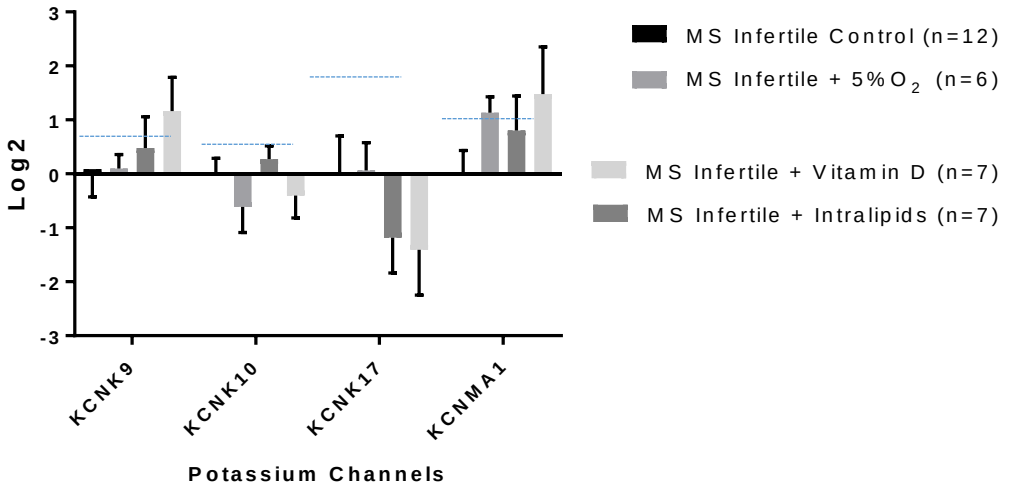
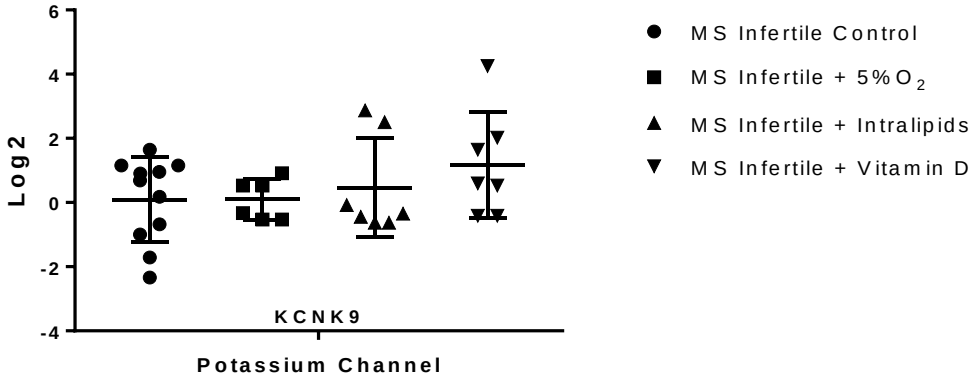
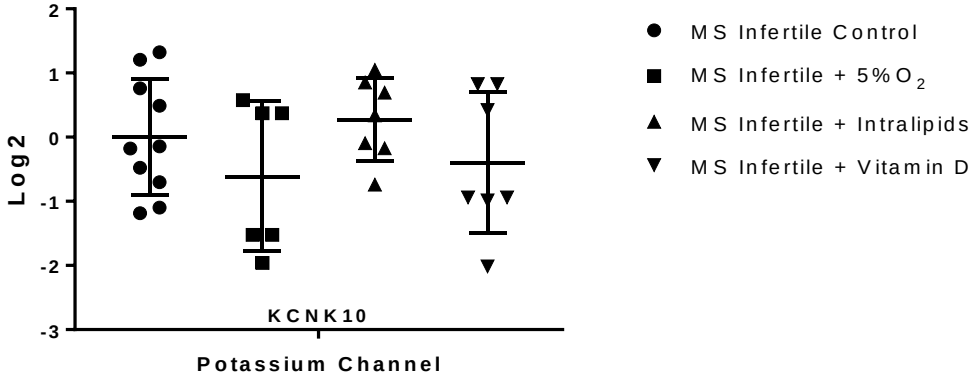



**Figure 66: PCR showing the $log_2$ gene expression of different treatments on infertile MS human endometrium with the dashed line showing the relative gene expression in MS fertile human endometrium**



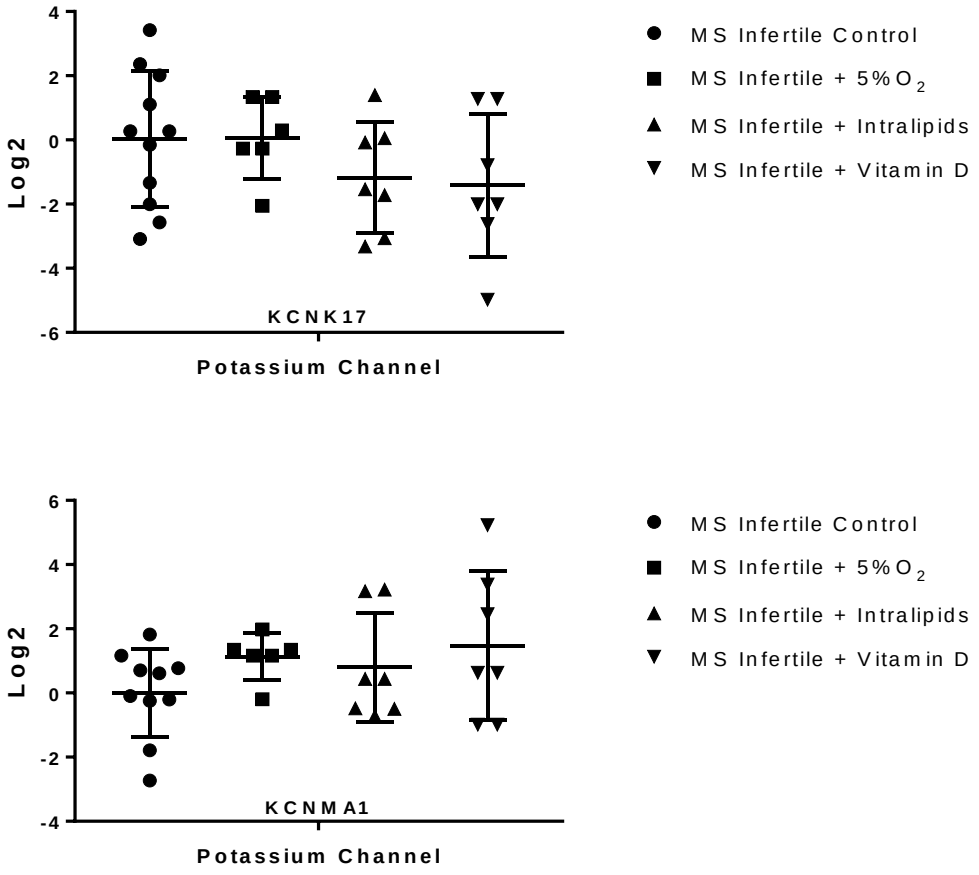



**Figure 67: PCR showing the $log_2$ gene expression with different treatments on each potassium channel separately in infertile MS human endometrium; bars represent mean data and standard deviation**

### 3.2.4 **Regular vs. irregular human menstrual cycle endometrium**

The $log_2$ mRNA expressions of KCNK9, KCNK10, KCNK17, and KCNMA1 were determined in regular cycle fertile (n = 13) and irregular cycle fertile endometrium (n = 7). No significant difference ($P > 0.05$) was found between the groups (Figure 68).

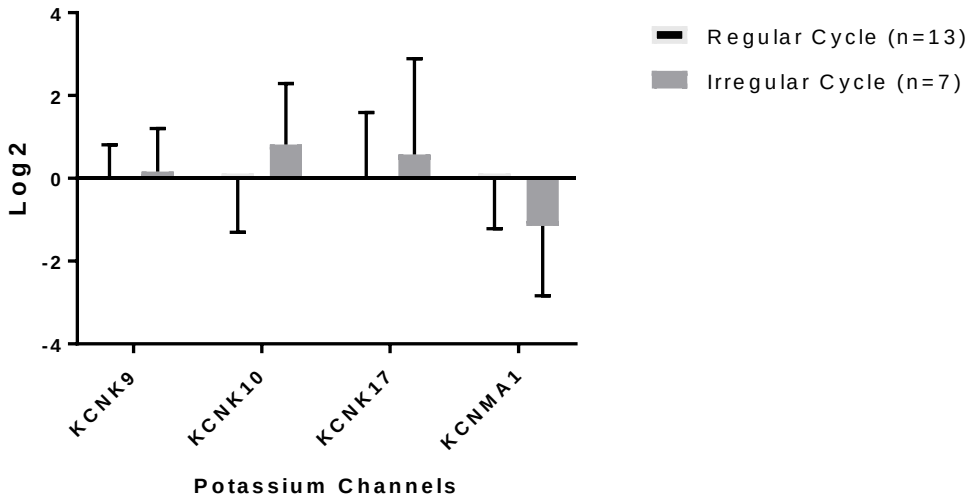



**Figure 68: PCR of regular vs. irregular fertile human endometrium**

## Decidua

### 3.2.5 Expression profiling of potassium channel genes in decidua

(Figure 69) shows the $log_2$ mRNA expressions of KCNK9, KCNK10, KCNK17, and KCNMA1 in decidual tissue from full-term placentas. KCNK17 was highly expressed relative to KCNK10, KCNK9, and KCNMA1.

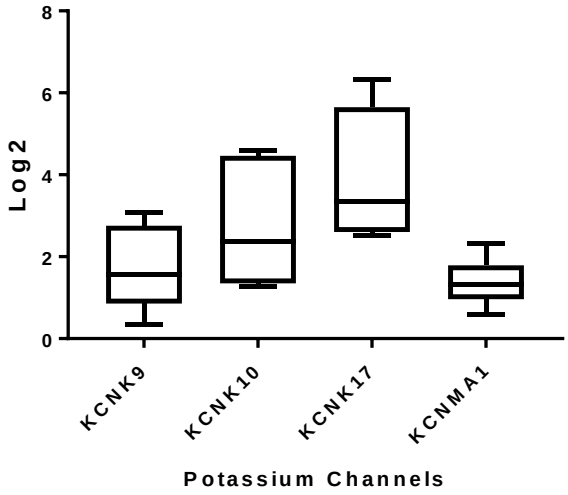



**Figure 69: $Log_2$ mRNA expressions of potassium channels in decidua**

### 3.2.6 **Effect of different treatments on decidual tissue**

Decidual tissue was used to compare potassium channel gene expression in the endometrium, since it is composed mainly of stromal cells and any changes in gene expression could be caused by the cell population.

The $log_2$ mRNA expressions of KCNK9, KCNK10, KCNK17, and KCNMA1 were determined in decidual tissue (n = 6) (control) vs. decidual tissues of the same patient; the cells were incubated with the following treatments for 24 h: 5% $O_2$ (n = 6), intralipids (n = 6), and vitamin D (n = 6). There is alteration in gene expression (Figure 70). Paired *t*-test was conducted for every gene separately; statistically significant difference ($P < 0.05$) was found only for the KCNK9 control decidua and the decidua treated with vitamin D and incubated in 5% $O_2$ (Figure 71).

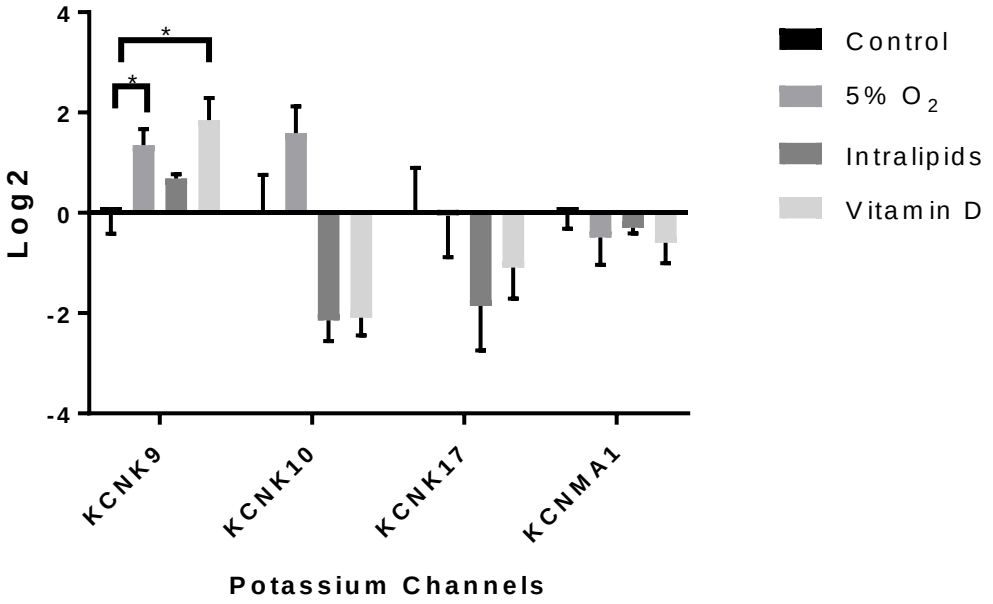
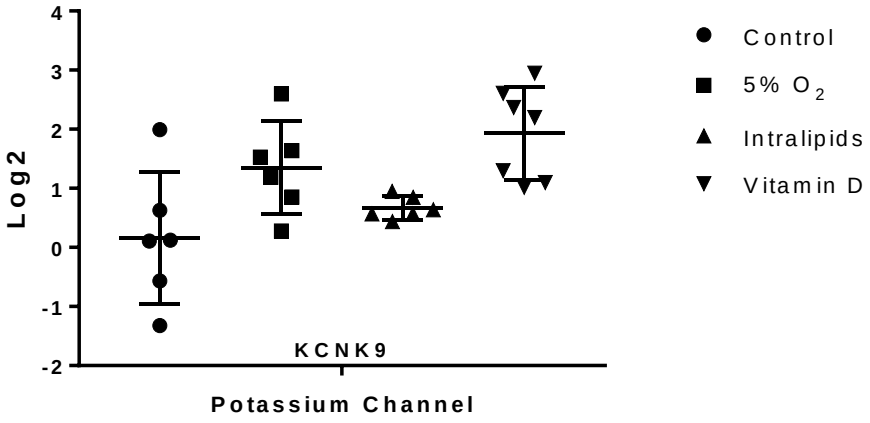
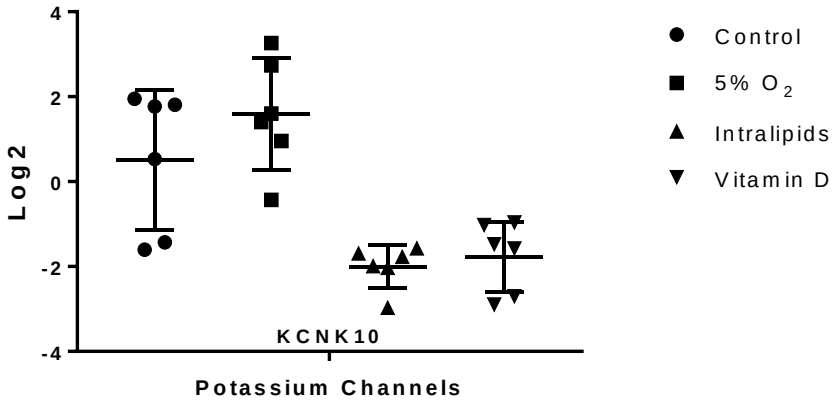
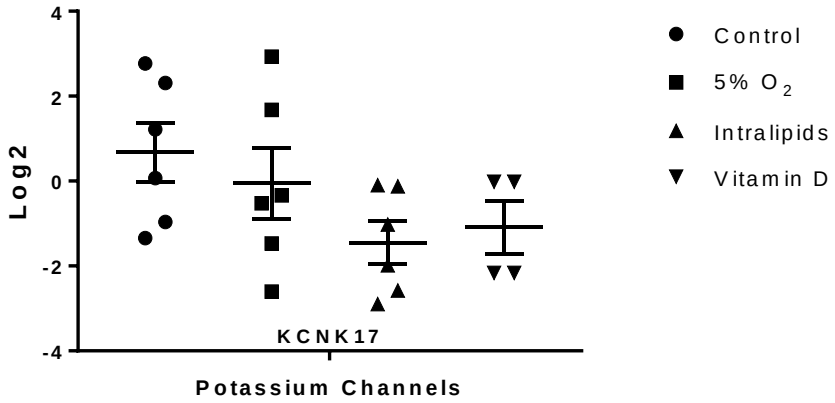



**Figure 70: PCR showing the $log_2$ gene expression of different treatments on potassium channel genes of decidual tissue.** Paired *t*-test was conducted for every gene separately; statistically significant difference ($P < 0.05$) was found only for the KCNK9 control decidua and the decidua treated with vitamin D and incubated in 5% $O_2$


4
3
2
1
0
-1
-2
Log2
KCNK9
Potassium Channel
Control
5% O2
Intralipids
Vitamin D


4
2
0
-2
-4
Log2
KCNK10
Potassium Channels
Control
5% O2
Intralipids
Vitamin D


4
2
0
-2
-4
Log2
KCNK17
Potassium Channels
Control
5% O2
Intralipids
Vitamin D

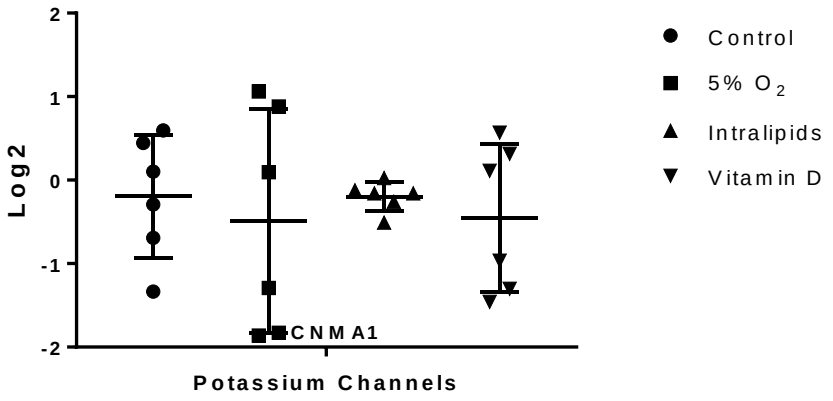



**Figure 71: PCR showing the $log_2$ gene expression of different treatments on each potassium channel separately in decidual tissue; bars represents mean data and standard deviation**

## 3.3 Flow cytometry

Flow cytometry was undertaken to detect potassium channel proteins at the single cell level. Flow cytometry is considered an efficient technique for quantifying the fluorescence signal of proteins and for correlating the intensity of the fluorescence to the number of proteins detected on the target cell surface (Suni and Maino, 2011, Hogg et al., 2015).

### Endometrium

### 3.3.1 Mid-secretory fertile vs. mid-secretory infertile

(Figure 72) shows the % gated potassium channels in both fertile secretory and infertile MS endometrial cells, where the % gated channels had slightly increased in the infertile group for the K2P (KCNK9, KCNK10, and KCNK17) channels, and only KCNMA1 showed less gated cells in the infertile group. Mann-Whitney test was carried out for each channel separately, showing no statistically significant difference between both groups across all channels.

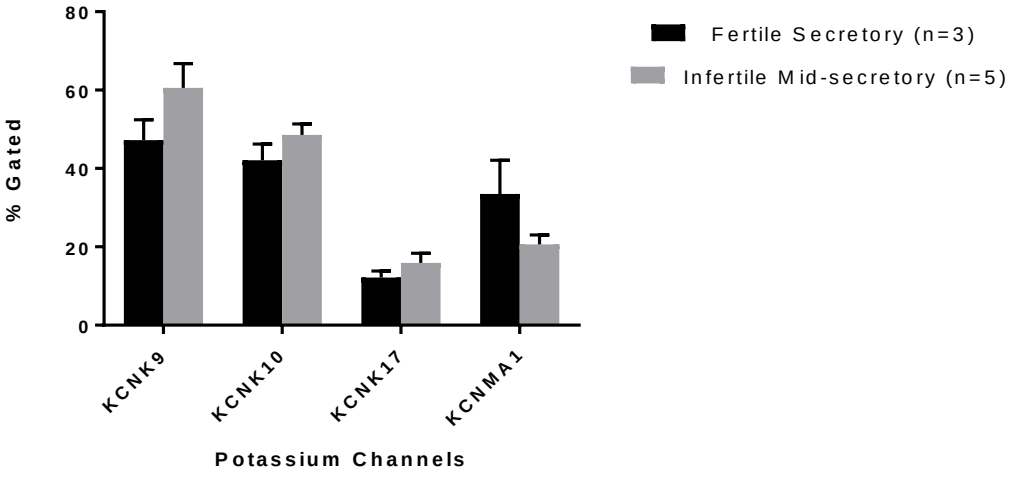



**Figure 72**: % gated potassium channels in fertile secretory vs. infertile mid-secretory endometrial cells

### 3.3.2 Effect of 5% $O_2$ incubation on potassium channels of MS infertile endometrium

In (Figure 73), K2P channels (KCNK9, KCNK10, and KCNK17) showed a decrease of % gated potassium channels in the treated infertile group. . A Mann-Whitney test was conducted for each gene separately, showing statistically significant difference ($P < 0.05$) for both KCNK9 and KCNK10 channels between both groups.

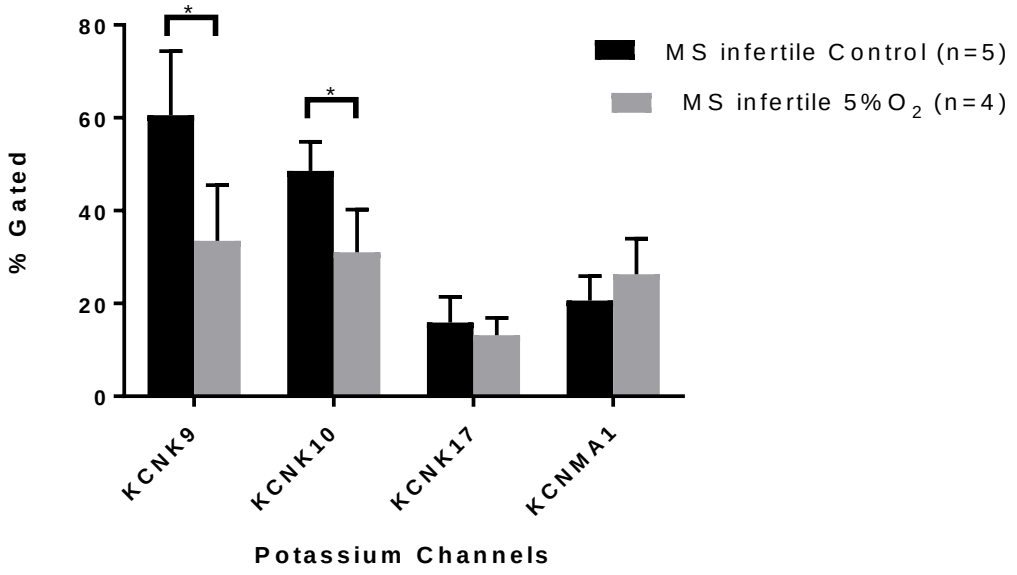



**Figure 73: % gated potassium channels in MS infertile control vs. MS infertile endometrial cells incubated in 5% O2.** A Mann-Whitney test was conducted for each gene separately, showing statistically significant difference ($P < 0.05$) for both KCNK9 and KCNK10 channels between both groups.

**Decidua**

### 3.3.3 Effect of acute and chronic 5% $O_2$ incubation on potassium channels of decidual cells

As mentioned in the materials and methods section, in acute 5% oxygen concentration, the oxygen concentration was changed in the last passage only (~24–36 hrs), while in chronic 5% oxygen concentration, the cells in the cell

culture were incubated from the first day (P0–D0) in 5% oxygen concentration (both samples from the same patient). (Figure 74) shows the effect of acute and chronic 5% $O_2$ on the percentage gating of potassium channels in decidual cells. All channels show a slight decrease in % gated under acute 5% $O_2$ incubation and a massive decrease under chronic incubation. Kruskal-Wallis test was done for each channel separately, and statistically significant difference ($P < 0.005$) was found between the control group and chronically incubated KCNK9 and KCNK10 channels and a statistically significant difference ($P< 0.001$) was found between the KCNK19 and KCNMA1 channels.

