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EXPRESSION AND HORMONAL REGULATION OF AQUAPORINS IN THE UTERUS

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To Carl-Adam,
Sarah, Philip, Nils and Knut

ABSTRACT

BACKGROUND

Aquaporins (AQPs) are water channels present in the cell wall, allowing water – and occasionally other molecules – to pass the cell membrane. So far 13 AQPs have been found in humans. Their function is to maintain the cell internal milieu by regulating water and ion equilibrium. AQPs can be found in virtually all cell types and tissues and are essential to the normal function of cells and organs. Malfunction of the water channels can cause disease.

The uterine wall consists of three layers of which the endometrium is the innermost portion, facing the uterine cavity. The endometrium undergoes structural changes during the menstrual cycle, influenced by the hormones estrogen (E) and progesterone (P), as a preparation for implantation of a fertilised egg.

Angiogenesis is the formation of new blood vessels from existing ones. This process is essential for regeneration of the endometrium after shedding during menstruation.

Excessive uterine bleeding, also called menorrhagia, is a common gynecological disorder, presenting with large menstrual bleedings and reduced quality of life for those affected. Several organic diseases can lead to excessive uterine bleeding, but in most of the cases the cause is unknown.

Mifepristone is a synthetic steroid which has the ability to bind to P receptors, thereby mainly blocking the effect of P. Mifepristone treatment improves menstrual bleeding pattern and has an effect on endometrial vessels.

OBJECTIVES OF THE STUDIES

The general objective of the studies was to gain knowledge about the expression and hormonal regulation of AQPs in the uterus as well as to investigate the connection between AQPs and uterine function in healthy subjects and patients diagnosed with excessive uterine bleeding.

RESULTS AND CONCLUSIONS

AQP1 and AQP2 are present in human endometrium; AQP1 in endothelial cells (lining the interior surface of blood vessels) and AQP2 in epithelial cells (lining cavities, surface structures, and glands). They can be assumed to be involved in events where water transport is essential, e.g. menstruation and reduction of uterine fluid volume at the time of implantation. AQP1 levels in the endometrium are reduced in women with excessive uterine bleeding, indicating that an impaired expression of AQP1 could be a cause to this condition. Mifepristone treatment increases AQP1 expression in human endometrium, which could imply a regulation by P. In cell culture, it was possible to show a hormonal regulation of certain AQPs: AQP1 protein expression was up-regulated in endothelial cells when exposed to E, as well as to P; AQP2 mRNA levels increased after exposure to E + IC, and P combined with mifepristone; and AQP7 protein-expression increased after treatment with E + IC, and E + P, suggesting a role for these AQPs in increased endometrial secretion after ovulation.

Keywords: endometrium, water channel, mifepristone, angiogenesis, edema, menorrhagia, dysfunctional uterine bleeding, estrogen, progesterone

ABSTRACT

BAKGRUND

Aquaporiner (AQPs) är vattenkanaler som finns i cellväggar. De tillåter vatten – och ibland andra molekyler – att passera igenom cellmembran. Hittills har 13 olika AQPs identifierats i människan. Deras funktion är att upprätthålla cellens inre miljö genom att reglera jämvikten av vatten och joner. AQPs finns i alla celltyper och vävnader och är livsviktiga för cellers och organs normala funktion. Dysfunktion hos vattenkanaler kan orsaka sjukdom.

Livmodern är uppbyggd av tre lager, av vilka endometriet bildar den inre delen mot livmoderkaviteten. Under menstruationscykeln genomgår endometriet, under inflytande av hormonerna östrogen (E) och progesteron (P), strukturella förändringar som förberedelse för implantationen av ett befruktat ägg.

Angiogenes är en process där nya blodkärl skapas från de redan existerande. Detta är av avgörande betydelse för regenerationen av endometriet efter att det stötts ut under menstruationen.

Menorragi är en vanlig gynekologisk åkomma, som innebär rikliga menstruationsblödningar och medför en försämrad livskvalitet. Ett flertal organiska sjukdomar kan leda till menorragi, men i de flesta fall är den bakomliggande orsaken okänd.

Mifepriston är en syntetiskt framställd steroid som har förmågan att binda till P-receptorer och därmed blockera effekten av P. Mifepristonbehandling påverkar bland annat kärlen i endometriet och kan förbättra blödningsmönstret.

MÅL MED STUDIERNA

Det övergripande målet med studierna var att få mer kunskap om uttryck och hormonell reglering av AQPs i livmodern samt att undersöka kopplingen mellan AQPs och endometriefunktion hos friska kvinnor och patienter diagnostiserade med menorragi.

RESULTAT OCH SLUTLEDNINGAR

AQP1 och AQP2 finns i humant endometrium; AQP1 i endotelceller (som täcker insidan av blodkärl) och AQP2 i epitelceller (som utkläder insidan av håligheter, ytstrukturer och körtlar). De kan antas vara inblandade i processer där vattentransport är av stor betydelse, exempelvis menstruation och minskningen av sekret i livmodern vid tiden för implantation. AQP1-nivåerna i endometriet är lägre hos kvinnor med menorragi, vilket tyder på att en rubbning i uttrycket av AQP1 skulle kunna vara en orsak till detta tillstånd. Behandling med mifepriston ökar mängden AQP1 som kan vara reglerat av P. I cellodlingsexperiment har vi kunnat visa att AQPs regleras på hormonell väg: AQP1-protein uppreglerades i endotelceller vid behandling med E samt P; AQP2-mRNA-nivåerna ökade efter exponering för E + IC samt P i kombination med mifepriston; mängden AQP7-protein ökade efter behandling med E och P i kombination och E + IC. Detta stöder teorin att dessa AQPs spelar en roll under tiden efter ägglossning då sekretionen ökar i endometriet.

ABSTRACT

THEORETISCHER HINTERGRUND

Aquaporine (AQPs) sind Wasserkanäle, die in den Zellwänden vorhanden sind. Sie lassen Wasser – und manchmal auch andere Moleküle – durch die Zellmembrane. Soweit sind 13 humane AQPs bekannt. Ihre Funktion besteht darin, das innere Milieu der Zelle durch Regulation von Wasser und Ionen zu bewahren. AQPs sind in allen Zell- und Gewebetypen vorhanden und für die normale Funktion der Zellen und Organe essentiell. Fehlfunktionen der Wasserkanäle können Krankheiten hervorrufen.

Die Gebärmutterwand besteht aus drei Schichten, von denen das Endometrium den innersten Teil – die Gebärmutterhöhle auskleidend - darstellt. Das Endometrium wird als Vorbereitung auf die Implantation eines befruchteten Eis während des Menstruationszyklus strukturellen Veränderungen unterworfen, die von den Hormonen Estrogen (E) und Progesteron (P) beeinflusst sind.

Angiogenese ist das Wachstum von kleinen Blutgefäßen (Kapillaren) aus einem vorgebildeten Kapillarsystem. Der Prozess ist für die Regeneration des Endometriums nach dem Abbau während der Menstruation von größter Bedeutung.

Menorrhagie ist ein häufiger gynäkologischer Zustand, der mit großen Menstruationsblutungen und reduzierter Lebensqualität einhergeht, und von anderen Krankheiten hervorgerufen werden kann, obwohl die Ursache meistens unbekannt ist.

Mifepriston ist ein synthetisches Steroid, das die Fähigkeit besitzt, P-Rezeptoren zu binden und dadurch den P-Effekt zu blockieren. Behandlung mit Mifepriston wirkt ausgleichend auf die Menstruationsblutung und wirkt auf Gefäße im Endometrium.

ZIEL DER STUDIEN

Hauptziel der Studien war, Kenntnis über den Ausdruck und die hormonelle Regulierung von AQPs in der Gebärmutter zu gewinnen, sowie den Zusammenhang zwischen AQPs und Gebärmutterfunktion sowohl bei gesunden Frauen als auch bei Patienten mit Menorrhagie zu erforschen.

RESULTATE UND SCHLUSSFOLGERUNGEN

AQP1 und AQP2 sind im menschlichen Endometrium vorhanden; AQP1 in Endothelzellen (innere Oberfläche von Blutgefäßen) und AQP2 in Epithelzellen (Körperhöhlen auskleidend, Oberflächenstrukturen und Drüsen). Es kann angenommen werden, dass sie in Geschehnissen, bei denen Wassertransport von Bedeutung ist, involviert sind, z.B. Reduktion von Flüssigkeit in der Gebärmutter zur Zeit der Implantation und Menstruation. AQP1-Niveaus im Endometrium sind bei Frauen mit Menorrhagie reduziert, was auf ein fehlerhaftes AQP1-Niveau zurückgeführt werden könnte. Behandlung mit Mifepriston steigert das AQP1-Niveau und könnte auf eine Regulierung durch P deuten. In der Zell-Kultur war es möglich eine hormonelle Regulierung von gewissen AQPs zu zeigen; AQP1-Protein wurde in Endothelzellen nach Behandlung mit sowohl E, als auch mit P gesteigert; AQP2-mRNA-Niveaus stiegen nach Behandlung mit E + IC, und P in Kombination mit Mifepriston; AQP7 wurde nach Behandlung mit E + P, und E + IC gesteigert, was eine Rolle dieser AQPs bei der erhöhten endometrischen Sekretion nach Ovulation aufzeigen könnte.

LIST OF PUBLICATIONS

I. **Anna Hildenbrand**, Luther Lalitkumar, Soren Nielsen, Kristina Gemzell-Danielsson, Anneli Stavreus-Evers
Expression of aquaporin 2 in human endometrium
Fertil Steril 2006; 86:1452–8

II. Miriam Mints, **Anna Hildenbrand**, Luther PG Lalitkumar, Sonia Andersson, Soren Nielsen, Kristina Gemzell Danielsson, Anneli Stavreus-Evers
Expression of aquaporin-1 in endometrial blood vessels in menorrhagia
Int Jour of Mol Med 2007; 19: 407-411

III. **Anna Hildenbrand**, Anneli Stavreus-Evers, PGL Lalitkumar, Soren Nielsen, Miriam Mints, Kristina Gemzell Danielsson
Aquaporin 1 is expressed in the human endometrium during normal cycle and increases after mifepristone treatment
Int Jour of Mol Med 2008; 22: 49-53

IV. **Anna Hildenbrand**, PGL Lalitkumar, Anneli Stavreus-Evers, Baiju Surendran, Kristina Gemzell-Danielsson
Hormonal control on aquaporins 1, 2, and 7 in human uterine endothelial cells by estrogen and progesterone: An *in vitro* study
Manuscript

CONTENTS

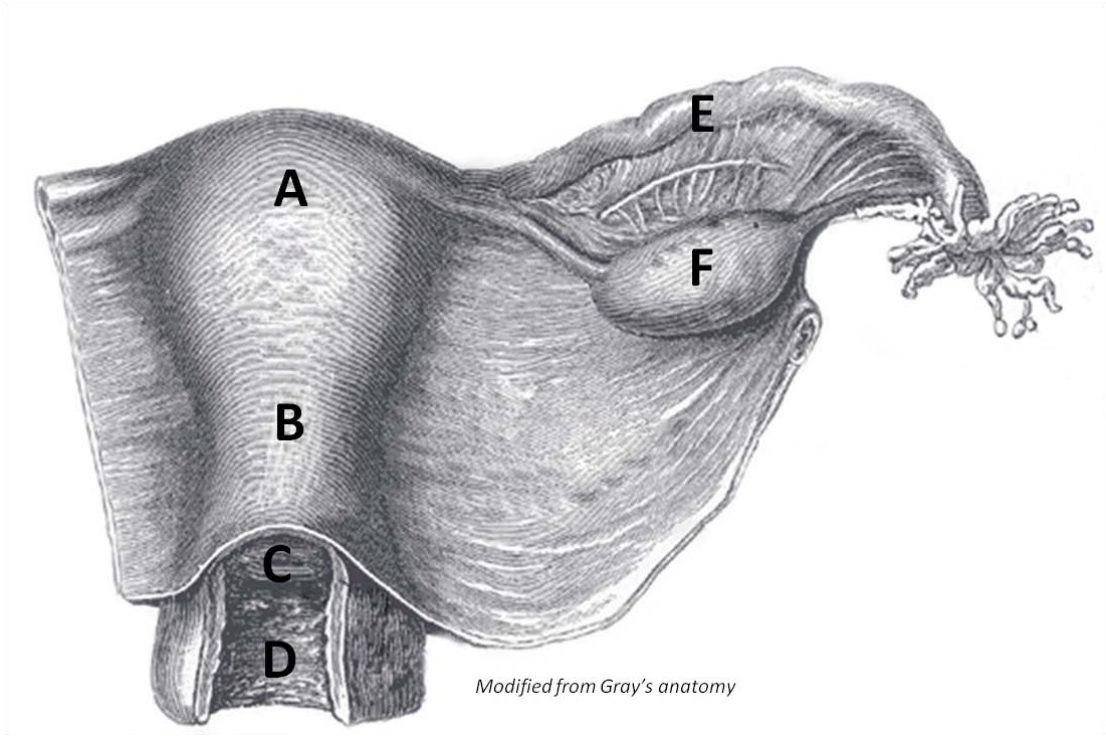
LIST OF ABBREVIATIONS

ABC	avidin-biotin-complex
ACTH	adrenocorticotropic hormone
AQP	aquaporin
ATP	adenosine triphosphate
Bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxy ribonucleic acid
DAB	diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DNA	deoxy ribonucleic acid
E	estrogen
ECM	extracellular matrix
ERα/β	estrogen receptor α/β
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
IGF	insulin like growth factor
Ig	immunoglobulin
IgG	immunoglobulin G
ICC	immunocytochemistry
IHC	immunohistochemistry
LH	luteinizing hormone
LIF	leukemia inhibitory factor
MMP	metalloproteinase
mRNA	messenger RNA
NK	natural killer (cells)
NPA	Asn-Pro-Ala
P	progesterone
PBS	phosphate buffered saline
PG	prostaglandin
PRA/PRB	progesterone receptor A/B
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	scanning electron microscopy
TGF	transforming growth factor
TIMP	total conversion of tissue inhibitor of metalloproteinase
VEGF	vascular endothelial growth factor
ZP	zona pellucida
17HSD	17β-hydroxysteroid dehydrogenase

1 INTRODUCTION

1.1 ANATOMY

The uterus is located in the pelvis, dorsal to the urinary bladder and ventral to the rectum, providing structural integrity and support to the adjacent organs. Anatomically, the uterus can be divided into three parts from cranial to caudal: fundus, corpus and cervix. The upper left and right parts of the uterus form the uterine horns, continuing in the fallopian tubes. The cervix opens up into the vagina through the cervical canal. The uterine wall consists of three layers; the endometrium which is the thin lining of the uterus; the muscular myometrium; and the serosa. The endometrium can be divided into two functionally different layers; the functional layer which is closest to the uterine cavity, and basal layer, located between the functional layer and the myometrium. The uterus receives its arterial blood from the uterine arteries (branches of the internal iliac artery), and the ovarian arteries (branches of aorta). Blood supply of the basal layer of the endometrium is provided by straight arteries, while the functional layer is supplied by coiled spiral arteries. The uterine blood drainage is conducted by the uterine veins, draining into the internal iliac vein. (Hall-Craggs 1990)



Picture 1: Anatomy of the female reproductive tract
A fundus, **B** corpus, **C** cervix/cervical canal, **D** vagina, **E** fallopian tube, **F** ovary

1.2 THE ENDOMETRIUM

1.2.1 Cells of the endometrium

The endometrium can be divided into two hormone responding tissue types: a single layer of columnar epithelium (lining of the uterine cavity and glands) and stromal connective tissue, of which the latter varies in thickness due to the hormonal levels and contains fibroblasts, endothelial cells and leukocytes. The luminal epithelium acts as a blood-uterine lumen barrier, preventing different substances to enter the uterus from the blood (McRae and Kennedy 1983), and it also provides the site of implantation for the developing embryo. The glandular epithelium secretes a number of autocrine and paracrine factors required for development of the endometrium and implantation of the embryo. Endometrial fibroblasts produce extracellular matrix (ECM), metalloproteinases (MMPs), and more. MMPs cleave proteins in the matrix, thereby taking part in remodelling endometrial ECM. Stromal endothelial cells, located in the wall of arteries and veins, are essential for angiogenesis (formation of new vessels from existing ones). Leukocytes are part of the immune system, mediating inflammatory response, and vary in number throughout the menstrual cycle. (Salamonsen and Lathbury 2000) The endometrial leukocytes vary in number and type during the menstrual cycle and include eosinophils, neutrophils, macrophages, mast cells, t-cells, b-cells, and natural killer (NK) cells. (Critchley et al. 1999)