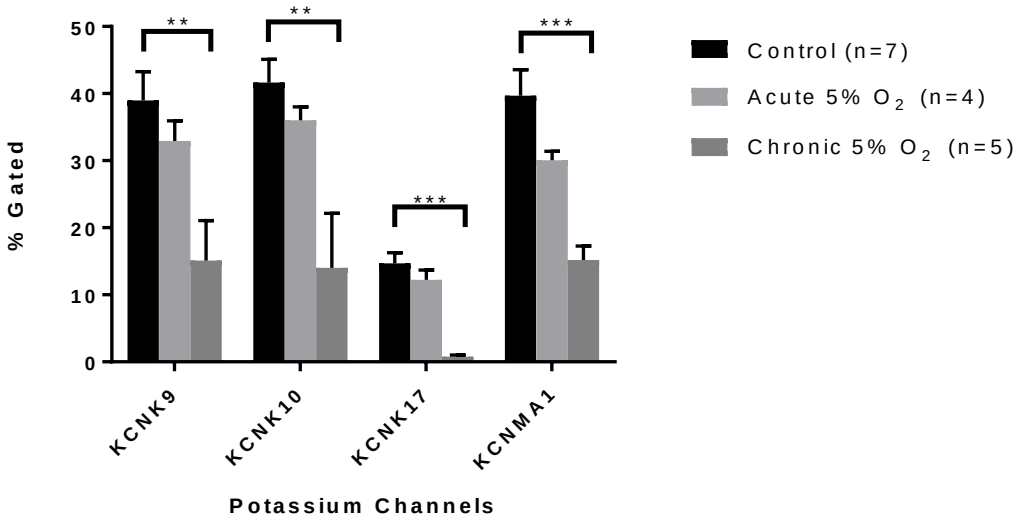



**Figure 74: % Gated potassium channels in decidual cells incubated in 5% O2.** Kruskal-Wallis test was done for each channel separately, and statistically significant difference ($P < 0.005$) was found between the control group and chronically incubated KCNK9 and KCNK10 channels and a statistically significant difference ($P< 0.001$) was found between the KCNK19 and KCNMA1 channels.

# 4 Discussion of validation and optimisation of techniques

## 4.1 Reference gene determination

RT-qPCR is a widely used technique to sensitively quantify genes expression. In order to normalise and optimise the PCR results between samples, laboratories use the housekeeping reference genes as internal controls (Hruz et al., 2011).

Housekeeping reference genes are specifically chosen for all the different conditions in a study. In our study, we referred to the 'Minimum Information for Publication of Quantitative Real-Time PCR Experiments' as our guidelines in order to deliver precise and reproducible results by reporting parameters such as RNA concentration, RNA integrity, reaction volume, and calibration curves (Bustin et al., 2009, Jacob et al., 2013).

**Endometrium:** In our experiment, it was found that *YWHAZ*, *TOP1*, and *ACTβ* are the most stable housekeeping reference genes out of six housekeeping reference genes were chosen to be tested from the available housekeeping genes (*ACTβ, CYC1, HPRT1, PPIA, TOP1*, and *YWHAZ*). These were the common genes used in the studies by Sadek et al., Lin et al. and Romani et al. Sadek et al. tested the variation in the stability of housekeeping genes in the endometrium of healthy and PCOS women. The study used nine housekeeping reference genes (*APTB, YWHAZ, 18S, CYC1, RPL13, SDHA, TOP1, GAPDH,* and *ACTβ*). The authors found that *YWHAZ*, *CYC1*, and *ACTB* were the most stable genes in both the control and PCOS endometrium groups (Sadek et al., 2011). These results supported our results concerning both YWHAZ and ACTβ.

Romani et al. tested housekeeping genes for human endometrioid endometrial carcinoma tissues. The study used the reference genes *GAPDH*, *B2M*, *ACTβ*, *POLR2A*, *UBC*, *PPIA*, *HPRT1*, *GUSB*, *TBP*, and *H3F3A*. The analysis of the

expression levels of all the used genes confirmed that *HPRT1* and *PPIA* were the most stably expressed and that *PPIA* could be the single best reference gene, regardless of the study group studied (Romani et al., 2014).

Lin et al. conducted a study on mouse uterus during the peri-implantation period, using the genes *PPIA, RPLP0, HPRT1, GAPDH, ACTB, TBP, B2M, 18S, UBC*, and *TUBA*. The study identified *RPLP0* and *GAPDH* as the most stable genes and *B2M* and *18S* as the least stable reference genes (Lin et al., 2013). These results did not support our findings for the most stable genes but gave similar opinion on the least stable genes.

A recent study published in 2017 by Stocker et al., identified stably expressed reference genes in the fertile human endometrium of women with recurrent implantation failure (RIF) and recurrent miscarriage (RM). The author tested 6 genes in the RIF group (*UBE2D2, ACTβ, PRDM4, ENOX2, SCLY*, and *GAPDH*), 10 genes in the RM group (*GAPDH, PRDM4, TYW1, ACTβ, SCLY, UBE4A, ENOX2, ERCC6, RNF20*, and *UBE2D2*), and 8 genes in the control group (*PRDM4, ENOX2, ERCC6, UBE2D2, UBE4A, RNF20, GAPDH*, and *ACTβ*). Stocker et al. found that the five most stable housekeeping genes in the endometrial tissues of the control group were *PRDM4, ENOX2, ERCC 6, UBE2D2*, and *UBE4A*. The five most stable housekeeping genes in the RIF group were *UBE2D, ACTβ, PRDM4, ENOX2*, and *SCLY*. In the RM group, *GAPDH, PRDM4, TYW1, ACTβ*, and *SCLY* were found to be the most stable genes (Stocker et al., 2017). These data do not support our findings since different housekeeping genes were tested other than *ACTβ* although *ACTβ* was found to be stable in all of the three groups.

**Decidua:** In our experiment, *YWHAZ, HPRT1*, and *ACTβ* were the most stable housekeeping reference genes. We chose eight housekeeping reference genes for further testing: *ACTβ, B2M, GAPDH, GUSB, HPRT1, PGK1, PPIA*, and *YWHAZ*. There is a dearth of literature on the decidual tissue of the placenta and most studies involved the whole placenta in general or without

identifying the area where the tissue was taken from in the placenta (Meller et al., 2005, Murthi et al., 2008, Cleal et al., 2009).

In the study conducted by Cleal et al., *ATP5B, ACTB, B2M, CYC1, EIF4A, GAPDH, SF3A1, TOP1, UBC, YWHAZ*, and *18s* genes were used and both the most stable genes were *UBC* and *TOP1* followed by *EIF4AT* then *YWHAZ* (Cleal et al., 2009). Other studies were carried out with unhealthy placentae, like the study by Meller et al. that examined biopsy specimens from diabetic mothers' placental tissues containing both maternal and fatal parts of the placenta. The housekeeping reference genes used were *B2M, GAPDH, HMBS, HPRT, SDHA, TBP*, and *YWHAZ*. *TBP* and *SDHA* showed to be the most stable genes followed by *YWHAZ, HPRT, HMBS, GAPDH*, and *B2M* (Meller et al., 2005).

In another study conducted by Murthi et al., placentae were obtained from foetal-restricted growth pregnancies and samples were randomly taken from parts of the central placental cotyledons. The housekeeping genes used were *ACTβ, GAPDH, 18S, TBP,* and *SDHA* and *YWHAZ, GAPDH* and *18S* were most stable, while *SDHA, ACTB*, and *TBP* were the least stable (Murthi et al., 2008).

The results of most of these studies were not consistent with our findings, except the fact that *ACTB* and *YWHAZ* were stable, but not the most stable genes. This could be explained by several reports indicating that the expression of *GAPDH* and *ACTβ* varies across tissues, during cell proliferation and development (Murthi et al., 2008).

## 4.2 Standard curves and assay efficiency

Housekeeping genes are used for the maintenance of basal function of cells, regardless the specific role it plays in the tissue or organism. Comparison of the target genes with these stable reference gene is important to adjust for potential experimental variables, such as the starting material quantity, enzymatic activity, differences in overall transcription between tissues, and the

effect of different conditions and treatments. Therefore, it is ideal to use housekeeping genes that are sufficiently and stably expressed in the tissue of interest, regardless of the physiological or pathological conditions (Stocker et al., 2017). It was reported that experimental treatment affects the expression of the internal control genes (Schmittgen and Zakrajsek, 2000), so it was crucial to test and confirm the stability of the chosen housekeeping genes with all the used treatments.

## 4.3 Validation for PCR

### Menstrual cycle phases

For PCR, only KCNK17 showed a statistically highly significant difference ($P < 0.001$) between the EP and MS endometrium. Also, KCNK17 showed statistically significant difference ($P < 0.05$) between the LP and MS endometrium as well as between ES and MS fertile human endometrium.

One way to determine gene expression through different menstrual cycle phases was to test and validate if we could add the ES (n = 6) and MS (n = 4) fertile patients forming one large (n = 10) secretory phase fertile group to have stronger statistical comparison with the infertile group. However, since KCNK17 gene expression showed a significant difference between ES and MS stages, the MS group was not mixed with the ES, and we used the small MS group (n = 4).

The KNCNK10, KCNK17, and KCNMA1 genes were more highly expressed in the secretory phase than in the proliferative phase. This data was supported by Zhang et al. who studied the large-conductance calcium-activated potassium channel in human endometrial epithelial cells (EECs), where KCNMA1 appeared to be expressed in a phase-related manner through the menstrual cycle, with a higher level in the MS phase than in the proliferative phase (Zhang et al., 2012). Patel et al. showed the opposite for KCNK17, which appeared to be more highly expressed in the proliferative phase than in the secretory phase (Patel et al., 2013). This observation could be linked to

oestrogen/progesterone through the cycle, as oestrogen stimulation of KCNMA1 channel activity occurs via cGMP-dependent phosphorylation and the effect of 17β-oestradiol on KCNMA1 is blocked when inhibiting cGMP-dependent protein kinase (PKG) activity, mirrored by exogenous cGMP or by PKG activity stimulation. This was shown by White et al. in coronary arteries, as they proposed that cGMP-dependent phosphorylation of KCNMA1 opens the channel leading to the relaxation of coronary arteries (White et al., 1995).

KCNMA1 channels play many roles in cellular functions, such as the synthesis and secretion of hormones (Braun et al., 2008). The phase-related expression of KCNMA1 channels through the menstrual cycle suggests that it plays a role in human endometrium and may be involved in both endometrial receptivity and embryo implantation (Zhang et al., 2012).

### Snap-frozen vs. incubated samples

The results showed that gene expression in the incubated tissue was lower by one or two folds at maximum, but with no statistically significant difference. Also, the RNA concentrations dropped drastically.

These data were taken for two validation decisions, i.e. to decide if we can combine snap-frozen data with incubated data as one group of MS infertile or if we should separate them, comparing only incubated MS infertile with incubated treated infertile samples. The other aspect was to determine whether it will be reasonable to relatively compare with the snap-frozen fertile data. In case there was significant difference between snap-frozen and incubated data, then we would not be able to predict whether the relative difference obtained was due to the difference between fertile and infertile (control and treated) groups or due to the incubation effect.

## 4.4 Validation of flow cytometry

### 4.4.1 Tissue cell population

The main aim of this thesis was to mimic the endometrial tissue in vitro as a group of different cell populations, without focusing on the role of every cell population separately, similar to the in vivo situation, where the embryo faces the whole endometrium during the implantation phase.

The problem arsing was mainly during cell culture, as the cells needed to be used later for flow cytometry and for determining the TEER (Chapter 4). The cell population changed with cell culture passages, as detected morphologically under the microscope. Chan et al. reported that by days 8–10 in cell culture, some of the cells are lifted off the plate surface after going through apoptosis. This is a group of cells formed by mature epithelial cells and they vanish after 15 days in culture (Chan et al., 2004).

Given the important role of both stromal and glandular epithelial cells in implantation, microarrays demonstrated that stromal and epithelial cells have unique mRNA signatures, each dependent on the day of the cycle (Evans et al., 2012). Therefore, we wanted to ensure that both cell populations were present throughout the experiments without identifying which cells are responsible for the results obtained from both endometrial and decidual cells.

Immunofluorescence after the end of the first passage showed positive staining for both vimentin (stromal cells) and cytokeratin (epithelial cells). For flow cytometry, since the cells were not sorted first before staining, we depended on both immunofluorescence and the assumption that the cell population in the flow was the result of passages, showing the presence of both cells populations and the percentage gated reduction through passages. It was important to clarify that the changes in the results were due to the effect of the treatment used and to exclude biases that could arise because of the changes in cell population during cell culture.

### 4.4.2 Excluding false-positive results

It is important to exclude any false-positive results that may arise from the false staining of lymphocytes (mainly NK cells) that express the voltage-gated potassium channels (Hu et al., 2009) and two-pore domain K2P channels (Schulte-Mecklenbeck et al., 2015). By the end of the cell culture (P0), the number of NK cells had significantly reduced preventing any false-positives on further passaging.

### 4.4.3 Optimum cell culture passage

This step was taken to reach the optimum passage at which we can use the potassium channel antibodies, especially since prolonged culturing (5–6 passages) alters their key functions and they cease to reflect their original source material (Hughes et al., 2007). From our results, we found that the optimum balance between having a good number of cells that enough to apply staining for four channels plus cells only and secondary antibodies only tubes on every sample and in the same time to be sure that the channels are not affected by prolonged passaging, so the end of passage two was chosen after considering the validation of the cell population ratio.

### 4.4.4 Optimum concentration of antibodies

This step was taken to validate the optimum concentration of antibodies used, especially as none of the potassium channel surface antibodies were commercially available for flow cytometry (provided as primary and secondary antibodies together). The antibody–antigen affinity can be affected by many factors including temperature, pH, and buffer constituents. Any changes in the concentrations of an antibody and an antigen solution can also affect the extent of the formation of antibody-antigen complex. Abcam immune fluorescence antibodies were used and from the four antibodies concentrations used, the 1:20 and 1:50 were ideal, generating specific staining with the least amount of background. There was not much difference between the two concentrations. The 1:50 concentration was chosen as it was sufficient for a good-quality

analysis without compromising the cost or quality. Abcam guidelines suggested 1:100 to be the ideal dilution for flow cytometry.

# 5 Discussion of results (target gene expression and protein translation)

All endometrial samples were taken by means of pipelle (EndoCell) sampling, which is a minimally invasive method with which it is possible to harvest an adequate amount of endometrial tissue (depending on the cycle phase and the surgeon's experience). This method yielded a sufficient number of cells to obtain good quantity and quality of RNA for gene expression. In relation to curettage, it should be considered for use in genomic studies (Kissel et al., 2013); pipelle sampling has fewer complications than curettage (e.g. perforation and anaesthesia complications).

## 5.1 Fertile vs. infertile

The results show that all target genes have lower gene expression in MS infertile endometrium, especially the KNCK17 gene, but none of the genes showed a statistically significant difference ($P < 0.05$). For protein translation, on flow cytometry, the % gated cells were slightly greater in the infertile group of the K2P (KCNK9, KCNK10, and KCNK17) channels and only KCNMA1 showed few gated cells in the infertile group.

The KCNMA1 result is consistent with that of Zhang et al. who showed lower KCNMA1 protein levels in the mid-secretory endometrial samples collected from failed IVF patients in comparison with those of successful pregnancies (Zhang et al., 2012). The activation of KCNMA1 channels leads to membrane hyperpolarisation and intracellular $Ca^2$ elevation, including the regulation of membrane potential and intracellular $Ca^2$ homeostasis in endometrial cells. The regulations lead to the synthesis and secretion of important factors (leukaemia inhibitory factor, integrin-3, and claudin-4) in the endometrium during the window phase of implantation, modulating the endometrial functions needed for embryo implantation (Zhang et al., 2012).

KCNK9 is an acid-sensitive potassium channel and there is a change in the pH through the female genital tract. Human cell homeostasis is highly sensitive to pH and regulated through intracellular pH (pHi). The pHi regulatory mechanism in the human pre-implantation embryo remains unknown (Lane and Bavister, 1999), but Spencer et al. reported a link between pH and fertility, stating that low endocervical mucus pH (generally pH ≤ 4.5) level may be associated with infertility (Spencer et al., 2013). This could explain the low expression of KCNK9 in the infertile group.

## 5.2 Expression of potassium channel genes in decidua

The results for the decidua potassium channel gene expression were consistent with literature and supported by the findings of Bai et al. In the cytotrophoblast cells, where mRNAs encoding KCNK10 and KCNK17 were detected, weak or no signals were observed for KCNK9 (Bai et al., 2005b). Also, another explanation for late gestation and diminishing of KCNMA1 current is attenuated membrane channel expression (Eghbali et al., 2003). However, this appears to be species-specific, as KCNMA1 acts differently in pregnant rats, unlike in mice, where KCNMA1 current inhibition occurs as a result of downregulation of channel transcript and protein expression. On the other hand, KCNMA1 channel levels increase in the late pregnant, non-labouring myometrium of humans, while decreased KCNMA1 channel expression is observed in pre-term and term labouring myometrium. This indicates that they play a role in the process of inducing spontaneous labour contraction (Brainard et al., 2007).

## 5.3 Effect of treatments on gene expression and protein translation

The overexpressed molecular functions in the MS endometrium involves oxidoreductase activity, carbohydrate binding, receptor binding, cellular components, and spindle microtubules (Diaz-Gimeno et al., 2011).

As mentioned before, the aim of using the decidual tissue, which has a similar composition to the endometrium, but with more stromal cells, was to determine whether the treatments have the same effect on potassium channels in both tissues or every type of tissue responds in a different way, since both tissues contain difference ratios of cells.

### Incubation in 5% $O_2$

The cell variation, composition and concentrations; and correlation between oxygen concentration, fluid osmolarity, dissolved chemicals, temperature, and pH within the female reproductive tract have not been well interpreted (Wale and Gardner, 2015). Elevated and non-physiological oxygen levels lead to the formation of ROS that have a negative impact on embryo development due to oxidative stress and higher rates of fragmentation. Also, very low oxygen levels may affect the oxidative metabolic processes leading to embryogenesis impairment (Ng et al., 2017).

Many potassium channels (voltage-dependent K channels, MaxiK channels and TASK-like channels) were observed to be affected by hypoxia and hypoxia-induced signals; hence, it is believed that potassium channels play an important role in sensing any reduction in oxygen concentration in the chemoreceptive cells of the carotid bodies or the lungs (Heitzmann and Warth, 2008).

**Endometrium:** The results showed lower expression of the K2P (KCNK9, KCNK10, and KCNK17) channels and higher expression of the KCNMA1, but with no statistical significance and under the effect of incubation in 5% oxygen concentration. Regarding flow cytometry, there was an increase in the % gated channels of KCNK9 and KCNK10 in the infertile control group, with a statistically significant difference ($P < 0.05$). The oxygen concentration used is considered the average physiological concertation, since oxygen concentration changes along the female genital track, ranging between 2% and 8% (Leese, 1995).

KCNK9 subunits play a functional role in chemo- and nutrient-sensing, taking part in many physiological stimuli, such as hypoxia (Duprat et al., 2007). The ROS formed as result of high oxygen concentration, disturb the KCNMA1 channels by changing the cysteine residue near the C-terminal of the channel's alpha subunit (Tang et al., 2004, Ghatta et al., 2006). Vascular KCNMA1 channel activation partly stimulates the vasodilator effects of endogenous nitric oxide (NO), where in hypertension, NO-mediated KCNMA1 signalling is either completely diminished or reduced (Ghatta et al., 2006, Fukami et al., 1998).

The changes in potassium channel expression could be linked to imbalance in the menstrual cycle hormones. Maas et al. reported that oxygen tension was lower during the follicular phase of the menstrual cycle (<10 mmHg) and that the oxygen tension in the oviduct increased after ovulation. This was explained by the hypothesis that ovulation stimulates local factors, such as an increase in blood flow increasing the oxygen flow, and changes through paracrine or autocrine signals – this remains to be proven (Maas et al., 1976).

Further evidence for increased oxygen tension during the ovulatory period is provided by the oestrogen effect. This is supported by the old studies of Resnik et al., who showed that administration of exogenous oestrogen after ovariectomy resulted in a significant increase in uterine blood flow, up to 10 times the basal level, and this was associated with a significant increase in the $pO_2$ level; however, progesterone was shown to hinder this increase (Resnik et al., 1974, Resnik et al., 1976).

Other studies have stated a contradictory opinion, namely that high intrauterine oxygen tension is observed during periods of low oestrogen and high progesterone (window of implantation period). This was explained by the role played by the functioning corpus luteum in the secretory phase in humans (Kigawa, 1981). The impact of oestrogen mediators on intrauterine oxygen tension is likely to be more complex. Oestrogen is a mediator of vascular response, inducing its effect through endothelial function with an increase in vascularity, and hyperaemia and oedema ensue (Storment et al., 2000).

Despite the evidence shown by the studies reporting the cyclic hormones linked to oxygen concentration and their influence in fertility, but this was not supported by our results that showed no statistically significant difference in the mRNA expression of the potassium channels in regular and irregular cycles, where hormones could play no role in their expression. Our assumption is consistent with that of Ng et al. who summarised the trends in the fluctuations of intrauterine oxygen tension within the menstrual cycle, stating that these fluctuations are not consistent amongst the studies, and there are no clear data on the early pregnancy state. Especially, in the presence of an embryo, the hypothesis will become more complicated as the oxygen requirement is low when the conceptus resides in the oviduct; then, the oxygen requirement increases drastically as it becomes a blastocyst in the intrauterine cavity (Ng et al., 2017).

This indicates that uterine contraction involves potassium channels, where oestrogen and progesterone influence the increase and decrease in uterine contractility, leading to a variation in oxygen tension. Hence, these hormones are linked and involved in the function of potassium channels (Ottosen et al., 2006). Uterine hyperperistalsis may contribute to subfertility and the use of oxytocin antagonists, such as atosiban, may be beneficial before and during embryo transfer to prepare the uterus for implantation (Ng et al., 2017).

The association of KCNMA1 channels with membrane and intracellular proteins is a novel mechanism to regulate uterine relaxation. This could be linked to contractions occurring during the window of implantation. Beta-receptor agonists are used widely in clinical practice to postpone pre-term labour. In the myometrium, activation of ß2-/ß3-receptors result in their functional complex with KCNMA1 channels, inducing channel activation and uterine relaxation (Chanrachakul et al., 2004, Doheny et al., 2005).

Expression of KCNMA1 channels is linked with chronic hypoxia as an important regulator of cell excitability (Kaczorowski et al., 1996). Studies have demonstrated that KCNMA1 channels are affected by acute hypoxia leading

to their suppression in the short term with a change in their properties, ending with marked physiological implications. These alterations are corrected with chronic hypoxia as a part of the channels' tolerance and remodelling by prolonged hypoxia (Peers and Kemp, 2004).

Another steroid hormone-regulated factor is the vascular endothelial growth factor (VEGF), which is a specific vascular endothelial mitogen, produced by the human endometrium. Vasoconstriction during menstruation leads to endometrial hypoxia, a possible stimulus for angiogenesis (Sharkey et al., 2000). In a study conducted by Popovici et al., they tested the hypothesis that hypoxia and cAMP, a known stimulus for endometrial decidualisation, induces VEGF in human endometrial stromal cells. These results demonstrate that both non-decidualized and decidualized endometrial stromal cells respond to hypoxia with increasing levels of VEGF and have an additive effect on VEGF production under hypoxic conditions (Popovici et al., 1999).