1.2.2 Endometrial dating

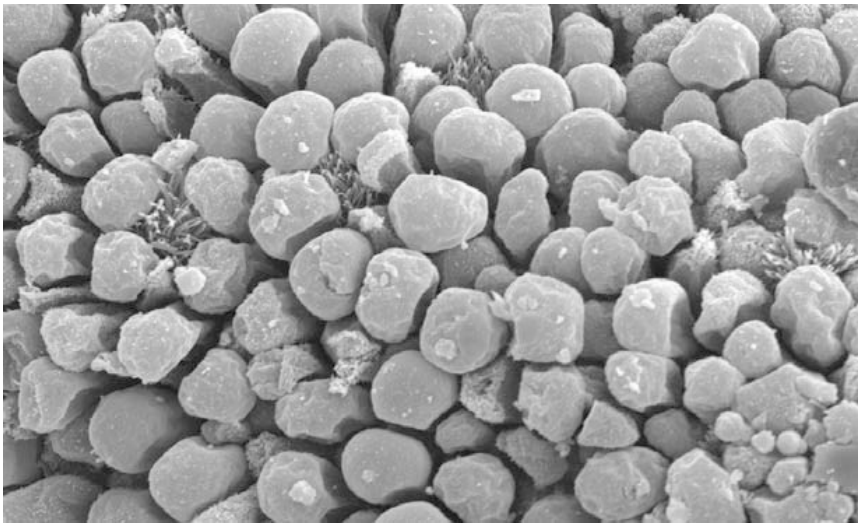
The endometrium undergoes characteristic and rapid histological changes, correlating to the different phases of the menstrual cycle, making it possible to determine different events, e.g. the implantation window, by means of the appearance of the tissue. (Noyes et al. 1975) Histological features, characteristic for the implantation window are increased volume of the glands, secretions, edema, coiling of spiral arteries with an accompanying accumulation of stromal cells around them (predecidual reaction), and the formation of decidual cells from stromal cells. (Speroff 1999)

1.2.3 Endometrial stem cells

Regeneration of endometrial glands and stroma, following menstruation, parturition and surgical procedures, takes place from their remnants in the basalis in order to form the new functional layer. Responsible for the regenerative capacity is a low number of epithelial and stromal clonogenic (with the ability to produce a colony from only one cell and at extremely low densities) stem cells. Little is known about these stem cells, but in other mature tissues, adult stem cells with high proliferative potential are present, responsible for maintenance of a specific tissue during the whole lifespan of the individual. Progenitor cells are derived from stem cells and restricted to one cell lineage in contrast to the adult stem cell, which has the potential to produce all cell types in a tissue or organ. Adult stem cells are identified by registration of their functional properties, rather than being recognised in the tissue, as no specific stem cell markers have been discovered so far. (Gargett 2006)

1.2.4 Pinopodes

Pinopodes are cytoplasmic protrusions of the endometrial surface, extending into the uterine cavity. They are found at the time when the endometrium is receptive for implantation of a developing embryo and have therefore been proposed to be structural markers of receptivity. In rodents, fluid uptake has been connected to pinopodes, but so far it has not been possible to prove that this would be the case in humans. (Enders and Nelson 1973) Pinopode formation and maintenance have been shown to be hormone dependent; progesterone (P) is crucial for the appearance, and estradiol (E), depending on administration day, interferes with the formation or induces regression. (Martel et al. 1991)



Picture 2: Pinopodes on the endometrial surface

1.3 THE MYOMETRIUM

In contrast to other smooth muscles, the myometrium is under hormonal control. The contractility changes during the menstrual cycle in a way related to tissue concentrations of hormones, rather than plasma levels. Myometrial activity is of importance for processes like discarding endometrial tissue and blood during menstruation, transportation of sperm cells and fertilised eggs, as well as implantation. Hyperactivity of the myometrium causes dysmenorrhoea. (Kissler et al. 2007; Åkerlund 1998) The myometrium is controlled by E and P, modulating contractility and relaxation together with other factors, e.g. oxytocin and prostaglandins (PG) mediating contractility while nitric oxide and prostacyclin mediate relaxation. (Hertelendy and Zakar 2004)

1.4 THE MENSTRUAL CYCLE

The first menstrual cycle (menarche) takes place during puberty and the last one will occur at the age of 40-60 (menopause). The average menstrual cycle in healthy, fertile women is 28 days (ranging from 21 to 35 days), counting from day one of menses. The menstrual cycle can be divided in different phases, depending on whether the endometrium or the ovary is in focus; the endometrial changes are recognised as menstrual, proliferative, and secretory phase, whereas the ovarian cycle is divided into the follicular and luteal phases. The purpose of the menstrual cycle is to prepare the endometrium for implantation of a fertilised egg. (Campbell-Monga 2006)

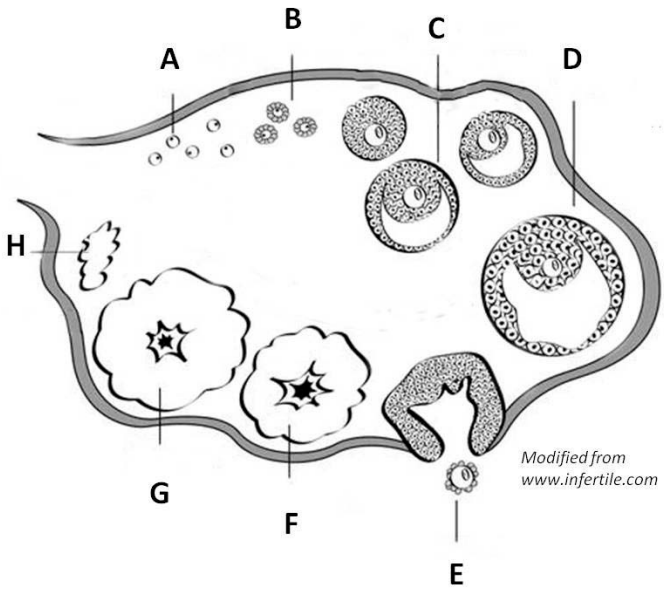
1.4.1 The menstrual phase

The menstrual phase, starting on day one of the menstrual cycle, lasts for 3-7 days in average and is characterised by the shedding of the functional layer of the endometrium, which results in menstrual flow consisting of blood, mucus, and tissue debris. Menstruation is triggered by a drop in P (and E) acting on P responsive decidual cells at the end of the previous menstrual cycle and only takes place if the woman is not pregnant. (Campbell-Monga 2006) The initialisation of menstruation also involves synthesis of PGE and PGF2α (stimulates uterine contractility and have vasoactive properties) (Elder 1983; Gemzell et al. 1990), vascular endothelial growth factor (VEGF) expression, influx of leukocytes, and constriction of spiral vessels. After this, the process is P dependent and becomes irreversible. MMPs are secreted by leukocytes, leading to breakdown of cell membranes and ECM. Prostaglandin levels increase and induce contraction of the myometrium and vasoconstriction of the spiral arteries. Finally, the functional layer of the endometrium will be shed. Parallel to this process, and under the influence of E, regeneration will start from the basalis layer. (Mihm et al. 2010)

1.4.2 The follicular phase and folliculogenesis

During the next phase of the menstrual cycle, the follicular phase (approximately day 4-14), increasing amounts of E are secreted, which stop the menstrual bleeding. Also due to the E effect, the lining of the uterus thickens. The follicular phase starts with gonadotropin releasing hormone (GnRH), secreted from hypothalamus, stimulating the pituitary to produce follicle stimulating hormone (FSH) and luteinizing hormone (LH). The latter two hormones, in turn, stimulate follicle growth in the ovary. The follicle is where the primary oocyte (egg cell), surrounded by granulosa cells, develops. The granulosa cells are covered by (counted from the inside) the follicular basement membrane, followed by theca interna, and theca externa. The maturation of a follicle, or folliculogenesis, takes place in several steps, starting with the primordial follicle, which is the stage where the immature oocyte rests from birth until initiation many years later. The folliculogenesis lasts for about 375 days, coinciding with thirteen menstrual cycles and starting continuously, meaning that there will be several follicles of all stages at any time. Towards the end of the folliculogenesis, the majority of the follicles which started growing at the same time have died; a process called atresia. The remaining follicles compete for FSH, which induces growth. In the end, only one dominant follicle, the one with the most FSH receptors, will remain. The growing follicles (after initiation, but before atresia) produce E and also P, although the major source of preovulatory P will be the adrenal gland, regulated by adrenocorticotropic hormone (ACTH). Ovulation (release of an ovum, or egg, from the dominant follicle)

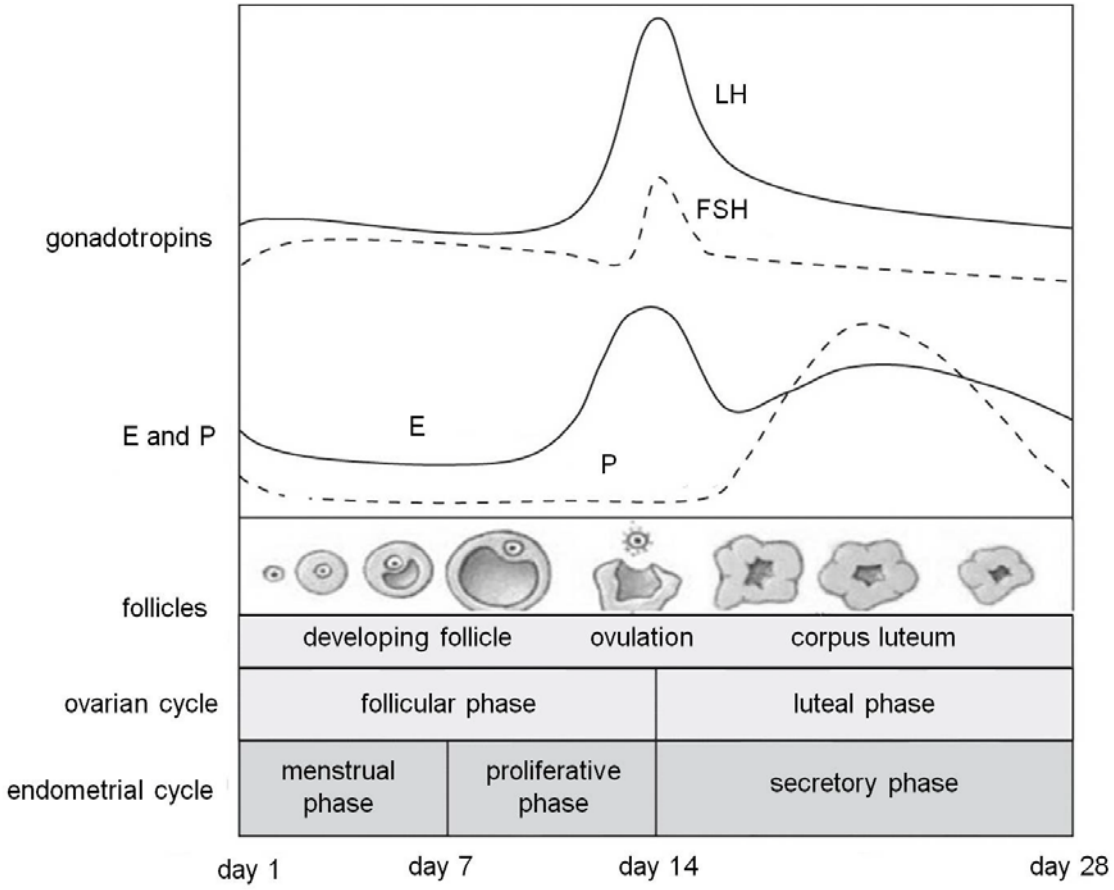
takes place on day 14 in a standardised cycle and marks the transition to the next phase of the menstrual cycle. It will be triggered by a peak in LH caused by a positive E feedback on hypothalamus. (Campbell-Monga 2006)



Picture 3: Folliculogenesis
A: primordial follicles, **B**: primary follicles, **C**: maturing follicles, **D**: dominant, preovulatory follicle, **E**: released ovum, **F**: early CL, **G**: mature CL, **H**: CA

1.4.3 The luteal phase

The luteal phase corresponds to day 14-28 and is characterised by P stimulating maturation of the endometrium, as a preparation for implantation and induction of pregnancy. After ovulation, the resulting empty follicle will become corpus luteum (CL) which secretes P. CL consists of two types of cells; small and large cells. The small cells secrete low levels of P when not stimulated, express LH receptors and produce increased amounts of P upon stimulation by LH and cAMP. The large cells secrete high levels of P in the absence of stimulation and are non-responding to LH or cAMP. Although they do not express receptors for LH, receptors for prostaglandin E_2 (PGE_2) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$) are present. (Hansel and Dowd 1986) During a few days (day LH+6 to LH+10; referring to days past LH surge), the so called implantation window, the endometrium is receptive to a blastocyst (the early developing embryo). If a pregnancy is not established, the egg will only survive for about 24 hours, the CL will vanish and the P levels will go down radically, causing the shedding of the endometrium and the start of the next menstrual cycle. On the other hand, if a blastocyst has implanted, the CL will remain for several weeks as a reaction of human chorionic gonadotropin (hCG) produced by embryonic chorionic cells until the luteo-placental shift (week 6-7 of pregnancy) when the placenta takes over the P production. (Campbell-Monga 2006)



Picture 4: The menstrual cycle
The menstrual cycle, showing the ovarian cycle, the endometrial cycle as well as hormone levels

1.5 HUMAN EMBRYOGENESIS WITH FOCUS ON IMPLANTATION

Human embryogenesis includes cell division and cell differentiation of the embryo from fertilisation to the end of the 8th week of development. During this period of time, all main organs are formed. After this, the embryo becomes a fetus. (Sandler 1985) The embryogenesis takes place in several steps:

1.5.1 Gametogenesis

Gametes are produced by spermatogenesis (sperm cells) and oogenesis (egg cells). During spermatogenesis, spermatogonia develop into sperm cells, a 64-day cycle taking place from puberty onwards. In the female, oogonia are developed into primary oocytes, which subsequently become primordial follicles; a stage in which the oocyte rests until initiation. All oocytes are arrested in the first meiosis at the end of the prophase (dictyotene) and can remain here until adulthood. Both the female and the male gametes have a haploid set of chromosomes (23 chromosomes). The oocyte is surrounded by a glycoprotein membrane, called zona pellucida (ZP). (Sandler 1985)

1.5.2 Fertilisation

Fertilisation occurs in the ampulla of the fallopian tube when a haploid oocyte gets impregnated by a haploid sperm. Prior to this process, ZP binds sperm and also contributes to the acrosome reaction, which helps a sperm to penetrate the egg cell. Fertilisation aims at replicating the diploid chromosome set (46 chromosomes) and determining the gender of the fertilised egg. The first cell division, creating two daughter cells, results in a zygote, which completes the fertilisation. (Sandler 1985)

1.5.3 Preimplantation

After fertilisation, the zygote migrates into the uterine cavity through the fallopian tube; a journey taking four days. Meanwhile the zygote undergoes cleavage, which will increase the number of cells, but not size as the ZP is still intact and will hinder any volume expansion. The zygote will enter the blastomere stage, then become a morula (16 cell-stage), which will undergo compaction where the trophoblasts (outer cell-layer) will form a compact epithelial structure, connected by tight junctions and microvilli. This way, the inner cells are protected from the outside milieu and differentiate into an embryoblast. Synchronically, the blastocyst cavity (a fluid-filled space) is created and a blastocyst is formed, which will hatch from the zona pellucida. The blastocyst cells are totipotent, meaning that every single cell has the ability to differentiate into any type of cell in the developing embryo. Secretions from the blastocyst contain a number of substances, including autocrine factors, (e.g. hCG and insulin-like growth factor 1, stimulating itself to growth and further invasion, respectively), factors degrading cell adhesions (e.g. tumour necrosis factor-alpha, inhibiting the adhesion molecule cadherin and thus loosening the connection between decidual cells and facilitating invasion), as well as endopeptidases and MMPs, e.g. collagenases (degrading the ECM between cells). (Merviel et al. 2001)

When the endometrium reaches its receptive stage it will become able to respond to signals from the early developing embryo. The so called cross-talk requires several different molecules secreted both in an autocrine and paracrine manner from both the

human trophoblast and the endometrium. The involved molecules include matrix MMPs, growth factors, and cytokines. (Nardo et al. 2003)

1.5.4 Implantation

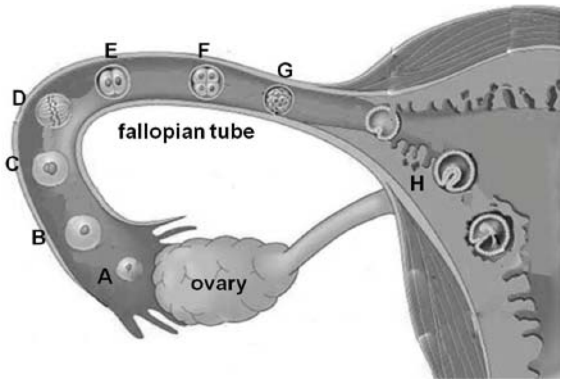
The implantation spans from the moment of hatching of the blastocyst (end of first week of embryonic development) to the formation of a primitive placental circulation system (mid second development week) and is initiated by the contact between the embryo and the endometrium. (Sandler 1985)

1.5.4.1 Endometrial structure and decidualisation

During the menstrual cycle, the endometrium undergoes changes aiming at creating a receptive stage to facilitate implantation. After ovulation, the uterine lining changes; a process called decidualisation and resulting in the decidua, characterised by large secretory decidual cells (containing glycogen and lipids) derived from fibroblasts, increased thickness, vascularity, and vascular permeability of the endometrium as well as longer, dilated glands. Subdecidual glands degenerate after the first trimester of pregnancy. However, some of them tend to produce more secrete than usual for a brief period of time before vanishing, an action called Arial-Stella phenomenon. (Sandler 1985)

1.5.4.2 Apposition, adhesion, and invasion

The blastocyst will get positioned at the endometrium and a loose connection is established (apposition). The contact between the blastocyst and the endometrium will increase in strength through penetration of the endometrium by protrusions of the trophoblast cells. When adhesion is established, the trophoblast will differentiate into syncytiotrophoblasts (ST) on the outside and cytotrophoblasts (CT) on the inside. Invasion starts with lytic activity of the ST, eroding structures in the endometrium and inducing the decidual reaction, which enables the embedding of the blastocyst. During the second week, vacuoles (membrane bound organelles, containing water and other molecules, e.g. enzymes) will appear in the ST, combining into lacunae which will be filled with maternal blood, creating the primitive utero-placental circulatory system. (Sandler 1985)