Hypoxia enhanced secreted immune-reactive VEGF levels by several folds in endometrial stromal and glandular cells, in vitro results provide a novel mechanism to account for both the peak in VEGF and angiogenesis in luteal phase human endometrium (Lockwood et al., 2002). VEGF expression was significantly higher in the women who became pregnant than in those who did not (Jinno et al., 2001).

**Decidua**: The channels in full-term decidua were different from those in the endometrium under the effect of 5% oxygen incubation that increased the expression of the P2K channels (statistically significant increase with the KCNK9) and decreased the expression of the KCNMA1.

Hypoxia in the endometrium is a physiologic event and may be seen as pathophysiologic event in the decidua of sever pre-eclampsia mothers and other vascular-related pregnancy disorders (Popovici et al., 1999)

As a consequence of these vascular adaptations and during a full-term pregnancy, major fluctuations in oxygen concentrations occur at the foeto-

maternal interface. Once the maternal perfusion of the placenta is established, the partial oxygen pressure increases sharply from around 20 mm Hg during the weeks 7–10 of pregnancy to reach 50 mm Hg during 11–14 weeks of pregnancy (Gellersen et al., 2007).

Such a drastic increase in oxygen tension at the utero-placental interface induces the production of ROS. In the absence of an effective defensive mechanisms, ROS (including superoxide anion, hydrogen peroxide, and hydroxyl radicals) cause damage to proteins, lipids, and nucleic acids. To overcome this oxidative stress, cells produce enzymes, which are able to neutralize ROS and repair their damage (Thannickal and Fanburg, 2000). Decidual cells are unique cells, adapted to cope and oppose the effect of ROS, preventing oxidative cell death; this emphasises their importance in maintaining tissue homeostasis during pregnancy (Gellersen et al., 2007).

### Intralipids

The mode of intralipid action was discussed in the systematic review (Chapter3), intralipid infusion is the current favourite immunotherapy of gynaecologists (Allahbadia, 2015). Recent research focused on their molecular biology and the parameters that play a role in the foeto-maternal cross-talk during the period of implantation (Allahbadia, 2015).

No direct link was reported between intralipids and potassium channels in any fertility-related publication despite them being used clinically worldwide (Lerchbaum and Obermayer-Pietsch, 2012). Hence, it was important to detect their effect on the potassium channel gene expression in both the endometrium and decidua.

In the endometrium, the intralipid showed upregulation in the expression of KCNK9, KCNK10, and KCNMA1 and downregulation in the expression of KCNK17, but with no statistical significance. While in the decidual tissue, intralipid incubation upregulated only KCNK9 and downregulated the expression of KCNK10, KCNK17, and KCNMA1.

The effect of the 20% intralipids (@Sigma Aldrich) on potassium channels could be explained by the effect of any of its components. Intralipids, according to the manufacturers' datasheet, consist of 18.0–22.0% soybean oil, 1.9–2.5% glycerine, 0.9–1.3% phospholipids, and free fatty acids <5.0 mEq/L, with a pH ranging between 8.0 and 8.9.

The upregulation of mRNA expression of KCNK10 channel in the endometrium could be due to the stimulation of KCNK10 by long-chain unsaturated fatty acids such as arachidonic acid (Bang et al., 2000). KCNK10 is also reversely activated by intracellular lysophosphatidic acid (LPA) (Chemin et al., 2004). There is strong evidence showing that arachidonic acid plays an important role in the activation of the C-terminus of KCNK10 channel (Kim et al., 2001). Fatty acid activates KCNK10 through the charged cluster (KKTKEE) of 25 amino acids (Patel et al., 1998) and through the production of phosphatidic acid and phospholipase D2 (Feliciangeli et al., 2015).

The results showed downregulation of KCNK17 mRNA expression in both infertile endometrium and decidua on incubation with intralipids. In humans, KCNK17 channel mRNA is expressed in many organs, such as the ovary, pancreas, and aorta (Kiyoshi et al., 2006), and in placental circulation (Wareing et al., 2006). Despite arachidonic acid causing the activation of some K2P channels, it still failed in altering or modulating KCNK17 channel currents (Girard et al., 2001).

The expression of KCNK9 mRNA in both endometrium and decidua was upregulated. Intralipid pH ranges between 8.0 and 8.9, which is higher than the intrauterine pH and since KCNK9 is an acid-sensitive potassium channel, the expression effect could be due to pH influence. In many tissues, potassium channels (KCNK2, KCNK3, KCNK4, KCNK5, KCNK9, KCNK10, KCNK16, KCNK17, and KCNK18) are found to be regulated by changes in the extracellular pH, leading to their inhibition by external acidification and their activation by external alkalinisation (Heitzmann and Warth, 2008).

Other components of the intralipids were reported to be involved in fertility and could have an effect on potassium channels, for example, the fatty acid composition of uterine phospholipids may affect the biophysical properties of the uterus and the cross-talk between the embryo and uterus (Hallé et al., 2015). These compounds are precursors of prostaglandins, influencing the immune system (Coyne et al., 2008, Sato et al., 2011) and modulating the expression of genes involved in uterine receptivity, providing the source of energy needed for decidualisation (Tsai et al., 2014). There was a positive correlation between the level of omega-6 fatty acids, especially arachidonic acid, and the implantation rate (Fattahi et al., 2018).

### Vitamin D

Vitamin D is a steroid hormone, playing a major role in the maintenance of calcium and phosphorus homeostasis (Holick, 2007). Despite the lack of evidence directly linking vitamin D and ion channels in the reproductive system, there is little evidence that vitamin D plays a role in the regulation of reproduction functions in both men and women, where some studies showed that vitamin D deficiency may result in reduced fertility potential in females (Anifandis et al., 2010).

It was reported that vitamin D deficiency is frequent in females undergoing IVF/ICSI (Vanni et al., 2014). In PCOS patients, vitamin D can improve insulin resistance and androgen levels (Wehr et al., 2010). Other studies reported higher pregnancy rates following IVF in women with sufficient levels of vitamin D in both serum and follicular fluid (Ozkan et al., 2010). This was explained in studies where target ablation of the 25-hydroxyvitamin D-1 hydroxylase enzyme could lead to uterine hypoplasia and absence of corpus luteum, contributing to infertility (Panda et al., 2001, Wang and Dey, 2006).

Besides the endometrium and implantation process, vitamin D plays a key role in the testis, showing a dual action of vitamin D, i.e. genomic or a non-genomic effect, resulting in amino-acid accumulation (Avila et al., 2007, Zanatta et al., 2011, Anagnostis et al., 2013).

The vitamin D used in our study was the active form of vitamin D, 1α,25-dihydroxyvitamin D3 (1,25-D3) (Sigma Aldric), which is also synthesised and metabolised in the human placenta (Avila et al., 2007, Barrera et al., 2008). This form of vitamin D was reported to be linked to potassium channels and reproduction in the study of Menegaz et al., showing for the first time the link between non-genomic 1,25-D3 potentiation of potassium currents and its coupling to exocytosis in the primary culture of Sertoli cells. This effect involves Ca2+ influx leading to K+ efflux and repolarisation, indicating that the steroid hormone 1,25-D3 appears to play a functional role in male fertility via stimulation of Sertoli cells in the testis (Menegaz et al., 2016), proven by addition of high K+ in the extracellular media that resulted in strong depolarisation of Sertoli cells followed by repolarisation with the efflux of potassium ions through the voltage-gated K+ channels (Menegaz et al., 2016).

# 6 Conclusion and future work

The current study provided direct knowledge of the potassium channels in the human endometrium in relation to infertility treatment. The study explored the modulation of potassium channels in the human endometrium through the menstrual cycle and in the infertile endometrium, how the channels could be important for decidualisation and timely embryo implantation, and how potassium channel regulation plays an important role in successful embryo implantation.

Using cultured stromal and epithelial cells separately could give a better understanding of each cell's function and role within the endometrium during the menstrual cycle and also in relation to different pathologies.

In future research, mutations within potassium channel genes could be considered, since scientific publications have reported mutations in subunits of potassium channels. These mutations are associated with specific diseases and disorders (Wulff et al., 2009). However, there are several studies on mutations whose clinical importance remains still unclear (Kocić, 2012).

# Chapter Six: Validation of EpiQuik™ Tissue Methylated DNA Immunoprecipitation Commercial Kit and Epigenetic (DNA Methylation) Regulation of Gene Expression of Potassium Channels in Endometrium

# 1 Introduction

As shown in the previous chapter, changes in the expression of the potassium channel genes (*KCNK9*, *KCNK10, KCNK17*, and *KCNMA1*) were observed in both the fertile and infertile groups and also through the menstrual cycles phases. As described in the main introduction (see section 1.x.y), epigenetic alterations such as DNA methylation that are brought about by environmental factors can often underlie changes in gene expression. In this chapter, we investigated the DNA methylation profile at the promoter region of the channel genes to determine whether the changes in expression observed in the endometrium at different stages of the menstrual cycle are associated with changes in DNA methylation. To do this, we chose the method of immunoprecipitating methylated DNA from tissues because methylated DNA immunoprecipitation (MeDIP) method, based on the quantification of methylated DNA fragments enriched using an antibody specific for methyl-cytosines (Tollefsbol, 2011). Hence, the methodology could only be employed to identify highly methylated genomic regions and can be used indirectly to map hypo-methylated ones (Pelizzola and Molinaro, 2011).

**Aims of the chapter**

The aim of this chapter was to characterise the changes in the epigenetic profile within the promoters of potassium ion channels in the endometrium using the EpiQuik kit because it is designed to work directly on tissue, and is ideal for the very small endometrial samples. This was achieved by:

1. Designing and optimising primers for the amplification of the promoter regions of the potassium channel genes.
2. Adapting the EpiQuik™ Tissue Methylated DNA Immunoprecipitation Kit to work optimally with endometrium and decidual tissue.
3. Using the optimised protocol to study the methylation profile of potassium channel gene promoters in primary tissues.

# 2 Results

The initial experiment set-up was prepared as per the manufacturer's recommendations and protocol (Figure 75). After lysis and sonication, the cell lysate was removed for agarose gel analysis. This revealed that the sonication had not worked as expected as no sheared DNA between 200 and 1000 bp was observed. Hence, the experiment was terminated to address the problems with the initial lysis and sonication steps.

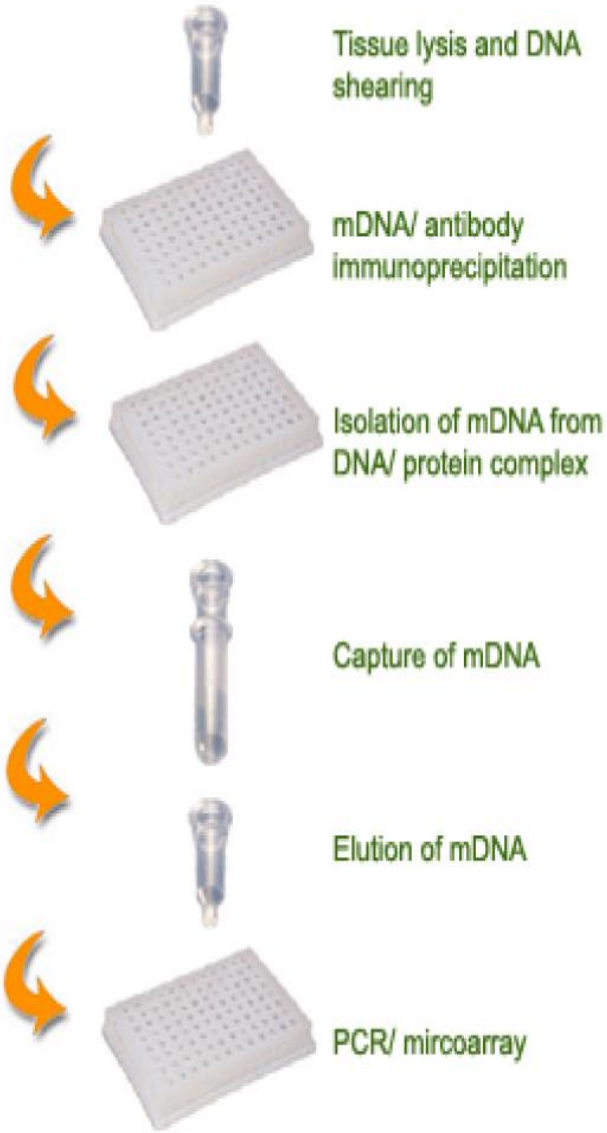
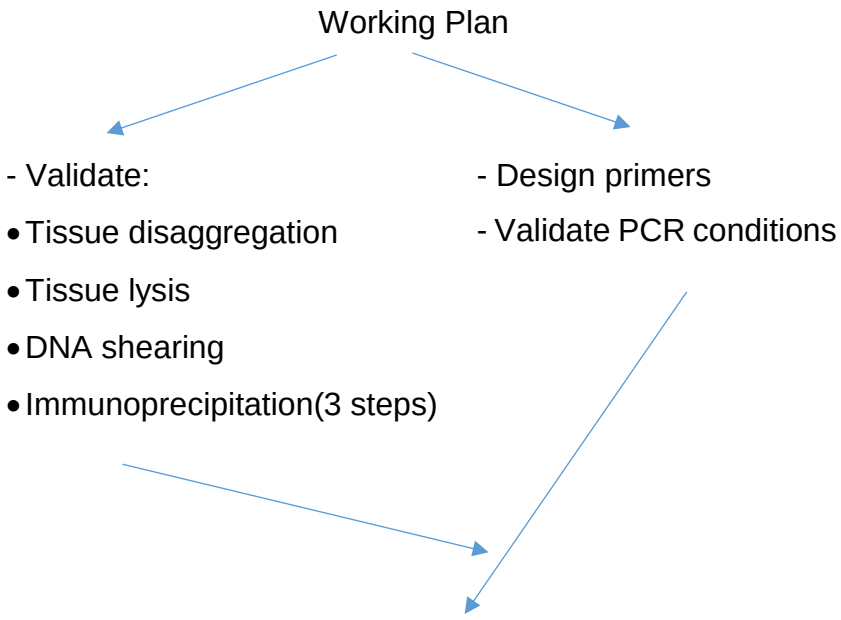



**Figure 75: EpiQuik™ Tissue Methylated DNA Immunoprecipitation Kit protocol**

Several other protocols were consulted, including the DNA methylation protocol (Mills and Ramsahoye, 2002) and methylated DNA immunoprecipitation (MeDIP) (Weber and Schübeler, 2007); (Mohn et al., 2009); (Sørensen and Collas, 2009); (Thu et al., 2009) and epigenetic protocols (Tollefsbol, 2011). It was difficult to find specific troubleshooting to address the failure in the EpiQuik™ protocol.

The target plan (Flowchart 3) was to validate this protocol to reach the optimum tissue disaggregation, tissue lysis, and DNA shearing steps before proceeding with the methylated DNA immunoprecipitation step. In parallel, I designed the primers and identified the best PCR conditions for amplification of the promoter regions of the potassium channel genes.




**Flowchart 3**: The working plan to validate the EpiQuik™ kit protocol

## 2.1 Designing of DNA primers

Previous studies showed that DNA methylation has functional roles especially when located within the regulatory regions (e.g. promoters) of the genes (Suzuki and Bird, 2008). Targeted CpG primers were designed using the UCSC Genome Browser and Primer-Blast software (Figure 76). Many factors were considered while choosing the most suitable primers. First, the length of the sonicated DNA used sets a limit to the length of the PCR amplicon as it is not possible to amplify fragments that are longer than the sonicated fragments. Second, to be located within the promoter region, CpG islands and histone modification tracks were used for identification. In addition to the potassium channel genes, in order to be able to interpret the result, positive controls (regions that are known to be methylated) and negative controls (regions that are known to be unmethylated) were needed. Based on published data, TSH2B and H19ICR were chosen as positive controls. This step was done in parallel to the validation step. Standard curves were plotted using 5 dilutions (100/10/1/0.1/0.01 ng/µL) on sheared DNA (after being optimised) for both the designed primers (*KCNK9, KCNK10, KCNK17*, and *KCNMA1*) and the two positive controls that were purchased (TSH2B and H19ICR) (Figure 77- 82). The standard curve ensures that the primers detect their target efficiently and precisely, which is critical.

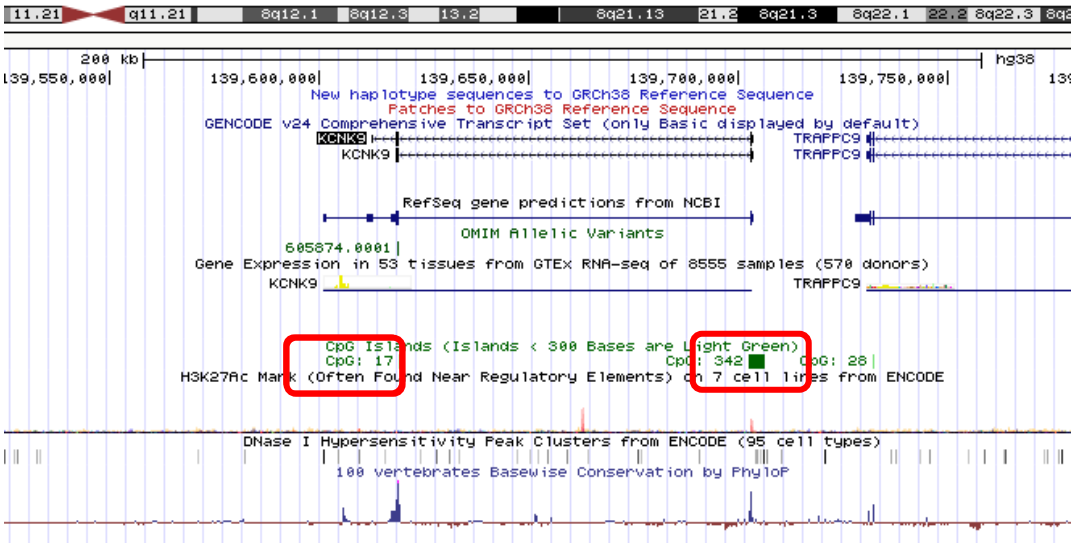



**Figure 76: CpG Islands on *KCNK9* using UCSC Genome**

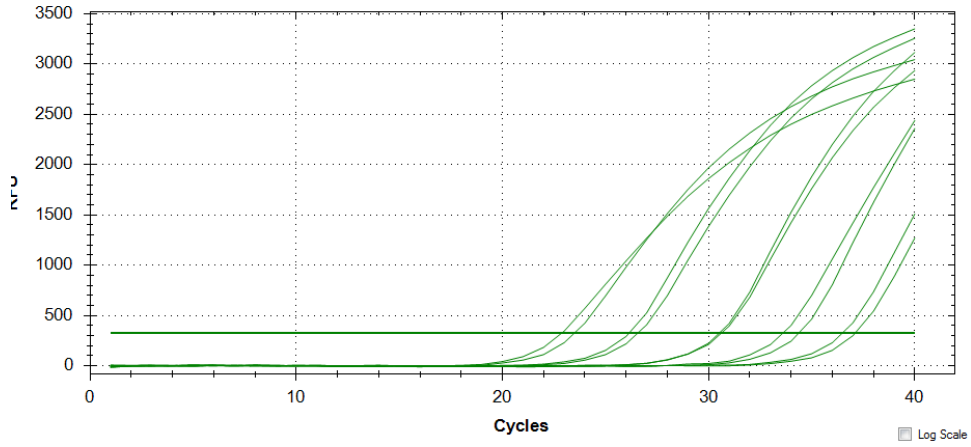



**Figure 77: Standard curve for TSH2B (positive control)**

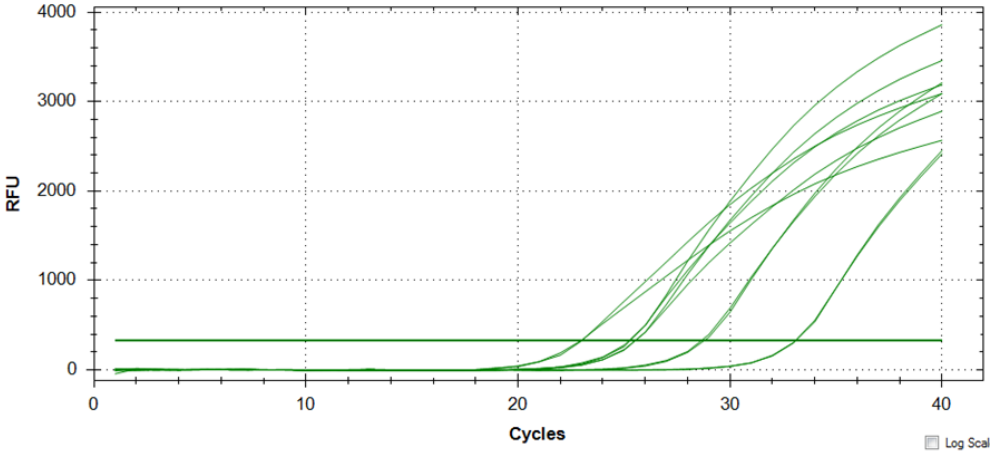



**Figure 78: Standard curve for H19ICR (positive control)**

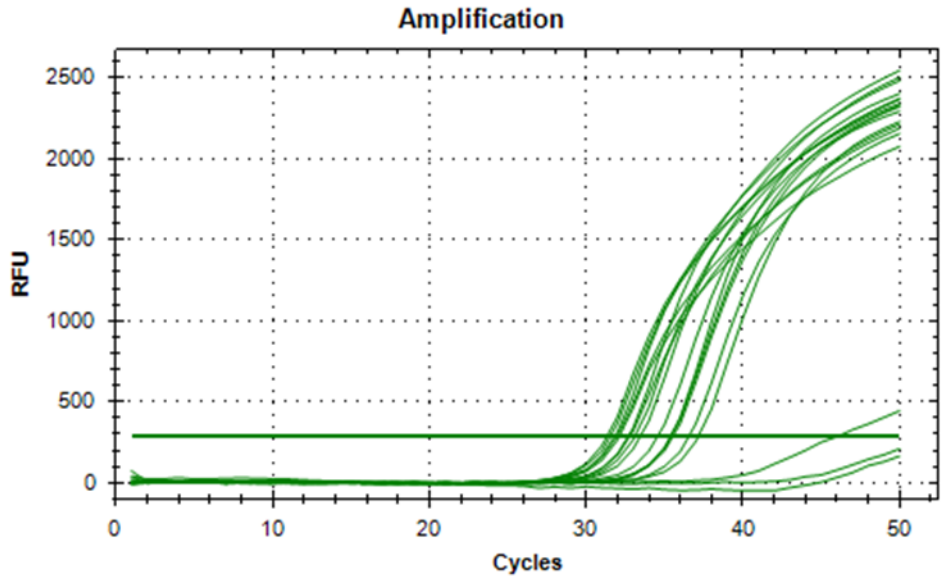



**Figure 79: Standard curve for the designed primer of *KCNK9***

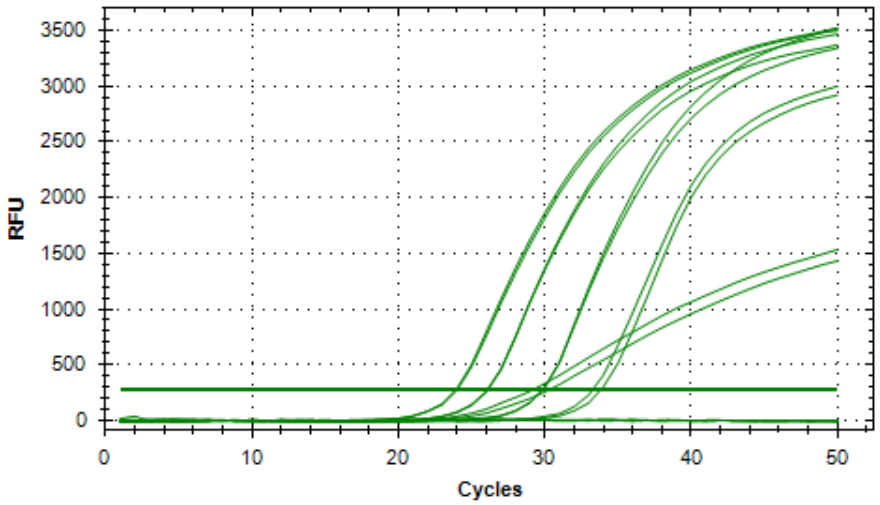



**Figure 80: Standard curve for the designed primer of *KCNK10***

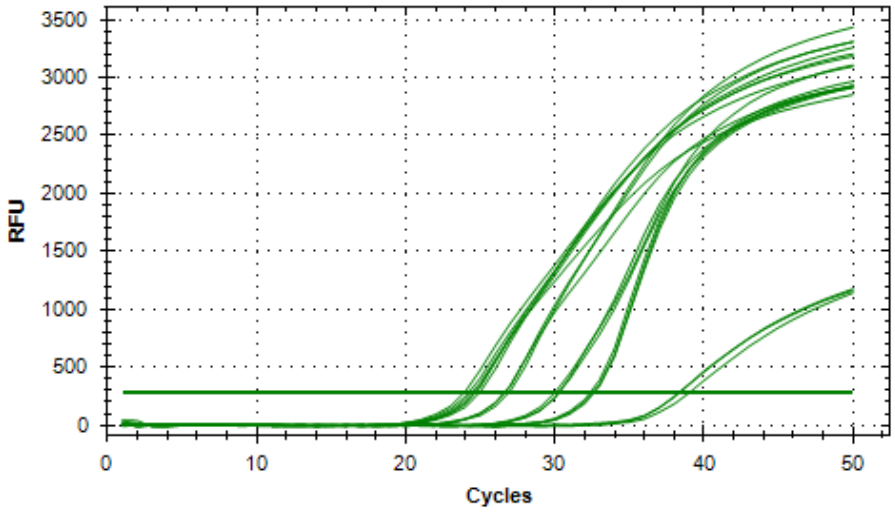



**Figure 81: Standard curve for the designed primer of *KCNK17***

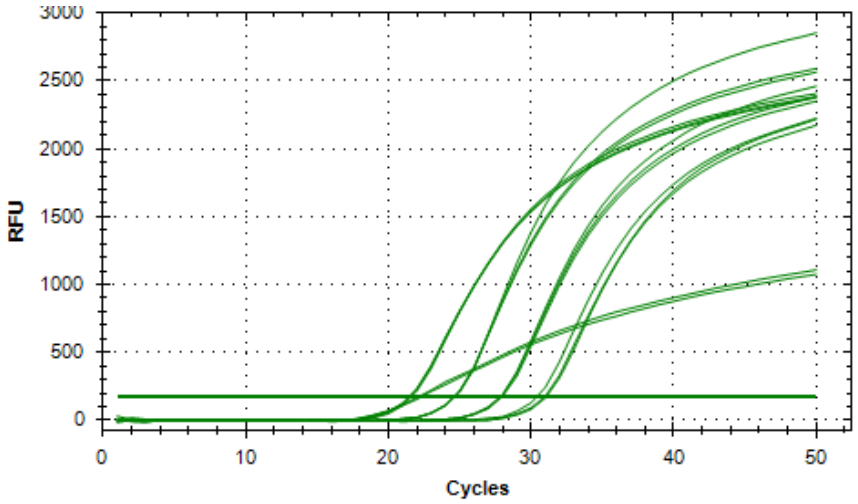



**Figure 82: Standard curve for the designed primer of *KCNMA1***

The *KCNK9* did not work well on different dilutions and we decided to study CpG islands *(CGI) 342*, because its CpG island was located at the annotated promoter end of the gene (note that the gene on the genome browser image was annotated on the minus strand so the promoter is to the right of the gene, associated with *CGI 342*).

On analysing the designed primers, it was found that:

1. When analysed by BLAST, they have the potential to bind to other regions in the human genome.

2. The expected product size was 862 bp in length and given that sheering the DNA by sonication yields fragments of about 500 bp (targeted) on average, it is very unlikely to see the product. Therefore, two new *KCNK9* primer pairs were designed (Table 20).

| | **Forward** | **Reverse** | **F1-R1: PCR product size** |
|---|---|---|---|
| Old *KCNK9* used | TCGCAAAGTAGAAGGAGCCG | GGTAAAAGGGGAAGCAGGTT | 862bp |
| New *KCNK9* (1) | CAGGCACTCTCCCCAGATTC | CCAAATCGAAGTGGCTCTGC | 389bp |
| New *KCNK9* (2) | TAGAAGGAGCCGGCGAATTT | CGTCTGCACCTTCACCTACC | 219bp |

**Table 20: The new *KCNK9* primers designed**

These primer pairs were tested on standard curves (Figure 83-84). Based on these results, primer pair 2 was selected for further experiments

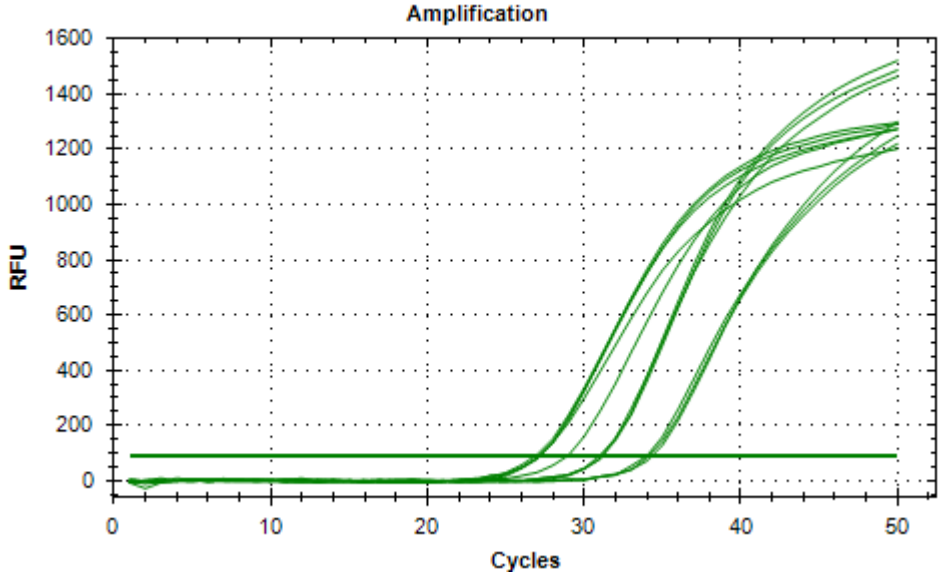



**Figure 83: Standard curve for the designed (1) primer of *KCNK9***

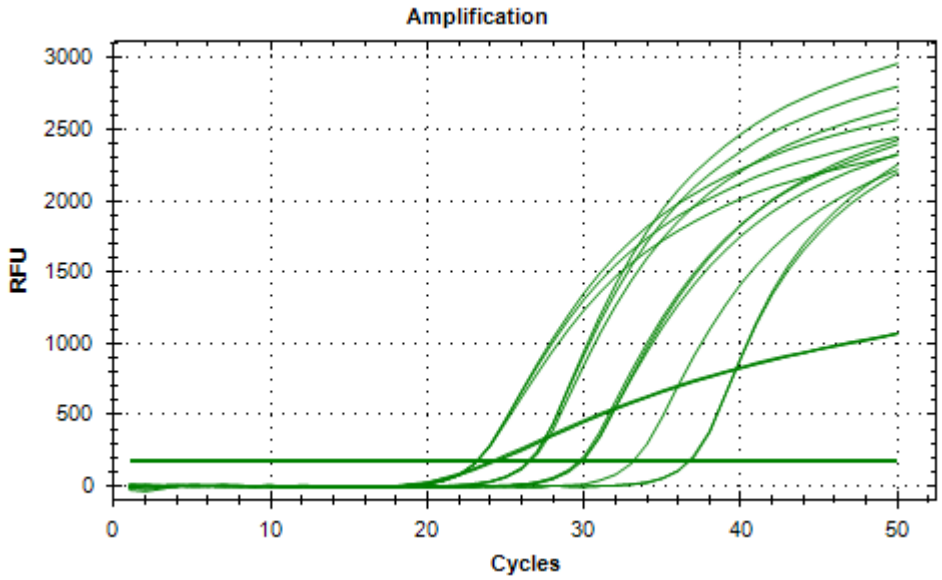



**Figure 84: Standard curve for the designed (2) primer of *KCNK9***

## 2.2 Validation of tissue disaggregation step

Per the original kit protocol, obtaining the optimum amount of tissue to start with, using the rest of the tissue in other experiments, and saving buffers supplied by the kit were some of the challenges. Different amounts of tissue were used.

The tissue pieces were weighted and transferred to a Dounce homogeniser with 1 mL of homogenising buffer per 200 mg of tissue. To determine the best tissue disaggregation, different number of strokes were applied (10, 20, and 30 strokes), proceeding with the normal protocol steps of lysis. Sonication was also performed at a variety of durations (10, 20, and 30 min) and the length of the sheared DNA was checked on agarose gel (Figure 85). A sample of the disaggregated tissue was also loaded on a slide and subjected to high magnification microscopy to check for nuclei.

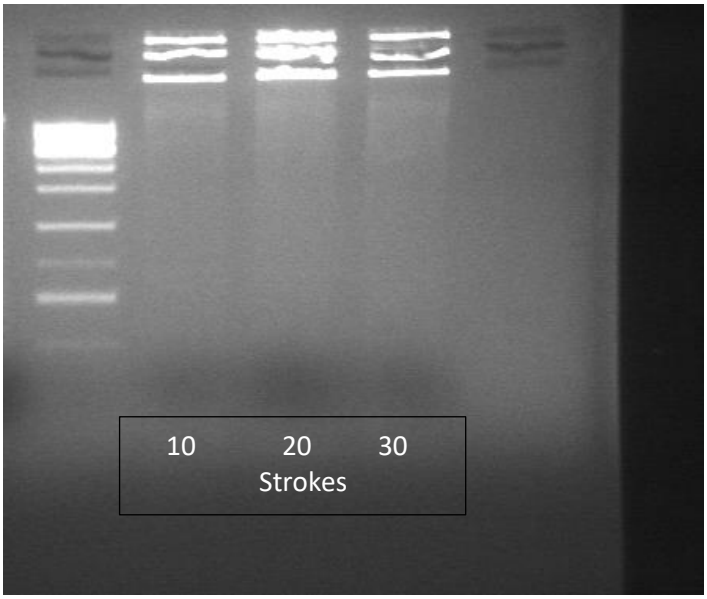



**Figure 85: The effect of different number of strokes on DNA yield**

The idea of using the tissue itself was tricky since the tissue was trapped in the gel wells after sonication. Hence, the supernatant of the disaggregated and homogenised tissue was used with no debris in the supernatant fluid. This step was performed using different amounts of starting tissue (50, 100, and 200 mg) (Figure 86).

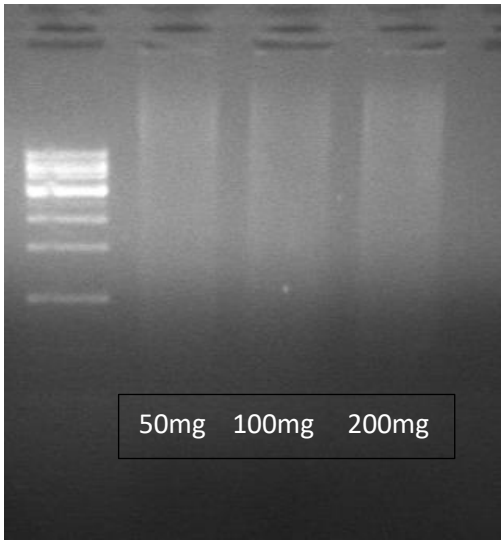



**Figure 86: The effect of different amounts of tissue on DNA yield**

Since the protocol was based on tissue lysis, another way of disaggregation was to homogenise the tissue with beads using a Rotor-stator homogeniser to guarantee the bursting of cells. Then, sonication for DNA shearing was done using the supernatant. This route did not show any positive results on running the sheared DNA on gel using 3 different amounts of tissue (50, 100, and 200 mg) as shown in (Figure 87).

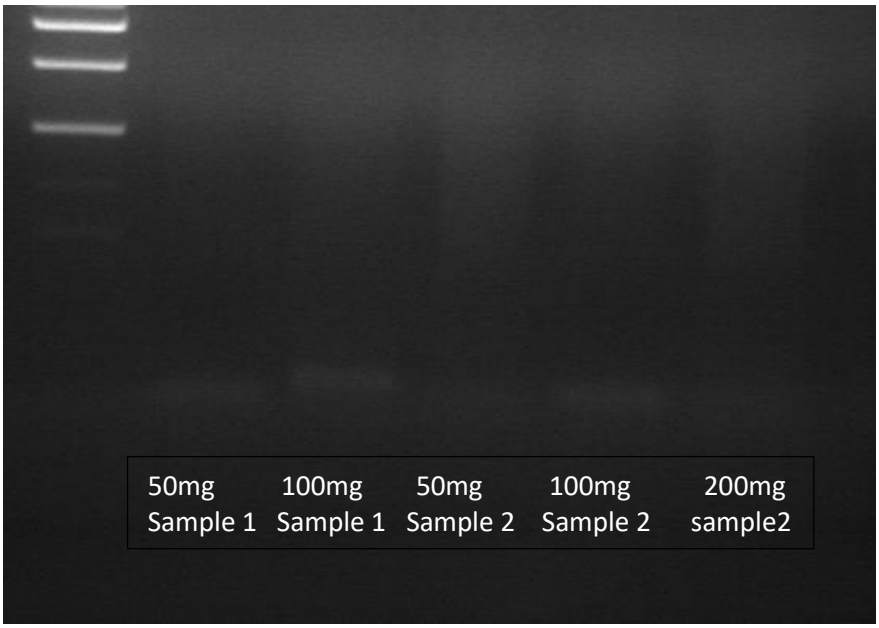



**Figure 87: The effect of using Rotor-stator homogeniser on DNA yield**

## 2.3 Validation of tissue lysis step

Obtaining the optimum sheared DNA was believed to be the main objective to proceed with the immunoprecipitation steps. Since the kit mainly focused on using the tissue as the start point through homogenisation and this did not work, the target at this point was to obtain DNA from the decidual/endometrial tissue using any of the commercially available kits and to adjust it to get the best length of sheared DNA on gel between 200 and 1000 bp.

DNA was extracted using a Qiagen RNA/DNA ALLPrep kit with 60 mg of tissue, as recommended in the protocol supplied with the kit. Then, the DNA was eluted using EB buffer and CP3 lysing buffer (100 µL/20 mg of tissue) and

sheared by sonication. Sonication was carried out with 4–5 pulses of 15–20 s each at level 2 using a Branson Microtip probe, followed by 30–40 s rest on ice between each pulse. Then, 5 µL of sonicated cell lysate was removed after every pulse for agarose gel analysis. This protocol was compared with EpiQuik™ kit's original protocol and EpiQuik™ kit using Rotor-stator homogeniser (Figure 88).

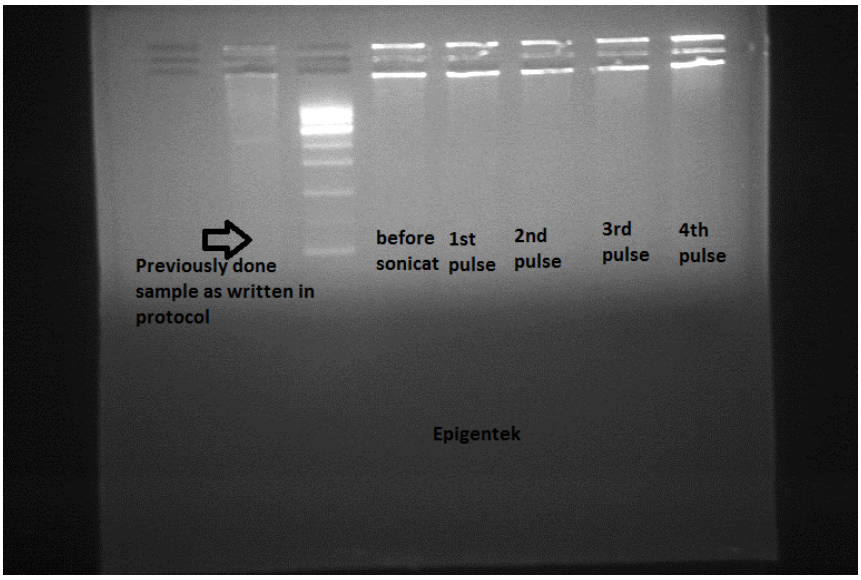



**Figure 88: Difference between DNA extracted by Qiagen ALLPrep kit and applying the EpiQuik™ kit protocol.**

Sonication using the extracted DNA with the Qiagen kit was effective but needed further optimisation for sonication. Also, adding CP3 lysis buffer to the extracted DNA diluted the sample. The next step of optimisation was to elute the DNA using the CP3 itself directly, and here, the issue that arose was how to blank the instrument when measuring the different samples, as CP3 has a high level of salt particulate, and this can be seen in the double peak when the instrument is blanked with EB, which disappears when the blanking is done with CP3 (Figure 89). In addition, the ratio for both blanking protocols is consistent with the presence of particulates. Within the original protocol, I did not have an estimate for the DNA amount to start with. When using Qiagen ALLPrep, two DNA concentrations (611 and 191 ng/µL) came from the same

sample (Figure 90), which still did not show the exact DNA concentration, but at least gave an estimate for the starting concentration before shearing.

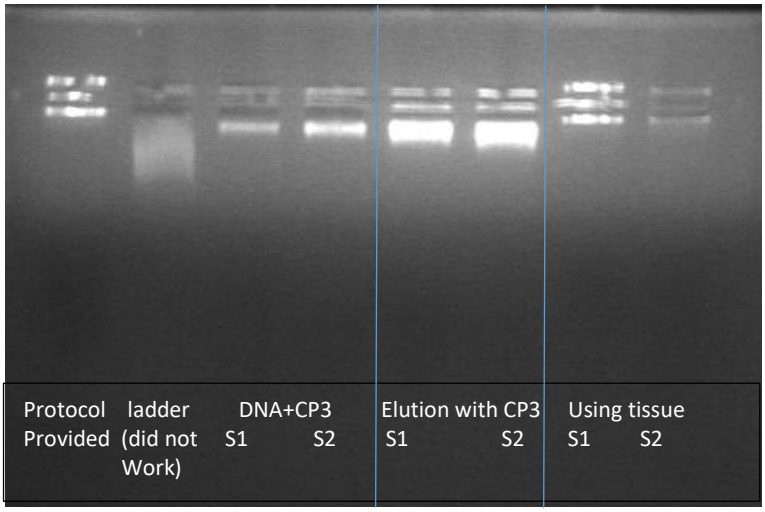



**Figure 89: The effect of CP3 lysis buffer on DNA shearing**

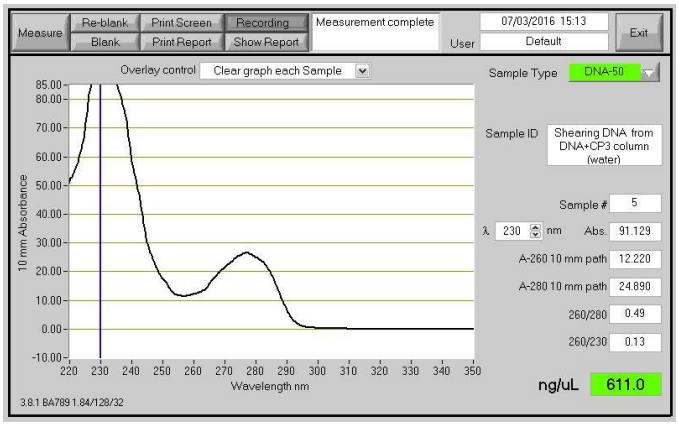



**Figure 90: Same sample eluted by CP3 lysis buffer without/with blanking nanodrop with CP3 lysis buffer**

DNA shearing showed better results with CP3 lysis buffer elution, but most fragments were still trapped in the wells and not sheared properly. Therefore, the next step was to optimise the shearing protocol.

## 2.4 Validation of DNA shearing step

Shearing of DNA became an important milestone to validate this protocol. To do this, more shearing time points were applied (15 and 30 min) with pulses of 15–20 seconds each at level 4 using a Branson Microtip probe, followed by 30–40 s rest on ice between each pulse. This step was applied to the 3 samples obtained from 3 different DNA lysis and extraction protocols (original protocol, DNA extracted using Qiagen kit with CP3 lysis buffer added to the DNA extracted, and CP3 eluted sample using the Qiagen kit) (Figure 91) The DNA eluted by CP3 using Qiagen kit and sheared for 30 min was found to be the best and was chosen for the further step of immunoprecipitation as the sonication reached its optimum target with DNA fragments of ~700 bp (Sambrook and Russell, 2006).

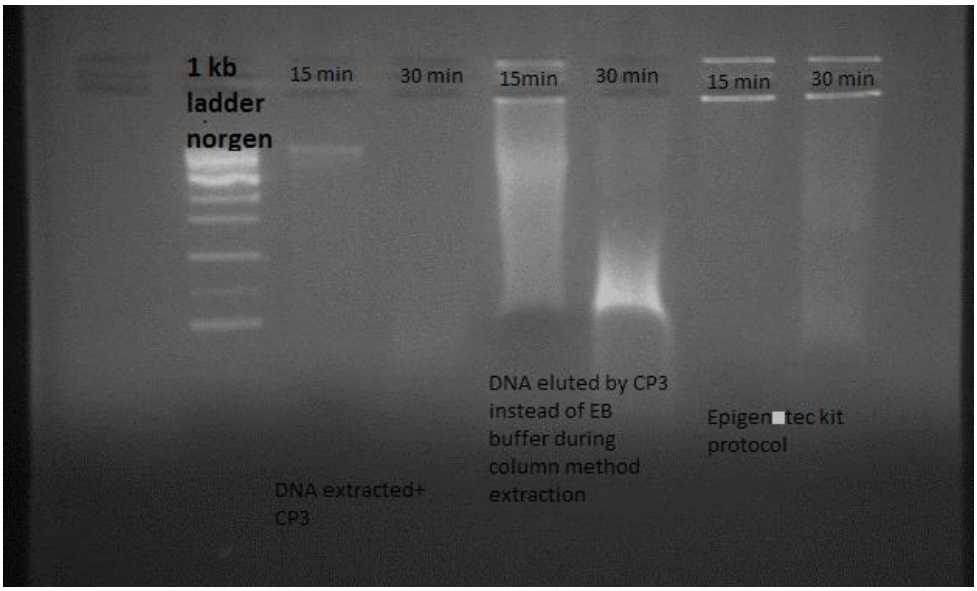



**Figure 91: The effect of different shearing timings on DNA extracted by different methods**

The DNA shearing was repeated without using the Branson microtip probe, but with the use of Eppendorf tubes. This showed a much better shearing effect when run on the gel (Figure 92).

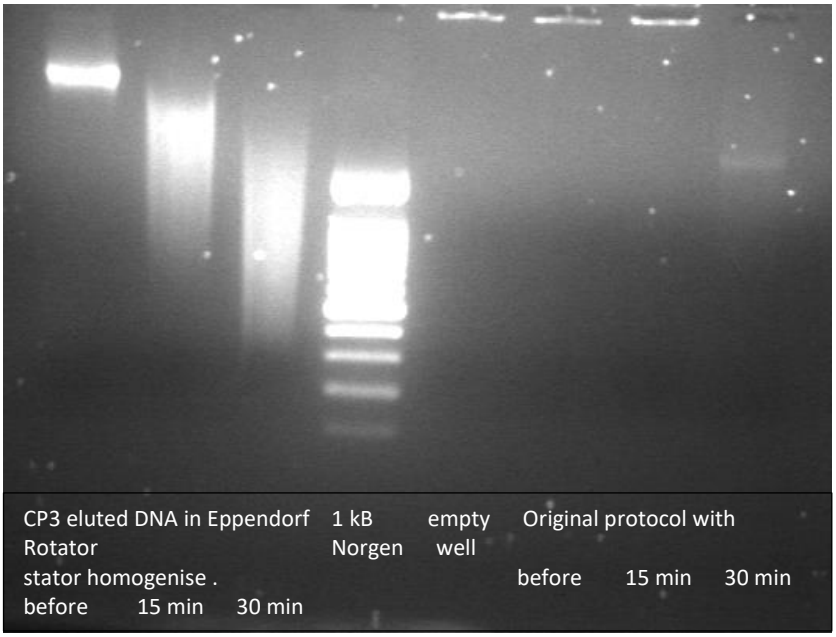



**Figure 92: The effect of sonication on DNA shearing without using the Branson microtip probe**

To finish this validation arm (Diagram 1), we used 50 mg of tissue/DNA extracted using Qiagen ALLPrep DNA/RNA kit/eluting with CP3/ sonicating in Eppendorf tubes for 30 min of 15–20 s pulses each at level 4 followed by 30–40 s rest on ice between each pulse.