Picture 6: The implantation process
A ovulated secondary oocyte, **B** day 0, **C** zygote (after fertilisation), **D** day 1, **E** day 2: 2-cellstage, **F** day 3: 4-cellstage, **G** day 4: morula, **H** day 7: implantation of blastocyst

1.5.5 Embryo nourishment

Prior to implantation, the blastocyst spends approximately 72 hours in the uterus without any possibility of nourishment from the maternal blood. During this period of time, it gets the necessary nutrients from uterine secretions. Also after this, maternal circulation to the human placenta is very limited until 10-12 weeks of pregnancy. During this time, the uptake of secretions, also called histiotroph, from the endometrial glands by the trophoblast continues being an essential source of nutrients, and also of substances for anabolic pathways between fetus and placenta. The secretions contain carbohydrates, glycoproteins, and lipids. Earlier on, the histiotroph was considered a plasma filtrate from the spiral arteries. The histiotroph also contains a wide variety of growth factors and cytokines, e.g. VEGF, TGF, IGF and LIF (discussed in section 1.6.4). (Hempstock et al. 2004)

Placenta formation begins with the formation of lacunae in the syncytiotrophoblast, filled with maternal blood from the spiral arteries. During the third week of pregnancy, the fetal vessels are connected to the placenta, starting the feto-placental circulation. Placental circulation can be divided into a fetal and maternal part, separated by the placental barrier, which controls the metabolic exchange processes between embryo and mother. (Sandler 1985)

1.5.6 Embryonic and fetal phases

The first eight weeks of pregnancy comprise the embryonic phase. Development occurs according to the genetic programming and environmental factors. During the second week, the embryoblast differentiates into two germinal layers; the epiblast (embryo to be) and the hypoblast (umbilical vesicle to be). The epiblast will differentiate further, resulting in three embryonic germinal layers; mesoblast, endoblast and epiblast, forming the trilaminar germ disk. The mesoblast will surround the neural tube and give rise to the urogenital system. During the same time, neurulation will take place, during which the notochord will induce differentiation of the neuroblast (neurons and glia to be). The median part of the epiblast will form a groove and subsequently a neural tube (nervous system to be). (Sandler 1985)

The fetal phase includes the two last trimesters of pregnancy and involve enlargement of the organ system, established during the embryonic phase. (Sandler 1985)

1.6 ENDOCRINOLOGY

1.6.1 Progesterone

P levels are low during the follicular phase of the menstrual cycle and the major source is the adrenal gland, controlled by ACTH. During the luteal phase, P is produced by the CL, until its obliteration, which will cause a sudden drop in P levels. In case of pregnancy, P levels will remain high as a result of stimulation of CL by hCG until the luteo-placental shift (week 6-7), when the placenta takes over P secretion. P is important for endometrial receptivity due to influence on epithelial and stromal cell proliferation and differentiation. Another function of P is to coordinate (using endocrine and paracrine pathways) the expression of many genes transcribed by endometrial cells. (Holinka and Gurpide 1981)

1.6.2 Estrogens

Plasma levels of E increase during two periods of the menstrual cycle. The first time is during the follicular phase, where E is secreted by the growing follicles, typically peaking about 48 hours prior to ovulation. During this period, E is synthesised by ovarian granulose cells (under the control of FSH) from cholesterol, using aromatase and p450 enzymes. The second time E levels increase in blood is during the mid-luteal phase, when the CL is responsible for the production. Plasma levels of E are the highest during pregnancy. After menopause, E is produced in peripheral tissues, like adipose tissue. E is mainly present as estradiol, which is metabolised by 17β-hydroxysteroid dehydrogenase (17HSD) to estrone, which is a less potent estrogen. 17HSD activity is induced by P, which makes P an important regulator of E. (Groothuis et al. 2007; Holinka and Gurpide 1981)

1.6.3 Steroid hormone receptors

Receptors for both E and P are located mainly in the cell nuclei. There are two types of P receptors, A and B (PRA, PRB), the latter being shorter, transcribed from the same gene, using different starting sites. A third PR has been identified in decidual cells in the later part of pregnancy. (Franco et al. 2008) So far, two E receptors, α and β (ERα, ERβ) have been identified. Steroid hormone receptor concentration and distribution vary during the menstrual cycle: During the mid-follicular phase, the number of PRs both in glands and stroma is low, whereas that of ER is high. (Bouchard et al. 1991) During the later part of the follicular phase and the beginning of the luteal phase, PRs in glands increase. After this both ERs and PRs disappear in glandular cells, but the levels in the stromal cells are still high. ERs and PRs in the myometrium change in a similar way, with the difference that PR-levels decrease less during the mid-luteal phase. Both ERs and PRs increase after administration of E and decrease by P. (Bouchard et al. 1991; Garcia et al. 1988) Thus, P can regulate E both by means of enzyme induction and receptor reduction. ERß has been shown present in endometrial endothelial cells, whereas PR has not been detected in arterioles (small arteries) in the endometrium.

1.7 UTERINE SECRETION

Uterine fluid consists of secretions not only from the endometrium but also from the fallopian tubes, follicles, peritoneum, and cervical mucus. (Casslen et al. 1981) The uterine fluid volume changes during the menstrual cycle, (Clemetson et al. 1973) with the greatest volume correlating to the time for ovulation. At mid-luteal phase there is an increased glandular secretory activity, however, at the same time the volume of uterine fluid is decreased (Gemzell-Danielsson and Hamberg 1994), indicating that there has to be some mechanisms for uptake of uterine fluid. In order to achieve a successful implantation, there should be a close proximity between the blastocyst and the receptive endometrium. The reduced fluid volume in the uterine cavity increases the probability of the blastocyst getting implanted. (Lindsay and Murphy 2004) The uterine fluid contains cytokines (multifunctional glycoproteins locally mediating biological actions by interaction with specific receptors) and a number of other factors:

1.7.1 Placental protein 14 (pp14 or glycodelin)

PP14 is produced by glandular epithelial cells during the last week of ovulatory cycles. Serum levels of PP14 vary throughout the menstrual cycle, typically showing the lowest levels at ovulation and increasing towards the menstrual phase, or in early pregnancy. PP14 is under control of P and participates in the preparation for blastocyst implantation. PP14 also has an immunosuppressive role in the endometrium and its serum levels are used to evaluate endometrial function. PP14 also works as a direct T-cell inhibitor. (Hempstock et al. 2004; Lalitkumar et al. 1998; Seppala et al. 1988)
In women with recurrent miscarriage and histologically both normal and retarded endometrial development, uterine fluid concentrations of PP14 in washings collected on day LH+6 were low compared to normal fertile women. (Dalton et al. 1995) Uterine secretion of PP14 is decreased after blocking P with mifepristone. (Lalitkumar et al. 1998)

1.7.2 Vascular endothelial growth factor (VEGF)

VEGF production takes place in endothelial cells, macrophages, and glandular epithelial cells and is induced by hCG. VEGF mediates formation of new vasculature in events like implantation and tissue regeneration after menstruation. (Artini et al. 2008) Antiprogestin treatment inhibits VEGF secretion during the luteal phase. (Ghosh et al. 1998)

1.7.3 Transforming growth factor (TGF)

TGF is involved in the cellular modulation of events like menstruation, proliferation, decidualisation, and pregnancy establishment. (Jones et al. 2006) Uterine secretion of TGF-β is known to be regulated by P. (Ghosh et al. 1998)

1.7.4 Insulin-like growth factor (IGF)

IGF1 is important in cell proliferation, differentiation, and prevention of apoptosis. In embryo development, IGF1 acts as both autocrine and paracrine growth-survival factor. It is also of importance for development of follicles, implantation, and pregnancy. (Inzunza et al. 2010) IGF2 is a product of the invading trophoblast, inhibiting metalloproteinase inhibitors (TIMP) and IGF binding protein. It can be assumed that the trophoblast modulates its own invasion by regulation of factors facilitating or impeding invasion in the decidua. (Hess et al. 2007)

1.7.5 Leukemia inhibitory factor (LIF)

An optimal level of LIF is an indicator of endometrial receptivity. LIF is involved in implantation, both in adhesion and invasion of the blastocyst. LIF is expressed in chorionic villi, first trimester decidua, decidual leukocytes, and uterine NK. Pinopodes release vesicles containing LIF, which is necessary for the cross-talk between the embryo (expressing LIF-receptors) and the endometrium. (Aghajanova 2010) LIF expression increases at the time of implantation, simultaneously with pinopode formation. (Aghajanova et al. 2003; Cullinan et al. 1996; Wanggren et al. 2007)

1.7.6 Interleukin 6 (IL-6)

IL-6 is mainly expressed in glandular and luminal epithelial cells during the mid-secretory phase of the menstrual cycle. It might contribute to trophoblast growth and development of placenta. (Dimitriadis et al. 2005)

1.7.7 Interleukin 11 (IL-11)

IL-11 is expressed in endometrial glandular and luminal epithelium, and is also secreted by invading trophoblast cells. IL-11 is involved in decidualisation and of importance for establishment of pregnancy. (Dimitriadis et al. 2005)

1.7.8 Mucin (MUC1)

MUC1 expression is high during the secretory phase and is thought to be involved in the process of guiding the blastocyst towards the implantation site. (Hild-Petito et al. 1996; Margarit et al. 2010)

1.8 PATHOLOGY

The field of gynecological pathology is very complex, and this text does not aim at covering it, rather on giving a brief orientation focusing at the topics of the thesis: dysfunctional uterine bleedings.

Cancer and pre-cancerous diseases of the female reproductive organs (ovaries, fallopian tubes, uterus, cervix, vagina, and vulva) refer to several types of malignancies and are a common cause of death. There are also several benign conditions, causing pain and sometimes increased bleedings, e.g. endometriosis and adenomyosis. Endometriosis is a condition where endometrial tissue grows outside the uterus, e.g. on ovaries, fallopian tubes, and pelvic sidewalls. The most common symptom of endometriosis is pelvic pain, which may or may not be synchronised with the menstrual cycle. Several causes have been suggested behind endometriosis, e.g. metaplasia (change from one tissue type to another), retrograde menstruation (backflow of menstrual fluid through the fallopian tubes into the peritoneal cavity), genetic disposition, environmental factors, and immune deficiencies. (Campbell-Monga 2006) Adenomyosis refers to ectopic (displaced) endometrial tissue in the myometrium. The condition can be painful and associated with increased menstrual bleedings. However, as endometrial glands can be trapped in the myometrium, pain can be present without accompanying bleedings. (Campbell-Monga 2006) Another condition causing reduced quality of life is infertility, or the inability to contribute to conception. Infertility can be caused by for example genetic disorders, hormonal disturbances, other diseases (e.g. diabetes, thyroid or adrenal disorders) and

environmental factors, but about 25 % of the cases the cause is unknown. (Campbell-Monga 2006)

1.8.1 Dysfunctional uterine bleedings

1.8.1.1 Excessive uterine bleedings

The definition of excessive uterine bleedings (menorrhagia) is menstrual bleedings with a blood loss greater than 80 ml. It is a common gynecological problem in women of reproductive age and accounts for more than 20 % of outpatient clinic visits. Common consequences of the disorder are iron deficiency anemia, hysterectomy as well as reduced quality of life. The etiology (medical reason) behind excessive uterine bleedings can be local (25 %), i.e. myomas (benign tumours of the myometrium), endometrial polyps (benign tumours of endometrium), an accompanying feature of systemic disorders (25 %), iatrogenic (a side effect of medical treatment) (5 %), but in about 45 % of the cases the cause is idiopathic (unknown), suggesting cellular defects or abnormalities of regulatory mechanisms of menstruation. Angiogenic disorders are also believed to contribute to excessive uterine bleedings. An increase in endothelial cell proliferation has been reported in menhorragic endometrium. (Abberton et al. 1996; Kooy et al. 1996) Prostaglandins have also been associated with menorrhagia due to a positive correlation between volume of blood loss and level of PGE2. (Cameron et al. 1987)

1.8.1.2 Amenorrhoea

Amenorrhoea is the absence of menstrual periods in women of reproductive age and it can be physiological (during pregnancy and lactation) or a symptom with several possible causes. Primary amenorrhoea, where the menstrual cycles have not started, can be due to e.g. congenital absence of uterus or delayed puberty. Secondary amenorrhoea, or ceasing of menstrual cycles, can be caused by hormonal disturbances from hypothalamus or the pituitary and early menopause. (Campbell-Monga 2006)

1.8.1.3 Dysmenorrhoea

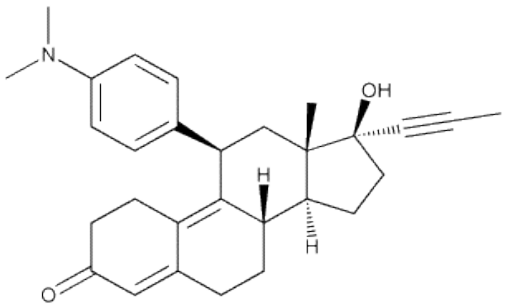
Severe pain during menstruation is termed dysmenorrhoea and can either be idiopathic (primary dysmenorrhoea) or caused by disease (secondary dysmenorrhoea). (Campbell-Monga 2006)

1.9 MIFEPRISTONE

Mifepristone is a 19-nor-steroid that binds with high affinity to the PR, thus inhibiting the effect of endogenous P. (Bygdeman et al. 1993) Mifepristone has different effects on endometrium and ovulation, depending on the dose given as well as the timing and duration of administration. When mifepristone is administered after ovulation, or in regimens that do not inhibit ovulation, there seems to be a dose dependent effect on endometrial receptivity and implantation. Mifepristone also reduces the fluid uptake at the time of implantation. (Gemzell-Danielsson and Hamberg 1994) Given once a month postovulatory, mifepristone is an effective contraceptive, acting by inhibition of endometrial development and thus also receptivity (Csapo and Resch 1979), which is observed when the normal down-regulation of PR in endometrium is inhibited. (Gemzell-Danielsson et al. 1993) Mifepristone in low, daily doses prevents ovulation

and also affects development of the endometrium, to a high extent inducing amenorrhoea. (Narvekar et al. 2006) An increased number of glandular glucocorticoid receptors and also increased microvessel density are accompanying events, suggesting that mifepristone-induced amenorrhoea might be due to regulation of vascular function. (Narvekar et al. 2006) If mifepristone (or other antiprogestins) are added to gestagen-only contraceptives, the bleeding pattern can be improved significantly (Gemzell-Danielsson et al. 2002), which has been suggested being a direct effect of mifepristone on endometrial arterioles (Johannisson et al. 1987). The positive effect on the bleeding pattern meets an increasing request for bleeding free contraceptive methods; unpredictable bleedings being the most common reason for interrupting a gestagen only contraceptive method. (Glasier et al. 2003)

The mechanism of action of mifepristone is complex and not all P effects are inhibited by mifepristone: The P/PR-complex binds to the DNA hormone response element (HRE), inducing the P effect. Mifepristone bound to PR also enters the cell nucleus, binding to a HRE, but different from the one binding P/PR, though partly overlapping, which can induce the same effect. (Bygdeman et al. 1993) Mifepristone is also known to interact with the glucocorticoid receptor. (Csaba and Inczefi-Gonda 2000) Glucocorticoids suppress angiogenesis in tumour cells. Mifepristone also decreases the expression of VEGF (important for angiogenesis). (Sengupta et al. 2003)



Picture 7: Mifepristone

1.10 ANGIOGENESIS

Angiogenesis is the formation of new microvessels from existing ones. In the adult human, angiogenesis only takes place during wound healing, endometrial regeneration, in the ovary, and during tumour growth. As a comparison, totally new vessels are formed directly from angioblastic precursors in a process called vasculogenesis. (Rafii et al. 2002) Angiogenesis is an essential component of the regeneration of the endometrium and occurs by non-sprouting mechanisms, mainly by intussusception and elongation. (Rogers et al. 1998) In the chick embryo, it has been possible to show that the endometrium possesses angiogenic potential during the whole cycle. (Maas et al. 2001) After induction of artificial cycles in primates, angiogenesis was shown most intense during the early proliferative phase. In humans, a second wave of angiogenesis, during the secretory phase, has been reported. (Maas et al. 2001) Endothelial cell migration peaks twice; during the early and mid/late proliferative phases. (Rogers et al. 1992) On the other hand, vessel density does not change during cycle, neither in

women with normal blood loss, nor in patients presenting with excessive menstrual bleedings. (Rees et al. 1984) Angiogenesis in tumour cells is down-regulated by glucocorticoids. (Yano et al. 2006) There are three different processes of angiogenesis: sprouting, intussusceptions and elongation.

1.10.1 Sprouting angiogenesis

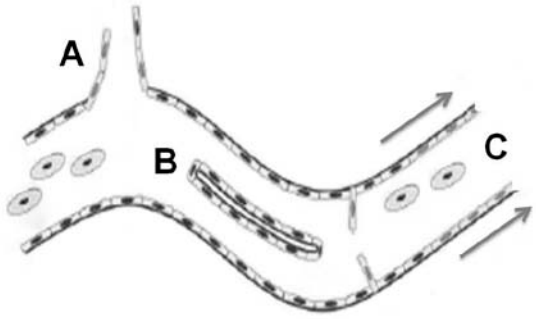
Angiogenesis through sprouting was the first mechanism identified. Sprouting is activated by angiogenic growth factors mediated by endothelial cells in the already existing blood vessels. The resulting activated endothelial cells release proteases (enzymes that break down the basement membrane), which enable endothelial cells to leave their original vessel wall. They proliferate into the surrounding matrix, forming sprouts which connect vessels close to each other through anastomoses. Sprouting occurs with a speed of several millimeters per day. (Risau 1997)

1.10.2 Intussusceptive angiogenesis

Intussusception ("growth within itself") is a non-sprouting angiogenesis where the formation of new vessels occurs by the splitting of existing ones. This process takes place in four steps: First, opposing capillary walls grow into the lumen, creating a contact zone. After this, intercellular junctions reorganise and the endothelial wall gets perforated. Then a cuff is formed by invading myofibroblasts and pericytes followed by deposition of matrix. Finally, there will be an enlargement in girth of the cuffs formed. Interestingly, intussusception occurs without an increase in the number of endothelial cells; the process is merely a reorganisation of existing cells. (Kurz et al. 2003)

1.10.3 Elongation

Elongation appears to be the most important angiogenic mechanism during the menstrual cycle, taking place mainly in the mid-late proliferative phase human endometrium. **(**Gambino et al. 2002) Elongation, and also vessel widening, is believed to occur through reconstruction of existing vessels in respond to increased metabolic needs of surrounding cells. (Risau 1997)



Picture 5: Angiogenesis
Angiogenic mechanisms: **A** sprouting, **B** intussusception, and **C** elongation.

1.11 WATER TRANSPORT

Water transport is essential in all human cells and tissues, as the proportion of water is high throughout the body. The major barrier to water transport is the plasma membrane. However, the mechanism behind water transport into and out of cells remained unknown a long time after most classes of membrane transport proteins had been discovered.

1.11.1 Active transport mechanisms

Active transport involves movement of a substance from areas with low concentration to others with high concentration, i.e. against its concentration energy, and takes place in cells accumulating high levels of molecules. The energy required for active transport can either be chemical (primary active transport), e.g. from adenosine triphosphate (ATP), or using an electrochemical gradient (secondary active transport). Particles that would not be able to cross the membrane without active transport mechanisms (e.g. because of membrane impermeability or movement against the concentration gradient) are recognised by membrane proteins and allowed to pass. An example of active transport is the transportation of sodium out of the cell and potassium into the cell by the sodium-potassium pump, where an ATPase acts as carrier, pumping three sodium ions out of the cell, at the same time as two potassium ions are pumped in. (Zeuthen 1995) In the case of primary active transport, the membrane proteins are acting as pumps, using chemical energy, typically from ATP. Secondary active transport involves proteins forming a membrane channel, and the energy source is an electrochemical gradient. Transportation can occur out of and into the cell at the same time, co-transporting one substance with another in opposite directions, a process known as antiport. When two substances are transported in the same direction, the process is called symport. In both antiport and symport, one of two substances is transported against its concentration gradient, using energy produced when the other substance is transported down its concentration gradient. (Zeuthen 1995)

1.11.2 Passive water mechanisms

Passive transport is the movement of particles across the cell membrane without using any chemical energy, and occurs in mainly four ways: diffusion, facilitated diffusion, filtration, and osmosis. In diffusion, particles move from regions with a high concentration to an area where the concentration is lower. In facilitated diffusion, substances spontaneously (without the use of energy) pass a membrane using transport proteins. When molecules or ions are filtered, those of a larger size will be retained, while the smaller ones will be able to pass through the membrane. In the case of osmosis, there is a net movement of solvent through a selectively permeable membrane from the hypotonic (lower concentration) to the hypertonic (higher concentration) solution, without use of energy. More specifically, water moves down a water potential gradient, meaning that it crosses a selectively permeable membrane from an area of high water potential (low solute concentration) to one of low water potential (high solute concentration), at which energy is released. (Zeuthen 1995)

1.12 AQUAPORINS (AQP)

Traditionally most scientists agreed that water transport through biological membranes occurs by simple diffusion through the lipid bilayer. However, some scientists questioned the diffusion theory. Based upon indirect observation, it was argued that specialised water-selective pores are necessary to explain the high water permeability of for example red blood cells and renal tubules. This water selective pores hypothesis was supported by mainly four facts. First, the amount of water in red blood cells and renal tubules is too high just to be explained by diffusion. Second, the water permeability of those structures can be reversibly inhibited by mercuric ions. The third argument was that activation energy is similar to diffusion of water in bulk solution. Finally, some structures exhibit fluctuations in water transport; for example the mammalian collecting duct, where it is regulated by the antidiuretic hormone, vasopressin. (Agre 2006)

1.12.1 Discovery of the AQPs

The controversies about the possible existence of water channels ended with the discovery of the AQPs. In 1988 Peter Agre and his group first described a 28 kDa integral membrane protein, which was a few years later known as AQP1. In the year 2003, Agre received the Nobel Prize in Chemistry for his discovery. The new protein was purified from red blood cells membranes and renal tubules. These are both structures with high water permeability and therefore Agre saw a possibility that the newly discovered protein might confer water permeability. This hypothesis was tested by injecting cRNA from AQP1 into African Clawed frogs' oocytes, which normally show low water permeability. The treated oocytes were transferred to hypo-osmotic buffer (distilled water), whereby an increased water permeability was noticed, which caused them to swell and burst. (Agre 2006; Preston et al. 1992)

1.12.2 Water selective pores hypothesis

The newly discovered protein was further studied in membranes, where it showed the same properties. The water selective pores hypothesis proved to be true as water permeability was reversibly inhibited by mercuric ions and it did exhibit low activation energy. Additional studies confirmed that membranes themselves possess some water permeability, but that it is up to 100 times greater in membranes containing AQP1 compared to those not expressing this water channel. (Agre 2006)

1.12.3 Structure of AQP1

AQP1 was the first water channel to be discovered. It has an hourglass structure and consists of three topologic elements: an extracellular and a cytoplasmic vestibule, connected by a pore. The protein is a tetramer formed by four AQP1 monomers, each containing an individual aqueous pore. Each AQP monomer contains 6 bilayer-spanning domains. The hemipore loops enter but do not span the bilayer, and each contains the signature motif Asn-Pro-Ala (NPA). Partial positive dipoles contributed by the short pore-lining alfa-helices and two highly conserved asparagines in the signature motif NPA cause a transient dipole reorientation of an isolated water molecule. (Agre 2006)

1.12.4 Water flow and channel selectivity

Water flow occurs from outside the cell to the inside through a narrow column. The water transport is facilitated by four bound water molecules which form 3 hydrophilic nodes. These nodes punctuate an otherwise extremely hydrophobic pore segment, making it more hydrophilic. AQPs are highly selective to water (and in some cases glycerol) and repel protons (hydronium ions, $H3O^{+}$). The latter is very important, for example in the renal tubules where water is reabsorbed at the same time as acid is secreted. Proton conduction is prevented by size restriction and electrostatic repulsion, mainly at two locations, one 8 Å above the center, and another one at the very center. Water selectivity is also achieved by a histidine-182 residue. (Agre 2006)

There are two principal mechanisms restricting AQP permeability to just water. First, there is a physical limit to the size of molecules that can pass, as the channel is only 2.8 Å at its narrowest par, combined with a highly conserved arginine residue creating a positively charged environment at this narrow passage of the channel. Second, the pair of dipoles at the NPA motifs interacts with individual water molecules, preventing them from forming hydrogen bonds to other water molecules. The resulting separation of water molecules eliminates the possibility of proton transfer through the channels. This combination of size and charge restriction is the basis for AQP permeability. (Agre 2006; King et al. 2004)

1.12.5 Expression in human tissues

So far, thirteen mammalian AQPs have been described, and they are divided into at least two subgroups: water-selective channels and channels permeated by water, glycerol, and other small molecules. AQPs have been found in virtually all kinds of organs and tissues throughout the human body. (Agre 2006)

1.12.6 Function

Peter Agre described the AQPs as being *"the plumbing system for cells"* (Dreifus 2009) AQPs are selective to water molecules, allowing passage in and out of the cell, while preventing passage of ions. Some AQPs are known as aquaglyceroporins due to their transporting glycerol, but also CO_2, ammonia, and urea, across the membrane. Determinant for this ability is the size of the pore. Common for all AQPs is that they are impermeable to charged molecules. AQP function has been connected to a number of tissues and physiological processes, e.g. in the reproductive tract (cervix ripening, placenta development, spermatogenesis, blastocyst development, and possibly fluid uptake in the endometrium), brain (production of cerebrospinal fluid), and kidney (renal concentration of urine). (Agre 2006)

To get back to the hypothesis mentioned earlier; that aquaporins might be involved in uptake of uterine fluid, there is evidence that AQPs could play this role in other species: In the mouse uterus there is a shift in AQP5 expression on the apical surface of epithelial cells at the time of implantation. This suggests that this AQP might be involved in the removal of uterine luminal fluid at the time of implantation in the

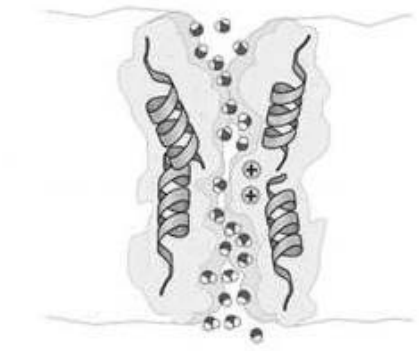
mouse. (Lindsay and Murphy 2004) AQP expression is, at least to some extent, hormone dependent. For example, an up-regulation of AQP2 in the mouse uterus as a response to E has been reported (Jablonski et al. 2003). In another study, AQP1 expression in the mouse myometrium was reported increased after exposure to E. (Richard et al. 2003).

1.12.7 Aquaporins and disease

"*If aquaporin could be manipulated, that could potentially solve medical problems such as fluid retention in heart disease and brain edema after stroke*", said Peter Agre. (Dreifus 2009) This statement indicates the important role of AQPs in disease - and as a possible cure. There are several examples of diseases identified as originating from mutations in AQPs, e.g. hereditary and acquired forms of nephrogenic diabetes insipidus in humans (mutations or impaired regulation of the AQP2 gene) (King and Agre 1996) and congenital cataracts in mice (mutations in AQP0). Humans deficient in AQP1 have been described as generally healthy, but lacking the ability to concentrate urine solutes and to conserve water when deprived of drinking water. (Agre 1998)

1.12.8 Aquaporins and angiogenesis

AQP1 has been shown to be involved in the endometrial angiogenesis. AQP1 is also involved in structural modifications of microvessels. One possible mechanism behind idiopathic menorrhagia could be an impaired angiogenesis and loss of structural integrity of microvessels, like coiling of blood vessels. This suggests a role for AQP1 in this process. (King et al. 2004) Saadoun et al have reported the involvement of AQP1 in cell migration, which has an important role in biological phenomena like angiogenesis, wound healing, and tumour spread. (Saadoun et al. 2005) In wound closure experiments, Lauffenburger et al were able to show that cell migration was enhanced upon AQP1 expression in cells, and also that growth and adhesion were not influenced by APQ1 expression (Lauffenburger and Horwitz 1996).



Picture 8: Aquaporin 1 water channel

2 AIM OF THE STUDIES

2.1 GENERAL AIM

The overall aim of the studies is to investigate a possible association between aquaporins and human endometrial function.

2.2 SPECIFIC AIMS

- To study the endometrial expression of AQP1 and 2, and their expression pattern throughout the menstrual cycle
- To investigate a possible progesterone dependent regulation of AQPs in the endometrium using the progesterone receptor modulator mifepristone
- To study the expression of AQP1 in endometrial samples from women with heavy menstrual bleeding (menorrhagia)
- To study the hormonal regulation of AQP1, 2, and 7 expression in uterine endothelial cells

3 MATERIALS AND METHODS

3.1 ETHICAL APPROVALS

All studies were approved by the Ethics Committee at Karolinska Institutet and all volunteers gave their written informed consent prior to inclusion in the studies.

3.2 MATERIALS

3.2.1 Endometrial tissue

3.2.1.1 Study 1

Endometrial biopsies were obtained from 34 healthy women, the mean age being 34 years. Prerequisites for the volunteers being enrolled to the study were having regular menstrual cycles, proven fertility, not using steroid hormone contraceptives or an intrauterine device for less than 3 months prior to the study and with no pregnancy or inflammatory pelvic disease within the previous 12 months. The biopsies were obtained from the anterior wall of the uterine cavity and only one biopsy was collected from each woman. The time of biopsy retrieval was randomly selected once between days LH+0 and LH+14, where the dating refers to the number of days past LH peak (LH peak corresponds to day 0). The endometrial histology was confirmed according to standard methods. From each biopsy one piece was processed for scanning electron microscopy and another one for immunohistochemistry. Scanning electron microscopy was used for identification and classification of pinopodes. When the size of the biopsy allowed, tissue samples were also frozen and kept for RNA extraction and reverse transcriptase–polymerase chain reaction (RT-PCR). The biopsies obtained for scanning electron microscopy were fixed in a mixture of glutaraldehyde and paraformaldehyde in sodium cacodylate buffer (pH 7.4) containing sucrose and calcium chloride. The samples to be investigated using immunohistochemistry were fixed in phosphate-buffered formaldehyde for a maximum of 24 hours and then stored in 70 % ethanol until embedded. The biopsies were divided into four groups according to LH peak.

3.2.1.2 Study :

Endometrial tissue was donated by 16 ovulating women (mean age 42 years) with a history of excessive uterine bleedings and 21 healthy ovulating women (mean age 38 years). Biopsies were obtained a using a Pipelle catheter for endometrium samples. The women in the control group were selected according to similar requirements as for study 1. Preoperative blood samples were collected and showed normal values for platelets, activated prothrombin thromboplastin time, INR, bleeding time, and von Willebrand factor, indicating normal coagulation. In the menorrhagia group, the uterine cavity was evaluated by hysteroscopy, showing no discernible endometrial pathology detected by this procedure or routine histological examination. The stage of the menstrual cycle was based on the date of the last menstruation, analyses of estradiol and progesterone, and the histological pattern of the biopsies. 7 women with

menorrhagia and 10 women from the control group were in the proliferative phase, and 9 women with menorrhagia and 11 from the control group were in the secretory phase when the biopsies were taken.

3.2.1.3 Study 3

Endometrial biopsies were obtained from 43 healthy women (mean age 34 years), according to the same criteria as for study 1. The biopsies were obtained in the same way as for study 1. From 28 women, the biopsies were taken at a randomly selected time between day LH<0 and LH+14. From an additional 15 women, biopsies were obtained on day LH+6 to LH+8 in either control cycles (no treatment), or following treatment with a single dose of 200 mg mifepristone administered on day LH+2. The endometrial samples obtained during the proliferative phase and luteal phase until LH+14 (n=28), were fixed in phosphate buffered formaldehyde for a maximum of 24 h and thereafter stored in 70 % ethanol until being embedded in paraffin. The endometrial samples obtained with or without mifepristone treatment (n=15) were snap frozen in liquid nitrogen. Larger biopsies were cut, and one piece was used for RT-PCR analysis. Eight samples were obtained after mifepristone treatment and seven samples were used as control. The paraffin-embedded biopsies were divided into four groups according to LH peak.

3.2.2 Myometrial uterine micro vascular endothelial cells

For cell culture, myometrial uterine micro vascular endothelial cells (passage three further multiplied until passage six) were used. Staining with vWF-factor was performed to confirm that the cells were endothelial cells.

3.3 METHODS

3.3.1 Immunohistochemistry (IHC)

Immunohistochemistry is a method to detect an antigen expressed by a cell and exploits the principle that an antibody binds specifically to its antigen. Antibodies, also known as immunoglobulins (Ig), are produced by plasma cells and used by the immune system to identify and neutralise antigens, for example viruses or bacteria. The general structure of all antibodies is very similar except for the antigen binding site, the hyper-variable region. The antigen binding site of the antibody recognises a unique part of an antigen, the epitope. The interaction between antibody and antigen is called induced fit, implicating a slight change of shape as the binding takes place. There are two types of antibodies, polyclonal and monoclonal. Monoclonal antibodies are derived from one single cell line and therefore only recognise one single epitope and thus more specific. Polyclonal antibodies are heterogeneous in specificity and derived by immunisation of a suitable host, such as a rabbit, mouse or goat. The injected antigen will provoke an immune response inducing production of a mixture of immunoglobulins, each recognizing a different epitope of the antigen

There is one direct and one indirect method of IHC, referring to the use of one or two antibodies. In the present studies the indirect method has been used, with an unlabelled primary antibody and a labelled secondary antibody. The primary antibodies are specific against the antigen to be studied and can be unconjugated, i.e. unlabelled (indirect IHC) or labelled (direct IHC). Secondary antibodies in turn are specific against the IgG of the animal species in which the primary antibody was raised and typically labelled with biotin, a reporter enzyme or fluorescent agents. The indirect method has the advantage that only a relatively small number of standard conjugated (labelled) secondary antibodies needs to be generated. For example, a labelled secondary antibody raised against rabbit IgG, which can be purchased "off the shelf", is useful with any primary antibody raised in rabbit. With the direct method, it would be necessary to make custom labelled antibodies against every antigen of interest.