## 2.5 Validation of antibody binding to assay plate, methylated DNA immunoprecipitation, and methylated DNA isolation/purification steps

DNA immunoprecipitation was performed as stated in the original kit protocol. The output was methylated DNA ready for PCR. For PCR, the plate was designed as shown in (Table 21), where each well contained 20 µL of the sample (Table 22):

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | |
|---|---|---|---|---|---|---|---|---|
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | ACTB (negative control) |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | GAPDH (negative control) |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | H19ICR (positive control) |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | TSH2B (positive control) |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | *KCNK9* |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | *KCNK10* |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | *KCNK17* |
| Input | Input | NoAb | NoAb | MeDIP | MeDIP | NO-DNA | NO-DNA | *KCNMA1* |

**Table 21: Plate design for PCR**

| | Power SYBR assay | 10X primer mix | $H_2O$ | template | Final volume in µL |
|---|---|---|---|---|---|
| Each 20 µL reaction | 10 | 0.8 | 7.2 | 2 | 20 |

**Table 22: Reagent mix in each well of the PCR plate**

PCR was set at 95°C for 10 min, 39 cycles of 95°C for 15 s, followed by 60°C for 1 min and a melting curve from 60°C to 95°C with 0.5°C increments. Only one wells of input and of MeDIP showed amplification for the H19ICR positive controls (Figure 93).

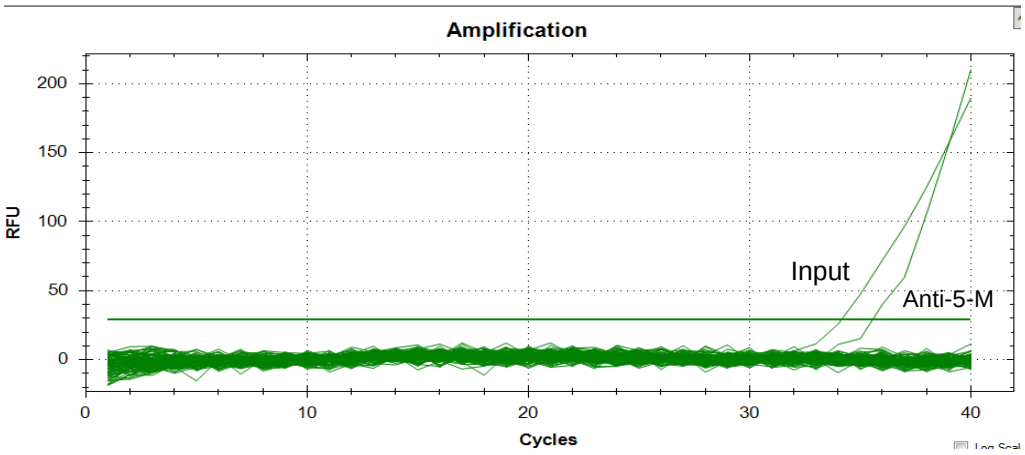



**Figure 93: PCR results after applying immunoprecipitation on sheared DNA**

Looking at both the amplification and the melting curves, one can determine which step needs troubleshooting. Some tips on troubleshooting are provided by the EpiQuik™ manual (Figure 94). The main problem at this stage is that it is hard to identify whether PCR failed to work as a result of errors in the immunoprecipitation step or the PCR condition settings. The fact that the input DNA did not amplify in conditions that were established as optimal for the primer pairs used suggest a problem with the PCR itself.

**TROUBLESHOOTING**

Little or No PCR Product

| | |
|---|---|
| 1. Insufficient tissues. | Increase tissue amount (ex: > 20 mg of tissue/per reaction). |
| 2. Insufficient tissue lysis. | Follow the guidelines in the protocol. Check the tissue lysis by observing a 5 µl portion of the tissue lysate under the microscope. |
| 3. Insufficient/too much sonication. | Follow the protocol instruction for obtaining the appropriate sized DNA. Keep the sample on ice during the sonication. |
| 4. Incorrect temperature/insufficient time for DNA release | Follow the guidelines in the protocol for appropriate temperature and time. |
| 5. Incorrect PCR conditions. | Check if all PCR components are added. Increase amount of DNA added to PCR reaction. Increase the number of cycles for PCR reaction. |
| 6. Incorrect or bad primers | Ensure the designed primers are specific to the target sequence. |
| 7. The column is not washed with 90% ethanol. | Ensure that wash solution is 90% ethanol. |

**Figure 94: EpiQuik™ Troubleshooting guide sheet**

Per the EpiQuik™ troubleshooting guide sheet and literature, the problem was either insufficient tissue or insufficient tissue lysis. This could not be confirmed since we were not aware of the initial DNA concentration as we were eluting with CP3 lysis buffer, which showed inaccurate DNA concentration due to the salt precipitates in the buffer, reflected by the wrong 160/280 ratio on the nanodrop OR, so the PCR conditions were not optimum.

The aim was now to take a step back for reaching a good sonicated DNA using the normal DNA extraction protocol of the AllPrep, eluting with EB buffer at the last step instead of CP3 buffer to be aware of the initial DNA concentration for the sonication step. Also, to avoid any salt accumulation that may affect the experiment, CP3 lysis buffer was not used for our experiment. The use of Qiagen EB buffer was supported by Sørensen and Collas, as using other reagents like sterilised $dH_2O$ overnight at 4°C (Thu et al., 2009) or TE containing 20 µg/mL RNAse (Mohn et al., 2009) may inhibit further enzymatic

reaction during sample labelling for immunoprecipitation(Sørensen and Collas, 2009).

To prove that 50 mg of the same tissue was used for 2 DNA extractions, one was eluted with EB buffer and the other, with CP3 lysis buffer. Each sample was sonicated for 15, 30, and 60 min to reach the best shearing sonication (Figure 95) and avoid insufficient or too much sonication.

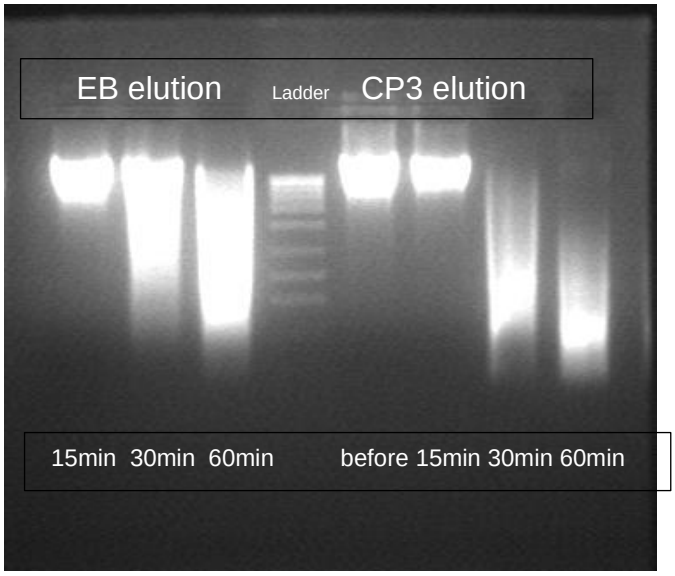



**Figure 95: The effect of sonication on DNA shearing when eluting DNA using EB buffer and CP3 lysis buffer.**

At this point, using the EB buffer-eluted DNA was the optimum step after sonication at 30 min and 60 min and could be used for immunoprecipitation steps instead of elution with CP3 lysis buffer, given the benefit of knowing our initial DNA concertation and being able to detect the dilution at every stage later.

At this stage, the aim was to set the best PCR conditions (time, cycles and temperature). Once PCR conditions are well set, it is easy to confirm that failure of PCR after immunoprecipitation step is due to the immunoprecipitation step only. PCR after immunoprecipitation did not work. The first condition to be used was the PCR condition suggested by each positive control protocol

using sheared DNA without the immunoprecipitation step (sample contained methylated and non-methylated DNA).

Two different PCR conditions were set for the SYBR green PCR using genomic DNA (concentration of 100 ng/µL). The first PCR condition (as provided by Diagenode TSH2B positive control) was incubation at 95°C for 3 min; 49 cycles (95°C for 15 s, 60°C for 45 sec) and 1 cycle at 72°C for 2 min. The melting of PCR products was performed from 55°C to 95°C, with 0.5°C increments. This was done it in duplicates (Figure 96).

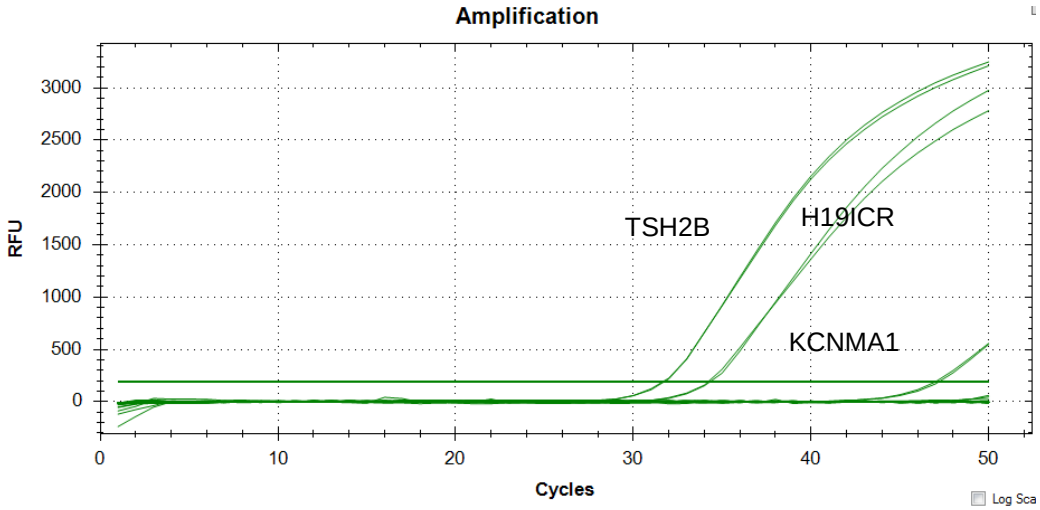



**Figure 96: PCR conditions provided by Diagenode TSH2B positive control**

The second PCR condition (as provided by Diagenode H19ICR positive control) was incubation at 95°C for 5 min; 49 cycles (95°C for 10 s, 60°C for 30 s, 72°C for 30 s). Melting of PCR products was performed from 55°C to 95°C, with 0.5°C increments. This was done it in duplicates (Figure 97). The positive control products from both PCR conditions were then run on gel electrophoresis (Figure 98).

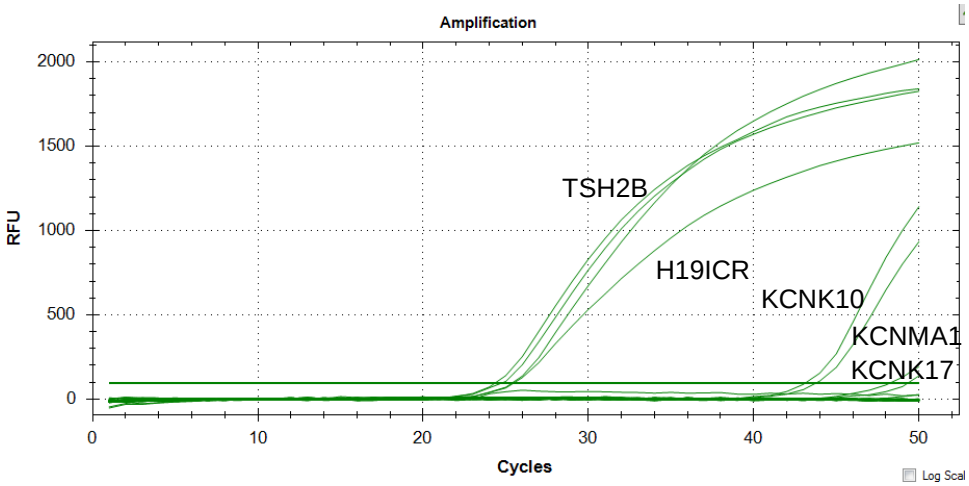



**Figure 97: PCR conditions provided by Diagenode H19ICR positive control**

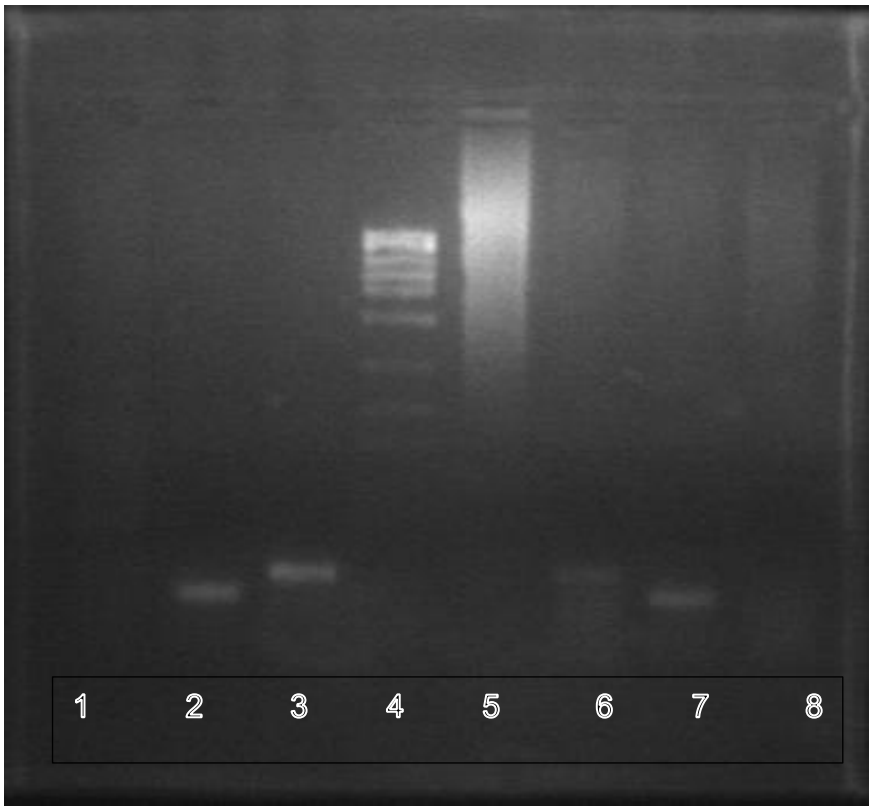



**Figure 98: PCR products from PCR validation steps run on gel**

1) GAPDH 1st PCR setup (showed no amplicon)

2) TSH2B (positive control) 1st PCR setup

3) H19ICR (positive control) 1st PCR setup

4) 1 kB ladder (Norgen)

5) Sheared DNA (from another validation that ran in parallel)

6) H19ICR (positive control) 2nd PCR setup

7) TSH2B (positive control) 2nd PCR setup

8) GAPDH 2nd PCR setup (showed no amplicon)

Finally, PCR condition 1 (Figure 99) was chosen, but with an annealing gradient of 50–60°C; incubation at 95°C for 3 min; 49 cycles (95°C for 15 s, 50–60 °C gradient for 15 s) and 1 cycle at 72°C for 2 min. The melting of PCR products was performed from 55°C to 95°C, with 0.5°C increments. This was done on the four target primers and in duplicate.

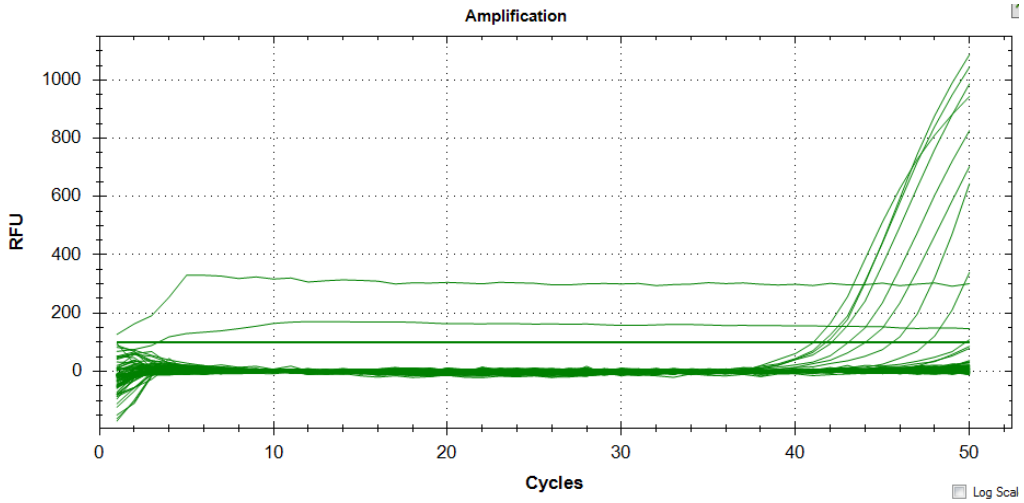



**Figure 99: PCR condition validation with an annealing gradient of 50–60°C**

| | *KCNK9* | *KCNK10* | *KCNK17* | *KCNMA1* |
|---|---|---|---|---|
| 60.0 C° | - | 47.6 | - | 41.80 |
| 59.4 C° | - | - | - | 41.56 |
| 58.3 C° | - | - | - | 43.06 |
| 56.3 C° | - | - | - | 45.60 |
| 53.9 C° | - | - | - | 49.81 |

| | | | | |
|---|---|---|---|---|
| 52.0 C° | - | - | - | - |
| 50.7 C° | - | - | - | - |
| 50.0 C° | - | - | - | - |

With this annealing gradient, we decided to use different DNA concentrations (other than the 100–120 ng/µL used in previous experiments). Using a temperature gradient was important to understand more about primer replication to reach the best PCR conditions. The samples were incubated at 95°C for 3 min; 49 cycles (95°C for 15 s, 50–60°C gradient for 15 s, 72°C for 15 s) and 1 cycle at 72°C for 2 min. This was done for each of the 4 target primers, using 4 serial DNA dilutions of 160, 80, 40, and 20 ng/µL and each sample was run in duplicate.

For primer *KCNMA1*

| | 160 ng | 80 ng | 40 ng | 20 ng |
|---|---|---|---|---|
| 60.0 C° | 41.63 | 43.04 | 46.74 | - |
| 59.4°C | 44.12 | 43.21 | 43.21 | - |
| 58.3°C | 44.56 | 42.36 | 49.83 | - |
| 56.3°C | 43.9 | 42,83 | 48.54 | - |
| 53.9°C | 45.73 | 46.67 | 48.93 | - |
| 52.0°C | 48.6 | 41,54 | 49.44 | - |
| 50.7°C | 48.03 | 44.9 | 49.24 | - |
| 50.0 C° | 45.09 | 44.99 | 0 | - |

For primer *KCNK17*

| | 160 ng | 80 ng | 40 ng | 20 ng |
|---|---|---|---|---|
| 60.0°C | 49.13 | - | - | - |
| 59.4°C | - | 48.92 | - | - |
| 58.3°C | - | 49.84 | 47.00 | - |
| 56.3°C | 49.79 | 49.68 | - | - |
| 53.9°C | - | - | - | - |
| 52.0°C | - | 49.57 | - | - |
| 50.7°C | 49.59 | - | - | - |
| 50.0°C | 49.75 | 48.75 | - | - |

For primer *KCNK10*

| | 160 ng | 80 ng | 40 ng | 20 ng |
|---|---|---|---|---|
| 60.0°C | 43.05 | 46.92 | 48.52 | - |
| 59.4°C | 44.48 | 44.95 | 49.62 | - |
| 58.3°C | 45.76 | 45.63 | 49.67 | - |
| 56.3°C | 46.45 | 45.20 | 49.08 | - |
| 53.9°C | 46.63 | 43.94 | 48.95 | - |
| 52.0°C | 45.73 | 42.91 | 47,46 | - |
| 50.7°C | 43.67 | 42.98 | - | - |
| 50.0°C | 43.02 | 42.12 | 46.01 | - |

For primer *KCNK9*

| | 160 ng | 80 ng | 40 ng | 20 ng |
|---|---|---|---|---|
| 60.0°C | - | - | - | - |
| 59.4°C | - | - | - | - |
| 58.3°C | - | - | - | - |
| 56.3°C | - | - | - | - |
| 53.9°C | - | - | - | - |
| 52.0°C | - | - | - | - |
| 50.7°C | - | - | - | - |
| 50.0°C | - | - | - | - |

There were no rhythmic changes between the annealing gradients and the dilution, and this led to messy results.

In case the problem was in the initial DNA starting concentration, so KCNKA 10 primer that showed relatively stable results through dilutions and annealing gradient, was for a PCR with a starting concentration of 536 ng/ µl, hoping to find an early acceleration. PCR was done in triplicates, but it did not show any results (Figure 100).

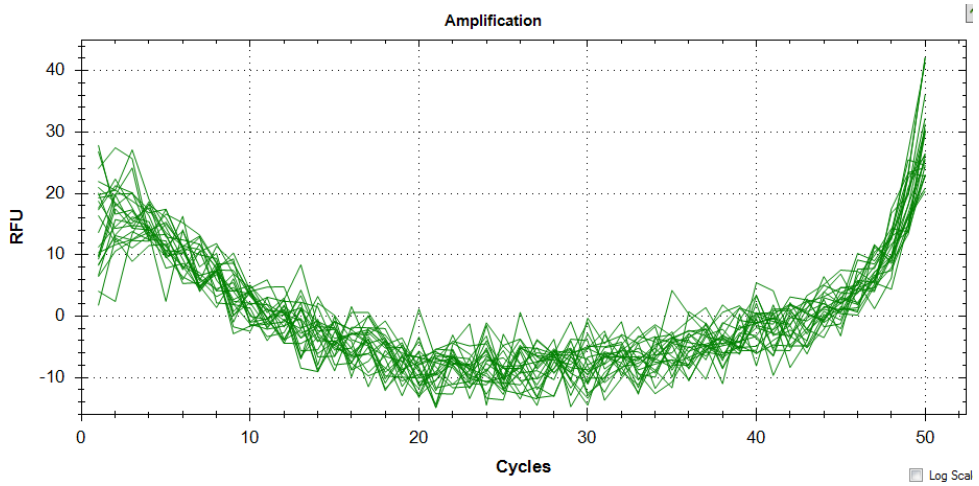



**Figure 100: PCR condition validation using high initial DNA concentration**

Using the same *KCNKA10* primer pair but going down to a concentration of 200 ng/µL appears to be effective (Figure 101). PCR was done in triplicates. This was consistent with the recommendation of Thu et al., who stated that 200 ng/µL of DNA is sufficient for antibody immunoprecipitation (Thu et al., 2009).

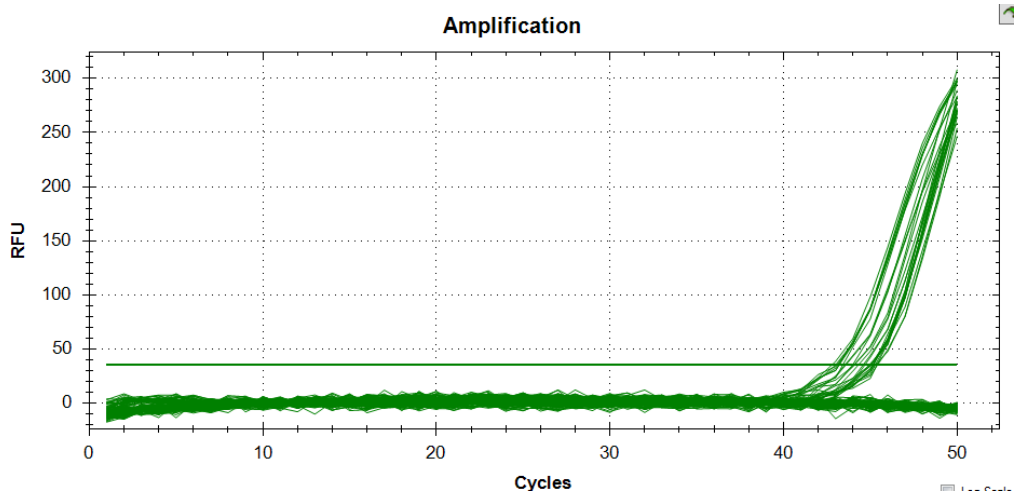
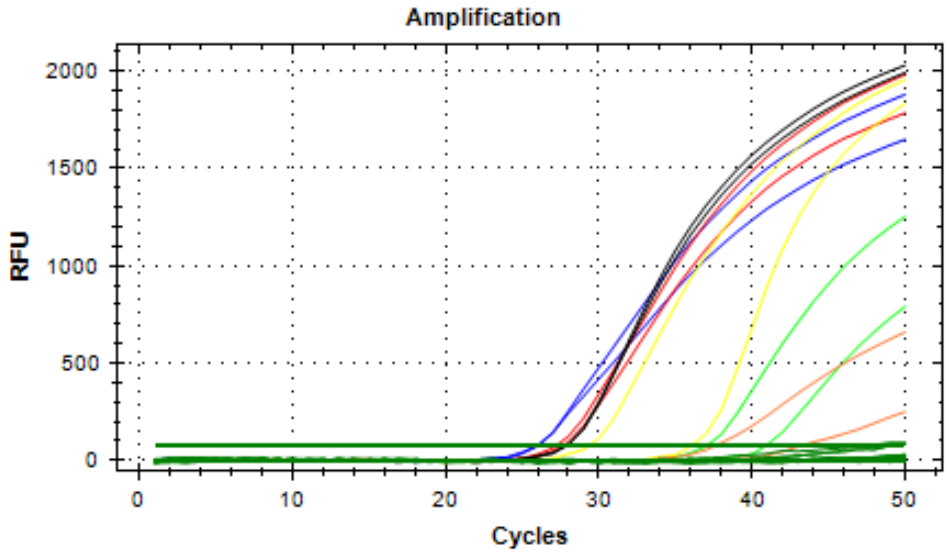



**Figure 101: PCR condition validation using DNA concentration of 200 ng/µL**

| Temp | 200 ng/µL |
|---|---|
| 60.0°C | 45.3 |
| 59.4°C | 45.2 |
| 58.3°C | 45,14 |
| 56.3°C | 44.6 |
| 53.9°C | 44.1 |
| 52.0°C | 43.23 |
| 50.7°C | 43.09 |
| 50.0°C | 42.81 |

As changing of the temperature on gradient annealing did not show any effect on the validation of PCR conditions, sheared DNA was set back to the initial PCR settings but for 49 cycles. This was done using the four primers and two positive controls primers and the PCR products were run on gel (Figure 102).



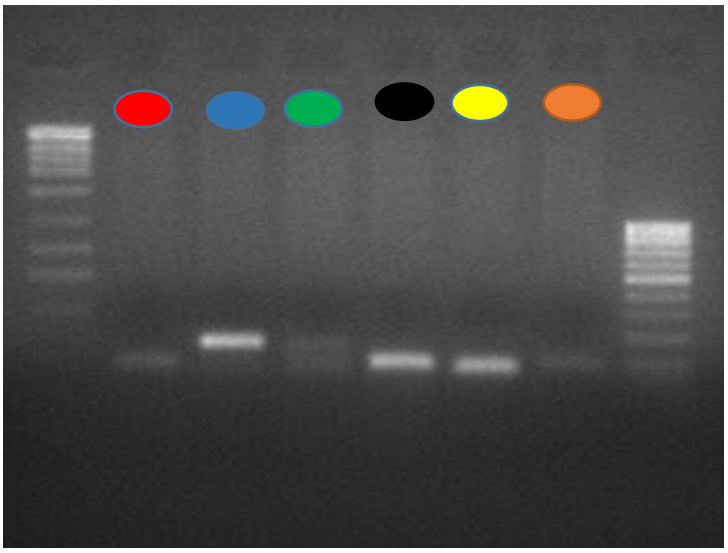



**Figure 102: Input sheared DNA amplification on PCR after setting PCR on initial settings**

Previously when PCR did not work, we were not sure whether that was due to the immunoprecipitation step or the PCR condition step. Therefore, once the PCR conditions were set, sheared DNA immunoprecipitation was repeated as mentioned in the EpiGentek kit protocol with the first PCR settings but with 49 cycles (95°C for 10 min followed by 95°C for 15 s, 60°C for 1 min × 49 cycles + melting of PCR products from 55°C to 95°C, with 0.5°C increments). This was done for the positive control only. If the positive controls worked, we could use the target primers.

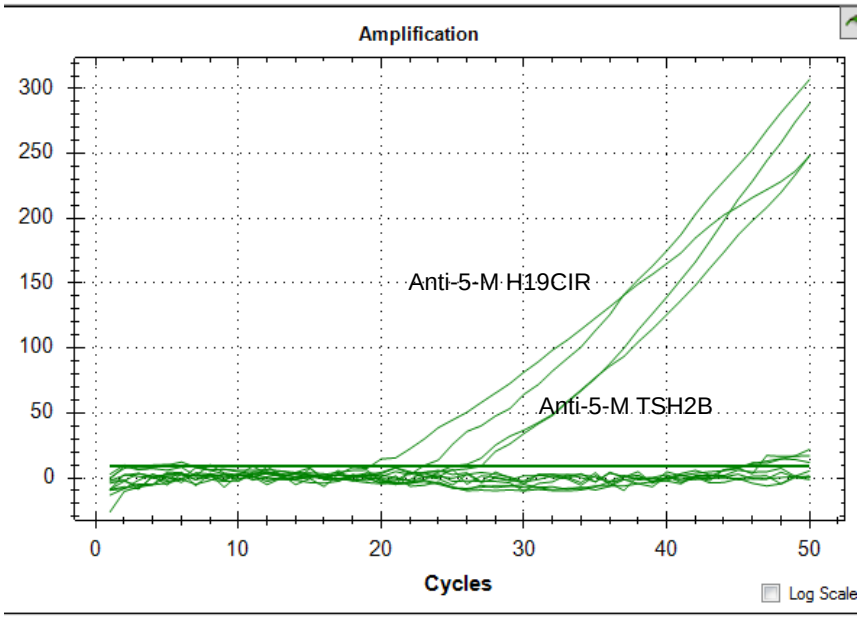



**Figure 103: Applying immunoprecipitation protocol after setting PCR conditions**

Immunoprecipitation worked and the melting curve showed corresponding peaks, but the input did not show anything because I treated it as per the protocol: 'Add 1 µL of proteinase K (used to overcome the viscosity of the cells lysis solution (Mohn et al., 2009) to each 40 µL of CP5 and mix. Add 40 µL of CP5 containing proteinase K to the samples (including the 'input DNA' vial). Cover the sample wells with strip caps and incubate at 65°C in a water bath for 15 minutes'. Then, applying all next steps and column on the input which is 10 µL in the start, resulting in into too much dilution of input.

However, on running the PCR products on gel, the anti-5-ethylcytosine samples showed no amplicons (Figure 104).

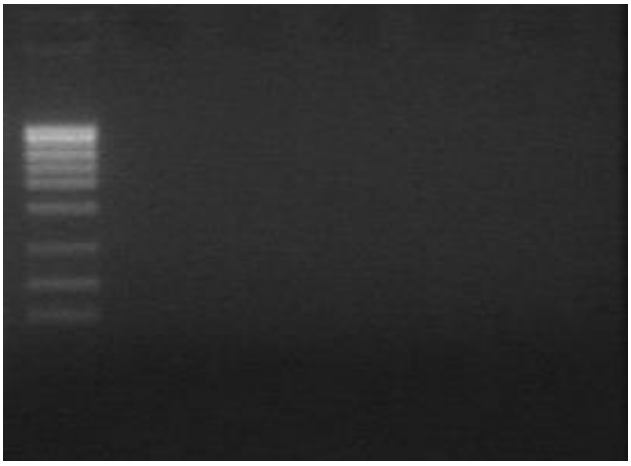


**Figure 104: Running PCR products on gel after applying immunoprecipitation protocol with the final PCR conditions set**

Since the PCR conditions were set at 49 cycles with no gradient annealing, and DNA extraction using EB buffer was found to be better than eluting with CP3 with the initial DNA concentration known. PCR was done using the best PCR condition but with both sheared DNA conditions (using EB and CP3 buffers) to reach the final optimum combination to be carried on to the further step. This was done on the positive controls. Again we took a step back and repeated the EpiGentek experiment, using DNA extracted with EB for elution and DNA extracted using CP3 (lysis buffer) for elution to 1) confirm that the previous experiment results were not caused by chance and 2) to compare the differences in amplification between the EB eluted sample and CP3 eluted sample. The input was not diluted as given in the protocol and retained as sheared DNA only (Figure 105).

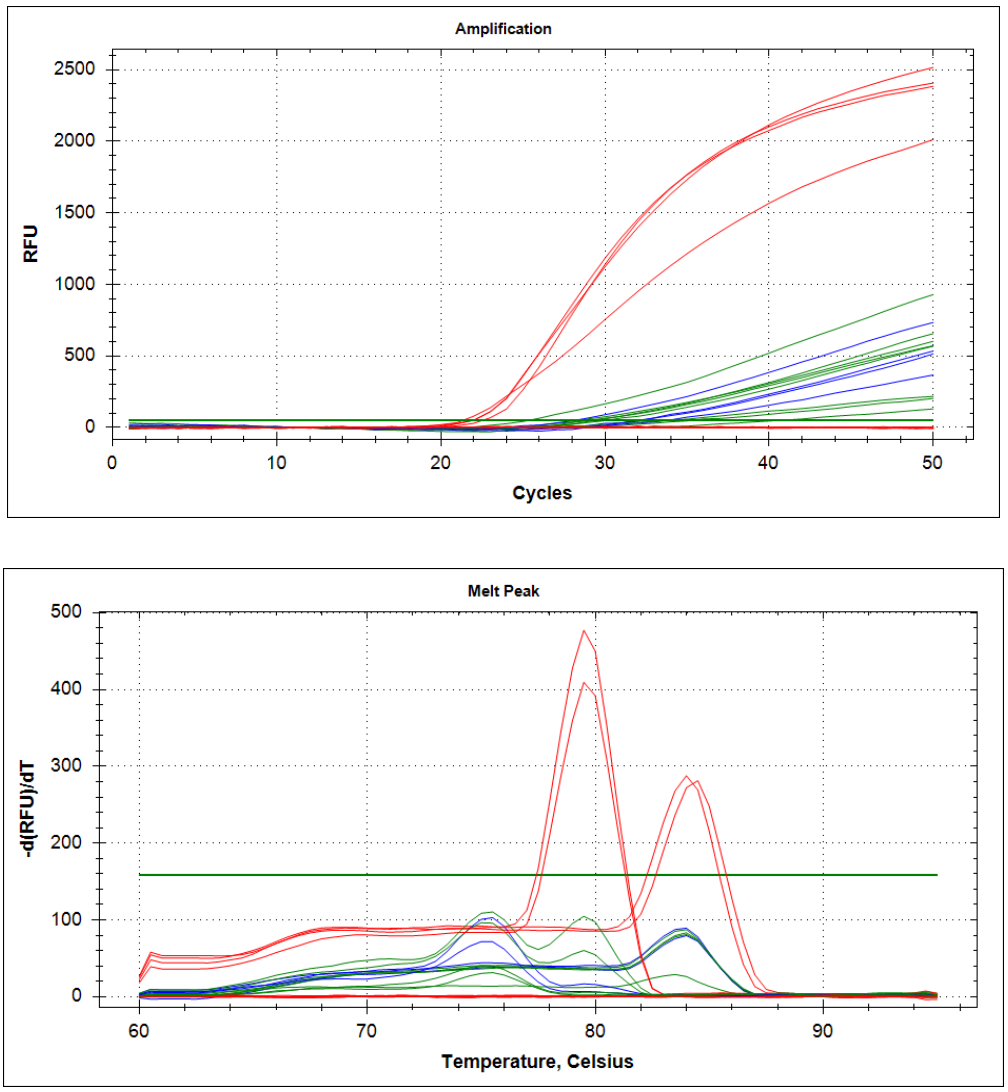



**Figure 105: PCR done using initial conditions, but EB buffer was used for elution**

The melting curve of some anti-5-methylcytosine samples and some normal mouse IgG samples show single peaks and double peaks.

In this experiment the input worked perfectly, but the mouse IgG samples showed amplification, although in the kit manual stated, 'Why would WGA amplify both DNA from sample and control IgG? 10% background from IgG precipitation, after whole genome amplification (WGA), it will also be amplified. It is not necessary for microarray use. Input should be used'.

The products were run (samples from DNA eluted with CP3) on 8-well gel (Figure 106).

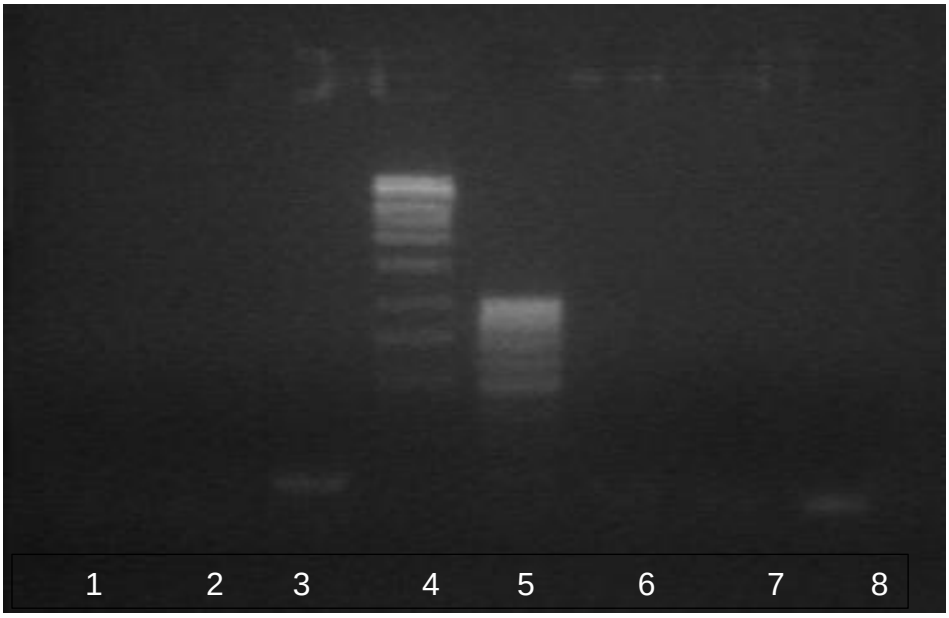



**Figure 106: PCR products run on gel for 2 positive controls**

1) TSH2B against anti-5-methylcytosine

2) TSH2B against normal mouse IgG

3) TSH2B against input (positive but faded amplicon)

4) 1 kB DNA ladder (Norgen)

5) 100 bp DNA ladder (Norgen)

6) H19CIR against anti-5-methylcytosine

7) H19CIR against normal mouse IgG

8) H19CIR against input (positive but fade amplicon)

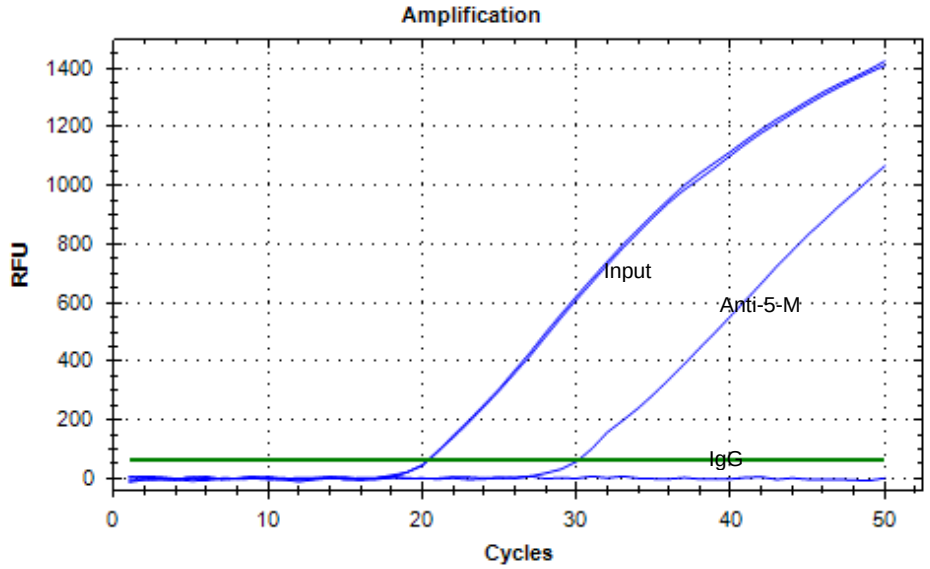
After optimum DNA extraction, elution, sonication, primer design, and PCR conditions were reached, the immunoprecipitation kit was used again, with this modified protocol for samples from 2 different patients (endometrial samples)

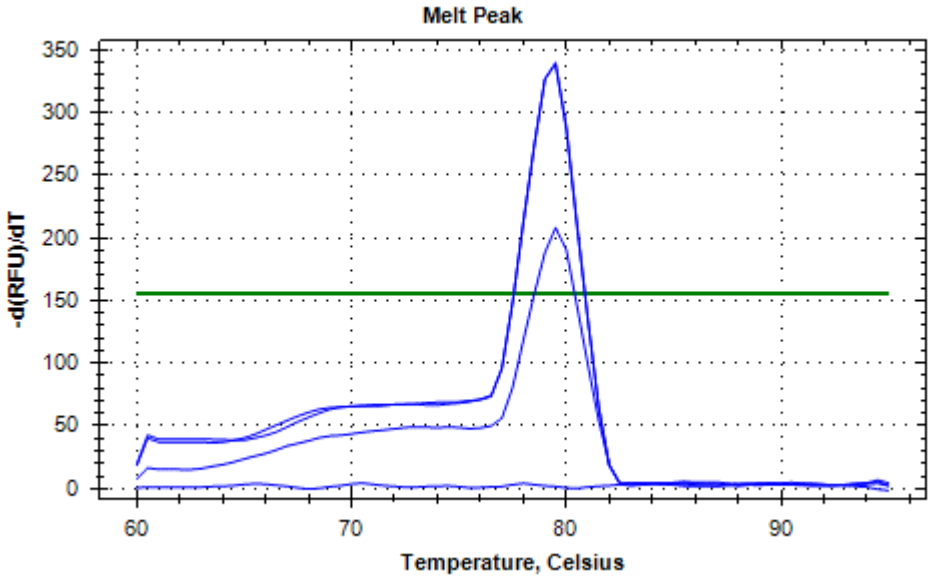
and on all the 6 genes (2 positive controls and 4 target genes) plus NTC for each gene since there was not enough DNA for the 2 negative controls.

The endometrial samples worked perfectly, with the 2 positive controls working in inputs and anti-5-M and not in the mouse IgG, and the target genes showed positive methylation of 1 gene (*KCNK17*) in the fertile patient with nothing in the negative IgG.

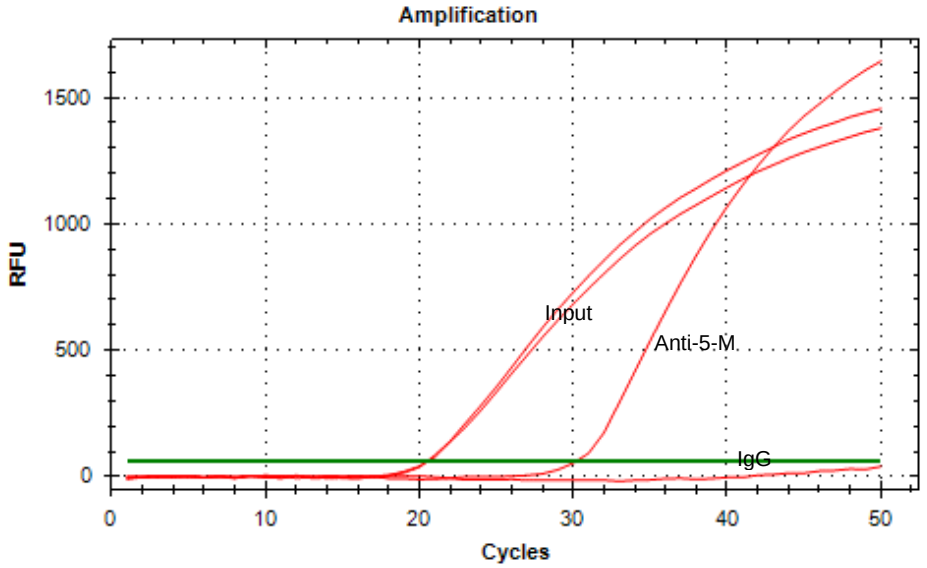
(Figure 107) shows the gene replication and melting curves for the input, anti-5-M and IgG

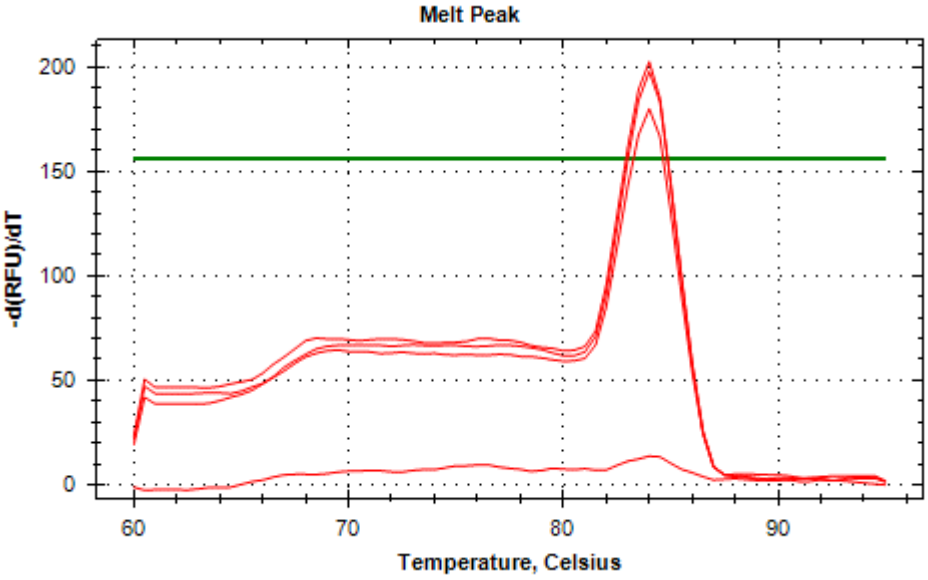
*TSH2B*


Amplification
RFU
1400
1200
1000
800
600
400
200
0
Input
Anti-5-M
IgG
0
10
20
30
40
50
Cycles