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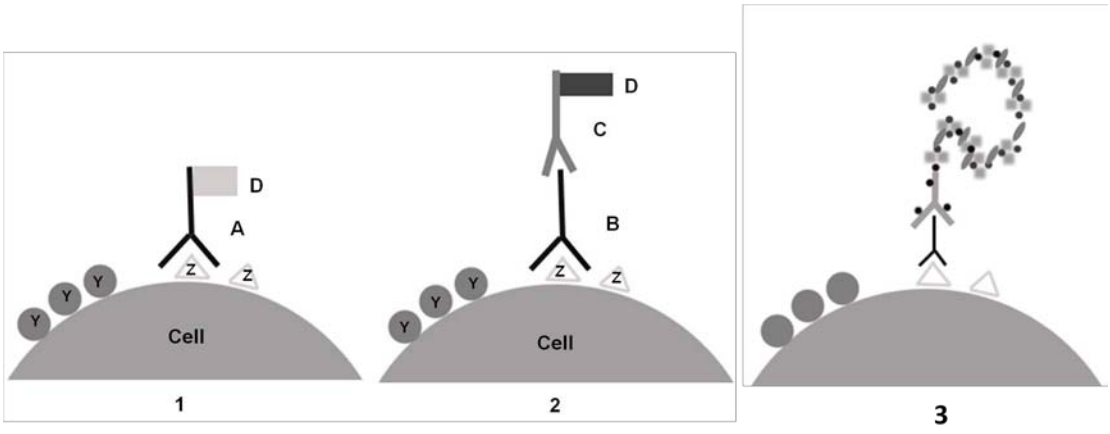
3.3.1.1 ABC-method

The ABC-method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. The procedure is conducted as follows: The tissue to be analyzed is cut into thin slices (about 4-40 µm) using a microtome and then mounted on slides. Paraffin is removed and rehydration is achieved by washing in decreasing ethanol concentrations. Non-specific background staining is reduced in three steps. First endogenous peroxidase activity is quenched using hydrogen peroxide (H_2O_2) in methanol in the dark (to slow down the thermal decomposition of H_2O_2). Then bovine serum albumin (BSA) is used to bind any proteins in a non-specific way, e.g. proteins with a disturbed affinity, preventing them from binding to the primary antibody. The last step aiming at reducing non-specific staining is incubation with normal (non-immune) serum from the host species providing the secondary antibody, preventing it from reacting with endogenous tissue Ig. The next step is incubation with first the primary antibody, and then the secondary antibody, which is coupled with avidin-peroxidase (an enzyme). As many avidins are able to bind to one biotin, an amplification of the signal is achieved. The enzyme-binding site is finally visualised when the complex reacts with 3,3'-diaminobenzidine (DAB), generating a brown staining. After counterstaining with hematoxylin the sections are mounted. Evaluation of staining intensity is performed in light microscopy.



Picture 9: Immunohistochemistry

1: IHC direct method**:** Labelled (D) primary AB (A), binds to its AG (Z).
2: IHC indirect method: Unlabelled primary AB (B), binds to its AG (Z), labeled (D) secondary AB (C) binds to the primary AB.
3: ABC-method

3.3.1.2 Study 1

Each biopsy was analysed in duplicates and treated according to the ABC-method described above. The primary antibody was polyclonal raised in rabbit and the secondary antibody was a biotinylated goat anti-rabbit antibody. Two sets of negative controls were prepared; one where the primary antibody was replaced by blocking serum, and the other one where the primary antibody was replaced by non-immune rabbit serum.

3.3.1.3 Study 2

IHC was conducted according to the ABC-method described above with minor changes (for details see article 2). The primary antibody was a polyclonal rabbit anti-goat AQP1 antibody. The secondary antibody was goat anti-rabbit antibody. In the negative control, the primary antibody was replaced by normal goat serum.

3.3.1.4 Study 3

IHC was conducted according to the ABC-method described above with minor changes (for details see article 3) and both frozen and paraffin embedded samples were used. The primary antibody was a monoclonal AQP1 antibody, raised in rabbit. The secondary antibody was a biotinylated goat anti-rabbit antibody. In the negative controls the primary antibody was replaced by blocking serum.

3.3.2 Immunocytochemistry (ICC)

3.3.2.1 Study 4

The slides containing formalin fixed endothelial cells were first treated against endogenous peroxidase activity. In order to reduce background staining, Background Sniper™ was added. The cells were incubated with the primary antibody for the respective AQP: AQP1 - mouse monoclonal; AQP2 - goat polyclonal; AQP7 - rabbit polyclonal IgG, and for vWF factor. As negative control mouse and rabbit immunoglobulins were used. The next steps included incubation with DaVinCi™ Green, MACH3™ Rabbit probe, MACH3™ Rabbit-HR polymer, and incubation with DAB. The slides were then dehydrated in graded alcohol and xylene, and finally mounted. In the negative controls the primary antibody was substituted by TBS.

3.3.3 RNA Isolation, extraction, and PCR

Total tissue-RNA is isolated and the RNA extracted is treated with DNase (an enzyme catalysing the hydrolytic cleavage of phosphodiester linkages of DNA). The RNA quality is checked by running on an agarose gel.

PCR is performed in three steps, which are repeated over and over again: denaturation (separation of the DNA-strands), annealing (DNA-polymerase attaches to the strand to

be copied) and elongation (synthesis of the new strand with the help of DNA-polymerase). Reverse transcriptase PCR is a variant of the method, in which the enzyme reverse transcriptase makes complementary DNA (cDNA) from an RNA template. The cDNA produced is then amplified using PCR.

3.3.3.1 Study 1

RNA was isolated and extracted as mentioned above. 2 µg of total RNA was reverse-transcribed into cDNA using the First-Strand cDNA Synthesis Kit. 2 µg of cDNA was then amplified using Taq DNA polymerase. AQP2 specific primers were generated from known sequences. The amplified region of the gene was between base pairs 3263 and 3485, which generates a product length of 222 base pairs (bp). The amplification cycle included denaturation at 95°C, 25 cycles of amplification at 94°C, annealing at 64°C, and extension at 72°C. Visualisation of the RT-PCR products was conducted by electrophoresis using an agarose gel followed by treatment with ethidium bromide. As a standard, a 100-bp ladder was chosen. To avoid nonspecific amplification, a negative control reaction was included in which no cDNA template had been added. Further, the PCR product was sequenced to confirm the specificity at the Center for Genomics and Bioinformatics at Karolinska Institutet

3.3.3.2 Study 2

Total tissue RNA was isolated using the SV Total RNA Isolation System. The extracted RNA was treated with DNase, and for quality check it was run on an agarose gel. Total RNA was reverse-transcribed into cDNA using the First-Strand cDNA Synthesis kit. Then the cDNA was amplified using TaqDNA polymerase. AQP2 specific primers were generated from known sequences. The amplification cycle included denaturation at 95˚C, 25 cycles of amplification at 94 ˚C, annealingat 64˚C, and extension at 72 ℃. Visualisation of RT-PCR products was conducted with electrophoresis using a 1.5 % agarose gel with a subsequent treatment with ethidium bromide. As a standard, a 100-bp ladder was chosen.

3.3.4 C-DNA synthesis and real-time PCR (study 4)

The endothelial cells flasks from the cell culture were harvested and the total RNA from the cells was extracted. 1µg of total RNA from each sample was used to synthesise complementary DNA (cDNA). This in turn was used to perform real time PCR. The internal control was 18S rRNA and TaqMan® Gene Expression Assays were used for analysis. Hippocampal tissue was the source of positive control for AQP1 and AQP2, whereas kidney was used for AQP7. TaqMan® Gene Expression Assays, consisting of a 20x mix of unlabelled oligonucleotide PCR primers, and TaqMan® MGB™ probes were used. Target gene probes were FAM™ dye-labelled and 18S ribosomal RNA probes were VIC dye-labelled. RT-PCR reactions were carried out in 96 well optical PCR plates with a reaction volume of 25 µl in a 7300 Real Time PCR System. For each primer / probe a single reaction master mix was prepared per plate. Each reaction well contained 12.5 µl 2x TaqMan® Universal PCR Master Mix, 6.25 µl water and 1.25 µl 20x TaqMan® Gene Expression Assays (primer / probe), plus 5 µl of cDNA template. All plates included 18S ribosomal RNA amplification of each sample as an endogenous control for data normalisation.

3.3.5 Cell culture (study 4)

An in vitro model was established, where myometrial uterine micro vascular endothelial cells (UtMVEC-myo) were treated with E, P, and the respective antagonists, mifepristone and IC. The expression of AQP1, 2, and 7 was detected using ICC and real time PCR. The culture medium contained endothelial cell growth medium with human recombinant epidermal growth factor, human fibroblast growth factor, vascular endothelial growth factor, ascorbic acid, hydrocortisone, human recombinant insulin-like growth factor, heparin, gentamicin, amphotericin-B, and 5 % fetal bovine serum. Harvesting occurred through trypsinisation, using trypsin / EDTA, followed by neutralisation. The cells were cultured in 25 cm^2 cell culture flasks or cell culture chambers with a seeding density of 5 000 cells/cm^2. After four days of culture, the cells were confluent to approximately 75 %. At this stage the cultures were changed to a medium containing charcoal / dextran treated FBS and the different groups were exposed to their respective treatment for 24 h as follows: i) E, ii) E + IC, iii) P, iv) E + P, v) P + mifepristone. After 24 h of culture, the cells in the flasks were washed in cold PBS and forwarded to RNA extraction. The cells in the culture chambers were fixed with cold formalin, washed with PBS and stored at 4º C until immunocytochemical analysis. There were six flasks and six culture chambers for each treatment group.

3.3.6 Light microscopy

The staining intensity after IHC was evaluated using light microscopy.

3.3.7 Scanning electron microscopy (study 1)

The purpose of using scanning electron microscopy (SEM) was to confirm the presence of pinopodes. These can be detected in light microscopy as well, but with lower accuracy. The SEM analysis also verifies the endometrial dating of the biopsies.
The tissue samples were prepared as follows. First the specimens were washed in a buffer containing cacodylate and calcium chloride, after that they were dehydrated using increasing concentrations of ethanol and acetone. Drying followed in a critical point dryer with carbon dioxide. For mounting the sections were coated with platinum.

3.3.8 Confocal Microscopy (Study 1)

The presence of AQP2 on pinopodes was visualised in confocal microscopy. The tissue samples were treated according to the IHC protocol, using the same primary AQP2 antibody, but a different secondary antibody; a Cy3-conjugated goat anti-rabbit IgG.

The fluorescence signal was localised using a confocal scanning laser microscope and the optical sections were recorded through the center of the tissue.

4 RESULTS

4.1 STUDY 1

4.1.1 Morphology

Morphologically all biopsies showed normal in-phase endometrium without signs of pathology. In the mid-luteal phase biopsies, pinopodes were present, which was shown using SEM.

4.1.2 Immunohistochemistry

Immunostaining for AQP2 was present in luminal and glandular epithelium as well as in vessels. The surface of the luminal epithelium was intensely stained during the whole cycle. In the samples where pinopodes were present, these were strongly stained. The cytoplasmic staining increased during the mid and late luteal phases. Nuclear staining was seen in only a few of the luminal epithelial cells. In the glandular epithelium, AQP2 was continuously increasing during the mid and late secretory phase towards the estimated time for menstruation. There was also immunostaining for AQP2 in the cytoplasm of the glandular epithelium, although this did not seem to differ between the groups. A few nuclei of the glandular epithelium stained positively for AQP2. There was a constant weak to moderate staining of some endometrial vessels in all slides. The stromal cells proper showed no or a very faint staining, while some other cells in the stroma, presumably leukocytes, were intensely stained. (Figures 11 and 12)
The inter-observer correlation for the immunohistochemical scoring was r_s <0.82, p<0.001.

4.1.3 Confocal Microscopy

Using confocal microscopy it was possible to detect an intense staining of AQP2 on the apical side of luminal and glandular epithelial cells, as well as on pinopodes, when present. (Figure 12 D)

4.1.4 RT-PCR

The presence of AQP2 in the human endometrium could be confirmed using RT-PCR and there was a positive band of 222 bp. (Figure 10)

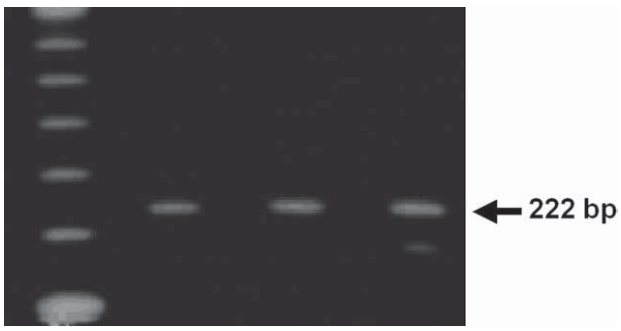


Figure 10: 222-bp band in the ethidium bromide-stained agarose gel of the RT-PCR product.

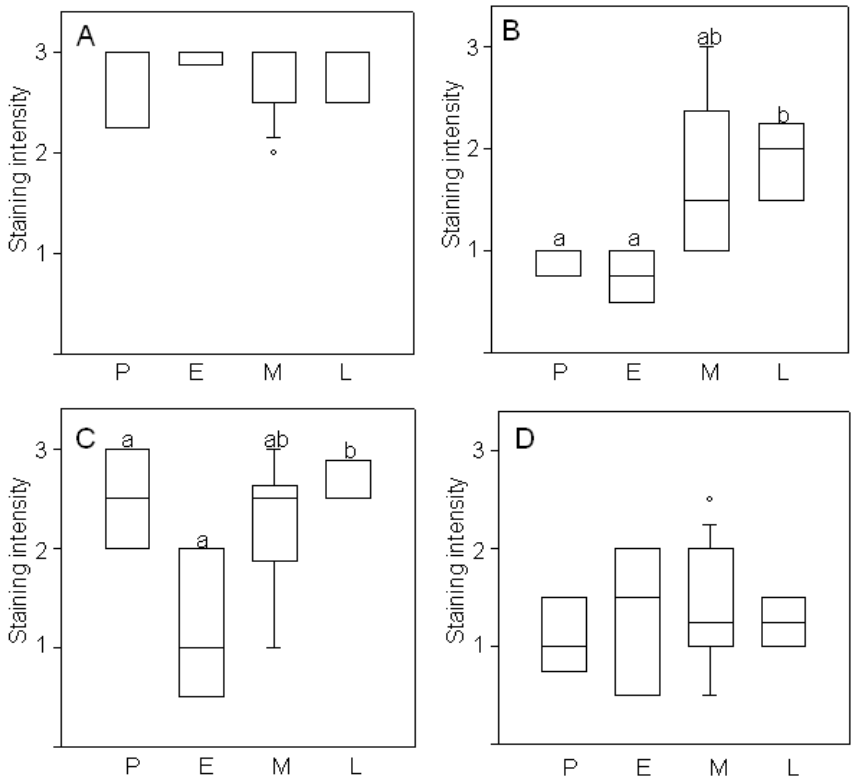


Figure 11: Box plots showing staining intensity of AQP2 during the menstrual cycle (P=proliferative phase; E=early luteal phase; M=mid-luteal phase; L=late luteal phase) at the following locations of the endometrium: **A:** the apical side of the luminal epithelial surface; **B:** the luminal epithelial cytoplasm; **C:** the apical side of glandular epithelial cell surface; **D:** the glandular epithelial cytoplasm.

Statistics according to Kruskal-Wallis analysis on variance in ranks; superscript “a” is statistically different from superscript “b”; $P<0.05$

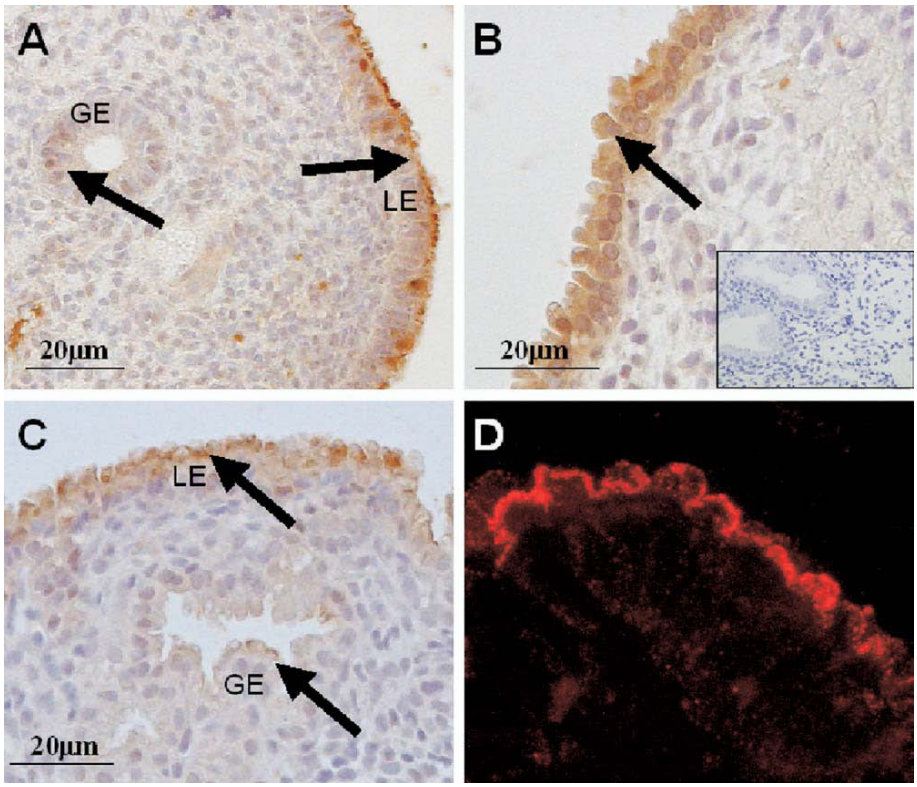


Figure 12: Immunohistochemistry (A–C) and confocal (D) images of AQP2 expression in human endometrium

A: Biopsy obtained on day LH+4 showing an intense immunostaining on the luminal epithelium surface, as well as in the glands (arrows). **B**: Biopsy obtained on day LH+7 showing pinopodes stained for AQP2. The inserted picture shows a negative control (primary antibody replaced by rabbit IgG), presenting no staining. **C**: Biopsy obtained on day LH+14 showing presence of AQP2 in the luminal (LE) and glandular (GE) epithelium; the staining being stronger in the latter. **D**: Pinopodes staining positively for AQP2.