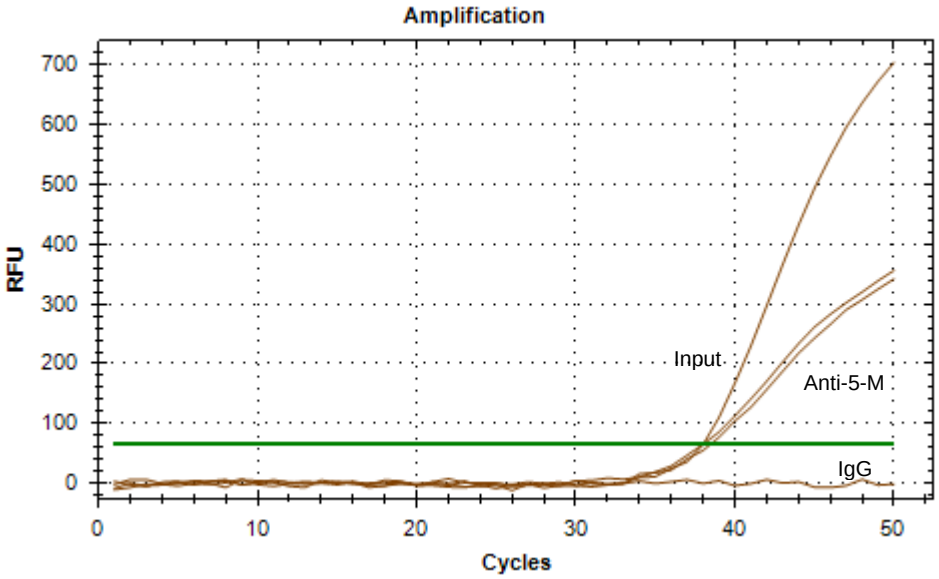

Melt Peak
-d(RFU)/dT
350
300
250
200
150
100
50
0
60
70
80
90
Temperature, Celsius

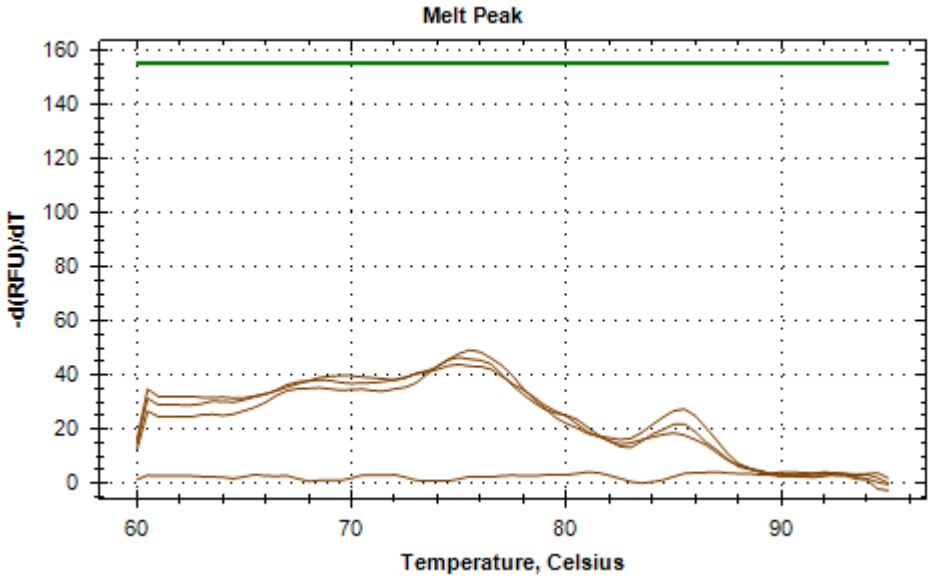
H19CIR (positive control)



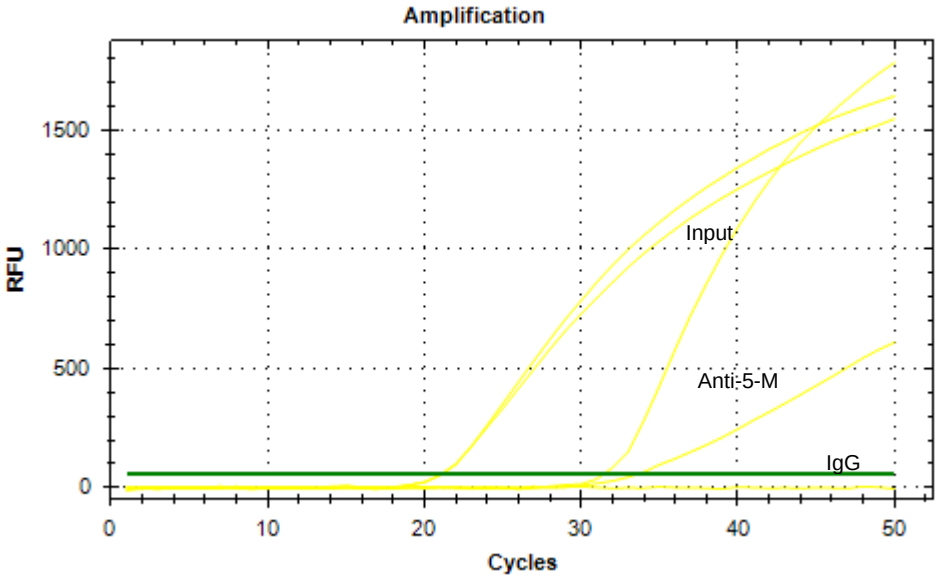



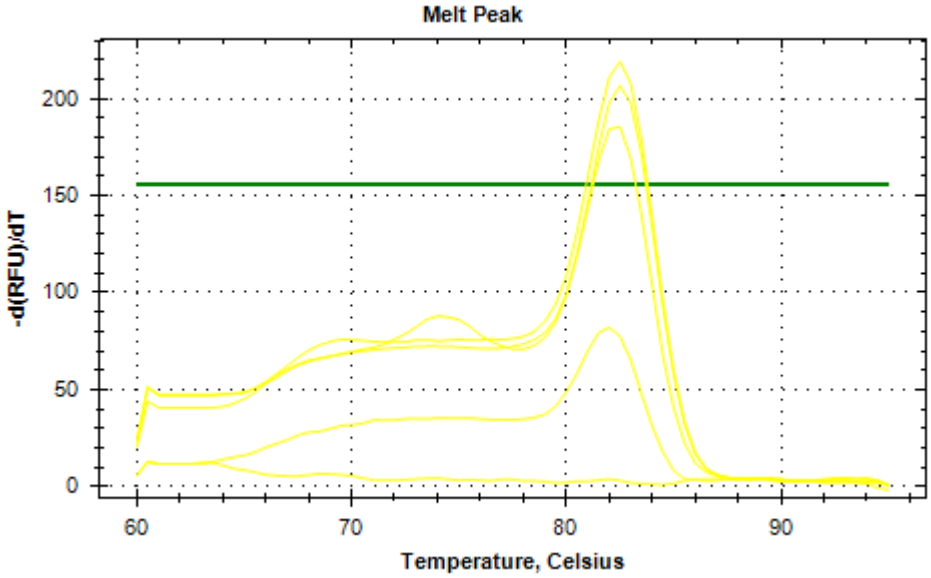
*KCNMA1*


Amplification
RFU
700
600
500
400
300
200
100
0
Input
Anti-5-M
IgG
0
10
20
30
40
50
Cycles

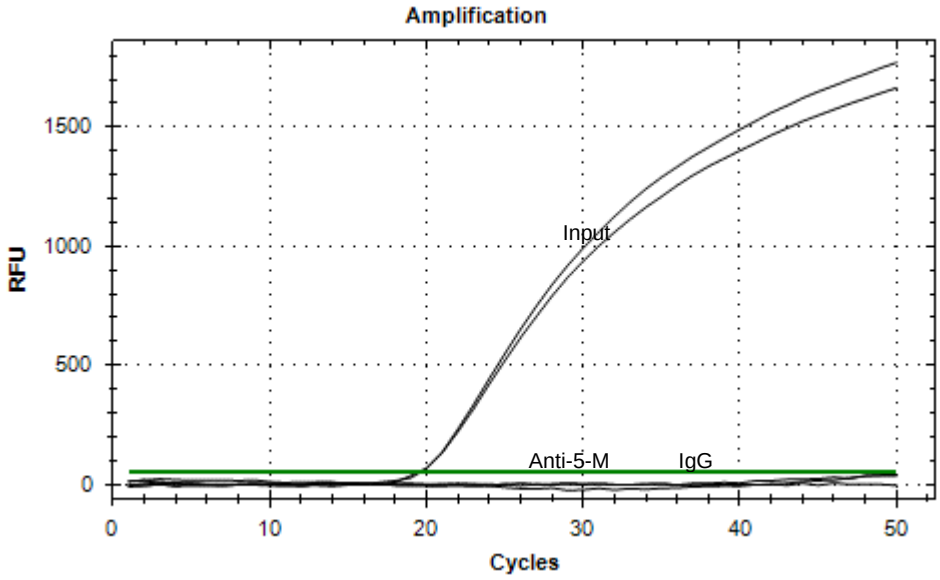

Melt Peak
-d(RFU)/dT
160
140
120
100
80
60
40
20
0
60
70
80
90
Temperature, Celsius

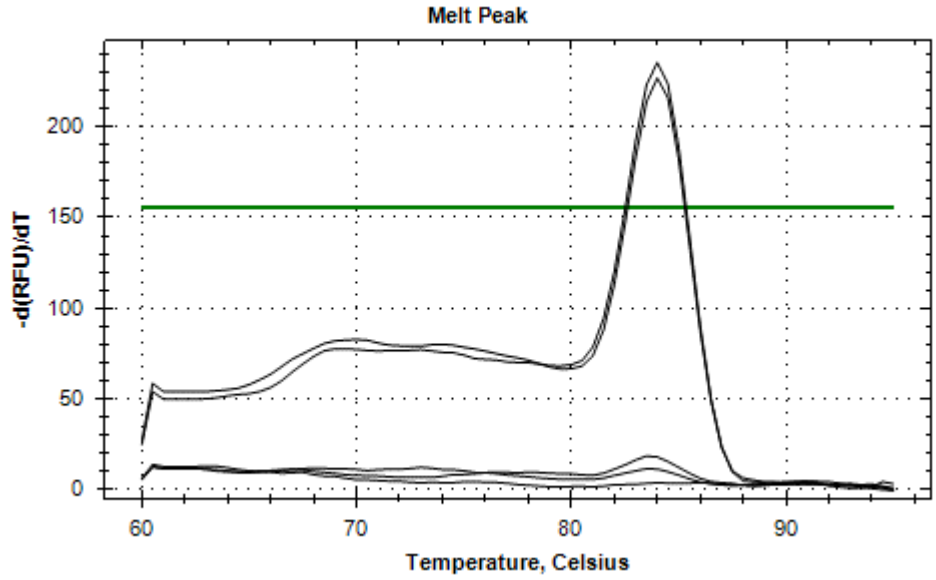
*KCNK17*


Amplification
RFU
1500
1000
500
0
0
10
20
30
40
50
Cycles
Input
Anti-5-M
IgG


Melt Peak
-d(RFU)/dT
200
150
100
50
0
60
70
80
90
Temperature, Celsius

*KCNK10*


Amplification
RFU
1500
1000
500
0
Input
Anti-5-M
IgG
0
10
20
30
40
50
Cycles


Melt Peak
-d(RFU)/dT
200
150
100
50
0
60
70
80
90
Temperature, Celsius

*KCNK9*

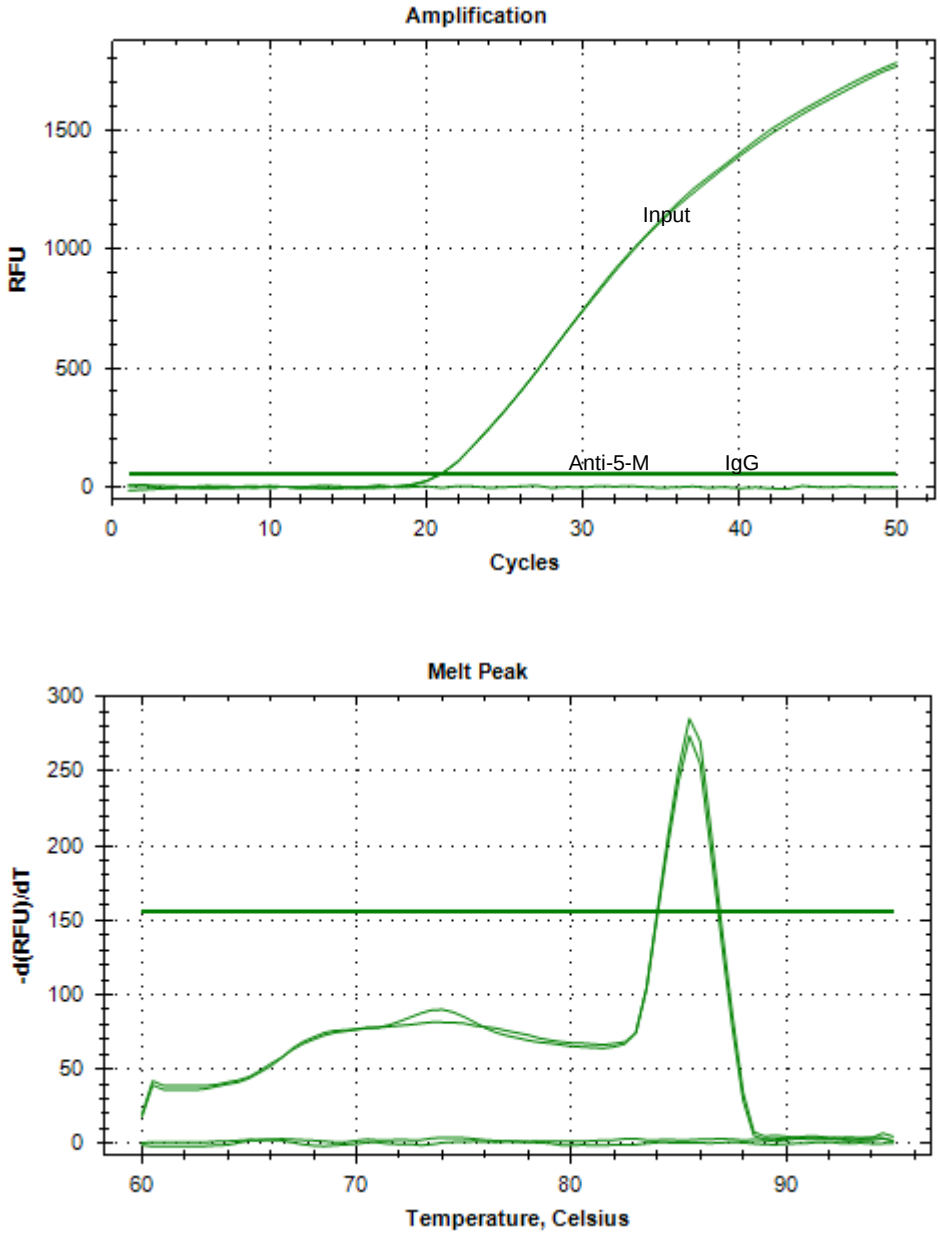



**Figure 107: PCR amplification of genes primers after final validation of EpiQuick kit**

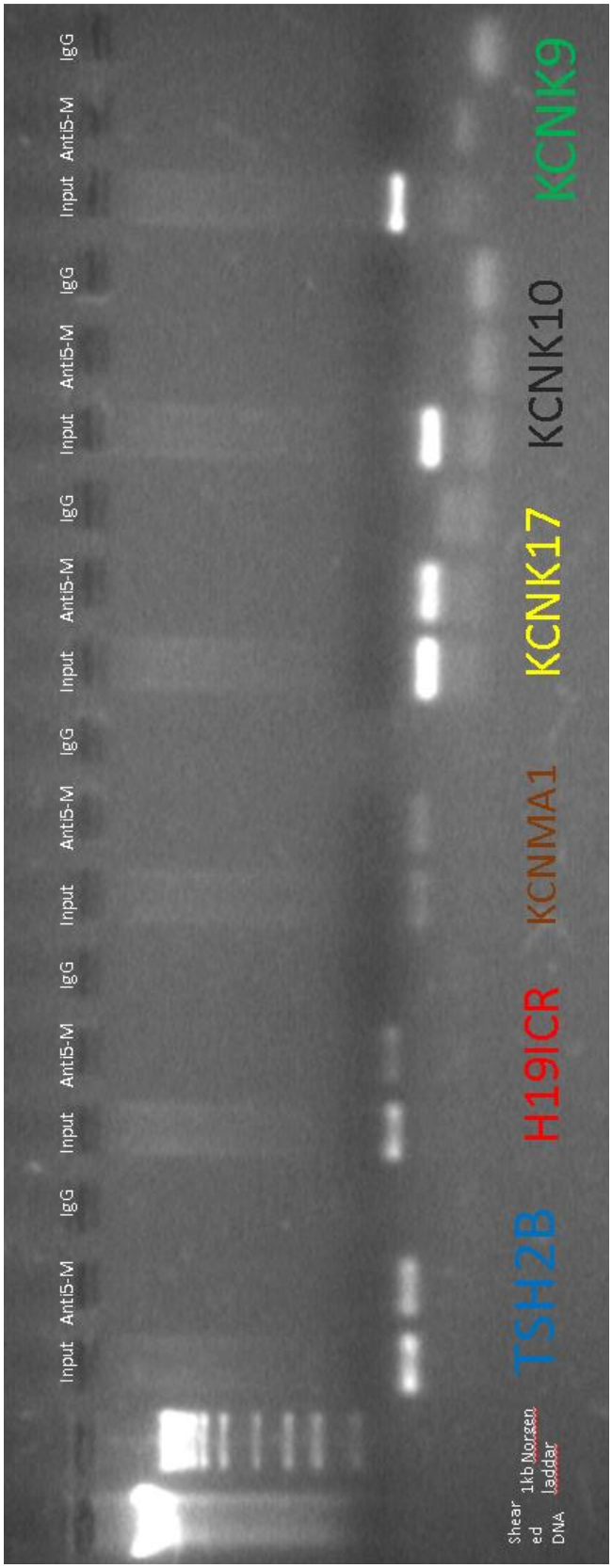



**Figure 108: PCR products on gel from the final validation of EpiQuick kit**

At this stage, it can be said that the EpiGentek EpiQuick immunoprecipitation validation was successful with the need to be repeated to confirm the results of the methylation of the target genes. This preliminary experiment suggests that *KCNMA1* and *KCNK17* are methylated in the tissue used for the DNA extraction and *KCNK10* and *KCNK9* are not.

# 3 Discussion

We set up a protocol for the analysis of DNA methylation of the potassium channel genes in the endometrium and decidua. We also applied this new protocol in a pilot study to check for methylation of the potassium channels in both fertile and infertile groups, and the results could be used for future projects.

Before starting the epigenetic experiment, it was important to choose the most suitable method to approach DNA methylation detection (Brosens et al., 2014) that could provide an unbiased result. This method needs to be simple and robust, with high sensitivity and specificity (Kurdyukov and Bullock, 2016). These requirements are necessary to identify de novo epigenetic methylation on the sites of the potassium genes studied in chapter X.

The new EpiQuik™ Tissue Methylated DNA Immunoprecipitation Kit by EpiGentek was the kit of choice since it was invented specially to be used with tissues. Methylated DNA immunoprecipitation is an unbiased analysis method in comparison to methods based on detection of methyl-cytosines in targeted regions of the genome (Pelizzola and Molinaro, 2011). The kit was developed to evaluate MeDIP enrichment measures and estimate the absolute or relative number of methyl-cytosines in a given sample (Pelizzola and Molinaro, 2011).

The findings are interesting, especially as *KCNK17* gene expression appeared to be significantly different ($P < 0.01$) between the proliferative and secretory phases of the menstrual cycle and also less expressed in the infertile group than in the fertile group ($P > 0.089$). All these differences needed an explanation in order to determine what could be influencing these genes and acting as a gene promoter (Berger et al., 2009).

DNA methylation plays an important role in the activation or repression of transcription and can influence gene regulation (Caplakova et al., 2016). If there was no difference in methylation between the studied groups, then the epigenetic promotors could be histone modification, histone variants, and

nucleosome positioning. Generally, CpG islands remain un-methylated in all tissues with the exception of the inactive X chromosome, nonessential genes in tissue culture cell lines, and imprinted genes (Cross, 2002). Therefore, methylation of the *KCNK17* gene highlights a cause of changes in the potassium channel genes expression across the different study groups.

# 4 Conclusion

The new EpiQuick kit was validated, reaching the optimum tissue disaggregation and lysis, DNA extraction, DNA shearing, primer designing and PCR conditions. This step enhanced the immunoprecipitation protocol to study the epigenetic changes in the potassium channel genes.

By reaching our final results, that initially showed positive methylation of KCNK17 genes and this can be a start of a future research to understand more about the potassium genes regulation and their role in fertility.

# Chapter Seven: General Discussion, Future Work and Conclusion

## General Discussion

-In this project, the main focus was on human endometrium for being responsible for the establishment of pregnancy and development. Despite the improved outcomes of ART, a new challenge has emerged, which is recurrent implantation failure (RIF) but evidence for the mechanisms underlying RIF and treatments used are limited. From this point, our first aim was to conduct a meta-analysis to interpret how may any of the available ‘’adds on’’ immune-modulators treatments available for RIF could improve live birth rate in women undergoing IVF.

The meta-analysis showed no clear evidence that administration of most of the immunotherapies studied improved live birth and miscarriage rate. However, sensitivity analysis (when abstract studies were included) showed some evidence that intralipid, PBMC, and GCSF may improve clinical pregnancy outcomes in RIF patients who are undergoing IVF. Most of the included studies were limited to abstracts lacking detailed information and the certainty of the evidence ranged from moderate to very low. Also other limitations were 1) limited patient number and heterogeneity across the eligible studies, 2) Variations in the dosage, regimen and use of placebos, 3) Lack of reporting adverse outcomes especially drug side effects, 4) Varying criteria for selecting patients with RIF prevented inclusion in the meta-analysis thus caution has to be exercised when applying the results to clinical practice. Therefore, the findings from this review must be interpreted with severe caution, especially that none of the studies reported any side effects despite the well-known and reported risks attached to these treatments, some of which are very serious.

-The second aim was to understand the molecular determinants that may hold important clues for implantation defects of the endometrial tissue in women with RIF. Endometrial cells are exposed to constantly changing microenvironmental factors, typically pH, lipids, oxygen tension, and electrolytes including a six-fold higher potassium (K+) concentration than is found in the plasma. We focused on the potassium channels in endometrium,

building the hypothesis that potassium channels of human endometrium are differentially expressed during the window of implantation. Modulation/dysregulation of these channels due to changes in the intrauterine microenvironment may alter conditions essential to implantation.

To reach an answer to this hypothesis, different lab approaches were utilised. First a physiological study was conducted to understand the role and regulation of potassium channels in fertile human endometrium through measuring the transepithelial electrical resistance (TEER) in a cultured endometrial monolayer and monitoring and observing any changes in the TEER results after using specific potassium channel inhibitors and other treatments (intralipids, vitamin D and low oxygen concentration) to regulate and control the potassium flux and hence understand their role of endometrium. While TEER recordings have been made for pig, mouse and human endometrium showing roles for ENaC (sodium channels) (Vetter et al., 1997, Enuka et al., 2012), CFTR (chloride channels) (Ahmad et al., 2013, Thorpe-Beeston et al., 2013, Zheng and Trudeau, 2015) and HERG (K+-channels) (Wang et al., 2007), a role for K2P channels has emerged from studies on bovine and human endometrium during implantation (Chan et al., 2013b, Patel et al., 2013).
The limitations for TEER experiments were: 1) using the electrodes and waiting for the readings to stabilise, makes the results subjective and not accurate. 2) Potassium channels inhibitors known are few and non-specific, as for KCNK17 channel and hence the TEER study was not designed as planned to block all the potassium channels used.

Despite these limitations, the physiological study using the TEER, showed that TEER readings were significantly decreased after adding the specific potassium channel blockers and on reducing the oxygen concentration, while no significant alteration was observed on adding intralipids nor vitamin D to the fertile endometrial monolayer cell culture.