4.2 STUDY 2

4.2.1 Immunohistochemistry

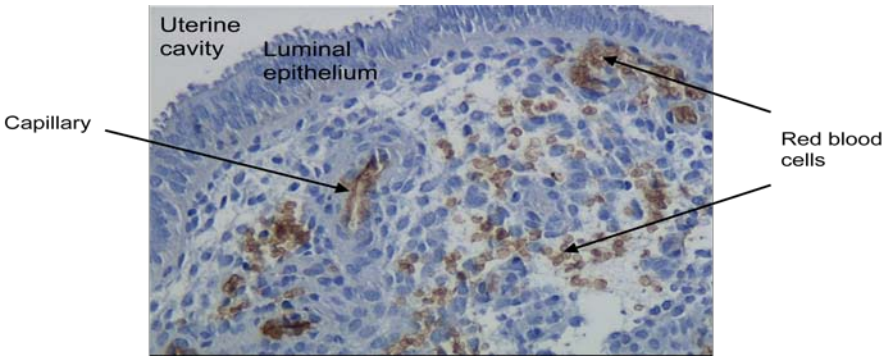


Figure 13: AQP1 expression in human endometrium

AQP1 was expressed solely in endometrial capillaries and erythrocytes (red blood cells), both in the control group, and in menorrhagic endometrium.

AQP1 was present in endometrial blood vessels only; no AQP1 expression could be detected in endometrial glands and stroma. (Figure 13) No differences in staining intensity could be distinguished between biopsies taken during proliferative and secretory phase. When assessing the AQP1 expression in vasculature (number of stained blood vessels per area), a significantly higher number of vessels expressing AQP1 could be detected in the control group compared to the menorrhagia group (p=0.006). (Figure 14) The number of vessels positive for AQP1 did not vary between different phases of the menstrual cycle, neither in the menorrhagia group, nor in the control group. The AQP1 staining intensity was significantly lower in the biopsies from menorrhagic endometrium compared to those from healthy controls (p=0.002) (Figure 14).

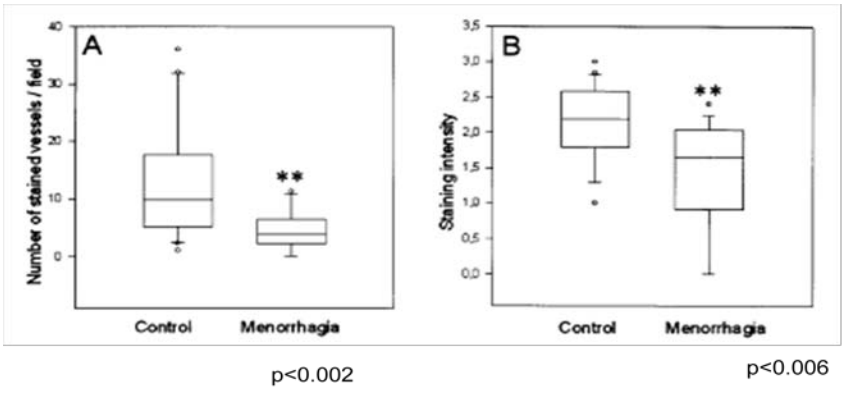


Figure 14:
The staining intensity for AQP1 in endometrial blood vessels was significantly lower in the menorrhagia group than in the controls (p=0.002). There were also a significantly lower number of stained vessels per unit area in the menorrhagia group than in the controls (p=0.006).

4.2.2 PCR

The PCR results confirm the presence of AQP1 in all control samples and in all biopsies from the patient group but one (M3). (Figure 15)

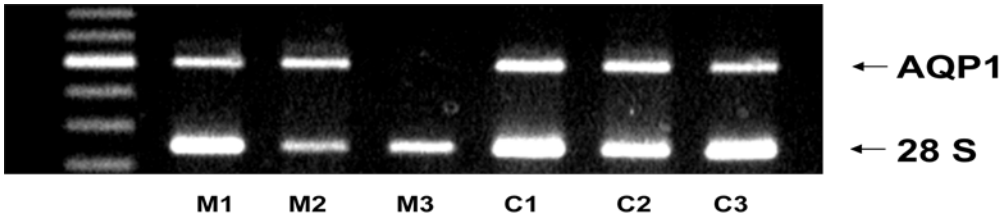


Figure 15: AQP1 mRNA in menorrhagia patients compared to controls.
In one of the menorrhagia patients (M3) no AQP1 was detected.

4.3 STUDY 3

4.3.1 Immunohistochemistry

AQP1 expression was detected in human endometrial vessels. (Figure 16A and B) Comparing biopsies originating from different phases of the menstrual cycle, no statistically significant differences, neither in expression of AQP1, nor in number of stained vessels, were detected. (Figure 17A and C) Mifepristone treatment increased the AQP1 staining intensity ($p<0.05$), whereas the number of stained vessels remained unchanged. (Figure 17B and D)

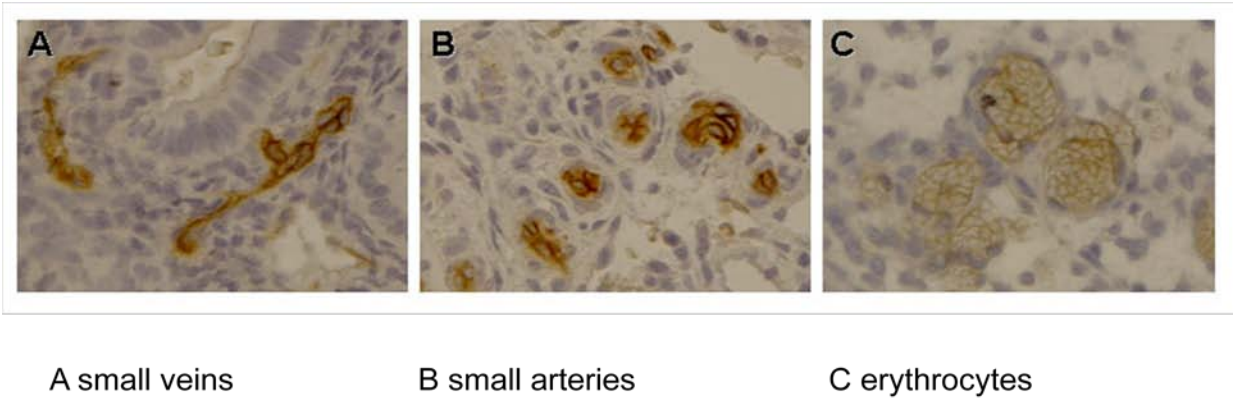


Figure 16: AQP1 expression in endometrial blood vessels
Immunostaining for AQP1 is present in endometrial blood vessels, showing small veins **(A)**, small arteries **(B)**, and red blood cells **(C)**.

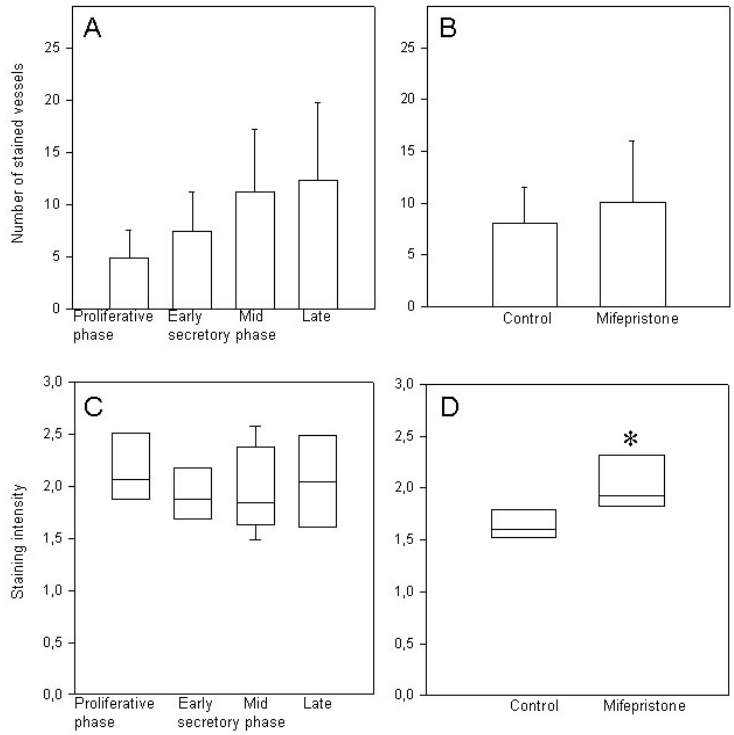
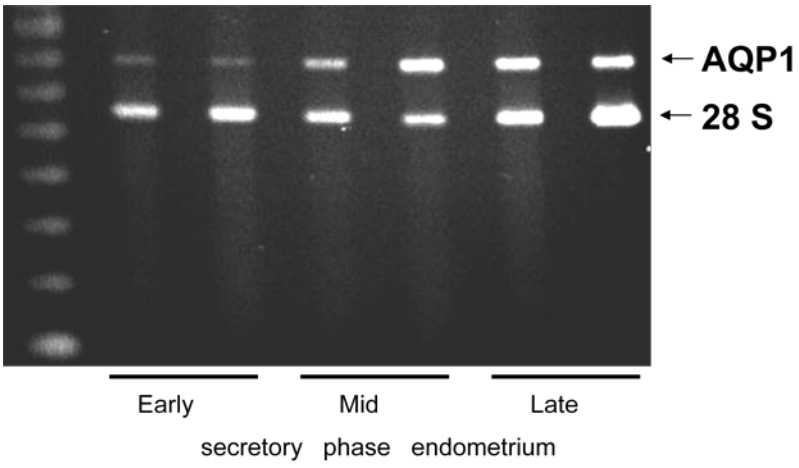


Figure 17: Picture **A** and **C** show biopsies obtained during the whole cycle, whereas **B** and **D** show the group where some participants had been treated with mifepristone. During the cycle there was an increase in number of stained vessels **(A)**, but not in staining intensity **(C)**. After mifepristone treatment, there was an increase in the staining intensity ($p<0.05$) **(C)** but not in the number of vessels stained **(A)**.

4.3.2 RT-PCR

AQP1 expression in human endometrium was verified using RT-PCR. A positive band of 701 bp could be detected. (Figure 18)



Picture 18: AQP1 expression confirmed in RT-PCR

4.4 STUDY 4

4.4.1 Protein and mRNA expression of AQP1 after treatment with E

AQP1 was expressed in endothelial cells (Figures 19A-F). The cytoplasmic staining for AQP1 increased after treatment with E ($p=0.001$), and there was a tendency towards am increased expression after treatment with E + IC, although not significant (Figure 20C).

AQP1 mRNA expression was increased in cells treated with E + ICI after 24 hours of culture ($p<0.001$). (Figures 20A and B)

4.4.2 Protein and mRNA expression of AQP1 after treatment with P

The immunostaining of AQP1 in the cytoplasm increased after P treatment ($p<0.001$). (Figure 20D)

The same results as in IHC were also achieved on the mRNA level after P treatment ($p<0.001$). (Figure 20 B)

4.4.3 Expression of AQP2 mRNA after treatment with E

AQP2 protein was present in endothelial cells. (Figure 21) The mRNA expression of AQP2 increased after treatment with a combination of E and IC ($p<0.01$). (Figure 21A) There was no immunocytochemistry performed for AQP2.

4.4.4 Expression of AQP2 mRNA after treatment with P

The AQP2 mRNA expression increased after exposure to P in combination with mifepristone ($p<0.01$). (Figure 21B)

4.4.5 Protein and mRNA expression of AQP7 after treatment with E

In the negative controls, where no steroid had been added, the expression of AQP7 in the cytoplasm was weak compared to other groups (figure 22C) The expression of AQP7 in cytoplasm was increased significantly after treatment with E in combination with IC ($p<0.01$) (Figure 22C).

AQP7 mRNA expression was increased in a statistically significant way after treatment with E and IC ($p<0.01$). (Figure 22A)

4.4.6 Protein and mRNA expression of AQP7 after treatment with P

The cells treated with estrogen in combination with its antagonist (E+IC) showed a tendency (not statistically significant) of increased expression of AQP7 in cytoplasm. There was a significant increase in AQP7 expression in the cytoplasm after treatment with estrogen in combination with progesterone, ($p<0.01$) (figure 22D).For the other groups there were no detectable differences in expression between controls and treatment groups.

AQP7 mRNA expression was significantly increased after treatment with E+ IC and E + P, respectively. (Figure 22 and B)

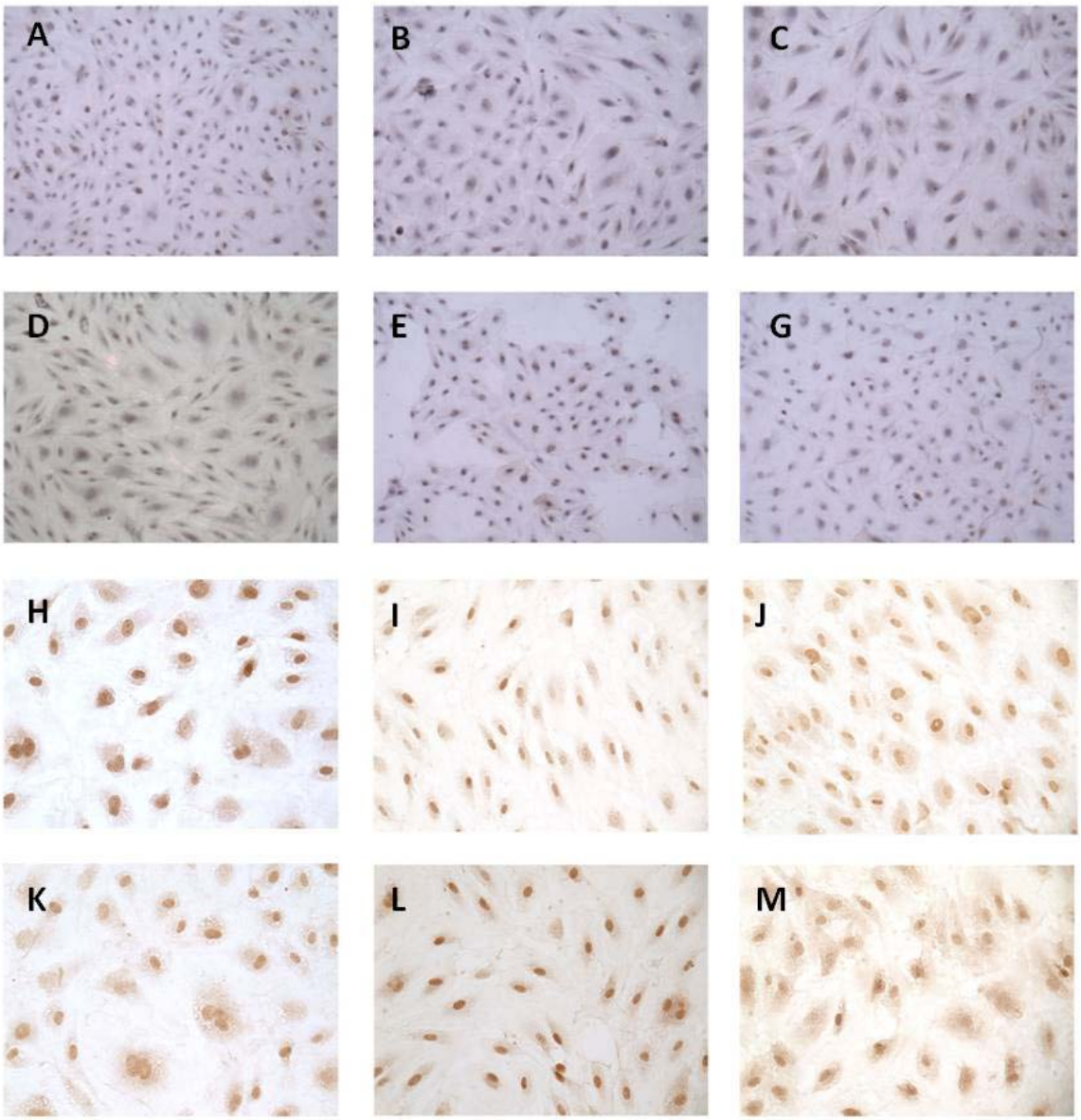


Figure 19: Immunocytochemistry staining of human uterine endothelial cells for **AQP1***:* compared with untreated control **(A)** and following exposure to E **(B)**, E + IC **(C)**, P **(D)**, P + mifepristone **(E),** E + P **(F)** and ***AQP7:*** compared with untreated control **(G)**, following exposure to E **(H)**, E + IC **(I)**, P **(J)**, P + mifepristone **(K)**, and E + P **(L)**

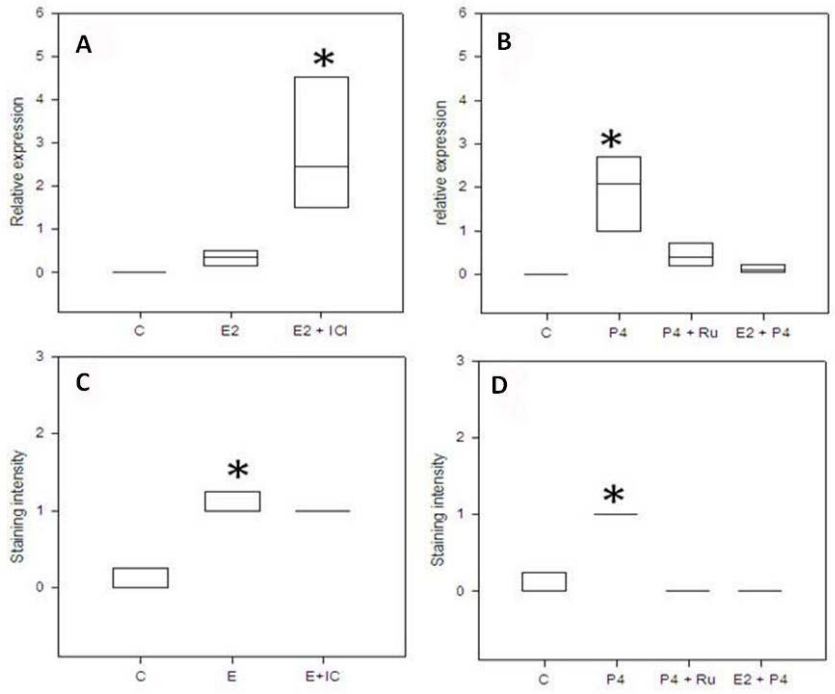


Figure 20: AQP1 mRNA expression (A and B)
Immunostaining intensity for AQP1 in cytoplasm (C and D)
AQP1 mRNA expression was increased in cells treated with E + ICI ($p<0.001$) **(A)**, and after P treatment ($p<0,001$) **(B),** cytoplasmic staining for AQP1 increased after treatment with E ($p=0.001$) **(C)**, and also after treatment with P ($p<0.001$) **(D)**

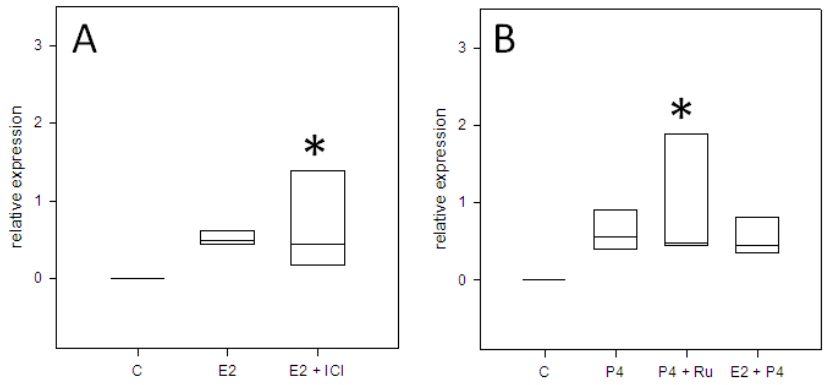


Figure 21: mRNA expression of AQP2 (A and B) increased after treatment with E + IC ($p<0.01$) **(A), AQP2** mRNA levels were increased after exposure to P + RU ($p<0.01$) **(B)**

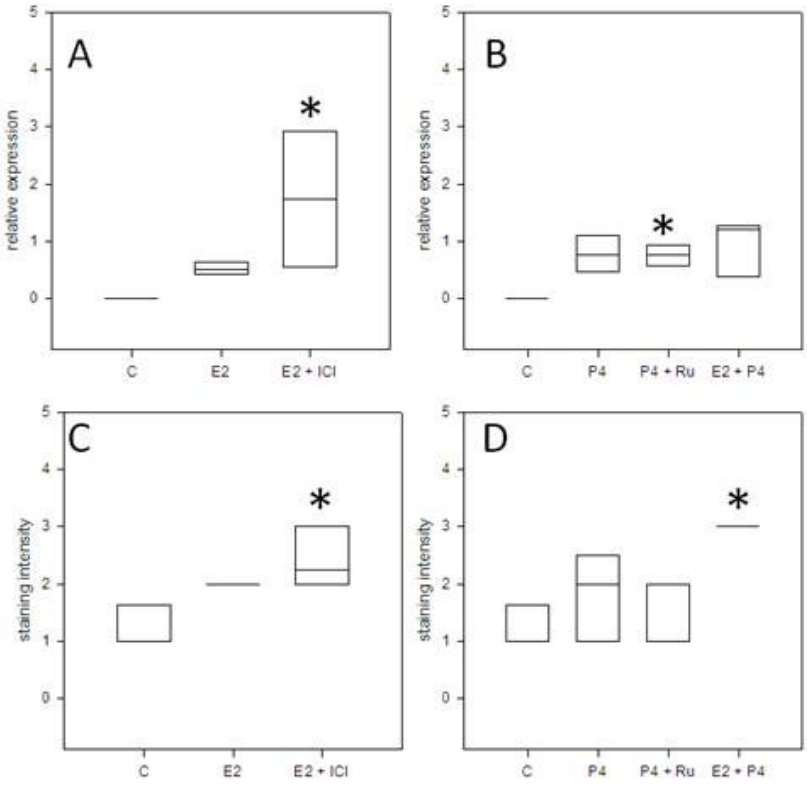


Figure 22: mRNA expression of AQP7 (A and B) and immunostaining intensity for AQP7 in cytoplasm (C and D): The mRNA expression of AQP7 was increased after treatment with E + IC ($p<0.01$) **(A)**, and after treatment with P + mifepristone ($p<0.01$) **(B)**, The expression of AQP7 in cytoplasm was increased after treatment with E in combination with IC ($p<0.01$) **(C)**, as well as after treatment with E in combination with P ($p<0.01$) **(D)**

5 DISCUSSION

5.1 STUDY 1: EXPRESSION OF AQUAPORIN 2 IN HUMAN ENDOMETRIUM

At the time of publication of study 1, the presence of AQP2 in human endometrium had not been reported before. This study describes an expression pattern for AQP2 in human endometrium with an increase in the mid and late secretory phases, suggesting a role for AQP2 in menstruation and possibly also in endometrial edema as well as in implantation.

Water and molecular transport over membranes is crucial to endometrial function and is an essential part of events like secretion, menstruation, and implantation. Implantation is dependent on the cyclic changes in the endometrium and its secretory pattern. As a preparation for implantation, the endometrium matures and there is only a limited period of time where the embryo can attach to the uterine wall; the implantation window. Determinant of this event is the receptive stage of the endometrium, which is characterised by stromal edema, epithelial cell mitosis, and proliferation of the vasculature and pinopode formation. The pinopodes only appear on the endometrial surface during the implantation window and are therefore considered ultra structural markers of endometrial receptivity. So far the function of pinopodes in humans is unknown, however, in mice they have a pinocytotic function, taking up substances on the endometrial surface in vacuoles.(Enders and Nelson 1973) In humans, the uterine secretion is also reduced at the time of implantation (Gemzell-Danielsson and Hamberg 1994), but, in contrast to rodents, there is no evidence that the pinopodes have a pinocytotic activity (Adams et al. 2002). Therefore there must be other mechanisms for uterine fluid reduction, i.e. an increase in uptake.

In this study, we could show an intense immunostaining for AQP2 on the apical epithelial surface, including pinopodes. There was no variation in surface staining during the menstrual cycle. However, the presence of AQP2 on pinopodes implies that the epithelial cells, from which these structures protrude, are involved in uterine secretion regulation. In the mouse uterus, there is a shift in AQP5 on the apical surface at the time of implantation, whereas AQP2 was not present. Apparently, the intense apical AQP2 staining on the luminal surface during the whole menstrual cycle in the human coincides with the shift in AQP5 expression in rodents (Lindsay and Murphy 2004) and it is possible that these two AQPs correlate with each other. However, it is also possible that additional AQPs are present in the human endometrium.

There is an up-regulation of AQP2 in the mouse uterus as a response to E (Jablonski et al. 2003). The increase of AQP2 in the apical surface of the glandular epithelium and the surface of the luminal epithelium in humans coincides with the increased P levels during the mid-luteal phase, indicating a hormonal regulation. However, AQP2 increases continuously during the late luteal phase, suggesting that factors other than the steroid hormones might be determinant for AQP2 expression.

Our results on AQP2 in human endometrium contradict a contemporary microarray study by Mobasheri et al where they aimed at detecting AQP2 and AQP3 in the human reproductive tract. They found AQP2 in the fallopian tube and AQP3 in the endometrium. (Mobasheri and Marples 2004) A possible reason for these contradictive results is that microarray studies have a high rate of false detection or fail to detect all mRNA in a sample.

At the implantation site, during onset of implantation, there is an increase in vascular permeability. (Horcajadas et al. 2004) In endometrial vessels AQP2 is involved in this process and this is possibly also the case at the implantation site. Endometrial edema changes during the menstrual cycle and reaches a peak just after the implantation window (Johannisson et al. 1987). In this study, AQP2 increased in the glandular epithelium during the mid and late secretory phases, which correlates with the increased endometrial edema, indicating an involvement of AQP2 in this process.

5.2 STUDY 2: EXPRESSION OF AQUAPORIN-1 IN ENDOMETRIAL BLOOD VESSELS IN MENORRHAGIA

The topic of the second study of this thesis was a direct consequence of the previous one. As AQP2 can be assumed to be involved in different events where water transport are essential, it was reasonable to assume that other AQPs could also play a role. From other studies it was known that AQP1 is expressed in vessels and crucial to angiogenesis, and also that malfunction in angiogenesis is one possible cause to idiopathic menorrhagia. Taking those facts and assumptions into account, investigating the distribution of AQP1 in menhorragic endometrium compared to normal endometrium was a natural next step.

The results of this study show that AQP1 is indeed present in the human endometrium; expressed only in blood vessels of the endometrial functional layer and not in endometrial glands and stroma. The number of blood vessels stained for AQP2 was significantly decreased in the menorrhagia group compared to the control group. The latter group also showed a significantly lower staining intensity of AQP1 compared to controls.

The role of AQP1 in angiogenesis has been reported in other studies, e.g. AQP1 protein expression in tumour microvessels. (Endo et al. 1999) Lauffenburger et al were able to show in wound closure experiments that cell migration was enhanced upon AQP1 expression in the cells, and also that growth and adhesion were not influenced by APQ1 expression (Lauffenburger and Horwitz 1996). Angiogenesis is of uttermost importance to endometrial reconstruction during the endometrial regeneration, and yet very little is known about the timing and mechanisms behind formation of new vessels. The chick embryo chorioallantoic membrane has been used to study the angiogenesis in vivo with the conclusion that the angiogenic potential is present throughout the menstrual cycle (Maas et al. 2001). Ruptured blood vessels are repaired during the menstrual phase of the cycle and after this, during the proliferative phase, the

endometrium grows fast, supported by the formation of new vessels. The secretory phase is characterised by differentiation, including the formation of spiral arteries and subepithelial capillary plexus. Endothelial cell migration peaks twice; during the early and mid / late proliferative phases (Rogers et al. 1992). The vessel density, on the other hand, is consistent throughout the cycle, both in women with normal blood loss and in patients presenting with excessive menstrual bleedings. (Rees et al. 1984)

In the present study, AQP1 expression was the same in the proliferative and the secretory phase endometrium. Studies on the hormonal regulation of AQP1 are contradictory; some showing E dependence, while others do not: Richards et al investigated the AQP1 expression in the inner cell layer of the myometrium in the mouse uterus after E exposure and found it to be increasing. (Richard et al. 2003) Other studies have focused on the role of AQP1 in disorders related to endothelial cell dysfunction, for example Saadoun et al looking into AQP1 expression in tumour angiogenesis: After implanting melanoma cells subcutaneously in wild-type and AQP1 knock-out mice, the speed of tumour growth was recorded.(Saadoun et al. 2005) In the mice deficient on AQP1, tumour growth was slowed down significantly. In our study we found the number of vessels staining positively for AQP1 significantly lower in the group with excessive bleedings than in the controls. The reason is not yet known. At the time of publication of this article, no study of the expression of AQP1 in menhorragic endometrium had yet been published. Excessive uterine bleedings have been associated with aberrant angiogenesis, e.g. an increase in endothelial cell proliferation. One of our previous studies showed a connection between vascular abnormalities of the endometrium and menorrhagia. (Mints et al. 2007a)

It could be assumed that the decreased AQP1 levels reported in this study are a result of changed transendothelial transportation due to changes in permeability. The presence of AQP1 in endometrial blood vessels suggests an involvement in the regulation of angiogenesis in human endometrium. Therefore, the aberrant angiogenesis in patients with excessive uterine bleedings could be AQP1 related.

5.3 STUDY 3: AQP1 IS EXPRESSED IN THE HUMAN ENDOMETRIUM DURING NORMAL CYCLE AND INCREASES AFTER MIFEPRISTONE TREATMENT

Prior to this third study of the thesis our group had reported a cyclic variation in AQP expression, which proposed the hypothesis that AQPs could be regulated by hormones. The expression of AQP1 increases towards the end of the cycle, correlating to P regulation. Therefore the setup chosen was to study P, by means of its antagonist mifepristone, as a possible candidate for hormonal regulation of AQP1. A deeper knowledge of a possible hormonal regulation of AQP1 in the human endometrium is of interest in order to understand endometrial physiology, including events like menstruation, implantation and angiogenesis, as well as P mediated receptivity.

Angiogenesis is essential for the menstrual cycle and there are studies showing that AQP1 has a role in this process, e.g. on AQP1 knockout mice showing that AQP1 is involved in angiogenesis and endothelial cell migration. (Saadoun et al. 2005) In study two, we came to the conclusion that AQP1 expression in women with excessive uterine bleedings is reduced compared to that of women with normal menstrual bleedings. In the present study we showed that AQP1 expression in endothelial cells is increased after mifepristone treatment.

An increasing number of women are requesting bleeding free contraceptive methods, and unpredictable bleedings are the most common reasons for interrupting a gestagen only contraceptive method (Glasier et al. 2003). Mifepristone has various effects on ovulation and endometrial development depending on the dose and duration of treatment. When given postovulatory once a month it acts as an effective contraceptive by inhibiting endometrial development and receptivity (Csapo and Resch 1979), which is seen when inhibiting the normal down-regulation of PR in the endometrium (Gemzell-Danielsson et al. 1993). Administered daily in low doses, mifepristone inhibits ovulation, affects endometrial development, and, as a result, amenorrhoea occurs in a high proportion of women. (Narvekar et al. 2006) There is also a connection between mifepristone-induced amenorrhoea, an increased number of glucocorticoid receptors in the glands, and microvessel density. Due to this, it has been proposed that the high incidence of amenorrhoea after mifepristone treatment might be a result of vascular functional regulation. The bleeding pattern is significantly improved if mifepristone, or other antiprogestins, are added to gestagen-only contraceptive methods. It has been suggested that mifepristone might have a direct effect on endometrial arterioles. However, PRs have not been detected in endometrial arterioles, indicating that the mifepristone effect would not be achieved through binding to PR. AQP1 is involved in endometrial angiogenesis and has been reported to be reduced in patients presenting with menorrhagia. Therefore the reduced bleeding seen after mifepristone treatment could be induced by mifepristone acting on AQP1 expression. AQP1 expression has been reported in both human and rodent uteri, located in endothelial cells only. In a previous study, we could not find any significant differences in staining of the vessels between cycle day 7 and cycle day 21, which was confirmed by our present study. For this study biopsy samples were obtained during different stages of the secretory phase, showing no statistically significant differences in AQP1 staining. Interestingly, the mRNA levels of AQP1 increased during the secretory phase. This discrepancy in AQP1 expression detection suggests further studies on the very recent discoveries on siRNA and its regulation in the translation of mRNA into AQP1 protein.

Physiological angiogenesis (in contrast to angiogenesis as a part of wound healing or in tumour growth) only takes place in the female reproductive tract in the adult human. (Gargett and Rogers 2001) Angiogenesis, increased vascular permeability, and spiral arteries remodelling are events essential to endometrial development, implantation, and placentation. The endometrium is capable of angiogenesis during the whole cycle, but the angiogenic pattern is mainly unknown. Endometrial angiogenesis during the reconstruction after the menstrual bleeding seems to occur mainly through elongation. (Gambino et al. 2002) After induction of artificial cycles in primates, angiogenesis was

shown to be most intense during the early proliferative phase. In humans, a second wave of angiogenesis, during the secretory phase, has been reported. (Maas et al. 2001)

In this study we show the distribution of AQP1 in human endometrium during normal cycle and after mifepristone treatment. Our group has previously reported the presence of AQP1 in human endometrium, with a significant reduction in expression in women with excessive uterine bleedings, indicating that AQP1 is important for normal endometrial function. The hormonal regulation of AQP1 has been studied in rats and found to be both E and P dependent. However, in humans this regulation was unclear when this study was conducted, and in order to gain more knowledge about this, mifepristone was used. Mifepristone binds with high affinity to the PR, thereby inhibiting the effect of endogenous P. The effect achieved by this treatment was an increase in AQP1 expression, suggesting that P could be involved in AQP1 regulation. The highest AQP1 levels were recorded during the late secretory phase, correlating to lower levels of P, which is contradictory to the changes shown during cycle. However, mifepristone does not only act through the PR, but is also responsible for a glucocorticoid effect, which might explain the discrepancy.