These important findings highlight a role in the transfer of potassium ions across the endometrium hence changing the potassium concentration in the intrauterine fluid and in turn could be responsible for the amount of intrauterine fluid. These are clinically important for the micro-environment surrounding the embryo during implantation and also helps in adherence of the embryo towards the endometrium. Further studies should be conducted using specific K2P channel blockers.

-In order to determine whether potassium channels differed between fertile and infertile (RIF) women, potassium channel gene expression (qPCR) and protein translation (flow cytometry) were measured in both groups. This was conducted by recruiting infertile RIF women who were undergoing IVF for endometrial biopsy to examine and determine altered expression of the studied potassium channels (KCNK9, KCNK10, KCNK17 and KCNMA1) during different phases of menstrual cycle using qPCR. Also, to study the expression in endometrium from fertile and infertile RIF groups, the effect of intralipids, low oxygen concentration and vitamin D were tested on endometrium of infertile RIF women.

The main limitation for this work was: 1) the heterogeneity of the endometrial tissue makes it difficult to identify which cell types showed the expression. 2) For flow cytometry, there was no specifically available potassium channels antibodies for flow cytometry (like those available for NKs with both primary and secondary antibodies together), so the antibodies used were the commercial antibodies available for immunofluorescence and immunohistochemistry.

Only one of the channels (KCNK17) showed significant upregulation through the menstrual cycle. Also, KCNK17 was significantly downregulated in infertile RIF mid-secretory endometrium in comparison to fertile mid-secretory endometrium (control). When mid-secretory infertile endometrial samples were tested after treating them with lower oxygen concentration, Intralipids and

Vitamin D, none of the channels showed significant altered expression in comparison to the control group. On the other hand, when same treatments were added to decidual tissue, one of the channels (KCNK9) showed significant upregulation. This could reflect the role of stromal cells, which the decidual tissue are rich in more than endometrial tissue.

-The next logical progression was to understand the outcomes from the qPCR results. This was important to have a better understanding of the regulation of potassium channels in endometrium and hence an epigenetic experiment was conducted to determine any association between the changes in expression observed in the endometrium with changes in DNA methylation.
To be able to investigate the DNA methylation profile at the promoter region of potassium channels genes, a new protocol was produced for the new EpiQuik™ Tissue Methylated DNA Immunoprecipitation commercial Kit, to work optimally with endometrium.

The protocol provided with the new commercial, tissue specific epigenetic kit took long time to be validated and ended up with producing a new protocol to apply and optimise this kit on endometrial and decidual samples. This new protocol for the new EpiQuik™ Tissue Methylated DNA Immunoprecipitation Kit was produced to perform epigenetic assessment of the endometrium to investigate the DNA methylation of the studied potassium channels. A pilot study was carried out, showing a hypermethylation of the KCNK17 gene in the fertile mid-secretory endometrium. This finding provides interesting insight of K channel regulation at the genomic level for methylation and is an under researched area

## Future Work

Each result and each technique in this thesis can be taken and expanded to focus on particular aspect, and to be applied clinically in the future. With regards to the endometrial pathophysiology, Potassium channels expression

can be tested for other causes of infertility in women endometriosis, irregular bleeding, adenomyomas and PCOs.

-In our study, endometrium and decidual tissue were used. For better understanding of the role of potassium channels in endometrial receiptivity and embryo implantation, it will be of high value to test for the channels expression in decidua from early abortion following D&C. This will give a full image for the potassium channels expression in endometrium/ decidua through menstrual cycle, followed by early pregnancy till full term decidua.

-Applying more treatments that may give more information on the regulation of potassium channels like atosiban, as recent studies have been using alternative or pharmacological drugs to reduce uterine contractility during IVF-embryo transfer, aiming to increase embryo implantation rates (Fanchin et al., 2001). This was proven by the data presented at the 74th annual meeting of the American Society of Reproductive Medicine (ASRM), held at Colorado in October 2018, where phase-three clinical studies with the oral oxytocin receptor antagonist, nolasiban, involving patients undergoing IVF showed an improvement in both on-going pregnancy and live birth rates (Visnova et al., 2018).

-More future work can be conducted in relation to both potassium channels and intrauterine pH. For pH Till now, all the recording for intrauterine pH are carried out in bovine and rabbits in early and mid 1970s (Ng et al., 2017). So it is important to develop a new study to understand the pH within the female reproductive tract as it may play a role in infertility. Studies observed that hyperactivated sperms requires Ca2+ influx into the sperm tail by an alkalinization-activated voltage-sensitive Ca2+-selective current (CatSper) (Qi et al., 2007). CatSper is actived (by raising pHi or progesterone stimulation) causing sustained intracellular calcium elevation (Alasmari et al., 2013).

Also studying new potassium channels involved in fertility can help to spot any reason that could be cause on unexplained infertility. Other potassium

channels can include pH-sensitive potassium channel of sperm (KSper), which is the most predominant presented in potassium conductance of mouse spermatozoa. KSper is important as it regulates the membrane potential of sperm (Smith et al., 2013). Any deletion or misregulation of CatSper gene may lead to infertility (Singh and Rajender, 2015). Future work can include knockout models for CatSper gene (after understanding the intrauterine pH) or knockout of KSper by transfection of exogenous siRNA can lead to infertility and this to be part of the era for developing gamete-based safe non-hormonal contraceptives.

-For TEER, fixation of cells at the end of experiments and carry on immunofluorescence staining to test for the potassium channels protein after applying potassium channels blockers and modulators.
-Also determine any alteration of functionally active potassium channels in endometrium, electrophysiology whole-cell voltage patch clamp recording
- Epigenetic regulation of potassium channels can be expanded after our new protocol was set up and optimised and applied on larger population. Also testing other epigenetic influences as histone modification will be important understand the potassium channels genes regulation, especially for genes that showed not to be regulated through DNA methylation.

## Conclusion

The main aim of this thesis was to ‘’go back to basics” and many techniques and approaches were carried out to arrange the puzzle with the target to reach a clearer image for better understanding the regulation of potassium channels in human endometrium and their role in endometrial receptivity and embryo implantation in women with RIF. At the end of this thesis, it was not possible to clearly answer the hypothesis.

There were many challenges through our work and limitations for the lab studies as: 1) Waiting for the ethical approval to start collecting human tissue

was one of the main limitations. 2) A larger (n) number of samples was needed and this was due to precise criteria of the target group, not allowing a high recruitment flow. 3) Limited amount of tissues obtained, especially if the sample was taken during the proliferative stage. This limited amount of tissue was a limitation to apply all the techniques or all treatments on the same sample and this made the strength of the statistics weaker as comparing paired samples is statistically stronger. 4) It was difficult to apply molecular biology on collected tissues, as samples need to be snap frozen or cultured as soon to avoid RNase activity and RNA degradation.

Despite these challenges, this work has demonstrated a new knowledge on the role of potassium channels in endometrium contributing to normal and infertile endometrial function and demonstrates the involvement of potassium channels in the uterine electrolyte transport and intra-uterine fluid amount and composition. These highlighted various fields, results and validation to be considered a concrete ground for future research within RIF endometrium that may have future application in improving implantation rates and identifying novel targets for fertility regulation. These current outcomes could be used within the context of assisted reproductive technologies to reach a better understanding of endometrial receptivity and with more future work, many clues could be used to predict the causes of success and failure of ART treatment.

# Appendices

The University of Nottingham
UNITED KINGDOM • CHINA • MALAYSIA

Derby Teaching Hospitals NHS
NHS Foundation Trust

## Participant Information Sheet

**IRAS Project ID: 218026**

**Title of Study:** Uterine Environment (REEM)

**Name of Researchers:** Professor Raheela Khan, Mr Saad Amer, Mr K Jayaprakasan

*We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. Talk to others about the study if you wish. Ask us if there is anything that is not clear. Take your time to decide whether you wish to take part or not.*

**What is the purpose of the study?**

Here at the Royal Derby Hospital we are looking at proteins present in cells that may play a role in becoming pregnant. These proteins have been described in cells lining the lung and stomach. They are also present in the endometrium (lining of the womb) and are regulated by local conditions and molecules. The purpose of our study is to find out more about the properties, function and behaviour of these proteins in relation to achieving pregnancy as well as in infertility.

**Why have I been invited?**

You are being invited to take part because you will be undergoing gynaecological investigation, hysterectomy or sterilisation. We are inviting 500 participants like you to take part. You may be asked if you would be willing to provide a biopsy taken from the lining of your womb (endometrium) and/or a blood sample at the time of investigation or sterilization. In the case of a hysterectomy, the endometrium will be removed directly from the uterus removed during surgery. The information obtained from experiments using your sample will be valuable in increasing our knowledge of the factors affecting pregnancy. We will also obtain scientific information that may help to explain why some women have difficulty becoming pregnant.

**Do I have to take part?** It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This would not affect your legal rights.

**What will happen to me if I take part?**

If you agree to take part, we will ask your permission to use a small piece (no more than the size of your thumb tip) of endometrium for use in laboratory-based research. You may also be asked to provide a blood sample (20mls; 4 teaspoons) which will be taken alongside any other blood samples being taken for surgery or investigation. The endometrial sample will be removed during the gynaecological procedure you will be undergoing. There will be no added discomfort or consequences to your recovery except possibly for a little vaginal bleeding afterwards. Taking the sample will not harm you, nor prolong the procedure significantly and will not affect the normal healing or function of your womb afterwards. In certain cases, you may be approached to provide a second endometrial biopsy but this time at a different stage of your menstrual cycle compared with the timing of the first biopsy. However this will be made clear to you very early on in discussions with the researcher.

**Expenses and payments:** Participants will not be paid to participate in the study.

**What are the possible disadvantages and risks of taking part?**

The risks of taking part are very low. They may include some vaginal bleeding afterwards or a sore arm from taking a blood sample. However you will be undergoing some of these procedures as part of your normal medical care and therefore there is little additional risk due to the research itself. This study will have no direct benefit for you.

**What are the possible benefits of taking part?** The information we get from this study may help in improving knowledge and future tests on important molecules involved in becoming pregnant.

**What happens when the research study stops?**

The data will be analysed and written up for publication in medical and scientific journals. It is anticipated that the findings from the present study will enable us to apply for further funding to continue this investigation.

V1.0; 05/2017

**What if there is a problem?**

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. The researchers contact details are given at the end of this information sheet. If you remain unhappy and wish to complain formally, you can do this by contacting Patient Advice and Liaison Service (PALS) of the Derby Teaching hospitals by Freephone 0800 7837691; Office 01332785156 or email: dhft.contactpals@nhs.net

**Will my taking part in the study be kept confidential?**

We will follow ethical and legal practice and all information about you will be handled in confidence.
If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.
All information which is collected about you during the course of the research will be kept **strictly confidential**, stored in a secure and locked office, and on a password protected database. Any information about you which leaves the hospital will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it.
**All data** will be securely locked away in a cabinet with access only by the CI and delegated staff. Your personal data (address, telephone number) will be kept for up to three years after the end of the study so that we are able to contact you about the findings of the study and possible follow-up studies (unless you advise us that you do not wish to be contacted). All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality, only members of the research team will have access to your personal data.

**What will happen if I don't want to carry on with the study?**

Your participation is voluntary and you are free to withdraw at any time, without giving any reason, and without your legal rights being affected.

**What will happen to any samples I give?**

We would also like to seek your consent so that any remaining samples may be stored and used in possible future research – this is optional (please indicate you agree to this on the consent form). The samples will be stored with a code unique to you and securely at the University of Nottingham under the University's Human Tissue Research Licence (no 12265).
Some of these future studies may be carried out by researchers other than current team of scientists, who ran the first study, including researchers working for commercial companies. Any samples or data used will be anonymised, and you will not be identified in anyway. If you do not agree to this any remaining samples will be disposed of in accordance with the Human Tissue Authority's codes of practice.

**Will any genetic tests be done?**

We will not carry out any genetic testing but we will be using DNA and RNA to understand how some genes of interest to us are involved in pathways that lead to pregnancy.

**What will happen to the results of the research study?**

The results will be published during the course of the study and up to three years later and may form part of a higher degree. Copies of published reports will be made available to participants of the study. Subjects will not be identified in any publication.

**Who is organising and funding the research?**

This research is being organised by the University of Nottingham and is being funded through our personal research accounts and a small amount of the fees paid by students carrying out this research.

**Who has reviewed the study?**

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by East Midlands-Derby Research Ethics Committee.

**Further information and contact details**

Professor Raheela N Khan, Division of Medical Science and Graduate Entry Medicine, School of Medicine,
University of Nottingham, Medical School, Royal Derby Hospital Centre, Derby DE22 3DT
**Tel:** +44 (0) 1332 724664;
**Email:** raheela.khan@nottingham.ac.uk

V1.0; 05/2017

# 5 Endometrial ethics consent form

The University of Nottingham
UNITED KINGDOM • CHINA • MALAYSIA

Derby Teaching Hospitals NHS
NHS Foundation Trust

**CONSENT FORM Endometrium**

**Title of Study: Uterine environment (REEM)**

**IRAS Project ID: 218026**

**Name of Researcher**: Professor Raheela Khan, Mr Amar, Mr Jayaprakasan

**Name of Participant:**

**Please initial box**

1. I confirm that I have read and understand the information sheet version number 1.01 dated 05/2017 for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected in the study may be looked at by authorised individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential. ☐

4. I understand and agree that an endometrial sample will be taken for analysis. ☐

   I understand and agree that a blood sample will be taken for analysis. ☐

5. **Consent for storage and use in possible future research:**
   I agree that the samples I have given and the information gathered about me can be stored by the University of Nottingham at the Medical school, Derby, for possible use in future studies. I understand that some of these studies may be carried out by researchers other than the current team who ran the first study, including researchers working for commercial companies. Any samples or data used will be anonymised, and I will not be identified in anyway.

   Agree ☐ Don't agree ☐

6. I agree to take part in the above study. ☐

| Name of participant | Date | Signature |
|---|---|---|
| Name of person taking consent | Date | Signature |

V1.0; 05/2017; 3 copies – 1 for participant, 1 for project file and 1 for medical notes

# 6 Placental Patient information sheet (PIS)

Derby Hospitals NHS
NHS Foundation Trust

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

## Patient Information Sheet

### Studies of Human Pregnancy and Preterm Birth

*You are being invited to take part in a research study of the University of Nottingham being carried out in Derby. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.*

**1. What is the purpose of the study?**

The switch from pregnancy to labour and how the uterus contracts for delivery is not understood. In some cases, labour may begin too early when the baby is not ready to be born (preterm). Preterm birth is delivery occurring before 37 completed weeks of pregnancy and affects about 8% of all pregnancies in the UK. Preterm labour is a factor in approximately 40% of all preterm deliveries. Two other pregnancy-specific disorders preeclampsia (high blood pressure with protein in the urine) and intra-uterine growth restriction (IUGR), where babies are born small for their size, may also lead to preterm birth. To date, there are still no effective drugs to treat preterm birth due to preterm labour nor any reliable markers that allow these disorders to be monitored throughout pregnancy.

Here at the Royal Derby Hospital, we are carrying out research into term and preterm birth with the aim of improving our understanding of the causes of preterm birth. We hope that new information from our studies will pave the way for the future development of new drugs and new ways designed to reduce the incidence of preterm delivery. The three projects we are involved with are as follows:

***Project 1: Inflammation in pregnancy:*** The uterus produces many important proteins which play a role in pregnancy and labour. It is thought that infection or inflammation of the female reproductive tract may activate the process of labour too early by causing uterine contractions through molecules produced by the placenta and cord blood.

***Project 2: Lipid-protein interactions in the pregnant human uterus.*** In recent years, there has been great interest in how certain fats affect pregnancy. We are studying how these fats (also known as lipids) present in the uterus (womb) influence labour. In addition, we know that the location of these lipids within cells may also indirectly activate pathways that control the production of proteins in the uterus.

***Project 3: Vascular problems in pregnancy:*** During pregnancy, blood flow to the baby provides food and oxygen essential to baby's growth and development. In some 10% of cases, poor blood flow to the placenta (afterbirth) may mean that the baby does not develop or grow properly, a condition known as intra-uterine growth restriction (IUGR). In addition, 1-2% of pregnancies result in preeclampsia, a leading cause of high blood pressure in pregnancy, which may endanger both mother's and baby's health if left undiagnosed. The aim of our project is to study the properties of the blood vessels in the uterus and placenta of women with preeclampsia or a growth-restricted pregnancy to identify whether blood vessel function is changed in these disorders compared with vessels of women with a normal pregnancy.

**2. Why have you been invited?**

We are recruiting patients from four different groups. You have been chosen as somebody who falls into one of the four following groups: (a) normal non-labouring, term pregnancy (b) labouring pregnancy, or you have been diagnosed with (c) a growth-restricted pregnancy, (d) preeclampsia. The 'normal pregnancy' group is a control group and including patients from this group will allow us to compare normal versus potentially difficult pregnancies. The groups will be divided further into term or preterm. We are recruiting patients for all three projects and it is likely that your tissue and cord blood samples will be used for any one of these 3 projects. We expect to recruit 3000 women in five years. There will be no direct benefit to you from this study but your participation may significantly aid our understanding of pregnancy and birth thus enabling us to make advances in the field of female and fetal health.

**3. Do I have to take part?**

*No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form.*

You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive. Any stored tissue samples that can still be identified as yours will be destroyed if you wish.

**4. What will happen to me if I take part?**

Should you decide to take part, if you will be undergoing a caesarean section, you may be asked to provide a myometrial (uterine muscle) biopsy which will be taken at caesarean section from the site of the operation. The biopsy is ~2g in weight and the size of a thumb tip. We will also ask you if we can take your placenta for research. In the case of a normal delivery, we will ask you to consider providing a placental and cord blood sample only after your baby has been delivered. Cord blood samples collected will not be available for any clinical needs such as subsequent treatment of your baby or other investigations. All samples will be taken after delivery of the baby. There is no further follow-up in relation to our study. This investigation is laboratory-based therefore samples will be taken to the lab and analysed using a variety of experimental techniques.

We would also wish to store any unused tissue and blood samples obtained from you for future unspecified research. Samples will only be used for studies approved by a research ethics committee. If however, you change your mind later about having your tissues stored for future research, then you can withdraw at any time without providing a reason. This should be done by contacting the Chief Investigator (contact details at bottom of this sheet).

**5. What are the other possible disadvantages and risks of taking part?**

There is minimal risk from taking the uterine muscle biopsy over and above that associated with the caesarean section. Taking the sample at caesarean section will not harm your baby, nor prolong the operation and will not affect the normal healing of your uterus. Nor will be there be any effect on the structure or function of your uterus afterwards, your future fertility or any risk to baby as the biopsy will be taken after delivery of your baby. If it is felt by the clinician in charge of your care that taking a uterine muscle biopsy is not appropriate, the sample will not be taken. Your wellbeing and that of your baby is paramount. There is no risk to you or your baby from the use of placental samples.

**6. What are the possible benefits of taking part?**

There is no direct benefit to you or your baby in taking part in this study. However the information obtained from using your uterine muscle biopsy and placenta may enable us to identify possible new pathways that will improve our understanding of uterine function.

**7. What happens when the research study stops?**

The data collected will be analyzed and prepared for scientific publication. We would hope to expand the study to understand more about the functions of the human uterus in normal and abnormal conditions. Participants will not be identified in any report, presentation or publication.

**8. Will my taking part in the study be kept confidential?**

Yes. All the information about your participation in this study will be kept confidential. If you consent to take part in the research, any of your medical records may be inspected by researchers directly involved with the project for purposes of analysing the results and all details will remain strictly confidential. They may also be looked at by people from regulatory authorities to check that the study is being carried out correctly.

**9. Who is organising and funding the research?**

This study is being funded through various sources including the Wellcome Trust, the Medical Research Council, British Heart Foundation, Action Medical Research and the University of Nottingham. The research is sponsored by the University of Nottingham.

**10. Who has reviewed the study?**

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by Derbyshire Research Ethics Committee.

**Complaints:**

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Dr Raheela Khan 01332 724664; raheela.khan@nottingham.ac.uk). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure or details can be obtained from the Royal Derby Hospital Patient Advice and Liaison Service, Uttoxeter Road, Derby, DE22 3NE, dhft.contactpals@nhs.net, tel: 0800 783 7691).

**Contact for further information:**

**Dr Raheela N Khan** 01332 724664; email: raheela.khan@nottingham.ac.uk
**Mr Daniel Hay** 01332 724842; email: daniel.hay@nottingham.ac.uk

For further information on the research of the group:
http:\\www.nottingham.ac.uk/research/groups/reproductivephysiology/index.aspx

**Thank you for taking the time to read this.**

RNK – V4/PTB 01/2014

# 7 Placental consent form

University of Nottingham
UK | CHINA | MALAYSIA

Derby Teaching Hospitals NHS
NHS Foundation Trust

**CONSENT FORM: Pregnancy**
**(Version 5MUPPS – 06/2018)**

**Title of Study: Studies of human pregnancy and preterm birth**

**REC ref: 09/H0401/90**

**Name of Researcher**: Professor Raheela Khan

**Name of Participant:** _____ **Please initial box**

1. I confirm that I have read and understand the information sheet version number 5 dated 06/2018 for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. I understand that should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis. ☐

3. I understand that relevant sections of my medical notes and data collected in the study may be looked at by authorised individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential. ☐

4. I understand and agree to a myometrial ☐ placental ☐ and cord blood sample to be taken for research investigations. ☐

5. I agree to the storage of uterine, placental and cord blood samples for any future, as yet, unspecified research studies relating to the study of pregnancy and pregnancy related disorders. ☐
*NB: Cord blood samples collected will not be available for any clinical needs such as subsequent treatment of your baby or other investigations.*

6. I agree to take part in the above study. ☐

| | | |
|---|---|---|
| Name of Participant | Date | Signature |
| Name of Person taking consent (if different from Principal Investigator) | Date | Signature |
| Name of Principal Investigator | Date | Signature |

3 copies: 1 for participant, 1 for the project notes and 1 for the medical notes

RNKV5 PTB Consent Form

# 8 Vitamin D specification sheet by manufacturer

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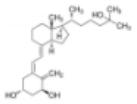
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## Product Specification

Product Name:
1α,25-Dihydroxyvitamin $D_3$ – ≥99% (HPLC)

**Product Number:** **D1530**
CAS Number: 32222-06-3

Formula: $C_{27}H_{44}O_3$
Formula Weight: 416.64 g/mol
Storage Temperature: -20 °C



| TEST | Specification |
|---|---|
| Appearance (Form)<br>Film (Vacuum Dried) | Conforms |
| Solubility (Solvent) | ACN (purged w/N2) |
| Solubility (Conc) | 0.90 - 1.10 mg/ml |
| Solubility (Turbidity) | Clear |
| Solubility (Color) | Colorless |
| Purity (HPLC) | ≥ 99.00 % |
| Overpack (By HPLC) | ≤ 10.00 % |
| Vial Content<br>Size | 10 µg or 0.1 mg |
| Vial Content (By HPLC) | |
| Storage Conditions | Under Argon |
| Recommended Retest Period<br>2 Years | ---------- |

Specification: PRE.1.ZQ5.10000009839

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

# 9 Intralipid specification sheet by manufacturer

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3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

## Product Specification

Product Name:
Intralipid – 20%, emulsion

| | |
|---|---|
| **Product Number:** | **I141** |
| CAS Number: | 68890-65-3 |
| MDL: | MFCD00287215 |
| Storage Temperature: | 2 - 8 °C |

| TEST | Specification |
|---|---|
| Component Content (%)<br>(WT % FAT) SOYBEAN OIL | 18.0 - 22.0 % wt |
| Component #2<br>GLYCERIN | 1.9 - 2.5 % |
| Component #3<br>PHOSPHOLIPID | 0.9 - 1.3 % |
| Free Fatty Acids | ≤ 5.0 MEQ/L |
| pH | 8.0 - 8.9 |

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**Title:** The genomics of the human endometrium
**Author:** Maria Ruiz-Alonso,David Blesa,Carlos Simón
**Publication:** Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease
**Publisher:** Elsevier
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