In this study, the number of stained vessels was counted after a single dose of mifepristone and found to be unchanged. Possibly, a mifepristone treatment of longer duration is needed in order to alter the number of vessels. Our previous studies showed that, among other aquaporins, AQP2 might play a role in water homeostasis in the human endometrium; AQP2 is mainly expressed in the glandular and luminal epithelium of the endometrium and it can, due to the location, be assumed that it is involved in uterine secretion during the menstrual cycle. In rodents, a shift in AQP5 expression on the apical surface of uterine epithelial cells at the time of implantation has been reported. (Lindsay and Murphy 2006) Mifepristone, when given as a single dose immediately after ovulation, converts the uterus into a non-receptive stage, similar to the one observed after inhibiting the normal down-regulation of PR in the endometrium. Except for binding to the PR, mifepristone also has a high affinity to the glucocorticoid receptor. (Csaba and Inczefi-Gonda 2000) Angiogenesis in tumour cells is down-regulated by glucocorticoids (Yano et al. 2006), which makes it reasonable to assume that the effect of mifepristone might be mediated through its action on the glucocorticoid receptor.

5.4 STUDY 4: HORMONAL CONTROL ON AQUAPORINS 1, 2, AND 7 IN HUMAN UTERINE ENDOTHELIAL CELLS BY ESTROGEN AND PROGESTERONE: AN IN VITRO STUDY.

Prior to this study it was known that i) AQPs are present in endothelial cells (Verkman 2002), ii) AQPs are hormonally regulated (Hildenbrand et al. 2008), and iii) the myometrium changes in accordance to hormone levels (Johannisson et al. 1982). The fourth study of this thesis evolved from these facts, further investigating the hormonal regulation of AQPs in endothelial cells.

In human endometrium, AQP1 is involved in angiogenesis and modification of micro vessels, as well as in the pathogenesis of idiopathic heavy menstrual bleeding (Mints et al. 2007b). AQP2 is also expressed in human endometrium; mainly in epithelial cells, but to some extent also in endothelial cells (Hildenbrand et al. 2006). It is likely that endometrial endothelial cells are different from myometrial endothelial cell, suggesting the possibility of a different AQP expression pattern than in the setting of this study. Cells behave differently in vitro, as in the actual study design, compared to in vivo. The presence of AQP7 has been reported in smooth muscle bundles (Mobasheri et al. 2009), but until the date of this study not in endothelial cells, which was the reason for including this AQP.

Our results show an E and P regulation of AQP1 in endothelial cells, where the expression was increased upon blocking of E (treatment with E + IC). Treatment with P alone gave the same effect. The hormone dependency in expression is further supported by the fact that AQP1 expression was up-regulated after treatment with E only. Mifepristone is a potent P antagonist in the presence of P, whereas mifepristone, at low doses, has a P agonistic effect on the endometrium in the absence of P. The cytoplasmic staining for AQP1 was low or close to zero in all groups, indicating a very low level of AQP1 transport through the cytoplasm.

AQP7 expression in endothelial cells was high after exposure to E plus IC, as well as E and P, suggesting that this AQP might be of importance during the postovulatory phase when the levels of E and P are high. Endometrial secretion is also increased during this phase, which correlates with increased AQP7 expression, which could indicate them being involved in this event.

As for AQP2, there is a negative result in the present setting. The same AQP2 antibody, that in earlier studies had given reliable immunohistochemical results, was not suitable for use in immunocytochemistry, and at time of the experiment, no other AQP2 antibody was commercially available for the setting and tissue chosen.

The connection between AQP1 and angiogenesis has been investigated in earlier studies. Angiogenesis is of vital importance to endometrial development, and therefore a deeper understanding of the accompanying events would be of great benefit. The endometrium is capable of physiological angiogenesis during the complete cycle; however, it seems to vary over time, though little is known about the actual angiogenetic pattern in human endometrium. (Goodger and Rogers 1995) In primates, angiogenesis has been shown to take place mainly during the early proliferative and secretory phases. (Maas et al. 2001) It could be assumed that the pattern of angiogenesis is similar in humans, which would correspond to the findings of this study, where the expression of AQP1 is high under influence of the hormones present during these phases.

6 CONCLUSIONS

Several AQPs are present in human endometrium, and the expression seems to be cycle dependent. AQP2 is mainly present in endometrial epithelial cells, but, to some extent, also in endothelial cells. The increased levels of AQP2 towards the end of the menstrual cycle coincide with the increasing edema observed in endometrium towards the menstrual period, indicating a role in physiological events like implantation, edema and menstruation. AQP2 could also be of importance for the reduction of uterine secretion seen at the time for implantation, which enables a close proximity between blastocyst and endometrium.

AQP1 is expressed in endometrial blood vessels, suggesting a role in angiogenesis. The reduced levels of AQP1 seen in menorrhagic endometrium might be connected to the impaired angiogenesis suggested as cause for idiopathic heavy menstrual bleedings. AQP1 being present in endometrial vessels can also be interpreted as an involvement in edema regulation. The mifepristone induced increase in AQP1 suggests P as a candidate for AQP1 regulation. As the spiral arteries of the endometrium do not possess any PR, the effect of mifepristone could be assumed to act directly on AQP1.

The in vitro studies on myometrial endothelial cells show a P and E regulation of AQP1, AQP2 and AQP7, indicating a role during the postovulatory phase of the menstrual cycle. It could be suggested that treatment with P or anti-estrogens would be a possible treatment for menorrhagia accompanied by low AQP1 levels.

7 SIGNIFICANCE

The significance of the studies is a deeper understanding of mechanisms behind, and an association of AQPs to, physiological events including water transport, e.g. endometrial edema, implantation, and menstruation, but also about the etiology behind idiopathic menorrhagia. More knowledge about the hormonal regulation of AQPs in human endometrium could provide us with tools to cure disease caused by impaired water transport.

8 CLINICAL APPLICATIONS

Assuming that AQP1 is involved in impaired angiogenesis as cause of idiopathic menorrhagia, mifepristone could be a possible treatment. The mechanism of action in this case could be through influence on the structural modifications of blood vessels, i.e. coiling of the spiral arteries. Another possible treatment for menorrhagia patients with low levels of AQP1 could be P or anti-E.

9 FUTURE PERSPECTIVES

9.1 MIFEPRISTONE TREATMENT OF MENORRHAGIA PATIENTS WITH LOW LEVELS OF AQP1

According to the results of the studies included in this thesis, AQP1 expression in human endometrium is induced as a result of exposure to mifepristone. AQP1 is involved in angiogenesis and an impaired vessel formation is suggested as one possible reason behind idiopathic heavy menstrual bleedings. Therefore it would be of great interest to study whether mifepristone is suitable as a drug for treatment of idiopathic menorrhagia in women with low AQP1 levels. The suggested mechanism of action would be an effect of mifepristone on AQP1 expression, leading to an improved and normalised formation of endometrial vessels.

9.2 TREATMENT WITH P AND ANTI-E FOR IDIOPATHIC EXCESSIVE UTERINE BLEEDINGS LOW IN AQP1

As mentioned above, some patients presenting with excessive uterine bleedings, where the cause is unknown, show a different AQP1 profile (typically with decreased levels of AQP1) in the endometrium compared to that of normal, fertile women. The last study of this thesis showed that P and E influence AQP expression. The next step is to see if these findings have a clinical significance as well, by studying the effect of treatment with P and anti-E, respectively. Ideally, exposure to P or anti-E would enhance AQP1 expression in endothelial cells in the uterus, and thereby improving angiogenesis and normalising the bleeding pattern.

9.3 AQP1 DEPENDENT ANGIOGENESIS AND THE INFLUCENCE ON ENDOMETRIAL REGENERATION

It is well documented that AQP1 is involved in angiogenesis. During the reconstruction of the endometrium after menstrual shedding angiogenesis is a crucial event. Clarification of whether AQP1 dependent angiogenesis is essential for endometrial regeneration would be of importance for the further understanding of the physiology of the menstrual cycle, and as a consequence, also other events like implantation and edema.

9.4 MECHANISM OF ACTION OF MIFEPRISTONE ON ENDOMETRIAL CELLS

Mifepristone is known to improve bleeding pattern in the endometrium and also to increase AQP1 expression. However, the mechanism of action has not yet been described. It could be speculated that mifepristone acts through binding to the PR, the glucocorticoid receptor or VEGF, but this needs to be further investigated.

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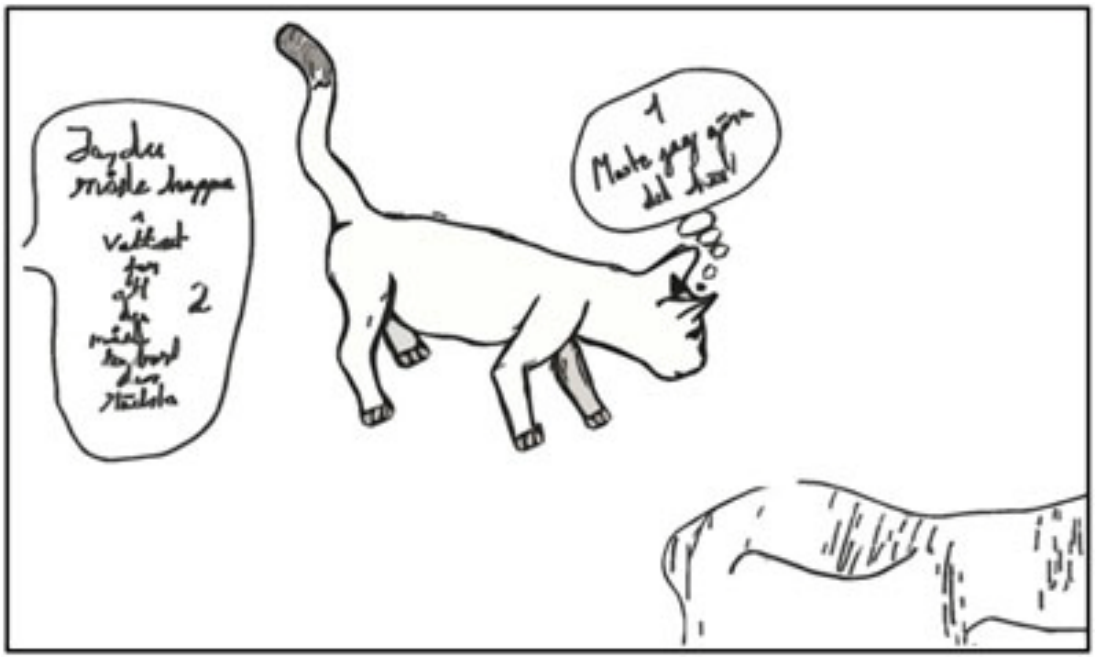
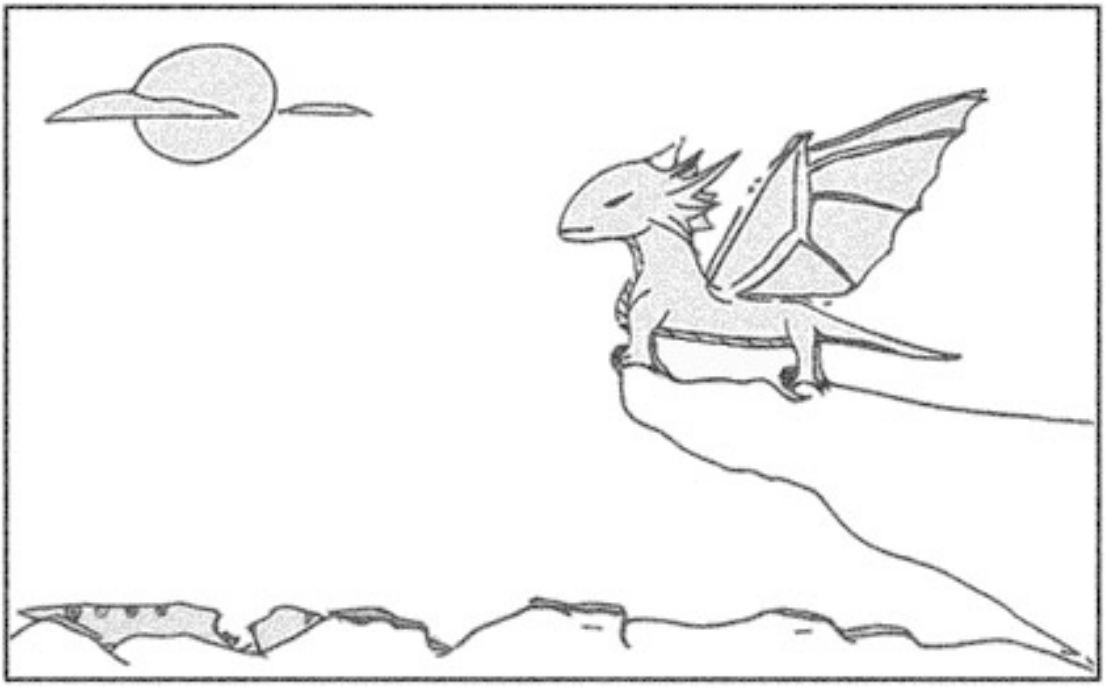
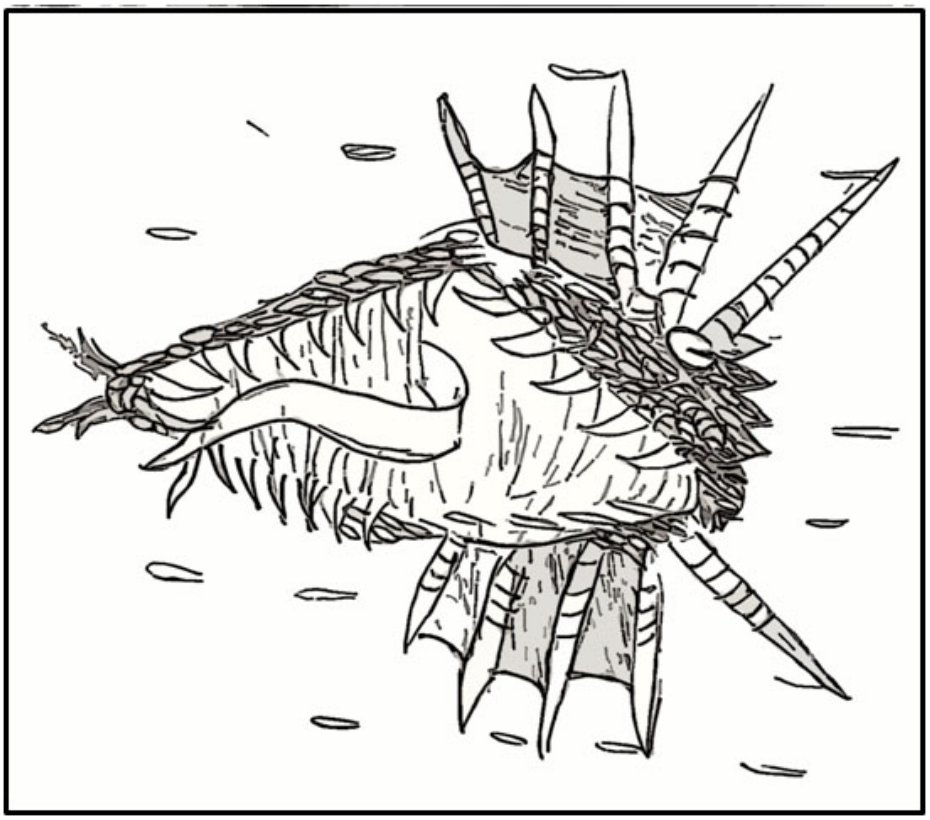
13 MORE ABOUT WATER



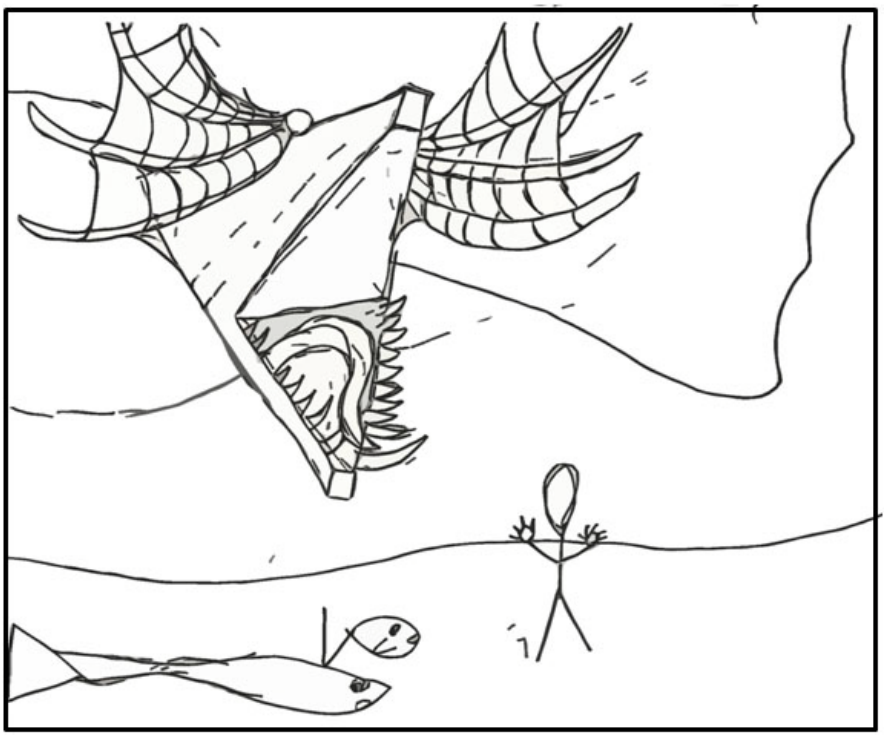
"Cat afraid of water"
Sarah Hildenbrand, 13.



"Ship going down and dragon on cliff"
Philip Hildenbrand, 10 år



”Dragon in rain”
Nils Wachtmeister, 10 år



”Dragon flying over sea”
Knut Wachtmeister, 8 år